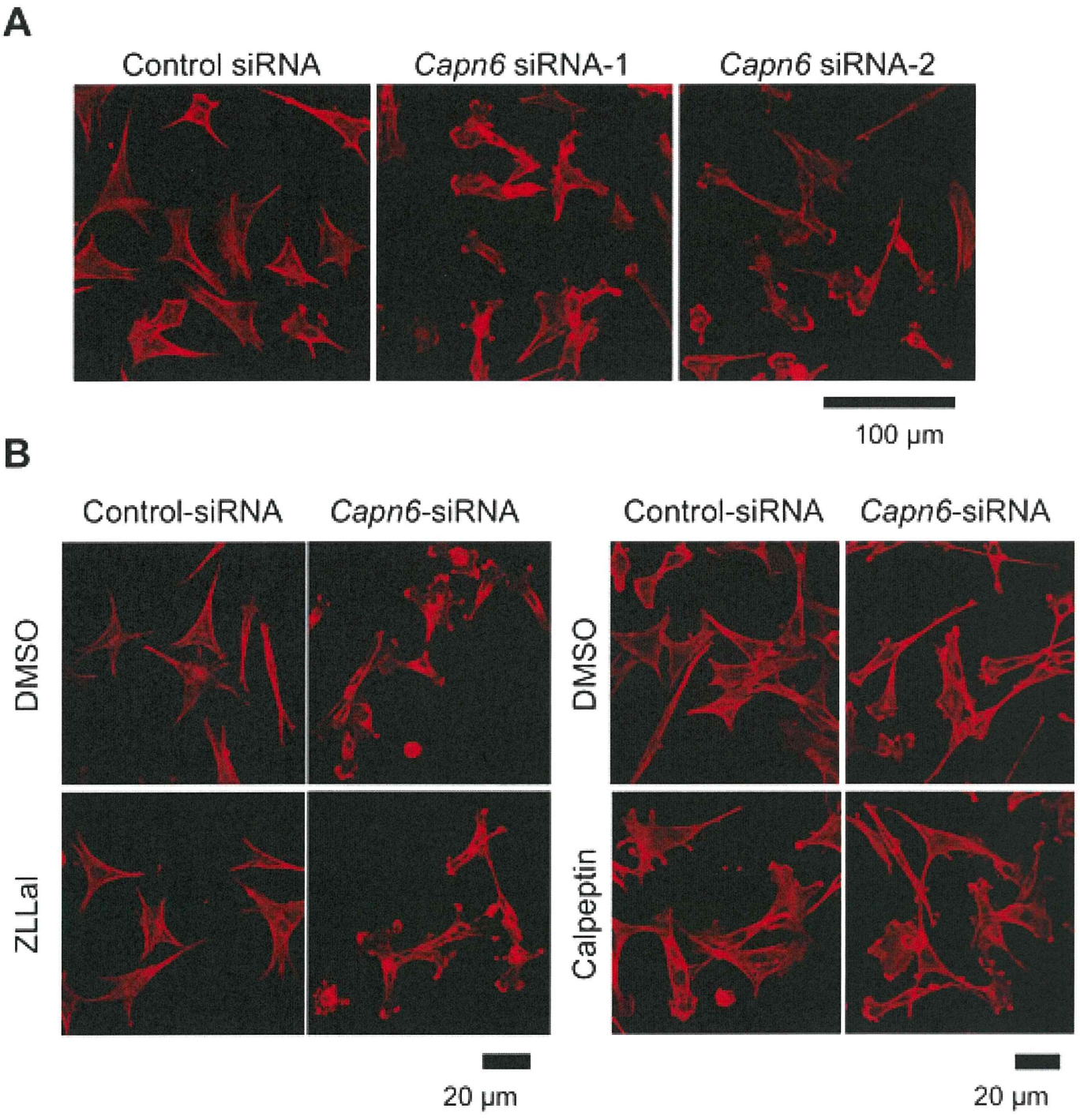
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Control siRNA
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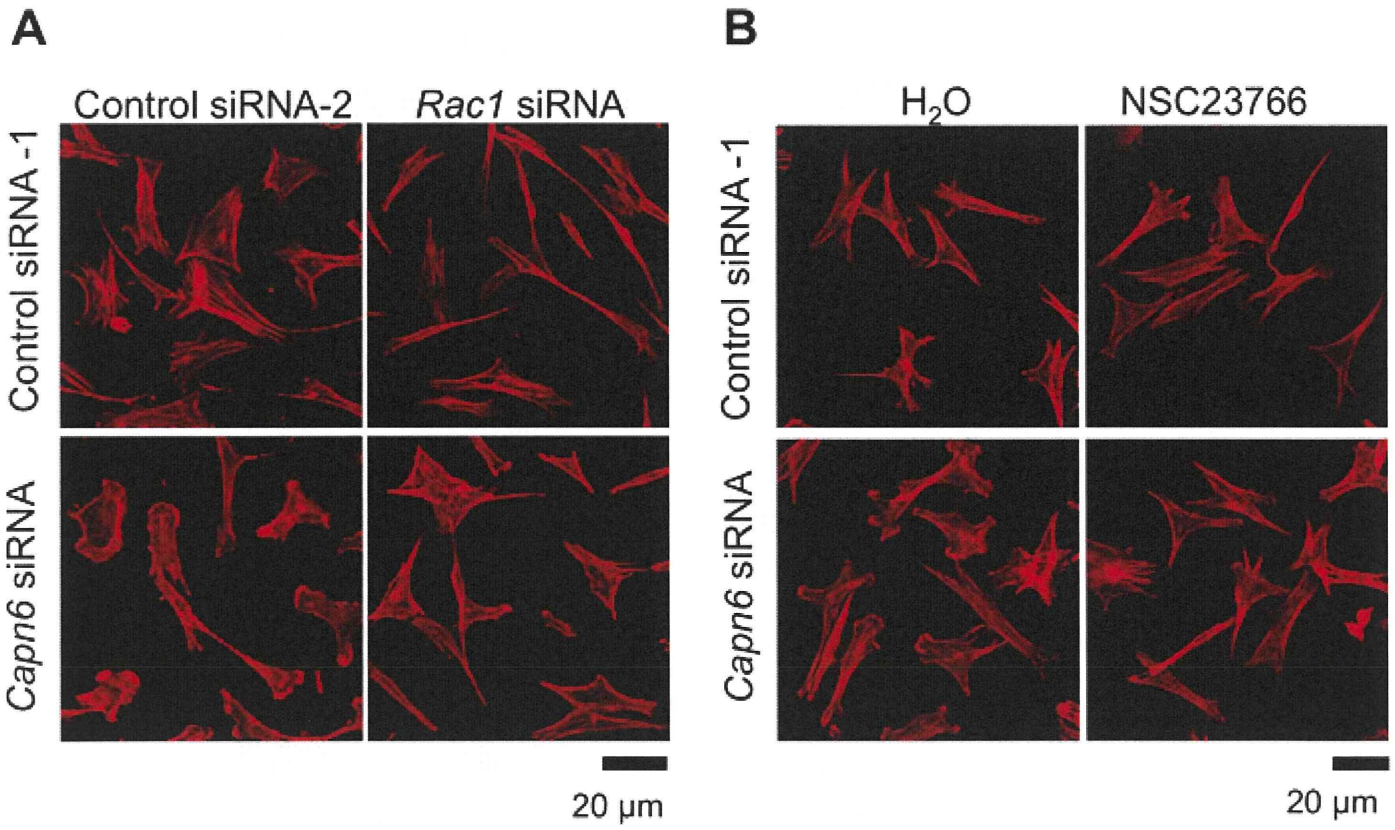
Capn6 siRNA-2
100 μm
B
Control-siRNA
Capn6-siRNA
Control-siRNA
Capn6-siRNA
DMSO
ZLLal
DMSO
Calpeptin
20 μm
20 μm
C
DMSO
ZLLal
Rac1-GTP
Total Rac1
siRNA
Capn6
Control-1
– + – +
+ – + –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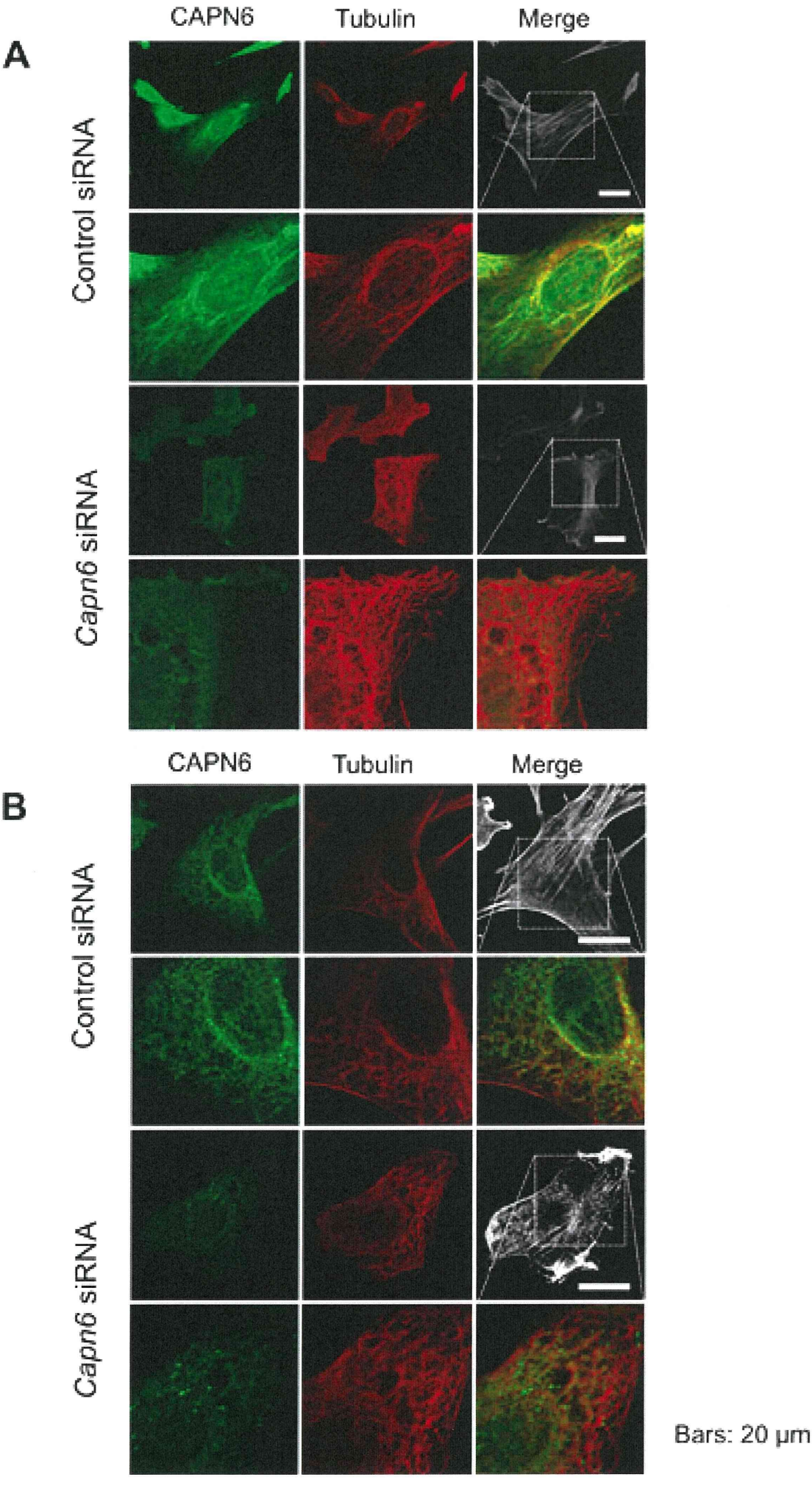
Rac1 siRNA
Control siRNA -1
Capn6 siRNA
20 μm
B
H2O
NSC23766
Control siRNA -1
Capn6 siRNA
20 μm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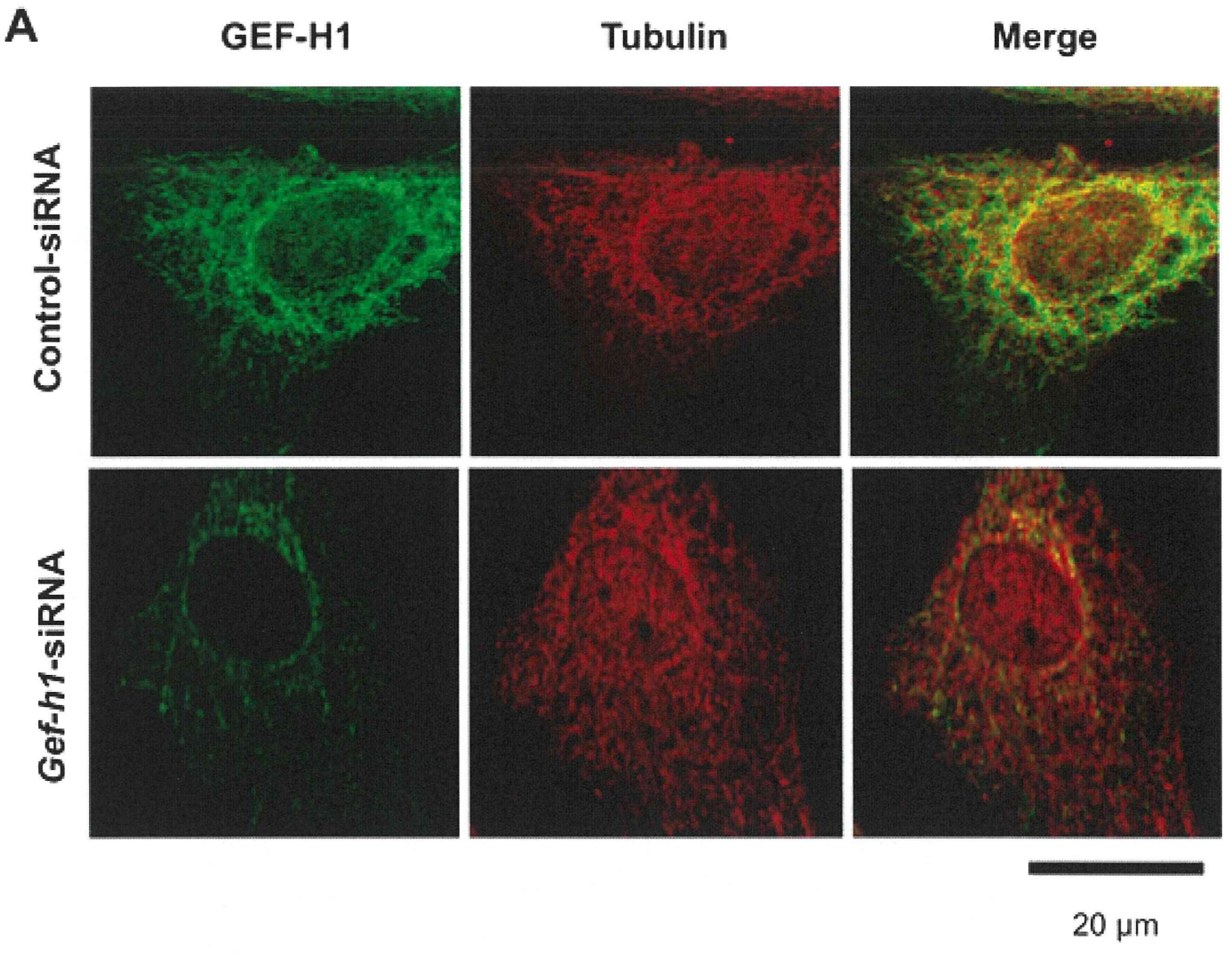
CAPN6
Tubulin
Merge
Control siRNA
Capn6 siRNA
B
CAPN6
Tubulin
Merge
Control siRNA
Capn6 siRNA
Bars: 20 μm


A
GEF-H1
Tubulin
Merge
Control-siRNA
Gef-h1-siRNA
20 μm
B
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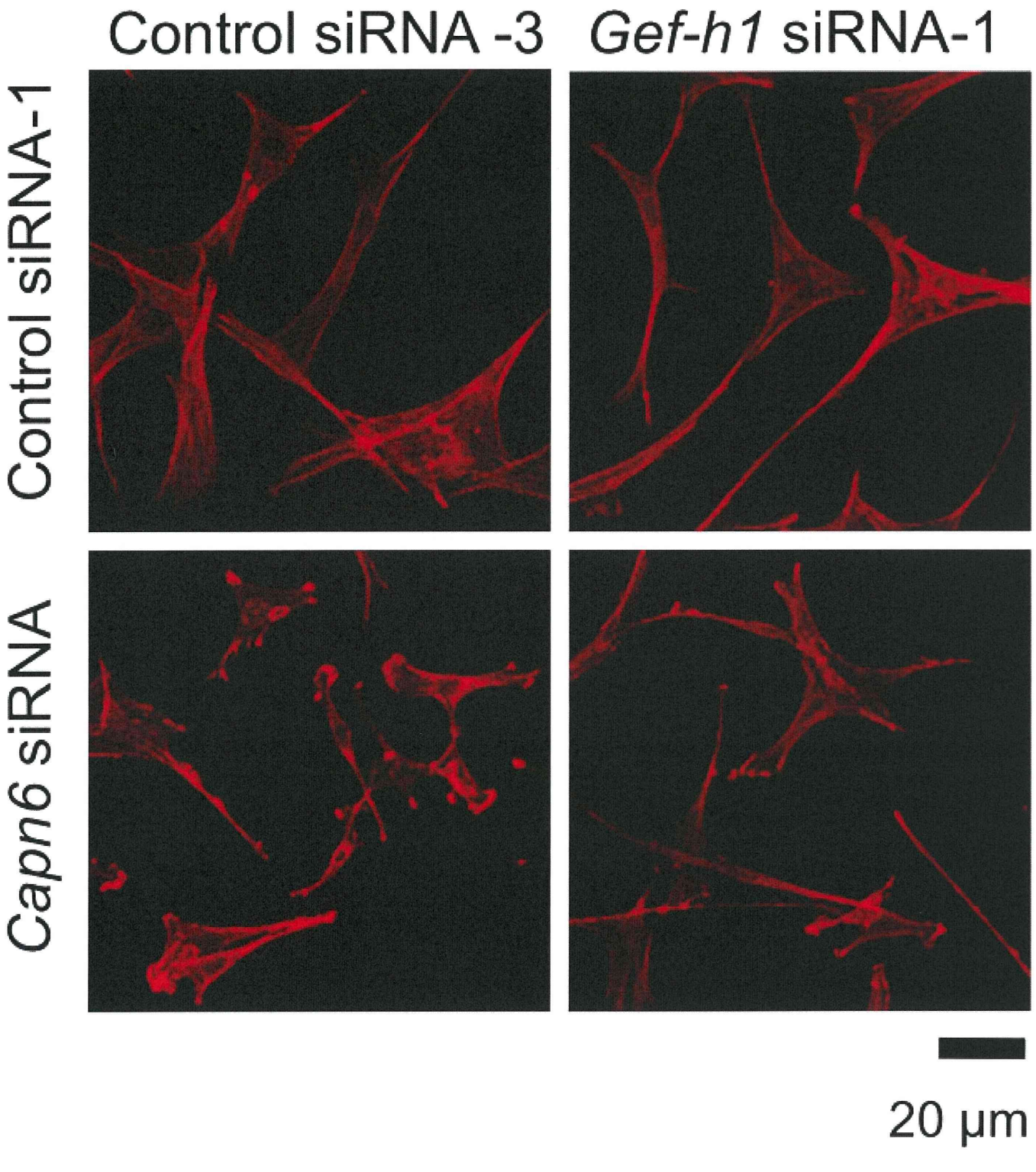
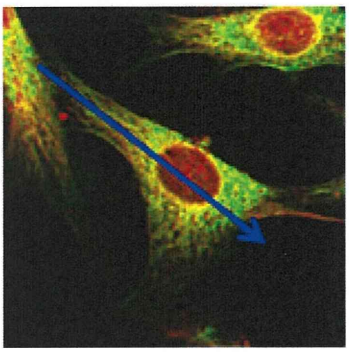
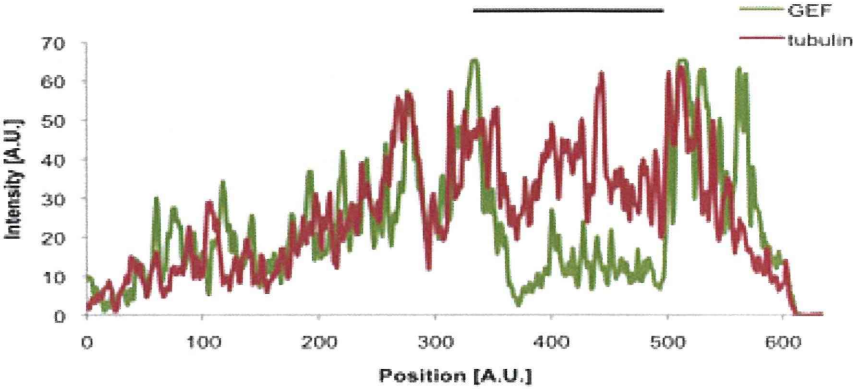
β-Tubulin
Control-siRNA
Gef-h1-siRNA

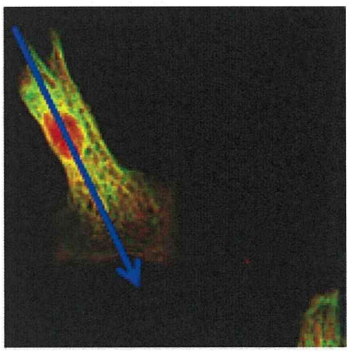

Control siRNA -3
Gef-h1 siRNA-1
Control siRNA-1
Capn6 siRNA
20 µm

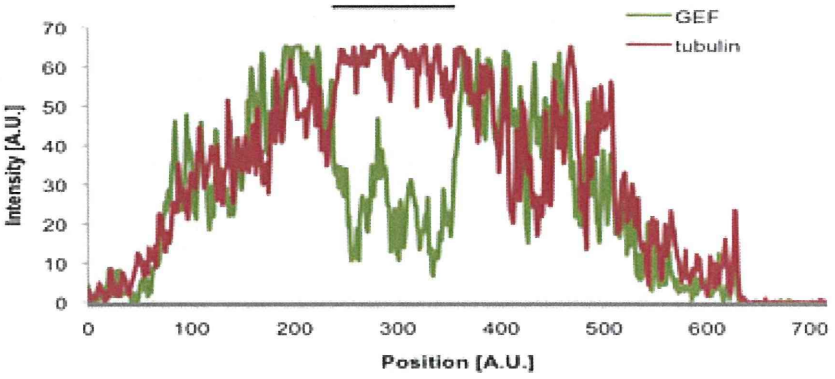
GEF-H1(Green)
Tubulin(Red)

Control siRNA

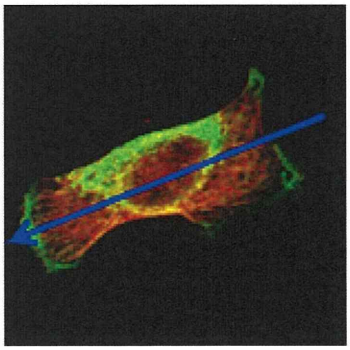


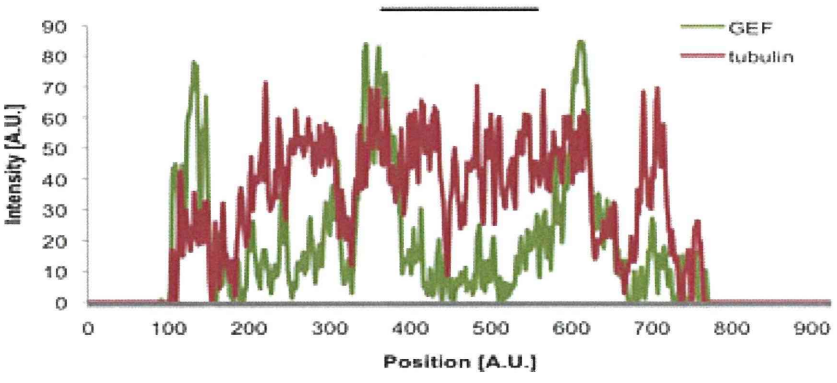


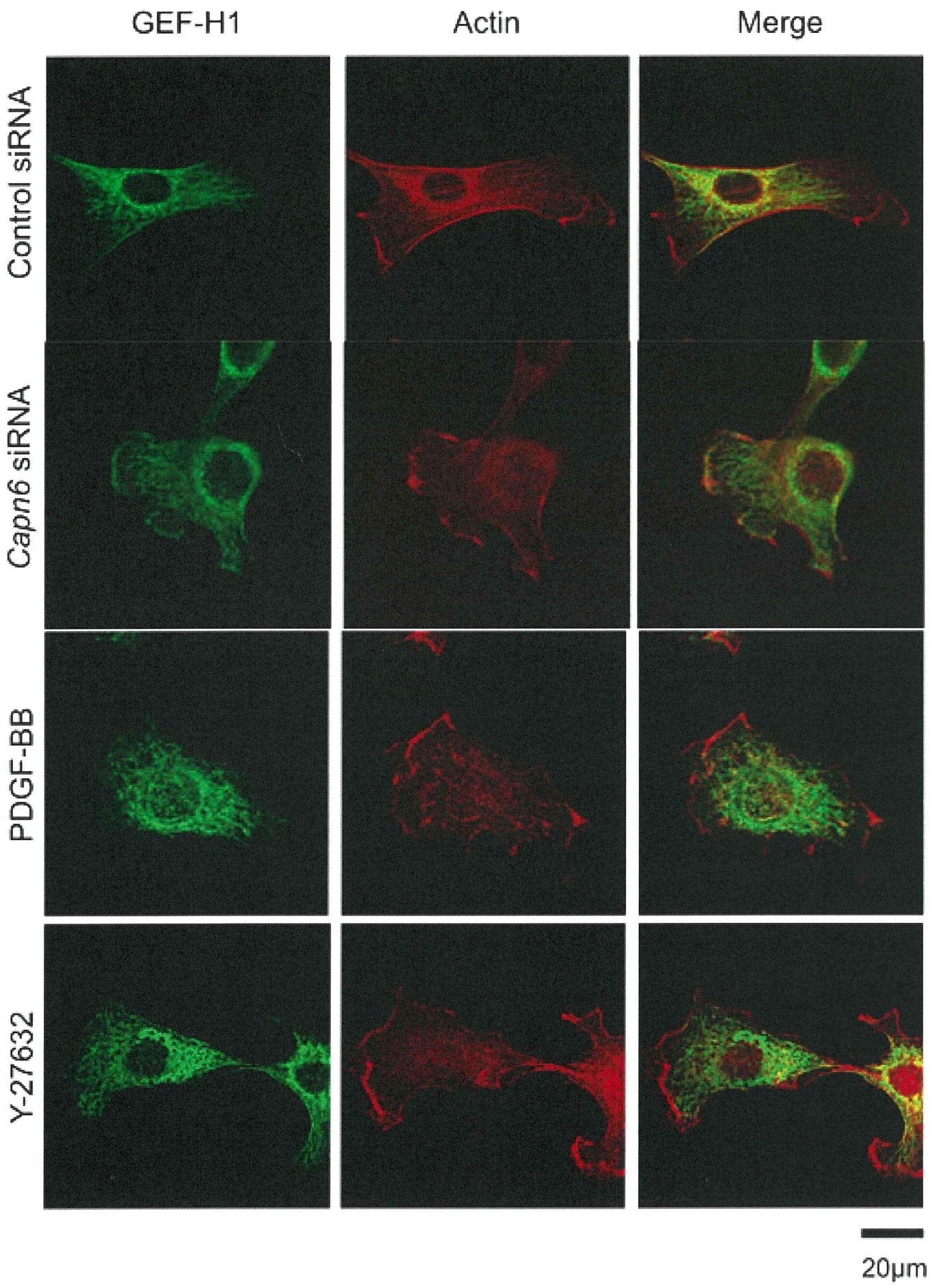


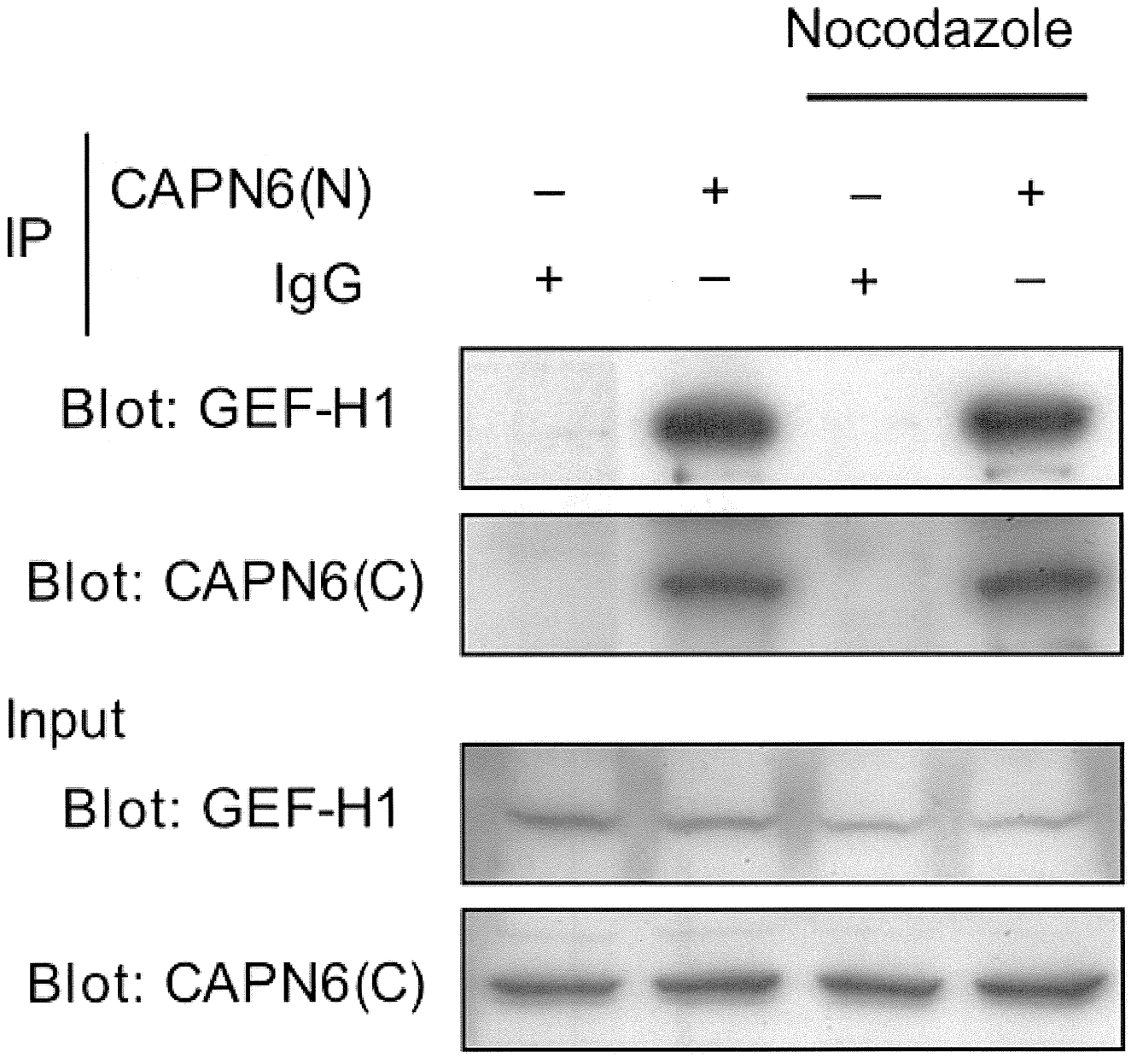


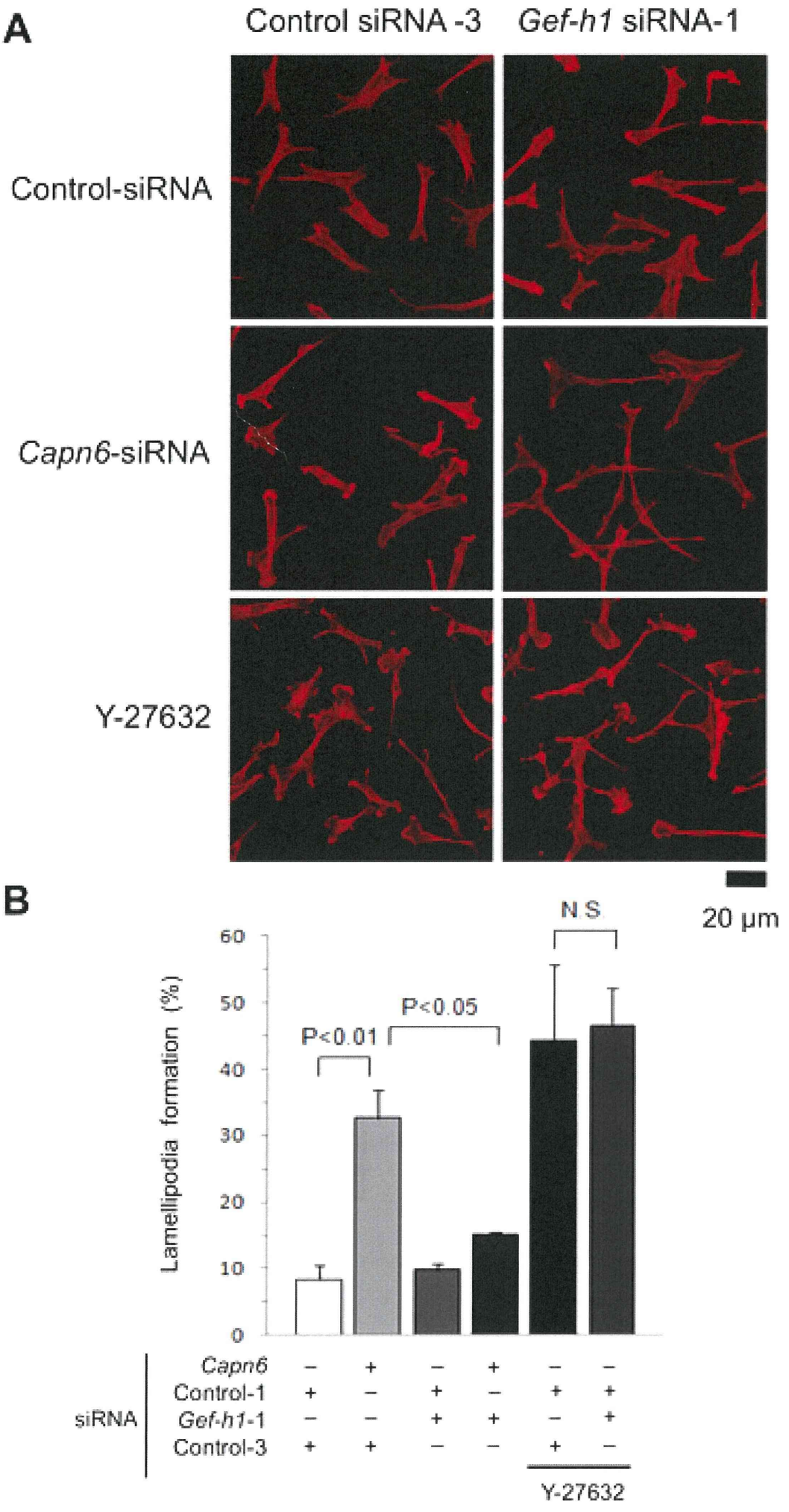
Capn6 siRNA






GEF-H1
Actin
Merge
Control siRNA
Capn6 siRNA
PDGF-BB
Y-27632
20μm


Nocodazole
IP
CAPN6(N)
IgG
–
+
–
+
+
–
+
–
Blot: GEF-H1
Blot: CAPN6(C)
Input
Blot: GEF-H1
Blot: CAPN6(C)


A
Control siRNA -3
Gef-h1 siRNA-1
Control-siRNA
Capn6-siRNA
Y-27632
20 μm
B
N.S
P<0.05
P<0.01
Lamellipodia formation (%)
60
50
40
30
20
10
0
Capn6 – + – + – –
siRNA Control-1 + – + – + +
Gef-h1-1 – – + + – +
Control-3 + + – – + –
Y-27632

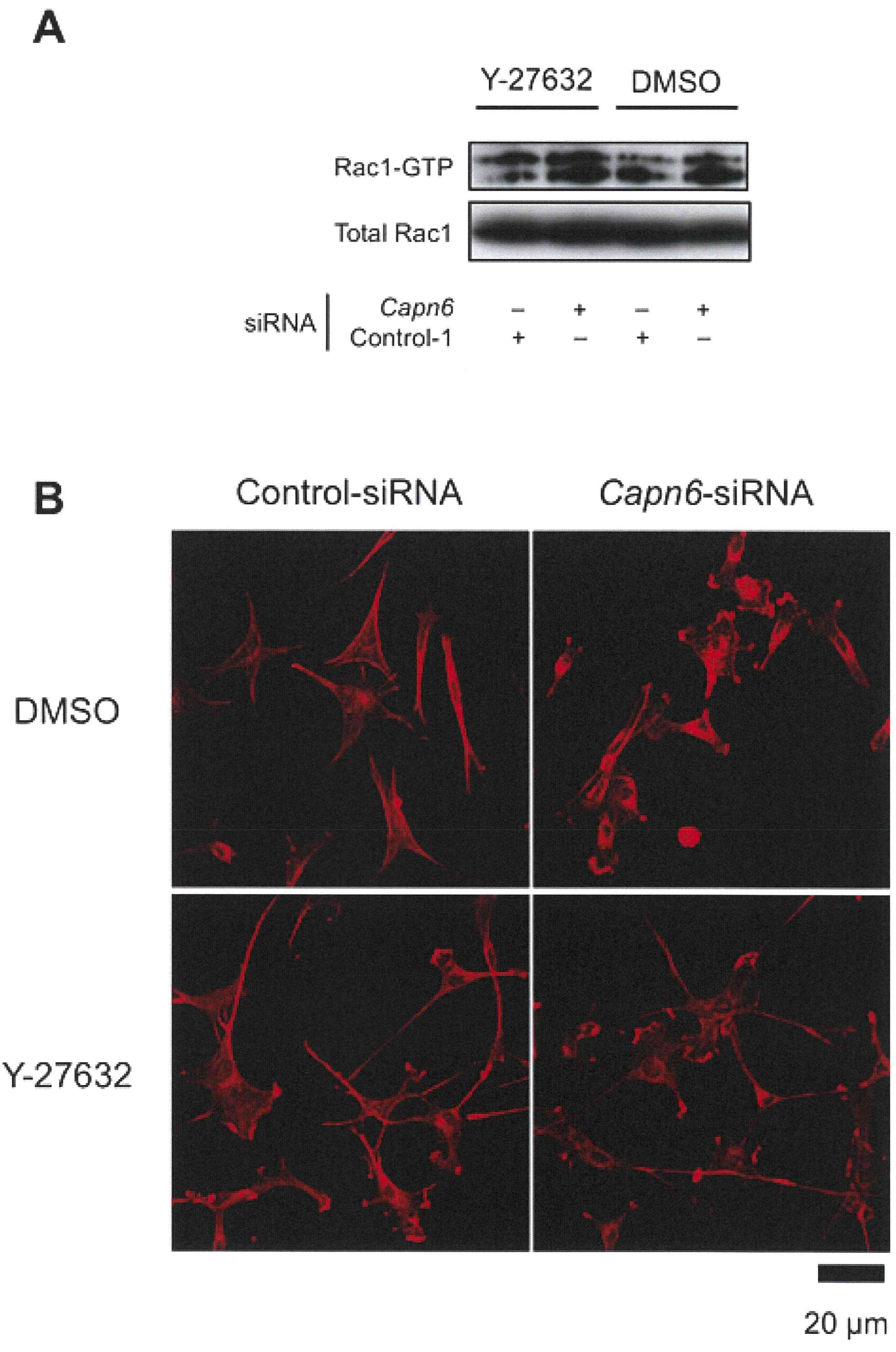

A
Y-27632
DMSO
Rac1-GTP
Total Rac1
siRNA
Capn6
Control-1
– + – +
+ – + –
B
Control-siRNA
Capn6-siRNA
DMSO
Y-27632
20 μm

Table S1. List of siRNAs used in this study

siRNA	Accession No.	Target nucleotides	Sequence
Capn6 siRNA -1	NM_007603	1518 to 1542	5'-GGUUCCGUCUUCACCAUCUGUAUAU-3'
Capn6 siRNA -2		1935 to 1959	5'-GGACCACUGACAUUCCUAUUAUCAU-3'
Rac1 siRNA	NM_009007	631 to 655	5'-GGCGAAAGAGAUCGGUGCUGUCAAA-3'
Gef-h1 siRNA -1	NM_008487	1085 to 1109	5'-CCCGGAACUUUGUCAUCCAUCGUUU-3'
Gef-h1 siRNA -2		3410 to 3434	5'-CCAUAAGUAUUGGAGAGCCUCUCCU-3'
RhoA siRNA	NM_016802	574 to 598	5'-GGGAAGCAGGUAGAGUUGGCUUUAU-3'

Control siRNA -1	Scrambled sequence of *Capn6* siRNA -1	5'-GGUUGCUUCCACUACGUCAUUCUAU-3'
Control siRNA -2	Scrambled sequence of *Rac1* siRNA	5'-GGCAGAACUAGGUGGUGUCCAGAAA-3'
Control siRNA -3	Scrambled sequence of *Gef-h1* siRNA -2	5'-CCAAUGAGGUUGAGACUCCUAUCCU-3'
Control siRNA-4	Scrambled sequence of *RhoA* siRNA	5'-GGGACGUGGAGAUUGCGGUUAAUAU-3'

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Tenascin C may regulate the recruitment of smooth muscle cells during coronary artery development

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ABSTRACT

Tenascin C (TNC) is an extracellular glycoprotein that is thought to be involved in tissue remodeling during organogenesis and regeneration. Using avian embryonic hearts, we investigated the spatiotemporal expression patterns of TNC during the formation of the proximal coronary artery. Immunohistochemistry showed that TNC was deposited around the developing coronary stem and that TNC colocalized with vascular smooth muscle α-actin. A quail-chick chimera, in which a quail proepicardial organ (PEO) had been transplanted, showed that quail tissue-derived cells contributed to the establishment of the endothelial and mural cells of the proximal coronary artery, and the quail tissue-derived mural cells displayed TNC. Proepicardial cells cultured in TNC showed the myofibroblast/smooth muscle cell phenotype and neutralizing anti-TNC antibody suppressed the expression of smooth muscle markers. These observations suggest that TNC plays a role in the mural smooth muscle development of the nascent proximal coronary artery.

1. Introduction

Tenascin C (TNC) is an extracellular matrix glycoprotein that regulates various cellular events such as cellular differentiation, migration, survival, and proliferation (reviews, Jones and Jones, 2000; Imanaka-Yoshida et al., 2004; Tucker and Chiquet-Ehrismann, 2009; Midwood and Orend, 2009). It has been reported that TNC is expressed in regions where extensive tissue remodeling occurs during embryogenesis and organogenesis. The expression of TNC in adult tissue is very limited; however, its expression is significant in pathological lesions, such as cancer invasion and wound healing. In the adult heart, the expression of TNC is low but its expression is upregulated in lesion of myocardial infarction to recruit myofibroblasts (Imanaka-Yoshida et al., 2001a, 2001b; Tamaoki et al., 2005). During cardiovascular development, TNC is reported to be expressed in the precardiac mesoderm, valvuloseptal endocardial cushion tissue, subepicardial mesenchyme, and nascent great arteries (Hurle et al., 1990; Crossin and Hoffman, 1991; Sugi and Markwald, 1996; Imanaka-Yoshida et al., 2003; Zhang et al., 2006). Despite our knowledge of the spatiotemporal expression of TNC in the developing heart, the role of TNC is largely unknown, because TNC knockout mutant mice have only subtle phenotypic changes (Saga et al., 1992; Ishii et al., 2008; Gurevicius et al., 2009). However, recent reports have proposed that TNC plays multiple important roles during tissue repair (Midwood and Orend, 2009; Nishioka et al., 2010). For example, the involvement of TNC in angiogenesis has been suggested based on its expression being highly associated with angiogenesis in a wide range of pathological states, such as cancer (Jallo et al., 1997), diabetes (Castellon et al., 2002), and aortic aneurysms (Paik et al., 2004). In the heart, Ballard et al. (2006) demonstrated the essential role of TNC in postnatal angiogenesis.

During cardiogenesis, the coronary vascular system develops after the ventricular compact layer has thickened because nutrition and oxygen are unable to penetrate cardiac muscle by simple diffusion from the endocardium. Among vertebrates, mammals, birds, and reptiles have complete coronary systems, in which the coronary arteries originate from the aorta and lead to veins. In the remaining vertebrates, most amphibians have no coronary vessels and the presence of coronary vessels is variable in fish (Reese et al., 2002). Recently, the developmental mechanisms of coronary vasculogenesis have been reevaluated (Reese et al., 2002; Olivey et al., 2004;

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Mu et al., 2005; Ratajska et al., 2008). The key events of coronary system development include the generation of the proepicardial organ (PEO); the formation of the epicardium; the generation of the subepicardial mesenchyme; the formation, remodeling and migration of the final vascular plexus; and connection to the aortic sinuses (Mu et al., 2005). The PEO, a progenitor of the epicardium and coronary vessels that develops on the dorsal surface of the splanchnic mesoderm just ventral to the sinus venosus, migrates across the ventricular surface to establish the initial epithelial epicardium (Hiruma and Hirakow, 1989). The nascent epicardium seeds mesenchymal cells into the subepicardial extracellular space, where they differentiate into vascular endothelial, cardiac fibroblasts, and smooth muscle cells (Poelmann et al., 1993; Mikawa and Gourdie, 1996). Blood islands develop from the subepicardial mesenchyme, and they connect with each other to form sinusoidal communications on the surface of the heart (Hirakow 1983; Kattan et al., 2004). Thereafter, endothelial strands, which extend from the sinusoids surrounding the outflow tract, invade into the aortic sinuses. Later, the endothelial strands that have invaded into the facing sinuses (the right and left coronary sinuses) fuse to form a single coronary stem (Bogers et al., 1989; Waldo et al., 1990; Ando et al., 2004). After the coronary plexus and the aorta have become interconnected with each other, the coronary arteries recruit vascular smooth muscle cells and the adventitia for histogenesis of the coronary arterial wall in the proximal to distal direction (Vrancken Peters et al., 1997; Landerholm et al., 1999).

In the present study, using avian embryonic hearts, we investigated the immunohistochemical localization of TNC in developing coronary artery stems and examined whether TNC is capable of facilitating proepicardium-derived cells to differentiate into the myofibroblast/smooth muscle cell phenotype.

2. Materials and methods

2.1. Avian embryos

Quail and chick embryos were staged according to the Hamburger and Hamilton staging system (1992). Then, the staged embryos (at least 3 embryos per stage were prepared) were collected in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS, which was injected into the ventricle using a fine glass needle. Their hearts were also fixed in 4% paraformaldehyde in PBS at 4 °C for 12 h. After being washed in PBS, the outflow tract regions were resected and embedded in paraffin. Serial 5 μm thick sections were cut, transferred to glass slides, and subjected to immunostaining.

2.2. Antibodies

The primary antibodies we used were as follows: rabbit polyclonal anti-TNC (Imanaka-Yoshida et al., 2001a, 2001b), monoclonal anti-TNC (clone 4F10, IBL, Gumma, Japan), anti-smooth muscle α-actin (SMA, clone 1A4, Sigma), anti-calponin (clone CP93, Sigma), anti-GAPDH (glyceraldehydes-3-phosphate dehydrogenase, CHEMICON), anti-quail cell (QCPN, Developmental Studies Hybridoma Bank), and anti-quail endothelial cell (QH1, Developmental Studies Hybridoma Bank). The following secondary antibodies were also used: TRITC-conjugated goat anti-mouse IgG2a, FITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology), FITC-conjugated goat anti-rabbit IgG (MBL, Nagoya, Japan), and peroxidase conjugated goat anti-mouse IgG (MBL, Nagoya, Japan).

2.3. Indirect immunostaining

Indirect immunostaining was performed as described elsewhere (Ando et al., 2004; Tamaoki et al., 2005). After deparaffinization, the sections were blocked with 1% bovine serum albumin (BSA) in PBS and incubated with the primary antibody (mixture) followed by secondary antibody (mixture). Diaminobenzidine/H_2O_2 solution was used to demonstrate peroxidase conjugated antibody binding. The samples were observed under a conventional fluorescent or light microscope, and images were recorded with a cooled CCD camera.

To create superimposed images (Fig. 1), the outflow tract regions from ED5-8 quail hearts were embedded in paraffin, and serial cross sections were prepared. Each serially sectioned sample was placed on two sets of glass slides (one set for TNC staining and the other for QH1 and 1A4). Using TRI/3D-SRF-II (Ratoc System Engineering) and Photoshop (Adobe), the signals for each stain were converted into pseudo-color, and then the resulting images from 40 to 70 sections were superimposed onto each other. Whole-mount antibody staining was performed as described elsewhere (Kataoka et al., 1997; Metzger et al., 2008). The fixed hearts were incubated with monoclonal anti-TNC antibody (4F10), processed with TSA kit with HRP-goat anti-mouse IgG and Alexa Fluor546 tyramide (Molecular Probes), and subsequently incubated with FITC-conjugated anti-smooth muscle α actin. Then, the samples were observed under a laser confocal microscope (Zeiss).

The cultured epicardium was fixed with 4% paraformaldehyde/PBS for 1 h at RT, rinsed with PBS, and blocked with 1% BSA/PBS containing 0.1% triton X-100. Samples were then incubated with a primary antibody mixture (QH1/1A4 or CP93/1A4) at 4 °C overnight, rinsed with PBS and incubated with a secondary antibody mixture for 1 h at RT. The nuclei were then stained with DAPI for 20 min, rinsed with PBS, and mounted. Then, the samples were observed under a laser confocal microscope (Zeiss) or conventional fluorescent microscope (Olympus).

2.4. Epicardial culture experiment

ED6 quail or chick hearts were collected in PBS, and the epicardium was cultured according to a modified version of the method described by Compton et al. (2006). The hearts were placed ventral side down on 8-well chamber slides (Nunc), which had been coated with type I collagen (50 μg/ml, 12 h, Becton Dickinson), followed by coated with purified TNC (0–10 μg/ml, 12 h; Tamaoki et al., 2005), supplemented with serum free defined medium (75% DMEM, 25% McCoy's medium, containing 10^{-7} M dexamethasone and penicillin-streptomycin; Matsui et al., 2008). After 20 h incubation, the hearts were removed, and the remaining epicardial explants were cultured for up to 48–72 h. In neutralizing experiment, slides were coated with type I collagen and 5 μg/ml TNC, and epicardial explants were cultured in the medium supplemented with normal rabbit IgG (Sigma) or polyclonal rabbit anti-TNC neutralizing antibody (Tamaoki et al., 2005). Resultant cultures were observed under a phase contrast microscope to examine any phenotypic changes and then subjected to immunohistochemistry and Western blotting.

2.5. Western blotting

Six epicardium cultures were homogenized in SDS sample buffer (2 μl/explant) (62.5 mM Tris–HCl, 10% glycerol, 2% SDS, 5% 2-β mercaptoethanol, and 1 mM Na_3VO_4; pH6.8). After heat denaturation at 95 °C for 5 min, samples (approximately 5 μg of protein) were subjected to 10 or 12% SDS-polyacrylamide gel electrophoresis and then transferred onto Immobilon-P membranes (Millipore). After being blocked with 5% non-fat dry milk, the membranes were incubated with primary antibody for 2 h at room temperature. After being washed, they were incubated with horseradish peroxidase-conjugated secondary antibodies.

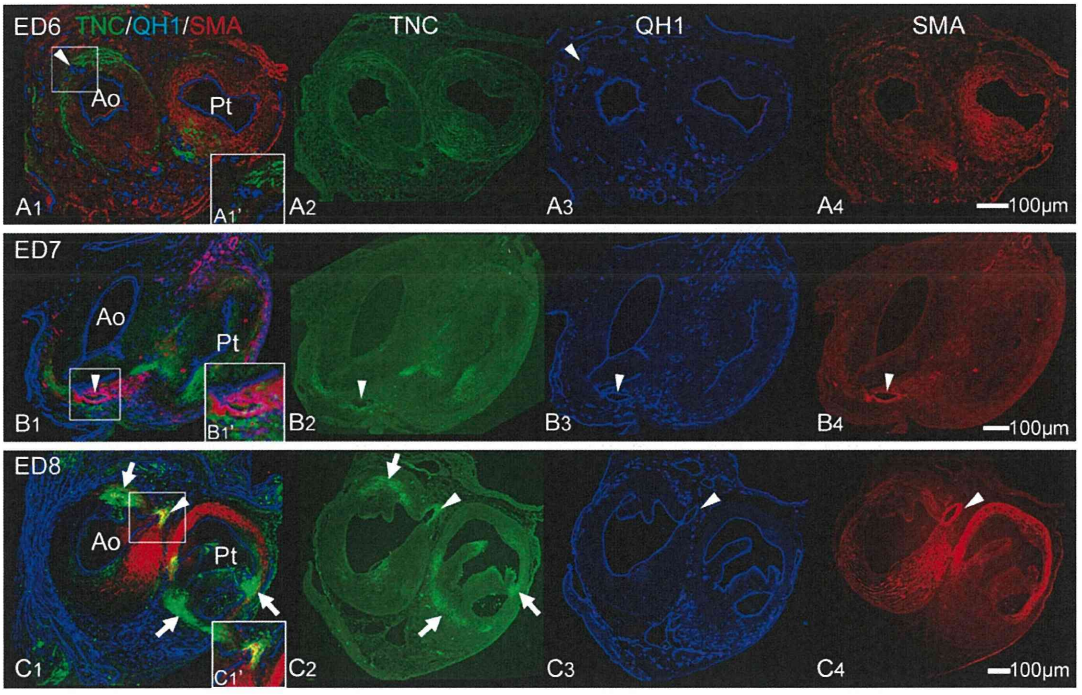

Fig. 1. Immunohistochemical localization of TNC, QH1, and SMA. *A1-4 (ED6)*: A QH1-positive endothelial strand, which is an anlage of the coronary stem, is invading the aortic wall (arrowheads). TNC was expressed around the outer wall of the aortic root but did not colocalize with the QH1-positive endothelial strand. A1' shows a high magnification of boxed area in A1. *B1-4 (ED7)*: At this stage, endothelial strands that penetrated the aortic sinus united to form a single coronary stem (arrowheads). TNC was deposited around the coronary stem, where SMA-positive mural cells accumulated (arrowheads). B1' shows a high magnification of boxed area in B1. *C1-4 (ED8)*: At this stage, the single coronary stem had fully developed, and both TNC and SMA were strongly expressed in the wall of the coronary artery (arrowheads). TNC was also deposited in the wall of the ascending aorta (Ao) and the pulmonary trunk (Pt), to which semilunar valves are connected (arrows). C1' shows a high magnification of boxed area in C1. Ao, ascending aorta; Pt, pulmonary trunk; QH1, quail endothelial marker; SMA, smooth muscle α-actin; TNC, tenascin C.

Immunoreactive bands were visualized using ECL detection reagent (Amersham).

2.6. Quall-chick PEO chimera

Quail-chick PEO chimeras were produced according to the method of Männer (1999). Stage 16–17 quail hearts were inspected to confirm that the PEO was not attached to the dorsal wall of the heart. Using an eggshell membrane, the quail PEO was isolated together with the sinus venosus and transplanted behind the chick heart. After an appropriate incubation period, the embryos were sacrificed, and their hearts were subjected to immunostaining for TNC and either QCPN or QH1.

3. Results

3.1. Immunohistochemical localization of tenascin C during coronary arterial stem development

We previously reported that during coronary arterial stem development in the quail embryonic heart, endothelial progenitor cells begin to invade into the aortic root at ED6, and the two-coronary system is completed at ED8 (Ando et al., 2004). To examine the spatiotemporal deposition of TNC during coronary artery stem development, we stained quail ED5-8 embryonic hearts with anti-TNC, QH1 (quail endothelial marker), and 1A4 (anti-smooth muscle α-actin, SMA) antibodies. At ED5, no QH1-positive endothelial strands (the anlagen of the coronary stem) penetrating the aortic wall were observed (not shown). At ED6-6.5, QH1-positive endothelial strands that penetrated the aortic wall were observed. TNC was distributed circumferentially in the wall of the aortic root; however, the TNC deposition did not coincide with the regions that the QH1-positive endothelial strands had penetrated (arrowheads in Figs. 1A and $3B_2$). At this stage, SMA was not detectable around the endothelial strands. At ED7, the endothelial strands penetrating the aortic sinus are unified and form a single coronary stem (Ando et al., 2004). At this stage, TNC was deposited around the coronary stem, where SMA-positive cells had accumulated (arrowheads in Fig. 1B). At ED8, the single orifice of the coronary artery had fully developed (arrowheads in Fig. 1C). TNC was deposited along the coronary arterial wall, in which SMA expression was extensive (arrowheads in Fig. 1C). TNC was also deposited in the aortic/pulmonary wall, to which semilunar valves were connected (arrows in Fig. 1C). Whole mount immunostaining of ED9 quail hearts showed that TNC was localized along the SMA-positive coronary artery (arrows in Fig. 2); and immunohistological tissue section showed that TNC was deposited around SMA-positive mural cells (arrowheads in Fig. 2). Our observations indicated that TNC was expressed at the developing proximal coronary artery, where SMA-positive cells, presumably prospective vascular smooth muscle cells of the tunica media, accumulated.

3.2. Quail-chick chimera experiments

It has been reported that both the endothelial and smooth muscle cells of coronary vessels originate from the PEO-derived subepicardial mesenchyme in avians (Mikawa and Gourdie, 1996; Männer, 1999), while the endothelial cells of coronary vessels are derived from the sinus venosus in mice (Red-Horse et al., 2010); thus, the origin of coronary endothelial cells is controversial. We next examined whether PEO-derived cells contribute to the proximal coronary artery. Quail-chick chimeras, in which quail

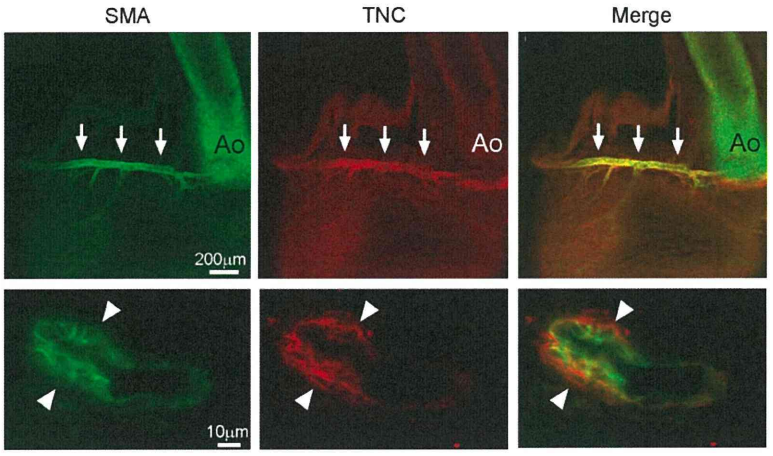

Fig. 2. A chick embryonic heart doubly stained for tenascin-C (TNC) and smooth muscle α-actin (SMA). Wholemount double immunostaining shows that TNC is localized along the SMA-positive coronary artery at the ED9 (arrows). Immunohistochemical staining of tissue section shows that the deposition of TNC is colocalized with SMA-positive mural cells in the developing coronary artery (arrowheads). Ao, ascending aorta.

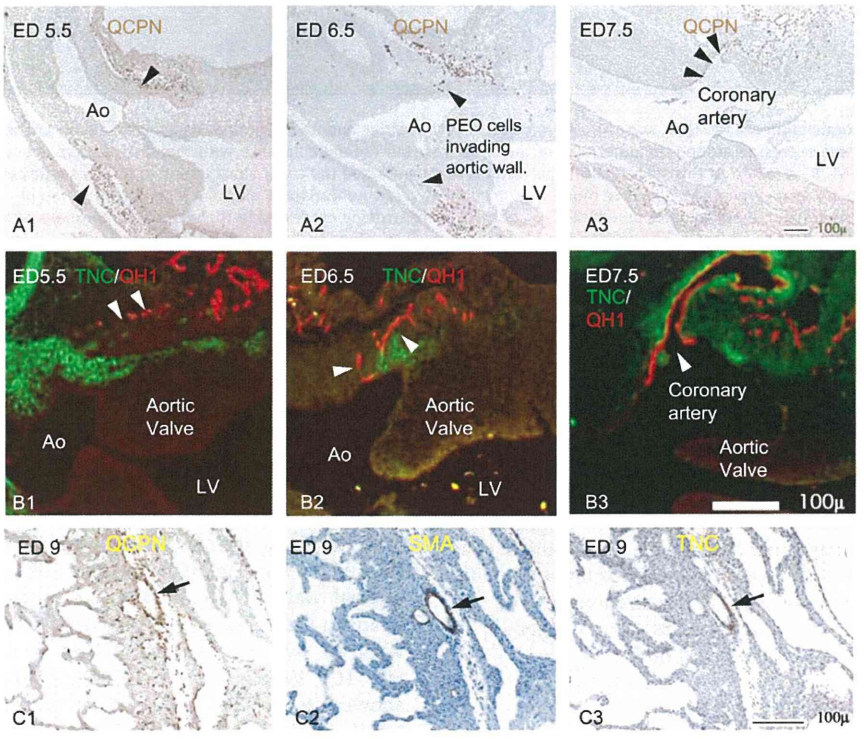

Fig. 3. PEO derived cells may develop into the endothelial and mural cells of the proximal coronary artery. Quail-chick chimeras, in which quail PEO were transplanted, were produced. After further incubation, the embryos were sacrificed, and the hearts were stained with antibodies for QCPN, QH1, 1A4, and TNC. *A1-3*: In chimeric chick hearts, QCPN-positive cells were found around the base of the ascending aorta (Ao) at ED 5.5 (arrowheads in A1), in the aortic wall at ED 6.5 (arrowheads in A2), and later in the endothelial lining of the proximal coronary artery (arrowheads in A3). *B1-3*: Transplanted PEO-derived QH1-positive endothelial cells are found around the aortic root at ED 5.5 (arrowheads in B1), in the aortic wall at ED 6.5 (arrowheads in B2), and in the endothelial lining of the coronary stem at ED7.5 (arrowheads in B3). From ED5.5-6.5, TNC was deposited in the aortic root but its association with QH1-positive cells was unclear (B1, 2). After the single coronary artery had fully developed, TNC was deposited subjacent to the QH1-positive endothelial cells (B3). *C1-3*: In the developing proximal coronary artery, both the QCPN and SMA-positive mural cells expressed TNC (arrows in C1-3). Ao, ascending aorta; ED, embryonic day; LV, left ventricle; QCPN, quail nuclear marker; QH1, quail endothelial marker; SMA, smooth muscle α-actin; TNC, tenascin C.

PEO had been transplanted, were produced and stained with QCPN (a quail nuclear marker), QH1 (a quail endothelial marker), 1A4, and anti-TNC. In chick embryonic hearts, in which quail PEO had been transplanted, QCPN-positive quail cells were found around the root of the ascending aorta (brown dots indicated by arrowheads in Fig. $3A_1$) and later in the aortic wall (arrowheads in Fig. $3A_2$). At ED 7.5, when the single coronary orifice had fully formed, both endothelial and subendothelial cells expressed the quail marker QCPN (arrowheads in Fig. $3A_3$). In the chimeric heart, TNC deposition (green in Fig. 3B) was observed in the root of the aorta, but no clear colocalization with QH1-positive quail PEO-derived endothelial progenitors was observed (red, Figs. $3B_1$, B_2). After the completion of the single coronary stem, strong anti-TNC staining became detectable subjacent to the QH1-positive

coronary endothelial cells (Fig. 3B$_3$). Immunostaining for TNC, QCPN and SMA in daughter sections prepared from chimeric chick heart showed that TNC, QCPN and SMA were deposited in the mural cells of the proximal coronary artery, suggesting PEO-derived mural cells (presumably nascent smooth muscle cells) express TNC (arrows in Fig. 3C). Similarly prepared sections, which were doubly stained with anti-SMA and QCPN, showed that SMA was expressed in QCPN-positive mural cells (CA in Supplemental Fig. 1). Observations suggest that PEO-derived cells contribute to the development/differentiation of both the endothelial and smooth muscle cells of the proximal coronary arteries and that TNC expression is closely associated with the recruitment of PEO-derived mural cells.

3.3. Cultured epicardial cells with TNC show SMC characteristics

We next examined whether TNC facilitates the differentiation of epicardial cells into smooth muscle cells in culture. The nascent epicardia from ED6 hearts were cultured on chamber slides with or without purified TNC. After 72 h, the cultures were examined for phenotypic changes under a phase-contrast microscope (Fig. 4A). In the epicardia cultured on slides coated with TNC, the cultures showed a whirl-like appearance, which was consisting of spindle-shaped cells and similar to hill-and-valley

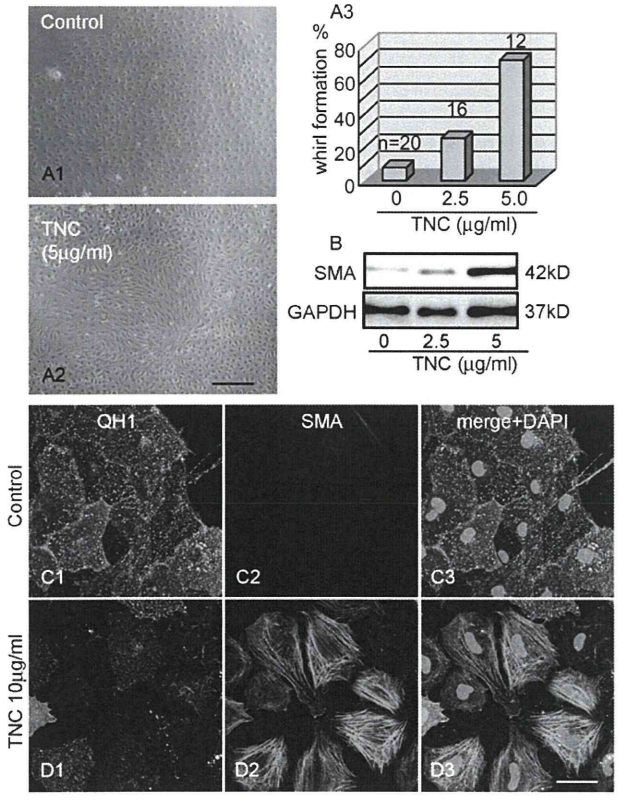

Fig. 4. The epicardium cultured in TNC showed smooth muscle cell characteristics. The nascent epicardia from ED6 quail hearts were cultured with or without TNC. After 72 h, the cells cultured in TNC showed a whirl-like appearance consisting of spindle-shaped cells (A2, 3), while the control cultures showed a typical cobble stone appearance (A1, 3). Western blotting showed that SMA was predominantly expressed in the cells cultured in TNC (B). Immunostaining showed that the epicardial cells cultured without TNC showed tight cell-cell contact and expressed the quail endothelial marker QH1 (C). On the other hand, the cells cultured in the TNC were separated and expressed SMA (D). A1 and 2, phase-contrast microscopic images; DAPI, nuclear staining; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; QH1, quail endothelial marker; SMA, smooth muscle α-actin; TNC, tenascin C. Bar, 250 μm (A); 10 μm (C and D).

morphology without thick multilayer (Figs. 4A$_2$ and A$_3$). On the other hand, the epicardial cells cultured without TNC showed a typical cobblestone appearance (Figs. 4A$_1$ and A$_3$). The resulting cultures were subjected to immunological detections for SMA expression. Western blot analysis showed that the amount of SMA was predominant in cells cultured with TNC in a dose dependent manner (Fig. 4B). Immunostaining showed that the epicardial cells cultured without TNC showed tight cell-cell contact and that many cells expressed the quail endothelial marker QH1 (Fig. 4C). On the other hand, the cells cultured with TNC were separated and expressed SMA (Fig. 4D). We next examined whether anti-TNC neutralizing antibody (Tamaoki et al., 2005) was capable of inhibiting the expression of smooth muscle markers. The epicardia from ED6 chick heart were cultured on TNC-coated slide supplemented with medium containing anti-TNC antibody for 48 h, and resultant cultures were subjected to immunological detections for smooth muscle markers (Fig. 5). As shown in Fig. 5A, epicardial cells cultured in control medium (containing normal rabbit IgG) expressed SMA and calponin (Fig. 5A). On the other hand, the epicardial cells cultured with anti-TNC antibody showed a weak staining for SMA and calponin (Fig. 5B). Western blot showed that the expression of SMA was reduced in cultures treated with ant-TNC antibody (Fig. 5C). These results suggest that TNC is capable of facilitating the differentiation of epicardial cells into myofibroblast/smooth muscle cells in culture. We further examined whether TNC is able to induce the migration of epicardium-derived mesenchymal cells into a TNC-rich environment. A transwell migration assay was performed, and the results showed that there was no significant difference in the number of cells that migrated across the wall between the control and TNC-rich cultures (Supplemental Table 1).

4. Discussion

4.1. Possible role of TNC in vascular development

During vascular development, vessels are initially established as endothelial tubes, which are subsequently supported by mural cells, such as the pericytes of capillary vessels and the vascular smooth muscle cells of the tunica media. In the present study, we showed that the deposition of extracellular TNC was closely associated with the onset of the formation of the thick SMA-positive vascular wall of the proximal coronary stem. When the single coronary stem has fully developed, both SMA and TNC are present in its mural cell layer. Furthermore, TNC altered the phenotype of the cultured proepicardium so that it resembled that of myofibroblasts or smooth muscle cells. These findings suggest that TNC may play a significant role in smooth muscle cell recruitment into the developing coronary arterial wall. Recently, our group has reported that TNC and PDGF collaboratively potentiate migration and proliferation of smooth muscle cell through crosstalk signal between integrin $\alpha v\beta 3$ and PDGFRβ by increased SRC recruitment and FAK activation (Ishigaki et al. 2010). During coronary development, endothelially-secreted PDGFB and mesenchymally expressed PDGFRβ interact with each other to recruit smooth muscle cells into the nascent tunica media (Hellström et al., 1999; Van Den Akker et al., 2005). Taking together, it is suggested that TNC may facilitate smooth muscle cell differentiation/organization in the wall of the coronary artery by modulating PDGF/PDGFRβ signaling via certain integrins.

Our immunohistochemistry showed that the deposition of TNC colocalized with the expression of SMA in mural cells; however, TNC did not coincide with endothelial progenitor cells. This observation suggested that TNC facilitates the recruitment of

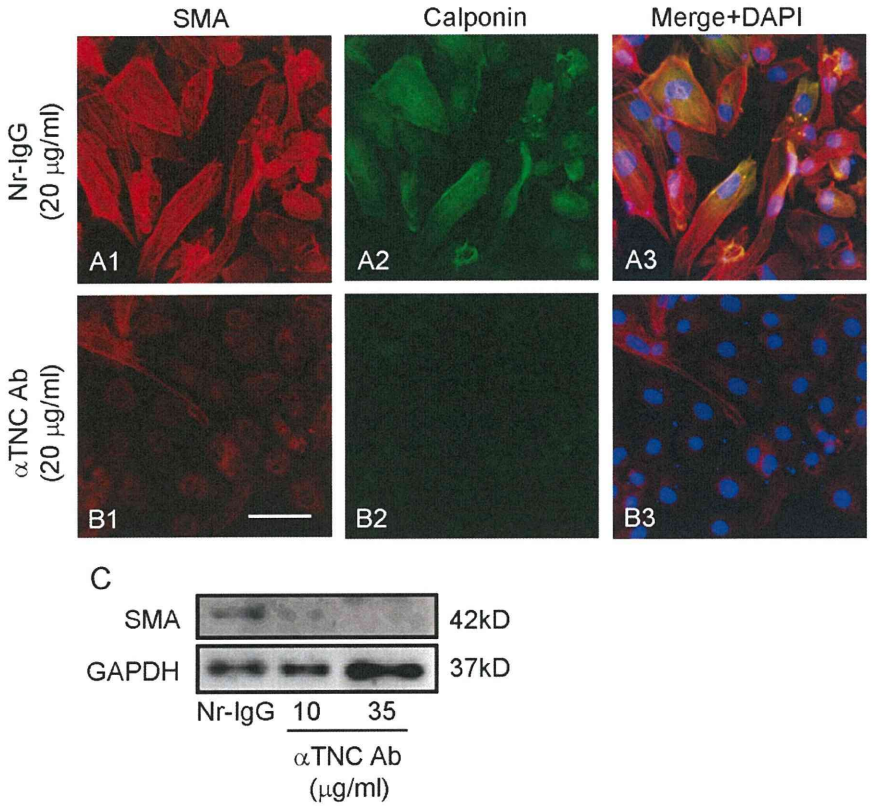

Fig. 5. Anti-TNC antibody suppressed the expression of SMA in cultured epicardium. The epicardia from ED6 chick hearts were cultured on TNC-coated slide with medium containing anti-TNC neutralizing antibody. After 48 h, the cells were subjected to immunological detection for SMA and calponin. Immunostaining showed that the epicardial cells cultured in control medium (containing normal rabbit IgG) expressed SMA and calponin (A). On the other hand, epicardial cells cultured with anti-TNC antibody showed that a weak staining for SMA and calponin (B). Western blot showed that the expression of SMA was reduced in cultures treated with anti-TNC antibody (C). αTNC Ab, anti-tenascin C neutralizing antibody; DAPI, nuclear staining; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nr-IgG, normal rabbit IgG; SMA, smooth muscle α-actin. Bar, 40 μm. Note that exposure times for SMA calponin were 125 ms and 1 s, respectively.

SMC progenitors during vascular development, but not guidance for invasion/migration of endothelial progenitors. In myocardial lesions in the adult, TNC is thought to induce SMA-positive myofibroblast accumulation during wound healing and tissue remodeling (Imanaka-Yoshida et al., 2001a, 2001b; Tamaoki et al., 2005). Cardiac fibroblasts obtained from TNC-null mutant mice showed lower migratory activity and reduced expression of SMA than wild type cells in culture, and the addition of purified TNC to TNC-null cardiac fibroblasts reversed both the migration and expression of SMA, suggesting that TNC facilitates the migration and differentiation of myofibroblast cells (Tamaoki et al., 2005). TNC is also expressed in neoplastic lesion and plays a significant role in angiogenesis during cancer invasion (Midwood and Orend, 2009). In glioblastoma multiforme, TNC is highly up-regulated at the invasive edge in association with vascular sprout (Zagzag et al., 2002). *In vivo* and *in vitro* experiments suggest that TNC in matrix micro-environment in cancer may potentiate angiogenesis in tumor development (Zagzag et al., 2002; Tanaka et al., 2004). These observations suggest that the role of TNC in coronary artery development may be different from that in tumor angiogenesis. It is also suggested that TNC is capable of playing a distinct role during vasculogenesis/angiogenesis in a context-dependent manner.

Extracellular TNC binds to several integrins at FN-III domains to mediate intracellular signaling (Jones and Jones, 2000). Therefore, other extracellular matrices that possess FN-III domains, such as vitronectin, tenascin-X, and -R, might interact with certain integrins and maintain integrin-mediated intracellular signaling during smooth muscle cell growth. This might be one of the reasons why TNC-null mutant mice do not show lethal abnormalities during embryogenesis (Saga et al., 1992). The spatiotemporal expression patterns of TNC in regions where smooth muscle cell/myofibroblast differentiation occurs suggest that TNC plays a role in the recruitment of smooth muscle cells during vascular development including development of the coronary artery.

4.2. Origin of the cells forming the proximal coronary artery

It has been reported that the proepicardium gives rise to cells that contribute to coronary endothelial and smooth muscle cells in chicks (Mikawa and Gourdie, 1996; Männer, 1999), while recent experiments in apelin-nlac-Z knock-in mice have shown that the coronary arteries are not derived from the proepicardium but rather from endothelial sprouts of the sinus venosus (Red-Horse et al., 2010). Quail-chick chimera experiments showed that both endothelial and mural cells possessed quail markers, suggesting that these cells are derived from transplanted quail tissue (Fig. 3; Männer, 1999). However, in chimera experiment quail PEO was transplanted together with the sinus venosus; thus, we could not rule out the hypothesis that coronary endothelial cells originate from the sinus venosus in avians. The quail PEO is reported to consist of two distinct cell types, which include a superficial mesothelial epithelium and an internal mesenchymal core (Nahirney et al., 2003). The internal mesenchymal core, which is adjacent to endothelial lining of the sinus venosus, possesses endothelial marker QH1 (Ishii et al., 2009). Therefore, it may be possible that the QH1-positive mesenchymal

cells in the PEO are derived from the endothelial cells of the sinus venosus. Further experiments should be necessary to elucidate the origin of the coronary endothelial cells in avians.

It has been reported that *PDGFRβ*$^{-/-}$ hearts failed to form coronary arterial tunica media, but that epicardial-specific deletion of *PDGFβ* resulted in a reduced number of coronary vascular smooth muscle cells, suggesting that two sources of coronary vascular smooth muscle cells exist (Mellgren et al., 2008). Neural crest-derived cells are found in the walls of the proximal coronary arteries in Wnt1-Cre/R26R mice (Jiang et al., 2000), but mesenchymal cells in the anterior heart field do not give rise to coronary vessels (Verzi et al., 2005). Therefore, neural crest cells might contribute to the formation of the proximal coronary arteries. Taken together with these observations, it is suggested that three distinct cell types are involved in the formation of the proximal coronary artery, PEO-derived subepicardial mesenchymal cells, endothelial cells from the sinus venosus, and neural crest-derived pharyngeal mesenchymal cells.

In conclusion, our results suggest that TNC may play a role in the recruitment of smooth muscle cells to the nascent coronary artery.

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Appendix A. supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.diff.2011.03.002.

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Supplemental Table 1

TNC (μg/ml)	Number of cells migrated
0 (n=14)	104±27
2 (n=14)	94±25
5 (n=9)	97±32

Transwell migration assay: The epicardia from ED6 chick hearts were prepared on cell culture insert (8-μm pore size, Becton Dickinson Labware) as described in materials and methods. The medium containing 2% chick serum was poured into the outer chamber (Falcon 24-well plate, Becton Dickinson Labware). To examine the effect of TNC on cell migration, purified TNC (0-5μg/ml) was added to serum-free medium of the upper chamber. The cells from the epicardium were allowed to migrate to the lower membrane surface for 48 h. The epicardial cells on the upper surface were wiped off and the inserts were fixed with 100% ethanol and stained with Coomassie Brilliant Blue. Stained cells on the lower membrane surface were counted under a ×20 objective lens (total cells/epicardial explant). Statistical analysis was performed using the unpaired *t*-test, and the significance was set at <5%. The results showed that there was no significant difference in the number of cells that migrated across the membrane between the control and TNC-rich cultures. n, number ofexplant examined.