Endothelial-mesenchymal transition in human atrial fibrillation

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Endothelial–Mesenchymal Transition in Human Atrial Fibrillation

Short title: Endothelial–Mesenchymal Transition and Atrial Fibrillation

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Abstract

Background: Atrial fibrosis is a hallmark of atrial structural remodeling leading to the persistence of atrial fibrillation. Although fibroblasts play a major role in atrial fibrosis, their source in the adult atrium is unclear. We tested the hypothesis that endothelial cells contribute to fibroblast accumulation through an endothelial–mesenchymal transition in the atrium of patients with atrial fibrillation.

Methods and Results: The study group consisted of patients with atrial fibrillation and valvular disease or atrial septal defect who underwent left atrial appendectomy during cardiac surgery (n = 38). The amount of fibrotic depositions in the left atrium positively correlated with left atrial dimension. Furthermore, snail and S100A4, indicative of endothelial–mesenchymal transition, were quantified in the left atrium using western blot analysis, which showed statistically significant correlations with left atrial dimension. Immunofluorescence assay of the left atrial tissue identified snail and S100A4 being expressed within the endocardium which is composed of $CD31^+$ cells. The snail-positive endocardium also showed the expression of membrane type 1-matrix metalloproteinase. Immunofluorescence multi-labeling experiments identified that heat

shock protein 47, prolyl-4-hydroxylase, and procollagen type 1 co-localized with snail and S100A4 within the endothelial cells of the left atrium, indicating the mesenchymal phenotype to produce collagen.

Conclusions: In this study, we showed that the endothelial–mesenchymal transition occurs in the atrium of patients with atrial fibrillation. This observation should help in constructing a novel therapeutic approach for preventing atrial structural remodeling.

Introduction

The first occurrence of atrial fibrillation (AF) is believed to be paroxysmal in nature; it then gradually perpetuates and finally develops into permanent AF with the progression of atrial remodeling [1,2]. Atrial fibrosis is a hallmark of atrial structural remodeling and leads to electrophysiological impairment of the atrium and persistence of AF [3]. Although fibroblasts play a major role in atrial fibrosis, their source in the adult atrium is unclear, and specific anti-fibrotic therapies are not currently available in clinical settings.

Formerly, adult fibroblasts were considered to have originated from embryonic mesenchymal cells [4,5]; these resident fibroblasts were believed to proliferate under pathological conditions. However, recent studies have shown that epithelial cells contribute to fibroblast accumulation through an epithelial–mesenchymal transition (EMT) in the kidney, lung, and liver, in addition to the proliferation of resident fibroblasts and bone marrow-derived fibroblasts [6]. Experiments in mice have demonstrated that endothelial cells associated with the microvasculature can also contribute to the formation of mesenchymal cells during the course of fibrosis via a

similar process known as endothelial–mesenchymal transition (EndMT) [7].

Herein, we tested the hypothesis that endothelial cells undergo EndMT in the human atrium during the development and progression of AF.

Methods

Patients

The study group consisted of 38 patients with AF (14 paroxysmal AF, 24 permanent AF) with valvular disease or atrial septal defect who underwent left atrial appendectomy during cardiac surgery. None of the patients had previous myocardial infarction, febrile disorders, systemic inflammatory diseases, malignancy, or chronic renal failure. During the cardiac surgery, atrial tissue samples were collected from all patients, and they were quickly frozen in liquid nitrogen and stored at −80 °C until use.

This investigation conforms to the principles outlined in the Declaration of Helsinki. All patients gave written informed consent, and an institutional review board approved the study.

Histology and Immunohistochemistry

Blocks of tissues were compound-embedded in an optimal cutting temperature and immediately frozen in liquid nitrogen after resection. Frozen cryostat sections (8 μm thick) were cut, air-dried, fixed in acetone, and then evaluated with standard protocols for Masson's trichrome staining. The images were digitized using a digital microscope (COOLSCOPE; Nikon). To quantify fibrotic deposition of the left atrium (LA), blue pixel content of the digitized photos was measured relative to the total tissue area using Image-Pro Plus (Media Cybernetics).

Immunostaining was performed by Dako EnVision+ Systems (Dako) using the primary antibodies listed in Table 1. Immunofluorescence labeling for microscopy was performed by treatment with Alexia Fluor 488- or 568-conjugated goat anti-rabbit antibodies or goat anti-mouse antibodies (Molecular Probes, 1:500 dilution). Immunofluorescence-labeled samples were examined with Axio Imager M1 and digitized with Axio Cam MRC5. The green channel had an excitation of 488 nm and an emission of 525 nm. The red channel had an excitation of 594 nm and an emission of

620 nm. Lack of any cross-talk between the channels was established. Control experiments performed by incubation with secondary antibodies only did not show positive staining under the same experimental conditions.

Western Blot Analysis

Total proteins (5 μg) extracted from the left atrial appendages were fractionated using SDS-PAGE gels, and transferred onto Amersham Hybond-P PVDF Transfer Membrane (GE Health Care). The primary antibodies used in this study were displayed in Table 1. The secondary antibodies used were goat anti-rabbit IgG HRP-linked antibody, horse anti-mouse IgG HRP-linked antibody, and goat anti-biotin HRP-linked antibody (all from Cell Signaling Technology).

Statistical Analysis

Data are expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism 5.0 for Macintosh (GraphPad Software). Left atrial dimension (LAD) was compared with fibrosis, snail, and S100A4 using linear regression analysis. $P <$

0.05 indicated statistical significance.

Results

Patient Characteristics

Table 2 lists the clinical data of the patients who participated in this study. Their mean age was 63.8 ± 9.1 years and there was male predominance (53%); their mean AF duration was 72.0 months. Of all patients, 14 had paroxysmal AF and 24 had persistent AF. In most cases, left ventricular systolic function was preserved (mean left ventricular ejection fraction: 63.1 ± 10.1%) and LA was dilated (range: 41–80 mm; mean: 54.9 ± 9.5 mm). Majority of the patients (82%) underwent cardiac surgery for mitral valve disease.

Atrial Structural Remodeling and EndMT Markers

Representative images of LA by Masson's trichrome staining are shown in Fig 1A. The amount of fibrotic depositions in LA was positively correlated with LAD ($y = 0.3004x - 4.395$, $R^2 = 0.3809$, $P < 0.0001$, Fig 1B). Furthermore, the amount of snail

and S100A4, indicative of EndMT, in LA showed weak but statistically significant correlations with LAD ($y = 0.1269x + 3.388$, $R^2 = 0.1387$, $P = 0.0213$ for snail, Fig 2A; and $y = 0.3728x + 2.793$, $R^2 = 0.1216$, $P = 0.0319$ for S100A4, Fig 2B). The amount of S100A4 was also positively correlated with the extent of fibrosis ($y = 0.9012x + 12.35$, $R^2 = 0.1683$, $P = 0.0105$, Fig 2D), whereas the amount of snail showed marginally insignificant correlation with the fibrosis ($y = 0.1984x + 7.955$, $R^2 = 0.08028$, $P = 0.0847$, Fig 2C).

Localizations of EndMT-Related Molecules in the Atrium

The immunofluorescence assay of the LA tissue revealed the expression of snail (Fig 3) and S100A4 (Fig 4) within the endocardium composed of $CD31^+$ endothelial cells. The staining indicative of snail and S100A4 was positive in 23.7% and 19.3% of $CD31^+$ cells, respectively. The snail-positive endocardium also showed the expression of membrane type 1-matrix metalloproteinase (MT1-MMP) (Fig 5).

The co-expressions of heat shock protein 47 (HSP47), prolyl 4-hydroxylase (P4HD), procollagen type 1 (ProCL1) with snail and S100A4 within endothelial cells in

LA were observed, indicating the mesenchymal phenotype producing collagen (Fig 6).

Discussion

Major Findings

The following were the major findings of this study: (1) in the atrium of patients with AF, the endothelial cells expressed snail, S100A4, HSP47, P4HD, and ProCL1, suggesting a process of EndMT. (2) The expression levels of snail and S100A4 were positively correlated with LAD.

Role of Fibroblasts in Developing AF

Atrial structural remodeling, particularly fibrosis in which fibroblasts play a key role, is important in many forms of AF [8,9,10]. In our study population, the amount of fibrotic deposition in LA was correlated with LAD, which is a strong predictor of recurrence of AF after catheter ablation or drug therapy [2,11]. Interstitial fibrosis separates cardiomyocytes, which interferes with electric continuity and slows the electrical conduction and thereby causes the progression of AF to permanent forms [3].

In addition, fibroblasts per se can electrically couple with cardiomyocytes and when their number increases, they promote reentry and/or ectopic activity [12]. Therefore, atrial fibroblasts are potentially a therapeutic target in AF.

EndMT Process in AF

EMT is a biological process that allows a polarized epithelial cell, which normally interacts with basement membrane, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of components of the extracellular matrix [13]. A central event in EMT is the downregulation of membranous E-cadherin expression, which leads to the loss of cell–cell contact and the consecutive progression of the cells toward a malignant phenotype [14]. Snail, a zinc-finger transcription factor, is a key EMT regulator [15]. Studies have shown that snail represses E-cadherin transcription by binding to the E-box site in the promoter of E-cadherin [16-18]. The completion of an EMT process is signaled by the degradation of underlying basement membrane by MMPs and the formation of a

mesenchymal cell that can migrate away from the epithelial layer in which it originated [13]. The co-expression of both snail and MT1-MMP within atrial endothelial cells in our study suggests an EndMT process in the human atrium.

To gather more evidence that atrial endothelial cells undergo EndMT during AF progression, we performed immunofluorescence multi-labeling experiments using antibodies against snail, S100A4, HSP47, P4HD, and ProCL1. S100A4 [19], also known as fibroblast-specific protein 1 (FSP1) [20,21], is a member of the S100 superfamily of EF-hand calcium-binding proteins. P4HD is an essential enzyme for the biosynthesis of procollagen [22] and HSP47 is an ER-resident stress protein that is believed to function as a molecular chaperone specific to the biosynthesis of procollagen [23,24]. We identified the co-expressions of these profibrotic molecules within the atrial endocardial cells, suggesting transition to fibroblast phenotype.

Limitations

The study has some limitations. First, it is still unknown whether there is a causal relationship between EndMT and AF as we could not obtain atrial samples from healthy

subjects. Second, we only collected samples of left atrial appendage; therefore, the present findings may not be representative of other areas of the atrium.

Conclusions

To the best of our knowledge, this is the first study to provide evidence that EndMT occurs in the atrium of patients with AF. Our findings should help researchers to construct a novel therapeutic approach for the prevention of atrial structural remodeling in the treatment of AF.

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Disclosures

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Figure Legends

Fig 1. Relationship between left atrial dimension (LAD) and fibrosis in the left atrium (LA). A: Representative images of Masson's trichrome staining. Fibrosis (blue-colored) was more prominent in larger LA. B: The amount of fibrotic depositions in LA was positively correlated with LAD.

Fig 2. Western blot analysis of the left atrial tissue for snail and S100A4. A: The expression of snail showed statistically significant correlation with left atrial dimension (LAD). B: The expression of S100A4 also showed correlation with LAD.

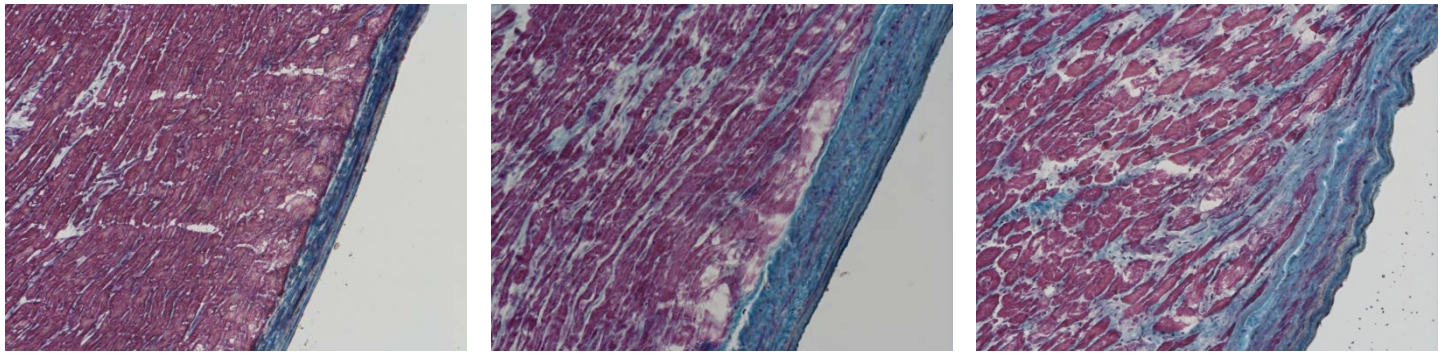
Fig 3. Immunofluorescence assay of left atrial tissue for snail and CD31. Snail was expressed within the endocardium composed of $CD31^+$ endothelial cells.

Fig 4. Immunofluorescence assay of left atrial tissue for S100A4 and CD31. S100A4 was expressed within the endocardium composed of $CD31^+$ endothelial cells.

Fig 5. Immunofluorescence assay of left atrial tissue for snail and membrane type 1-matrix metalloproteinase (MT1-MMP). The snail-positive endocardium showed the expression of MT1-MMP.

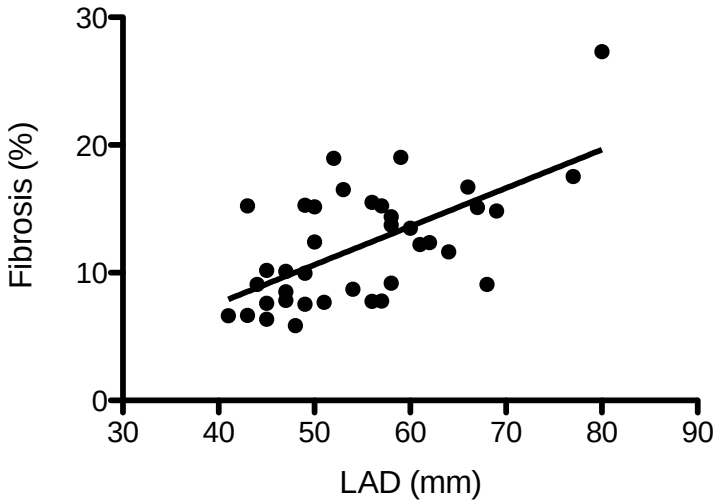
Fig 6. Immunofluorescence multi-labeling experiments using antibodies against snail, S100A4, heat shock protein 47 (HSP47), prolyl 4-hydroxylase (P4HD), and procollagen type 1 (ProCL1). Co-expressions of HSP47, P4HD, and ProCL1 with snail and S100A4 within left atrial endothelial cells were observed.

A



LAD (mm) 43 60 73

B



$y = 0.3004x - 4.395$

$R^2 = 0.3809$

$P < 0.0001$

Figure 1

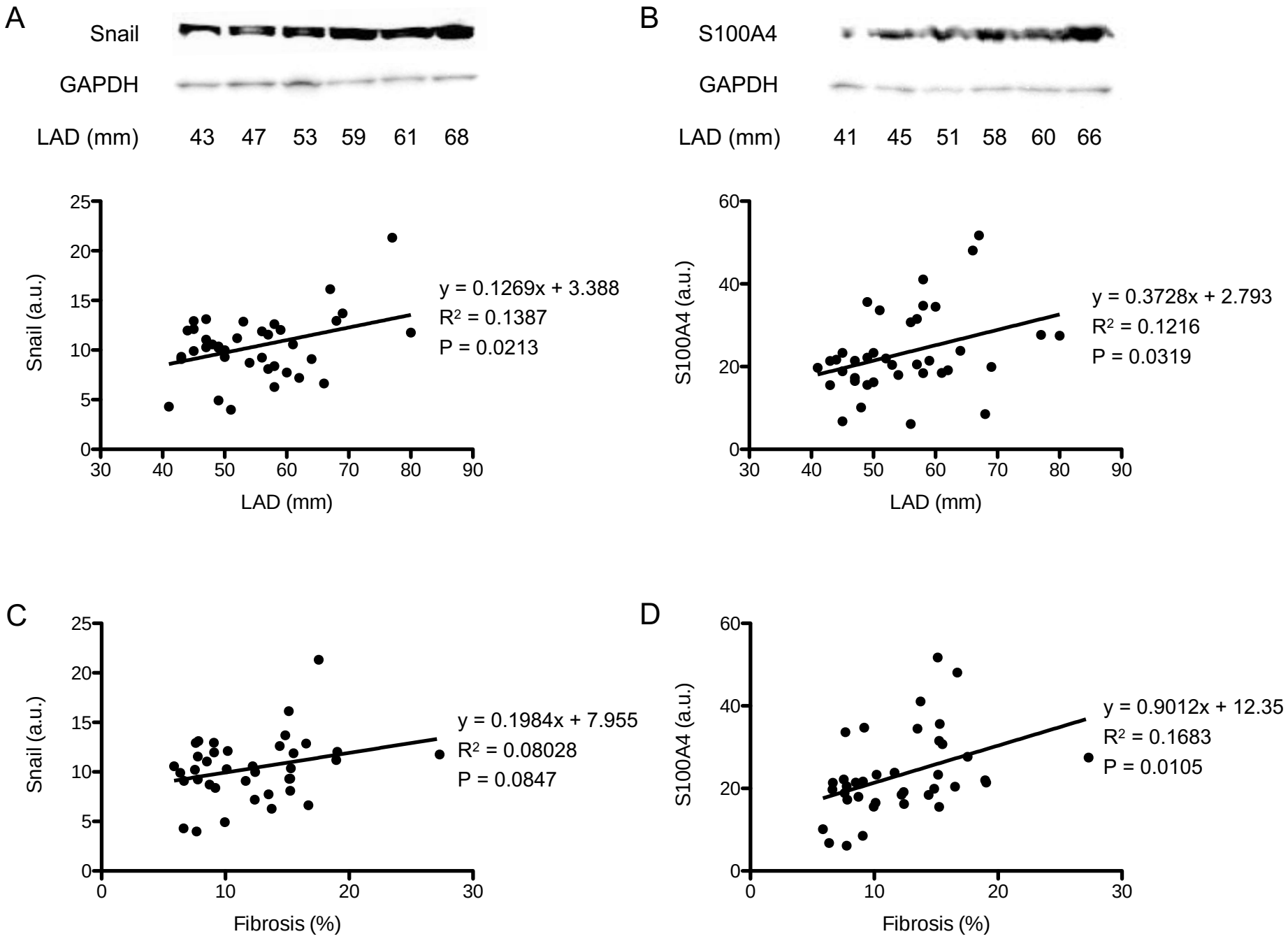

A
Snail
GAPDH
LAD (mm) 43 47 53 59 61 68
Snail (a.u.)
LAD (mm)
y = 0.1269x + 3.388
R² = 0.1387
P = 0.0213
B
S100A4
GAPDH
LAD (mm) 41 45 51 58 60 66
S100A4 (a.u.)
LAD (mm)
y = 0.3728x + 2.793
R² = 0.1216
P = 0.0319
C
Snail (a.u.)
Fibrosis (%)
y = 0.1984x + 7.955
R² = 0.08028
P = 0.0847
D
S100A4 (a.u.)
Fibrosis (%)
y = 0.9012x + 12.35
R² = 0.1683
P = 0.0105

Figure 2

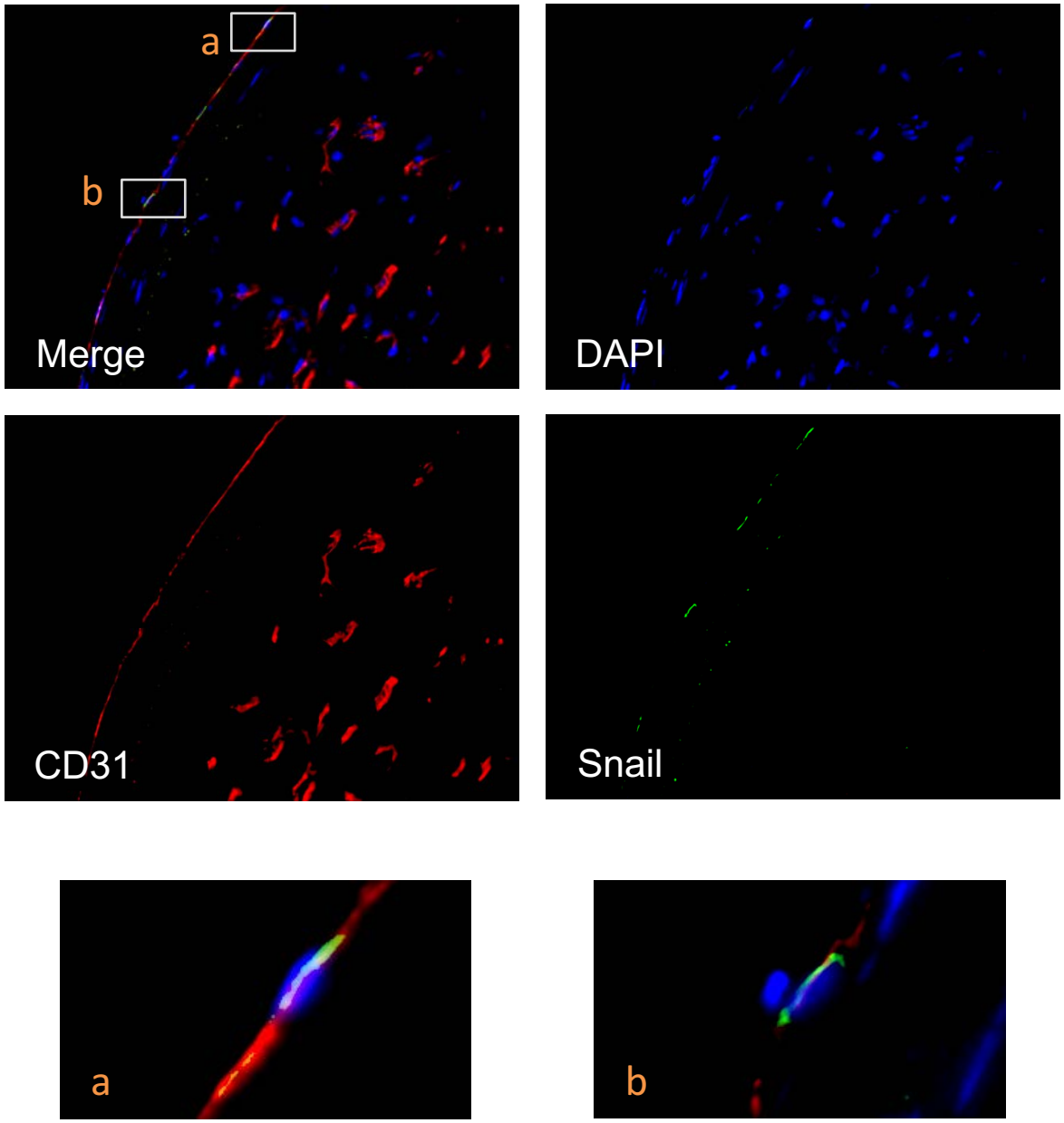


Figure 3

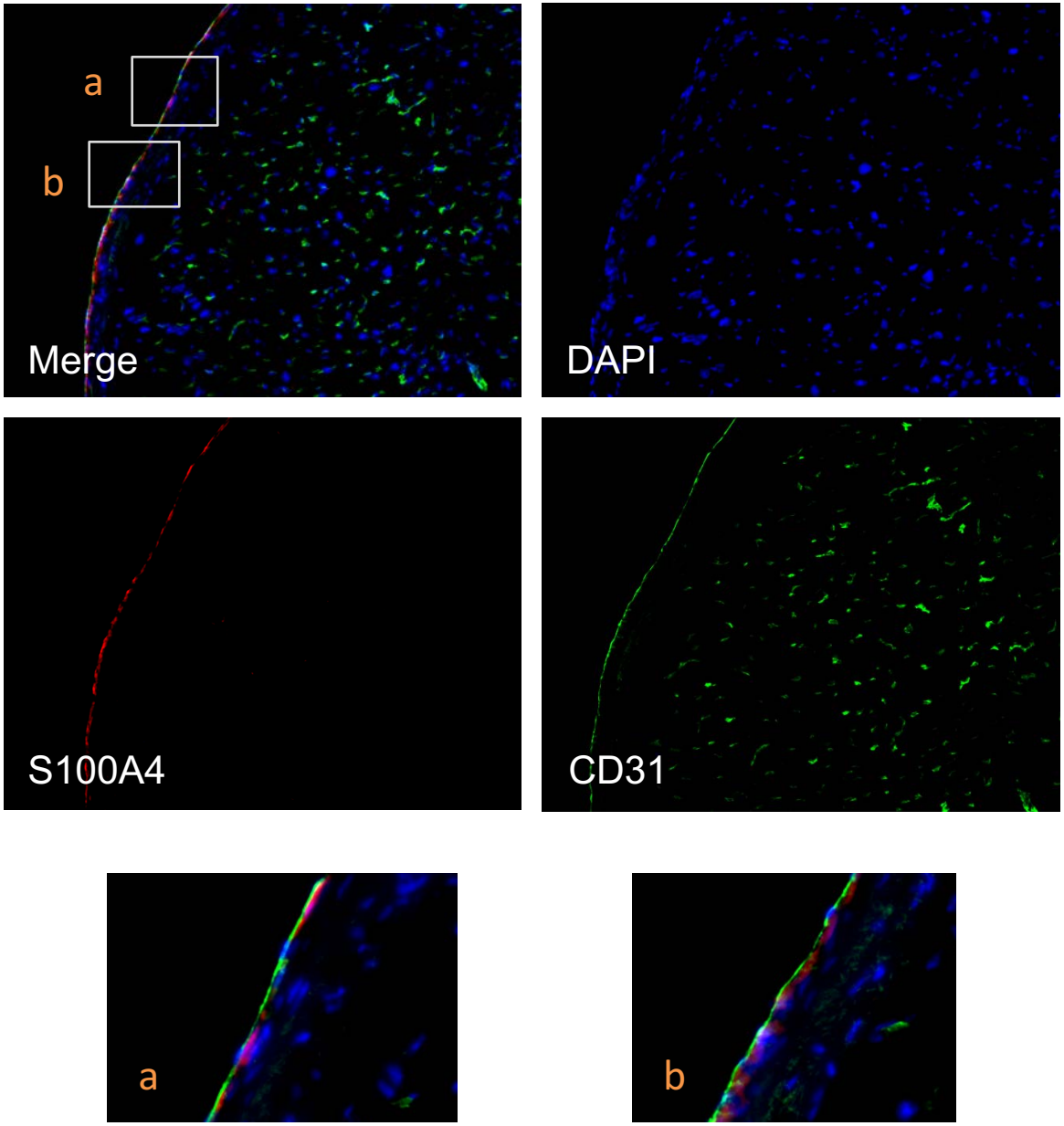
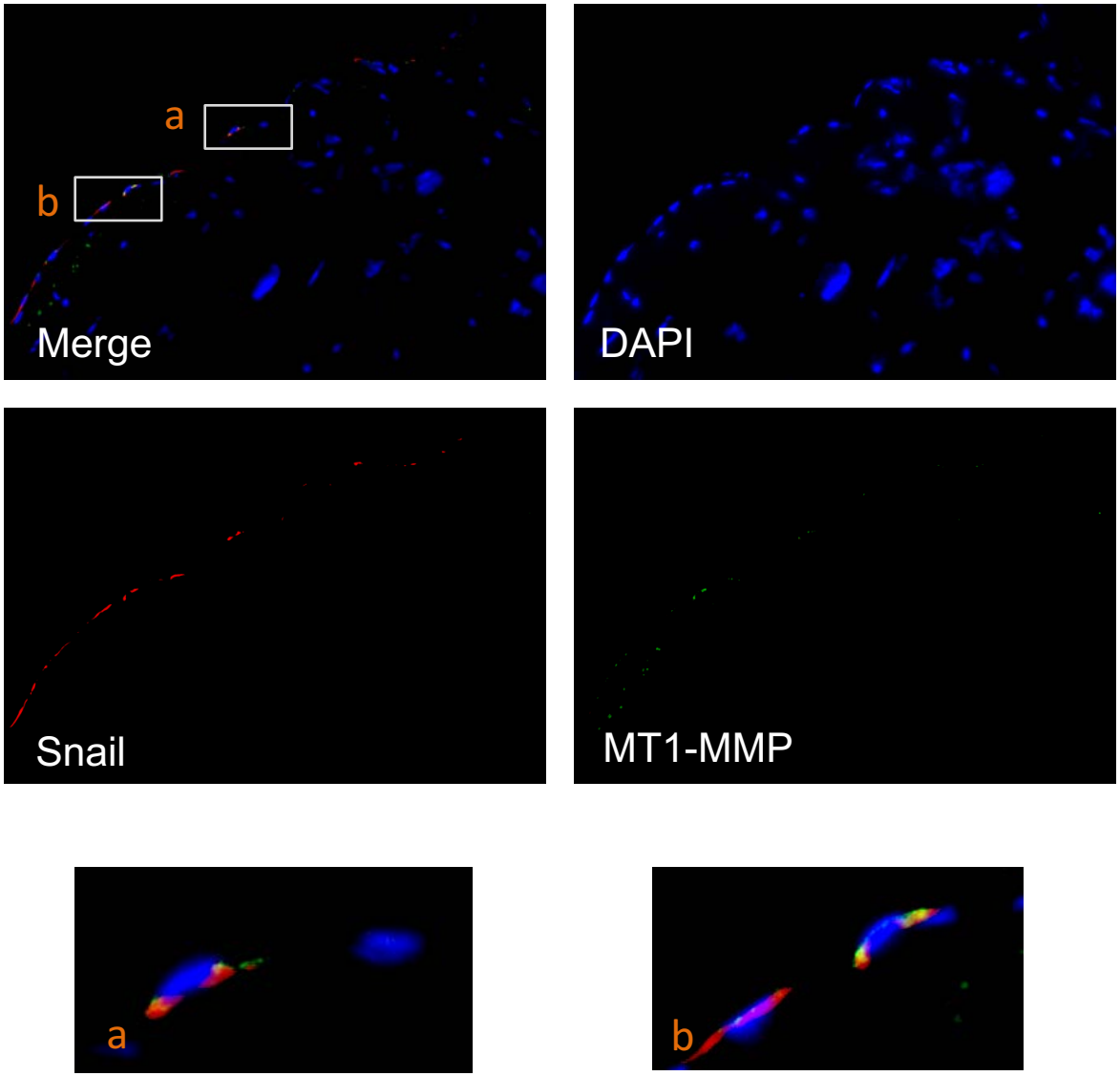


Figure 4



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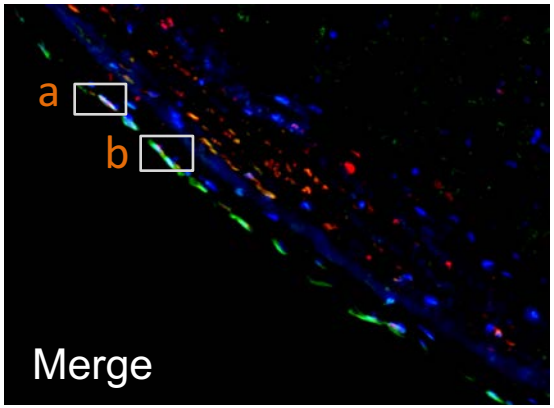
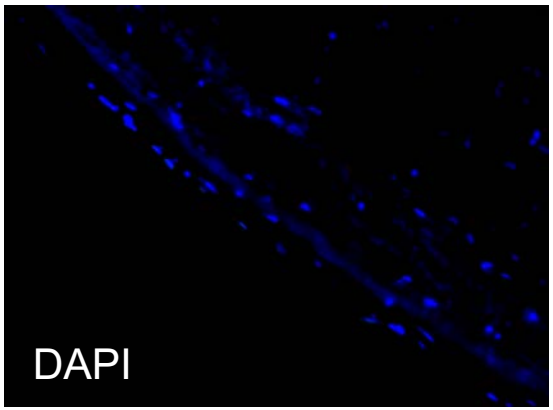
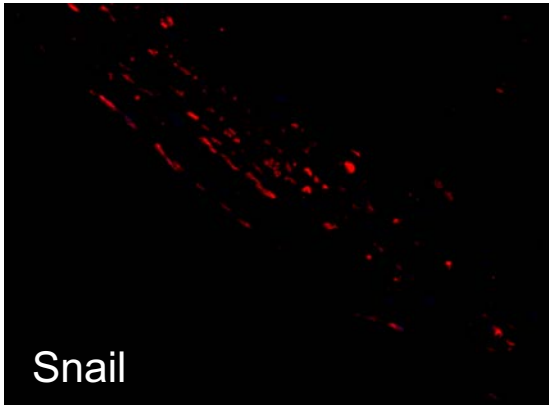
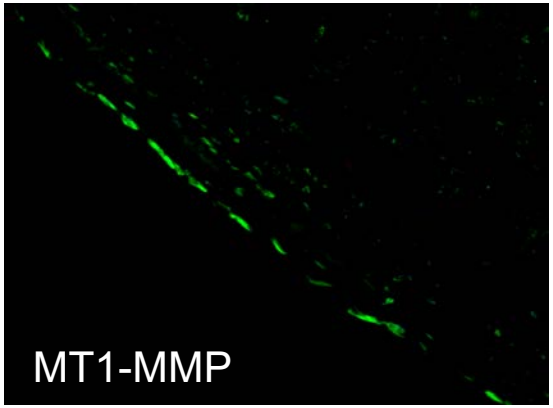








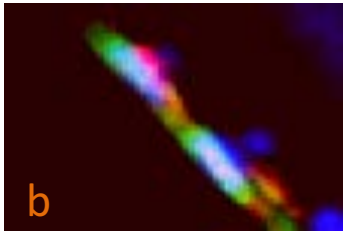


Figure 5

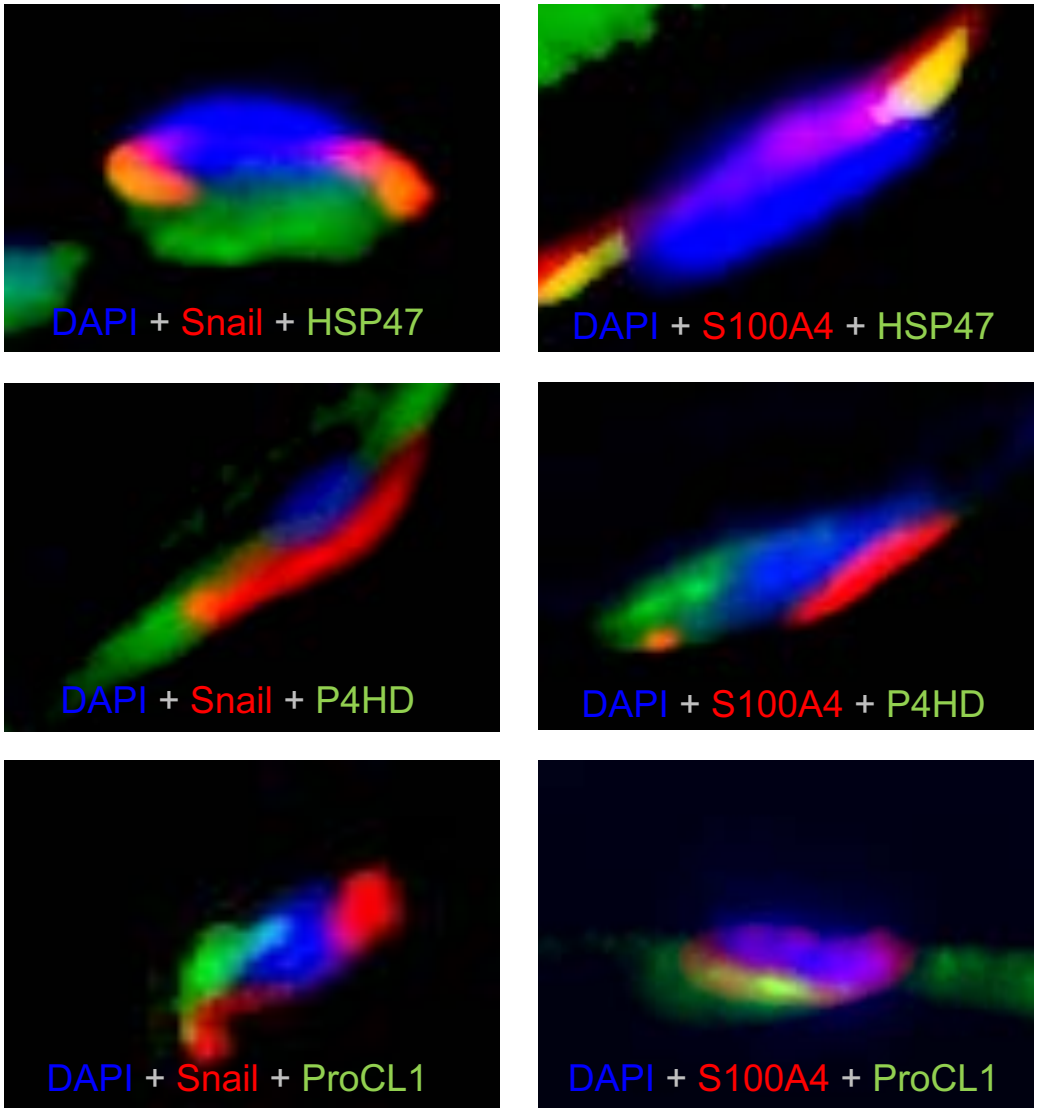
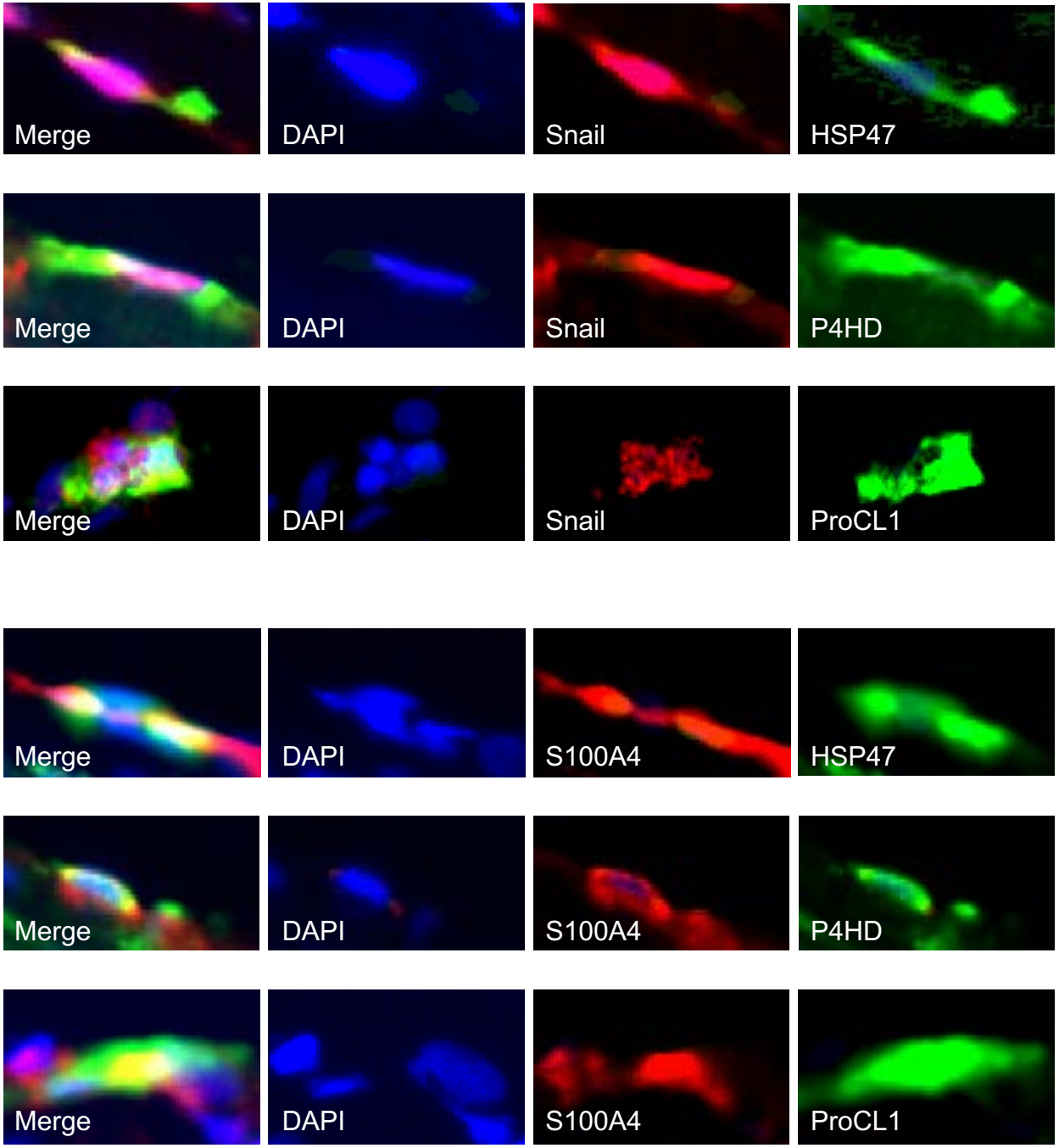

DAPI + Snail + HSP47
DAPI + S100A4 + HSP47
DAPI + Snail + P4HD
DAPI + S100A4 + P4HD
DAPI + Snail + ProCL1
DAPI + S100A4 + ProCL1

Figure 6 old


Merge
DAPI
Snail
HSP47
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DAPI
Snail
P4HD
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DAPI
Snail
ProCL1
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S100A4
HSP47
Merge
DAPI
S100A4
P4HD
Merge
DAPI
S100A4
ProCL1

Figure 6

Table 1. Antibodies used in this study.

Antibody	Manufacturer	Cat#
Snail1 + Snail2	Abcam	ab85931
S100A4	Abcam	ab27957
MT1-MMP	DKF	F-84
Heat shock protein 47	Stressgen	SPA-470
Prolyl-4-hydroxylase	Abcam	ab44971
Procollagen type I	American Research Products	01-20107
CD31	Dako Cytomation	N1596

Table 2. Clinical data of patients included in this study.

	No.	Sex	Age (years)	AF Duration (months)	Heart Disease	LVEF (%)	LAD (mm)	Drug Therapy
Par AF	1	M	36	66	MR	55	68	A, B, L, W
	2	M	63	15	MR	61	45	A, B, L, W
	3	M	63	12	AR	76	48	A, C, L, W
	4	M	69	27	ASD	60	47	B, L, W
	5	M	80	1	AS/MS	39	64	B, L, W
	6	M	71	10	MR	71	54	L, W
	7	F	57	1	ASD	77	41	B, L, W
	8	F	81	1	ASD	55	51	A, B, L, W
	9	F	50	12	AR	47	47	B, L, W
	10	F	58	13	AR/MR	69	50	C, L, W, AD
	11	F	56	1	AR	64	43	B, L, W
	12	M	76	61	MR	73	49	B, L, W
	13	F	68	1	MS	70	47	A, C, L, W
	14	F	63	1	MS	50	50	C, L, W
Per AF	15	M	56	12	MR	65	43	A, B, L, W
	16	F	73	71	MR	57	62	A, L, W
	17	F	59	48	MR	64	56	A, B, L, W, AD
	18	M	56	31	MR	57	45	A, B, C, L, W
	19	F	49	12	MR	67	44	L, N, W
	20	M	63	26	MR	62	57	A, B, L, W
	21	M	59	28	MR	76	58	A, C, L, W, AD
	22	F	73	117	MR	67	58	B, C, L, W

23	M	77	180	MR	59	66	A, C, D, L, W
24	M	55	180	MR	51	60	A, C, L, W
25	M	66	135	MR	72	61	A, B, W
26	F	59	132	MS	56	67	A, L, W
27	F	72	276	MS	67	77	A, L, W
28	F	68	120	MS	73	69	L, W
29	F	74	2	MR	76	57	L, W
30	M	65	108	ASR/MS	58	49	B, L, W
31	M	70	192	MS/TR	50	49	A, B, L, W
32	M	71	36	MR	51	52	L, W
33	F	61	240	MSR	66	80	L, W
34	F	63	312	MSR/ASR/TR	80	56	C, L, W
35	F	62	96	MS	61	58	B, C, L, W
36	M	63	32	MR/ASD	75	53	A, L, W
37	M	57	43	AR	46	59	A, B, C, L, W
38	M	64	84	AS/MR	74	45	A, B, L, W

Par = paroxysmal; Per = permanent; LVEF = left ventricular ejection fraction; LAD = left atrial dimension; MR = mitral regurgitation; AR = aortic regurgitation; AS = aortic stenosis; MS = mitral stenosis; TR = tricuspid regurgitation; ASD = atrial septal defect; A = angiotensin-converting enzyme inhibitor or angiotensin receptor blockers; B = β-blockers; C = calcium channel blockers; L = loop diuretics; N = nitrates; W = warfarin; AD = anti-arrhythmic drug. AF duration is defined as the time