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p38 Mitogen-activated protein kinase and c-Jun NH_2-terminal protein kinase regulate the accumulation of a tight junction protein, ZO-1, in cell-cell contacts in HaCaT cells

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Running title: Involvement of JNK and p38 MAPK in tight junction formation

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Abstract

To investigate the involvement of stress-activated protein kinases, JNK and p38 MAPK, in the assembly of tight junctions in keratinocytes, we treated HaCaT cells with various combinations of SP600125 (an inhibitor of JNK), SB202190 (an inhibitor of p38 MAPK) and anisomycin (an activator of both JNK and p38 MAPK) and examined the localization of ZO-1, an undercoat constitutive protein of the tight junction. Short-term (8 h) incubation with SP600125, SB202190 or anisomycin induced the accumulation of ZO-1 in the cell-cell contacts, with reduced ZO-1 staining in the cytoplasm, while only long-term (24 h) incubation with SP600125 induced cytoplasmic staining. SP600125, SB202190 or SP600125 plus SB202190 treatment induced thin string-like staining for ZO-1 in the cell-cell contacts. Anisomycin treatment induced bold line-like staining for ZO-1, while anisomycin plus SP600125 treatment induced zipper-like staining for ZO-1. Anisomycin plus SB202190 treatment or anisomycin plus both SP600125 and SB202190 treatment for 8 h failed to lead to the accumulation of ZO-1 in cell-cell contacts, but induced string-like staining with several gaps 16 h after removal of these agents. These results suggest that the localization of ZO-1 in cell-cell contacts is differently regulated by the inhibition of JNK and/or p38 MAPK, activation of both

JNK and p38 MAPK and activation of p38 MAPK depending on the incubation period.

Introduction

The epidermis is a stratified squamous epithelium comprising four layers; the basal layer, spinous layer, granular layer and cornified layer. Keratinocytes proliferate in the basal layer attached to the basement membrane, and differentiate and migrate toward the surface of the skin.

Tight junctions (TJs) create a barrier to both the diffusion of solutes through the paracellular pathway and to the movement of proteins and lipids between the apical and basolateral plasma membrane domains. TJs are located at the uppermost portion of the lateral plasma membrane, and consist of transmembrane proteins such as occludin, claudins, tricellulin and junction adhesion molecules (Ikenouchi et al., 2005; Schneeberger and Lynch, 2004). Claudins formed TJ-strands observed by freeze-fracture electron microscopy when were expressed in cells without TJs, such as fibroblasts and HEK293 cells (Furuse et al., 1998; Inai et al., 2010; Inai et al., 2009). They are associated with actin cytoskeleton-interacting proteins, including zonula occludens proteins, ZO-1 and ZO-2 (Schneeberger and Lynch, 2004). It was previously believed that TJs are formed only in simple epithelium, but not in stratified epithelium.

However, claudin-1-deficient mice died within one day of birth because of severe defects in the permeability barrier of the epidermis (Furuse et al., 2002). This finding led to the discovery of TJs in stratified epithelia, including the epidermis.

The localization of TJ proteins, including claudn-1, claudin-4, occludin and ZO-1, was reported previously (Brandner et al., 2002; Furuse et al., 2002; Morita et al., 1998; Pummi et al., 2001; Tunggal et al., 2005). Occludin is concentrated as dots in the most apical region of the lateral membranes of the granular cells in the second layer. Claudin-4 is distributed mainly in the second/third layers of granular layer. ZO-1 is mainly localized to the granular layer, and some labeling is present in the uppermost spinous layer. Although occludin, claudin-4 and ZO-1 are mainly localized to the granular layer, claudin-1 is distributed diffusely throughout plasma membranes of keratinocytes, from the basal to granular layers. Electron microscopy revealed that a typical TJ structure (characterized by fusion of the outer leaflets of the adjoining cell membranes) was found in the most apical region of the lateral plasma membranes of the granular cells in the second layer (Brandner et al., 2002; Furuse et al., 2002).

Mitogen-activated protein kinases (MAPKs) are a family of serine-threonine kinases that are activated by various stimuli, including growth factors, cytokines and phorbol esters, and are involved in modulating cellular responses (Bogoyevitch and

Court, 2004; Hagemann and Blank, 2001; Pearson et al., 2001; Schaeffer and Weber, 1999). MAPKs consist of extracellular signal-regulated kinase-1 and -2 (ERK1/2, also known as p44 and p42 MAPKs), c-Jun NH_2-terminal kinases (JNK1/2/3), p38 MAPKs (p38α/β/γ/δ), ERK3/4, ERK5 and ERK7/8 in mammalian cells (Kyriakis and Avruch, 2001). The MAPK cascades consist of MAPKK kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. JNK and p38 MAPK are also called stress-activated protein kinases, and have been implicated in a variety of cellular processes, including cell proliferation, differentiation and death.

DNA microarrays revealed that the inhibition of JNK induced the transcription of cornification markers, inhibition of motility, withdrawal from the cell cycle, stratification and production of cornified envelopes, suggesting that inhibition of JNK may be involved in the induction of epidermal differentiation (Gazel et al., 2006). TJs are formed in keratinocytes of the granular layer in the skin (Furuse et al., 2002) and are considered to be one of the hallmark structures of keratinocyte differentiation. In consistent, the formation of TJs was induced by SP600125 (a JNK inhibitor) in HaCaT cells derived from histologically normal skin (Aono and Hirai, 2008; Kitagawa et al., 2014). JNK and p38 MAPK are both involved in the assembly and disassembly of TJs in keratinocytes (Siljamaki et al., 2014) and other cells (Kojima et al., 2010; Naydenov

et al., 2009). In this study, we investigated the expression and localization of ZO-1, one of the undercoat constitutive proteins of the TJ, in HaCaT cells treated with various combinations of SP600125 (an inhibitor of JNK), SB202190 (an inhibitor of p38 MAPK) and anisomycin (an activator of both JNK and p38 MAPK) to determine the effects of these agents on the formation of TJs. We found that the localization and accumulation patterns of ZO-1 in cell-cell contacts were differentially regulated by inhibition of JNK and/or p38 MAPK, activation of both JNK and p38 MAPK and activation of p38 MAPK, depending on the incubation period.

Materials and methods

Cell culture and treatment

HaCaT cells (Boukamp et al., 1988) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Equitech Bio, Inc., Kerrville, TX, USA), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA). This growth medium contained approximately 2.2 mM calcium. Cells were seeded at 2× confluent density (~4.0×10^5 cells/cm^2) in the wells (6 mm in diameter) of glass slides printed with a highly water-repellent mark (Matsunami Glass Ind., Ltd., Osaka, Japan), and were cultured for two days with a daily

medium change. SP600125 (Merck KGaA, Darmstadt, Germany), SB202190 (Sigma-Aldrich, St. Louis, MO, USA) and anisomycin (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) to make a 20 mM stock solution. To determine the optimal concentration of agents, confluent cells were treated with either 0.2, 0.5, 1.0, 2.0 or 5.0 μM anisomycin, 10, 20 or 40 μM SP600125, or 10, 20 or 40 μM SB202190 in growth medium supplemented with 7.6 mM $CaCl_2$ (final calcium concentration, 9.8 mM), referred to as high calcium medium, for 8 h, were washed once with DMEM and were further cultured in growth medium for 16 h. To examine the effects of the incubation period for the different agents, confluent cells were treated with either 40 μM SP600125, 40 μM SB202190 or 0.5 μM anisomycin in high calcium medium for 3, 6, 8, 10, 12 or 24 h, washed once with DMEM and further cultured in growth medium for 21, 18, 16, 14, 12 or 0 h (total 24 h), respectively. Confluent cells were treated with either 0.2% DMSO (control), 40 μM SP600125, 40 μM SB202190 or 40 μM SP600125 plus 40 μM SB202190 in high calcium medium in the absence or presence of 0.5 μM anisomycin for 8 h. After an 8 h incubation with these agents, some cells were fixed with 1% paraformaldehyde in PBS. All other cells were washed once with DMEM, and further cultured in growth medium for 16 h.

Immunofluorescence microscopy

The cells were fixed with 1% paraformaldehyde in PBS for 10 min, washed in PBS and incubated in 0.2% Triton-X 100 in PBS for 15 min for permeabilization. Subsequently, the cells were washed in PBS, and then incubated with 1% bovine serum albumin in PBS (BSA-PBS) for 15 min to block nonspecific binding. The cells were incubated with primary antibodies for 1 h. A mouse anti-ZO-1 monoclonal antibody (mAb) (1:1,000) and a mouse anti-claudin-4 mAb (1:100) were obtained from Zymed Laboratories (South San Francisco, CA, USA). After rinsing them four times in PBS, the cells were incubated with anti-mouse Ig conjugated with Alexa 488 (Molecular Probes, Eugene, OR, USA) at a 1:400 dilution in BSA-PBS for 30 min in the dark. The cells were then washed four times in PBS and mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Images were taken using a LSM710 confocal laser scanning microscope (Oberkochen, Germany) with a ×20/0.5 NA objective or a ×63/1.4 NA oil objective.

Results

To determine the optimal concentrations of anisomycin, SP600125 and

SB202190, HaCaT cells were treated with high calcium medium (9.8 mM calcium) containing 0.2, 0.5, 1.0, 2.0 or 5.0 μM of anisomycin, 10, 20 or 40 μM of SP600125, or 10, 20 or 40 μM of SB202190 for 8 h, washed once with DMEM and further cultured in growth medium for 16 h. Twenty-four hours after the addition of these agents, the cells were fixed and stained with an anti-ZO-1 antibody. Treatment with 0.2, 0.5 or 1.0 μM of anisomycin induced string-like staining for ZO-1 in the cell-cell contacts in some cells, with reduced staining for ZO-1 in the cytoplasm (Suppl. Fig. 1). The induction of string-like staining for ZO-1 was less noticeable when the cells were treated with 2.0 or 5.0 μM of anisomycin (Suppl. Fig. 1). To induce string-like staining for ZO-1, treatment with 40 μM SP600125 or 40 μM SB202190 was optimal (data not shown).

We then examined the effect of the incubation period with these agents with regard to their induction of string-like staining for ZO-1. HaCaT cells were treated with high calcium medium containing either 40 μM SP600125, 40 μM SB202190 or 0.5 μM anisomycin for 3, 6, 8, 10, 12 or 24 h, were washed once with DMEM and then were further cultured in growth medium for 21, 18, 16, 14, 12 or 0 h (total 24 h), respectively. Twenty-four hours after the addition of these agents, the cells were fixed and stained with an anti-ZO-1 antibody. SP600125 treatment induced string-like staining for ZO-1 in cell-cell contact by 3 h, but SB202190 or anisomycin treatment induced it by 6 h (Fig.

1). The number of cells with string-like staining for ZO-1 gradually increased over time for up to 12 h by these agents.

There were differences in the string-like staining for ZO-1 after 24 h of treatment with these agents. The string-like staining for ZO-1 was present after the 24 h SP600125 treatment, was markedly reduced by 24 h SB202190 treatment and disappeared after 24 h of anisomycin treatment. Note that the cells with string-like staining for ZO-1 in cell-cell contacts showed reduced ZO-1 staining in their cytoplasm.

Next, we examined the localization of ZO-1 in HaCaT cells treated with various combinations of SP600125, SB202190 and/or anisomycin for 8 h (Fig. 2). In the control cells (treated with 0.2% DMSO), ZO-1 was localized in the cytoplasm, and was diffusely localized along cell-cell contacts. SP600125 treatment for 8 h induced string-like staining for ZO-1 in cell-cell contacts in many, but not all, cells. SB202190 treatment and SP600125 plus SB202190 treatment induced string-like staining for ZO-1 in a relatively large number of cells, but SP600125 plus SB202190 treatment induced more string-like staining than SB202190 treatment did. Anisomycin treatment induced string-like staining in the cell-cell contacts in most cells. Anisomycin plus SP600125 treatment reduced the number of cells with string-like staining, but did not completely eliminate it. In contrast, the string-like staining almost completely disappeared from the

cell-cell contacts following anisomycin plus SB202190 or anisomycin plus both SP600125 and SB202190 treatment.

We next observed the ZO-1 staining in Fig. 2 in detail with a ×63/1.4 NA oil objective (Fig 3). Thin and continuous string-like staining (uniform in thickness) for ZO-1 was observed in some cells treated with SP600125, SB202190 and SP600125 plus SB202190. In contrast, anisomycin treatment induced bold line-like staining for ZO-1, which was non-uniform in thickness (arrows in Fig. 3). Anisomycin plus SP600125 treatment induced zipper-like staining for ZO-1 (arrowheads in Fig. 3) along most cell-cell contacts, in addition to bold line-like staining in a few cells. Zipper-like staining for ZO-1 was also observed in the outermost boundaries of some cells with string-like staining for ZO-1. Anisomycin plus SB202190 treatment or anisomycin plus SP600125 and SB202190 treatment induced sparse dot-like or short line-like staining for ZO-1, and occasional string-like staining for ZO-1 in only a few cells.

Next, we examined the changes in the localization of ZO-1 in HaCaT cells cultured for 16 h in normal growth medium after incubation with various combinations of SP600125, SB202190 and/or anisomycin for 8 h (Fig. 4). All treatments except for the control induced string-like staining for ZO-1. The cells with string-like staining for ZO-1 had markedly decreased cytoplasmic staining for ZO-1 (arrows in Fig. 4)

following treatment with SP600125, SB202190, SP600125 plus SB202190, anisomycin or anisomycin plus SP600125. In contrast, the cells with string-like staining for ZO-1 in cell-cell contacts did not exhibit reduced cytoplasmic staining for ZO-1 (arrowheads in Fig. 4) when they were treated with anisomycin plus SB202190 or anisomycin plus SP600125 and SB202190.

We observed the ZO-1 staining in Fig. 4 in detail with a ×63/1.4 NA oil objective (Fig 5). SP600125, SB202190, SP600125 plus SB202190, anisomycin and anisomycin plus SP600125 treatments all induced continuous and straight string-like staining for ZO-1 in the cell-cell contacts. However, anisomycin plus SB202190 treatment or anisomycin plus SP600125 and SB202190 treatment induced irregular and discontinuous staining for ZO-1 (arrowheads in Fig. 5) in some cells, in addition to continuous and straight string-like staining for ZO-1.

Finally, we examined the localization of claudin-4, an integral membrane protein at the TJ, which forms the backbone of TJ strands, in HaCaT cells that were treated with 0.2% DMSO, SP600125, SB202190, SP600125 plus SB202190 or anisomycin for 8 h, washed once with DMEM and further cultured in growth medium for 16 h (Fig. 6). String-like staining for claudin-4 was observed in the cell-cell contacts following all treatments except for the control.

Discussion

The accumulation of ZO-1 in cell-cell contacts in HaCaT cells was induced by short-term treatment (8 to 12 h) with anisomycin (an activator of JNK and p38 MAPK) but was lost after long-term treatment (24 h) with anisomycin. Similarly, dual effects of anisomycin on the expression of tricellulin, which is concentrated at the tricellular TJ (in addition to the bicellular TJ), were reported in human pancreatic duct epithelial cells and were dependent on the incubation period. Treatment with 1 μM anisomycin for 4 to 8 h increased the expression of tricellulin, but treatment for 24 h decreased it (Kojima et al., 2010). Short-term treatment with SB202190 (an inhibitor of p38 MAPK) also induced string-like staining for ZO-1 in cell-cell contacts, but long-term treatment markedly reduced the string-like staining. In contrast to anisomycin or SB202190 treatment, SP600125 (an inhibitor of JNK) treatment still showed string-like staining for at least 24 h in HaCaT cells. These results indicate that short-term inhibition of either JNK or p38 MAPK and short-term activation of both JNK and p38 MAPK induced the assembly of TJs in HaCaT cells. In contrast to the long-term inhibition of JNK, long-term inhibition of p38 MAPK or long-term activation of both JNK and p38 MAPK induced disassembly of the TJs. These results suggest that stress-activated protein

kinases, JNK and p38 MAPK, regulate the assembly and disassembly of TJs in HaCaT cells, depending on the period of activation or inhibition of these kinases.

The present study showed that the accumulation of ZO-1 in cell-cell contacts was induced by the inhibition of JNK and/or p38 MAPK, activation of both JNK and p38 MAPK or activation of p38 MAPK, although the patterns of localization of ZO-1 in cell-cell contacts were different (Fig. 3). In agreement with the findings of the present study, activation of p38 MAPK by high Ca^{2+} (1.4 mM) for up to 72 h induced the expression and accumulation of ZO-1 in cell-cell contacts in human keratinocytes cultured under low Ca^{2+} conditions (0.06 mM) (Siljamaki et al., 2014). In contrast to the activation of p38 MAPK, the activation of JNK (by treatment with anisomycin plus SB202190) failed to induce the accumulation of ZO-1 in cell-cell contacts. This result is consistent with a previous report that the disassembly of ZO-1 from cell-cell contacts triggered by calcium depletion in intestinal epithelial cells was inhibited by SP600125 (Naydenov et al., 2009). We herein provide the first evidence that the patterns of ZO-1 localization in the cell-cell contacts in HaCaT cells is differentially regulated by the inhibition of JNK and/or p38 MAPK, activation of both JNK and p38 MAPK or activation of p38 MAPK.

Anisomycin treatment for 8 h induced bold line-like staining for ZO-1 in

cell-cell contacts, while SP600125 and/or SB202190 treatment for 8 h induced thin line-like staining. ZO-1 is a peripheral membrane adaptor protein that links TJ transmembrane proteins, such as claudins (Itoh et al., 1999a) and occludin (Fanning et al., 1998; Furuse et al., 1994; Itoh et al., 1999b), to the actin cytoskeleton (Itoh et al., 1997; Matter and Balda, 2007). In addition to TJs, ZO-1 also localizes in adherens junctions in non-epithelial cells, such as fibroblasts and cardiac myocytes (Itoh et al., 1993) by binding to α-catenin (Imamura et al., 1999). Furthermore, ZO-1 binds to connexins in gap junctions (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998). Therefore, it is possible that anisomycin treatment or SB202190 and/or SP600125 treatment may differently affect the formation of adherens junctions, gap junctions and TJs, and these cell-cell adhesion molecules may exhibit different associations with ZO-1. We are now examining the localization of these cell-cell adhesion molecules in HaCaT cells treated with various combinations of these agents for 8 h to address this possibility.

The bold line-like staining for ZO-1 induced by anisomycin treatment for 8 h was altered to zipper-like staining by the addition of SP600125, or to sparse dot-like staining by the addition of either SB202190 or SP600125 plus SB202190. These results indicate that the activation of both p38 MAPK and JNK (anisomycin treatment) and activation of p38 MAPK (anisomycin plus SP60125 treatment) led to the accumulation

of ZO-1 in cell-cell contacts, with different appearances (bold line vs zipper), and that the activation of JNK (anisomycin plus SB202190 treatment) failed to induce major accumulation of ZO-1 in cell-cell contacts. The zipper-like staining for ZO-1 induced by the activation of p38 MAPK resembles an adhesion zipper or spot-like adherens junction, which is observed in differentiating primary keratinocytes (Vaezi et al., 2002) or polarizing epithelial cells (Suzuki et al., 2002; Vasioukhin et al., 2000; Yonemura et al., 1995).

Calcium switch after 4 h and 24 h induced zipper-like staining for ZO-1, which was composed of distinct dots, and line-like staining for ZO-1 in keratinocytes, respectively (Pummi et al., 2001), revealing the maturation steps of TJs. Based on these previous results, the activation of p38 MAPK induced immature TJs, but the activation of both p38 MAPK and JNK induced more mature, but not fully mature, TJs. Mature TJs, characterized by continuous and thin linear staining for ZO-1, were formed by 16 h after 8 h incubation of anisomycin or anisomycin plus SP600125 (Fig. 5). Eight hours of incubation with anisomycin plus SB202190 or anisomycin plus SP600125 and SB202190 failed to induce the accumulation of ZO-1 in cell-cell contacts, but linear staining for ZO-1, albeit with several gaps, was observed in cell-cell contacts 16 h after the removal of these agents. In spite of the insufficient accumulation of ZO-1 in the

cell-cell contacts following an 8 h incubation with anisomycin plus SB202190 or anisomycin plus SP600125 and SB202190, the TJs automatically matured to some extent by 16 h after the removal of these agents. Therefore, even if the accumulation of ZO-1 in cell-cell contacts is insufficient, TJ formation may proceed after the removal of the agents.

Freeze-fracture replica electron microscopy revealed that there is an anastomosing network of TJ strands consisting of intramembranous particles on the lateral plasma membranes. Previous studies have shown that when one of the claudin members is expressed in TJ-free cells, such as fibroblasts and HEK293 cells, TJ strands are formed (Furuse et al., 1998; Inai et al., 2010; Inai et al., 2009). Thus, claudins are the essential component of TJ strands, and can reconstitute the TJ strands. The observation of string-like staining for claudin-4 in the cell-cell contacts 16 h after the removal of SP600125, SB202190, SP600125 plus SB202190 or anisomycin suggests that TJ strands may be formed by claudin-4.

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Figure legends

Fig. 1 The effects of SP600125, SB202190 or anisomycin treatment for different time periods on the localization of ZO-1 in HaCaT cells. Cells were treated with high calcium medium containing either 40 μM SP600125, 40 μM SB202190 or 0.5 μM anisomycin for 3, 6, 8, 10, 12 or 24 h, were washed once with DMEM and then were

further cultured in growth medium for 21, 18, 16, 14, 12 or 0 h (total 24 h), respectively. Twenty-four hours after the addition of these agents, the cells were fixed and stained with an anti-ZO-1 antibody. Images were taken with a ×20/0.5 NA air objective. Three hours of SP600125 treatment induces string-like staining for ZO-1 in cell-cell contacts in a few cells (arrow). The number of cells with string-like staining for ZO-1 increased during the incubation when cells were treated for up to 10 h or 12 h, and this lasted for at least 24 h in the presence of SP600125. Six hours of SB202190 treatment, but not 3 h of treatment, induces string-like staining for ZO-1 in the cell-cell contacts in a few cells (arrow). The number of cells with string-like staining for ZO-1 increase over the incubation period for up to 12 h, but markedly decreased when cells were treated for 24 h. Six hours of anisomycin treatment, but not 3 h of treatment, induced string-like staining for ZO-1 in the cell-cell contacts in a few cells (arrow). The cells with string-like staining for ZO-1 increased after incubation for up to 12 h, but disappeared when the cells were treated for 24 h. Note that the cells with string-like staining for ZO-1 in the cell-cell contacts had reduced ZO-1 staining in the cytoplasm. The scale bar represents 100 μm.

Fig. 2 The localization of ZO-1 in HaCaT cells treated with the indicated combinations

of agents for 8 h. Cells were treated with high calcium medium containing the indicated combinations of 40 μM SP600125, 40 μM SB202190 and/or 0.5 μM anisomycin for 8 h, and then were fixed and stained with an anti-ZO-1 antibody. Images were taken with a ×20/0.5 NA air objective. In control cells (treated with 0.2% DMSO), ZO-1 was localized in the cytoplasm and was diffusely localized along cell-cell contacts. SP600125 treatment for 8 h induced string-like staining for ZO-1 in the cell-cell contacts in many cells. SB202190 treatment and SP600125 plus SB202190 treatment induced string-like staining for ZO-1 in a relatively large number of cells, but SP600125 plus SB202190 treatment induced more string-like staining than did treatment with SB202190 alone. Treatment with anisomycin for 8 h induced string-like staining for ZO-1 in the cell-cell contacts in most cells. Treatment with anisomycin plus SP600125 reduced the number of cells with string-like staining for ZO-1, but some cells still had the string-like staining. The string-like staining for ZO-1 almost disappeared from the cell-cell contacts following treatment with anisomycin plus SB202190 or anisomycin plus SP600125 and SB202190. The scale bar represents 100 μm.

Fig. 3 The detailed localization of ZO-1 in HaCaT cells treated with the indicated combinations of agents for 8 h. Cells were treated with high calcium medium containing

the indicated combinations of 40 μM SP600125, 40 μM SB202190 and/or 0.5 μM anisomycin for 8 h, and then were fixed and stained with an anti-ZO-1 antibody. Images were taken with a ×63/1.4 NA oil objective. Thin and continuous string-like staining for ZO-1, which was uniform in thickness, was detected in some cells treated with SP600125, SB202190 and SP600125 plus SB202190. Zipper-like staining for ZO-1 (arrowhead) was observed in the outermost boundaries of some cells with string-like staining for ZO-1. In contrast, anisomycin treatment induced bold line-like staining for ZO-1, which was non-uniform in thickness (arrows). Anisomycin plus SP600125 treatment induced zipper-like staining for ZO-1 along most cell-cell contacts (arrowheads) and bold line-like staining for ZO-1 along some cell-cell contacts (arrows). Treatment with anisomycin plus SB202190 or anisomycin plus SP600125 and SB202190 induced sparse dot-like or short line-like staining for ZO-1 along cell-cell contacts, and occasionally string-like staining for ZO-1 in a few cells. The scale bar represents 20 μm.

Fig. 4 The localization of ZO-1 in HaCaT cells treated with the indicated combinations of agents for 8 h, followed by further culture in growth medium for 16 h. Cells were

treated with high calcium medium containing the indicated combinations of 40 μM SP600125, 40 μM SB202190 and/or 0.5 μM anisomycin for 8 h, were washed once with DMEM and were further cultured in growth medium for 16 h. Twenty-four hours after the addition of these agents, the cells were fixed and stained with an anti-ZO-1 antibody. Images were taken with a ×20/0.5 NA air objective. In the control cells (treated with 0.2% DMSO), string-like staining for ZO-1 was only occasionally observed (arrow). All treatments, except for the control, induced string-like staining for ZO-1 (arrows and arrowheads). Note that the cells with string-like staining for ZO-1 had markedly reduced cytoplasmic staining for ZO-1 (arrows) following treatment with SP600125, SB202190, SP600125 plus SB202190, anisomycin or anisomycin plus SP600125. In contrast, cells with string-like staining for ZO-1 did not reduce cytoplasmic staining for ZO-1 (arrowheads) when they were treated with anisomycin plus SB202190 or anisomycin plus SP600125 and SB202190. The scale bar represents 100 μm.

Fig. 5 The detailed localization of ZO-1 in HaCaT cells that were treated with the indicated combinations of agents for 8 h and further cultured in growth medium for 16 h. The cells were treated with high calcium medium containing the indicated combinations

of 40 μM SP600125, 40 μM SB202190 and/or 0.5 μM anisomycin for 8 h, were washed once with DMEM and were further cultured in growth medium for 16 h. Twenty-four hours after the addition of these agents, the cells were fixed and stained with an anti-ZO-1 antibody. Images were taken with a ×63/1.4 NA oil objective. Continuous and straight string-like staining for ZO-1 was detected in the cells treated with SP600125, SB202190, SP600125 plus SB202190, anisomycin and anisomycin plus SP600125. However, treatment with anisomycin plus SB202190 or anisomycin plus SP600125 and SB202190 induced irregular and discontinuous staining (arrowheads) in some cells, in addition to the continuous and straight string-like staining for ZO-1.

Fig. 6 The localization of claudin-4 in HaCaT cells treated with SP600125, SB202190, SP600125 plus SB202190 or anisomycin. Cells were treated with high calcium medium containing 0.2% DMSO (control), 40 μM SP600125, 40 μM SB202190, 40 μM SP600125 plus 40 μM SB202190 or 0.5 μM anisomycin for 8 h, were washed once with DMEM and were further cultured in growth medium for 16 h. Twenty-four hours after the addition of the agents, the cells were fixed and stained with an anti-claudin-4 antibody. Images were taken with a ×63/1.4 NA oil objective. String-like staining for

claudin-4 was observed in the cell-cell contacts following all treatments except for the control. The scale bar represents 20 μm.

Suppl. Fig. 1 The effects of treatment with different concentrations of anisomycin on the localization of ZO-1 in HaCaT cells. Cells were treated with high calcium medium (9.8 mM calcium) containing either 0.2% DMSO (control) or 0.2, 0.5, 1.0, 2.0 or 5.0 μM anisomycin (AM) for 8 h, were washed once with DMEM and were further cultured in growth medium for 16 h to determine the optimal concentration of AM. Twenty-four hours after the addition of anisomycin, the cells were fixed and stained with an anti-ZO-1 antibody. Images were taken with a ×20/0.5 NA air objective. In the control cells, ZO-1 was localized in the cytoplasm, and was diffusely localized along cell-cell contacts. Treatment with 0.2, 0.5 or 1.0 μM AM induced string-like staining for ZO-1 in the cell-cell contacts in some cells with reduced staining for ZO-1 in the cytoplasm. The induction of string-like staining for ZO-1 decreased when cells were treated with 2.0 or 5.0 μM AM. The scale bar represents 100 μm.

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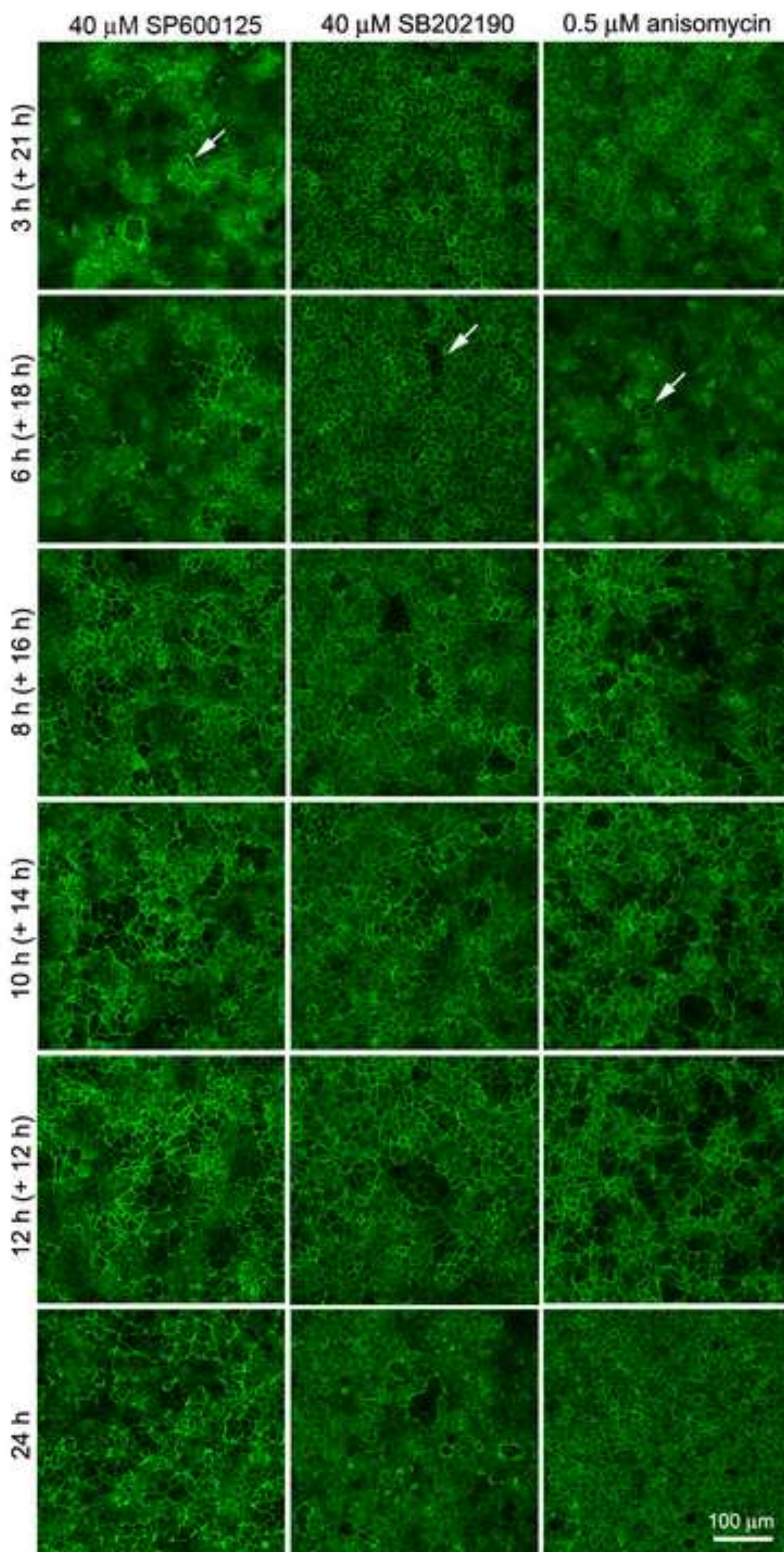

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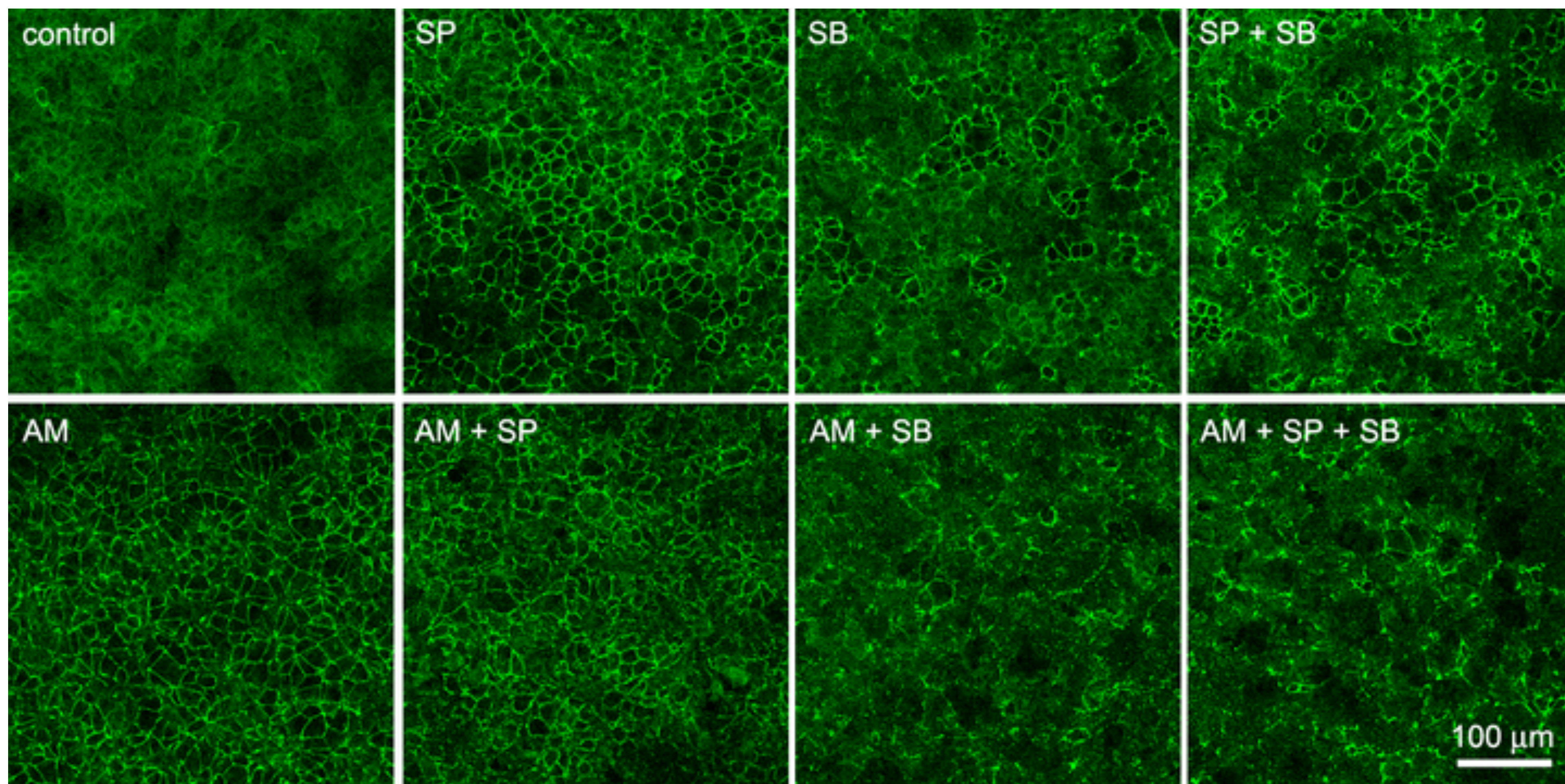

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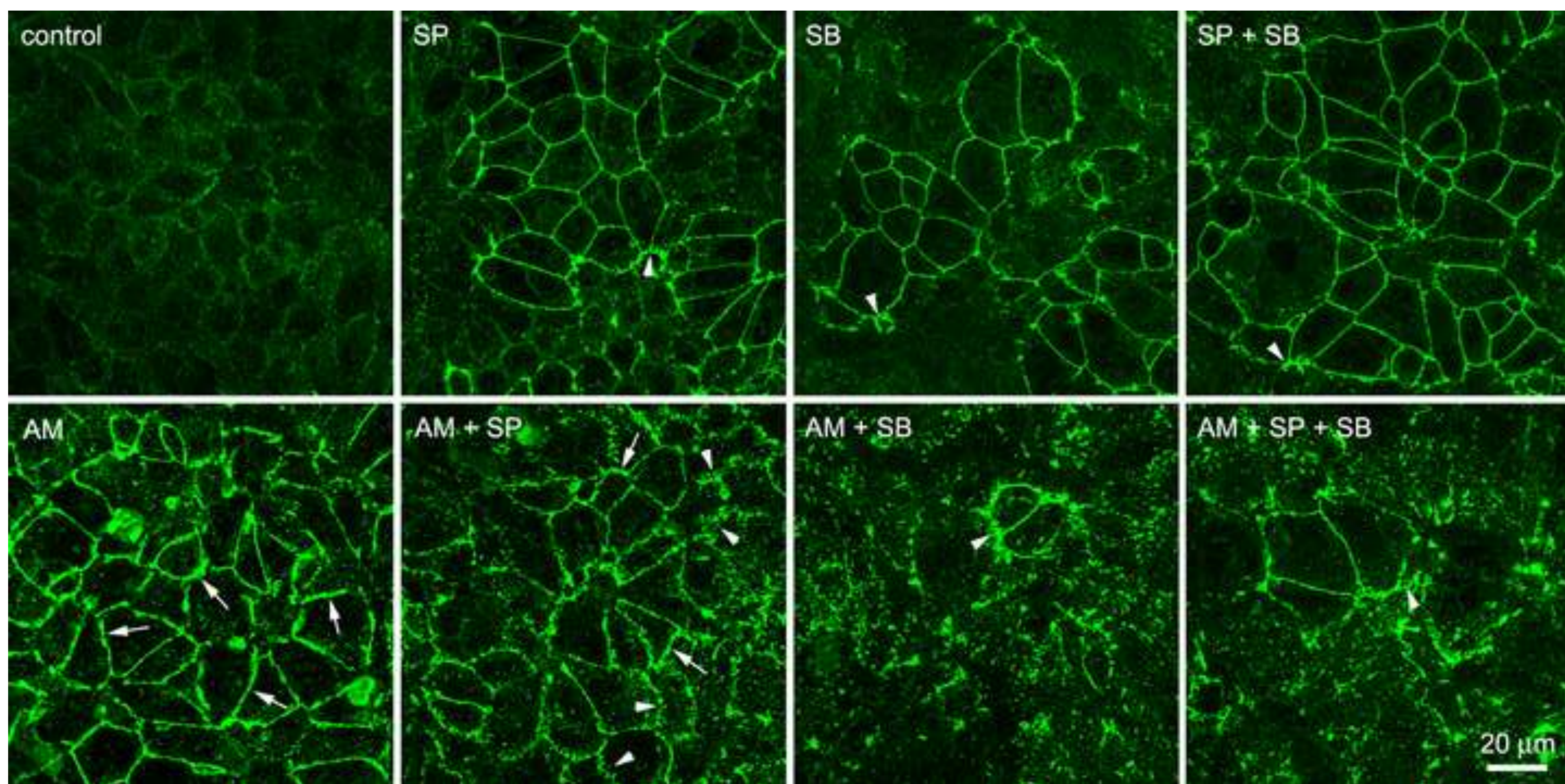

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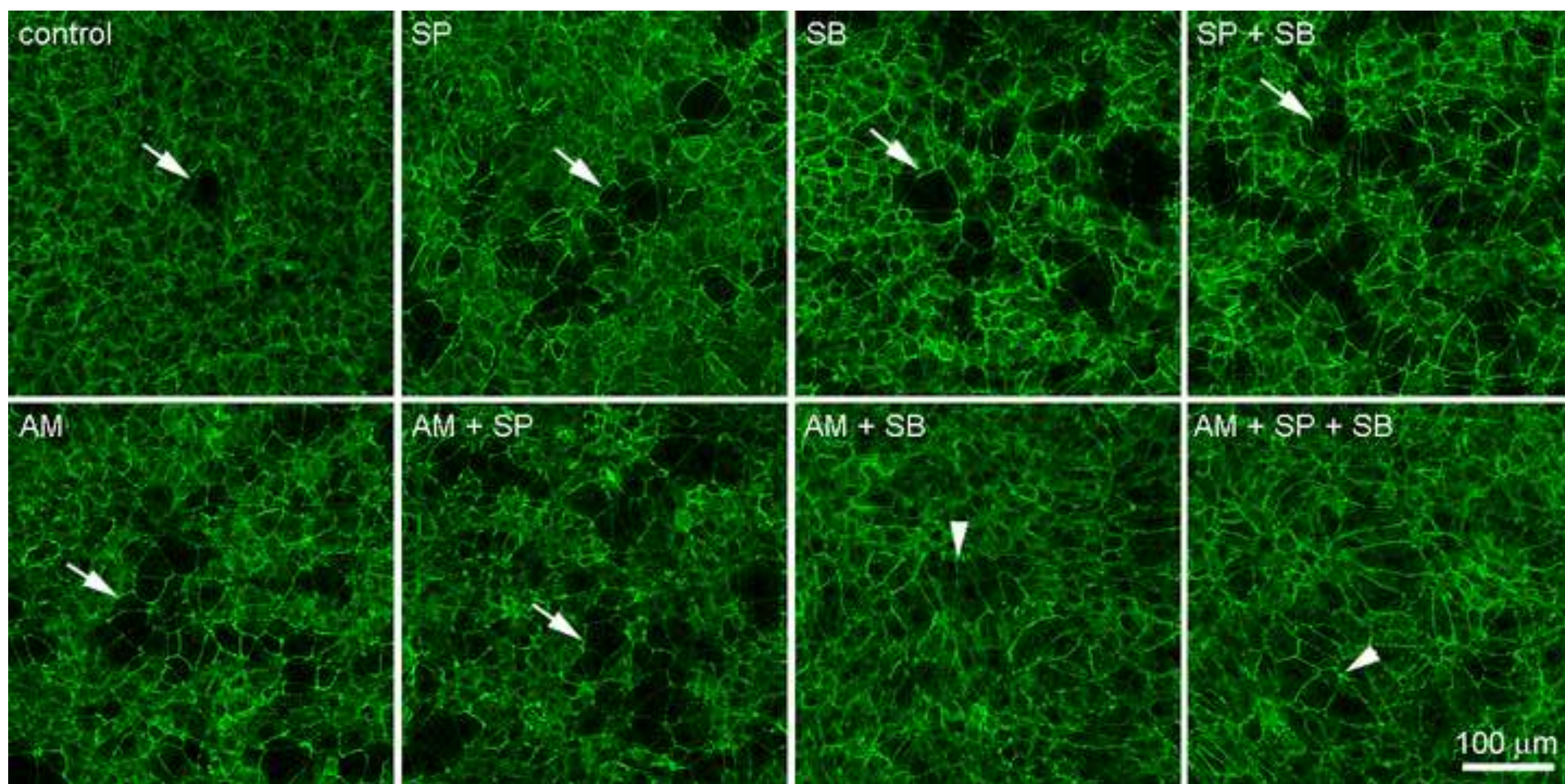

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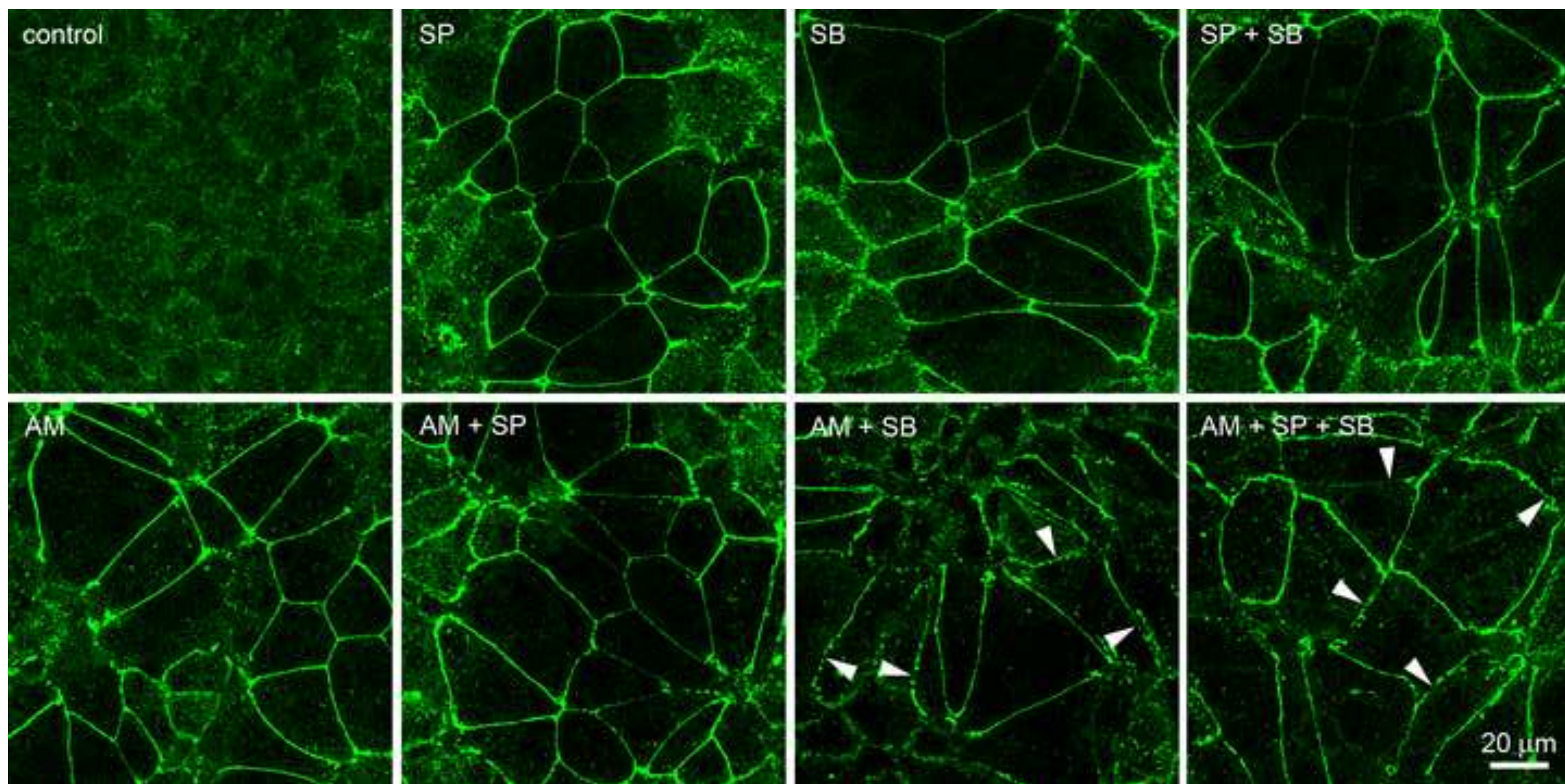

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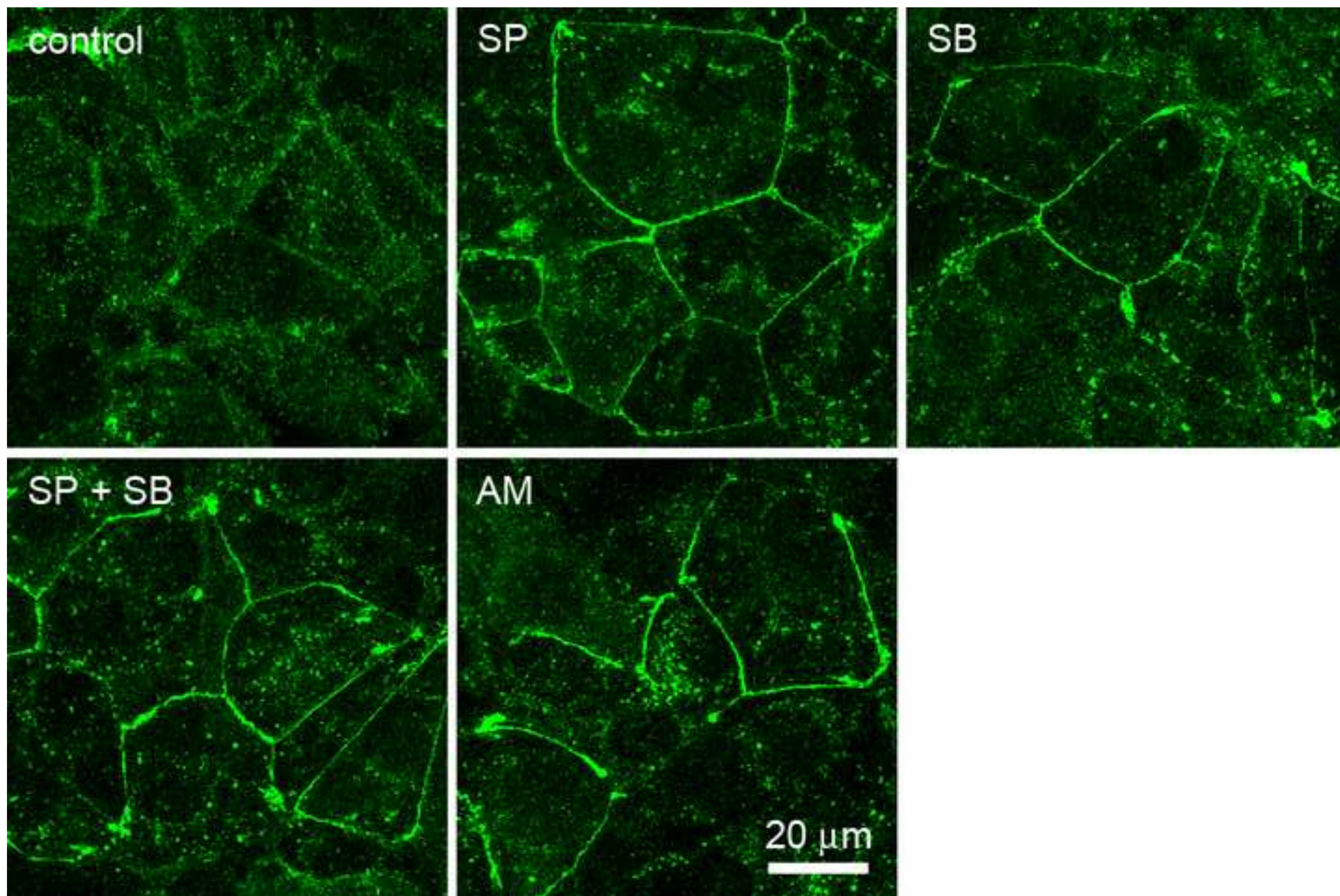

Supplemental Figure 1

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