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Mesenchymal stem cell-conditioned medium accelerates regeneration of human renal proximal tubule epithelial cells after gentamicin toxicity

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ABSTRACT

Bone marrow-derived mesenchymal stem cells (MSCs) have the capacity to regenerate renal tubule epithelia and repair renal function without fusing with resident tubular cells. The goal of the present project was to investigate the role of MSCs secreted cytokines on tubule cell viability and regeneration after a toxic insult, using a conditionally immortalized human proximal tubule epithelial cell (ciPTEC) line. Gentamicin was used to induce nephrotoxicity, and cell viability and migration were studied in absence and presence of human MSC-conditioned medium (hMSC-CM) *i.e.* medium containing soluble factors produced and secreted by MSCs. Exposure of ciPTEC to 0–3000 μg/ml gentamicin for 24 h caused a significant dose-dependent increase in cell death. We further demonstrated that the nephrotoxic effect of 2000 μg/ml gentamicin was recovered partially by exposing cells to hMSC-CM. Moreover, exposure of ciPTEC to gentamicin (1500–3000 μg/ml) for 7 days completely attenuated the migratory capacity of the cells. In addition, following scrape-wounding, cell migration of both untreated and gentamicin-exposed cells was increased in the presence of hMSC-CM, as compared to exposures to normal medium, indicating improved cell recovery. Our data suggest that cytokines secreted by MSCs stimulate renal tubule cell regeneration after nephrotoxicity.

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1. Introduction

The proximal tubule epithelium in the kidney is essential in the clearance of endogenous waste products, exogenously administered compounds, such as drugs, as well as environmental toxicants (Ferguson et al., 2008; Mutsaers et al., 2011). Due to the unique physiological role of the kidney, the tubular epithelium is exposed to high concentrations of potential toxic compounds and therefore a frequent target of injury due to toxicity. This can lead to acute kidney injury (AKI) or acute renal failure (ARF), which is a frequently occurring clinical problem that affects up to 7% of hospitalized patients. ARF is potentially reversible; however, it can be a determining element of multiple organ failure as well. Hence, the mortality rate in hospital-acquired ARF ranges from 30 to 80% (Schrier et al., 2004).

While tubular cell death due to necrosis or apoptosis is widespread following injury, less severely injured cells can survive and are believed to be the principal source for tubule regeneration (Humphreys et al., 2011; Lin et al., 2005). Surviving tubular cells can spread and migrate to cover the exposed areas of the basement membrane followed by redifferentiation into an epithelial phenotype (Duffield et al., 2005). Additionally, resident epithelial cells may lose their characteristic features of mature renal tubular epithelia, *e.g.* the apical brush border membrane and tight-junctions. As a consequence, the cells acquire a flat and more spread morphological phenotype, resembling undifferentiated mesenchyme or fibroblasts. This loss in phenotype, *via* the process of epithelial-to-mesenchymal transition (EMT), is supposed to be an essential factor in promoting regeneration through activation of cell migration and proliferation pathways (Guo and Cantley, 2010; Witzgall et al., 1994). Yet, such resolution may occur only when the tubular basement membrane is still intact (Fragiadaki and Mason, 2011).

In addition to EMT, bone marrow-derived mesenchymal stem cells (MSCs) may have the capacity to migrate to the injured kidney and contribute to tubule epithelium regeneration and renal function repair without fusing with resident tubular cells (Huls

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et al., 2010). The exact mechanism through which MSCs mitigate renal damage is not fully elucidated. One possible explanation is that MSCs produce cytokines and growth factors that promote anti-inflammatory, immunosuppressive, anti-apoptotic and proliferative effects (Humphreys and Bonventre, 2008). This hypothesis is supported by other studies demonstrating that conditioned medium, in which MSCs secreted growth factors and cytokines are present, could ameliorate renal tubular injury (Humphreys and Bonventre, 2008). Yet, most studies that investigated the role of MSCs in the regeneration of kidney injury used rodent or other mammalian animal models.

Therefore, this study was designed to investigate whether soluble factors produced and secreted by MSCs could provide protection against gentamicin-induced nephrotoxicity using a human *in vitro* model. We hypothesized that human MSC-derived conditioned medium would prevent renal proximal tubular cells to undergo nephrotoxicant-induced cell death thereby protecting against tubular injury. We used a recently developed conditionally immortalized human proximal tubular epithelial cell line (ciPTEC) (Wilmer et al., 2009), which was exposed to the nephrotoxic drug gentamicin, and studied the cell viability and cell migration in absence and presence of human MSC-conditioned medium (hMSC-CM).

2. Materials and methods

2.1. Proximal tubular epithelial cells

The ciPTEC line was generated by culturing cells exfoliated in the urine of a healthy volunteer, followed by immortalization using both the temperature-sensitive mutant U19tsA58 of SV40 large T antigen (SV40T) and the essential catalytic subunit of human telomerase (hTERT), as previously described (Wilmer et al., 2009). The cells were cultured in ciPTEC medium containing phenol red-free DMEM/F12 medium (Gibco/Invitrogen, Breda, the Netherlands) supplemented with 10% (v/v) fetal calf serum (FCS; MP Biomedicals, Uden, the Netherlands), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), epithelial growth factor (10 ng/ml) and tri-iodothyronine (40 pg/ml) at 33 °C in a 5% (v/v) CO_2 atmosphere. Propagation of cells was maintained by subculturing the cells at a dilution of 1:3 to 1:6 at 33 °C. For experiments, cells were cultured at 33 °C to 40% confluency, followed by maturation for 7 days at 37 °C during which the cells formed a confluent monolayer. Furthermore, expression of SV40T decreases gradually in ciPTEC cultured at 37 °C, minimizing the influence of the transfection on cellular phenotype and allowing cells to differentiate. Routinely, cell morphology was monitored by phase-contrast microscopy at each passage and showed no marked difference from passage 35 up to 40, during which experiments were performed. Moreover, it was previously reported that ciPTECs maintain their proximal tubular characteristics, including expression of CD13, ZO-1, megalin-mediated albumin uptake, and functional expressions of organic cation transporter 2 and P-glycoprotein over a prolonged period of culturing time (Wilmer et al., 2009).

2.2. Stem cell isolation and culture, and preparation of hMSC-CM

Bone marrow-derived MSCs were isolated from healthy donors at the Radboud University Nijmegen Medical Centre, as described before (Jansen et al., 2010). In short, MSCs were isolated from each bone marrow sample by Ficoll density gradient centrifugation. Afterwards, the cells were seeded into polystyrene culture flasks (Becton Dickinson, Bedford, Mass, United States) at a density of 1×10^6 cells/cm^2 in alpha-Minimum Essential Medium (alpha MEM), with 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco BRL), 2 μM L-glutamine (Gibco BRL) and 10% (v/v) FCS selected for MSC growth (Hyclone, characterized, Lot ALF 14015). Cultures, maintained in a humidified atmosphere with 5% (v/v) CO_2 at 37 °C, had their medium changed twice weekly thereafter. On reaching 60–80% confluency, adherent cells were detached after treatment with 0.05% (v/v) trypsin/1 μM EDTA solution (Gibco, BRL) for reseeding at 10^3 cells per/cm^2. To obtain human MSC-conditioned medium (hMSC-CM), cells were cultured in DMEM medium containing low glucose (1 g/l), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% (v/v) FCS of a selected batch. We cultured hMSCs to confluency and collected the medium after approximately 24–48 h after refreshing medium. hMSC-CM was centrifuged at $800 \times g$ for 5 min to remove detached MSCs and stored at −80 °C until further use, hMSC-CM was diluted (1:1) in ciPTEC culture medium prior to incubation.

2.3. Cell viability

Sensitivity of cells to gentamicin (100–3000 μg/ml) was determined by a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma) assay. ciPTECs were grown in 96-well plates and exposed to gentamicin for 24 h in 100 μl of ciPTEC medium (control) or hMSC-CM. Next, medium was removed, 20 μl preheated (37 °C) MTT solution (5 mg/ml PBS) was added and cells were incubated for 4 h at 37 °C. Afterwards, MTT solution was removed, followed by the addition of 200 μl DMSO. The extinction of the solution was measured at 570 nm using a Benchmark Plus Microplate Spectrophotometer (Biorad, Veenendaal, the Netherlands).

2.4. Cell migration

The effect of hMSC-CM on migration of unexposed and gentamicin-exposed ciPTECs was determined using the scratch assay. Cells were cultured in 6-well plates and the confluent monolayers were treated with ciPTEC medium, hMSC-CM or gentamicin (1500–3000 μg/ml) for 24 h followed by scrape-wounding the cells using a plastic pipette tip. Following scraping, the medium containing detached cells was removed and replaced with either ciPTEC medium without or with gentamicin (1500–3000 μg/ml) or hMSC-CM, and incubated until the monolayer was restored (5–7 days). To replenish nutrients, medium was refreshed every 2 days. Cell migration and cell monolayer recovery was studied by phase contrast microscopy over time, as described in detail by Liang et al. (2007). Length of scratching area was measured on stored images using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

2.5. Statistics

Statistics were performed using GraphPad Prism 5.02 via a One-way analysis of variance (ANOVA) followed by the Dunnett's or Bonferroni's Multiple Comparison Test. Differences between groups were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Gentamicin-induced cell death can be partially rescued by hMSC-CM

First the cytotoxic effect of gentamicin without co-exposure to hMSC-CM was evaluated, after which the protective effect of hMSC-CM on a toxic concentration of gentamicin was studied (see Fig. 1A for experimental design). A dose-dependent reduction

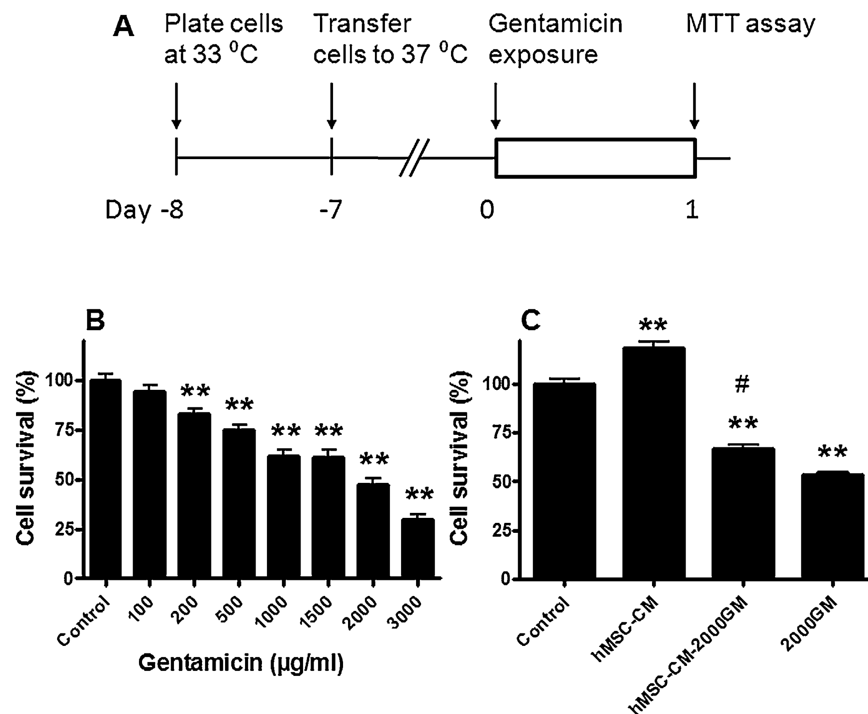

Fig. 1. Reduction in cell viability of ciPTECs exposed to gentamicin and partial recovery by hMSC-CM. Cell viability was determined using the MTT assay and data are shown as the percentage of viable cells. (A) Schematic overview of experimental set-up. Cells were plated at 33 °C at day-8 and transferred to 37 °C to enable maturation. Medium was replaced every other day. At day 0, medium was replaced with ciPTEC medium supplemented with gentamicin for 24 h. At day 1, an MTT assay was performed. (B) There is a significant increase in cell death with increasing doses of gentamicin. (C) The increase in cell death by 2000 μg/ml gentamicin was partially restored by co-exposing cells to hMSC-CM. Cells incubated with ciPTEC medium are indicated as control. $^{**}P<0.001$ as compared to control; $^{\#}P<0.01$ as compared to 2000 μg/ml gentamicin.

in cell viability was observed after exposing cells to gentamicin for 24 h as assessed by the MTT assay, with an apparent LC_{50} of 1765 ± 25 μg/ml (Fig. 1B). At a concentration of 2000 μg/ml, gentamicin reduced cell viability by 53% ($P<0.001$). In the presence of hMSC-CM, viability was only reduced by 33%, suggesting a protective effect of conditioned medium on gentamicin-induced nephrotoxicity (Fig. 1C).

3.2. *Effect of the hMSC-CM on migration of ciPTECs exposed to gentamicin*

The scratch assay is a straightforward method to study cell migration *in vitro* (see Fig. 2A for experimental set-up). Long term, *i.e.* 7 days, exposure of ciPTEC to high doses of gentamicin, ranging from 1500 to 3000 μg/ml, completely attenuated the migratory capacity of the cells (Fig. 2B, D, F, H, J and L). However, ciPTECs exposed to gentamicin (1500 and 2000 μg/ml) for 24 h, followed by a change toward fresh medium without the nephrotoxicant, showed a tremendous increase in cell migration and cell transformation with monolayer restoration for up to 80% (Fig. 2C, E, I and K). For the highest concentration tested, 3000 μg/ml, no cell migration was observed during 7 days using fresh medium after 24 h gentamicin exposure, suggesting severe toxicity (Fig. 2G and M). Fig. 2N shows a summary of three independent experiments, demonstrating that restoration of the monolayer was possible after 24 h gentamicin exposure at concentrations up to 2000 μg/ml. When hMSC-CM was used instead of normal medium the recovery rate after intermediate toxic concentration of gentamicin (1500 μg/ml) was accelerated by 25% on day 5 (Fig. 3; $P<0.05$). Treatment with conditioned medium solely resulted in a 96% restoration of the scratch area on day 3 and 100% on day 5, whereas normal medium resulted in a restoration of the scratch wound by 87% and 96% on day 3 and 5, respectively.

4. Discussion

The results of the present study indicate that MSC-derived conditioned medium increased cell viability and accelerated cell migration after gentamicin-induced cell toxicity in a unique human proximal tubular epithelial cell line. Gentamicin is well known for its nephrotoxicity in various *in vitro* and *in vivo* investigations, which hampers its clinical use. In accordance with previous studies using Madin–Darby canine kidney cells type II (Notenboom et al., 2006), we observed a concentration-dependent increase in cell toxicity after gentamicin exposure, indicating that ciPTECs are an ideal, human-derived, tool to study gentamicin-induced renal toxicity. Concentrations of ≥2000 μg/ml gentamicin and multiple days dosing of ciPTECs caused irreversible cell damage and a lack of ability to migrate into a scratch-wounded area.

Wound healing assays have been carried out in tissue culture studies to estimate the migration and proliferation rates of cells and the impact of culture conditions on these parameters. Upon scratching a line through a monolayer, the open gap is inspected microscopically over time as the cells move in and fill the damaged area. This healing can take from several hours to over a day depending on the cell type, conditions, and the extent of the wounded region. Here, the ciPTEC monolayer was almost completely recovered within five days using normal medium. This process was significantly accelerated when cells were exposed to hMSC-CM. In presence of gentamicin, no cell monolayer regeneration was observed for up to seven days, however, when cells were exposed for 24 h to gentamicin followed by a replacement with normal medium or hMSC-CM the monolayer was restored in a great part within seven days. Again, the recovery rate was accelerated by 25% by hMSC-CM.

During this study, hMSC-CM contained penicillin and streptomycin, due to the cell culture requirements of primary human

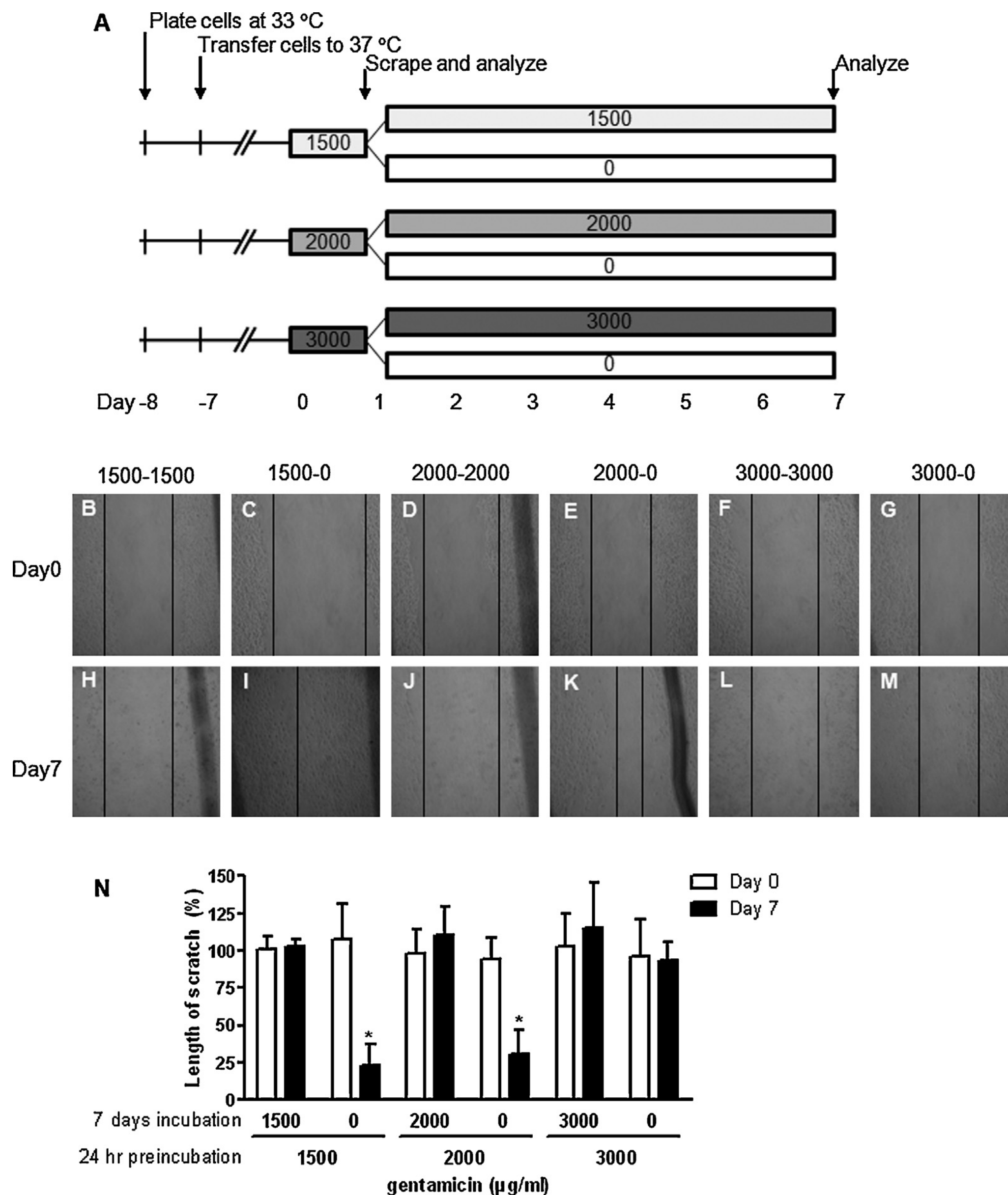

Fig. 2. Effect of gentamicin on cell migration. (A) Schematic overview of experimental set-up. Cells were plated at 33 °C at day-8 and transferred to 37 °C to enable maturation. Medium is replaced every other day. At day 0, medium was replaced with ciPTEC medium supplemented with gentamicin for 24 h. At day 1, the cell monolayer was disrupted by scraping the cells, following medium replacement without (0) or with the indicated concentration (μg/ml) gentamicin. At day 7, length of scratching area was analyzed by phase contrast microscopy. (B–M) Representative images are shown of confluent monolayers of ciPTECs exposed to gentamicin (1500 μg/ml (B and C), 2000 μg/ml (D and E) and 3000 μg/ml (F and G)). The wound-induced cell motility was measured after treatment with gentamicin without (H, J, and L) or with (I, K, and M) a change to control medium (0). Magnification, 10×. (N) Mean of three independent experiments performed in triplicate. *$P < 0.05$ as compared to same treatment on day 0.

MSCs. Previously, it has been reported that antibiotics can negatively influence epithelial physiology (Shen et al., 2003; Zietse et al., 2009). This indicates that our results possibly underestimate the potential beneficial effect of hMSC-CM on tubular regeneration.

The functional importance of bone marrow-derived cells in the kidney has been studied *in vivo* in multiple rodent models of kidney injury as well as in larger mammals (Masereeuw, 2009). Following kidney injury, the number of bone marrow-derived cells in the circulation slightly increases. Trans-differentiation of bone marrow-derived cells to functional tubular epithelium has been demonstrated, however, beneficial effects of bone marrow-derived transplantations may have been accelerated by irradiation of the animals prior to transplantation and kidney injury (Fang et al., 2008). Indeed, other groups failed to detect any evidence of bone marrow-derived cells in sections of injured kidneys, excluding their trans-differentiation (Duffield and Bonventre, 2005). Recent studies support a paracrine or endocrine role of MSCs, in which an improvement of renal function is observed without direct involvement in tubular epithelial engraftment.

It has been proposed that MSCs must provide paracrine and/or endocrine factors that explain their positive effects on kidney repair following injury (Togel and Westenfelder, 2011). Evidence for this paracrine/endocrine process was provided by Bi et al. (2007), using a model of cisplatin-induced renal damage. The apparent reparative function of MSCs could be achieved *via* an intraperitoneal injection of the MSC-conditioned medium alone. Our study provides supporting evidence of such a paracrine effect of MSCs, which have been shown to secrete a number of growth factors (Togel and Westenfelder, 2011). Imberti et al. (2007) suggested that this humoral function results from insulin-like growth

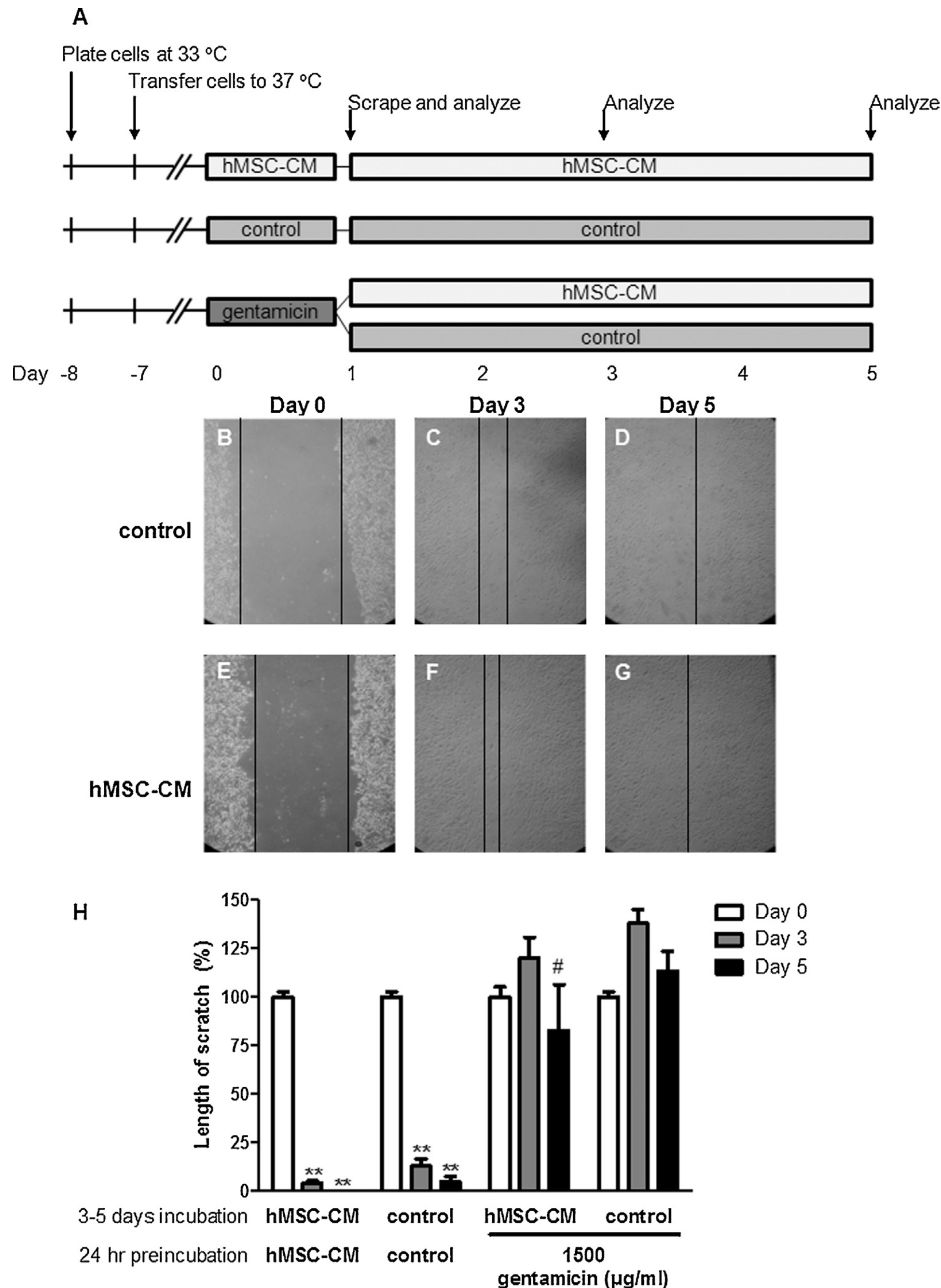

Fig. 3. MSC-derived conditioned medium increases cell migration. (A) Schematic overview of experimental set-up. Cells were plated at 33 °C at day-8 and transferred to 37 °C to enable maturation. Medium is replaced every other day. At day 0, medium was replaced with hMSC-CM or control medium in presence or absence of gentamicin (1500 μg/ml) for 24 h. At day 1, the cell monolayer was disrupted by scraping the cells, following medium replacement with either hMSC-CM or ciPTEC medium (control). At day 3 and 5, length of scratching area was analyzed by phase contrast microscopy. (B–G) Representative images of confluent monolayers of ciPTECs exposed to medium (control; B), hMSC-CM (E), and gentamicin for 24 h and then were scratched by a plastic pipette. The wound-induced cell motility was measured after recovery in control medium (C and D) and hMSC-CM (F and G). Magnification, 10×. (H) Mean of three independent experiments performed in triplicate. $^{**}P < 0.001$ as compared to day 0 of same treatment; $^{\#}P < 0.05$ hMSC-CM as compared to ciPTEC medium on same day.

factor 1 (IGF1), whereas others attributed it to a combination of hepatocyte growth factor (HGF), IGF1 and epidermal growth factor. Vascular endothelial growth factor can be an additional factor in the renoprotection by MSCs (Togel et al., 2009). It has long been known that IGF1 and HGF can play reparative roles in the kidney following acute injury (Humphreys and Bonventre, 2008). Bone morphogenetic protein 7 has also been implicated in the protection against fibrosis (Zeisberg and Kalluri, 2008). The current study, demonstrating hMSC-CM accelerates repair after gentamicin-induced nephrotoxicity in ciPTECs, is in concordance with the theory that not MSCs themselves, but secretory proteins dominate regeneration processes. Moreover, the human origin of ciPTECs in this study underscores the promising effective therapeutic interventions using MSCs in tissue regeneration in case of ARF and AKI. Still, a number of uncertainties exist in understanding which (patho)physiological triggers cause activation of MSCs and the subsequent production of soluble factors. Most likely, the inflammatory conditions after an injury provide key signals. Togel and Westenfelder (2011) suggested that MSCs exert their renal protection through inhibition of proinflammatory cytokines. This

implies that the reparative role of MSCs may be multifactorial and include production of anti-inflammatory cytokines to limit apoptosis, enhance proliferation and dampen the inflammatory response. In addition, the renal interstitium itself likely contributes to the renal regeneration process as well, by controlling fibrosis alongside preserving the kidney's architecture (Kaissling and Le, 2008). Future *in vivo* studies should be directed at identifying the multiple factors that contribute to renal restoration after acute injury.

In conclusion, hMSC-conditioned medium accelerates monolayer restoration after gentamicin-induced toxicity in a human renal proximal tubular epithelial cell line, demonstrating that human MSCs can play an important role in renal repair mechanisms after acute injury. These findings warrant further investigation into the specific set of cytokines and growth factors excreted by MSCs that contribute to the positive effect of these cells on renal repair *in vivo*. Identification of these factors could aid in the development of novel therapies.

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