The followings were published in 'Biochemical and Biophysical Research Communications' 435 (2013) 327-333

Cellular interactions via conditioned media induce *in vivo* nephron generation from tubular epithelial cells or mesenchymal stem cells

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ABSTRACT
There are some successful reports of kidney generation by utilizing the natural course of kidney development, namely, the use of an artificially treated metanephros, blastocyst or ureteric bud. Under a novel concept of cellular interactions via conditioned media (CMs), we have attempted *in vivo* nephron generation from tubular epithelial cells (TECs) or mesenchymal stem cells (MSCs). Here we used 10X CMs of vascular endothelial cells (VECs) and TECs, which is the first to introduce a CM into the field of organ regeneration. We first present stimulative cross-talks induced by these CMs between VECs and TECs on cell proliferation and morphological changes. In MSCs, TEC-CM suppressed these changes, however, induced cytokeratin expression, indicating the differentiation of MSCs into TECs. As a result, glomerular and tubular structures were created following the implantation of TECs or MSCs with both CMs. Our findings suggest that the cellular interactions via CMs might induce *in vivo* nephron generation from TECs or MSCs. As a promoting factor, CMs could also be applied to the regeneration of other organs and tissues.

Keywords:
conditioned medium, cross-talk, glomerulogenesis, nephron generation, mesenchymal stem cell, tubular epithelial cell

1. Introduction

Organ regeneration is a novel and attractive therapeutic strategy for intractable diseases. However, despite an increase in the number of renal failure patients, a kidney remains one of the most difficult organs to regenerate due to its complicated structure composed of several different cell types. Some successful techniques for kidney generation have been presented. Hammerman *et al*. obtained kidneys through a series of metanephros transplantations into other sites or animals [1,2]. Yokoo *et al*. advanced the method of metanephros transplantation by injecting human mesenchymal stem cells (hMSCs) into the site of ureteric bud sprouting prior to the formation of metanephros in rodent embryos. This method resulted in the formation of chimeric metanephros containing hMSCs and rodent embryonic cells after cultivation of the whole embryo. They created urine-producing kidney tissues through the transplantation of these artificial metanephroi [3,4]. Using a concept of blastocyst complementation, Nakauchi *et al*. have successfully generated kidneys by injecting mouse pluripotent stem cells (PSCs), including embryonic stem cells and induced (i) PSCs, into a mouse Sall1$^{-/-}$ blastocyst [5,6]. Although the mechanisms of kidney development have become clear [7-9], the genes and factors that act at each stage of kidney development are not completely understood. Therefore, Yokoo *et al*. and Nakauchi *et al*. may have utilized the natural course of kidney development. Vessels and collecting ducts (CDs) they obtained were chimeras composed of embryo- or blastocyst-derived cells and injected stem cells. Their techniques are subject to difficult issues, most notably ethical and immunological concerns. Nigam *et al*. have also successfully generated a kidney *in vivo* by reconstructing the developmental course of kidney. There are 3 components of a kidney: vessels, metanephric mesenchyme (MM)-derived tubules and ureteric bud (UB)-derived CDs. They initiated the formation of CDs from a UB cell and transplanted the composite tissue of *in vitro* regenerated CDs and MM [10,11]. Osafune *et al*. recently developed the method for differentiating iPSCs into nephrogenic intermediate mesoderm (IM) [12]. Because the MM can be replaced by the patient's nephrogenic IM, the technique of Nigam *et al*. will avoid the issues that Yokoo *et al*. and Nakauchi *et al*. face. Alternatively, there are some successful reports of *in vitro* kidney tissue generation and *in vivo*

kidney repair by using fetal renal cells and/or UB cells [13,14]. Their techniques also have a possibility of being free of these issues.

We have also attempted *in vivo* kidney generation using a novel concept of cellular interactions, or cross-talks, via conditioned media (CMs). In this study, we focused on *in vivo* nephron generation, also known as glomerulogenesis and tubulogenesis. We used differentiated mouse vascular endothelial cells (VECs); MILE SVEN 1 (MS1) cells, canine tubular epithelial cells (TECs); Madin-Darby canine kidney (MDCK) cells and hMSCs. A 10X concentration of MS1-CM and MDCK-CM was used as a regeneration-promoting factor. Our preliminary study using 1X CMs showed mild morphological changes in 3-dimensional (3-D) culture and very immature glomerular structures following the implantation of MDCK cells. First, we tested the effect of cellular interactions on cell proliferation and morphological changes between MS1 and MDCK cells and between hMSCs and MS1 or MDCK cells using 2-D and 3-D cultures with 10X MS1-CM and MDCK-CM. In addition, the effect of these CMs on the differentiation of hMSCs into TECs was examined by an immunohistochemical (IHC) assay of cytokeratin and by 3-D cultures. Finally, MDCK cells or hMSCs mixed with type 1 collagen (Col-1) gel and both 10X MS1-CM and MDCK-CM were implanted into the subcutaneous spaces of immunodeficient rats. Twelve weeks after implantation, the explants were performed microscopic studies including specific IHC assessments for characterizing the glomerular and renal tubular cells.

2. Materials and methods

2.1. Conditioned media

MS1 cells (ATCC, USA) and MDCK cells (ECACC, UK) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Lonza, USA) containing 5% fetal bovine serum (FBS; Lonza, USA). Before these cells reached confluency, they were treated twice with DMEM lacking FBS. The CMs without FBS were collected once the cells reached confluency, and they were centrifuged at 1400 rpm for 5 minutes. Then, the CMs were concentrated 10 times by dialysis using the Pellicon XL Device with a pore size of 0.22 μm (Millipore, USA).

The concentrations of kidney-associated cytokines such as vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) in both 10X MS1-CM and MDCK-CM were measured by an enzyme-linked immunosorbent assay (ELISA) using the following commercial kits: Quantikine Human VEGF

Immunoassay (R&D Systems, USA), Quantikine Human PDGF-BB Immunoassay (R&D Systems, USA), Human IL-6 CLEIA Fujirebio (Fujirebio, Japan), Quantikine Human FGF basic Immunoassay (R&D Systems, USA) and HGF Otsuka ELISA kit (Otsuka Pharmaceutical Co., Japan) [15-17].

2.2. 2-D cultures

To study how the cellular interactions between VECs and TECs and between MSCs and VECs or TECs influence cell proliferation, MS1 cells, MDCK cells and hMSCs (Lonza, USA) were each divided into the following 3 groups: the 10X MS1-CM-added group, the 10X MDCK-CM-added group and the CM-free control group (n=10 for each group). MS1 and MDCK cells were cultivated in 10 cm dishes using 10 ml of DMEM containing 5% FBS, and hMSCs were cultivated using 10 ml of Lonza's Mesenchymal Stem Cell Basal Medium (MSCBM) with MSCGM SingleQuots growth supplement, which is composed of FBS, L-glutamine and penicillin/streptomycin. One ml of 10X CM was added to each of the CM-added groups. Each dish was seeded with $0.1X10^6$ cells. When several dishes from each cell group reached 90-95% confluency, the cell cultures were discontinued. The cell numbers were counted and expressed as the mean ± SD. The significance was determined using an unpaired, two-tailed Student's *t* test. *P* values less than 0.05 were considered to be significant.

2.3. 3-D cultures

To examine the effect of cellular interactions on morphological changes, 3-D cultures of MS1 cells, MDCK cells and hMSCs were grown with Col-1 gel complex containing 10X MS1-CM or MDCK-CM. The gel complex was composed of rat tail Col-1 solution (60%/v) (4.08 mg/ml, Lot no. 59802; BD Biosciences, USA), 10X Medium 199 (10%/v) (Invitrogen, USA), basal medium; DMEM or MSCBM without FBS and supplement (15%/v), 1N NaOH (23 μl/ml gel), and 10X MS1-CM or MDCK-CM (15%/v). When both CMs were used, the basal medium was excluded, and 15%/v of each CM was added. The controls contained 30%/v basal medium excluding CM. Each cell line was mixed with the gel complex at a concentration of $1.0X10^6$ cells/ml gel. A 48-well plate was seeded with 250 μl of the cell/gel mixture, and it was incubated at 37 °C with 5% CO_2 for 14 days before observation.

2.4. Differentiation of MSCs into TECs

To verify the differentiation of MSCs into TECs, hMSCs were grown in 2-D culture for 4 weeks in a 6-well plate with 2 ml of supplemented MSCBM and 0.2 ml of 10X MS1-CM, MDCK-CM or without CM.

These cultivated hMSCs were transferred to a microscope slide, and cytokeratin was assessed by an IHC assay using monoclonal mouse anti-human cytokeratin antibody (DakoCytomation, Denmark). As a positive control, the IHC assay was also performed using MDCK cells. Because it is unclear whether aquaporin 1 (AQP1)-negative cells are distal TECs or undifferentiated MSCs, we chose the staining for cytokeratin.

Furthermore, to verify the formation of tubular structures, hMSCs that were cultivated in 2-D condition with 10X MDCK-CM for 4 weeks were used for 3-D cultures. The 3-D cultures were grown with 10X MS1-CM, 10X MDCK-CM or without CM, as previously described for 3-D cultures.

2.5. Implantation of TECs or MSCs

For the implantation of MDCK cells and hMSCs, a gel complex was prepared with Col-1 solution (60%/v), both 10X MS1-CM and MDCK-CM (15%/v of each), 10X Medium 199 (10%/v) and 1N NaOH (23 μl/ml gel), as previously described for the 3-D culture model. Instead of CM, the control group were used basal medium; DMEM or MSCBM (30%/v), without FBS or supplements. Two million MDCK cells or hMSCs were mixed with 1 ml of the gel complex. The next day after incubating overnight, 300 μl of the cell/gel mixtures were implanted into the abdominal subcutaneous spaces of 7-week-old immunodeficient hairless rats, HWY-SLC (Shimizu Laboratory Supplies, Japan). Twelve weeks later, the rats were sacrificed and the implanted tissues were retrieved for analysis. All procedures were approved by the Committee for Animal Experiments of Institute for Frontier Medical Sciences, Kyoto University.

2.6. Histology of the explants

Glomerular structures formed in the retrieved tissues were observed under a microscope after hematoxylin and eosin (H&E) and periodic acid-methenamine-silver (PAM) stainings and IHC assays using the following antibodies: goat polyclonal anti-mouse platelet/endothelial cell adhesion molecule-1 (PECAM-1) antibody (Santa Cruz Biotechnology, USA), mouse monoclonal anti-human α-smooth muscle actin (α-SMA) and vimentin antibodies (both, DakoCytomation, Denmark) and anti-rat synaptopodin antibody (Progen Biotechnik, Germany). To detect proximal and distal tubules, the tissues were stained for AQP1 and cytokeratin using a mouse monoclonal anti-human AQP1 antibody (Santa Cruz Biotechnology, USA) and the previously described cytokeratin antibody.

3. Results

3.1. Cytokine levels in 10X VEC-CM and TEC-CM

The concentrations of VEGF, PDGF-BB, IL-6, bFGF and HGF in 10X MS1-CM were <20 pg/ml, 1020 pg/ml, <0.2 pg/ml, <10 pg/ml and <0.30 ng/ml, respectively, and those in 10X MDCK-CM were 35 pg/ml, 530 pg/ml, <0.2 pg/ml, 27 pg/ml and <0.30 ng/ml, respectively. The symbol '<' means 'below the measurable limit'. The limit of PDGF-BB is 31.2 pg/ml. Although the PDGF-BB levels were high, it is uncertain which cytokine was responsible for the following cross-talks. There is also a possibility that other unknown factors played pivotal roles in the observed phenomena.

3.2. Cell proliferation

On day 9 in 2-D cultures, the numbers of MS1 cells cultivated with 10X MS1-CM, 10X MDCK-CM and without CM, as a control, were 3.42±0.34, 4.03±0.30 and 2.97±0.28 $X10^6$ cells/dish, respectively (n=10 for each). On day 6, the numbers of MDCK cells under the same conditions were 4.15±0.34, 3.37±0.36 and 2.68±0.40 $X10^6$ cells/dish, respectively (n=10 for each). MS1 cell proliferation significantly increased when cultured in 10X MDCK-CM compared to 10X MS1-CM (p<0.0005). A similar trend was observed for MDCK cells, as 10X MS1-CM accelerated their proliferation compared to 10X MDCK-CM (p<0.0001). These results indicate that there is a cross-talk between VECs and TECs that promotes cell proliferation. In addition, the proliferation of MS1 and MDCK cells was enhanced in an autocrine/paracrine manner by their own CMs compared to their controls (p<0.005 and p<0.001) (Fig. 1A and B).

On day 19, hMSCs in 2-D cultures with 10X MS1-CM, 10X MDCK-CM or without CM demonstrated proliferation rates of 2.68±0.26, 1.80±0.24 and 2.05±0.26 $X10^6$ cells/dish, respectively (n=10 for each). Compared to 10X MDCK-CM and the CM-free control group, 10X MS1-CM greatly enhanced the proliferation of hMSCs (p<0.0001 for each group). Alternatively, 10X MDCK-CM significantly suppressed the proliferation of hMSCs compared to the control (p<0.05) (Fig. 1C).

3.3. Morphological changes

After 14 days in 3-D cultures, 10X MS1-CM caused small tubular changes in 20-30% of MS1 cells, whereas larger tubular structures, a process known as vasculogenesis, were formed with 10X MDCK-CM in more than 50% of MS1 cells. In 10-20% of MDCK cells, 10X MDCK-CM caused small tubular changes, whereas 10X MS1-CM induced the formation of larger tubular structures, a process known as tubulogenesis, in 50-60% of MDCK cells. In addition, MS1 and MDCK cells cultivated with both 10X CMs showed the greatest tubular changes. However, MS1

and MDCK cells scarcely showed any changes in CM-free cultures. A cross-talk between VECs and TECs was also observed in the formation of tubular structures (Fig. 2a-h).

The 10X MS1-CM changed 30-40% of hMSCs into tubular structures, which was almost the same percentage and degree of change observed with the mixture of 10X MS1-CM and MDCK-CM. The CM-free control culture turned hMSCs into spindle-like cells. However, 10X MDCK-CM was unable to change hMSCs. Indicative of the possibility of a cross-talk between MSCs and TECs, both the proliferation and morphological changes observed in hMSCs were evidently suppressed by 10X MDCK-CM (Fig. 2i-l).

3.4. Differentiation of MSCs into TECs

After 4 weeks in 2-D culture with 10X MDCK-CM, cytokeratin expression was observed in 40-50% of hMSCs, indicating the differentiation into TECs. 10X MS1-CM did not show a similar expression pattern. MDCK cells expressing cytokeratin were used as a positive control, and hMSCs cultivated without CM were used as a negative control (Fig. 3A).

Following 4 weeks in 2-D culture with 10X MDCK-CM, hMSCs were cultivated for 14 days in 3-D conditions. Human MSCs that were cultivated with 10X MS1-CM formed stronger tubular structures than hMSCs cultivated with 10X MDCK-CM. Human MSCs without CM showed few changes (Fig. 3B). These tubular changes were similar to those observed in MDCK cells (Fig. 2e-g), but not in hMSCs (Fig. 2i-k), confirming the differentiation of hMSCs into TECs.

3.5. In vivo nephron generation from TECs or MSCs

In the tissue from MDCK cell implantation, approximately 30 glomerular structures with tubule-connected Bowman's-like capsules were generated adjacent to angiogenic vessels. PAM staining revealed the walls of various-sized glomerular capillary-like structures, which included several red blood cells (RBCs). IHC assays showed PECAM-1-positive capillary endothelial cells, α-SMA-positive or vimentin-positive mesangial cells and endothelial cells, and synaptopodin-positive podocytes. PECAM-1 was not expressed in all capillary structures, as determined by PAM staining. Much fewer α-SMA-positive mesangial cells were present compared to vimentin-positive mesangial cells. Additionally, cytokeratin-positive tubules were scattered throughout the tissue. Some of them were AQP1-positive proximal tubules, while the rest were AQP1-negative distal tubules (Fig. 4A).

Approximately 40 glomerular structures were detected in hMSC-implanted tissue. The glomerular findings were very similar to what were

observed after MDCK cell implantation. However, the number of tubular structures was much less than the number in MDCK cell-implanted tissue. Additionally, AQP1-negative distal tubules were more rare than AQP1-positive proximal tubules (Fig. 4B).

Glomerular and tubular structures were not observed in both the control tissues lacking CMs.

4. Discussion

We first demonstrated the cross-talks between VECs and TECs that stimulated both cell proliferation and morphological changes. In MSCs, VEC-CM also enhanced the proliferation and formation of tubular structures, whereas TEC-CM significantly suppressed these changes. When renal tubules are not damaged, TECs might act to inhibit changes in MSCs. Alternatively, we observed that TEC-CM, but not VEC-CM, changed MSCs into cytokeratin-positive cells. Despite the ability of MSCs to differentiate into various cell types such as bone, cartilage, tendon, smooth muscle cells, cardiomyocytes, fibroblasts and adipocytes [18], our finding indicates that TEC-CM, and therefore TECs, may have the ability to differentiate MSCs into TECs.

Based on these cellular interactions induced by CMs, we implanted individual TECs or MSCs with both VEC-CM and TEC-CM, and we easily obtained many glomerular and renal tubular structures. Xinaris *et al*. implanted renal organoids constructed *in vitro* from fetal renal cells below the host kidney capsule, resulting in further maturation of kidney tissue with vascularized glomeruli [14]. Which implantation of individual cells and *in vitro* generated organoids is more practical would be clarified by data accumulated in the future. It was noted that differentiated TECs might contribute to glomerulogenesis. With respect to the characterization of glomerular cells, specific IHC assays identified capillary endothelial cells, mesangial cells and podocytes. PECAM-1 expression was negative in many capillary-like structures, especially in smaller capillary-like structures. The further identification of capillary endothelial cells, in addition to podocytes, may require an electron microscopic study. However, the presence of RBCs in these structures seems to certify that these structures are just capillaries. α-SMA-positive mesangial cells were rare compared to vimentin-positive mesangial cells, which may suggest that the glomerular structures are fresh or growing. Although we did not examine the origins of these cells, Yokoo *et al*. previously used the LacZ gene to demonstrate that implanted hMSCs differentiate into TECs, Bowman’s capsule cells and glomerular podocytes [3,4]. Using specific stainings against cell markers, Osafune *et*

al. also showed that nephrogenic IM differentiated into TECs and podocytes [12]. The shape of glomerular structures we obtained seemed to be similar to normal glomeruli. However, further analysis of filtration function using a mix of fluorescent labeled-inulin and albumin or collected urine is needed to determine whether our glomerular structures are functional glomeruli [19].

AQP1-positive proximal tubules and AQP1-negative distal tubules were obtained after implantation of TECs or MSCs. However, the number of these tubules was less than that of glomerular structures. Yokoo *et al*., Nakauchi *et al*. and Nigam *et al*. showed rich tubules in their regenerated tissues [3,4,5,11]. Other factors that are not present in VEC-CM and TEC-CM might be responsible for the enhanced tubulogenesis observed in their studies. The decisive difference between the methods previously published by other authors and our method is the participation of UB. A cross-talk between UB and fetal mesenchyme appears to play an initial role in tubulogenesis during kidney development [7,8]. Thus, we need to examine the cellular interactions between UB cells and MSCs or TECs using their CMs to create more extensive tubules/nephrons.

We are the first to use CMs as a promoting factor in organ regeneration. Because many factors are included in a CM, it was very difficult to analyze the presence and specific function of each constituent of the CM. As a reference, we measured the concentrations of 5 kidney-associated cytokines. The presence of bFGF in TEC-CM indicates the cellular interaction between VECs and TECs, as bFGF is an angiogenic cytokine [20]. In contrast, patients with POEMS syndrome, which is characterized by the formation of glomerular and tubular structures in the skin lesions [21,22] or angiofollicular hyperplasia in the renal interstitium [23], have high serum levels of VEGF [24,25]. Therefore, we attempted *in vivo* glomerulogenesis from hMSCs using 100 pg/ml VEGF, which is 3 times higher than the concentration found in TEC-CM. However, we could not obtain any glomerular structures (data not shown). Although individual factors such as VEGF, bFGF and HGF have been used successfully as a growth factor for organ/tissue regeneration [26-29], the regeneration of complicated organs including a kidney would require many unknown mechanisms, such as plural growth factors. We intend to use CMs as a basal growth factor, because human cell-CMs, similar to insulin and erythropoietin, could be applied to other patients without severe immunorejection. In the near future, we will also be able to obtain CMs of patient iPSC-derived MSCs, TECs and CD/UB cells. Furthermore, if necessary, growth factors such as bFGF and HGF can be added to the CM to enhance angiogenesis and tubulogenesis [17,20,26].

In summary, using a novel concept of cellular interactions via CMs, we demonstrated stimulative cross-talks between VECs and TECs and suppressive actions of TECs against MSCs on cell proliferation and morphological changes. It was also noted that TECs might have induced the differentiation of MSCs into TECs. As a result, we obtained nephron-like structures after implantation of TECs or MSCs, manifesting the utility of CMs as a regeneration-promoting factor. Our technique using CMs would be useful for the regeneration of not only a kidney but also other organs/tissues.

Acknowledgments

T.M. thanks former Prof. Haruyoshi Yoshida of Fukui University School of Medicine for inspiring T.M.'s interest in kidney regeneration. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Figure Legends

Fig. 1. Cross-talks via CMs on cell proliferation.
The numbers of (A) MS1 cells (day 9), (B) MDCK cells (day 6) and (C) hMSCs (day 19) cultivated without CM (left bar), with 10X MS1-CM (center bar) or with 10X MDCK-CM (right bar in each graph). n=10 for each. *p<0.005, **p<0.0001, ***p<0.001 and ****p<0.05 to CM (-).

Fig. 2. Cellular interactions via CMs on morphological changes.
(a-d) MS1 cells, (e-h) MDCK cells and (i-l) hMSCs cultivated in Col-1 gel (a,e,i) without CM, (b,f,j) with 10X MS1-CM, (c,g,k) with 10X MDCK-CM or (d,h,l) with both 10X CMs. Scale bars: (a-l): 40 μm.

Fig. 3. Differentiation of MSCs into TECs by TEC-CM.
(A) Cytokeratin stainings of hMSCs cultivated in 2-D for 4 weeks (a) with 10X MS1-CM, (b) with 10X MDCK-CM, or (c) without CM, as a negative control. (d) MDCK cells as a cytokeratin-positive control.
(B) Human MSCs cultivated in 2-D condition with 10X MDCK-CM for 4 weeks were moved to 3-D cultures for 14 days (e) without CM, (f) with 10X MS1-CM, or (g) with 10X MDCK-CM. Scale bars: (a-g): 40 μm.

Fig. 4. *In vivo* nephron generation from TECs or MSCs.
Nephron-like structures in (A) MDCK cell-implanted and (B) hMSC-implanted tissues. (a,i) H&E and (b,j) PAM stainings demonstrating glomerular structures with Bowman's-like capsule and RBC-included capillary structures. The characterization of glomerular cells by IHC assays for (c,k) PECAM-1 (arrows), (d,l) α-SMA, (e,m) vimentin, and (f,n) synaptopodin. (g,o) Aquaporin 1-positive proximal tubules and (h,p) cytokeratin-positive, but aquaporin 1-negative, distal tubules. Scale bars: (a-p): 40 μm.

Figure 1. (A,B)

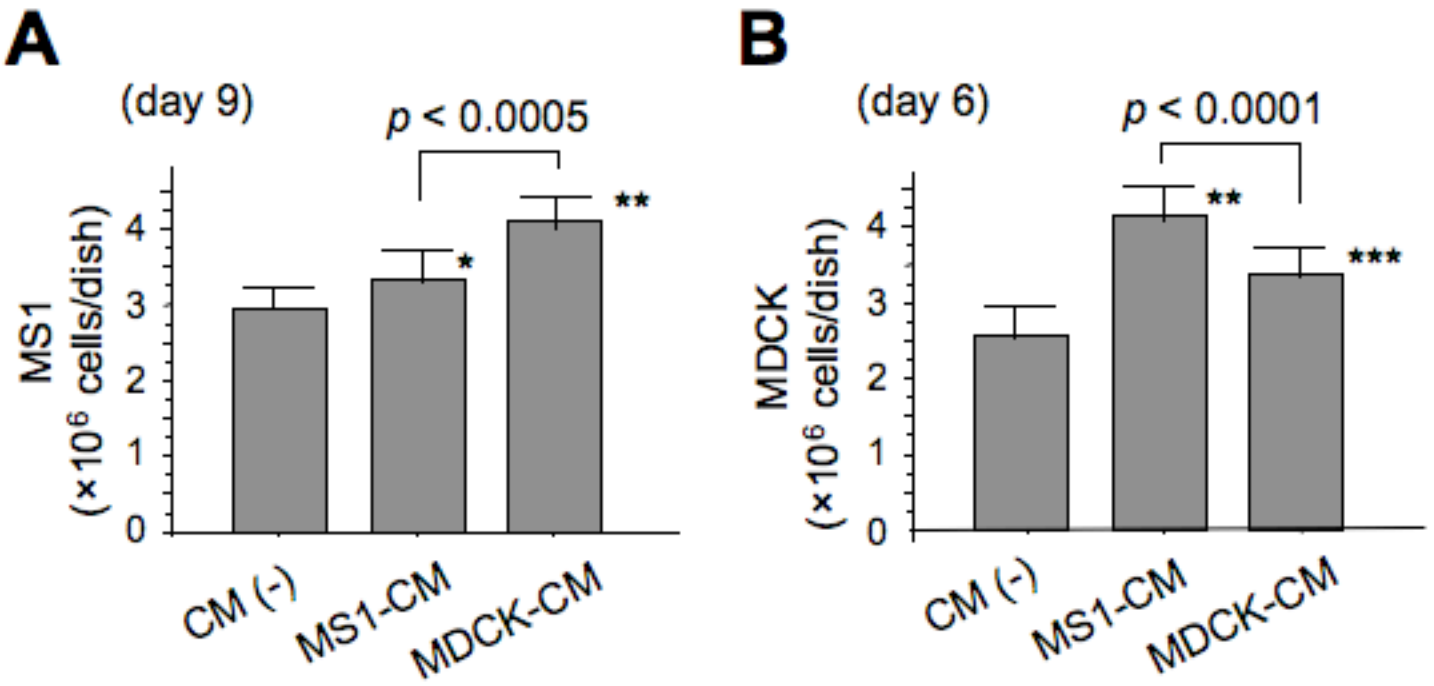

A
(day 9)
p < 0.0005
MS1
(×10⁶ cells/dish)
0
1
2
3
4
*
**
CM (-)
MS1-CM
MDCK-CM
B
(day 6)
p < 0.0001
MDCK
(×10⁶ cells/dish)
0
1
2
3
4
**
***
CM (-)
MS1-CM
MDCK-CM

Figure 1. (C)

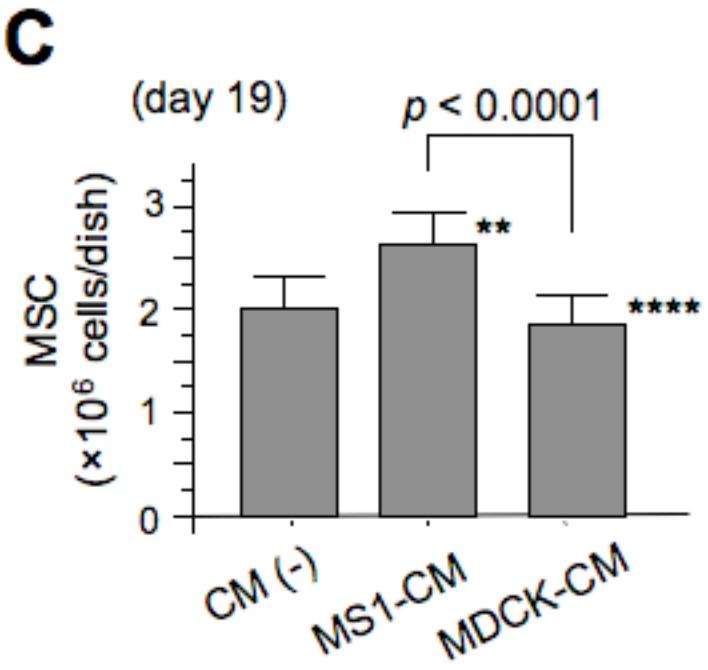


Figure 2.

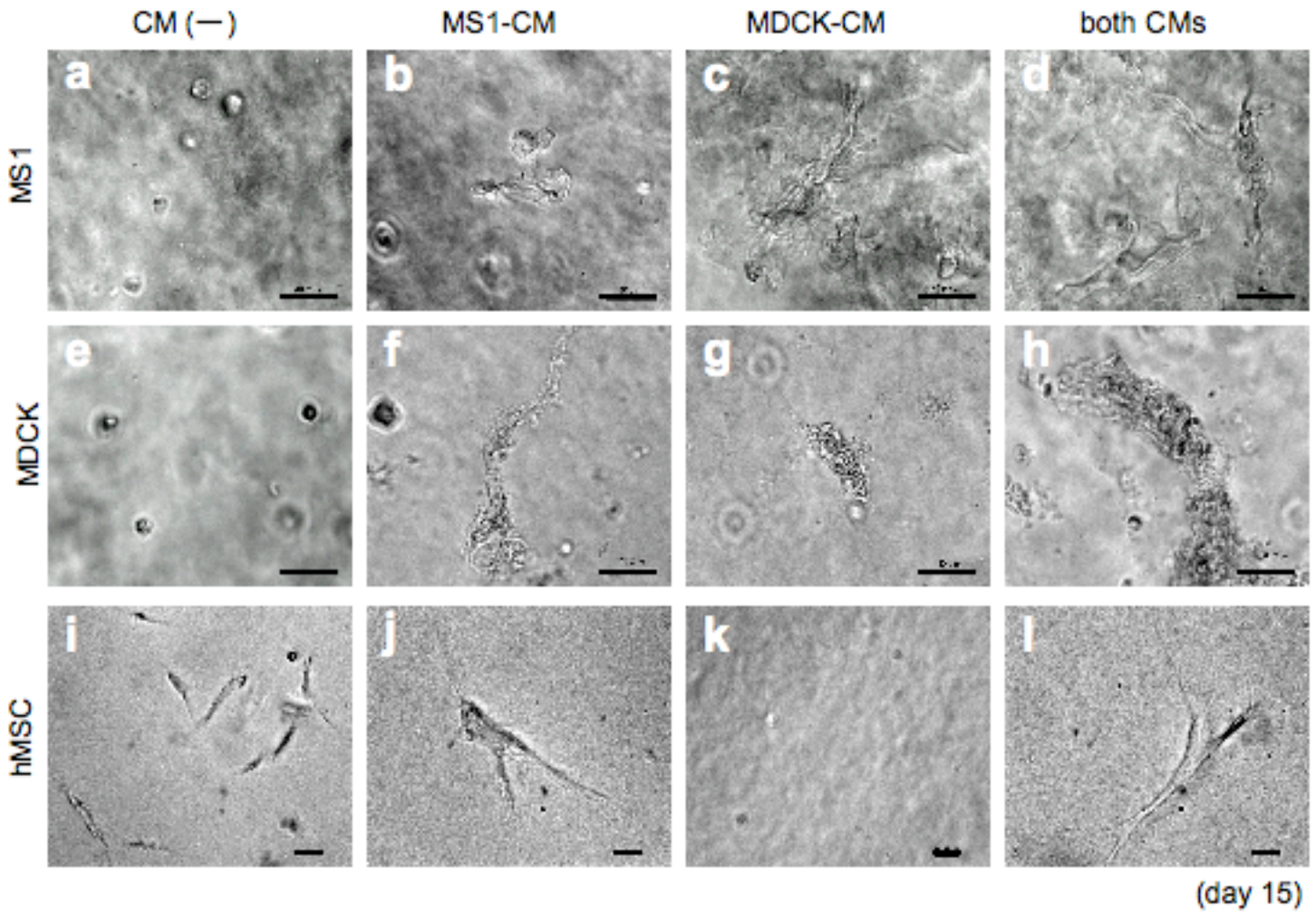


Figure 3 (A)

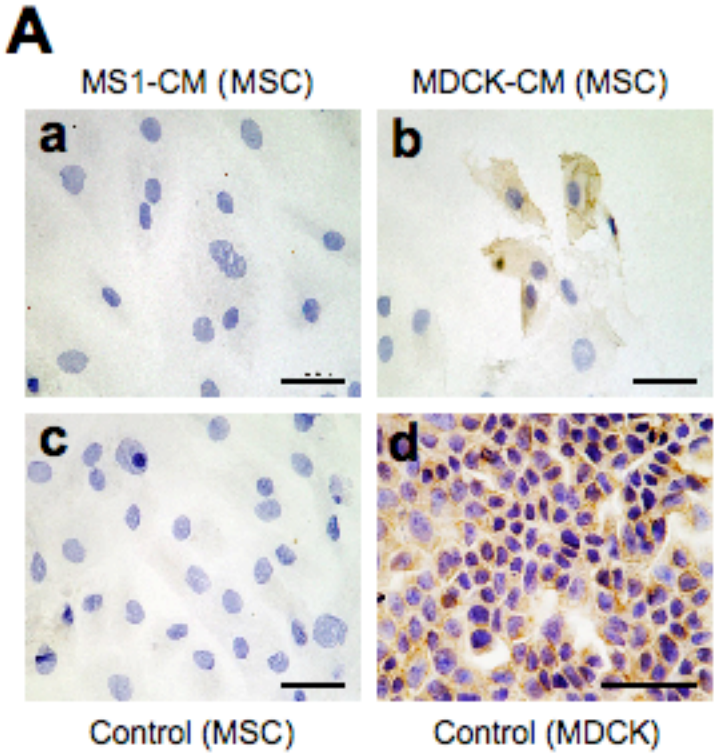


Figure 3 (B)

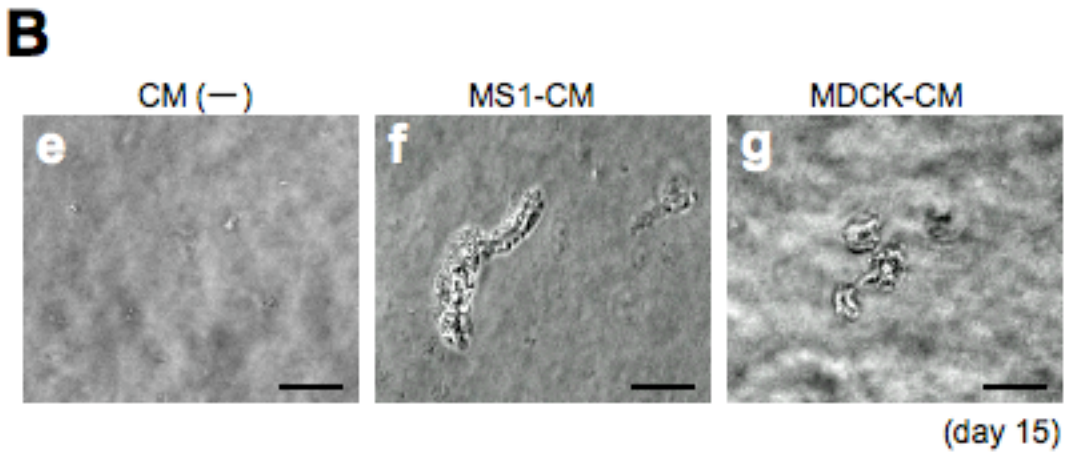


Figure 4 (A).

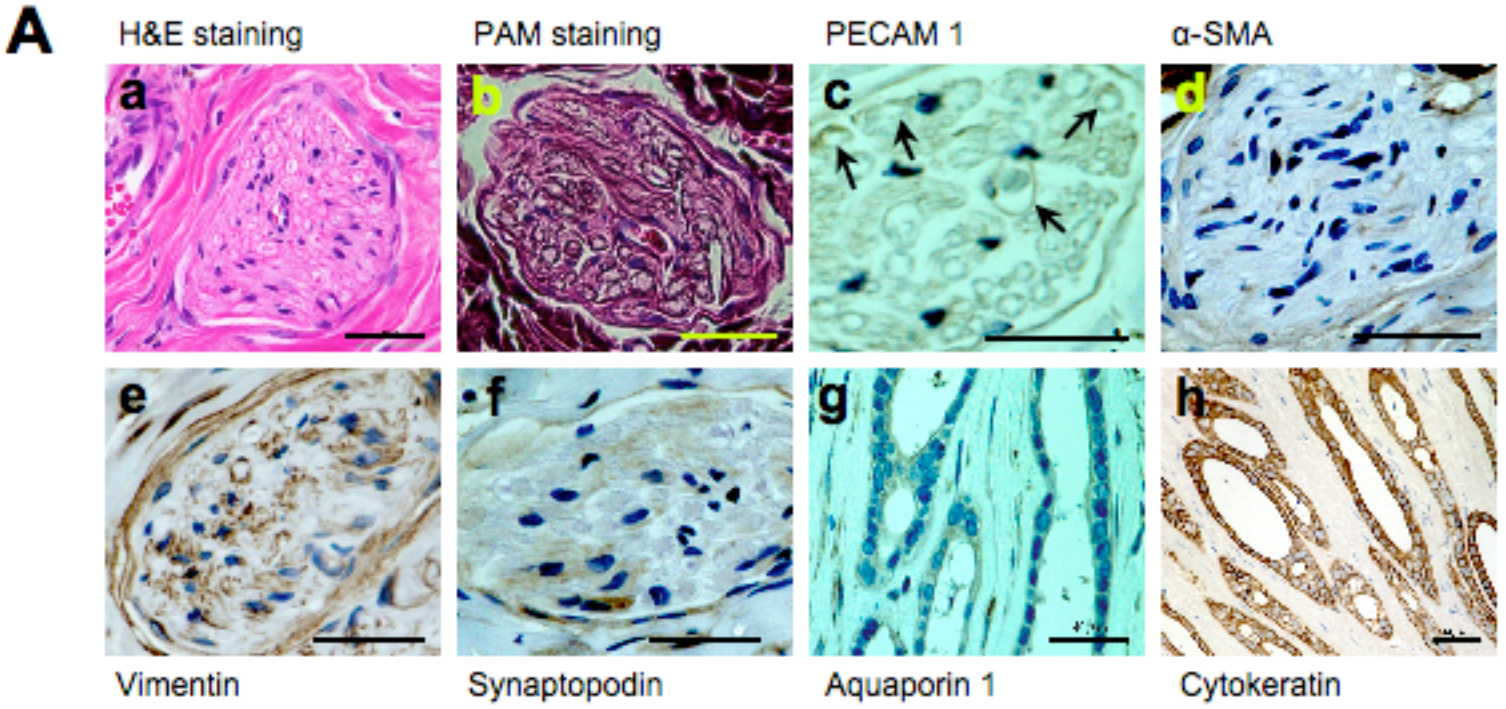
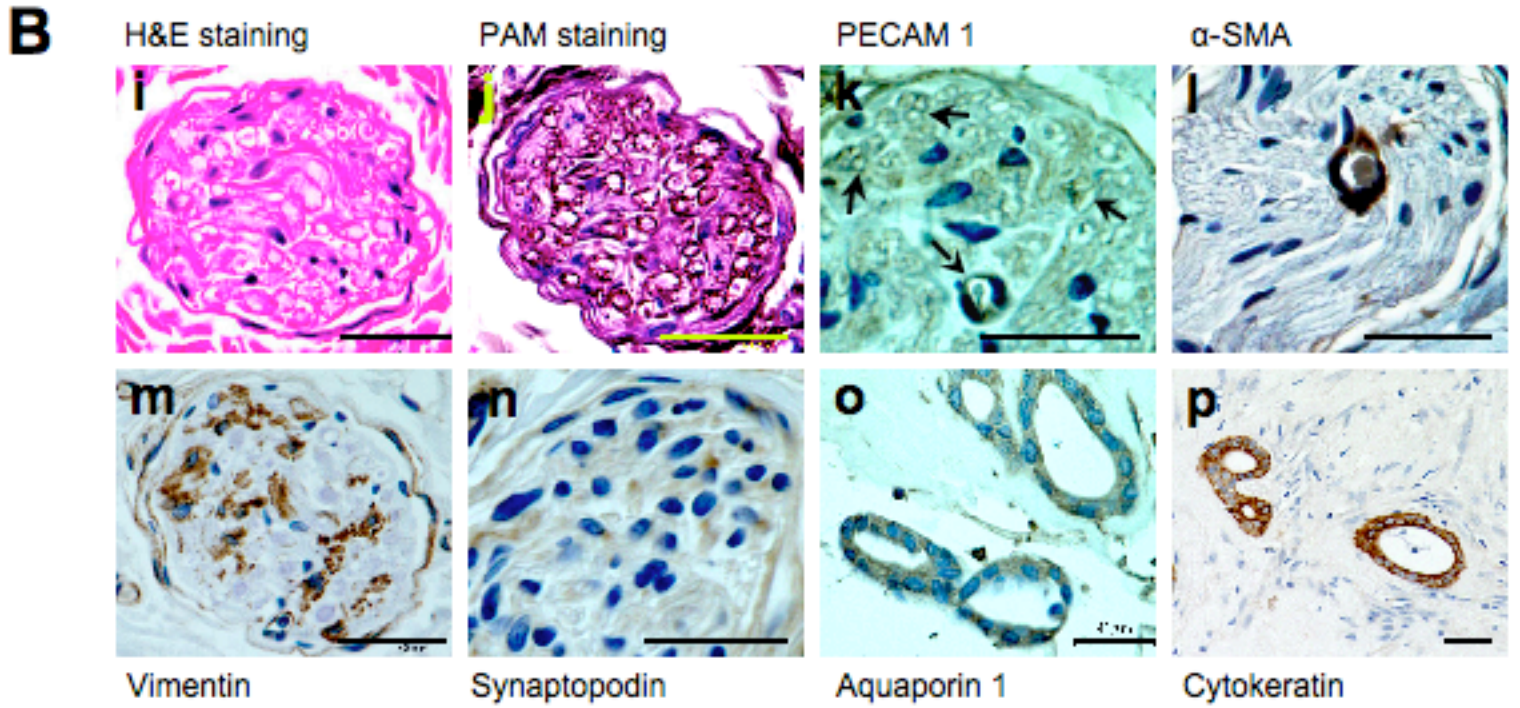

A
H&E staining
PAM staining
PECAM 1
α-SMA
a
b
c
d
e
f
g
h
Vimentin
Synaptopodin
Aquaporin 1
Cytokeratin

Figure 4 (B).


B
H&E staining
PAM staining
PECAM 1
α-SMA
i
j
k
l
m
n
o
p
Vimentin
Synaptopodin
Aquaporin 1
Cytokeratin