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Micropatterning control of tubular commitment in human adult renal stem cells

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ABSTRACT

The treatment of renal injury by autologous, patient-specific adult stem cells is still an unmet need. Unsolved issues remain the spatial integration of stem cells into damaged areas of the organ, the commitment in the required cell type and the development of improved bioengineered devices. In this respect, biomaterials and architectures have to be specialized to control stem cell differentiation. Here, we perform an extensive study on micropatterned extracellular matrix proteins, which constitute a simple and non-invasive approach to drive the differentiation of adult renal progenitor/stem cells (ARPCs) from human donors. ARPCs are interfaced with fibronectin (FN) micropatterns, in the absence of exogenous chemicals or cellular reprogramming. We obtain the differentiation towards tubular cells of ARPCs cultured in *basal* medium conditions, the tubular commitment thus being specifically induced by micropatterned substrates. We characterize the stability of the tubular differentiation as well as the induction of a polarized phenotype in micropatterned ARPCs. Thus, the developed cues, driving the functional commitment of ARPCs, offer a route to recreate the microenvironment of the stem cell niche *in vitro*, that may serve, in perspective, for the development of ARPC-based bioengineered devices.

1. Introduction

The intrinsic features of adult stem cells such as multipotency, self-renewability and hypoimmunogenicity have raised a continuously increasing interest, potentially allowing for developing therapies for degenerative diseases and for creating stem cell-based systems for drug screening [1–3]. Nowadays, adult stem cells represent a potentially groundbreaking strategy for defining new cell-based therapies, since preventing ethical issues associated to embryonic cells, and reducing the possibility of teratomas and other tumor formation [4]. Being directly isolated from patients for autologous transplantation, these cells are also suited to avoiding the risk of immunological rejection [5].

It is estimated that over 2 million patients world-wide currently suffer from end-stage kidney disease and, in spite of the advances in kidney transplantation, the shortage of donors leads many patients to dialytic treatments for long periods [6]. Consequently, the use of stem cells for the development of renal tissue engineering methods and for the embedment in bioengineered and patterned devices represents a relevant goal of current biomedical research [7]. For example, a previous work has highlighted an increased response in terms of adhesion, proliferation and alignment in modified human embryonic kidney cells on laser-nanostructured polystyrene [8].

A very promising population of adult renal stem/progenitor cells (ARPCs) has been recently identified in the human kidney [9–11]. These cells are self-renewable, and they can differentiate into

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multiple renal cell types [10–12] and express proteins typical of immune system cells as a sensor of tissue damage [9]. The injection of ARPCs in mice affected by severe combined immunodeficiency with acute renal failure has promoted a high regeneration of damaged tubular structures [10]. These findings suggest that ARPCs have a great potential in view of developing future treatments for both acute and chronic renal injury. However, to fully exploit such potential for bioengineering applications, it is mandatory to recapitulate *in vitro* the conditions that regulate the stem cell behavior, with the ultimate aim of effectively controlling the differentiation on properly designed biomaterials. These issues still represent a bottleneck to achieve renal tissue regeneration and to realize effective bioengineered kidney-mimicking devices.

The most used method to differentiate stem cells currently consists in culturing them within conditioned mediums, enriched by differentiation inducing agents, cytokines and chemicals [2]. However, the investigation of factors that affect stem cells *in vivo* has highlighted the importance of the extracellular microenvironment as a highly heterogeneous and dynamic domain, in which stem cells are controlled not only by the presence of growth factors and cytokines, but also through the interactions with the surrounding extracellular matrix (ECM) and with other neighboring cells [13–15]. Micropatterning yields smart routes to mimic such a complexity *in vitro*, through relevant chemical and topographical cues that might influence the fate of stem cells. Several studies have shown that micro- and nanostructured substrates colonized by stem cells can induce a cellular adaptive response in terms of alignment, elongation, polarization, and eventually differentiation [16–18]. Differently from traditional strategies, these methods might even allow the addition of exogenous biomolecules to be avoided, thus being highly non-invasive while affecting cell differentiation towards desired lineages.

For instance, microcontact printing (μCP) allows precise protein patterns to be produced [19,20], which serve as adhesive islands to guide cell growth and spreading [21,22]. These well-known, straightforward technologies make use of elastomeric stamps to print peptides or proteins with a resolution in the range of a few micrometers [21,23,24]. Mammalian cells plated on the aforesaid substrates selectively grow onto the adhesive regions coated with the transferred protein, whereas uncoated regions could be passivated against cell attachment. A recent application of such a technique has been addressed to understand how ECM proteins could impact stem cell response, leading to find that chemically micropatterned substrates could directly influence not only the shape and the cytoskeletal architecture of stem cells, but even their differentiation profile [25–29]. For example, poly(lactic-*co*-glycolic acid) micropatterned with fibronectin (FN) has been found to drive the myogenic differentiation of human mesenchymal stem cells cultured in the absence of any induction media [2,30]. While patterning ARPCs is still entirely unexplored, this raises the fascinating idea that printed micropatterns might be employed to coax the differentiation of adult renal stem cells *in vitro*, and possibly to control the degree of specialized tissue commitment depending on the specific pattern geometry.

Herein, we investigate for the first time the differentiation of ARPCs into the tubular lineage driven by micropatterned surfaces. In detail, we employed μCP to print FN onto tissue culture polystyrene (TCPS) substrates, forming spatially-defined stripes of different widths as a mean to control the morphology of ARPCs. We found that cells cultured on unpatterned substrates largely flattened, whereas those on micropatterned stripes showed a smaller spreading area and were highly elongated and confined in correspondence to the underlying FN features. By extensive immunofluorescence assays with the stem marker CD133, the epithelial markers Cytokeratin 19 (CK19), E-cadherin (ECAD) and Glucose Transporter 1 (GLUT1), and the markers of cell polarization Aquaporin-2 (AQP2), Aquaporin 1 (AQP1) and Na^+/K^+ ATPase (NKA), we found that the alteration of cell morphology is accompanied by the induction of the tubular differentiation which is stable in the long-term, and by the induction of cell polarity in ARPCs cultured in basal medium conditions. These experiments, also involving Western blotting and Real-time PCR assays, provide a remarkable insight into the differentiation of adult renal stem cells, and evidence that ECM micropatterns serve as effective guidance for spatially directing not only the growth of ARPCs, but also, and more importantly, the tubular differentiation in the absence of chemical induction factors or cellular reprogramming. These findings can open new routes for ARPC-based bioengineering targeting the development of novel devices and therapies.

2. Materials and methods

2.1. Materials

For photolithographic processes, wafer of Si/SiO_2 (Si-Mat) with an oxide thickness of 100 nm and the positive photoresist AZ 5214E were purchased from Clariant Corporation AZ Electronic Materials. To carry out soft lithography, polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit) was purchased from Dow Corning and TCPS coverslips were supplied by Agar Scientific. FN, Bovine Serum Albumin (BSA), and Phosphate Buffer Saline (PBS) were provided by Sigma-Aldrich.

For cell culture, Endothelial Cell Growth Medium (EGM) and Renal Epithelial Cell Growth Medium (REGM) were purchased from Lonza. Hepatocyte growth factor (HGF), 0.05% trypsin/0.2% ethylenediaminetetracetic acid (EDTA) solution and Fetal Bovine Serum (FBS) were provided by Sigma Aldrich. The synthetic lipopeptide [S-[2,3-bis(palmitoyloxy)-(2R)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine × 3 trifluoracetic acid (TFA)] (Pam_2Cys-Ser-$(Lys)_4$ × 3 TFA) was purchased from Emc Microcollections.

For viability and immunofluorescence assays, trypan blue, the nuclear stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), the dyes phalloidin-tetramethylrhodamine B isothiocyanate labeled (phalloidin-TRITC) and phalloidin-fluorescein isothiocyanate labeled (phalloidin-FITC), the antibodies rabbit anti-human FN (anti-FN), rabbit anti-goat-IgG-FITC, goat anti-rabbit IgG-FITC, goat anti-rabbit IgG-TRITC, rabbit anti-human CD133, goat anti-mouse IgG-TRITC were purchased from Sigma Aldrich. Rabbit anti-human CK19 was provided by Novus Biologicals. Rabbit anti-human AQP1 was purchased from Millipore. Rabbit anti-human GLUT1, goat anti-human NKA and mouse anti-human ECAD, mouse anti-human AQP2 were purchased from Santa Cruz Biotechnology. CD133 MicroBead Kit was purchased from Miltenyi Biotec GmbH.

For the Western blotting we used rabbit anti-CK19 (1:500), rabbit anti-AQP1 (1:500), and mouse anti-α-tubulin (1:10,000, clone B512, Sigma-Aldrich) as primary antibodies. Secondary antibodies conjugated to horseradish peroxidase were from Invitrogen (diluted 1:5000). For RNA purification and retrotranscription we used the RNeasy mini kit (Qiagen), DNase enzyme (Promega Corporation) and SuperScript II Reverse Transcriptase (Invitrogen). Quantitative Real-time PCR was performed by Power SYBR Green (Applied Biosystems) and by the Applied Biosystems 7900HT Fast Real-time PCR System. The used primers were GAPDH *Forward*: 5′-GGTGGTCTCCTCTGACTTCAACA-3′ *Reverse*: 5′-GTTGCTGTAGCCAAATTCGTTGT-3′; CK19 *Forward:* 5′-GAAGAAGAACCATGAGGAGGAAATC-3′ *Reverse*: 5′-CTTCGCATGTCACTCAGGATCTTG-3′; ECAD *Forward*: 5′-CAACGACCCAACCCAAGAATCTATC-3′ *Reverse*: 5′-TCACTTGGTCTTTATTCTGGTTATCC-3′. All primers were