主論文の要旨

Pharmacologic conversion of cancer-associated fibroblasts from a protumor phenotype to an antitumor phenotype improves the sensitivity of pancreatic cancer to chemotherapeutics

（癌関連線維芽細胞における腫瘍促進性から腫瘍抑制性への形質変換は膵臓癌の化学療法に対する感受性を改善させる）

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【緒言】

難治癌と言われる膵癌の特徴に間質線維化とそれに伴う組織硬化がある。組織硬化による内圧上昇は腫瘍血管を虚脱させ、その結果、抗癌剤の間質浸透と癌細胞への到達を阻害することが知られている。この癌間質の線維化を形成する上で中心的な役割を果たすのが癌関連線維芽細胞(CAF)である。癌組織では活性化 CAF がコラーゲン等の細胞外基質およびそれらを架橋するリシルオキシダーゼ(LOX)ファミリー分子群を産生する。コラーゲン等の基質は LOX によって架橋されると直線的な重合線維を形成し、その結果として組織は硬くなる。組織硬化は内圧の上昇により血管虚脱を誘導することで抗がん剤の腫瘍内への送達を阻害することが多くの研究により明らかになっている。

CAF の分野では平滑筋アクチン(SMA)陽性 CAF に関する研究が主に行われてきたが、2019 年我々はそれとは異なる新しい CAF 亜群として Meflin 陽性 CAF(SMA 弱陽性)を同定した。Meflin 欠損マウス及び膵癌自然発症マウス等を用いた解析により、Meflin 陽性 CAF は従来の癌促進性 SMA 陽性 CAF と異なる癌抑制性 CAF であることを報告している。また系統追跡実験によりこの Meflin 陽性 CAF は癌の発育とともに癌促進性 SMA 陽性 CAF へと形質転換することも明らかになっている。今回我々はこれまで不明であった Meflin の機能に加え、癌間質における Meflin の発現と化学療法感受性との関連性を検討した。さらに臨床応用を見据え CAF における Meflin の発現上昇を誘導する化合物をスクリーニングし、得られた化合物の有用性を検証した。

【方法】

Meflin の機能

免疫沈降法と質量分析を組み合わせた手法で Meflin の結合分子を探索し、次に Meflin の結合による結合分子の機能の変化を検討した。

Meflin の発現と化学療法感受性との関連性

当院で化学療法を受けた膵癌患者から採取した EUS-FNA 検体における Meflin の発現と奏効率(ORR)との関連性を評価した。さらに野生型マウス、Meflin 遺伝子欠損マウスそれぞれにマウス膵癌細胞株(mT5)を皮下移植し、その後抗がん剤(Gem)による治療を行い腫瘍径の比較をした。次に CAF における Meflin の発現を誘導するために、Meflin をコードするセンダイウイルス(SeV)ベクターを作製した。野生型マウスの皮下腫瘍(mT5)に SeV-Meflin を感染させ腫瘍内で Meflin の過剰発現が誘導させることで抗がん剤への感受性の変化を評価した。

Meflin の発現上昇を誘導する化合物のスクリーニングと有用性の検証

これまでに Meflin の発現上昇を誘導する物質としてビタミン D を同定している。ビタミン D が核内受容体に作用する分子であることからヒト膵癌 CAF を用いて同受容体のリガンドライブラリーをスクリーニングし、マウス膵癌(mT5)皮下移植モデル

を使用しその化合物の効果を評価した。

【結果】

Meflin の機能

Meflin の結合分子を探索したところ、LOX を同定した。組換え分子を用いた実験により、Meflin-LOX 間結合は直接的な結合であり、また Meflin は LOX のコラーゲン架橋活性を阻害することが判明した(Fig1 a-e)。LOX は一般的にコラーゲン等の細胞外基質の架橋と組織硬化を引き起こす酵素と言われており、その活性を抑制することが示唆された。

Meflin の発現と化学療法感受性との関連性

膵癌 EUS-FNA 検体における Meflin 高発現群は低発現群に比べ有意に奏効率が高かった(Fig2 a,b)。また、皮下移植マウスモデルを使用した実験では、野生型マウスと比べ Meflin 欠失マウスでは有意に抗癌剤感受性が減弱した。また興味深いことに腫瘍組織を評価すると Meflin 遺伝子欠失マウスに移植した mT5 腫瘍は、腫瘍血管内径および CD31 陽性内皮細胞領域が野生型マウスに比べ減少した(Fig2 c-f)。SeV ベクターを用いた実験では Meflin 過剰発現が誘導された腫瘍では腫瘍血管径の有意な増加および抗癌剤に対する感受性が増強した(Fig3 a-f)。このことは、腫瘍における Meflin の発現が腫瘍の抗癌剤感受性に関与していることを示している。

Meflin の発現上昇を誘導する化合物のスクリーニングと有用性の検証

ビタミン D 誘導体よりも Meflin の発現を誘導する物質として AM580 を同定した。さらに、AM580 と同時に開発され、急性前骨髄球性白血病(APL)に対して認可されている AM80(一般名:タミバロテン)も AM580 と同様の効果を示すことを見出した(Fig4 a)。皮下移植(mT5)マウスモデルで検証したところ、AM80 単剤投与は抗腫瘍効果を示さなかったが、CAF における Meflin の発現上昇、腫瘍血管面積の増加(Fig4 b-g)、コラーゲン架橋の低下によるコラーゲン線維の配向の変化、組織の軟化が認められた(Fig1 f-i)。さらに AM80 と抗がん剤(Gem)の併用を行ったところ、抗がん剤単独と比較して有意に腫瘍内抗がん剤濃度の上昇と腫瘍縮小効果が示された(Fig5 a-h)。また、この効果が Meflin 遺伝子欠損マウスでは確認されなかったことから、AM80 は Meflin を介してこの効果をもたらしている可能性が示唆された(Fig6 a-h)。

【考察】

今回の研究では、まず Meflin の機能が LOX のコラーゲン架橋活性を抑制することが示された。LOX は組織硬度、間質内圧等の腫瘍間質の物理学的特性に関する重要な調節因子であり、Meflin 高発現の腫瘍では組織の軟化、腫瘍血管の拡張、抗がん剤の送達が改善される可能性が示唆された。そこで膵癌患者の臨床検体と様々なマウスモデルを用い検証した結果、膵癌の CAF における Meflin の発現上昇は腫瘍血管の拡張、

化学療法への感受性を増強させることを見出した。これらの実験をふまえ CAF におけるメフリンの発現を誘導する化合物のスクリーニングを行い、Am80 の同定へとつながった。Am80 投与は腫瘍内 CAF における Meflin の発現上昇をもたらし、コラーゲンの配向の改善、組織の軟化、腫瘍血管の拡張の結果、化学療法に対する感受性改善が示された。現在、米国では、AM80 と同様に Meflin の発現を誘導する化合物であるビタミン D と既存の化学療法とを組み合わせた治験が複数進行中である。それらの結果との関連性が期待されると同時に、現在 AM80 を用いた化学療法との併用治験が実施されており今後本研究結果をうけて CAF を標的とした治療法の開発がすすむことが期待される。

【結論】

CAF における Meflin の発現が化学療法に対する腫瘍感受性を改善することを示した。

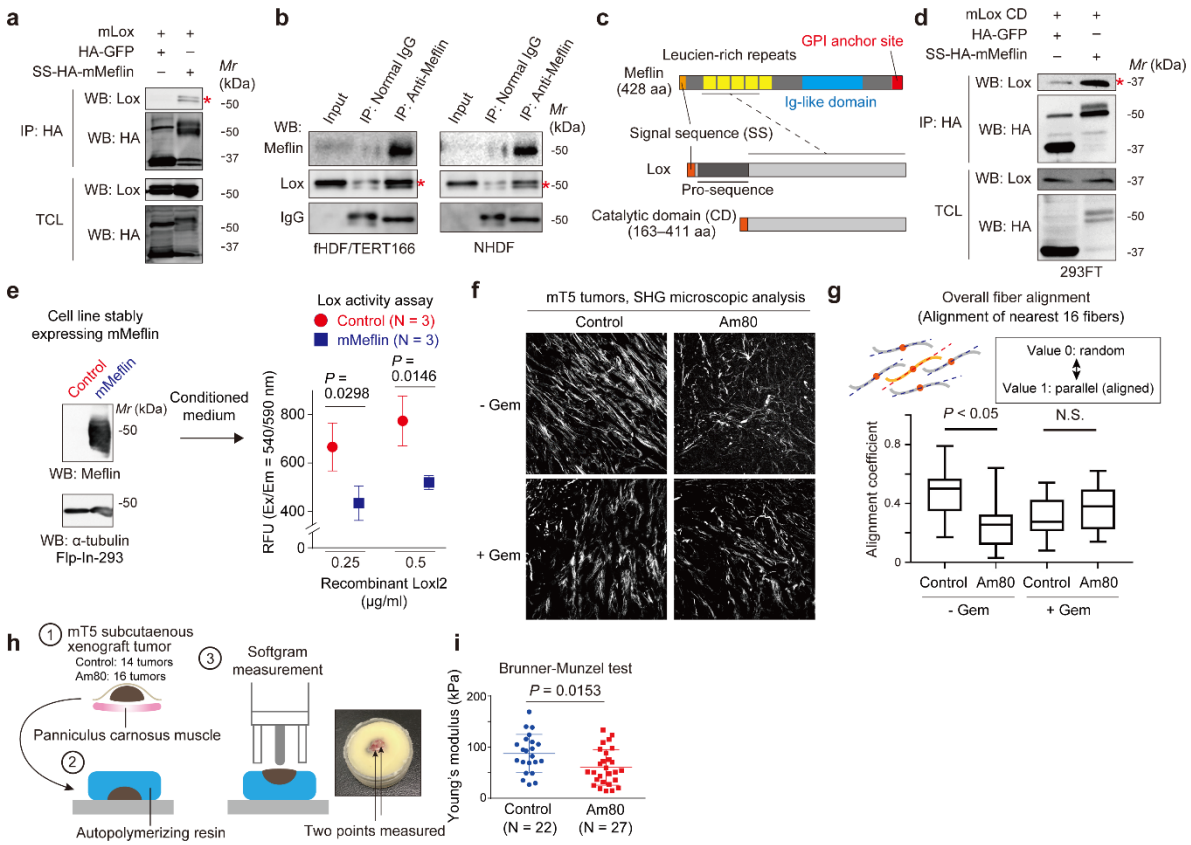

Figure 1. Meflin was involved in the regulation of Lox activity and collagen remodeling.

(a) The indicated plasmids were transfected into HEK293 cells, followed by IP with anti-HA antibodis and WB analysis. Lox coprecipitated with HA-Meflin (asterisk). mMeflin, mouse Meflin; mLox, mouse Lox; SS, signal sequence; TCL, total cell lysate.

(b) Interaction of endogenous Meflin and Lox proteins. Cell lysates prepared from the fHDF/TERT166 fibroblast cell line (left) or primary cultured human fibroblasts (NHDFs, right) were immunoprecipitated with anti-Meflin antibodies, followed by analysis of eluates by WB with the indicated antibodies. Lox proteins coprecipitated with Meflin (asterisk).

(c) Primary structure of human Meflin (top) and mouse Lox (middle). Lox comprises an amino-terminal SS, a pro-sequence, and a catalytic domain (CD). (Bottom) Construct encoding the CD of Lox with an SS at the amino terminus. Dashed line: protein domains responsible for the Lox/Meflin interaction.

(d) The indicated plasmids were cotransfected into 293FT cells, followed by IP with anti-HA antibodies and WB analysis. Meflin interacted with the CD of mLox (asterisk).

(e) Conditioned medium from control Flp-In-293 cells or Flp-In-293 cells stably expressing mMeflin was mixed with recombinant Loxl2 (0.25 and 0.5 μg/mL), followed by measurement of hydrogen peroxide using a fluorometric-based method. (Left) WB analysis of Meflin expression in Flp-In-293 cells. RFU, relative fluorescence unit.

(f, g) Measurement of collagen alignment in the stroma of subcutaneous mT5 tumors developed in mice by SHG microscopy. Eight to sixteen images from tissue sections of control tumors and tumors treated with Am80 were analyzed. Representative images **(f)** and quantification of collagen alignment **(g)**.

(h, i) Measurement of the stiffness of mT5 tumors developed in control mice (n = 14) and those treated with Am80 (n = 16). The Young's modulus of the flat surfaces of tumors contacting the fascia of the panniculus carnosus muscle were measured (**h**). One or two points were selected in each tumor, and each point was measured more than 5 times; quantification was performed (**i**).

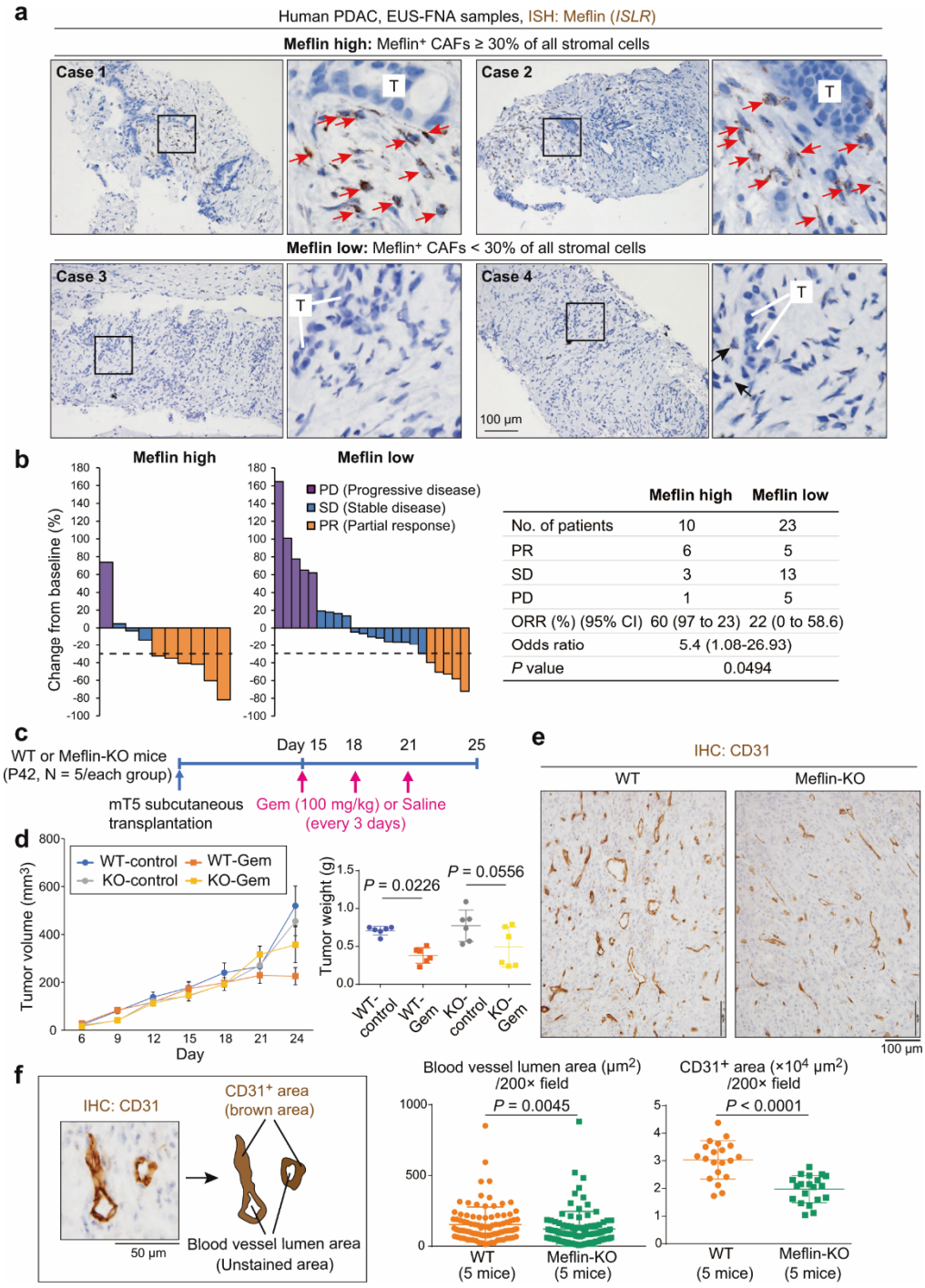

Figure 2. Numbers of Meflin$^+$ stromal cells correlated with response of patients with PDAC to chemotherapeutics and tumor vessel area.

(a) Representative images of Meflin ISH analysis on formalin-fixed and paraffin-embedded (FFPE) sections of biopsy samples obtained from patients with PDAC by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). Cases with 30% or more of stromal cells positive for Meflin were classified as Meflin high (cases 1 and 2, upper panel), whereas others were classified as Meflin low (cases 3 and 4, lower panel). Boxed areas were magnified in adjacent panels. Red arrows indicate Meflin$^+$ CAFs. Black arrows indicate CAFs weakly positive for Meflin. T, tumor cells.

(b) Waterfall plots depicting tumor responses of patients with PDAC (N = 33), who received Gem, nabPTX plus Gem (GnP), or FOLFIRINOX, evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. The table in the right panel shows the numbers of patients classified by their response status and Meflin expression. The results of statistical analysis are also shown.

(c) WT and Meflin-KO female mice at postnatal day (P) 42 were subcutaneously transplanted with mT5 mouse PDAC cells (1×10^6 cells/mouse), followed by i.p. administration of Gem or saline three times every 2 days and sacrifice at day 25.

(d) Time course of the volumes of tumors developed in WT and Meflin-KO mice administered saline or Gem (left panel) and quantification of tumor weight at the end of experiment (right panel).

(e) Representative images of tissue sections of WT and Meflin-KO mT5 tumors stained for CD31. Brown color denotes positivity. IHC, immunohistochemistry.

(f) Schematic illustration depicting the method of quantification of CD31-stained blood vessel area and lumen of mT5 tumors in WT and Meflin-KO mice, performed using ImageJ software (left panel). Quantification data are shown in middle and right panels.

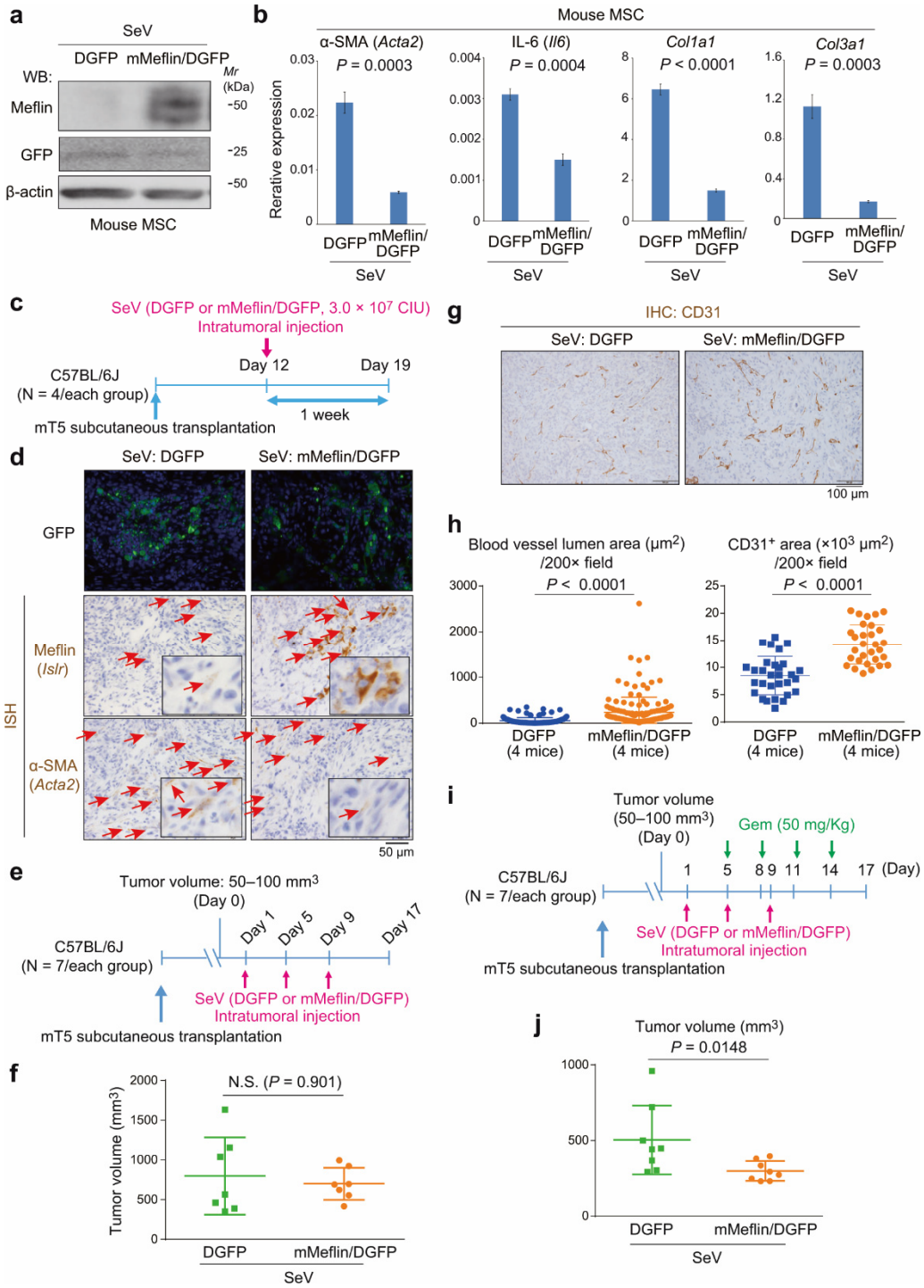

Figure 3. SeV-mediated transduction of Meflin induced increases in tumor vessel area and sensitivity to Gem.

(a, b) Recombinant SeV encoding DasherGFP (DGFP) or mouse Meflin and DGFP (mMeflin/DGFP) was used to infect mouse MSCs, followed by western blot (WB) analysis with the indicated antibodies **(a)** and quantitative PCR (qPCR) for the indicated genes **(b)**. *Mr*, molecular marker.

(c) WT female mice (P42) were subcutaneously transplanted with mT5 mouse PDAC cells (1×10^6 cells/mouse), followed by intratumoral injection of the indicated SeV (3×10^7 CIU) at day 12 and sacrifice at day 19.

(d) Observation of GFP fluorescence in frozen sections prepared from mT5 tumors infected with SeV encoding DGFP (left) and Meflin/DGFP (right) (upper panel). FFPE sections were also obtained from tumors and stained for Meflin (*Islr*) and α-SMA (*Acta2*) by ISH (middle and lower panels). Arrows indicate Meflin$^+$ or *Acta2*$^+$ CAFs.

(e–h) mT5 tumors transplanted into WT female mice (P42) were allowed to grow until reaching 50–100 mm^3 in volume. Mice were then injected with SeV encoding DGFP or Meflin/DGFP three times every 4 days and sacrificed on day 17 **(e)**. Tumor volume was measured **(f),** and IHC for CD31 was performed to quantify the tumor vessel lumen and CD31$^+$ area **(g, h)**.

(i, j) mT5 tumors transplanted into WT female mice (P42) were allowed to grow until reaching 50–100 mm^3 in volume (day 0). Mice were then injected with SeV encoding DGFP or Meflin/DGFP three times every 4 days (days 1, 5, and 9). The mice were i.p. administered Gem four times on days 5, 8, 11, and 14 and then sacrificed on day 17 **(i)**. Tumor volume was measured **(j)**.

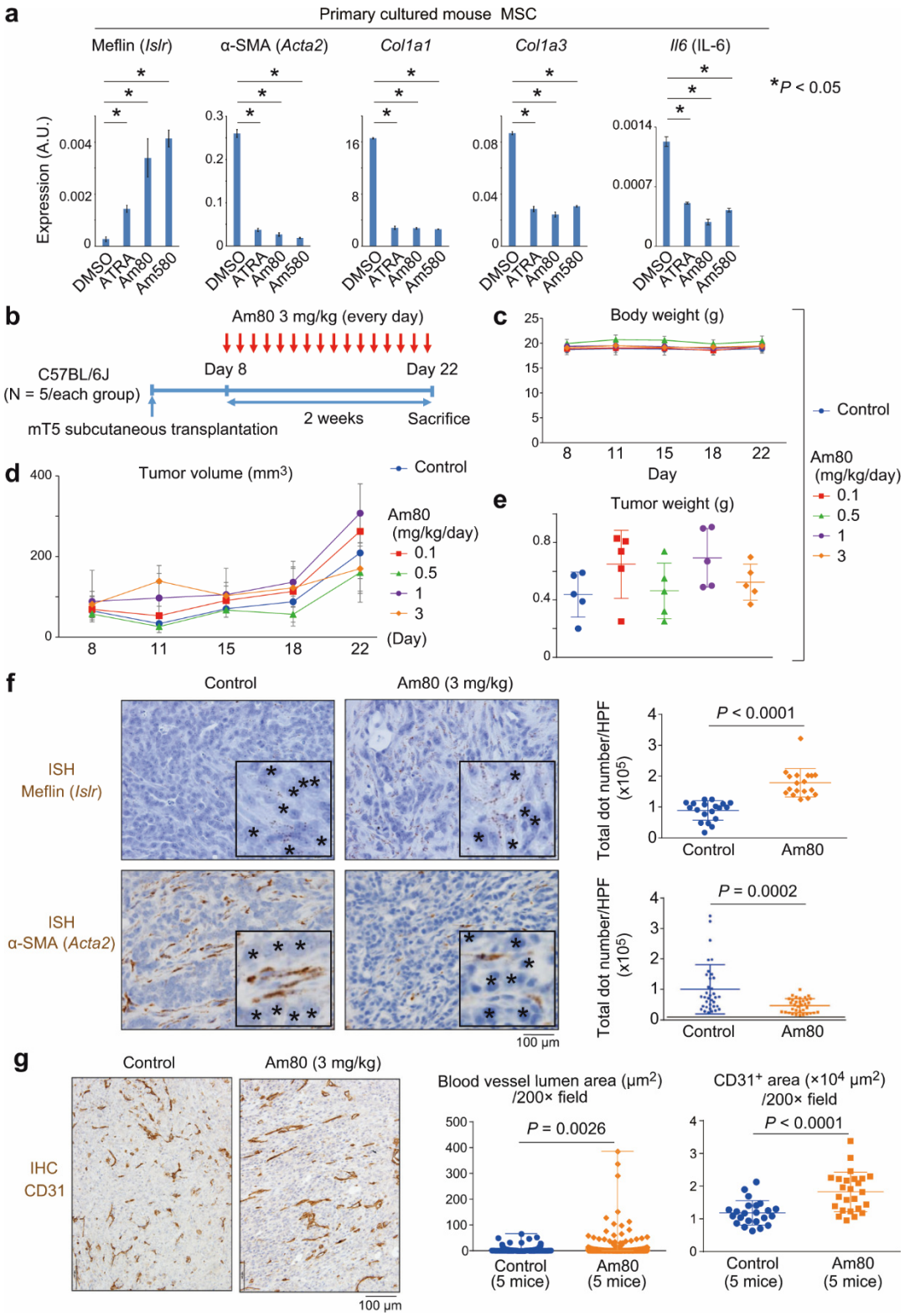

Figure 4. Effects of Am80 administration on the expression of Meflin in CAFs and on tumor vessel area in a PDAC xenograft mouse model.

(a) Primary cultured mouse MSCs were plated in the presence of DMSO, ATRA, Am80, and AM580 for 48 h, and qPCR of the indicated genes was performed.

(b–e) WT female mice (P42) were subcutaneously transplanted with mT5 mouse PDAC cells (1×10^6 cells/mouse), followed by oral administration of Am80 (3 mg/kg, 0.5% carboxymethylcellulose [CMC] solution) every day for 14 days **(b)**. Body weights of mice **(c)** and tumor volumes **(d)** were measured during the observation period. **(e)** Tumor weight on the day of sacrifice (day 22).

(f, g) Tissue sections prepared from control mT5 tumors (left) and those administered Am80 (right) were examined for Meflin and α-SMA expression by ISH **(f)** and for CD31 expression by IHC **(g)**. Meflin and α-SMA expression levels **(f)** and tumor vessel lumen and $CD31^+$ areas **(g)** were calculated. For quantification of ISH signals, the number of dots per high-power (400×) microscopic field (HPF) was counted, and 20 HPFs randomly selected from 5 animals were evaluated for each group. Asterisks denote tumor cells that were negative for Meflin and α-SMA expression.

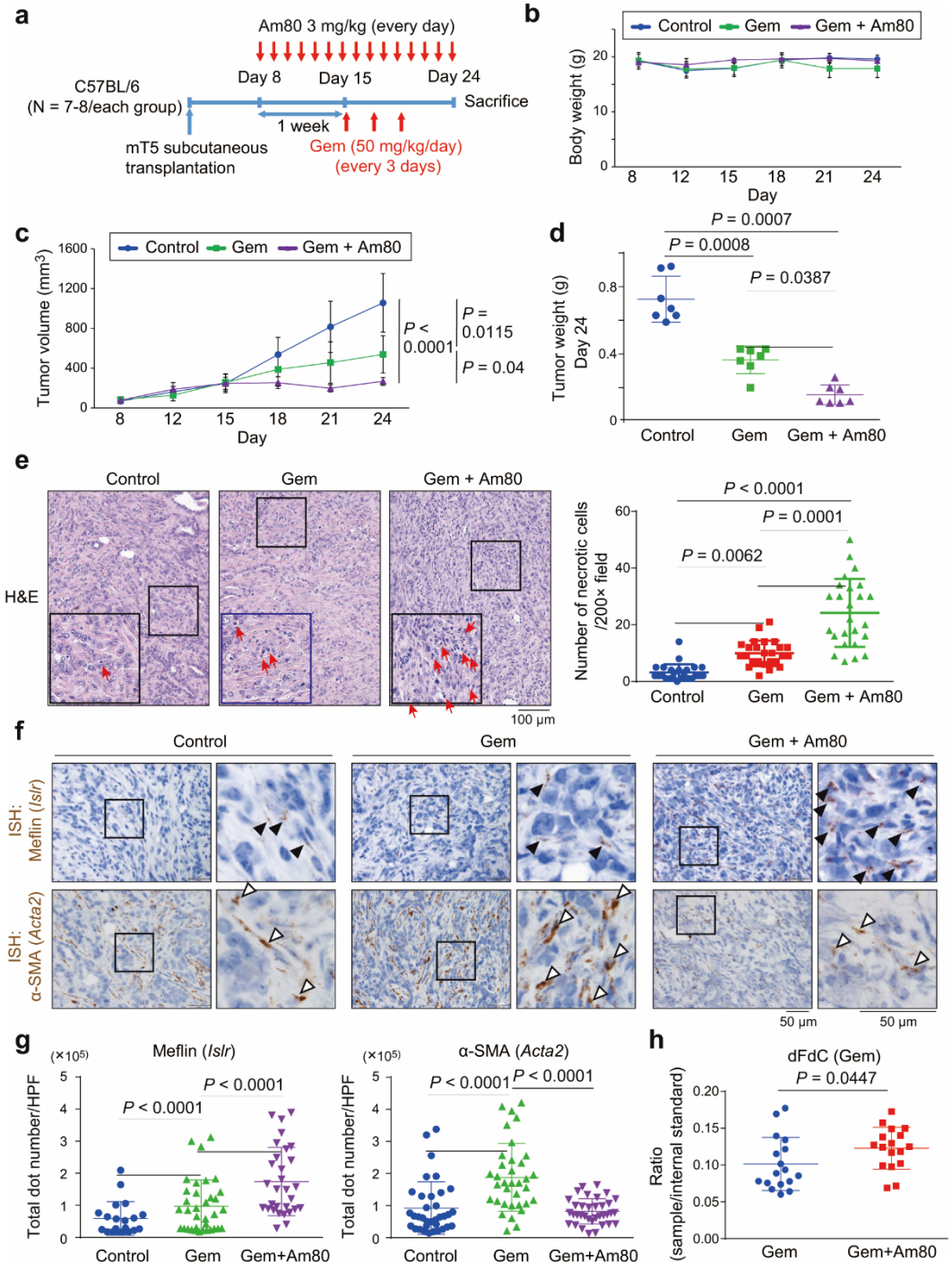

Figure 5. Am80 administration improved tumor sensitivity to Gem and induced Meflin expression in CAFs in a subcutaneous PDAC xenograft mouse model.

(a) WT female mice (P42) were subcutaneously transplanted with mT5 mouse PDAC cells (1×10^6 cells/mouse) on day 0, followed by oral administration of Am80 from day 8 for 16 consecutive days. The mice were administered Gem from day 15 three times every 3 days and sacrificed on day 24.

(b–d) Measurement of the body weights of mice **(b)** and tumor volumes **(c)** during the observed period and of tumor weights on day 24 **(d)**.

(e) Representative images of H&E-stained tissue sections of tumors prepared from the indicated groups. Boxed regions are magnified in insets. Arrows denote necrotic cells with pyknotic nuclei. The numbers of necrotic cells were counted and quantified in the graph shown in the right panel. Twenty-five images obtained from five tumors per group using a 20× objective lens were analyzed and quantified.

(f, g) Tissue sections of tumors from the indicated groups were stained for Meflin and α-SMA by ISH **(f)**, followed by quantification of total dot numbers **(g)**. Open and solid arrowheads denote Meflin$^+$ and α-SMA$^+$ CAFs, respectively.

(h) Quantification of dFdC (Gem) delivered to tumors in the indicated groups after administration of Gem three times. Lysates prepared from mT5 tumors treated with Gem (n = 17) and Gem plus Am80 (n =17) were added with stable isotope-labeled dFdC (internal control) subjected to liquid chromatography-mass spectrometry analysis, followed by quantification of dFdC and the internal control. The data are expressed as relative ratios of dFdC to the internal control.

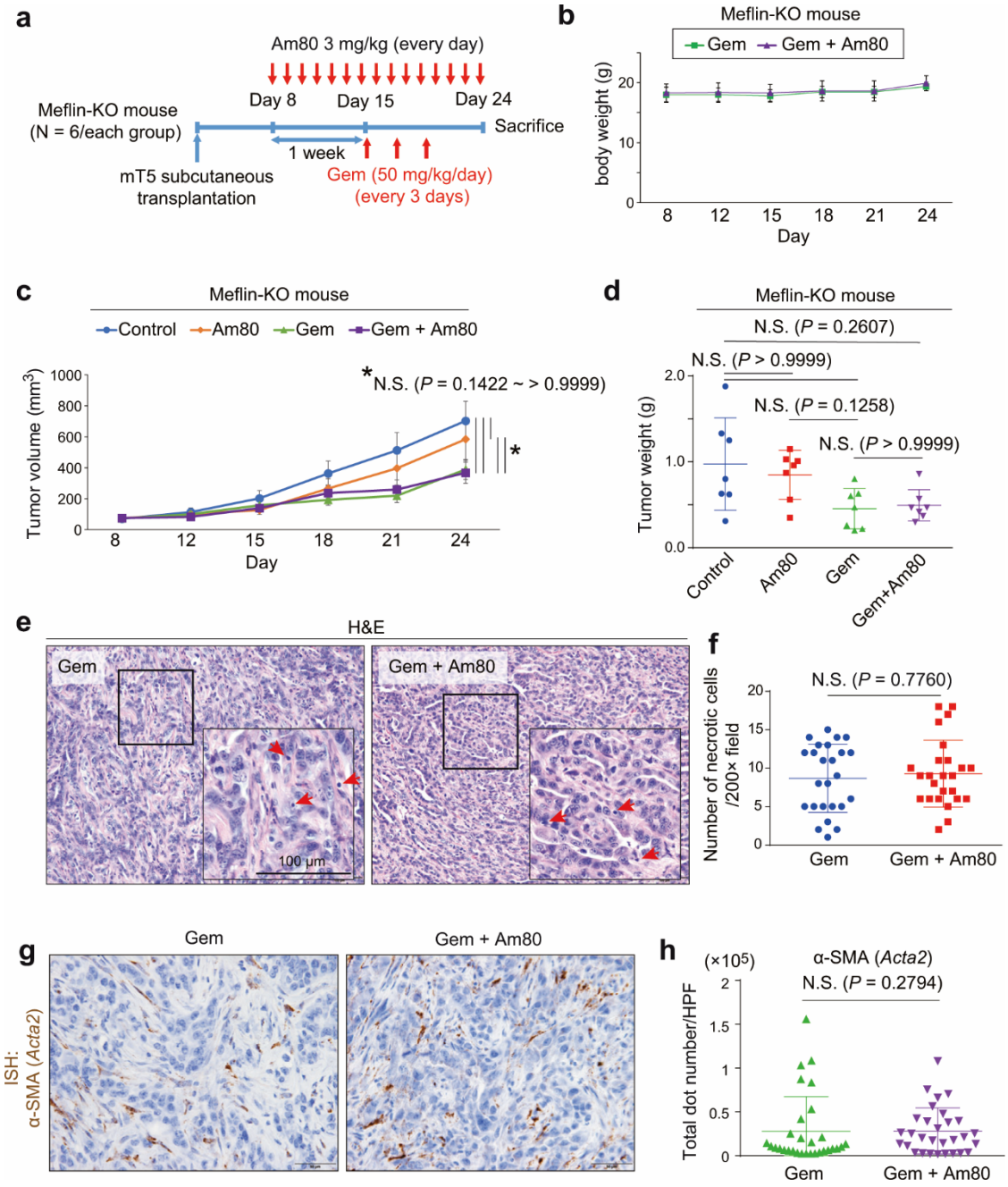

Figure 6. Increased tumor chemosensitivity by Am80 administration was mediated by alterations in Meflin expression in CAFs.
(a) Meflin-KO female mice (P42) were subcutaneously transplanted with mT5 mouse PDAC cells (1×10^6 cells/mouse) on day 0, followed by oral administration of Am80 from day 8 for 16 consecutive days. Mice were administered Gem from day 15 three times every 3 days and sacrificed on day 24.
(b–d) Measurement of the body weights of mice **(b)** and tumor volumes **(c)** during the observation period, and tumor weights on day 24 **(d)**.
(e, f) Representative images of H&E-stained tissue sections of tumors prepared from the indicated groups **(e)**. Boxed regions are magnified in insets. Arrows denote necrotic cells with pyknotic nuclei. The numbers of necrotic cells were counted and quantified in the graph shown in **(f)**.
(g, h) Tissue sections of tumors from the indicated groups were stained for α-SMA by ISH **(g)**, followed by quantification of the total dot numbers **(h)**.