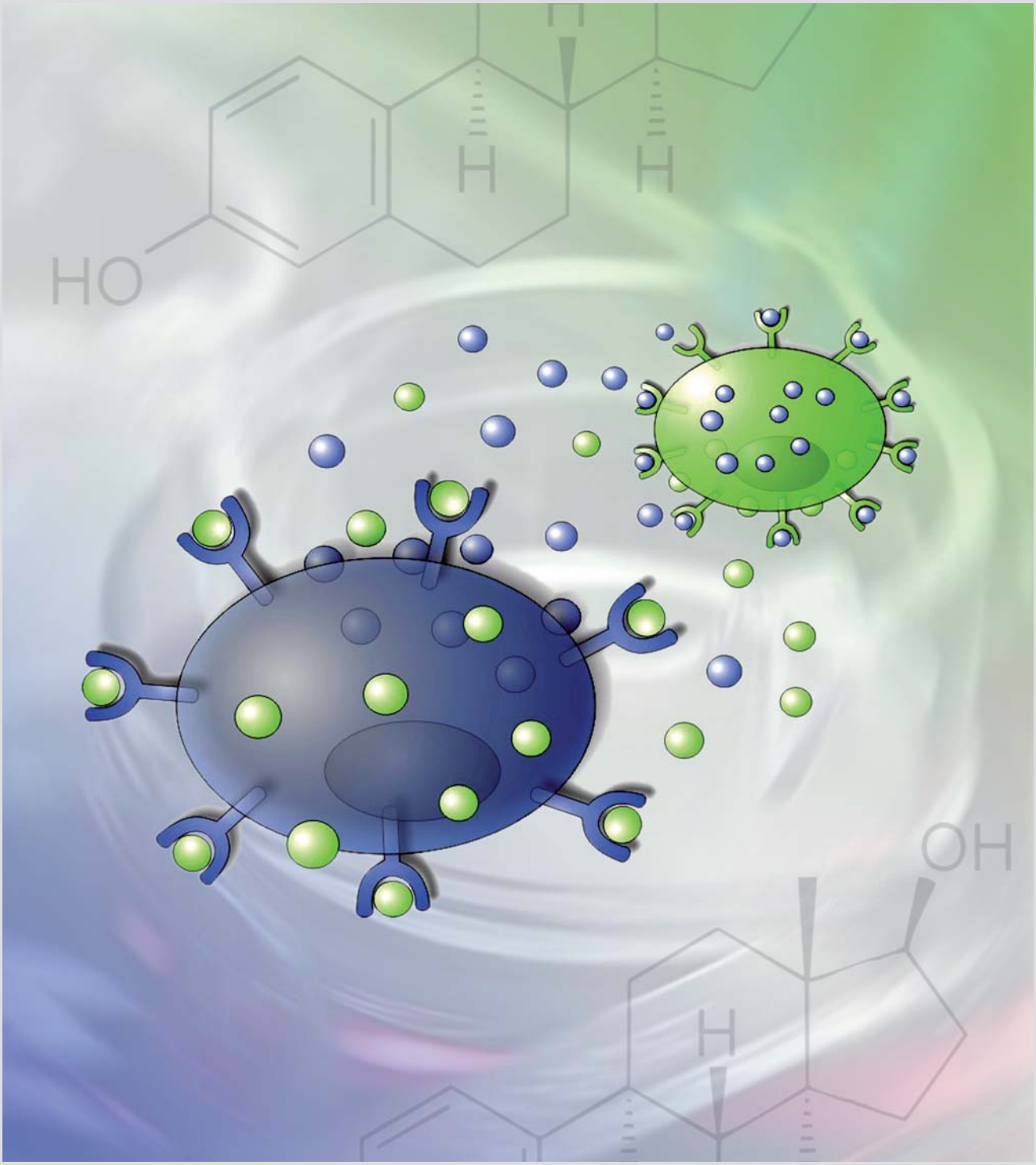
Application Note

Co-culture in ThinCert™ cell culture inserts



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Introduction

Co-culture describes various techniques where different cell populations are cultivated in close proximity in the same cell culture environment. The applications of co-cultures are multi-facetted and include:

- stimulation and maintenance of cell function and differentiation,
- cultivation of embryonic stem cells on feeder cells,
- applications in reproductive medicine (e.g. autologous endometrial co-culture),
- investigation of immune cell interactions,
- investigation of paracrine mesenchymal-epithelial interactions,
- restoration of heterocellular functions *in vitro* (e.g. blood-brain-barrier).

Direct co-culture can be performed in nearly all cell culture dishes, for instance by layering two cell types one on top of the other. In contrast, indirect co-culture takes advantage of cell culture inserts with porous membranes, to keep the co-cultivated cell populations separated (**Fig. 1**).

Material

Item	Manufacturer	Cat.-No.
Alexa Fluor 488 anti rabbit IgG antibody	Invitrogen GmbH	A11008
Beta estradiol	Sigma-Aldrich Chemie GmbH	E8875
Cell proliferation kit I (MTT)	Roche Diagnostics	1465007
CELLSTAR® 24 well cell culture plate	Greiner Bio-One GmbH	662 160
DakoCytomation Fluorescent Mounting Medium	Dako Deutschland GmbH	S3023
DAPI, dilactate	Sigma-Aldrich Chemie GmbH	D9564
DMEM medium	Biochrom AG	F0435
Fetal calf serum	Invitrogen Life Technologies	10270-106
Formalin	Sigma-Aldrich Chemie GmbH	HT5014
Insulin (bovine)	Biochrom AG	K 3510
Ki67 antibody	Abcam	Ab15580
L-alanyl-L-glutamine	Biochrom AG	K0302
PBS-Dulbeco	Biochrom AG	L1825
RPMI medium	Biochrom AG	F1295
ThinCert™ 24 well cell culture insert with 0.4 µm pores	Greiner Bio-One GmbH	662 640
Tritron X100	Sigma-Adrich Chemie GmbH	T8787

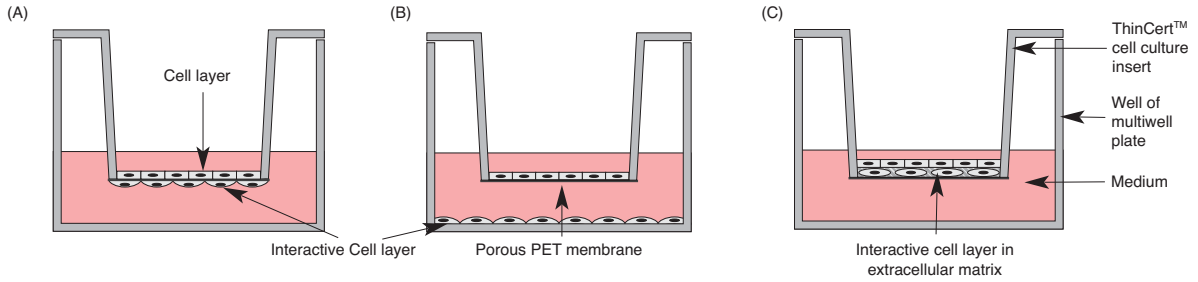

Figure 1
Different modes of co-culture using ThinCert™ cell culture inserts.
Two cell populations that are co-cultivated in different compartments (insert and well) stay physically separated, but may communicate via paracrine signalling through the pores of the membrane (**A, B**). Alternatively, both cell populations may be co-cultivated in the upper compartment (insert), thus allowing extensive and direct cell-cell interactions (**C**).

Co-cultures with cell culture inserts have been widely used to study mesenchymal-epithelial interactions during normal and tumoral development (Hofland et al., 1995; Gache et al., 1998). Here, such a co-culture model has been established using MCF7 mammary carcinoma cells, human fibroblasts and ThinCert™ cell culture inserts. These experiments illustrate the excellent suitability of ThinCert™ cell culture inserts for co-culture applications.

The following protocol presents technical details for co-culture and may be easily adapted to match individual requirements and research interests other than paracrine growth regulation.

Methods

Seeding of fibroblasts onto the underside of the membrane and co-culture

24 well ThinCert™ cell culture inserts with translucent membranes and 0.4 µm pores were inverted and placed in a 12 well plate (**Fig. 2/1**). The well bottom was humidified with 100 µl sterile water (**Fig. 2/2**). 60 µl of a cell suspension containing 0 (control); 83,000; 167,000 or 418,000 human juvenile foreskin fibroblasts per ml DMEM medium (supplemented with 10% FCS, 4 mM L-alanyl-glutamine) was pipetted into the inner circle of the underside of the membrane (**Fig. 2/3, 2/4**).

The plate was covered with a lid, thus holding the cell suspension to the underside of the membrane by capillary forces (**Fig. 2/5**). The cells were allowed to adhere overnight at 37°C and 5% CO_2. Subsequently, the insert was placed in the well of a 24 well plate pre-filled with 800 µl RPMI medium containing 10 µg/ml insulin, 110 pM estradiol, 10% FCS and 4 mM L-alanyl-glutamine (**Fig. 2/6**). 200 µl MCF7 suspension containing 25,000 cells per ml supplemented RPMI medium was added to each insert. Co-cultures were maintained for 2 days at 37°C and 5% CO_2.

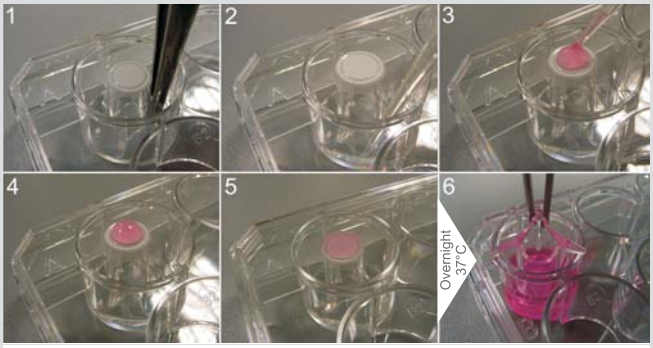

Figure 2
Cell seeding onto the underside of the insert membrane. A 24 well insert is placed upside down into a humidified well of a 12 well plate (**1, 2**). Cell suspension is pipetted onto the membrane underside (**3, 4**). The plate is closed with a lid (**5**). After overnight incubation (37ºC, 5% CO_2) the insert is placed in a 24 well plate. A second cell population may now be seeded into the upper compartment (**6**).

Quantification of proliferative cells by Ki67 immunocytochemistry

The immunocytochemistry protocol used here is described in our corresponding application note (No. 073 100) (www.gbo.com/bioscience/thincert). In brief, cells in the insert were fixed with 500 µl 4% formalin, washed twice with 500 µl PBS and permeabilised for 25 min with 500 µl 0.5% Triton/PBS. After washing with PBS, non-specific protein binding sites were blocked with 500 µl 10% FCS/PBS for 1.5 h.

Cells were washed with PBS and incubated for 1 h with 100 µl rabbit anti Ki67 antibody (1:100 in 1% FCS/PBS). After washes with PBS, the cells were incubated for 1 h with 100 µl Alexa 488 anti rabbit IgG antibody (1:250 in 1% FCS/PBS). Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). Insert membranes were cut out and mounted on microscopy slides using fluorescence mounting medium.

For analysis, the number of Ki67 positive cells and the total cell number (DAPI) per microscopic field were counted using an inverted microscope at a 250x magnification. A two-tailed t-test was used for comparison between two experimental data sets. A value of $P<0.05$ was considered statistically significant.

MTT[1] assay

The MTT assay was performed with the Cell Proliferation Kit I from Roche. After removal of the cell culture medium from the inserts, the fibroblasts were wiped off the underside of the insert using a cotton swab. The inserts were placed in a freshly prepared 24 well plate containing 400 µl MTT medium (0.5 mg/mL) per well.

100 µl MTT medium was added to each insert. After an incubation of 4 h at 37 °C and 5% CO_2, 400 and 100 µl solubilization solution was added to each well and insert, respectively. The next day, 200 µl of the combined and mixed solutions from the insert and well were transferred to a clear bottom 96 well plate. The absorbance was measured with a TECAN Safire plate reader at 570 nm.

Results and discussion

Mesenchymal-epithelial interactions have been shown to play an important role in normal breast development and breast tumorigenesis. *In vivo*, positive feedback loops between hormone responsive breast tumor cells and their surrounding fibroblasts seem to account for enhanced tumor growth and are likely mediated by hormones and growth factors exchanged between both cell populations (reviewed in Clarke et al., 1992). Previously, indirect and direct co-cultures of breast tumor cells and fibroblasts have been extensively used to study mesenchymal-epithelial interactions in breast tumor formation *in vitro* (Hofland et al., 1995; Gache et al., 1998; Heneweer et al., 2005). Indirect co-cultures using cell culture inserts revealed the paracrine growth promoting effect exerted by fibroblasts on breast tumor cells (Hofland et al., 1995; Gache et al., 1998).

Here, such a co-culture model has been established using MCF7 breast cancer cells and human fibroblasts that were co-cultivated on the upper and lower sides of the membrane of ThinCert™ cell culture inserts, respectively. Proliferative MCF7 cells could be identified on the basis of positive Ki67 immunoreactivity (**Fig. 3**).

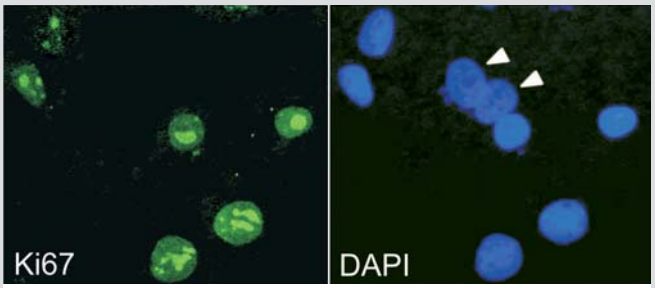

Figure 3
Identification of non-proliferative cells (arrowheads) based on lack of Ki67 immunoreactivity.

[1] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

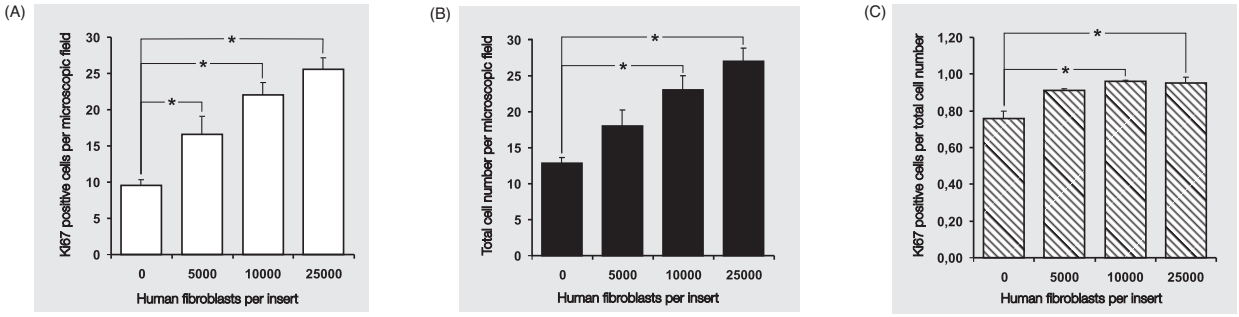

Figure 4
Growth promoting effect of primary human fibroblasts on MCF7 cells. Fibroblasts cultivated on the underside of ThinCert™ cell culture inserts exert a growth promoting effect on MCF7 cells cultivated on the upper side of the membrane. The effect manifests in (**A**) the number of proliferating (Ki67 positive) MCF7 cells, (**B**) the total number of MCF7 cells and (**C**) the ratio between proliferative vs. total cell numbers. Averages and standard errors are shown. Statistically significant differences are marked with asterisks (P<0.05).

Human fibroblasts demonstrated a clear growth promoting effect on MCF7 cells. This effect increased in a dose dependent manner with the number of applied fibroblasts. It was reflected in:

(1) an increased number of Ki67 positive MCF7 cells (**Fig. 4/A**),
(2) an increased total number of MCF7 cells (**Fig. 4/B**),
(3) an elevated ratio of Ki67 positive cells vs. total cell number (**Fig. 4/C**), and
(4) an increased MTT metabolism of the MCF7 cell population (**Fig. 5**).

For instance, as compared to single culture conditions, the co-cultivation of MCF7 cells with 25,000 fibroblasts yielded a 2.2-fold higher final cell number and a 1.2-fold larger fraction of proliferative cells (**Fig. 4**).

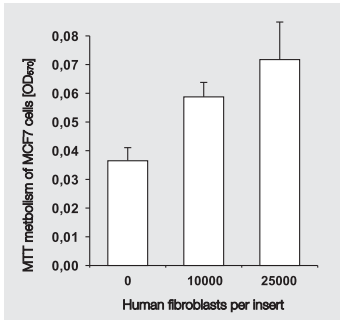

Figure 5
MTT metabolism of MCF7 cells under single culture and co-culture conditions. Averages and standard errors are shown.

All parameters were assessed after two days in co-culture with identical original seeding densities of 5,000 MCF7 cells per insert.

The co-culture model established here proved to be a helpful tool for studying the paracrine interaction of different cell populations in vitro. The use of small pores (0.4 µm) guarantees that primarily paracrine signaling and not direct cell-cell contact accounts for the observed effects. Many variations of this model are conceivable, such as the application of larger pores (3.0 µm) that may allow direct cell-cell contact and therefore additional interaction to occur between the co-cultivated cell populations.

References

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