Conditioned medium from renal tubular epithelial cells initiates differentiation of human mesenchymal stem cells

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Abstract

Objectives: Mesenchymal–epithelial interactions play a pivotal role in tubular morphogenesis and in maintaining the integrity of the kidney. During renal repair, similar mechanisms may regulate cellular reorganization and differentiation. We have hypothesized that soluble factors from proximal tubular epithelial cells (PTC) induce differentiation of adipose-derived adult mesenchymal stem cells (ASC). This hypothesis has been tested using cultured ASC and PTC.

Material and methods: Conditioned medium was prepared from injured PTC and transferred to ASC cultures. ASC proliferation was analysed by a fluorometric and photometric assay. Signal transduction was analysed by phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/ERK2). Grade of ASC differentiation was assessed by morphological analysis and cell expression of characteristic markers.

Results: Conditioned medium significantly induced proliferation and phosphorylation of ERK1/ERK2 of ASC. After 12 days of incubation, cell morphology changed to an epithelial-like monolayer. Expression of cytokeratin 18 was induced by conditioned medium, while α -smooth muscle actin, CD49a and CD90 expression decreased. These alterations strongly indicate onset of the differentiation process to the epithelial lineage. In summary, soluble factors from PTC induce signal transduction and differentiation of ASC.

Conclusions: Our study shows that conditioned medium from renal tubular epithelial cells provides a convenient source of inductive signals to initiate differentiation of ASC towards epithelial lineage. We deduce that these interactions may play an important role during renal repair mechanisms.

Introduction

Reciprocal interactions between epithelial and mesenchymal cells are a fundamental process in nephrogenesis, the maintenance of organ integrity in the adult, and in renal regeneration. The kidney is derived from two primordial structures: the metanephric mesenchyme and the ureteric bud. Epithelial cells of the nephron are derived from the metanephric mesenchyme, whereas epithelial cells of the collecting duct are built from the ureteric bud (1,2). Knowledge of the cellular interactions, together with a growing understanding of mechanisms underlying kidney regeneration and repair, should help to exploit the biological potential of mesenchymal stem cells (MSC) for new therapeutic approaches after renal injury (3,4).

One of the major pathways to induce stem cell differentiation is contact with differentiated but impaired cell layers. Differentiation may either be initiated by direct cell–cell contacts, or chemically via secreted factors such as cytokines, interleukins or growth factors. Both these mechanisms probably work in concert. Thus, as a first approach, the influence of conditioned medium (CM) has to be investigated as it contains all these growth- and differentiation-regulating factors released by cells in a nonconfluent state, mimicking the *in vivo* situation of an incomplete and injured epithelial layer.

The recovery of renal integrity by replacement of damaged tubular cells and by recreating cells is initiated by activation of processes that have yet to be understood (3). The renal tubular epithelium exists in a relatively quiescent, slowly replicative state, but has a great potential for regeneration (3). Whether this is a result of proliferation

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of resident tubular cells, renal stem cells or recruitment of circulating stem cells is still under debate. Several studies provide evidence that stem cells either migrate to the injured kidney and integrate into the tubular system, or they just facilitate regeneration by endocrine effects (5–9); others describe intrarenal cells as the only source of the restoration of tubular epithelium (10,11). *In vitro* culture of human MSC and renal tubular cells of well-defined nephronal origin is a suitable model for investigation of these mesenchymal–epithelial interactions. The goal of our study was to initiate differentiation of adipose-derived adult mesenchymal stem cells (ASC) into epithelial cells, and as a first step we analysed the effects of CM from human renal tubular epithelial cells on human MSC.

Materials and methods

ASC

Human ASC were isolated from lipoaspirate cells of patients undergoing cosmetic liposuction, as described by Zuk and coworkers (12); they demonstrated that fractionation of adipose tissue can provide a population of multipotent MSCs (12). Our study was approved by the ethics committee of the clinic of the Johann Wolfgang Goethe-University (Frankfurt, Germany). Briefly, the tissue was digested for 45 min with 0.075% collagenase I (CellSystems, St. Katharinen, Germany) and the stromal–vascular fraction was separated from remaining fibrous material and floating adipocytes by centrifugation at 300 g. Sedimented cells were filtered through a 100- μ m pore filter. For cell culture, erythrocyte contamination was reduced by density gradient centrifugation with Bicol (Biochrom, Berlin, Germany); high contamination with erythrocytes was found to markedly decrease cell adherence and proliferation. Preceding density gradient separation provided a better yield of adherent cells than treatment with erythrocyte lysing buffer. For initial cell culture and expansion of cell numbers, medium 199 (M199; Sigma, Taufkirchen, Germany) with physiological glucose concentration (100 mg/dL) supplemented with 10% foetal calf serum (FCS; PAA, Pasching, Austria) was used. Primary cell isolates and cultured cells were characterized as previously described (13). Cultured ASC were CD29⁺, CD44⁺, CD49a⁺, CD73⁺, CD90⁺, CD105⁺, and CD166⁺ and CD14[−], CD31[−], and CD45[−]. For our experiments, we used 2nd–5th passages of ASC.

In vitro differentiation potential of ASC was proven in specific media (14–16). Osteogenic differentiation was induced in osteogenic medium containing ascorbic acid (50 μ M, Merck), β -glycerophosphate (10 mM, Sigma), dexamethasone (0.1 μ M, Ratiopharm, Ulm, Germany) and 15% FCS for 12 days. Osteogenic phenotype was assessed through the observation of calcium hydroxyapatite by von

Kossa staining (Sigma). Adipogenic differentiation was induced in adipogenic medium containing insulin (10 μ M, Novo Nordisk, Bagsvaerd, Denmark), dexamethasone (1 μ M, Ratiopharm), isobutyl-methylxanthine (1 mM, Sigma), indomethacin (200 μ M, Fluka, Taufkirchen, Germany) and 10% FCS for 12 days. Oil Red O (Sigma) staining revealed accumulation of lipid droplets in intracellular vacuoles, indicating adipogenic differentiation.

Renal tubular epithelial cells and media conditioning

Human renal proximal tubular epithelial cells (PTC) were separated from renal tissue using anti-CD13 antibody (ImmunoTools, Friesoythe, Germany) and coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described and characterized previously (17,18). CM was produced from 2nd–5th passage cultures of PTC isolated from different donors. Cells were subcultured by trypsinization. After 24 h, the medium was changed either into serum-free M199 or supplemented M199 (MS) for 4 days. Thus, CM was produced by nonconfluent PTC, which could therefore be regarded as injured cells. The medium after conditioning was termed pM199/pMS.

Supplemented M199 contains hydrocortisone (36 ng/mL), adrenaline (500 ng/mL), insulin (5 μ g/mL), triiodothyronine (6.5 ng/mL), transferrin (10 μ g/mL) and selenium (5 ng/mL), as described for PTC (19). After 4 days of conditioning, the medium was centrifuged, filtrated through a 0.22- μ m filter, and directly transferred onto ASC cultures. M199, MS or M199 with 10% FCS (MF) that had not been in contact with tubular cells was used as control. Time-course experiments to evaluate the best conditioning time were performed, and tested by maximal ERK1/ERK2 stimulation in ASC. As negative controls, we used CM from A431 cells (skin carcinoma cells, ATCC CRL-1555) in selected experiments. Furthermore, we tested the glucose content of CM after conditioning by a routine method and substituted glucose to 100 mg/dL in all CM. Medium osmolarity after conditioning was measured using a cryometric technique with a microosmometer (Knauer, Berlin, Germany). The pH value of the medium after conditioning was measured by a standard routine method using a pH meter (Metrohm, Herisau, Switzerland).

Determination of cell proliferation

Cell proliferation was determined by a fluorometric assay using 4,6-diamino-2-phenylindole (DAPI) (20) and by a photometric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, 1.5×10^4 cells were seeded in 96-well plates, serum-depleted, and cultured in CM or control medium (M199, MS, MF) for 24, 48 or 72 h. For the DAPI assay, cells were permeabilized

using 0.02% sodium dodecyl sulphate (SDS), 150 mM NaCl and 15 mM sodium citrate. Finally, DAPI (2 µg/mL) was added to each well. Fluorescence was measured in a fluorescence reader (355 nm ex/460 nm em; FluoStar, BMG Labtech, Offenburg, Germany). Results were calculated as percentage of the control. For the MTT assay, cells were incubated in phosphate-buffered saline (PBS) containing MTT at 37 °C for 4 h. Afterwards, the medium was removed, 0.04 N HCl was added, and absorbance was measured in a microplate reader at 550 nm vs. 650 nm. Results were calculated as percentage of the control.

Western blot analysis

Mesenchymal stem cells were grown to subconfluence, washed, and kept serum-starved for 2 h. In selected experiments, cells were pre-incubated with the MEK inhibitor PD 98059 (10 µM, Sigma) for 2 h prior to stimulation with CM. Cells were incubated with CM or control at 37 °C for 10 min and processed for Western blotting as described previously (18). In brief, cells were lysed using 10 mM Tris (pH 7.4), 0.1% SDS, 0.1% Tween 20, 0.5% Triton X-100, 150 mM NaCl, 10 mM EDTA, 1 M urea, 10 mM NEM, 4 mM benzamidine and 1 mM phenylmethanesulphonylfluoride and collected by scraping. After centrifugation, the pellet was suspended in Laemmli's buffer and heated at 95 °C for 5 min prior to electrophoresis on a 10% SDS–polyacrylamide gel. Protein content was determined by standard assay and equal volume of protein was loaded on each lane. Separated proteins were electrophoretically transferred to Immobilon transfer membrane (Millipore, Schwalbach, Germany) and membranes were blocked for 2 h. Immunoblotting was performed by incubating with antibody against activated dual phosphorylated ERK1/ERK2 (Thr 183/Tyr 185, anti-active mitogen-activated protein kinase (ERK1/ERK2) resulting in a 44/42 kDa band; Promega, Mannheim, Germany), whole ERK (Santa Cruz, Heidelberg, Germany), cytokeratin 18 (resulting in a 45 kDa band, Dako, Glostrup, Denmark), α -smooth muscle actin (resulting in a 42 kDa band, Dako), or vimentin (resulting in a 58 kDa band, Sigma), and secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG; Amersham Pharmacia, Freiburg, Germany). Protein bands were made visible using an enhanced chemiluminescence system (Amersham Pharmacia) and recorded on radiographic film followed by densitometric evaluation using ImageJ 1.36 (National Institutes of Health, <http://www.nih.gov>).

Flow cytometry

To characterize phenotypes of ASC after incubation with CM, flow cytometric analysis was performed. Cells were

detached using 0.05% trypsin and 0.02% EDTA, and were stained with antibodies against CD49a, CD73, CD166 (all BD Pharmingen, Heidelberg, Germany), CD90 (Dianova), or CD105 (ImmunoTools). After fixation, labelled cells were analysed using a FACScan flow cytometer (BD Pharmingen) and subsequently with WinMDI 2.8 (<http://facs.scripps.edu/software.html>). All experiments included negative controls without antibodies or with isotype controls.

Fluorescence microscopy

Cells were rinsed three times with PBS, and fixed in ice-cold methanol/acetone (1 : 1) for 5 min. Non-specific binding sites were blocked by PBS containing 5% normal goat serum and human IgG for 20 min. Primary anti-cytokeratin 18 (Dako) antibody was applied without washing, and was incubated for 30 min at 37 °C with gentle shaking. After washing, cells were incubated with a Cy3-conjugated secondary monoclonal antibody for 30 min at 37 °C. All dilutions of antibodies were made in PBS containing 1% goat serum and human IgG. Controls for autofluorescence or nonspecific fluorescence were performed on fixed cells processed without the secondary antibody or with a non-relevant primary antibody. Monolayers were mounted in mounting medium and examined using Zeiss fluorescence microscope.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation. For statistical analysis, the Mann–Whitney rank sum test was used. *P*-values of < 0.05 were considered significant, and *P*-values of < 0.01 were considered highly significant.

Results

Based on the hypothesis that factors secreted from PTC represent at least one control factor that has the ability to awaken stem cells from their quiescence, the potency of medium conditioned by PTC to stimulate ASC signal transduction and differentiation was investigated. This required four steps: (i) determination of optimal time for conditioning; (ii) adequate replenishment of glucose after conditioning; (iii) proof of activity of the growth signals secreted by PTC; and (iv) proof of initiation of differentiation steps by a common signal transduction pathway, and changes in cell morphology and differentiation marker expression.

First, we tested the optimal conditioning time as assessed by ASC signal transduction in time-course experiments (24 h to 5 days of conditioning). Maximum ERK1/ERK2 phosphorylation in ASC was reached after 4 days of conditioning (data not shown). Therefore, we used this

conditioning time for all experiments. Then, due to glucose consumption during conditioning, glucose content of CM was replenished to 100 mg/dL glucose. Glucose contents after conditioning were 32.6 ± 7.1 mg/dL after 24 h, 22.7 ± 13.3 mg/dL after 2 days, 1.4 ± 1.4 mg/dL after 3 days, and 0.6 ± 0.1 mg/dL after 4 days of conditioning (control pure M199 97.5 ± 0.7 mg/dL). Medium osmolarity and pH value remained unchanged after conditioning. Osmolarity after conditioning was 295 ± 7.1 mosm/L (M199 without conditioning: 284 ± 5.8 mosm/L) ($n = 5$). Medium pH value after conditioning was 7.34 ± 0.05 (M199 without conditioning: 7.44 ± 0.06) ($n = 5$).

Conditioned medium significantly stimulated proliferation of ASC, as demonstrated by the DAPI and MTT assays (Table 1). The carcinoma cell line A431 was used as a control cell system, which should not affect ASC. CM from A431 cells induced no proliferation of ASC as proven by the MTT assay (data not shown).

Additionally, significant phosphorylation of ERK1/ERK2 was induced by CM (pM199 or pMS) in comparison with M199 ($n = 4-6$; Fig. 1a, Table 2). This phosphorylation was time-dependent and inhibitable by mitogen-activated protein kinase inhibitor PD98059 as revealed by additional experiments (Fig. 1c, Table 2). CM from MS induced phosphorylation of ERK1/ERK2, whereas stimulation by unconditioned MS was higher than in unconditioned M199 (Fig. 1a, Table 2), probably due to culture supplements. MF or epidermal growth factor (EGF) (10 ng/mL) as controls stimulated approximately the same amount (Fig. 1a, Table 2).

As assessed by phase contrast microscopy after 12 days of culture, undifferentiated ASC (controls) had an elongated 'fibroblastoid' phenotype (Fig. 2a). CM induced a change in morphology of the cells to a more epithelial-like monolayer (Fig. 2b). To elucidate the influence of CM on ASC differentiation, expression of different markers was

Table 1. Proliferation assays. Cells were cultured for the indicated time in control (M199, medium 199; MS, supplemented M199; MF, M199 $\pm$ 10% foetal calf serum) or conditioned media (pM199/pMS). Results are expressed as percentage of the control (mean values and standard deviations)

	DAPI			MTT		
	24 h	48 h	72 h	24 h	48 h	72 h
M199	100%	100%	100%	ND	ND	ND
pM199	$106\% \pm 2$	$125\% \pm 12^a$	$123\% \pm 10^a$	ND	ND	ND
MS	100%	100%	100%	100%	100%	100%
pMS	$125\% \pm 12^a$	$134\% \pm 18^a$	$137\% \pm 14^a$	$120\% \pm 10^a$	$126\% \pm 20^+$	$118\% \pm 24^a$
MF	$146\% \pm 12^a$	$155\% \pm 17^a$	$169\% \pm 31^a$	$132\% \pm 7^a$	$136\% \pm 26^a$	$139\% \pm 31^a$

$n = 6-8$ for DAPI (4,6-diamino-2-phenylindole), $n = 3$ for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), $^aP < 0.05$, ND, not done.

Figure 1. Characteristic Western blot analysis. (a) Phospho-ERK1/ERK2 (upper blot) and whole ERK (lower blot) in adipose-derived adult mesenchymal stem cells (ASC) cultured in conditioned or control medium. Lane 1, medium 199 (M199); lane 2, M199 with 10% foetal calf serum (MF); lane 3, supplemented M199 (MS); lane 4, conditioned MS (pMS); lane 5, conditioned M199 (pM199). (b) Time-dependent phosphorylation of ERK1/ERK2. Phospho-ERK1/ERK2 (upper blot) and whole ERK (lower blot). Lane 1, M199 10 min; lane 2, pM199 10 min; lane 3, pM199 30 min; lane 4, pM199 60 min; lane 5, pM199 120 min. (c) Influence of PD98059 (PD) on ERK1/ERK2 phosphorylation. Phospho-ERK1/ERK2 (upper blot) and whole ERK (lower blot). Lane 1, M199 + PD; lane 2, pM199 + PD; lane 3, pM199 10 min. Densitometric analysis is listed in Table 2.

Table 2. Densitometric analysis of stimulation experiments. (A) Effect of conditioned medium and controls. (B) Time-dependent phosphorylation of ERK1/ERK2. (C) Influence of mitogen-activated protein kinase inhibitor PD98059 (PD). Results are expressed as mean values and standard deviations of the ratio P-ERK/total ERK

A	M199 0.28 ± 0.05	MF 1.09 ± 0.22 ^{ab}	MS 0.43 ± 0.06	pMS 0.87 ± 0.03 ^b	pM199 1.1 ± 0.63 ^a	EGF 1.18 ± 0.47 ^{a,b}
B	M199 10' 0.34 ± 0.06	pM199 10' 1.04 ± 0.48 ^a	pM199 30' 0.84 ± 0.32 ^a	pM199 60' 0.61 ± 0.17	pM199 120' 0.47 ± 0.09	
C	M199 + PD 0.09 ± 0.06	pM199 + PD 0.25 ± 0.03 ^c	pM199 1.18 ± 0.45			

$n = 4-6$, ^a $P < 0.05$ vs. M199, ^b $P < 0.05$ vs. MS, ^c $P < 0.05$ vs. pM199. M199, medium 199; MS, supplemented M199; MF, M199 + 10% foetal calf serum; pM199/pMS, PTC-conditioned media from M199 or MS.

Figure 2. Cell morphology. Six thousand five hundred adipose-derived adult mesenchymal stem cells (ASC) per square centimetre were seeded and cultured for 12 days in conditioned or control medium. Cells were from the same isolation, trypsinized at the same time and seeded at the same cell density. Undifferentiated ASC displayed an elongated 'fibroblastoid' morphology (a), whereas conditioned medium 199 (pM199) (b) induced a change in cell morphology to a more epithelial-like monolayer (bar: 100 μm).

evaluated by flow cytometry (Fig. 3), Western blotting (Fig. 4) and immunofluorescence stainings (Fig. 5). After incubation with pM199 for 12 days, expression of cytokeratin 18 was induced (calculated in percent after densitometric evaluation: M199, 100% (background signal); pM199, 301% ± 70; pMS, 283% ± 164; $n = 5$). Our previous studies with ASC have revealed differentiation to epithelial lineage by expression of cytokeratin 18, an early epithelial marker, after incubation with retinoic acid (13). Therefore, we used all-*trans* retinoic acid (5 μM, 436% ± 270) as a positive control. On the other hand, expression of α-smooth muscle actin, which is expressed in undifferentiated ASC, was severely decreased (densitometric evaluation: M199, 100%; pM199, 20% ± 2; pMS, 16% ± 2; $n = 3$). Vimentin expression remained almost unaffected (densitometric evaluation: M199, 100%; pM199, 106% ± 12; pMS 94% ± 8; $n = 3$). Additionally, we demonstrated induction of cytokeratin 18 expression by immunofluorescence staining. Controls in standard medium did not show any presence of cytokeratin 18 (Fig. 5a), whereas after incubation with CM for 12 days, nearly 50% of the cells expressed this epithelial marker (Fig. 5b,c).

Expression of markers CD49a and CD90 was decreased by pM199 ($n = 5$, Fig. 3), whereas other markers (CD73, CD105, CD166) remained unchanged ($n = 4$, Fig. 3).

Furthermore, to show differentiation potential of ASC, differentiation into adipocytes and osteoblasts was induced by adipogenic and osteogenic media. After 12 days of incubation, differentiation was shown by Oil Red O and von Kossa staining. Cells cultured in adipogenic medium had lipid droplets, whereas cells cultured in osteogenic medium exhibited massive hydroxyapatite deposits (figures not shown).

Discussion

Intercellular crosstalk between cells of mesenchymal and epithelial origin via soluble factors or direct cell-cell

Figure 3. Flow cytometry. Representative overlay histograms of the expression of adipose-derived adult mesenchymal stem cell (ASC) markers after incubation with conditioned medium 199 (pM199) for 12 days ($n = 5$). Solid filled grey histogram (pM199), black histogram (M199), dashed-line grey histogram (isotype control, only in the histograms on the left side). Dot blot shows forward and sideward scatter analysis of ASC.

Figure 4. Characteristic Western blot analysis. Expression of cytokeratin 18 (45 kDa, upper blot) after incubation in conditioned medium for 12 days (lane 1, M199; lane 2, pM199; lane 3, pMS). In contrast, α -smooth muscle actin expression was lowered (42 kDa, middle blot), while vimentin expression was almost unaffected (58 kDa, lower blot).

contact is a key factor in nephrogenesis, tubulogenesis and renal regeneration after renal failure (2). Our study addressed the question, whether these interactions could be simulated by an *in vitro* system using cultured human

ASC and renal epithelial cells. We hypothesized that soluble factors from impaired PTC induce differentiation of ASC. To answer this question, stem cells from adipose tissue were cultured with CM from PTC.

Adipose-derived adult mesenchymal stem cells are multipotent stem cells that have characteristics similar to bone marrow-derived MSCs (21,22), and possess powerful *ex vivo* expansion potential and versatile differentiation potential, placing themselves in the centre of stem cell-based therapies and transplantation. It has been shown that ASC, induced by soluble molecules, can differentiate into multiple cell types of mesodermal, endodermal and epidermal origin, induced by soluble signals, has been shown. Recent studies have described plasticity towards chondrocytes, osteoblasts, fibroblasts, adipocytes, smooth muscle cells, neurones, cardiomyocytes, hepatocytes and endothelial cells (16,23–25). Our earlier study showed differentiation of ASC towards epithelial lineage after addition of retinoic acid (13). Different *in vitro* studies addressed the question of whether CM itself or cocultures with appropriate cells induce stem cell differentiation. It has been shown that CM from osteocytes stimulates proliferation and differentiation of bone marrow MSC into osteoblasts (26). Furthermore, bone marrow MSC were induced to express neural markers in cocultures with neural cells (27,28) or after incubation with neural cell CM (28). Others showed complete differentiation of isolated metanephric

Figure 5. Immunofluorescent staining of cytokeratin 18. Expression of the epithelial marker cytokeratin 18 could be demonstrated after incubation with conditioned medium for 12 days (b, c), whereas controls in standard medium were negative (a).

mesenchyme induced by CM from ureteric bud cells (29). Our studies showed different effects of CM from epithelial cells: short-term (proliferation in the first days of incubation) and long-term (change in morphology and differentiation after 12 days of incubation). We speculate that these effects of CM, induction of proliferation and

differentiation mechanisms, which became apparent at different time points, depend on different factors secreted from PTC. In the case of normal culture methods (passaging cells by trypsination), subconfluent epithelial cells can be considered as injured cells. Potential factors released by injured PTC include different growth factors, cytokines, chemokines and interleukins. Nevertheless, we have not tested which factors are secreted in the CM because of the huge variety of factors released by epithelial cells.

The common signal transduction pathway via mitogen-activated protein kinase ERK1/ERK2 is activated by various stimuli, which points out the rationale to measure ERK1/ERK2 as signal transduction messenger. Different studies suggest that differences in properties of the transmitted signals stimulated by different factors in the same cell system may determine whether these cells proliferate, migrate, differentiate or express cytokines or other factors (30,31). It has been shown that different factors modulate ERK activation in terms of magnitude and duration (32), whereas a transient or a sustained activation of ERK may result in a different cellular response in the same cell system (30,31). In our study, all used CM had a strong stimulatory (but transient) effect on signal transduction of ASC.

We used full-supplemented medium, because renal tubular cells show better growth and differentiation in this medium. Nevertheless, to exclude the effects of culture supplements on ASC, we used pure M199 for conditioning. A higher basal stimulation of ERK1/ERK2 by MS compared to pure M199 could be detected. We did not detect any other significant differences between the conditioned media (pM199 and pMS).

Our long-term studies show that CM changes cell morphology from a multilayer, elongated 'fibroblastoid' cell type into a more epithelial-like cell monolayer. Furthermore, differentiation of ASC was shown by induced cytokeratin 18 expression and a decrease of CD49a, CD90 and α -smooth muscle actin expression after 12 days in CM. Cytokeratin filament proteins are one of the first epithelial-specific structural proteins to be synthesized in a differentiation program. In differentiating epithelia, cytokeratin expression proceeds through the initial expression of 'primary' keratins. Simple epithelia, such as glandular, intestinal or kidney epithelia, express solely their primary keratins (for instance, cytokeratin 18). Therefore, a limitation of our study is that we only could show the beginning of an epithelial differentiation. Recently, we have not detected another epithelial marker with increased expression after incubation with CM (e.g. ZO-1, E-cadherin), probably due to the beginning of differentiation towards the epithelial lineage or due to the lack of additional stimuli, such as cell-matrix or cell-cell interactions.

On the other hand, CD90 (Thy-1), a molecule involved in cell–cell and cell–matrix interactions and in cell proliferation, adhesion and migration is widely expressed in undifferentiated stem cells and stromal tissues (33). Likewise, α 1-integrin (CD49a) has been shown to be expressed on undifferentiated ASC (34) and bone marrow MSC, where it has recently been described as a tool for cell sorting (35). Furthermore, α -smooth muscle actin is expressed by undifferentiated ASC and its regulation has been shown by differentiation agents, such as TGF- β 1 and retinoic acid (36). CD49a, CD90 or α -smooth muscle actin – together with a panel of markers – are commonly used as markers for undifferentiated MSCs and are down-regulated upon differentiation (14,36,37). However, no marker specifically and unequivocally identifies undifferentiated ASC. At present, these cells are defined by their immunophenotypic profile (e.g. CD29⁺, CD44⁺, CD73⁺, CD90⁺, CD105⁺, CD166⁺, and CD14⁺, CD31⁺, CD45⁺, HLA-DR⁺), their cell morphology, and their multilineage differentiation potential (14–16).

In summary, our study shows that CM from renal tubular epithelial cells provides a convenient source of inductive signals to initiate differentiation of ASC towards the epithelial lineage.

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