

Aberrantly Expressed Galectin-9 is Involved in the Immunopathogenesis of anti-MDA5-Positive Dermatomyositis-Associated Interstitial Lung Disease

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Research

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Abstract

Background: Rapidly progressive interstitial lung disease (RP-ILD) has high mortality rate and poor prognosis. Galectin-9 (Gal-9) plays multiple functions in immune regulation. We investigated Gal-9 expression in patients with dermatomyositis (DM) and the impact of Gal-9 on the development of DM-ILD.

Methods: Enzyme-linked immunosorbent assay and qRT-PCR were used to examine Gal-9 expression in the sera and isolated peripheral blood mononuclear cells (PBMCs) from patients with DM. Immunohistochemistry was performed to analyze the expression of Gal-9 and its ligand (T-cell immunoglobulin mucin (Tim)-3 and CD44) in lung tissues from patients who were positive for anti-melanoma differentiation-associated gene 5 (MDA5). The effect of Gal-9 on human lung fibroblasts (MRC-5) was also investigated *in vitro*.

Results: Serum Gal-9 levels were significantly higher in patients with DM than in those with immune-mediated necrotizing myopathy and healthy controls ($p < 0.001$). Higher levels of serum Gal-9 were observed in anti-MDA5-positive patients with DM than in anti-MDA5-negative patients with DM (33.8 (21.9–44.7) vs 16.2 (10.0–26.9) ng/mL, $p < 0.001$). Among the anti-MDA5-positive patients with DM, serum Gal-9 levels were associated with ILD severity. Serum Gal-9 levels were significantly correlated with disease activity in anti-MDA5-positive patients with DM in both cross-sectional and longitudinal studies. PBMCs isolated from anti-MDA5-positive patients with DM (3.7 ± 2.3 ng/mL) produced higher levels of Gal-9 than those from patients with immune-mediated necrotizing myopathy (1.1 ± 0.3 ng/mL, $p = 0.022$) and healthy controls (1.4 ± 1.2 ng/mL, $p = 0.045$). The mRNA levels of Gal-9 were positively correlated with levels of type-I interferon-inducible genes MX1 ($r = 0.659$, $p = 0.020$) and IFIH1 ($r = 0.787$, $p = 0.002$) in PBMCs from anti-MDA5-positive patients with DM. Immunohistochemistry revealed increased Gal-9 and Tim-3 expression in the lung tissues of patients with DM and RP-ILD. *In vitro* stimulation with Gal-9 protein increased CCL2 mRNA expression in MRC-5 fibroblasts.

Conclusions: Among anti-MDA5-positive patients with DM, Gal-9 could be a promising biomarker for monitoring disease activity, particularly for RP-ILD severity. Aberrant expression of the Gal-9/Tim-3 axis may be involved in the immunopathogenesis of DM-ILD.

Background

Dermatomyositis (DM) is a subgroup of idiopathic inflammatory myopathies (IIMs) which is frequent involved in muscle, skin, lung and other organs [1]. Myositis-specific autoantibodies (MSAs) are common in patients with DM and can help define the disease into more homogeneous groups [2]. Patients carrying anti-melanoma differentiation-associated gene 5 (MDA5) antibodies are likely to develop interstitial lung disease (ILD), particularly rapidly progressive (RP)-ILD, which has a high mortality rate and poor prognosis [3–5]. Previous studies suggested that activation of monocytes, macrophages, neutrophils,

and CD4⁺ T helper (Th) 1 cells, and increased expression of CD4⁺CXCR4⁺ T cells and interferon (IFN)- γ contribute to the development of DM-ILD [6–9]. However, the pathogenesis of DM-ILD remains unclear.

Galectins are a family of proteins that bind to β -galactoside-containing glycans [10]. In this family, galectin-9 (Gal-9) has been detected in monocytes, macrophages, endothelial cells, fibroblasts, and Kupffer cells [11, 12]. Gal-9 plays multiple functions in immune regulation such as inducing cell migration, activation, and apoptosis [13, 14]. Several ligands of Gal-9 such as T-cell immunoglobulin mucin (Tim)-3, CD44, and protein disulfide isomerase have been identified [15–17]. Th1 cells, Th17 cells, and alveolar macrophages were reported to express Tim-3 [18–20]. Lu and colleagues [21] demonstrated that the Gal-9/Tim-3 pathway suppressed the respiratory syncytial virus-induced lung inflammation by inhibiting the Th1 and Th17 immune response in mice. Additionally, it has been reported that the CD44-dependent interaction with hyaluronan was inhibited by Gal-9 on human lung fibroblast cells, thus protecting against lung fibrosis in patients with cryptogenic organizing pneumonia [22]. A growing body of reports have suggested the importance Gal-9 in a variety of diseases such as cancer and autoimmune diseases. It has been demonstrated that Gal-9 is correlated with disease activity and strongly correlated with IFN score in patients with systemic lupus erythematosus [23]. Furthermore, Wiersma et al. [24] reported that Gal-9 activates peptidyl arginine deiminase 4 in granulocytes and promotes immunopathology in patients with rheumatoid arthritis.

Increased serum Gal-9 levels were reported in juvenile DM and were demonstrated to correlate with disease activity [25, 26]. However, very limited data is available in the relation between the serum levels of Gal-9 and MSA types or DM-ILD. In addition, the effect of Gal-9 has not been established in the pathogenesis of DM-ILD. Therefore, in this study, we systemically investigated expression of Gal-9 in patients with DM, and its impact on the development of DM-ILD.

Methods

Patient sampling

A total of 154 patients with IIM including 129 patients with DM and 25 patients with immune-mediated necrotizing myopathy (IMNM) from China-Japan Friendship Hospital were enrolled in this study. DM was diagnosed based on the 2017 ACR/EULAR IIM criteria [27], and IMNM was diagnosed using ENMC IMNM criteria [28]. Patients younger than 16 years of age and those exhibiting complications or other connective tissue diseases were excluded from the study. Additionally, we enrolled 30 healthy, age- and sex-matched volunteers as healthy controls (HCs). Informed consent was obtained from all participants. The Ethical Review Committee of the China-Japan Friendship Hospital (2019-25-K19) approved this study.

We collected the demographic features, clinical features, and laboratory data of the patients from electronic medical records. In the longitudinal study, 21 anti-MDA5-positive patients with DM were followed up for 1–42 months. The median follow-up duration was 22 months. We collected the blood

samples during the follow-up period at each hospitalization. The interval between the two sample collections from a single patient was 1–25 months. ILD was confirmed by computed tomography and pulmonary function analyses [29]. RP-ILD was diagnosed by a worsening radiologic interstitial status and the presence of respiratory symptoms such as progressive dyspnea and hypoxemia [29, 30]. For pulmonary function examination, the results of forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1) and diffusing capacity of carbon monoxide (DLco) were collected. The myositis disease activity was assessed by 10-cm visual analog scales (VAS) for muscle, six extramuscular organ systems including constitutional, cutaneous, joint, gastrointestinal, pulmonary, and cardiac, and the physician's global assessment (PGA).

Detection of serum Gal-9 and MSA

An enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) was used to measure the serum Gal-9 levels. Additionally, MSAs were detected using an immunoblot assay kit (Euroimmun, Lübeck, Germany). Anti-3-hydroxy-3-methyl coenzyme A reductase protein autoantibodies were measured by ELISA (Inova Diagnostics Inc., San Diego, CA, USA). These assays were performed according to the manufacturer's instructions.

Cell culture and treatment

1. Peripheral blood mononuclear cells (PBMCs) culture and treatment

PBMCs were isolated by centrifugation on a Histopaque density gradient (Sigma-Aldrich, St. Louis, MO, USA). The isolated PBMCs were seeded into 96-well plates at 5×10^5 cells/mL in Roswell Park Memorial Institute 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 U/ μ g/mL penicillin/streptomycin (Gibco) at 37°C and 5% CO₂ for 48 h. The supernatant was collected by centrifugation. An ELISA kit was used to determine Gal-9 levels in the supernatant, as described.

2. MRC-5 fibroblasts culture and treatment

MRC-5 human lung fibroblasts were obtained from American Type Culture Collection (Manassas, VA, USA) and seeded into 6-well plates at 1×10^5 cells/mL in minimum essential medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco), 1% non-essential amino acids (Gibco), and 100 U/ μ g/mL penicillin/streptomycin (Gibco) at 37°C and 5% CO₂ for 8 h. Next, Gal-9 (Biolegend, San Diego, CA, USA) or transforming growth factor- β (TGF- β) (Peprotech, Rocky Hill, NJ, USA) were added. The proliferation of MRC-5 fibroblasts stimulated with Gal-9 for 24 or 48 h was tested with a luminescent cell viability assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from PBMCs or cultured MRC-5 fibroblasts stimulated with Gal-9 for 24 h using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The mRNA levels were tested by SYBR-Green-based qRT-

PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primers of target genes [Gal-9, Tim-3, CD44, MX1, IFIH1, monocyte chemoattractant protein-1 (CCL2), interleukin 1 β (IL-1 β), IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, tumor necrosis factor- α (TNF- α), IFN γ , CCL18, C-X-C motif chemokine ligand 4 (CXCL4), and CXCL10] are shown in Supplementary Table 1. The $2^{-\Delta\text{Ct}}$ method was used to calculate the relative gene levels.

Immunohistochemistry

Lung sections were obtained from anti-MDA5-positive patients with DM. Diagnosis was obtained by surgical resection or percutaneous lung biopsy. Tissues were fixed in 10% formalin and embedded in paraffin, and subjected to antigen retrieval by heating and treatment with 3% hydrogen peroxide for 15 min. After incubation with rabbit anti-Gal-9 monoclonal antibody (1:500 dilution; Abcam, Cambridge, UK), anti-Tim-3 (1:400 dilution; Proteintech, Rocky Hill, NJ, USA), and anti-CD44 (1:50 dilution; Biolegend) overnight at 4°C, goat anti-rabbit IgG antibody (Gene Tech Shanghai Company Limited, Shanghai, China) was incubated with the tissue sections for 30 min at room temperature. 3,3'-Diaminobenzidine (Gene Tech Shanghai Company Limited) was used as a chromogenic reagent and hematoxylin was used for counterstaining.

Western blot analysis

MRC-5 fibroblasts were stimulated with Gal-9 or TGF- β for 48 h. Total protein from the cells was extracted by adding protein lysis buffer to the cells. Western blotting was conducted using primary antibodies of rabbit polyclonal anti-smooth muscle actin (SMA) (1:1000 dilution; Proteintech) and mouse monoclonal anti-GAPDH (1:1000 dilution; Abcam), followed by secondary antibodies including peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution; Abcam) and peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution; Abcam). Enhanced chemiluminescence substrate (Thermo Fisher Scientific, Waltham, MA, USA) was added to the membranes. Quantitative protein densitometry was performed with ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

Data analysis was performed using GraphPad Prism V.7.01 (GraphPad, Inc., San Diego, CA, USA) and SPSS Version 22 (SPSS, Inc., Chicago, IL, USA). Numbers (percentages), mean \pm standard deviation, or median values and interquartile range (IQR) were used to express the data. Student's *t*-test or Mann-Whitney *U*-test was used for two-group comparisons. For comparison among multiple groups, the Kruskal-Wallis *H*-test was performed. The correlations of normally and non-normally distributed data were measured using Pearson's correlation and Spearman's correlation, respectively. Longitudinal data were analyzed with the generalized estimating equation model. *p*-Values below 0.05 were considered to indicate statistical significance.

Results

Patient characteristics

Patients with IIM (n = 154) were enrolled in the present study. The demographics, clinical manifestations, and laboratory characteristics of patients are shown in Table 1. Approximately 84.4% of patients (130 of 154) were administered corticosteroids and immunosuppressive agents (methotrexate, cyclophosphamide, ribavirin, intravenous immunoglobulin, hydroxychloroquine, and mycophenolate mofetil). The corticosteroid dosages were between 0.5–1 mg/kg.

Table 1
Baseline characteristics observed in DM patients and IMNM patients

Characteristics	DM patients	IMNM patients
	(n = 129)	(n = 25)
Gender (Female/Male)	89/40	14/11
Age of onset, median (IQR), years	49 (39–57)	47 (31.5–57.5)
Disease duration, median (IQR), months	6 (2–24)	10 (4.5–24)
Clinical features, no. (%)		
ILD	89 (69.0%)	0 (0%)
RP-ILD	26 (20.2%) %)	0 (0%)
Muscle weakness	55 (42.6%)	20 (80%)
Myalgia	36 (27.9%)	6 (24%)
Mechanic's hands	37 (28.7%)	0 (0%)
Raynaud's phenomenon	10 (7.8%)	0 (0%)
Heliotrope rash	95 (73.6%)	3 (12%)
Gottron's papules	69 (53.5%)	1 (4%)
Skin ulceration	25 (19.4%)	0 (0%)
Calcinosis	5 (3.9%)	0 (0%)
Arthritis/arthritis	35 (27.1%)	1 (4%)
Dysphagia	18 (14.0%)	3 (12%)
Malignancy	1 (0.8%)	0 (0%)
Pulmonary function test, median (IQR)		

DM: dermatomyositis; IMNM: immune-mediated necrotizing myopathy; IQR: interquartile range; ILD: interstitial lung disease; RP-ILD: rapidly progressive interstitial lung disease; FVC: forced vital capacity; FEV₁: forced expiratory volume in 1 s; DLco: diffusing capacity of carbon monoxide; MSA: myositis-specific antibody; MDA5: melanoma differentiation-associated gene 5; ARS: aminoacyl-tRNA synthetase; TIF1- γ : transcriptional intermediary factor 1 γ ; NXP-2: nuclear matrix protein 2; SAE: small ubiquitin-like modifier activating enzyme; SRP: signal recognition particle; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; CK: creatine kinase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate. ^aData available for 53 patients; ^bdata available for 46 patients; ^cdata available for 127 patients; ^ddata available for 128 patients; ^edata available for 124 patients; ^fdata available for 109 patients; ^gavailable for 9 patients; ^havailable for 8 patients; ⁱavailable for 24 patients; ^javailable for 15 patients.

Characteristics	DM patients	IMNM patients
FVC %	84.9 (74.1-104.1) ^a	77.0 (62.8–88.7) ^g
FEV ₁ %	79.9 (76.0-83.6) ^a	80.1 (73.6–83.9) ^g
DLco %	63.1 (50.8–77.9) ^b	74.3 (68.9–91.1) ^h
Laboratory features		
MSA, no. (%)	116 (89.9%)	17 (68%)
Anti-MDA5-positive	56 (43.4%)	0 (0%)
Anti-ARS-positive	24 (18.6%)	0 (0%)
Anti-TIF1-γ-positive	12 (9.3%)	0 (0%)
Anti-NXP-2-positive	11 (8.5%)	0 (0%)
Anti-Mi-2-positive	7 (5.4%)	0 (0%)
Anti-SAE-positive	6 (4.7%)	0 (0%)
Anti-SRP-positive	0 (0%)	10 (40%)
Anti-HMGCR-positive	0 (0%)	7 (28%)
CK (IU/L), median (IQR)	78 (36–214) ^c	1900 (745–5723)
ALT (IU/L), median (IQR)	35 (20–73) ^c	72 (30–223)
AST (IU/L), median (IQR)	27 (17–57) ^c	51 (25–117)
LDH (IU/L), median (IQR)	279 (202–356) ^d	417 (239–698) ⁱ
CRP (mg/dL), median (IQR)	0.42 (0.18-1.00) ^d	0.29 (0.13–0.60) ⁱ
ESR (mm/h), median (IQR)	12.5 (7.0-33.8) ^e	6.5 (3.3–9.8) ⁱ

DM: dermatomyositis; IMNM: immune-mediated necrotizing myopathy; IQR: interquartile range; ILD: interstitial lung disease; RP-ILD: rapidly progressive interstitial lung disease; FVC: forced vital capacity; FEV₁: forced expiratory volume in 1 s; DLco: diffusing capacity of carbon monoxide; MSA: myositis-specific antibody; MDA5: melanoma differentiation-associated gene 5; ARS: aminoacyl-tRNA synthetase; TIF1-γ: transcriptional intermediary factor 1 γ; NXP-2: nuclear matrix protein 2; SAE: small ubiquitin-like modifier activating enzyme; SRP: signal recognition particle; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; CK: creatine kinase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate. ^aData available for 53 patients; ^bdata available for 46 patients; ^cdata available for 127 patients; ^ddata available for 128 patients; ^edata available for 124 patients; ^fdata available for 109 patients; ^gavailable for 9 patients; ^havailable for 8 patients; ⁱavailable for 24 patients; ^javailable for 15 patients.

Characteristics	DM patients	IMNM patients
Ferritin (ng/mL), median (IQR)	226.9 (89.5-696.3) ^f	109.9 (41.2-168.8) ^j

DM: dermatomyositis; IMNM: immune-mediated necrotizing myopathy; IQR: interquartile range; ILD: interstitial lung disease; RP-ILD: rapidly progressive interstitial lung disease; FVC: forced vital capacity; FEV₁: forced expiratory volume in 1 s; DLco: diffusing capacity of carbon monoxide; MSA: myositis-specific antibody; MDA5: melanoma differentiation-associated gene 5; ARS: aminoacyl-tRNA synthetase; TIF1- γ : transcriptional intermediary factor 1 γ ; NXP-2: nuclear matrix protein 2; SAE: small ubiquitin-like modifier activating enzyme; SRP: signal recognition particle; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; CK: creatine kinase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate. ^aData available for 53 patients; ^bdata available for 46 patients; ^cdata available for 127 patients; ^ddata available for 128 patients; ^edata available for 124 patients; ^fdata available for 109 patients; ^gavailable for 9 patients; ^havailable for 8 patients; ⁱavailable for 24 patients; ^javailable for 15 patients.

Increased serum Gal-9 levels in patients with IIM, particularly in anti-MDA5-positive patients with DM

Serum Gal-9 levels were significantly higher in patients with IIM than in HCs (19.8 (10.0–33.6) vs 4.9 (3.5–6.3) ng/mL, $p < 0.001$). Additionally, serum Gal-9 levels in patients with DM were more than 3-fold higher than in patients with IMNM (23.7 (12.3–35.9) vs 7.4 (5.2–10.8) ng/mL, $p < 0.001$) (Fig. 1a). Furthermore, after dividing the patients with DM by MSAs, significantly higher serum levels of Gal-9 were observed in the anti-MDA5-positive group than in the anti-MDA5-negative group (33.8 (21.9–44.7) vs 16.2 (10.0–26.9) ng/mL, $p < 0.001$) (Fig. 1b). However, the Kruskal–Wallis H -test ($p = 0.494$) revealed no significant difference between the patients with other MSAs aside from anti-MDA5-antibodies (Fig. 1c).

Significant association of serum Gal-9 levels with RP-ILD and disease activity in anti-MDA5-positive patients with DM

We analyzed the association between serum levels of Gal-9 and RP-ILD in anti-MDA5-positive patients with DM. A significant difference was found in serum levels of Gal-9 between patients with RP-ILD and those with non-RP-ILD (42.4 (34.9–68.5) vs 26.4 (17.0–39.8) ng/mL, $p < 0.001$) (Fig. 2a). Furthermore, the association between the serum levels of Gal-9 and pulmonary function was analyzed in 23 anti-MDA5-positive DM patients with pulmonary function tests. We observed a negative correlation between serum levels of Gal-9 and FVC% ($r = -0.575$, $p = 0.004$) (Fig. 2b). Serum levels of Gal-9 did not correlate with FEV₁% ($p = 0.668$) or DL_{CO}% ($p = 0.249$) (Fig. 2c, d).

To determine the relationship between serum Gal-9 levels and disease activity, a cross-sectional study of 56 anti-MDA5-positive patients with DM was conducted. Significant positive correlations were observed between serum Gal-9 levels and PGA VAS scores ($r = 0.635$, $p < 0.001$) (Fig. 2e) and pulmonary VAS scores ($r = 0.503$, $p < 0.001$) (Fig. 2f). Serum Gal-9 levels were also correlated with muscle VAS scores ($r = 0.462$, $p < 0.001$), cardiac VAS scores ($r = 0.314$, $p = 0.019$), joint VAS scores ($r = 0.266$, $p = 0.047$), and constitutional VAS scores ($r = 0.380$, $p = 0.004$) (Supplementary Fig. 1a–d). However, no correlation was

observed between the serum levels of Gal-9 and cutaneous VAS scores ($p = 0.123$) or gastrointestinal VAS scores ($p = 0.084$) (Supplementary Fig. 1e, f).

Additionally, a longitudinal study was conducted in 21 anti-MDA5-positive patients with DM to further explore the association between serum Gal-9 levels and disease activity. We collected 89 serum samples and their corresponding clinical data. The serum levels of Gal-9 were significantly correlated with PGA VAS scores using the generalized estimating equation model ($\beta = 0.041$, $p < 0.001$) (Fig. 2g).

PBMCs isolated from patients with DM produced high Gal-9 levels and Gal-9 mRNA levels correlated with type-I IFN-inducible gene expression

To determine whether PBMCs produce Gal-9 protein, we isolated PBMCs from 7 anti-MDA5-positive patients with DM, 3 patients with IMNM, and 8 HCs. After culturing the PBMCs for 48 h, the levels of Gal-9 in the supernatant were measured by ELISA. As shown in Fig. 3a, the levels of Gal-9 in the DM group (3.7 ± 2.3 ng/mL) were higher than those in the IMNM group (1.1 ± 0.3 ng/mL, $p = 0.022$) and HC group (1.4 ± 1.2 ng/mL, $p = 0.045$).

To explore the relation between Gal-9 and type-I IFN, the mRNA levels of Gal-9 and two type-I IFN-inducible genes (MX1 and IFIH1) from PBMCs were measured. PBMCs were isolated from 12 anti-MDA5-positive patients with DM, 7 patients with IMNM, and 6 HCs. The MX1 mRNA levels in patients with DM were higher than those in patients with IMNM ($p = 0.004$) and HCs ($p = 0.008$) (Fig. 3b). Similarly, IFIH1 mRNA levels were higher in patients with DM than in patients with IMNM ($p = 0.001$) and HCs ($p = 0.006$) (Fig. 3c). Interestingly, Gal-9 mRNA expression was correlated with the mRNA levels of type-I IFN-inducible genes MX1 ($r = 0.659$, $p = 0.020$) and IFIH1 ($r = 0.787$, $p = 0.002$) (Fig. 3d, e) in patients with DM.

Enhanced Gal-9 and Tim-3 expression in the lung tissue of patients with DM-ILD

Immunohistochemistry revealed that Gal-9 expression was more substantial in patients with non-RP-ILD (Fig. 4g, h) and RP-ILD (Fig. 4m, n) than those with no ILD (Fig. 4a, b). Furthermore, the expression of Tim-3 was elevated in patients with non-RP-ILD (Fig. 4i, j) and RP-ILD (Fig. 4o, p) compared to those with no ILD (Fig. 4c, d), whereas similar CD44 expression levels were observed in patients with no ILD (Fig. 4e, f), non-RP-ILD (Fig. 4k, l), and RP-ILD (Fig. 4q, r).

Fibroblast expressed increased levels of inflammatory cytokines following stimulation of Gal-9 in vitro

To identify the potential roles of Gal-9 in the pathogenesis of DM-ILD, MRC-5 human fibroblasts were stimulated with Gal-9 *in vitro*. We found that MRC-5 fibroblasts stimulated with Gal-9 expressed higher CCL2 ($p = 0.024$) mRNA levels (Fig. 5a) compared to control fibroblasts, but no differences were observed in IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, TNF- α , IFN- γ , CCL-18, CXCL4, and CXCL10 (all $p > 0.05$, data not shown). Additionally, the effect of Gal-9 on the proliferation of MRC-5 fibroblasts was explored. Cell viability analysis showed no differences between the control group and Gal-9-treated groups in MRC-5 fibroblasts stimulated for 24 h (all $p > 0.05$, Fig. 5b) or 48 h (all $p > 0.05$, data not shown). Massive deposition of the extracellular matrix caused fibrosis, and α -SMA protein expression was the primary

moderator of fibrosis. TGF- β , a central mediator of fibrosis, has been reported to induce the α -SMA expression. In our study, although TGF- β promoted the expression of α -SMA protein in MRC-5 fibroblasts ($p = 0.046$), Gal-9 did not ($p = 0.189$) (Fig. 5c). Overall, these results indicate that MRC-5 fibroblasts expressed increased levels of CCL2 following Gal-9 stimulation. In addition, Gal-9 did not promote proliferation and showed no pro-fibrotic effect on MRC-5 fibroblasts.

Discussion

This study demonstrated that the serum levels of Gal-9 were significantly increased in patients with IIM, particularly in anti-MDA5-positive patients with DM. Among patients carrying anti-MDA5 antibodies, Gal-9 expression was increased in both the sera and lung tissues of patients with RP-ILD. In addition, a significant correlation was observed between the serum levels of Gal-9 and disease activity in anti-MDA5 positive patients with DM. High serum Gal-9 levels in patients with DM may, at least in part, be derived from PBMCs and were correlated with type-I IFN-inducible gene expression. Furthermore, Gal-9 modulated the production of CCL2 mRNA in MRC-5 fibroblasts *in vitro*.

Elevated serum Gal-9 levels have been detected in various autoimmune disease [31–33]. Consistent with the previous studies in patients with juvenile DM [25, 26], our study demonstrated that adult patients with DM exhibited increased serum Gal-9 levels compared to those in HCs. Additionally, the serum levels of Gal-9 were higher in the DM group than in the IMNM group. Serum Gal-9 levels were analyzed by subgrouping patients based on MSAs. Interestingly, anti-MDA5-positive patients with DM exhibited high serum Gal-9 levels. These results suggest that Gal-9 is involved in DM pathogenesis.

As a complex and fatal complication, RP-ILD is important in the clinical treatment of patients with DM, and anti-MDA5 antibodies were demonstrated to be closely linked to RP-ILD [34, 35]. In our study, the serum levels of Gal-9 in the RP-ILD group were higher than those in the non-RP-ILD group in anti-MDA5-positive patients with DM. We also identified an association between serum Gal-9 levels and pulmonary function impairments in patients with RP-ILD. This suggests that Gal-9 is associated with RP-ILD. Furthermore, in anti-MDA5-positive patients with DM, the serum levels of Gal-9 were linked to disease activity in both cross-sectional and longitudinal studies. Recently, Wienke et al. [26] demonstrated that serum levels of Gal-9 can be used to distinguish active disease and remission in patients with DM. Thus, the association between serum Gal-9 and clinical features suggests that Gal-9 is an easily detected biomarker of RP-ILD severity. Furthermore, implementation of serum Gal-9 level analysis into clinical practice may improve the diagnosis and evaluation of disease activity of anti-MDA5-positive patients with DM.

Gal-9 was shown to express in many immune cells and tissue cells [36, 37]. We demonstrated that PBMCs isolated from patients with DM produced high levels of Gal-9, suggesting that PBMCs are among the sources of serum Gal-9 in patients with DM. IFNs are a large family of cytokines that participate in antiviral response and regulate innate and adaptive immunity, and type-I IFNs were shown to be significantly increased in patients with DM [38]. Zhang et al. [39] demonstrated that PBMCs from anti-

MDA5-positive patients exhibited significantly upregulated IFN-inducible genes. Similarly, in our study, the type-I IFN-inducible genes MX1 and IFIH1 were enhanced in PBMCs from anti-MDA5-positive patients with DM compared to patients with IMNM and HCs. Additionally, among anti-MDA5-positive patients with DM, we observed positive correlations between Gal-9 mRNA and IFN-inducible genes. Furthermore, previous studies showed inhibition of Gal-9 following TLR7- and TLR9-mediated activation of plasmacytoid dendritic cells and B cells in murine lupus models. Gal-9 also inhibited the expression of IFN- α , TNF- α , and IL-6 [40]. Therefore, Gal-9 may be involved in regulating type-I IFN levels in patients with DM.

Furthermore, we investigated Gal-9 expression in the lung tissues of patients with ILD and contribution of Gal-9 to the immunopathogenesis of DM-ILD. Gal-9 expression was upregulated in the lung tissues of RP-ILD. Similarly, a recent study used immunohistochemistry to show that Gal-3 expression is more obvious in patients with DM-ILD than in HCs [41]. Additionally, we confirmed that Tim-3, a Gal-9 ligand, was upregulated in the lung tissues of patients with RP-ILD. A previous study indicated that alveolar macrophage Tim-3 expression was elevated in patients with idiopathic pulmonary fibrosis [19]. These results suggest that the interaction of Gal-9 with Tim-3 is a driving force in the pathogenesis of RP-ILD. Particularly, we showed that Gal-9 upregulates CCL2 mRNA levels in MRC-5 fibroblasts *in vitro*. CCL2, a member of the CC chemokine subfamily, was shown to be a potent chemotactic factor for monocytes and macrophages [42]. As the major mediator of the initiation and development of inflammatory response, CCL2 was previously associated with pulmonary inflammation [43, 44]. Moreover, previous studies have reported the importance of macrophage activation in the pathophysiology of DM-ILD [45]. In conclusion, our results suggest that Gal-9 upregulated the expression level of CCL2, leading to macrophage activation and accelerated inflammation, thus influencing the pathology of DM-ILD. Gal-9 secretion and macrophage activation led to persistent inflammation. It may suggest that Gal-9 is not only a potential biomarker, but also plays a role in the pathogenesis of DM-ILD.

There were some limitations to this study. First, this was a retrospective study and the number of patients was relatively small, particularly in the follow-up study. Second, although PBMCs represent an important source of Gal-9, the source of serum Gal-9 is not completely understood.

Conclusions

In conclusion, we described that serum Gal-9 levels were associated with RP-ILD and disease activity in anti-MDA5-positive patients with DM, and Gal-9 upregulated CCL2 mRNA levels in MRC-5 fibroblasts *in vitro*. Finally, Gal-9 may not only represent a new biomarker but also participate in the immunopathogenesis of DM-ILD in patients with DM carrying anti-MDA5 antibodies.

Abbreviations

DMs, dermatomyositis; IIMs, idiopathic inflammatory myopathies; MSAs, myositis-specific autoantibodies; MDA5, melanoma differentiation-associated gene 5; ILD, interstitial lung disease; RP-ILD,

rapidly progressive ILD; Th, T helper; IFN, interferon; Gal-9, galectin-9; Tim, T-cell immunoglobulin mucin; IMNM, immune-mediated necrotizing myopathy; HCs, healthy controls; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; DLco, diffusing capacity of carbon monoxide; VAS, visual analog scales; PGA, physician's global assessment; ELISA, enzyme-linked immunosorbent assay; PBMCs, peripheral blood mononuclear cells; TGF- β , transforming growth factor- β ; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CCL2, monocyte chemoattractant protein-1; IL, interleukin; TNF- α , tumor necrosis factor- α ; CXCL, C-X-C motif chemokine ligand; SMA, smooth muscle actin; IQR, interquartile range.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

LL, QLP and GCW designed the studies. LL, YMZ, APS, WLL and XL participated in ELISA, qRT-PCR, immunohistochemistry and cell culture. LL, YMZ and YWS performed data analysis. LL edited the manuscript. XL, QLP and GCW reviewed the study. All authors read and approved the final manuscript.

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Availability of data and materials

All data are available in the manuscript or upon request to the authors.

Ethics approval and consent to participate

Informed consent was obtained from all participants. The Ethical Review Committee of the China-Japan Friendship Hospital (2019-25-K19) approved this study.

Consent for publication

Not applicable.

Competing Interest

The authors declare that they have no competing interests.

Author details

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Figures

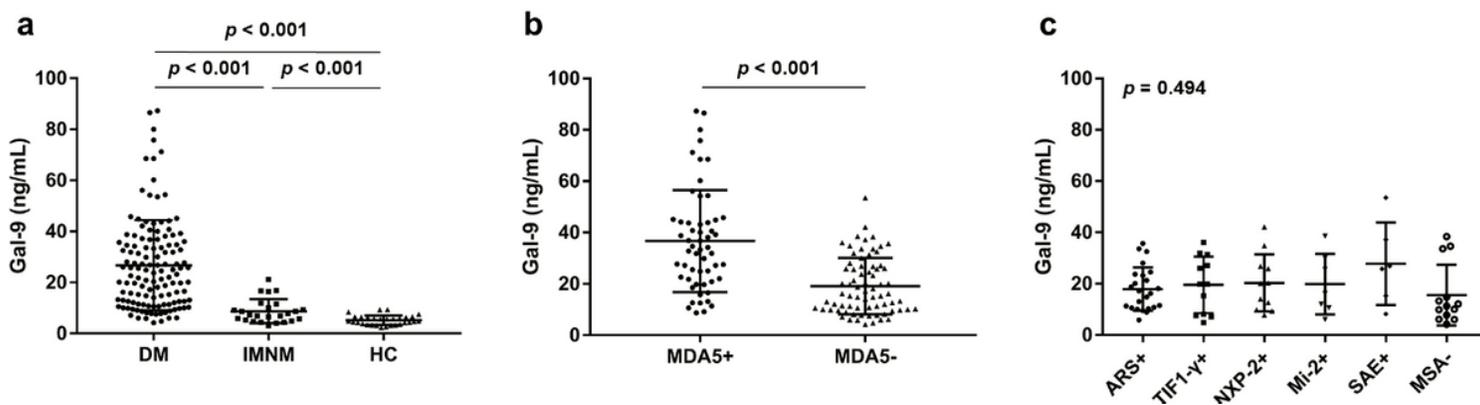


Figure 1

Serum galectin-9 (Gal-9) levels in patients with idiopathic inflammatory myopathies and healthy controls (HCs). a Patients with dermatomyositis (DM), patients with immune-mediated necrotizing myopathy (IMNM), and HCs. b Patients with DM divided into anti-melanoma differentiation-associated gene 5 (MDA5)-positive and anti-MDA5-negative group. c Anti-MDA5-negative patients stratified by myositis-

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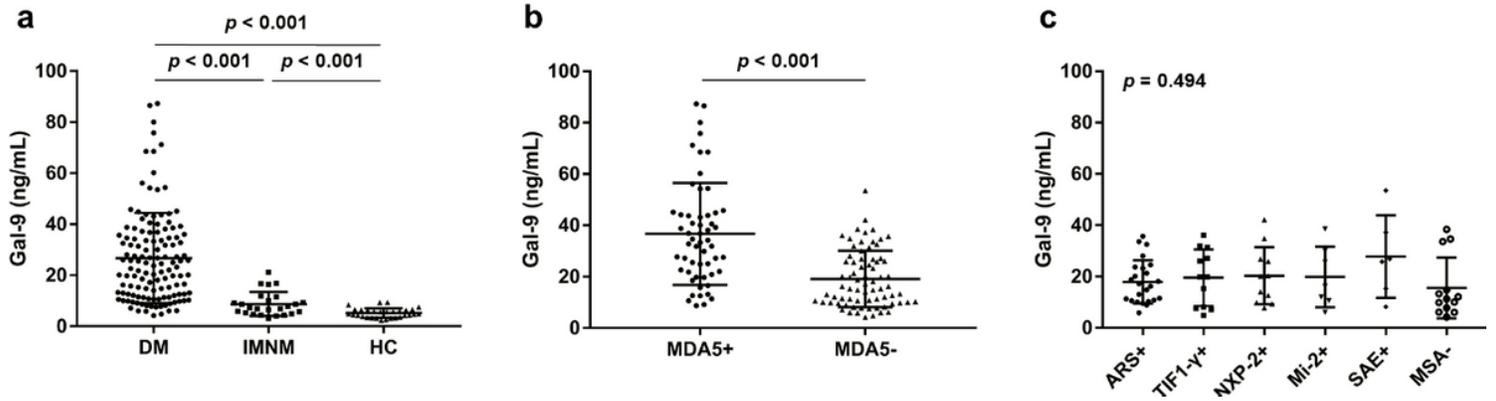


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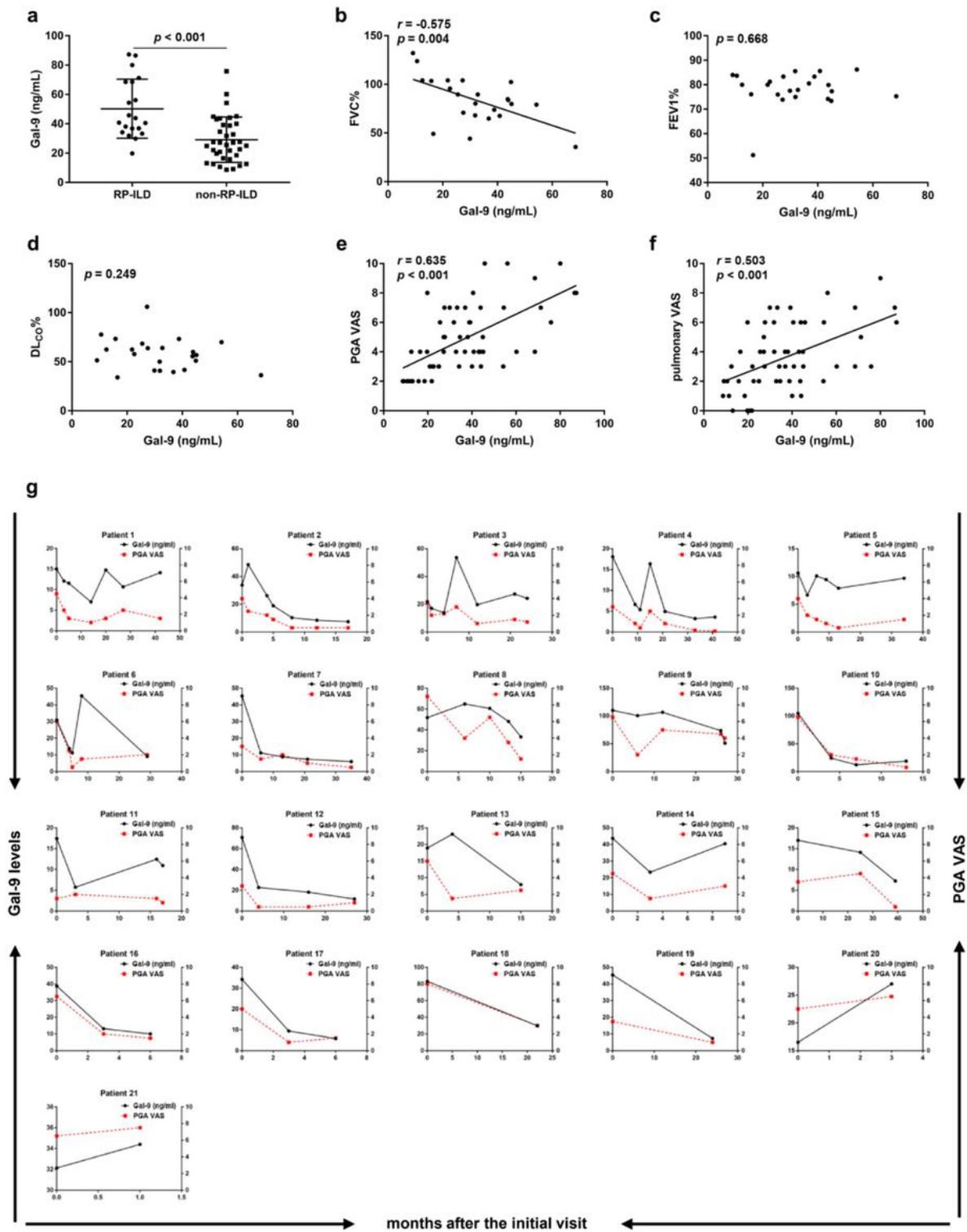


Figure 2

Correlations between serum galactin-9 (Gal-9) levels and clinical features in anti-melanoma differentiation-associated gene 5 (MDA5)-positive patients. a Serum levels of Gal-9 in patients with rapidly progressive interstitial lung disease (RP-ILD) and patients with non-RP-ILD. b-d Correlations between serum Gal-9 levels and pulmonary function impairment parameters. e-f Correlations between serum Gal-9 levels and physician's global assessment (PGA) visual analog scale (VAS) scores (e) and

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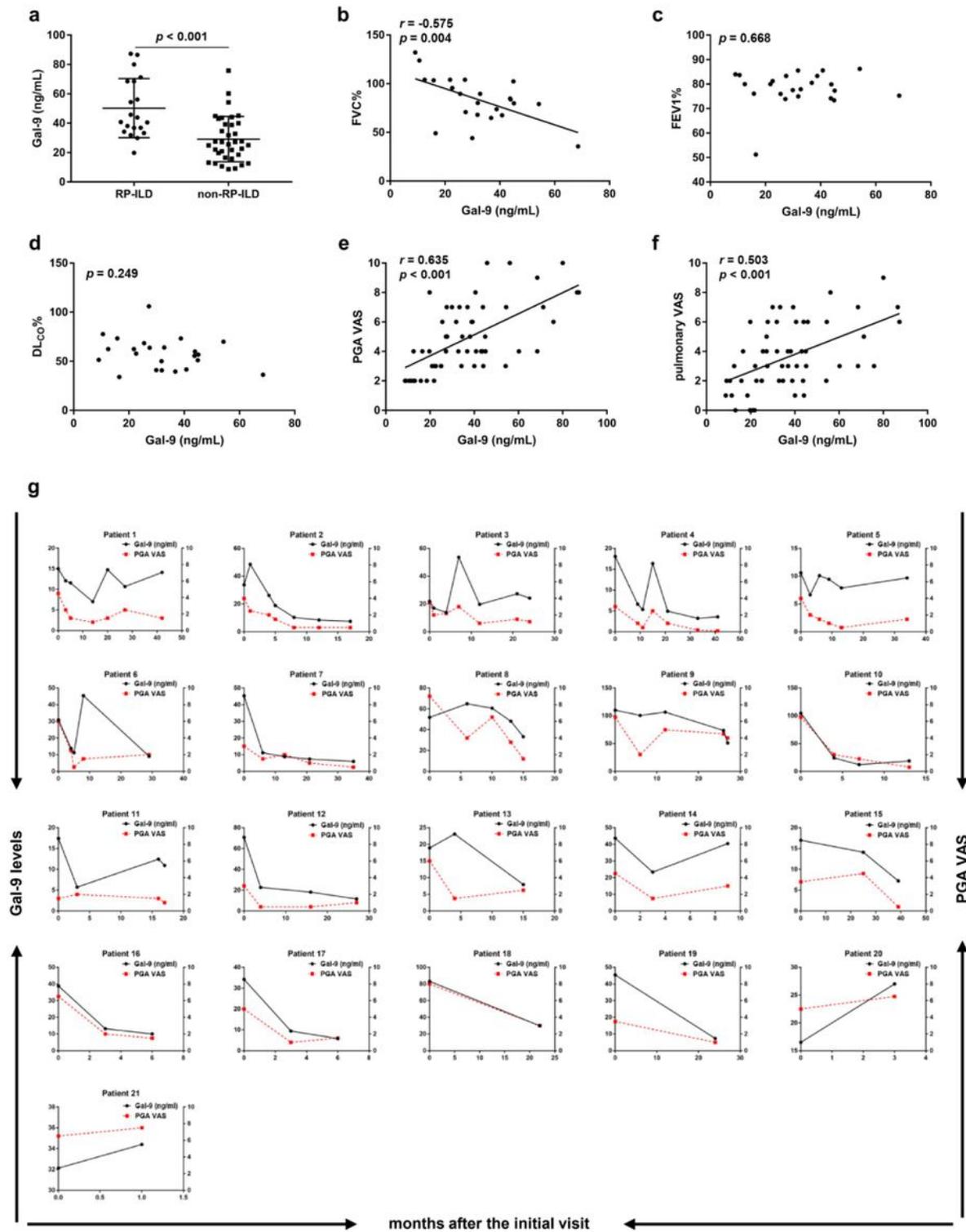


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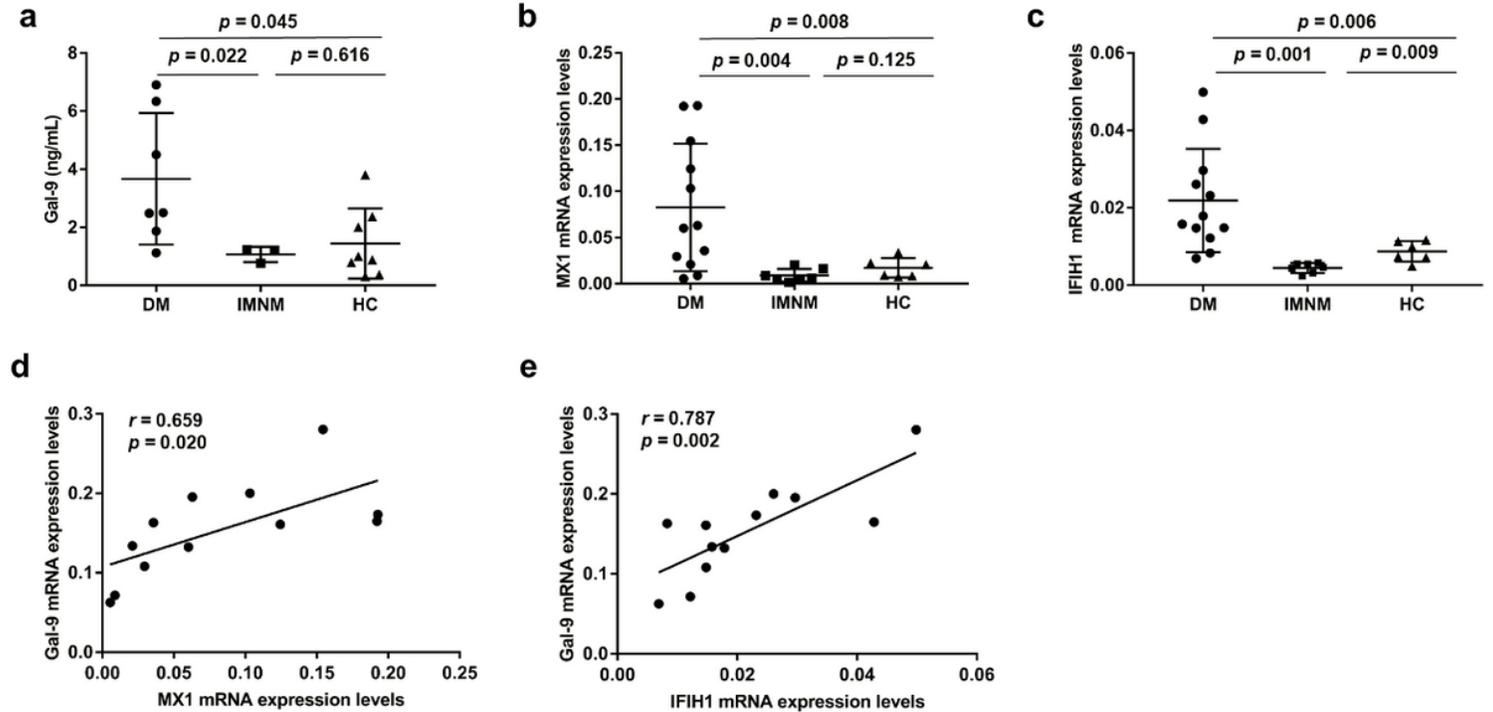


Figure 3

Concentrations of galectin-9 (Gal-9) in peripheral blood mononuclear cells (PBMCs). PBMCs derived from patients with idiopathic inflammatory myopathies and healthy controls (HCs). a Gal-9 levels in PBMCs derived from patients with dermatomyositis (DM), patients with immune-mediated necrotizing myopathy (IMNM), and HCs. b-c mRNA expression of type-I interferon-inducible gene MX1 (b) and IFIH1 (c) in patients with DM, patients with IMNM, and HCs. d-e Correlations between Gal-9 and MX1 (d) and IFIH1 (e) mRNA expression levels in patients with DM.

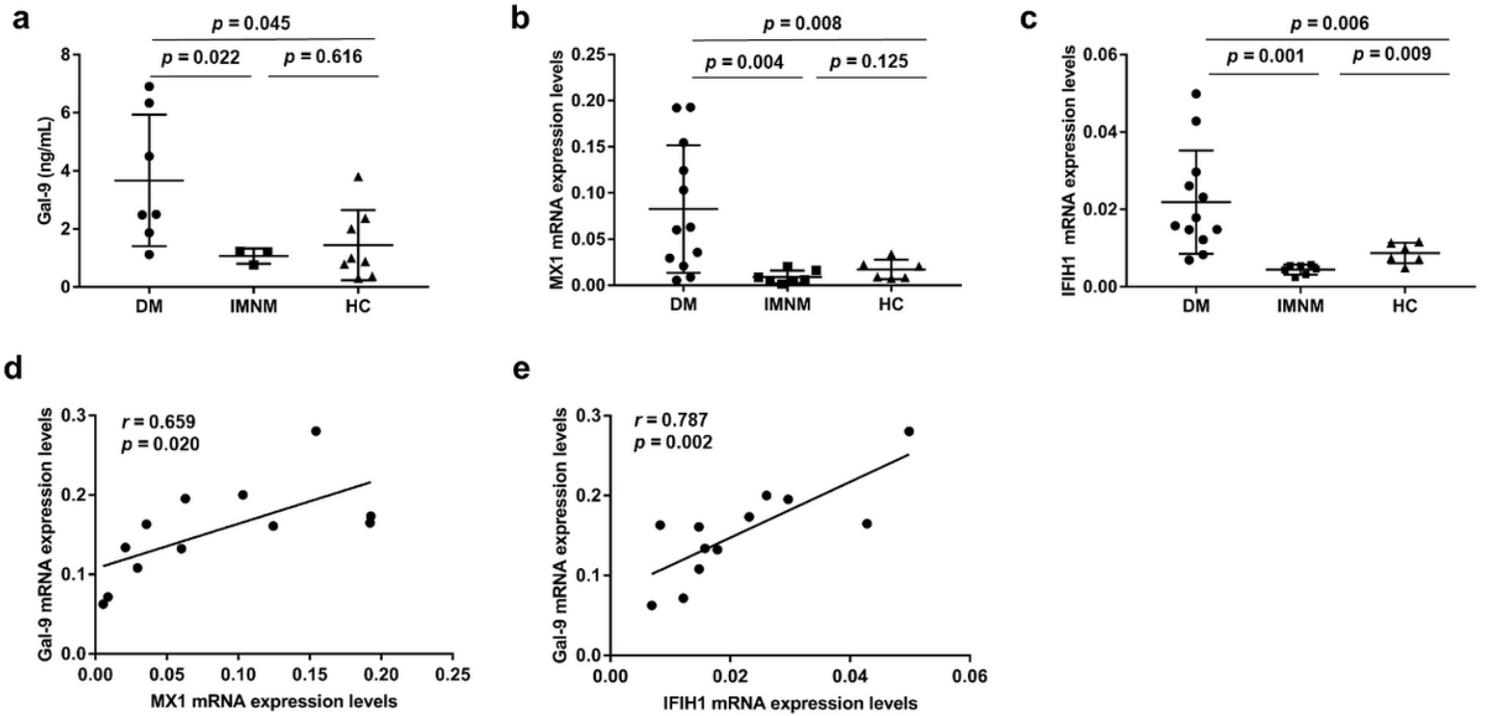


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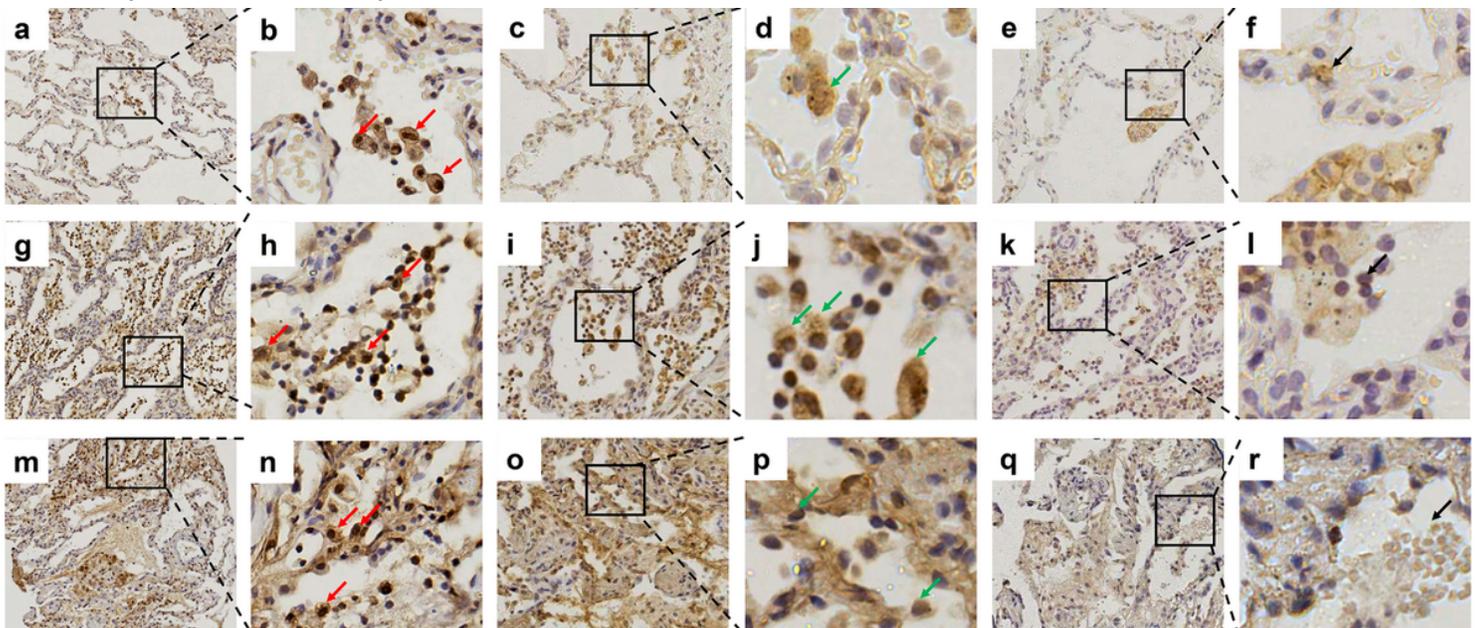


Figure 4

Immunohistochemical staining of galectin-9 (Gal-9) in lung sections. Lung sections from anti-melanoma differentiation-associated gene 5 (MDA5)-positive dermatomyositis (DM) patient with no interstitial lung disease (ILD) (a–f: 39-year-old female patient), surviving anti-MDA5-positive DM patient with non-rapidly progressive (RP)-ILD (g–l: 27-year-old male patient), and non-surviving anti-MDA5-positive DM patient with RP-ILD (m–r: 55-year-old male patient). (a, b, g, h, m, and n) Staining with anti-Gal-9 antibody (red arrowhead); (c, d, i, j, o, and p) Staining with anti-T-cell immunoglobulin mucin-3 antibody (green arrowhead). (e, f, k, l, q, and r) Staining with anti-CD44 antibody (black arrowhead).

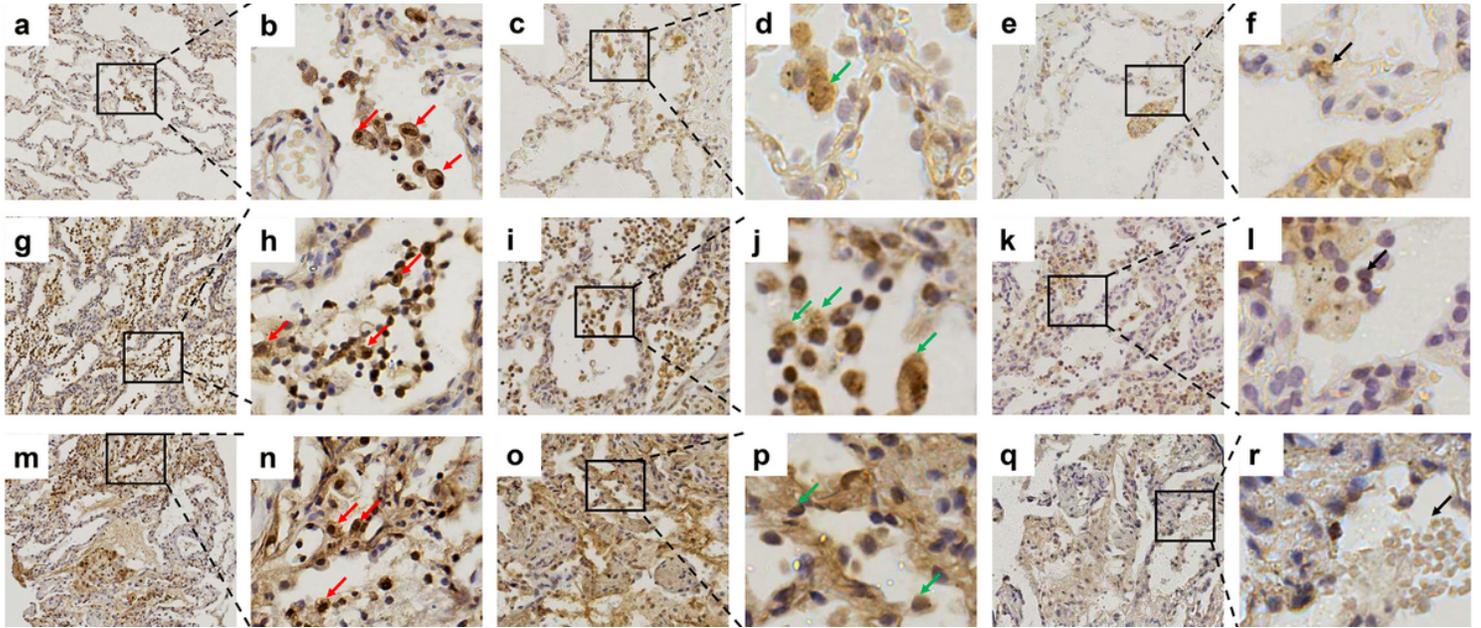


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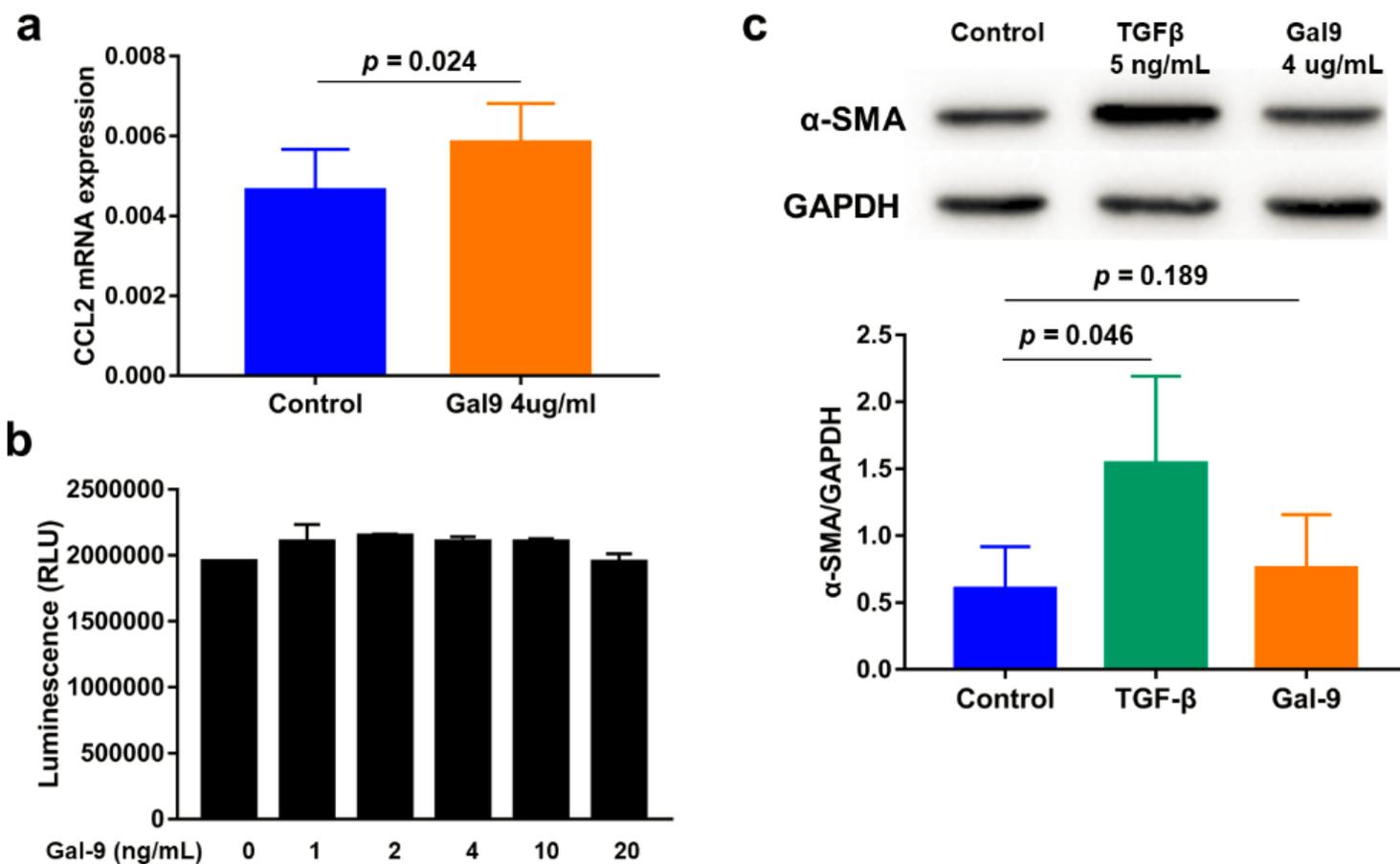


Figure 5

Effects of galectin-9 (Gal-9) on the function of MRC-5 fibroblasts. a monocyte chemoattractant protein-1 (CCL2) mRNA levels in Gal-9-treated MRC-5 fibroblasts compared to the control group. b Proliferation of MRC-5 fibroblasts stimulated with Gal-9 for 24 h. c α - smooth muscle actin (SMA) protein expression of MRC-5 fibroblasts treated with transforming growth factor- β (TGF- β) or Gal-9. The graphs show the mean \pm standard deviation of and each graph determined from at least 3 independent experiments. GAPDH: glyceraldehyde-3-phosphate dehydrogenase

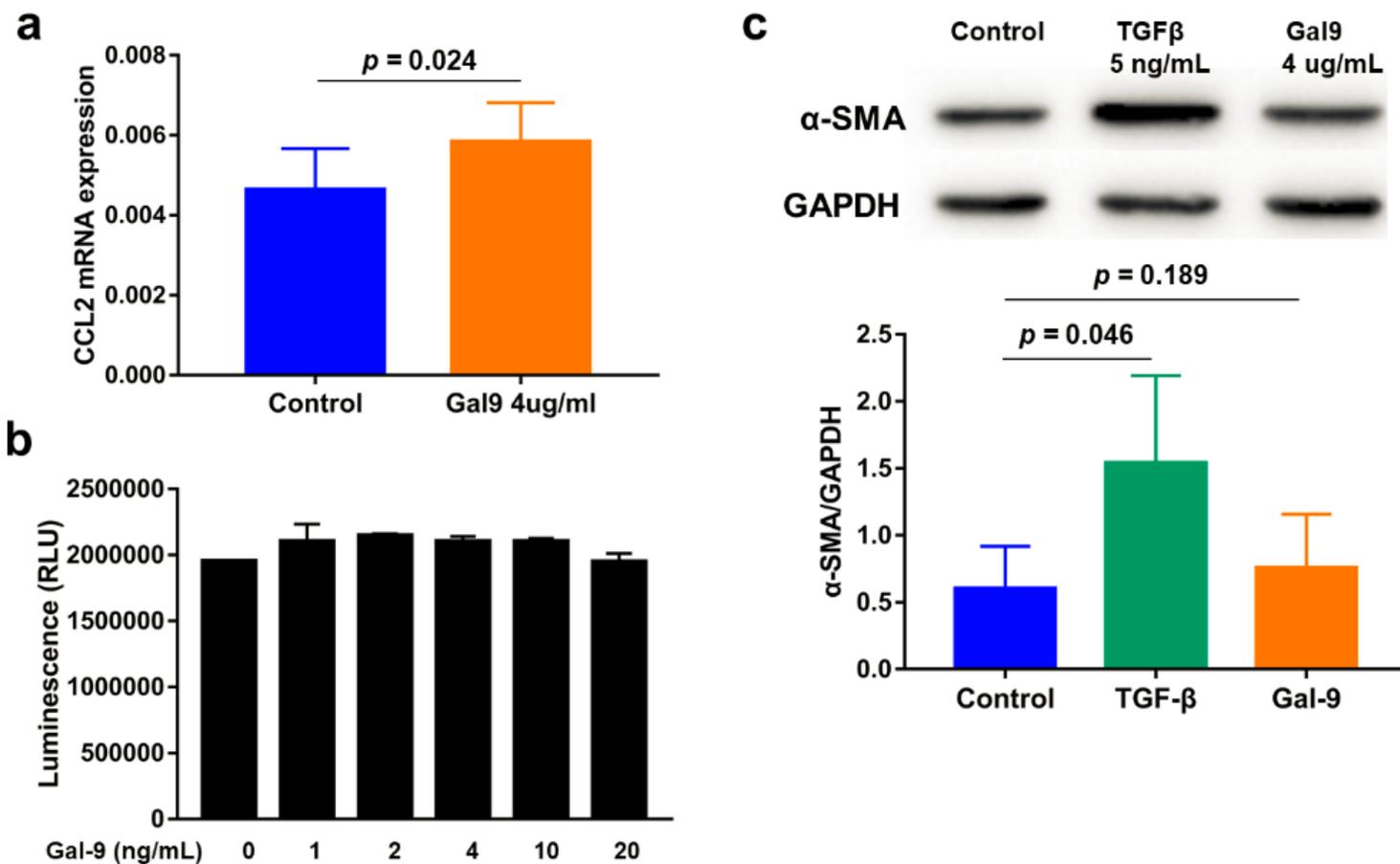


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