Abstract:

The present invention relates to a new three-dimensional co-culture method of podocytes and endothelial cells, and a relative co-culture system. Furthermore, the invention relates to the use of said Co-culture system as an in vitro study model of pathologies affecting the kidneys, and in particular the renal glomerular filtration barrier.

Title: METHOD FOR THE THREE-DIMENSIONAL CO-CULTURE OF PODOCYTES AND ENDOTHELIAL CELLS AND RELATIVE IN VITRO CO-CULTURE SYSTEM
METHOD FOR THE THREE-DIMENSIONAL CO-CULTURE OF
PODOCYTES AND ENDOTHELIAL CELLS AND RELATIVE IN VITRO
CO-CULTURE SYSTEM

The present invention relates to a new three-dimensional co-culture method of podocytes and endothelial cells, and a relative in vitro co-culture system. Furthermore, the invention relates to the use of said co-culture system as a screening model of drugs or in vitro study of pathologies which affect the kidneys, and in particular the renal glomerular filtration barrier.

Conventional cell culture methods are generally used for defining the base properties of single cell types. Cells, however, live in a three-dimensional microenvironment which deeply influences their function. In particular, in the renal glomerulus, the podocytes adhere to the outer side of the glomerular basement membrane, whereas the endothelial cells adhere to its internal side. The three-dimensional organization of podocytes, basement membrane and endothelial cells allow the system to operate the filtration barrier function, as illustrated in Figure 1.

When the filtration barrier is damaged, proteins are lost in the urine. This phenomenon arises in primary diseases of the various glomerular components, and also as a consequence of extremely widespread systemic pathologies, such as obesity, diabetes and hypertension. In spite of the numerous efforts made in
scientific research, and recent investigative progress, there are few explanations on the aetiology and pathogenesis of proteinuric pathologies with negative consequences on diagnostic and therapeutic possibilities, which have remained limited.

The possibility of recreating, in vitro, a structure similar to the filtration barrier would therefore be extremely useful in studying proteinuric pathologies and understanding the mechanisms which are at the basis of the protein loss on the part of the glomerular filter.

Numerous technical problems, however, have always prevented the creation of this system, above all due to the extreme differentiation and specialization of the cells composing it, and probably the interdependence of the various components of the system itself.

The cells which compose the filtration barrier are, in fact, extremely differentiated and specialized. It is also evident that a study of their function cannot disregard the context of the barrier itself due to various functional aspects.

Various authors in the past have used organotypic cultures of renal tissue in continuous perfusion [1-3], but the request for a simple co-culture system which reproduces the structure of the renal filtration barrier remains current.

Goligorsky et al. have described a sandwich co-culture method [4,5]. The podocyte line used consists of primary podocytes of rats transfected with SV-40.
The endothelial line used is HUVEC obtained by Clonetics Corp. The podocytes are grown on the bottom of 24-well plates, and are subsequently covered by Matrigel (extracellular matrix, Fisher Scientific) which is gelified at 37°C for 30 minutes. After adding the medium, the endothelial cells are added to each well, completing the sandwich. With respect to the physiological configuration of the filtration barrier, it is evident that the system differs in two substantial elements: a) the podocyte cell is compelled to adhere to both the base of the well and to the matrix covering it; b) there is no possibility of reproducing glomerular filtration, as there is no supernatant on the podocyte side. In the recent work published by Hirschberg R. et al. [6], the co-culture system is thus composed: the podocytes (conditional immortalized mouse line obtained from the so-called Immortomouse) are cultivated on membranes positioned in 96-well plates and induced to differentiation. The endothelial cells (in this case they are differentiated endothelial cells starting from circulating progenitors) are cultivated on the base of wells in 96-well plates. The membranes with the podocytes are then positioned in the wells containing the endothelial cells. It is not clear whether the membrane rests on the endothelial cells or whether it remains suspended, but in both cases the system is clearly different from the physiological condition of the glomerular filter, as the space ratios between the elements forming it are
altered.

Another co-culture system is described in EP1437147A1, which is mainly designed for the growth of alveolar epithelial cells and endothelial cells. The experimental reproduction of this system, however, effected by the authors of the present invention according to the method described in EP1437147A1, did not allow an artificial glomerulus to be reconstructed, i.e. the co-culture system when podocytes and glomerular endothelial cells are used.

On the basis of what is indicated above, there is an evident necessity for availing of a new co-culture method of podocytes and endothelial cells, and a relative three-dimensional in vitro system which reproduces in a simplified but complete manner, the characteristics and space ratios of the glomerular filter, and allows the application of numerous research methods to both of the populations in the culture and their respective supernatants, overcoming the disadvantages of the techniques so far used.

The authors of the present invention have set up a new three-dimensional co-culture method of podocytes and endothelial cells, together with the relative in vitro co-culture system.

An object of the present invention therefore relates to an in vitro co-culture system of podocytes and endothelial cells comprising a solid support containing a first culture medium inside which a semi-permeable membrane consisting of plastic material with
pores having a diameter ranging from 0.3 µm to 1.2 µm, preferably 1 µm, is kept in suspension through a second solid support, said membrane being coated on both sides with collagen type IV, wherein on the lower side of the membrane a microvascular endothelial mammal cell line is grown in the first culture medium and on the upper side of the membrane a mammal podocyte line is placed in culture in a second culture medium.

The membrane is preferably polyethyleneterephthalate (PET). In a particularly preferred embodiment of the invention, the Millipore membrane (Millicell Hanging Cell Culture Insert) having pores with a diameter of 1 µm, is used.

The line of mammal podocytes is preferably the immortalized line obtained from the authors of the present invention, from the mouse Immortomouse (Charles River, St Louis, MO, USA), a transgenic mouse for a sensitive temperature variant of "SV40 large T antigen" under the control of the promoter H-2Kb interferon-gamma-inducible (as described in [7]). In alternative embodiments, the podocyte line can be mutated (i.e. by means of gene silencing), or primary podocyte cultures can be used coming from genetically normal animals or transgenic animals.

In a preferred embodiment of the invention, the solid support is a multi-well plate. Figure 8 schematically illustrates the configuration of the co-culture system of podocytes and endothelial cells inside a well.
According to an alternative embodiment, the podocyte line can be replaced with a line of pericytes to simulate, in vitro, the system of pericytes - microvascular endothelial cells which exists on the level of all the capillaries of the organism.

According to another alternative embodiment, the system according to the invention also comprises a third cell population situated on the bottom of the first solid support, and selected according to the tissue to be reproduced from mesangial cells (in the case of renal glomerulus), fibroblasts (connective tissue), astrocytes (hemato-encephalic barrier), or specific parenchymal cells (myocardiocytes, hepatocytes, neuronal cells, etc.).

A further object of the present invention relates to a method for the in vitro co-culture of podocytes and endothelial cells comprising the following phases:

a) culture of a microvascular endothelial mammal cells line on the lower side of a semi-permeable membrane coated on both sides with collagen type IV consisting of plastic material with pores of diameter between 0.3 µm and 1.2 µm kept in suspension inside a solid support containing a first culture medium in the presence of VEGF at a concentration of between 3 and 50 ng/ml, preferably 5 ng/ml, for one week;

b) culture of a mammal podocytes line under "permissive conditions", i.e. in the presence of gamma-interferon at a temperature of 33°C (7), for one week;

c) transfer of the mammal podocytes line obtained from
step b) on the upper side of the membrane of step a) and culture in a second culture medium under "non-permissive conditions", i.e. in the absence of gamma-interferon at a temperature of 37°C (7);

d) co-culture of the two cell lines in the two environments separated by the membrane.

Said solid support is preferably a multi-well plate.

According to a preferred embodiment of the method of the invention, said membrane is PET with pores having a diameter of 1 µm. In a particularly preferred embodiment of the invention, the membrane Millipore (Millicell Hanging Cell Culture Insert) having pores with a diameter equal to 1 µm, is used.

The mammal podocytes line is preferably that obtained in our laboratory from the mouse Immortomouse (Charles River, St Louis, MO, USA), a transgenic mouse for a sensitive temperature variant of "SV40 large T antigen" under the control of the promoter H-2Kb interferon-gamma-inducible [7]. In alternative applications, the podocyte line can be mutated (i.e. by means of gene silencing), or primary podocyte cultures can be used coming from genetically normal animals or transgenic animals.

When an interferon-gamma-inducible conditionally immortalized podocytes line previously grown for a week in "permissive conditions", i.e. in the presence of interferon-gamma at 33°C, is used, the culture in a second medium of step c) in "non-permissive conditions"
takes place in the absence of interferon-gamma and at 37°C.

When a primary culture is used, the initial phase comprises the isolation of the glomeruli, which are put in culture until the podocytes begin to grow. Cells and glomeruli are then trypsinized and the glomeruli are eliminated by filtration, to obtain the primary podocyte culture (method described in [7]).

According to a preferred embodiment of the invention, the line of microvascular endothelial mammal cells is the murine line ATCC Number CRL-2586™.

The method according to the invention can also comprise the introduction of a third cell line on the bottom of the solid support, selected according to the tissue which is to be reproduced from mesangial cells (in the case of renal glomerulus), fibroblasts (connective tissue), astrocytes (hemato-encephalic barrier), or specific parenchymal cells (myocardiocytes, hepatocytes, neuronal cells, etc.).

According to an alternative embodiment of the method of the invention, the line of podocytes can be substituted with a line of pericytes and the culture medium of step a) contains PDGF-B (specific growth factor for pericytes) instead of VEGF.

The invention also relates to the use of the in vitro co-culture system as defined above in a screening method of drugs or substances suitable for modifying the functionality of the renal glomerulus comprising the evaluation of one or more parameters selected from
permeability of the capillary barrier, cell morphology, immunodetection of the specific cell expression markers and markers released in the supernatants of the cell populations.

It is evident that the advantage of operating in a three-dimensional context, even if simplified with respect to the in vivo structure, is essentially due to the possibility of applying stimuli and variations on one side of the membrane and studying the consequences produced on the other side.

The evaluation of the permeability barrier can be effected through spectrophotometric measurements of the transmembrane passage of proteins or polymers with a defined molecular weight (such as albumin or dextrans), also conjugated with a fluorescence marker.

The morphology of the cells of the co-culture system according to the invention or their confluence degree can be easily controlled by means of optical microscopy, transmission and scanning electron microscopy.

The system also enables the qualitative evaluation of specific markers of the two cell populations by means of immunocytochemistry, immunofluorescence, and immunogold electron microscopy. In order to quantitatively evaluate the above expression markers it is also possible to carry out quantitative methods, such as the so-called "in cell ELISA" which allows information to be obtained on the protein expression relating to the number of cells present on the
membrane.

Each type of biochemical or molecular test (e.g. ELISA and Western Blot), can be subsequently applied to the study of the respective supernatants.

Finally, the invention relates to the use of the co-culture system according to the invention, as an in vitro study model of the dysfunctions or pathologies affecting the renal glomeruli.

The three-dimensional co-culture system of podocytes and endothelial cells according to the invention can therefore be effectively applied in pharmacological studies, in the discovery of diagnostic markers, and in analyses of biological samples coming from individual subjects, with the perspective of a personalization of the diagnosis and treatment.

The present invention will now be described for illustrative but non-limiting purposes, according to its preferred embodiments with particular reference to the figures of the enclosed drawings, wherein:

figure 1 shows the structure of the renal glomerulus, an entanglement of glomerular capillaries whose basal membrane is externally covered by the extensions of the podocytes (foot processes) and internally by the endothelial cells, forming the filtration barrier. The glomerular capillaries are sustained by the mesangial axis, composed of mesangial cells and extracellular matrix.

figure 2 shows the image obtained by optical microscopy of the co-culture system, whereby it is
possible to control the presence of the two cell populations on the two sides of the membrane. A toluidine blue-coloured semifine section is represented in the figure.

Figure 3 shows the image of the co-culture system obtained by transmission electron microscopy. Transmission electron microscopy allows the morphological details of the two cell types to be examined. In the image the interaction between cell extensions of podocytes is evident.

Figure 4 shows the image of the co-culture system obtained by scanning electron microscopy. With scanning electron microscopy, it is possible to add further information on the morphology and cell density. An image obtained from the side of the endothelial cells is represented in the figure.

Figure 5 shows the expression of CD31 on the part of the endothelial cells grown in co-culture (IF, 200X) by means of immunofluorescence.

Figure 6 shows the expression of VCAM-1 on the part of the endothelial cells, put in co-culture with primary podocytes coming from the null mouse for Rab3A (IHC, 400X) by immunohistochemistry.

Figure 7 shows the Western Blots carried out on podocyte line cultures after two weeks of growth under non-permissive conditions. The differentiated cells are positive for specific podocyte markers of mature cells.

Figure 8 schematically shows the co-culture system of podocytes and endothelial cells according to the
invention.

Figure 9 shows the differences between cells grown on collagen type IV and collagen type I. The image shows the phenotype change of the podocyte line depending on the type of collagen on which the cells are grown. The images on the left are examples of cells grown on type IV collagen: the actin is organized in stress fibres which pass through the cell body and continue along the extensions. Nefrin is prevalently expressed along the cell extensions. The two images on the right show the podocytes grown on type I collagen. The reduction in stress fibres and general remodelling of actin can be observed. Furthermore, nefrin is exclusively present in the cell body, whereas it is absent in the cell extensions.

Figure 10 shows the expression of specific podocyte proteins in the maturation phases of the conditional immortalized podocyte mouse line used, and the further action favouring cell differentiation on the part of retinoic acid. Synaptopodin, a podocyte marker present in mature podocytes, is completely absent in cells cultivated under "permissive conditions" (image on the left). The positivity for synaptopodin is evident in cells matured means of by temperature variation and absence of interferon-gamma (image in the centre), and the marker is more fully expressed if the cells are differentiated in the presence of retinoic acid (image on the right).

Figure 11 illustrates the use of a different
membrane (PTFE membrane, polytetrafluoroethylene) from that selected and used according to the present invention.

It should be noted how the structure of the membrane is less homogeneous with respect to that represented in figures 2 and 3, and prevents the adhesion of the podocyte population. The characteristics of non-homogeneity of the membrane and poor resistance to processing methods are particularly evident in the image in transmission electron microscopy.

An embodiment example of the co-culture system, object of the present invention, is provided hereunder for illustrative but non-limiting purposes of the present invention.

EXAMPLE 1

MATERIALS

The following were used for obtaining the co-culture system:

a) A conditional immortalized mouse podocyte line obtained in our laboratory [method described in 7], following, with some modifications, the method described by Mundel P et al [8]. In short, the glomeruli were isolated from the kidneys of transgenic mice H-2I\&-tsA58 (Immortomouse, Charles River, St Louis, MO, USA) aged 6-8 weeks. These are transgenic mice for a sensitive temperature variant of "SV40 large T antigen" under the control of the promoter H-2Kb interferon-gamma-inducible. The glomeruli were
separated from the renal tissue by sieving the tissue through filters with a decreasing pore diameter (from 100 to 36 µm) and grown in a standard medium at 37°C. When the podocyte cells began to grow, cells and glomeruli were detached by treatment with trypsin and the glomeruli eliminated by filtration. The cells were re-plated and propagated at 33°C in a medium containing 20U/ml of recombinant mouse interferon-gamma (so-called "permissive conditions"). The cells were then purified according to the limiting dilution method [8], i.e. cultivated at decreasing concentrations (10, 1, 0.1 cells/vial). The clones obtained were characterized by immunocytochemistry and western blot and only the 5 clones which expressed WT1 and nephrin in over 90% of the cells, were selected and propagated. A cell aliquot from each clone was brought to complete maturation, whereas most of the cells were frozen. The maturation was obtained by growth under "non-permissive conditions", i.e. at 37°C in a medium not containing interferon-gamma (7). This podocyte line is therefore characterized by a high proliferative capacity when brought to 33°C in the presence of interferon-gamma (so-called "permissive conditions") (7). The cells, brought to 37°C in the absence of interferon for about two weeks (so-called "non-permissive conditions") differ in mature podocytes, having cell extensions and expressing the complete panel of specific markers of these cells, such as nephrin, podocin, synaptopodin, alpha-actinin (Figure ...
Alternatively, the podocyte line can be substituted by a culture of primary podocytes. The latter offer the advantage of a better differentiation which makes them functionally more similar to the in vivo situation, and allow cells coming from transgenic animals of interest to be used. The method used (see reference [7]) envisages the isolation of glomeruli from newly-born animals (maximum 10 days of age), and was borrowed from methods for obtaining cultures of primary neurons. Potential disadvantages, which should be taken into consideration, are determined by the shorter cell survival and necessity for a complete characterization of the cells at each isolation.

b) A mouse endothelial cell line (EOMA-Mus musculus, ATCC-CRL-2586), characterized by functional aspects typical of microvascular endothelial cells. This is a particularly important detail, as microvascular endothelial cells differ in numerous functional aspects from cells lines commonly used, such as HUVEC, which derive from the venous endothelium, or from other derivation lines from vessels having a greater calibre than capillaries. It is difficult to replace the cell line with primary glomerular cells, as the glomerular endothelium is particularly differentiated and non-proliferous when put in a culture.

c) A semi-permeable membrane with pores having a diameter ranging from 0.3 µm to 1.2 µm, preferably a membrane of PET with pores of 1 µm (Millicell Hanging
Cell Culture Insert, Millipore) which can be allocated in suspension inside wells in the culture plates, so that the base of the membrane is suspended in the medium and does not touch the bottom of the well. The selection of the membrane was determined by particular properties of the material forming it (PET) which is ideal for the growth of the cell types considered herein.

PET was selected as it is the material used in vascular grafts (12). The use of a polycarbonate membrane was discarded as this material is not transparent to microscopes (Millicell Technical Guide, 2004, Lit. No.: TN2004EN00, page 4 Millipore, http://www.millipore.com/publications.nsf/a736,64f9f981af8c852569b9005b4eee/2984f0525bf6e23285256e820061bd4e/$FILE/TN2004EN00.pdf). The use of a PTFE membrane is not suitable for the purposes of electron microscopy, as indicated in figure 11.

Furthermore the same material offers various advantages such as an improved consistency and resistance when the system is removed from the well and processed by transmission electron microscopy, the improved shear quality of ultrathin sections (the membrane does not exfoliate), and the excellent visibility of the cells in both bright field microscopy and in immunofluorescence and immunocytochemistry.

The use of a different type of membrane led to a series of drawbacks, as represented in Figure 11. d) the membrane is covered on both sides with type IV
collagen (12). The choice of collagen is extremely important, as type IV collagen is that which is physiologically present in basal membranes of glomerular capillaries. The use, for example, of type I collagen produces important variations in the cell phenotype, as represented in Figure 9.

METHODS

When placed on the two sides of the membrane, the two cell types are cultivated in specific mediums which allow their differentiation and maturation. In particular, Vascular Endothelial Growth Factor (VEGF) [9] is added to the culture medium of the endothelial cells and the endothelial cells are left to grow alone on the lower side of the membrane for a week. Only at this point are the podocytes added under "non-permissive conditions".

RESULTS

The system conceived by the present invention is schematized in Figure 8. The system consists of a membrane suspended in a well of a plate for cell cultures. The endothelial cells are grown on the lower side of the membrane, whereas the podocytes are put in culture on the upper side.

The endothelial cells are put in culture on the lower side of the membrane, and the growth factor VEGF is added to the culture medium at a concentration of 5ng/ml.

This phase is critical as VEGF is produced by the podocytes in the glomerulus, and the endothelial cells
have the specific receptors of this growth factor. Knock-out animals for VEGF have endothelial alterations which do not allow a normal development of the glomerular structure [10] and it has been demonstrated that the addition of VEGF to endothelial cell lines is capable of causing the fenestration typical of the glomerular endothelium [11].

After a week, the podocytes (maintained up until this moment under "permissive conditions", i.e. at 33°C with the addition of interferon-gamma) are placed on the upper side of the membrane and are left to differentiate for two weeks under "non-permissive conditions" (i.e. in the absence of interferon and at a temperature of 37°C).

In a further variant, retinoic acid is added to the medium which covers the podocytes on the membrane, which allows a further differentiation of the podocyte cell, making it even more similar both morphologically and functionally to the primary cell and in vivo cell (Figure 10).

The system is formed so as to keep the two cell environments and respective supernatants separate. The communication between the two micro-environments takes place exclusively through the membrane itself.

The presence of the cells and their confluence degree can be easily controlled by means of optical microscopy (Figure 2), transmission electron microscopy (Figure 3) and scanning electron microscopy (Figure 4).

The system allows the study of the two cell
components by means of immunofluorescence (Figure 5) and immunohistochemistry (Figure 6).

In short, the cells are fixed in cold acetone or paraformaldehyde, depending on the markers to be analyzed. This is followed by a permeabilization with Triton, a pre-incubation with BSA, the cells are then sequentially incubated with the primary antibody, the secondary marked antibody, and if necessary the detection system. At this point, the membrane is detached from the support and adhered to a plate, so that the cell type of interest is on the surface. The plate is covered with a cover glass plate and the results are analyzed by optical or fluorescence microscopy.

It is also possible to substitute the cell lines indicated herein with cells obtained from transgenic animals (Figure 6). There are currently numerous proteinuric animal models obtained by mutation of podocyte molecules. The co-culture system according to the invention can be used complementarily with respect to the in vivo study of animals, as it allows the co-culture of primary podocytes coming from the experimental model in a system which reproduces the situation of the filtration barrier in vitro.

Alternatively, with respect to the primary culture coming from mutated animals, the conditional immortalized cell line preferably used herein can be further mutated or silenced into genes of interest. The same also applies to the endothelial line. Furthermore,
modified/mutated components of the glomerular basal membrane can substitute/be added to type IV collagen, to reproduce other pathological situations.

BIBLIOGRAPHY


[12] Liu Y, He T, Song H, Gao C. Layer-by-layer assembly of biomacromolecules on poly (ethylene terephthalate) films and fiber fabrics to promote
CLAIMS

1. An in vitro co-culture system of podocytes and endothelial cells comprising a solid support containing a first culture medium inside which a semi-permeable membrane consisting of plastic material with pores of diameter between 0.3 µm and 1.2 µm is kept in suspension through a second solid support, said membrane being coated on both sides with collagen type IV, wherein on the lower side of the membrane a microvascular endothelial mammal cell line is grown in the first culture medium and on the upper side of the membrane a mammal podocyte line is placed in culture in a second culture medium.

2. The system according to claim 1, wherein said plastic material is polyethylene terephthalate (PET).

3. The system according to each of claims 1-2, wherein said membrane has pores having a diameter equal to 1 µm.

4. The system according to each of claims 1-3, wherein said line of mammal podocytes is conditionally immortalized.

5. The system according to each of claims 1-4, wherein said solid support is a multi-well plate.

6. The system according to each of claims 1-5, also comprising a third cell population on the bottom of the first solid support selected from the group consisting of mesangial cells, fibroblasts, astrocytes and parenchymal cells.

7. A method for the in vitro co-culture of podocytes
and endothelial cells comprising the following steps:

a) culture of a microvascular endothelial mammal cells
line on the lower side of a semi-permeable membrane
coated on both sides with collagen type IV consisting
of plastic material with pores having a diameter
ranging from 0.3 μm to 1.2 μm kept in suspension inside
a solid support containing a first culture medium in
the presence of VEGF at a concentration of 3 to 50
ng/ml, preferably 5 ng/ml, for one week;

b) culture of a line of mammal podocytes under
"permissive conditions", i.e. in the presence of
interferon-gamma at 33°C, for one week;

c) transfer of the mammal podocytes line obtained from
step b) on the upper side of the membrane of step a);

d) co-culture of the two cell lines in the two
environments separated by the membrane.

8. The method according to claim 7, wherein said solid
support is a multi-well plate.

9. The method according to each of claims 7-8, wherein
said membrane is made of PET with pores having a
diameter of 1 μm.

10. The method according to each of claims 7-9, wherein
said line of podocytes is conditionally immortalized.

11. Method according to claim 10, wherein when an
interferon-gamma-inducible conditionally immortalized
podocytes line grown under "permissive conditions", 
i.e. in the presence of interferon-gamma at 33°C, is used, the culture in a second medium of step c) under "non-permissive conditions" takes place in the absence of gamma interferon and at 37°C.

12. The method according to each of claims 7-11, wherein under item b) a primary culture of mammal podocytes is used.

13. Method according to each of claims 7-12, wherein said microvascular endothelial cells line is the murine line ATCC CRL-2586.

14. The method according to each of claims 7-13, comprising the introduction of a third cell line on the bottom of the solid support selected from the group consisting of mesangial cells, fibroblasts, astrocytes and parenchymal cells.

15. Use of the in vitro co-culture system as defined according to claims 1-6, in a screening method of drugs or substances suitable for modifying the functionality of the renal glomerulus comprising the evaluation of one or more parameters selected from cell permeability, cell morphology, immunodetection of the expression markers released in the supernatants of the cell populations.

16. Use of the in vitro co-culture system according to claim 15, wherein said cell permeability is evaluated through the use of albumin or dextrans, also marked.

17. Use of the in vitro co-culture system according to claim 15, wherein the cell morphology is analysed through optical or electronic microscopy.
18. Use of the in vitro co-culture system according to claim 15, wherein the expression markers released in the supernatants are detected through the ELISA or Western blot test.

19. Use of the in vitro co-culture system as defined according to claims 1-6, as an in vitro study model of the dysfunctions or pathologies affecting the renal glomeruli.

20. An in vitro co-culture system obtained according to claims 7-14.

21. Use of the in vitro co-culture system obtained according to claims 7-14, in a screening method of drugs or substances suitable for modifying the functionality of the renal glomerulus comprising the evaluation of one or more parameters selected from cell permeability, cell morphology, immunodetection of the expression markers released in the supernatants of the cell populations.
Fig. 1

Fig. 2
Fig. 6

Fig. 7
Fig. 8

Fig. 9
### A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC.

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

- EPO-Internal
- EMBASE
- FSTA
- WPI Data
- BIOSIS

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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* Further documents are listed in the continuation of Box C.  

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Date of the actual completion of the international search: 12 April 2011

Date of mailing of the international search report: 18/04/2011

Authorized officer: Bonello, Steve

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