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Supplemental Information

TET-dependent GDF7 hypomethylation impairs aqueous humor outflow and serves as a potential therapeutic target in glaucoma

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SUPPLEMENTARY INFORMATION

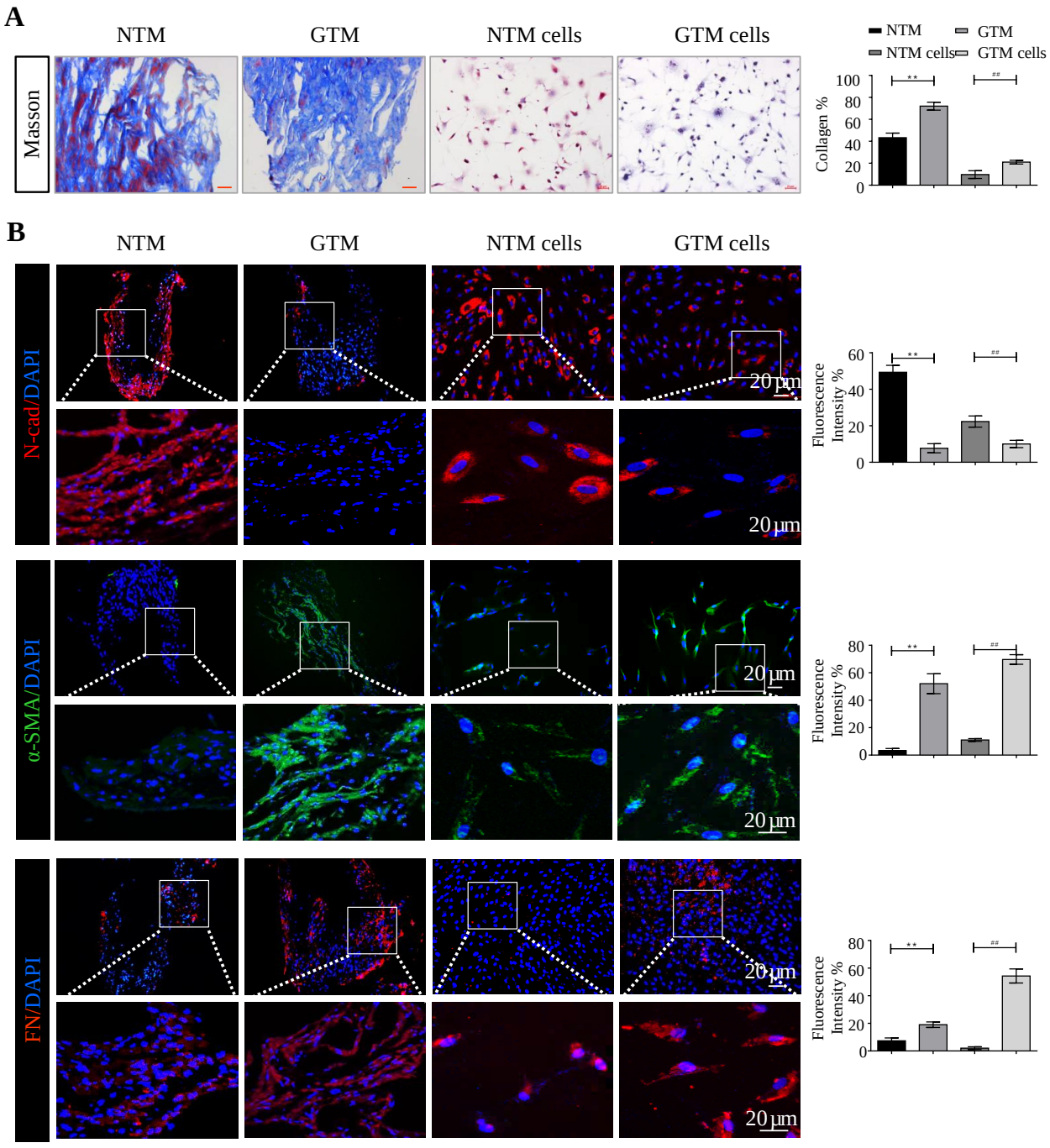

Figure S1. Trabecular meshwork fibrosis in glaucoma

A. Masson staining presented an increase of collagen (blue) in GTM samples and GTM cells compared with the normal ones (n=3 per group).

B. As shown by IF, N-cad expression decreased significantly in GTM samples and

cells. Meanwhile, GTM samples and cells had increased level of pro-fibrotic markers, α-SMA and FN (n=3 per group).

Scale bar=20 μm. The data represent the means ± SD. Compared with NTM samples: **p<0.01. Compared with NTM cells: ##p<0.01. GTM=glaucomatous trabecular meshwork; NTM=normal trabecular meshwork; FN=fibronectin; α-SMA=α-smooth muscle actin; N-cad=N-cadherin.

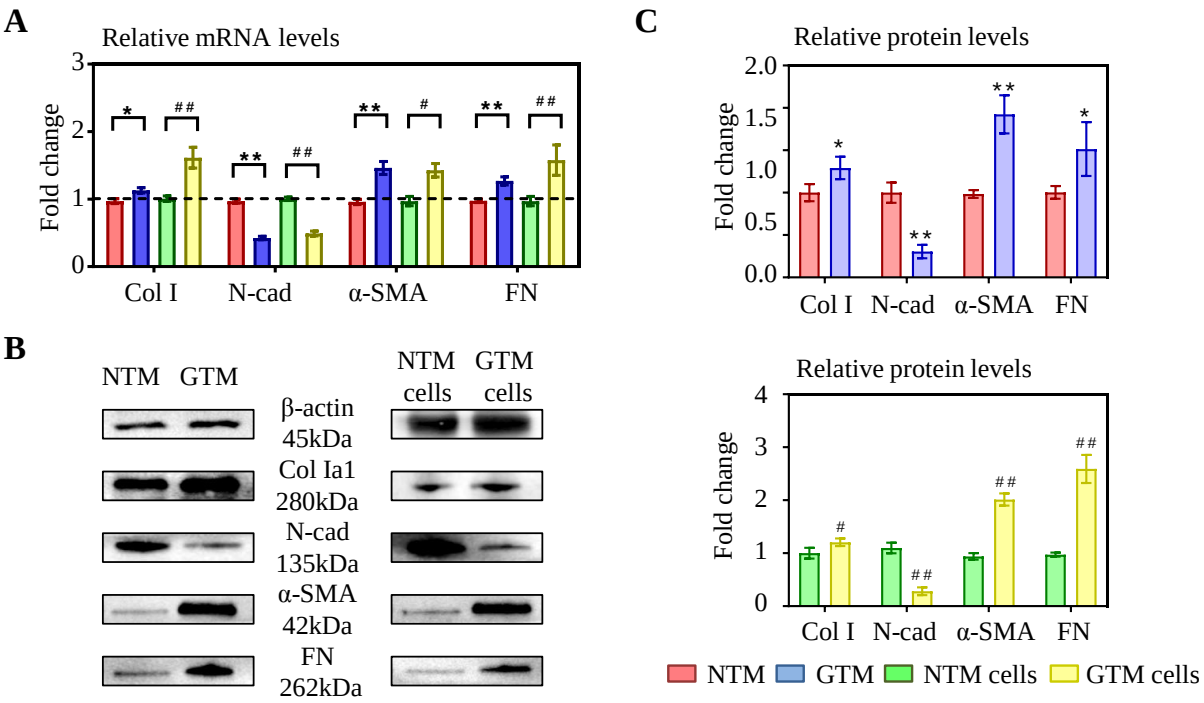

Figure S2. Increase of pro-fibrotic markers in glaucoma

A. The mRNA levels of fibrosis related markers were measured by real-time PCR. N-cad was decreased, whereas Col I, α-SMA, and FN were increased in GTM samples and GTM cells compared with healthy controls (n=3 per group).

B and C. As shown in immunoblot assays, the protein levels of Col I, α-SMA, and FN were significantly up-regulated in GTM samples and GTM cells. And there was a decrease in N-cad in GTM samples and GTM cells compared with the ones from normal controls (n=3 per group).

The data represent the means ± SD. Compared with NTM samples: *p<0.05, **p<0.01. Compared with NTM cells: $^{\#}$p<0.05, $^{\#\#}$p<0.01. GTM=glaucomatous trabecular meshwork; NTM=normal trabecular meshwork; FN=fibronectin; Col I=collagen I; α-SMA=α-smooth muscle actin; N-cad=N-cadherin.

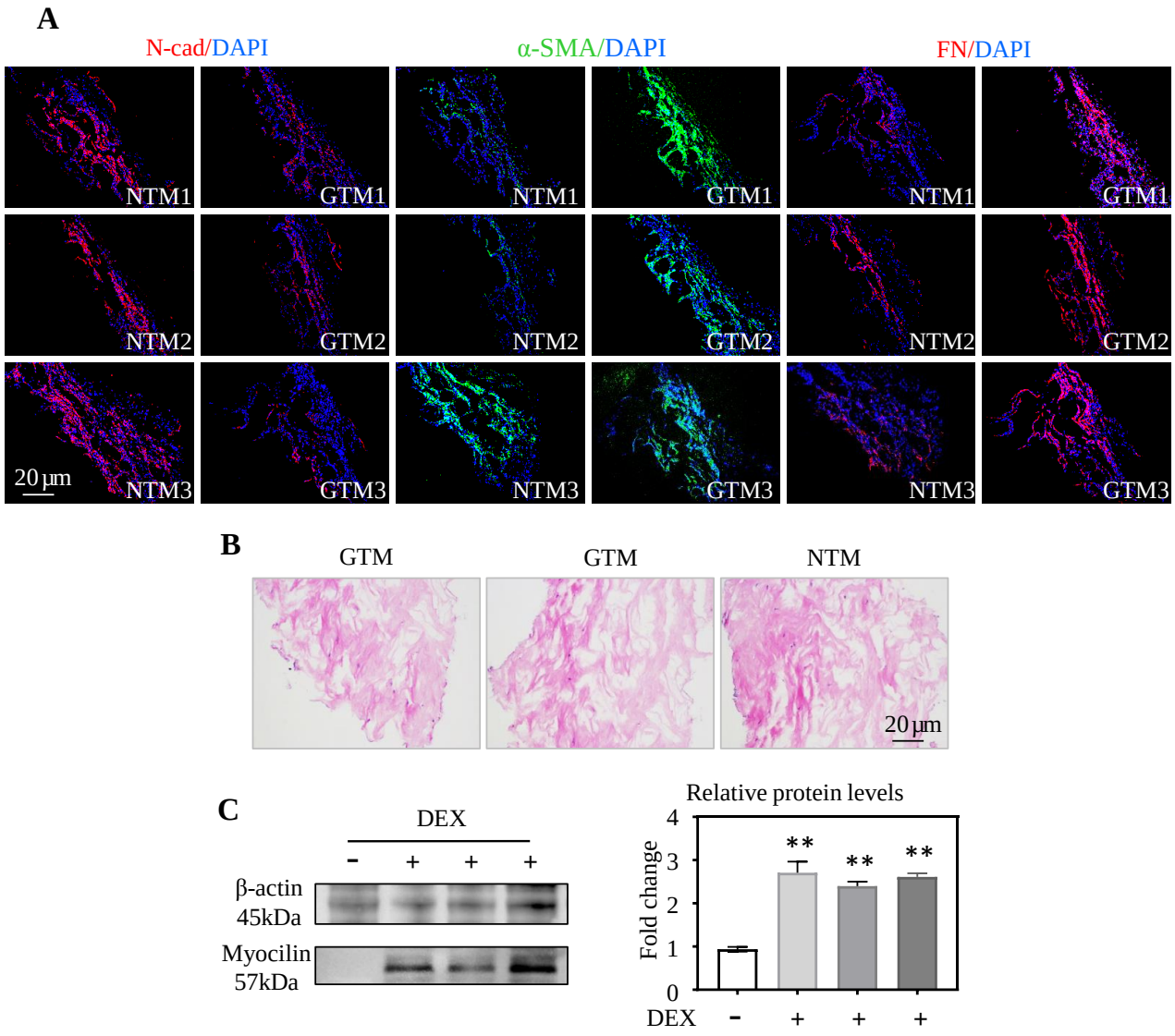

Figure S3. Validation of TM samples and cell strains

A. A gallery of immunofluorescence data presenting the level of N-cad, α-SMA, and FN in the NTM and GTM samples. The expression of α-SMA and FN was greatly increased with decreased N-cad in GTM tissue compared with NTM tissue (n=3 per group).

B. Histology validation of two random GTM samples and one NTM sample. The morphology of GTM samples is comparable to NTM with HE stain.

C. Validation origin of TM cells. After treated with 100nM dexamethasone for 4 days, the myocilin expression in all three NTM cell strains were dramatically increased as

measured by Western blot (n=3 per group).

Scale bar=20 μm. The data represent the means ± SD. Compared with NTM: **p<0.01. NTM=normal trabecular meshwork; GTM=glaucomatous trabecular meshwork; DEX=Dexamethasone; α-SMA=α-smooth muscle actin; N-cad=N-cadherin; FN=fibronectin.

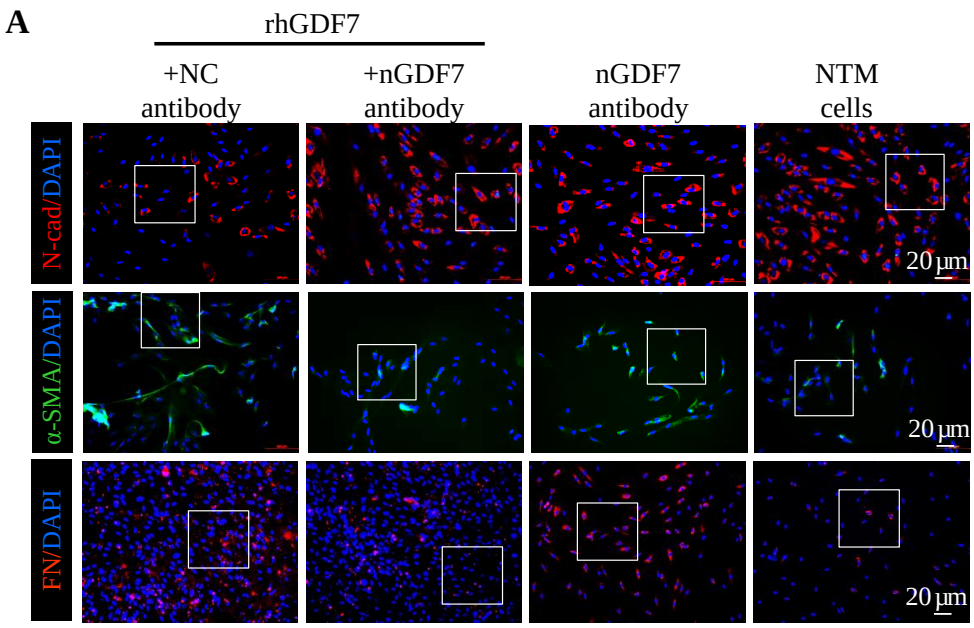

Fig. S4 Recombinant GDF7 protein promoted fibrosis in cultured TM cells

A. NTM cells were treated with 5 ng/ml rhGDF7 for 72 hours. As measured by IF labeling, rhGDF7 significantly decreased N-cad and increased α-SMA and FN expression in TM cells. This rhGDF7-induced fibrotic transition was inhibited by the use of GDF7 neutralizing antibody (n=3 per group).

Scale bar=20 μm. rhGDF7=recombinant human GDF7; NC=normal control; nGDF7=GDF7 neutralizing antibody; NTM=normal trabecular meshwork; FN=fibronectin; Col I=collagen I; α-SMA=α-smooth muscle actin; N-cad=N-cadherin.

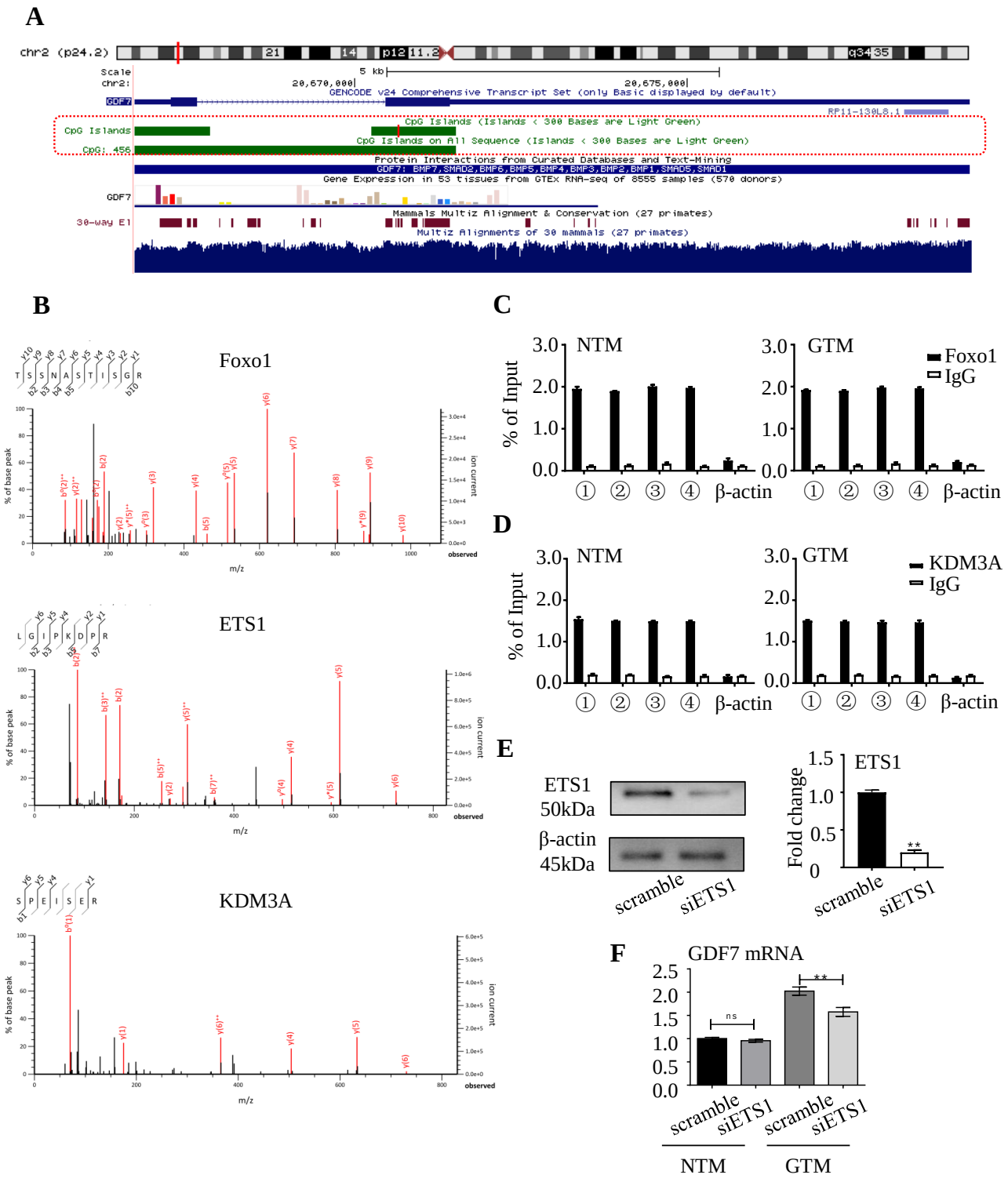

Figure S5. Methylation diagram of GDF7 promoter and associated transcription factors

A. Diagram of GDF7 gene location, transcription site, CpG islands, protein interactions, and conservation between species. The target site was located in CpG

143, as marked by the red tag.

B. Identification of transcription factors binding to GDF7 promoter by reverse-ChIP. The proteins bond to GDF7 promoter were pulled down with reverse-ChIP and analyzed by mass spectra assay. Three candidate transcription factors which might bind to the GDF7 promoter were presented.

C and D. The RNA molecules bond to Foxo1 or KDM3A protein were pulled down with ChIP. Then the pulled down RNA was analyzed by qPCR with the primers targeting different regions of GDF7 promoter. Foxo1 or KDM3A showed comparable binding affinity with glaucomatous and normal GDF7 promoter ($p>0.10$).

E. The knock down efficiency of ETS1 siRNA was confirmed by Western Blot in TM cells.

F. ETS1 knock down had little effect on the expression of GDF7 mRNA in NTM cells ($p>0.10$). But transcription of GDF7 mRNA was decreased in GTM cells compared with scramble (fold change=1.37 ± 0.65, $p<0.01$, n=3 per group).

The data represent the means ± SD. Compared with scramble: **p<0.01.CpG=5'-C-phosphate-G-3'; NTM=normal trabecular meshwork; GTM=glaucomatous trabecular meshwork; siETS1= siRNA of ETS1.

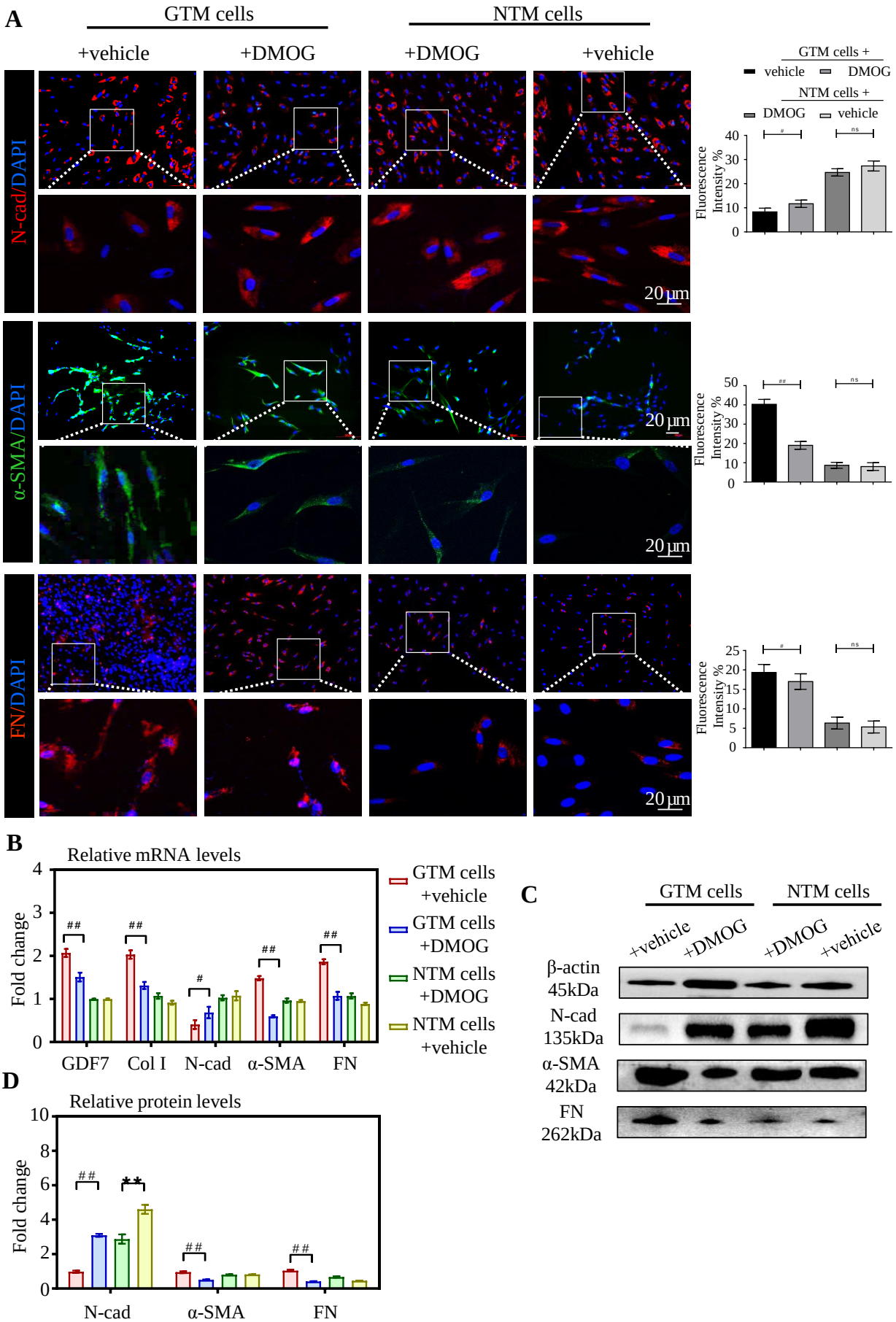
A
GTM cells
NTM cells
+vehicle
+DMOG
+DMOG
+vehicle
N-cad/DAPI
α-SMA/DAPI
FN/DAPI
20 μm
GTM cells +
vehicle
DMOG
NTM cells +
DMOG
vehicle
Fluorescence Intensity %
ns
B
Relative mRNA levels
Fold change
GDF7
Col I
N-cad
α-SMA
FN
GTM cells +vehicle
GTM cells +DMOG
NTM cells +DMOG
NTM cells +vehicle
C
GTM cells
NTM cells
+vehicle
+DMOG
+DMOG
+vehicle
β-actin 45kDa
N-cad 135kDa
α-SMA 42kDa
FN 262kDa
D
Relative protein levels
Fold change
N-cad
α-SMA
FN

Figure S6. DMOG prevented TM fibrosis

A. In GTM cells, the expression of α-SMA and FN was up-regulated with decreased LN as determined by immunofluorescence. These disturbances in fibrosis related markers were prevented by DMOG with decreased α-SMA and FN in GTM cells. DMOG treatment exerted no significant effect in NTM cells regarding the expression of α-SMA, FN, and N-cad ($p>0.05$, n=3 per group).

B. As measured by real-time PCR, DMOG inhibited the increase of GDF7 in GTM cells. Also, the mRNA levels of Col I, α-SMA and FN were decreased in response to DMOG in GTM cells. And the expression of N-cad was rescued by DMOG compared with vehicle (n=3 per group).

C and D. As measured by immunoblot, DMOG suppressed the increase of α-SMA and FN proteins in GTM cells. And glaucoma induced decrease in N-cad was inhibited by DMOG. The expression levels of these fibrosis related genes remained no change in NTM cells in response to DMOG (n=3 per group).

Scale bar=20 μm. The data represent the means ± SD. Compared with NTM cells: *$p<0.05$, **$p<0.01$. Compared with GTM cells: #$p<0.05$, ##$p<0.01$. DMOG=dimethyloxallyl glycine; FN=fibronectin; Col I=collagen I; α-SMA=α-smooth muscle actin; N-cad=N-cadherin.

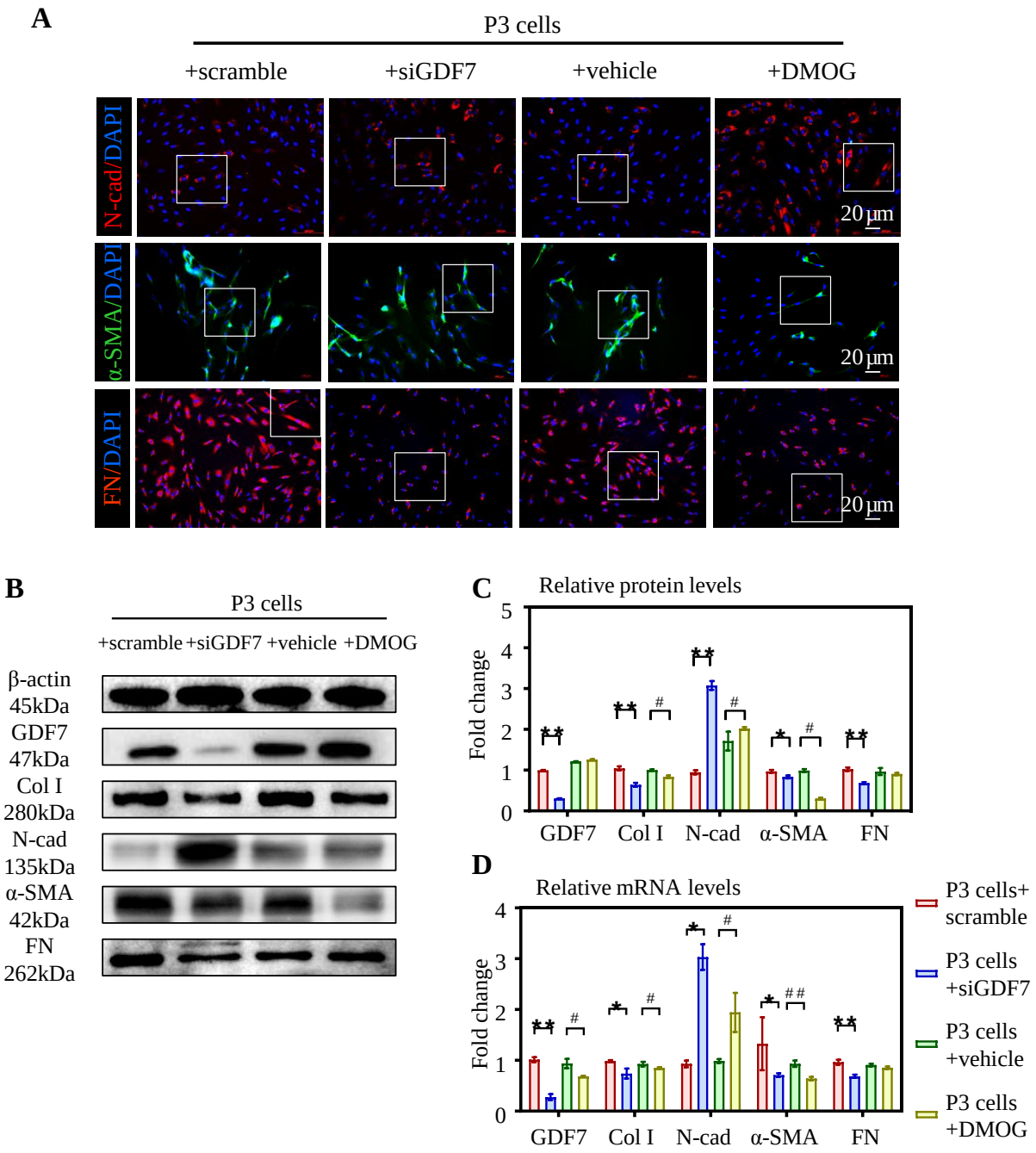

Figure S7. DMOG blocked GDF7 positive feedback loop and TM fibrosis

A-C. The knock down efficiency of siGDF7 was confirmed by Western blot and real-time PCR. In P3 cells, siGDF7 effectively inhibited the increase of α-SMA and FN, and regained expression of N-cad. Also, DMOG treatment blocked the GDF7-induced pro-fibrotic effects by up-regulating N-cad and down-regulating α-SMA and FN in P3 cells (n=3 per group).

Scale bar=20 μm. The data represent the means ± SD. Compared with scramble: *p<0.05, **p<0.01. Compared with vehicle: #p<0.05, ##p<0.01. siGDF7=siRNA of GDF7; DMOG= dimethyloxallyl glycine; FN=fibronectin; Col I=collagen I; α-SMA=α-smooth muscle actin; N-cad=N-cadherin.

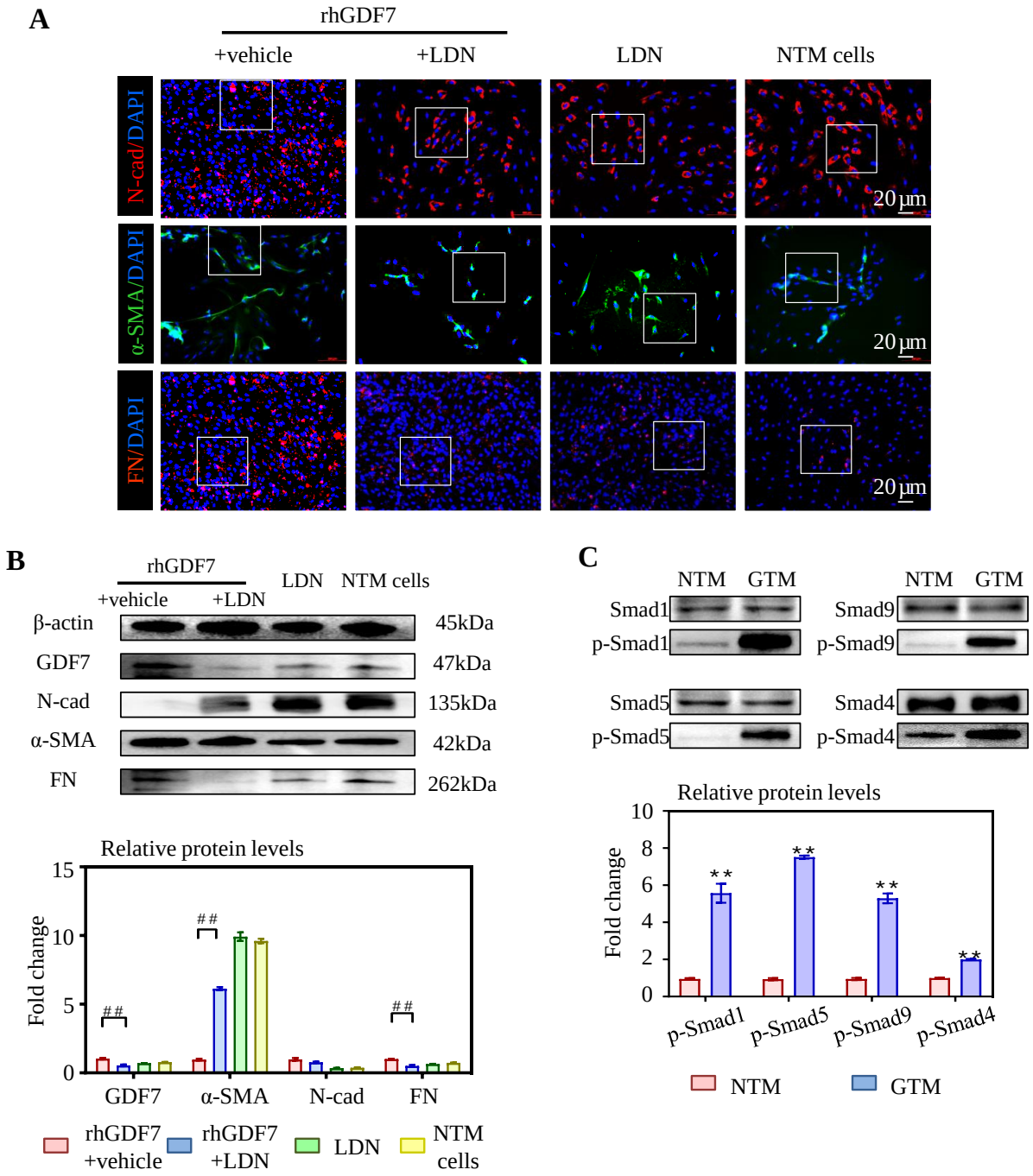

Figure S8. GDF7 promoted TM fibrosis via BMPR2/Smad signaling

A. LDN inhibited increase of α-SMA and FN in rhGDF7 treated cells. And the loss of N-cad was prevented by LDN. LDN treatment alone didn't affect the level of these fibrosis-related proteins in TM cells as labeled by IF (n=3 per group).

B. The rhGDF7-induced increase in the fibrotic markers, α-SMA and FN, was

significantly inhibited by LDN. The protein level of the N-cad was increased in response to LDN in rhGDF7 treated cells. LDN treatment alone didn't affect the expression of GDF7, α-SMA, FN, or N-Cad in NTM cells (n=3 per group).

C. As measured by the immunoblot assay, the ratio between phosphorylated Smad1, 5, 9 and Smad4 and their total protein level were dramatically increased in GTM samples compared with healthy controls (n=3 per group).

Scale bar=20 μm. The data represent the means ± SD. Compared with NTM: **p<0.01. Compared with rhGDF7 treated cells: #p<0.05, ##p<0.01. NTM=normal trabecular meshwork; GTM=glaucomatous trabecular meshwork; rhDF7=recombinant human GDF7; FN=fibronectin; α-SMA=α-smooth muscle actin; N-cad=N-cadherin.

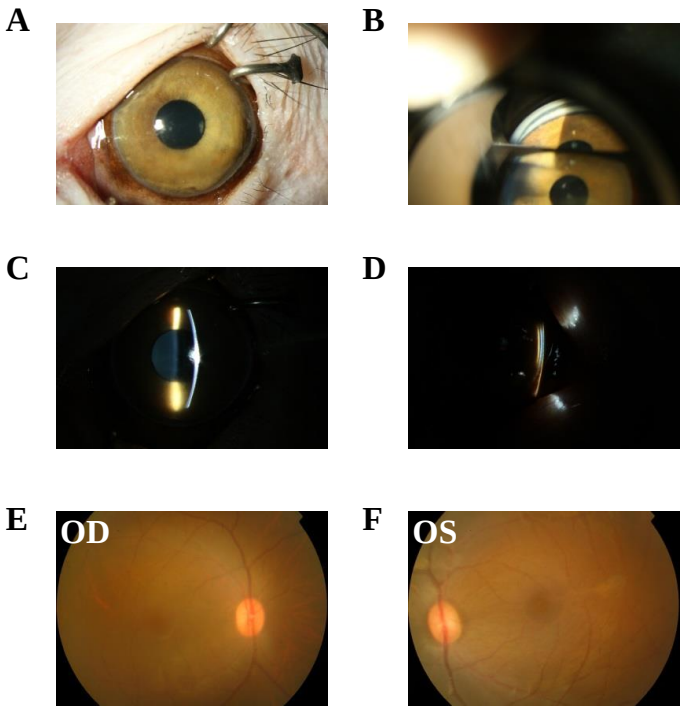

Figure S9. Baseline ocular manifestations in rhesus monkeys

A. Anterior segment.

B. Anterior chamber angle.

C. Central anterior chamber.

D. Peripheral anterior chamber.

E and F. Normal fundus appearance of both eyes.

OD=right eye; OS=left eye.

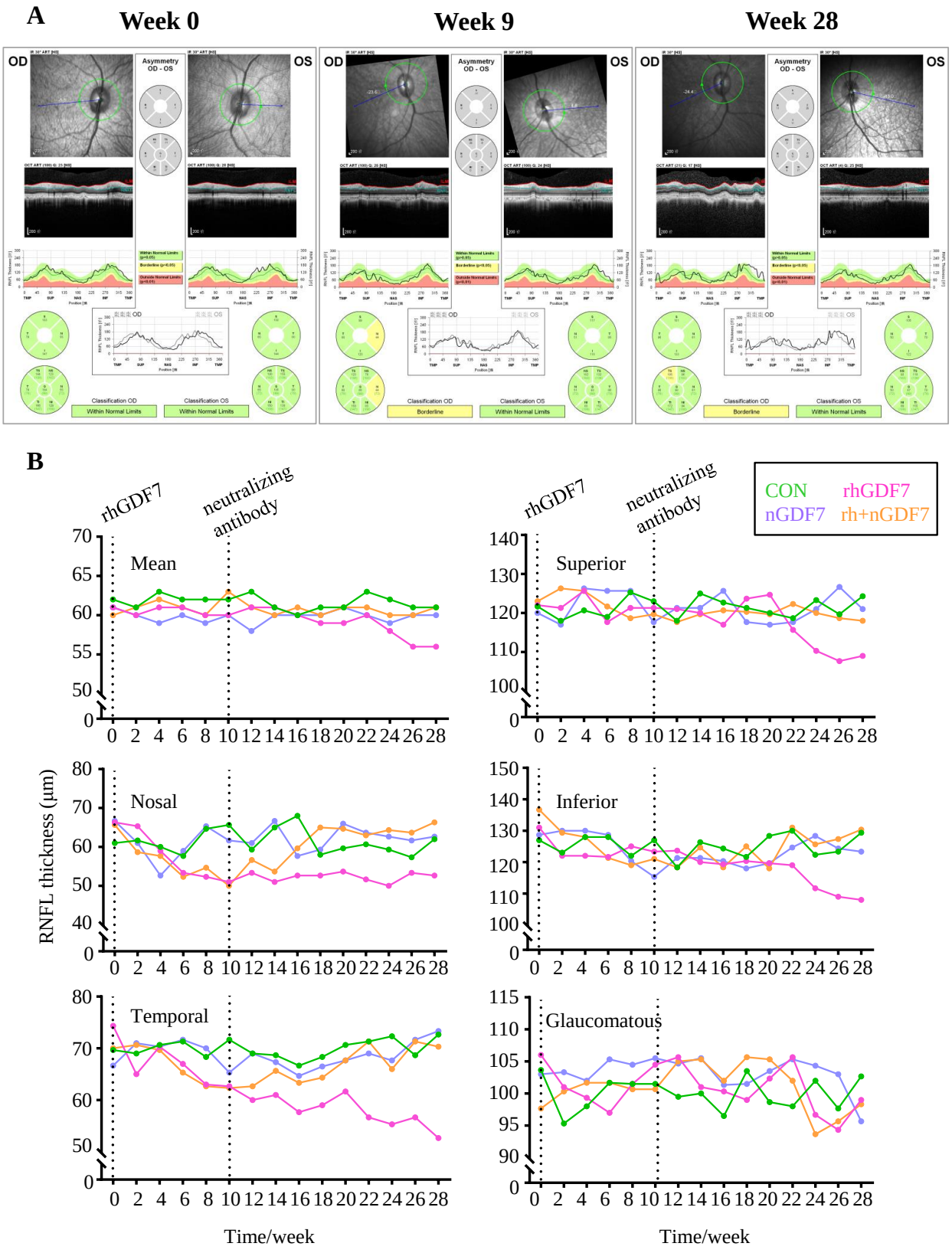

Figure S10. GDF7 neutralization exerted a neuroprotective effect in rhesus monkeys

A. A decrease in RNFL thickness was observed in rhGDF7-treated eyes compared with the control eyes after 10 weeks' treatment. The RNFL attenuation was ameliorated by the GDF7 neutralizing antibody.

B. RNFL thickness was attenuated in different regions (mean, superior part and nosal part) in response to rhGDF7 treatment. The GDF7 neutralizing antibody effectively protected RNFL from attenuation in whole retina (n=4 per group).

OD=right eye; OS=left eye; RNFL=retinal nerve fiber layer; rhGDF7=recombinant human GDF7; nGDF7=GDF7 neutralizing antibody.

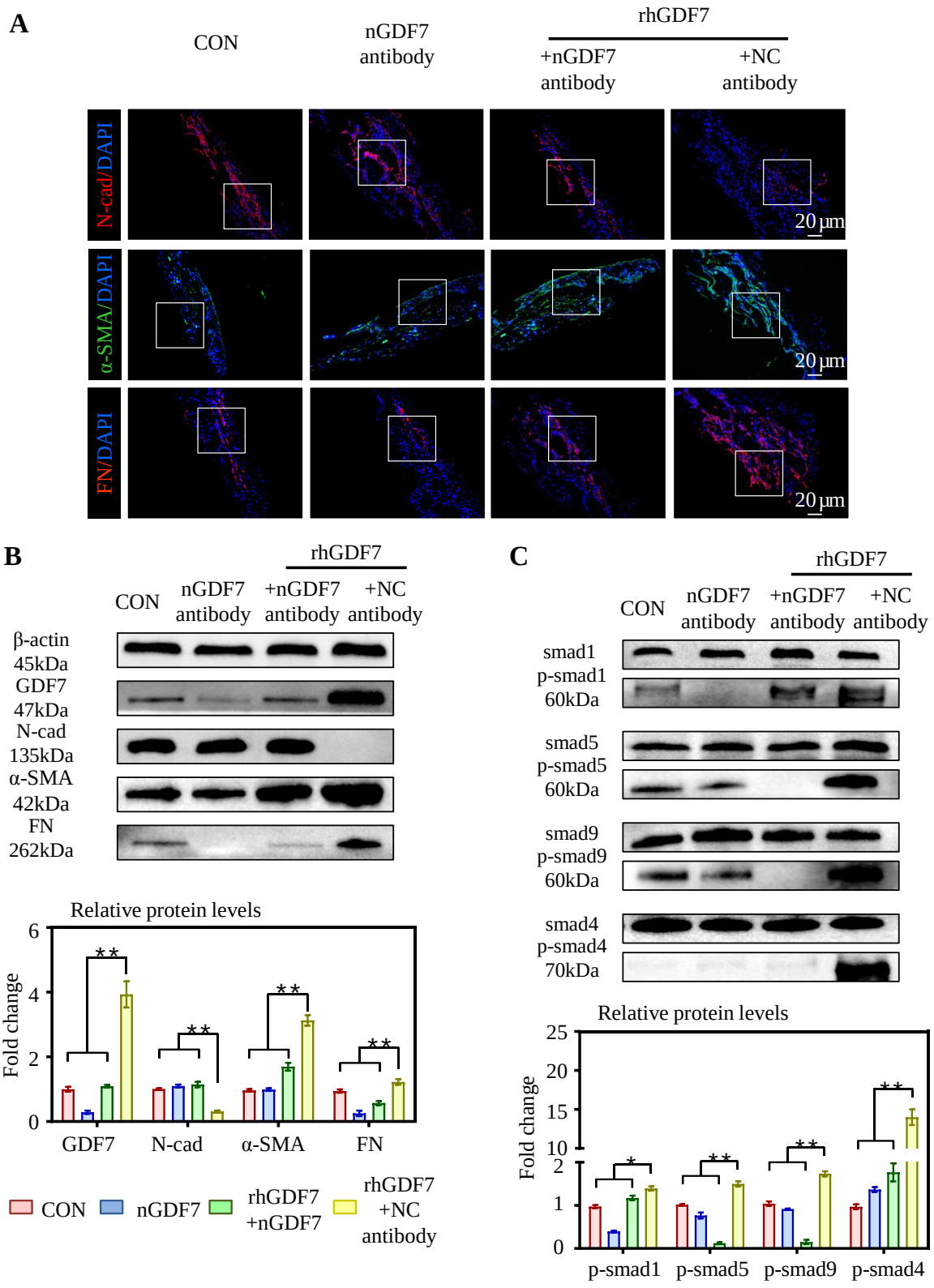

Figure S11. Pivotal role of GDF7/BMPR2/Smads signaling in the TM fibrosis

A. Intracameral delivery of rhGDF7 increased α-SMA and FN in monkey eyes. RhGDF7 also decreased the expression of N-cad in monkey TM. The GDF7

neutralizing antibody effectively prevented the rhGDF7-induced changes in these fibrosis related markers (n=4 per group).

B. RhGDF7 increased the protein levels of fibrotic markers, α-SMA and FN and decreased the expression of N-cad in monkey TM. The GDF7 neutralizing antibody inhibited the changes of α-SMA, FN and N-cad in rhGDF7 treated cells (n=4 per group).

C. The activation of BMPR2/Smad signaling was observed in rhGDF7 treated TM samples with more phosphorylated Smad1, 5, 9 and Smad 4 proteins. The GDF7 neutralizing antibody effectively inhibited the phosphorylation of Smad proteins as measured by immunoblot (n=4 per group).

Scale bar=20 μm. The data represent the means ± SD. Compared with rhGDF7 group: $^{*}p<0.05$, $^{**}p<0.01$. rhGDF7=recombinant human GDF7; nGDF7=neutralizing antibody of GDF7; FN=fibronectin; Col I=collagen I; α-SMA=α-smooth muscle actin; N-cad=N-cadherin.

Table S1. Demographic and baseline ocular characteristics of POAG patients

A

Factor	POAG n=8	
	Unilateral n=3	Bilateral n=5
Gender(male/female)	6/2	
Age(years)	57.4±7.2	
Area of visual field loss (%)	36.6±2.2	35.4±3.7
Average RNFL thickness (μm)	64.6±6.3	64.0±7.9
Cup / disc ratio	0.80±0.05	0.79±0.03
IOP (mmHg)	22.9±0.9	23.1±0.8

B

No.	Gender	Age	Affected eye	IOP (mmHg)	Area of visual field loss (%)	Average RNFL thickness (μm)	Cup/disc ratio	Medication
1	Male	70	OU	23	35	58	0.78	Timolol, Brimonidine
2	Female	62	OU	23.5	32.7	68.5	0.76	Timolol, Latanoprost
3	Male	59	OU	23.7	30.2	75	0.75	Timolol, Latanoprost
4	Male	43	OD	23.3	33.5	71	0.74	Bimatoprost
5	Male	57	OU	24	39.4	60.5	0.87	Timolol, Brimonidine, Brinzolamide
6	Female	54	OD	22.8	38.6	68	0.8	Latanoprost
7	Male	59	OU	22.3	39.8	61	0.86	Timoxan, Brimonidine
8	Male	55	OS	23.7	37.8	53	0.82	Brimonidine

A. The summarized characteristics of the demographic and ocular examinations of the eight patients recruited in DNA methylation microarray. The detailed description of each characteristic or result is expressed as the mean ± SD.

B. The detailed information of the eight POAG patients recruited in DNA methylation microarray. POAG=primary open angle glaucoma; RNFL=retinal nerve fiber layer; IOP=intraocular pressure.

Table S2. Patient demographic and baseline ocular characteristics in the expanded validation

Factor	POAG n=90	
	Unilateral n=7	Bilateral n=83
Gender(male/female)	64/26	
Age(years)	52.0±6.0	
Area of visual field loss (%)	35.21±6.5	37.82±7.61
Average RNFL thickness (μm)	69.89±16.8	70.21±23.67
Cup / disc ratio	0.79±0.12	0.80±0.08
IOP (mmHg)	24.42±1.33	23.73±0.61

Characteristics of the demographic and ocular examinations in 90 POAG patients for expanded validation of GDF7 expression. The detailed description of each characteristic or result is expressed as the mean ± SD. POAG=primary open angle glaucoma; RNFL=retinal nerve fiber layer; IOP=intraocular pressure.

Table S3. Summary of the statistical indices of the ANN prediction model

Indices	High IOP	Attenuated RNFL	High CDR	VF defects
ACC	81.25%	87.50%	93.75%	87.50%
SEN	75.00%	100.00%	100.00%	87.50%
SPE	87.50%	75.00%	87.50%	87.50%
PPV	85.70%	80.00%	88.90%	87.50%
NPV	77.80%	100.00%	100.00%	87.50%
F-Measure	80.00%	88.90%	94.10%	87.50%

The evaluation indices for the ANN-based model are presented. Accuracy (ACC) = (TP + TN) / (TP + TN + FP + FN); Sensitivity (SEN) = TP / (TP + FN); Specificity (SPE) = TN / (TN + FP); Positive Predictive Value (PPV) = TP / (TP + FP); Negative Predictive Value (NPV) = TN / (TN + FN); F-Measure = 2TP / (2TP + FP + FN); TP=true positive; TN=true negative; FP=false positive; FN=false negative; IOP=intraocular pressure; RNFL=retinal nerve fiber layer; CDR=cup/disc ratio; VF=visual field.

Table S4. Simplified diagnostic criteria of POAG for patient inclusion

Diagnostic Criteria of POAG
Open anterior chamber angle
Presence of highest daily IOP greater than 21 mm Hg
Abnormal visual field loss according to Hodapp-Parrish-Anderson criteria for diagnosing glaucomatous damage
Glaucomatous optic disc changes, CDR or CDR asymmetry> 97.5th percentile for the normal population
Without signs of secondary glaucoma or non-glaucomatous cause of optic neuropathy

POAG=primary open angle glaucoma; IOP=intraocular pressure; CDR=cup/disc ratio.

Table S5. Information of the BSP targets and primers

A

Probe ID	Gene symbol	Mean POAG	Mean NC	P-Value	CpG ISLANDS	Promoter associated
cg00060287	COL1A1	0.038125	0.043125	0.02264	chr17:48276877-48279008	yes
cg00386551	FGF12	0.042125	0.053	0.034082	chr3:192125821-192127994	no
cg05481257	GDF7	0.401125	0.566125	0.023901	chr2:20870006-20871280	no
cg06259570	MMP27	0.437625	0.44475	0.048095	no	no
cg00643111	TGFb1	0.100625	0.108	0.043427	chr16:31483276-31483646	no

B

Primer	Sequence	Target size
COL11a1_F	ATTTAGGTTGGAGGAAGGAG	273bp
COL11a1_R	ATATATTTACTTCTCCCCCACC	
FGF12_F	ATGAGGATATTTTTTTTGTTGAA	262bp
FGF12_R	TAAAAAAAAACACAACAAATAATAACC	
GDF7 _F	TGGATTTTAAGGAGTTYGGT	265bp
GDF7 _R	ACCACCATATCCTCRTATTACTTAT	
MMP27 _F	GTTTTAGTTGAAGAAAGAGAGGAATG	259bp
MMP27 _R	AACAATTCCTTCTCACAAAAAAAT	
TGFb1 _F	GTAGGTGTGGATGGTTGTTAGTTG	287bp
TGFb1 _R	CTCCTAACACATAAACCCACCC	

A. Detailed information of the five candidate methylated regions detected in microarray. Mean POAG=average methylation level of the target region among POAG patients; Mean NC=average methylation level of the target region among healthy controls. The specific positions of the targeted CpG islands are listed.

B. The BSP primers of the five differentially methylated regions in POAG patients. F=Forward primer; R=reverse primer. BSP=bisulfite sequencing PCR.

Table S6. Probes used for reverse ChIP

Probe NO.	Sequence	Length	Site
1	CCTGTGCTTCTTGGTCTGTATG	22	117
2	CCAACCACCAGTGTGTTCCC	20	282
3	CTTAGCCTCTTGGAGACCGA	20	395
4	GTAGAGCAGAGAGCGGAAGG	20	533
5	CTCGGCTAAGATTTGTGCAAAC	22	617
6	TGCTTCTAGGACTGGGATTGG	21	782
7	TTGAGCCGCATAACACTCGA	20	1002
8	CCCCTAGATCGGAAACTTTCAG	22	1353
9	CTAAGAGTTTGGCTGTGCGT	20	1706
10	ACTCAGCCACAGACCCCAAA	20	1949
11	CAGAGAAACACCCGCCCCCT	20	2138
12	CCCGGCCTAGAGTGGAGACC	20	2354

Twelve probes designed to pull down the proteins binding to different regions of GDF7 promoter.

Table S7. Primers used for ChIP-qPCR

Gene	Primer NO.	Sequence	Site	Product length
GDF7	1	TGGCAGGGGTGTCATGTAATC	470	23
		AAGTCGCTTGCCTTGTCTTG		
	2	ACCTAGGCGGCTTTGGATTC	1177	57
		AAACGAACTGCCGAAGAAGG		
	3	CGTCCTCATTTCGAGCTCTTTC	1764	12
		AGGCCTGTAAGAGACCTTTGG		
	4	TGCAATCCTTTGGGGTCTGTG	1941	94
		TGCCCTGGTTCCCAATGC		
β-actin	1	TGACAAGGACAGGGTCTTCC	1009	211
		CACCGTCCGTTGTATGTCTG		

Four pairs of primers used for amplification of four different regions in GDF7 promoter.

Table S8. Primary antibodies and their dilutions

Primary antibody	Manufacture	Product size	Dilution
Anti-Collagen I antibody	Abcam	200-300 kDa	WB(1:1000)
Anti-Fibronection antibody	Abcam	262 kDa	IF(1:200)
			WB(1:1000)
Anti-α-SMA antibody	Abcam	42 kDa	IF(1:200)
			WB(1:1000)
Anti-GDF7 antibody	Abcam	47 kDa	IF(1:200),
			WB(1:1000)
β-actin antibody	CST	45 kDa	WB(1:1000)
Smad1 antibody	CST	60 kDa	WB(1:1000)
Phospho-Smad1 (Ser206)	CST	60 kDa	WB(1:500)
Smad5 antibody	CST	60 kDa	WB(1:1000)
Phospho-Smad5 (Ser463/465)	CST	60 kDa	WB(1:500)
Anti-Smad9 antibody	Abcam	52 kDa	WB(1:1000)
Phospho-Smad9 (Ser465/467)	CST	60 kDa	WB(1:500)
Smad4 antibody	CST	70 kDa	WB(1:1000)
Phospho-Smad4 (Thr276)	SAB	60 kDa	WB(1:1000)

IF=immunofluorescence; WB= western blot.

Table S9. Primer sequences used for real-time PCR

Primer name	Sequence (5'to3')	Nucleotides
β-actin-forward primer	TTCTGCTCTTCGGTTCTGCC	20
β-actin-forward primer	GCCGTGTAGGTCGAAACAGA	20
GDF7-forward primer	CTCCTTCATCAGCAGTCTTCC	21
GDF7-reverse primer	ATCGGCAACACCTCACAATG	20
COL I-forward primer	CAATGCTGCCCTTTCTGCTCCTTT	24
COL I-reverse primer	ATTGCCTTTGATTGCTGGGCAGAC	24
α-SMA-forward primer	CCGACCGAATGCAGAAGGA	20
α-SMA-reverse primer	ACAGAGTATTTGCGCTCCGAA	21
Fibronectin-forward primer	GCCACTTCAAAGTTCTCATTCCT	23
Fibronectin-reverse primer	GAAGATCCCCACCTCCCACAA	21
N-cadherin-forward primer	ACAGTGGCCACCTACAAAGG	20
N-cadherin-reverse primer	TGATCCCTCAGGAACTGTCC	19

Table S10. Definitions of the POAG-related outcomes

Definitions of POAG-related outcomes	
High IOP	Presence of highest daily IOP greater than 21 mm Hg
Attenuated RNFL	Thickness of RNFL<97.5th percentile for the age and sex-corrected average value of healthy population
High CDR	Glaucomatous optic disc changes, CDR or CDR asymmetry> 97.5th percentile for the normal population
Visual field Defects	Abnormal visual field loss according to Hodapp-Parrish-Anderson criteria for diagnosing glaucomatous damage