Localization and Expression of Nuclear Factor of Activated T-cells 5 in Myoblasts Exposed to Pro-inflammatory Cytokines or Hyperosmolar Stress and in Biopsies from Myositis Patients

Running title: NFAT5 in Myoblasts and Myositis

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Supplementary Table 1. Cell culture data

Not applicable (N/A).

Primary myoblast culture	Status	Provided by	Age (years)	Gender	Mutation/ Deletion
UMyo 1	Unaffected	Myobank (F)	16	Unknown	N/A
UMyo 2	Unaffected	Myobank (F)	16	Unknown	N/A
UMyo 7	Unaffected	Göttingen (D)	Unknown	Unknown	N/A
UMyo 8	Unaffected	Göttingen (D)	Unknown	Unknown	N/A

Supplementary Table 2. Primary antibodies used in immunohistochemistry

NFAT5= Nuclear Factor of Activated T-cells 5; NCAM= neural cell adhesion molecule; vWF= von Willebrand Factor; HDAC6= histone deacetylase 6. Antibodies directed to CD56 (NCAM) were applied to identify regenerating muscle fibers. CD3 stained T-cells, CD4+ and CD8+ provided a distinction between CD4+ cells and CD8+ cytotoxic T-cells. CD68 localized macrophages alongside with dendritic cells, vWF binds to endothelial cells and HDAC6 (histone deacetylase 6) is involved in cell motility.

Antigen	Primary antibody	Clone	Concentration	Source
CD3 (all T cells)	Mouse monoclonal IgG1	F7.2.38	1/100	Dako
CD4	Mouse monoclonal IgG1	MT310	2 µg/mL	Dako
CD8	Mouse monoclonal IgG1	C8/144B	9 µg/mL	Dako
CD56 (=NCAM)	Rabbit polyclonal	/	1 µg/mL	Merck
CD68	Mouse monoclonal IgG1	KP1	2,2 µg/mL	Abcam
HDAC6	Rabbit monoclonal IgG	D2E5	1/200	Cell Signaling
NFAT5 (V-18)	Goat polyclonal (IgG)	/	10 µg/mL	Santa-Cruz
NFAT5 (F-9)	Mouse monoclonal IgG2a	F-9	2µg/mL	Santa-Cruz
NFAT5 SP5110P	Rabbit polyclonal (IgG)	/	0,5µg/µL	Acris Antibodies
vWF	Mouse monoclonal IgG1	F8/86	2,3µg/mL	Dako

Supplementary Table 3. Patient data

Polymyositis (PM); dermatomyositis (DM); inclusion body myositis (IBM); non-necrotic invaded fiber (NNIF).

Patient	Muscle pathology	Age (years)	Gender	Muscle damage	Inflammation	Concomitant disease
Patient 1	PM (NNIF +)	51	Female	Severe	Severe	Graves, SLE
Patient 2	PM (NNIF +)	50	Female	Mild	Mild	Diabetes
Patient 3	PM (NNIF +)	38	Male	Weak	Weak	None
Patient 4	PM (NNIF +)	37	Female	Mild	Mild	None

Patient 5	PM (NNIF +)	73	Female	Severe	Severe	Diabetes,Hypo-thyroidism
Patient 6	PM (NNIF +)	55	Male	Mild	Mild	Rheumatoid arthritis
Patient 7	DM	66	Male	Mild	Mild	Unknown
Patient 8	DM	65	Female	Severe	Severe	Unknown
Patient 9	DM	45	Male	Severe	Severe	Unknown
Patient 10	DM	42	Female	Severe	Weak	Unknown
Patient 11	DM	57	Male	Weak	Weak	Unknown
Patient 12	DM	49	Female	Unknown	Unknown	None
Patient 13	DM	73	Male	Unknown	Unknown	None
Patient 14	DM	56	Male	Unknown	Unknown	Unknown
Patient 15	DM	31	Female	Unknown	Unknown	Unknown
Patient 16	IBM	70	Female	Severe	Severe	None
Patient 17	IBM	63	Male	Mild	Weak	None
Patient 18	IBM	73	Male	Severe	Severe	None
Patient 19	IBM	59	Male	Severe	Severe	Diabetes+ACE inhibitors
Patient 20	IBM	70	Male	Severe	Severe	None
Patient 20	IBM	68	Male	Severe	Severe	None
Patient 22	IBM	71	Male	Severe	Severe	Arterial hypertension
Patient 23	IBM	81	Female	Severe	Severe	None
Patient 24	IBM	59	Female	Severe	Severe	None

Patient 25	IBM	57	Female	Severe	Severe	None
Patient 26	IBM	62	Female	Severe	Severe	Neuropathy

Supplementary Table 4. Primary antibodies used in Western-blotting

GAPDH= glyceraldehyde 3-phosphate dehydrogenase, PARP= poly (ADP-ribose) polymerase

Antigen	Primary antibody	Clone	Concentration	Source
GAPDH	Mouse monoclonal IgM	71.1	0.4µg/mL	Sigma- Aldrich
Histone H3	Mouse monoclonal IgG3	24834	5µg/mL	Cell Signaling
NFAT5 (F-9)	Mouse monoclonal IgG2a	F-9	2µg/mL	Santa-Cruz
PARP	Mouse monoclonal IgG1	4C10-5	0.5µg/mL	BD Biosciences

Supplementary Table 5. Genes used in RT-qPCR

^{UBC}= ubiquitin C; ^{HPRT1}= hypoxanthine phosphoribosyltransferase 1; ^{B2M}= beta-2 microglobulin; ^{RPL13A}= 60S ribosomal protein L13a; ^{YWHAZ}= Tyrosine 3-Mono-oxygenase/Tryptophan 5-Mono-oxygenase Activation Protein, Zeta; ^{SDHA}= Succinate dehydrogenase complex, subunit A, ^{HMBS}= hydroxymethylbilane synthase, ^{TBP}= TATA-binding protein, ^{AluSq}= Alu restriction enzym, ^{AluSx1}= interspersed repeat subfamily.

Gene	Primer	Concentration	Source
NFAT5	PrimePCR SYBRGreen Assay qHsaCID0015734 intron-spanning	1x	Bio-Rad
UBC	F:ATTTGGGTCGCGGTTCTTG	1,25pmol	IDT
	R:TGCCTTGACATTCTCGATGGT	1,25pmol	IDT
HPRT1	F: TGACACTGGCAAAACAATGCA	1,25pmol	IDT
	R: GGTCCTTTTCACCAGCAAGCT	1,25pmol	IDT
B2M	F: TGCTGTCTCCATGTTTGATGTATCT	1,25pmol	IDT
	R: TCTCTGCTCCCCACCTCTAAGT	1,25pmol	IDT
RPL13A	F: CCTGGAGGAGAAGAGGAAAGAGA	1,25pmol	IDT
	R: CCTGGAGGAGAAGAGGAAAGAGA	1,25pmol	IDT
YWHAZ	F: ACTTTTGGTACATTGTGGCTTCAA	1,25pmol	IDT
	R: CCGCCAGGACAAACCAGTAT	1,25pmol	IDT
SDHA	F: TGGGAACAAGAGGGCATCTG	1,25pmol	IDT
	R: CCACCACTGCATCAAATTCATG	1,25pmol	IDT
HMBS	F: GGCAATGCGGCTGCAA	1,25pmol	IDT
	R: GGGTACCCACGCGAATCAC	1,25pmol	IDT
TBP	Unknown	1,25pmol	[49]
AluSq	Unknown	1,25pmol	[49]
AluSx1	Unknown	1,25pmol	[49]

Supplementary Table 6. NFAT5 immunofluorescent staining in patients' muscle biopsies

Dermatomyositis (DM); inclusion body myositis (IBM); polymyositis (PM); scoring: - = absent, + = weak positive, ++ = mild positive, +++ = strongly positive, ( ) = minority of cells, N/A = not applicable/not present.

Staining	Control	DM	IBM	PM
Myonuclei/Perinuclear	+	+++	+	++
Sarcolemma	-/(+)	- /(+)	+/-	-/+
CD3+ T-cells	-	-	+/-	-
CD4+ T-cells	-	-/(+++)	+/-	-/+
CD8+ T-cells	-	-	-	-
CD68 + cells	N/A	-/+	-/+	N/A
NCAM+ muscle fibers	N/A	-	-/+	-/(+)
Endothelial cells	-	-/+	-/(+)	-/(+)

Supplementary Figure 1 Additional tests

Immunofluorescence was performed in UMyo (unaffected myoblasts) with NFAT5 Goat antibody and NCAM antibody, followed respectively by a Cy3 (red)- labelled secondary antibody and Alexa-488 (green)- labelled secondary antibody. Myonulei were counterstained with DAPI staining (blue). **(A):** In UMyo 1 control myoblasts, NCAM and NFAT5 are expressed in almost all cells. (n=3). **(B):** Myoblasts were exposed to DMEM60 (DMEM with a total increase in osmolarity of 60mM), DMEM60 + NFAT5 siRNA and to DMEM60 + NFAT5 scrRNA, assessing NFAT5 antibody specificity. NFAT5 Rabbit shows aspecific binding in the myonuclei. NFAT5 Mouse binds aspecifically in the cytoplasm. NFAT5 Goat is the most suited antibody for IHC, albeit some minor punctate signals remain at long exposure time (n=1). **(C):** NFAT5 expression is not influenced by addition of heat-treated horse serum in UMyo8 after addition of DMEM18 or DMEM60 (n=3).

Supplementary Figure 2 Confocal microscopy

By confocal microscopy, untreated UMyo1 myoblasts have a spindle-shaped morphology (n=3).

Supplementary Figure 3 Variation in NFAT5 localization and expression amongst unaffected cell cultures

(A): Immunofluorescence was performed in UMyo (unaffected myoblasts) with NFAT5 Goat antibody, followed by a Cy3 (red)-labelled secondary antibody. Myonulei were counterstained with DAPI staining (blue). In all UMyo, NFAT5 nuclear staining is induced after adding DMEM60 (DMEM with a total increase in osmolarity of 60mM) (n=1). **(B):** By WB, an increase in NFAT5 protein levels is observed in all tested UMyo (n=3).