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A novel method for successful induction of interdigitating process formation in conditionally immortalized podocytes from mice, rats, and humans

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ABSTRACT

Formation of processes in podocytes is regarded as the hallmark of maturity and normal physical condition for the cell. There are many accumulated findings about molecular mechanisms that cause retraction of podocyte processes; however, there is little knowledge of the positive mechanisms that promote process formation in vitro, and most previous reports about this topic have been limited to low-density cultures. Here, we found that process formation can be induced in 100% confluent cultures of conditionally immortalized podocytes in mouse, rat, and human species by combining serum depletion and Y-27632 ROCK inhibitor supplementation on the scaffold of laminin-521(L521). We noted the cytoskeletal reorganization of the radial extension pattern of vimentin filaments and downregulation of actin stress fiber formation under that condition. We also found that additional standard amount of serum, depletion of ROCK inhibitor, or slight mismatch of the scaffold as laminin-511(L511) hinder process formation. These findings suggest that the combination of reduced serum, podocyte-specific scaffold, and intracellular signaling to reduce the overexpression of ROCK are required factors for process formation.

1. Introduction

In vivo formation of foot processes in podocytes is characterized by highly arborized processes from the cell body and the interdigitation of neighboring processes at high confluence [1,2]. The cytoskeleton in in vivo foot process formation is a primary hallmark used to evaluate the maturity and pathophysiology of kidney diseases, especially proteinuria [3]. The reproduction of podocyte foot process formation in vitro would be significant for the development of a powerful evaluation system for drug discovery research. It would allow the evaluation of the drug cytotoxicity of candidate compounds in podocytes by phase contrast observation. Several methods of podocyte induction from pluripotent stem cells have been reported, and these methods have produced high expression of slit diaphragm proteins (e.g., nephrin) from podocytes in vitro. However, there have been no reports on the successful induction of process formation in high-confluence in vitro cultures of podocytes induced from pluripotent stem cells [4–6]. In 2018, Yaoita et al. reported a novel culture method to reproduce the interdigitating process formation of podocytes, at 100% confluence, whereas until then, process induction methods were only successful under sparse cell density conditions [7–10]. Although the culture method developed by Yaoita et al. has high novelty, the applicable cell source is limited to rat primary podocytes; thus, the method could not be applied to conditionally immortalized podocytes [9].

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Generally, conditionally immortalized podocytes have lost many of their phenotypes in vivo, including process formation at high cell density with conventional culture methods [11]. However, these cell lines have the benefit of providing unlimited cell growth, unlike primary podocytes. Hence, it is a significant advantage to reproduce podocyte process formation in conditionally immortalized podocytes. Here, we aimed to investigate whether conditionally immortalized podocytes retain the ability to reproduce process formation under the confluent conditions in multiple species.

2. Materials and methods

2.1. Cell culture

We employed three different species of conditionally immortalized podocyte cell lines; heat-sensitive mouse podocyte (HSMP) in mouse [12], 2DNA1D7 in rat [13] and AB8/13 in human [14]. All podocyte cell lines were cultured in a 5% CO2 incubator under permissive growth conditions (PGC) at 33 °C for cell propagation. Culture of HSMP required the supplementation of 50 U/mL murine interferon (IFN)-γ (PeproTech, USA). HSMP was cultured in RPMI 1640 medium (Nacalai Tesque, Japan), 10% FBS (Sigma-Aldrich, Japan), 1 mmol/L sodium pyruvate (ThermoFisher Scientific, USA), 10 mmol/L HEPES buffer (Dojindo, Japan), penicillin (50 units/ml), and streptomycin (50 mg/ml). 2DNA1D7 was cultured in D-MEM/Ham's F-12 medium (ThermoFisher Scientific, USA), 5% FBS, 0.1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X: ThermoFisher Scientific, USA), penicillin (50 units/ml), and streptomycin (50 mg/ml). AB8/13 was cultivated in RPMI 1640 medium, 10% FBS, 1.0% Insulin-Transferrin-Selenium (Fujifilm Wako, Japan), penicillin (50 units/ml), and streptomycin (50 mg/ml). To dissociate cells in subculture, Accutase (Nacalai Tesque, Japan) was used for all cell lines.

2.2. Induction of process formation and phase contrast imaging

For all cell lines, dissociated cell pellets were diluted with FBS-free RPMI 1640 medium containing 1 mmol/L sodium pyruvate, 10 mmol/L HEPES buffer, 0.1% ITS-X, 1.0 ng/mL human IGF-1 (PeproTech, USA), 0.2 ng/mL human basic FGF (PeproTech, USA), and 10 μM Y-27632 (Fujifilm Wako, Japan). The tissue culture-treated well plates (AGC glass techno, Japan) and cover slips (Matsunami, Japan) were coated with laminin-521 (L521) (Corning, USA) in FBS-free RPMI 1640 medium. The cell suspension was seeded at a density of 1.25×10^5 cells/cm^2 for HSMP, 1.0×10^6 cells/cm^2 for 2DNA1D7, and 9.6×10^5 cells/cm^2 for AB8/13 to ensure high confluence at seeding time, depending on the cell size. Both standard (with 10% FBS) and induction (with non-FBS, 10 μM Y-27632 and L521) culture methods were used under non-permissive growth conditions (NPGC) at 37 °C. IFN-γ was removed from only HSMP. The cytoskeleton was observed 2 h after cell seeding. All phase contrast images were acquired using an Olympus IX71N-22 PH microscope.

2.3. Immunofluorescence imaging

For immunofluorescence images, cells cultured on cover slips were fixed with 4% paraformaldehyde in PBS for 10 min and subsequently permeabilized with 0.3% Triton X-100 in PBS for 5 min at room temperature. Anti-vimentin antibody (1:300) (ab92547, Abcam, UK) and Acti-Stain 488 Phalloidin (98 nM final concentration) (PHDG1-A, Cytoskeleton, Inc., USA) were used to evaluate intermediate filament and F-actin localization patterns, respectively. Primary antibody staining was performed via blocking preparation using an animal-free blocker (Vector Labs, USA) diluted with Milli-Q water (1:5) overnight at 4 °C. Secondary antibody staining was performed using donkey anti-rabbit IgG, Alexa Fluor 568 (A10042, Thermo Fisher Scientific, USA) diluted with PBS together with Acti-stain and DAPI (1:1000) (D523, DOJINDO, Japan) overnight at 4 °C. Samples were washed out with PBS after each antigen-antibody reaction at room temperature for 5 min three times. Fluorescence imaging was performed using a Keyence BZ-X700 fluorescence microscope with optical sectioning and Z-stack modes. Z-stacking images were processed using ImageJ Fiji (NIH).

2.4. Quantitative PCR

To clarify the mechanism of podocyte process formation, gene expression levels in standard and induction culture conditions were compared using real-time PCR. HSMP was used for the assay because immunocytochemistry showed that, of the three cell lines, it had the most branching of processes and thus, the highest induction efficiency of process formation. Target genes for analysis were limited to those related to cell morphology. RNA was extracted from cells using ReliaPrep™ RNA Miniprep Systems (Promega, Japan) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan) and Verti™ Thermal Cycler (Applied Biosystems, USA). Real-time PCR was performed using THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Japan). The primers used were designed using Primer-BLAST (NIH, USA), as shown in Table 1. All used kits for quantitative PCR were carried out according to the manufacturer's instructions.

2.5. Statistical analysis

All values are expressed as the mean ± S.D. from technical triplicates obtained from two biologically independent experiments. To perform the statistical analysis, two groups, standard and induction, were compared using Student's t-test by Microsoft Excel.

3. Results

3.1. Optimizing culture condition for process formation

When HSMP was cultured with high confluence on L521 with

Table 1
Primers used for quantitative RT-PCR.

Gene		Sequence (5′-3′)
Gapdh	F	AGGTCGGTGTGAACGGATTTG
	R	TGTAGACCATGTAGTTGAGGTCA
PODXL	F	GCTGCTACTGTCGCCTGCAT
	R	TGTTGTGGCACTTTGGTGGCT
SYNPO	F	GGCCGATTGACAGAGCATCC
	R	TTCGGTGAAGCTTGTGCTCCG
ACTN4	F	GCCCGACGAGAAGGCCATAA
	R	ACCTTGCAGATCCGGTTGGC
Vim	F	TCCAGAGAGAGGAAGCCGAA
	R	AAGGTCAAGACGTGCCAGAG
ITGA3	F	CCTGATCGCCTCAAGCTCGG
	R	GTTGTCTGGCCCGCACTCTT
ITGB1	F	TGCAGGTTGCGGTTTGTGGA
	R	GCATCCGTGGAAAACACCAGC
ZO-1	F	TGAGGCAGCTCACGTAGGTCT
	R	CTGGGTGACCAAGAGCTGGTT
PCAD	F	TATTCGTCCCCGAGAATGGC
	R	TCTCCCTGTCCAGTGGCATA
KCAD	F	GCTGTGGCACAACATCACAG
	R	CATCCTTGTCAACAGCACGC
NCAD	F	GGAATGCTGCGGTACAGGAT
	R	GGCCATAAGTGGGATTGCCT

10 μM serum-depleted Y-27632 medium (induction culture conditions), we observed the cell process extended from the cell body, with arrowhead branching patterns (Fig. 1). Therefore, we verified the induction culture conditions contained the elements required for process formation in HSMP. HSMP was also cultured with or without Y-27632 and 10% FBS; and with L511 (Nippi) instead of L521, using the induction culture conditions as the base. Phase contrast images showed that, in contrast to the induction group, conditions without Y-27632, with 10% FBS or L511, instead of L521, did not permit podocyte process formation (Fig. 1).

3.2. Applying the induction culture condition to multi-species conditionally immortalized podocytes

We verified whether the induction culture conditions permit process formation in the podocytes of other species. In phase contrast images (Fig. 2), the morphology of induction groups showed an arborized process formation pattern, in contrast to the cobble stone pattern seen in standard groups with 10% FBS at high cell density. Podocytes from mouse, rat and human species all showed a difference in morphology between the standard and induction culture conditions.

3.3. Localization patterns of cytoskeletal proteins

We focused on the localization of intermediate vimentin filaments, and F-actin microfilaments related to cell morphology. In immunofluorescent images (Fig. 3), we observed in the induction groups that F-actin localized in a spotty pattern, and vimentin filaments formed a pattern with significant arborization from the nucleus. Moreover, branching of the vimentin bundle was seen in the induction groups, mostly in mouse species (HSMP). In the standard groups, F-actin stress fibers and vimentin filaments showed a scattering pattern. These differences are observed in all three species. Based on these findings, morphological differences in podocytes are accompanied by evident differences in cytoskeletal protein localization, suggesting reorganization of the cytoskeleton.

3.4. Comparison of mRNA expression levels in HSMP between standard and induction culture condition

We compared the mRNA expression levels of HSMP between standard and induction culture conditions with respect to phenotypic genes related to cell morphology (Fig. 4). The induction group had a higher expression level of podocalyxin (PODXL), an apical glycoprotein, than the standard group. In contrast, synaptopodin (SYNPO) and α-actinin-4 (ACTN4), actin-binding proteins in the induction group, showed lower expression in the induction group than in the standard group. The expression level of vimentin, one of the intermediate filaments, was not significantly different between the standard and induction group. The expression levels of integrin α3 (ITGA3), a heterodimer with high affinity to L521, which is highly expressed in mature podocytes in vivo, had a higher expression level in the induction group, whereas another similar heterodimer, integrin β1 (ITGB1), had a lower expression in the induction group. The induction group had lower expression of tight junction protein 1 (ZO1) and cadherin.

4. Discussion

This study provides the new insight that the combination of serum depletion, the scaffold of L521, and the inhibiting Rho kinase cascade promotes process formation in conditionally immortalized podocytes. Focusing on each factor, it was previously reported that protein overload in cultured podocytes results in actin reorganization and that the major component of the matured glomerular basement membrane is L521 instead of L511 in the developing stage of glomeruli [15,16]. In detail, for optimized laminin isoforms, although L511 has been selected for cultured podocytes as an alternative to L521, we observed poor podocyte process formation in our study compared with that for L521. Detailed observation of the location of the laminin isoform in matured glomeruli showed that the laminin β1 subunit is observed in the mesangial area, but not in capillaries. Notably, abnormal wider expression in the glomerulus is observed in several nephrotic syndromes [17]. Serum depletion and selection of L521 for the scaffold of cultured podocytes in this study is a factor common with the study by Yaoita et al. Therefore, we conclude that the slight difference in extracellular matrix isoforms has a great influence on podocyte process formation, and L521 is more suitable for process formation in cultured podocytes than L511.

The inhibition of the Rho kinase pathway has several positive roles, including attenuation of podocyte foot process effacement in abnormal conditions. Indeed, it has been reported that the activation of this pathway causes foot process effacement [18–20]. In this study, the inhibition of the Rho kinase pathway by Y-27632 with serum depletion and the L521 scaffold was the crucial element for process formation. Hence, we suggest that in cultured podocytes, the Rho kinase pathway is more highly activated than in podocytes in vivo, and its inhibition is essential for process formation in cultured podocytes

However, some limitations of this study should be noted. The duration of process formation using the method in this study was limited to only several hours. We also observed deformation, with active migration and the subsequent boosted proliferation of cells. In addition, vacuoles in the cytoplasm were remarkably observed in

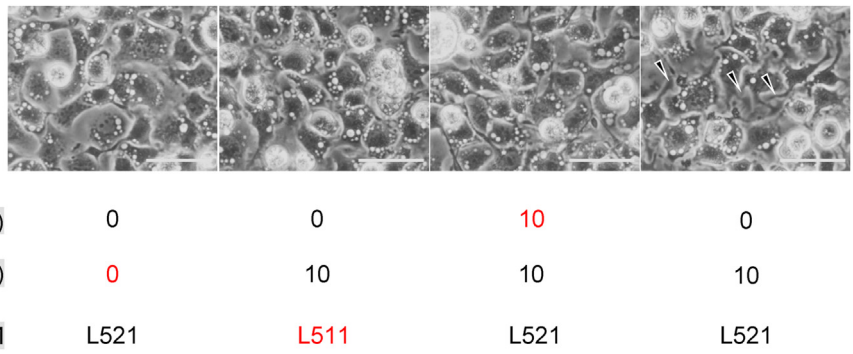

Fig. 1. Optimizing culture condition for process formation Interdigitating process formation (arrowheads) was observed only in cultured podocytes with F0, Y10, and L521, but not in podocytes without Y, with L511 scaffold instead of L521, or with F10 supplementation. Scale bar, 50 μm.

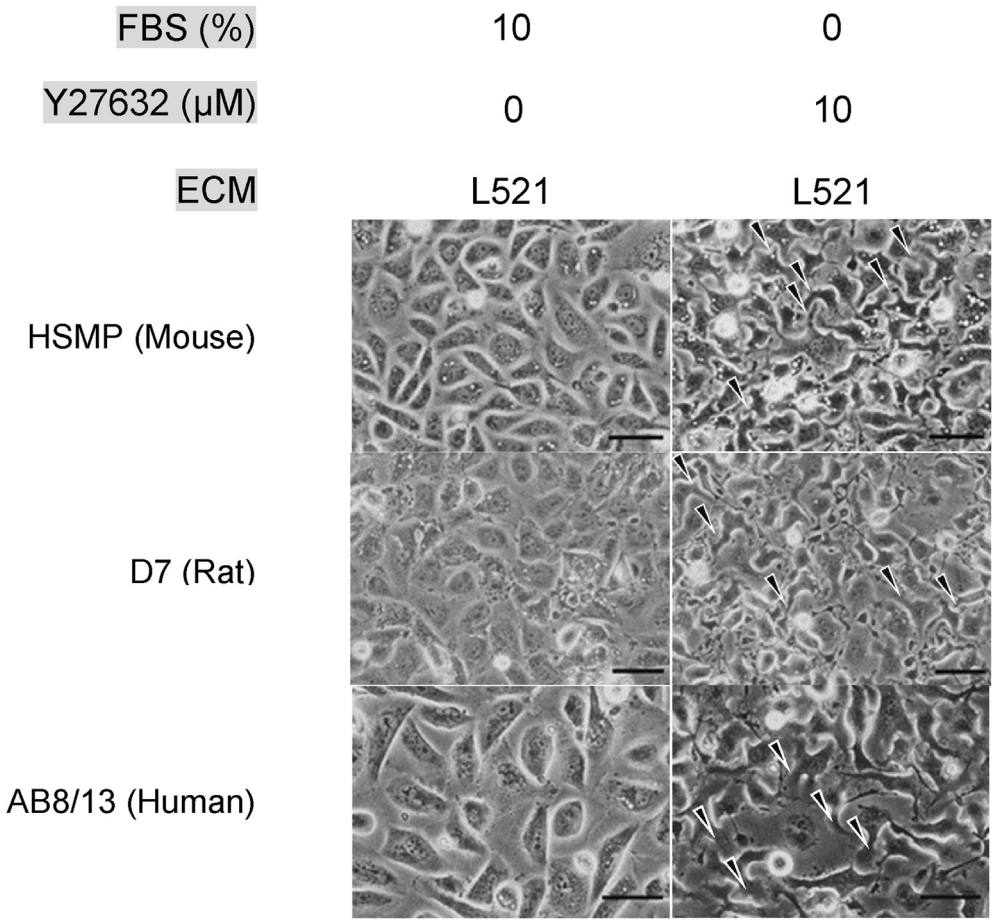

Fig. 2. Phase contrast images of process formation in conditionally immortalized podocytes Every image was taken 2 h after seeding. Interdigitated process formation (arrowheads) was observed in HSMP, D7, and AB8/13 in the induction group and not in the standard group. Scale bar, 50 μm.

HSMP. Previous study reported that L521 can stimulate integrin signaling cascade of small GTPase Rac activation pathway or increasing reactive oxygen species (ROS) production [21,22], and those cause cell migration, overgrowth, and vacuoles [23,24]. Moreover, harmful effects of Rac and ROS for podocytes also have been reported [25,26]. Therefore, we consider that current induction culture condition has any adverse effects. Clarifying and regulating possible adverse signal cascade would improve this limitation.

Although the comparison of mRNA expression levels in the induction group was performed while under possible oxidative stress, we observed higher expression levels of PODXL and lower expression levels of N- and K-cadherin, suggesting that this induction method improves polarity, differentiates podocytes to some extent [27], and contributes to podocyte process formation via the suppression of cell junction differentiation [28]. On the other hand, ZO-1 and P-cadherin (major cell junction proteins for mature podocytes); and SYNPO and ACTN4 (highly expressed in mature podocytes), had lower expression than in the standard group. Based on a previous report that oxidative stress has a negative effect on cell junctions comprising ZO-1 or P-cadherin [29,30], exploring the effect of antioxidants as a serum substitute might lead to overcoming this problem. While it has been reported that SYNPO induces expression of actin stress fibers [31] and that α-actinin has the role of bundling the actin filament in vitro [32], the downregulation of SYNPO and ACTN4 in the induction group might be linked to attenuation of actin stress fibers. In the induction group, inversely related expression levels of ITGA3 and ITGB1 were observed. It has been reported that the α3 subunit is bound mostly with laminin alone, while the β1 subunit also interacts with collagen, among several types of integrin heterodimers [33]. In this study, the ITGA3 expression ratio to GAPDH was lower than that of ITGB1 [ITGA3 (0.048 ± 0.0084: standard, 0.063 ± 0.0061: induction), ITGB1 (0.286 ± 0.029: standard, 0.152 ± 0.012: induction)]. Therefore, the correction of the imbalance between ITGA3 and ITGB1 might be a natural consequence of the laminin-rich subunits in the induction group.

In summary, this study showed that the combined culture conditions of serum depletion, specialized L521 scaffold, and Rho kinase pathway inhibition had significant effects on process formation in conditionally immortalized podocytes. As alteration of podocyte foot process formation is traditionally one of the pathological hallmarks of nephrotic syndrome, these findings elucidate the potential mechanism by which this symptom manifests. However, podocyte process formation has not been realized on the planar base before 2017, even with podocytes induced from pluripotent stem cells. Although the novel culture method developed by Yaoita et al. was remarkable for being able to reproduce interdigitating process formation under highly confluent conditions on a planar base, the method is limited in that it is only applied to rat primary podocytes, and is therefore not capable of being applied to conditionally immortalized podocytes, which provide unlimited cell growth. Hence, the findings of this study provide us with the prospect that conditionally immortalized

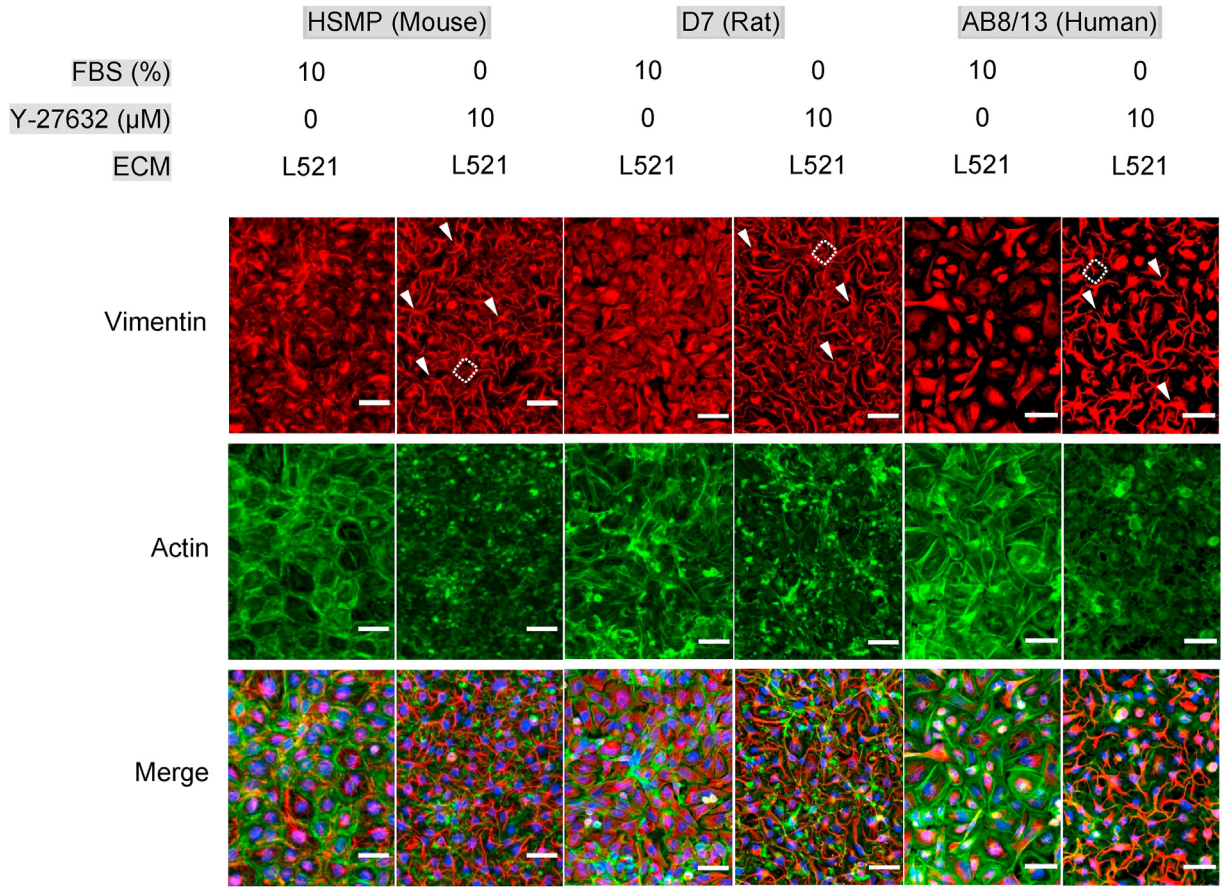

Fig. 3. Localization patterns of cytoskeletal proteins Localizations of vimentin (red) and F-actin (green) were obviously different between cultured podocytes HSMP, D7 and AB8/13 in the standard (10% FBS) and induction (10 μM Y-27632) groups. In the standard groups, vimentin filaments form a disseminated pattern and actin stress fibers are evident. In the induction groups, vimentin filaments form a highly arborized pattern stemming from the nucleus (arrowheads), and actin stress fibers are absent. Branching of vimentin bundles (rectangle of dotted line) was also seen, especially in HSMP. Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

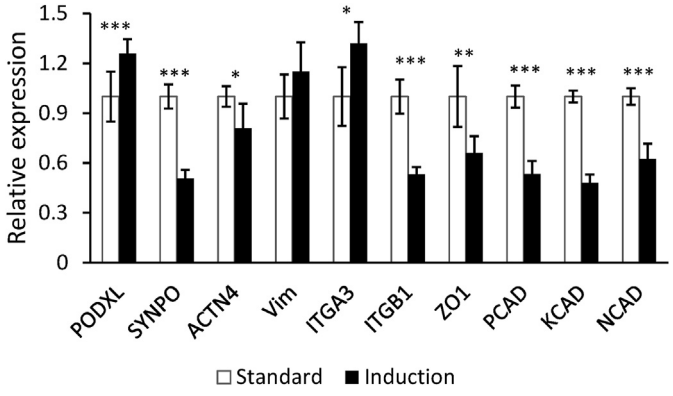

Fig. 4. Comparison of mRNA expression levels in HSMP between standard and induction culture groups.
PODXL expression levels in the induction groups were higher than in the standard groups, and vice versa for SYNPO and ACTN4 expression levels. There is no significant difference in vimentin(Vim) expression levels between the groups. ITGA3 and ITGB1 expression levels in the induction groups were higher and lower than in the standard groups, respectively. Expression levels of tight junction protein 1 (ZO1) and cadherin in the induction groups were lower than those in the standard groups. All data were normalized to that of GAPDH and are shown as the ratio with the standard group expression level. ($n = 6$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

podocytes could be useful cell sources for drug discovery and the study of proteinuria syndromes.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Teruo Fujii reports financial support was provided by the Japanese Agency for Medical Research and Development, Tokyo, Japan. Kotaro Doi, Hiroshi Kimura, Tetsuhiro Tanaka, Reiko Inagi, Masaomi Nangaku reports financial support was provided by the Japanese Agency for Medical Research and Development, Tokyo, Japan.

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