Technical Report

Chemically defined medium environment for the development of renal stem cells into tubules

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The use of stem cells is a valuable therapeutical option for the regeneration of diseased tissues and organs. However, the involved cellular processes are hardly known. To gain detailed information about their development, a new culture technology was developed. Embryonic renal tissue containing stem/progenitor cells was mounted within a perfusion culture container at the interface of an artificial interstitium made of polyester. Using this innovative approach we show that renal tubules develop in chemically defined Iscove's modified Dulbecco's medium without serum addition and without coating by extracellular matrix proteins. The development of tubules depends on the administration of aldosterone, and can be visualized by immunohistochemical labeling. The presented technology makes the exact analysis of developmental steps now possible, and provides a new powerful tool to optimize growth and differentiation of renal stem cells. It may also enable many other kinds of stem cells to steer their development into functional tissues under clearly defined *in vitro* conditions.

Received 17 January 2007
Accepted 2 March 2007

Keywords: Renal stem · progenitor cells · Tubules · Aldosterone

Despite intensive investigations in the last decade, the mechanism of tubulogenesis in developing mammalian kidney is still hardly known [1–3]. This fact hampers stem cell-mediated repair of diseased kidney [4]. Our aim is to obtain information about how to generate structured tubules derived from renal stem cells and to learn how to trigger this process. To investigate this development, an efficient *in vitro* culture model is needed. Analyzing renal stem cell development by conventional, stagnant culture conditions did not result in the generation of tubules. Furthermore, numerous growth factors described in literature to promote tubulogenesis have been tested; however, none of them induced recognizable development [5]. Despite improving culture conditions with the help of different perfusion containers, the tubules could not be generated during these experiments. Consequently, the microenvironment within the culture containers was further analyzed [6]. In all the culture conditions mentioned above, the volume of the medium was found to be a number of times larger than the volume of the cultivated tissue (Fig. 1a). It is striking that such a "dead space volume" between blood and tissue does not occur in an organism. Experimentally, reducing the dead space volume within a perfusion culture container by decreasing the internal size, we observed that each geometrical change resulted in an alteration of the development of cultured tissue.

While reducing the dead space volume within a culture container, two major problems have to be considered, the minimal amount of medium required for a successful culture of tissues and the material used for the inner wall of the culture container for favoring an optimal interaction with developing tissue. The technical solution was to place a fleece between the inner wall of a perfusion culture container and the growing tissue as an artificial interstitium (Fig. 1b). The fleece should consist of a 3-D mesh of fibers, and must have excellent biophysical properties, optimal biocompatibility, sufficient hydrophilic surface for the culture medium and elastic deformability. By screening various suitable materials that can be used

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Abbreviations: ECM, extracellular matrix proteins ; **IMDM,** Iscove's modified Dulbecco's medium ; **MCR**, mineralocorticoid receptor

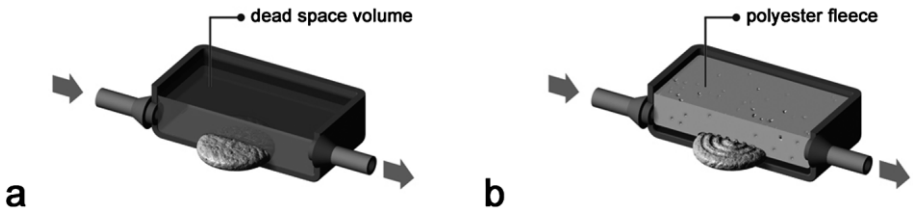

Figure 1. Dead space volume and artificial interstitium in a perfusion container. (a) Normally, a perfusion culture container contains a larger volume of medium compared to tissue. Thus, it forms a large dead space volume. (b) Placing a polyester fleece as an artificial interstitium reduces the dead space volume inside the perfusion culture container and creates a growth-promoting surface for the development of stem cells.

for an artificial interstitium, we finally found a fleece made of polyester to be the best material (Company WALRAF, Grevenbroich, Germany). This kind of fleece was originally employed as supporting material for pond foils, wiping cloths, table cloths or as stemming material for automobile industry. In the area of biomedicine it was not used for culture experiments until now.

The currently used perfusion container for the culture experiments was composed of a base and a lid. The space between the inner wall and the growing tissue was filled with an artificial interstitium made of a polyester fleece to reduce the dead volume space. During the entire culture period the fleece was in contact with the mounted tissue. In this way, the culture medium flowed through the fleece as in natural capillaries and ensured an equally distributed liquid exchange. Thus, the dead space volume was minimized and a constant fluid environment was created within the culture container. Additionally, mechanical protection was provided to the growing tissue.

The new culture technology consists of a storage bottle connected over a silicon tube with the perfusion container. Using a peristaltic pump (IPC-N8, Ismatec, Wertheim, Germany) the fresh medium is transported to the tissue. In the current experiments renal stem/progenitor cells derived from neonatal rabbit kidney were placed between two layers of a polyester fleece within the container. For a 14-day culture period fresh, chemically defined Iscove's modified Dulbecco's medium (IMDM) supplemented with 50 mM HEPES was perfused constantly. The medium leaving the container was not recycled but collected into a waste bottle. To ensure the constant temperature of 37°C, the perfusion culture container was incubated on a heating plate (Medax-Nagel, Kiel, Germany) covered by a removable lid under atmospheric air on a laboratory table. In this way an absolutely controllable environment was created.

Avoiding formation of gas bubbles

For perfusion culture, a low transport rate of the medium was necessary to guarantee an optimal nutrition and provision with respiratory gas. By testing, we determined the optimal transportation rate to be 1 mL/h. However, when applying such slow transport of medium we observed that gas bubbles arose frequently and unpredictably. The bubbles were formed along the fluid path preferentially at material transitions, where tubes, fittings and the culture container were connected. During the 14-day culture period such bubbles constantly increased in diameter by the fusion with smaller ones. When bubbles were released in random intervals into the transported medium, they formed an embolus resulting in an erratic interruption of the fluid continuum. Thus, when gas bubbles reached the

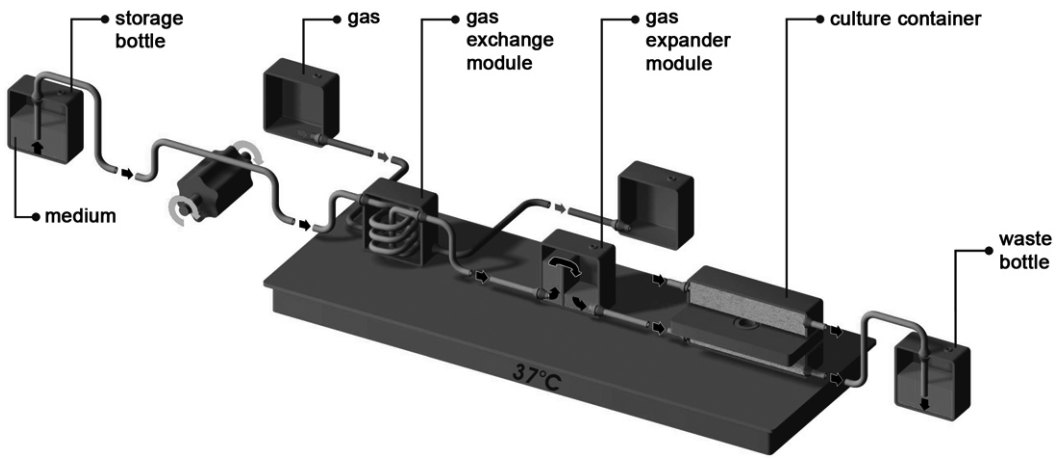

Figure 2. Schematic illustration of the newly established culture system for the generation of tubules derived from renal stem cells.

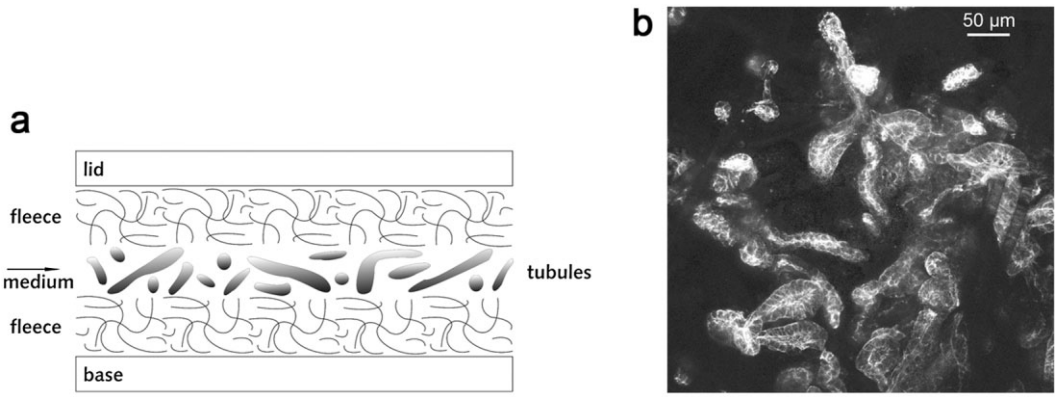

Figure 3. Development of tubules derived from renal stem/progenitor cells of neonatal rabbit kidneys. (a) Schematic illustration of the culture set-up, indicating the tubules that develop between two layers of fleece at the interface of an artificial interstitium. (b) Confocal laser-scanning microscopic view showing developed renal tubules labeled with the antibody against cytokeratin 19 after a 14-day culture period in IMDM plus 0.1 µM aldosterone.

artificial interstitium made of a polyester fleece inside the perfusion culture container, they prevented an evenly distributed exchange of the culture medium. This again led to changes of liquid pressure and caused a regional shortage of medium supply. Together, these represent a fatal consequence for the nutritive supply and respiratory gases, which are the most essential factors for the development of stem cells.

To prevent the formation of gas bubbles, special tools and tubes for medium transport were constructed consisting of silicone, with a constant diameter and without material transitions (Fig. 2). Furthermore, to avoid bubble formation during suction of the medium, specific cover caps for bottles were designed to allow medium to contact only the tube and not cap materials. In addition, spontaneously arising gas bubbles were eliminated using a newly developed gas expander module. Inside the module, the culture medium crosses a barrier and the existing gas bubbles, but not the solubilized oxygen, are separated at this point from the liquid phase. Thus, the culture medium entering the perfusion container is rich in oxygen but free of gas bubbles. Furthermore, by the use of a gas exchange module the content of respiratory gas could be modulated. The gas exchange module works via diffusion through long thin-walled and highly gas permeable silicone tubes. In contrast to the conventional gas injection method with pressurized oxygen, no gas bubbles arose using such a gas exchange module.

Tubulogenic action of aldosterone

Exposing renal stem cells to the interface of an artificial interstitium in IMDM resulted in tissue growth, but showed a disappointing degree of differentiation. Thus, the technical condition for tissue growth had been created, but it still lacked a sufficiently inductive stimulus for structured tubules to develop. Therefore, several growth factors and hormones were tested. It was discovered that the steroid hormone aldosterone exerted a surprisingly unexpected tubulogenic action on the development of renal stem cells ([5, 7] and W. W. Minuth, L. Denk and K. Hu, Involvement of the mineralocorticoid receptor in the development of renal tubules, submitted). If 0.1 µM aldosterone was added to IMDM medium, stem cells derived from neonatal rabbit kidney developed numerous tubules over the 14-day culture period (Fig. 3). The tubules were immunopositive with anti-cytokeratin 19 (Fig. 3b). The inductive action of aldosterone was concentration dependent, and 0.1 µM proved to be the optimal concentration. The action of aldosterone could be blocked by antagonists such as 0.1 mM spironolactone or 0.1 mM canrenoate. In contrast, the glucocorticoid dexamethasone did not show any comparable tubulogenic effect, although the glucocorticoid hormone is able to bind to mineralocorticoid receptor (MCR). The results suggest that the tubulogenic action of aldosterone was specifically mediated by the MCR, which is now being further analyzed.

The amount and degree of differentiation of the developed tubules induced by aldosterone is impressive (Fig. 3b). Labeling with anti-laminin γ1 showed consistent development of a basal lamina. Immunohistochemistry for Na/K-ATPase further demonstrated a positive label at the basolateral plasma membrane, indicating polarization of the developed tubules. Occludin was found at the border between the apical and lateral plasma membrane, revealing the development of tight junctions [5].

In summary, we developed a new culture technology for growing renal stem cells into tubules under absolutely controllable *in vitro* conditions. Using this approach, we showed for the first time that renal stem cells developed at the interface of an artificial interstitium into 3-D structured tubules, which depended on the administration of aldosterone. Although these cultures could be maintained for weeks in chemically defined medium, the quality and quantity of generated tubules look promising for future

laboratory work. Currently, it is still unclear which biophysical factors support the development of the 3-D structured tubules at the artificial interstitium. Thus, a new field is opened for the study of growth-promoting factors at the interface of the artificial interstitium.

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