Endothelial and epithelial differentiation potential of human adult stem cells PSC and MSC and the relation of ZO-1 isoform ratio to epithelial differentiation in Caco-2

Dissertation
zur Erlangung des Grades
des Doktors der Naturwissenschaften
der Naturwissenschaftlich-Technischen Fakultät III
Chemie, Pharmazie, Bio- und Werkstoffwissenschaften
der Universität des Saarlandes

von
Katharina Meier

Leverkusen
2013

Tag des Kolloquiums:	13. Januar 2014
Dekan:	Prof. Dr. Volkhard Helms
Mitglieder des Prüfungsausschusses:	
Vorsitzende:	Prof. Dr. Alexandra Kiemer
1. Gutachter:	Prof. Dr. Claus-Michael Lehr
2. Gutachter:	Prof. Dr. Ingolf Bernhardt
Akademischer Mitarbeiter:	Dr. Stefan Boettcher

CONTENTS

1 ABSTRACT

First part of this thesis dealt with the potential of human adult stem cells PSC and MSC to differentiate in epithelial- and endothelial-like cell types. Before starting differentiation experiments, PSC as a new source of stem cells, were analyzed for their endogenous expression profile of endothelial, epithelial and stem cell markers. Changes in expression profile induced by various matrices, changes in morphology during air-interface culture and generation of organoid bodies were also determined. For provoking endothelial differentiation, stem cells were incubated on different surfaces and with various combinations of bFGF and VEGF. Both cell types showed already an immanent endothelial potential, which was not enhanced by the applied protocols. Epithelial differentiation was initiated by coculture with the epithelial cell lines A549 and Calu-3 in either direct or indirect contact or in a combination. Not only type of coculture but also choice of epithelial cell type had a partially contrary influence on the expression of CDH-1.

Second part of this thesis monitored changes in ZO-1 isoform ratio in Caco-2, T84, Calu-3, A549 and PSC. There existed no correlation to proliferation, cell number and density, TEER or expression of occludin. However, for the first time it was observed that ZO-1 isoform ratio was not constant but varied in nearly all cell types. In Caco-2 there was even a complete turn-over observed, which correlates with proceeding functional differentiation.

2 KURZZUSAMMENFASSUNG

Im ersten Teil der vorliegenden Arbeit wurden endotheliales und epitheliales Differenzierungspotential der humanen adulten Stammzellen PSC und MSC untersucht. Da es sich bei PSC um eine sehr neue Stammzellquelle handelte, wurden diese zunächst genau hinsichtlich ihrer bereits innewohnenden endothelialen, epithelialen und auch Stammzellmerkmale charakterisiert. Um eine endotheliale Differenzierung zu erreichen wurden die Stammzellen auf verschiedenen Oberflächen mit unterschiedlichen Kombinationen von bFGF und VEGF inkubiert. Keines der angewendeten Protokolle war in der Lage eine weitergehende Expression endothelialer Marker zu bewirken. Für die epitheliale Differenzierung wurden die Stammzellen mit den Lungenzelllinien A549 und Calu-3 cokultiviert. Dies geschah entweder in direkter oder indirekter Cokultur oder in einer Kombination. Nicht nur der verwendete epitheliale Zelltyp sondern auch die Art der Cokultur hatte einen teilweise gegensätzlichen Einfluss auf den Marker CDH-1.

Im zweiten Teil wurden Änderungen im ZO-1-Isoformverhältnis in Caco-2, T84, Calu-3, A549 und PSC beobachtet. Das bisher als fix postulierte Verhältnis variierte über die Kulturdauer in fast allen Zellarten. Eine Korrelation zu Proliferation, Zellzahl und -dichte, TEER oder Occludinexpression gab es nicht. Bei Caco-2 jedoch wurde sogar eine komplette Umkehr des Verhältnisses beobachtet, die einher ging mit der voranschreitenden funktionalen Differenzierung.

3 ABBREVIATIONS

AIF	Air Interface Culture
AJ	Adherens Junctions
APN	Aminopeptidase
bFGF	Basic Fibroblastic Growth Factor
BrdU	5'-Bromo-2'-deoxy-uridine
BSA	Bovine Serum Albumine
C I	Collagen I
C IV	Collagen IV
CD	Cluster of Differentiation
CDH 1	Cadherin 1
COPD	Chronic Obstructive Pulmonary Disease
DM	Differentiation Medium
EB	Embroid Bodies
ECM	Extracellular Matrix
(h)ESC	(human) Embryonic Stem Cell
ECGM	Endothelial Cell Growth Medium 2
EMT	Epithelial to Mesenchymal Transitions
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FN	Fibronectin
hAEpC	Human Alveolar Epithelial Cells
HUVEC	Human Umbilical Vein Endothelial Cells
ICM	Inner Cell Mass
iPS	Induced Pluripotent Stem Cells
LN	Laminin
MSC	Mesenchymal Stem Cell
OB	Organoid Body
PBS	Phosphate Buffered Saline
(RT-) PCR	(Reverse Transcriptase-) Polymer Chain Reaction
PDL	Poly-D-Lysine
PET	Polyethylene Therephtalate
PM	Primary Medium
PSC	Pancreatic Stem Cells
RFI	Relative Fluorescence Intensity
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
TCP	Normal Tissue Culture Plastic
TEER	Transepithelial Electrical Resistance
TJ	Tight Junction
VCAM 1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF Receptor
vWF	von Willebrand Factor
ZO	Zona Occludens

4 MOTIVATION

Differentiation in biology is defined as a process by which a less specialized cell undergoes a change to a more specialized cell type. This process dramatically changes a cell's size, shape, membrane potential, metabolic activity, and responsiveness to signals. These changes are largely due to highly controlled modifications in gene expression.

Differentiation is pivotal during ontogenesis, when the zygote becomes an embryo with many specialised cell and tissue types. But also in the adult organism differentiation takes place in many different cell types. First of all there is the differentiation of adult stem cells. They serve as the body's own maintenance and repair system. They divide and create daughter cells for use in normal cell turn over and tissue damage. Moreover, the term differentiation comprises the further functional specialisation of already dedicated somatic cells. This thesis was interested in both aspects.

Directed differentiation of adult stem cells is of great interest for medical and pharmaceutical research. Stem cells from a patient could generate cells, tissues and organs for later transplantation without risk of rejection. Furthermore, autologous stem cells could differentiate into target cells for individual drug response studies *in vitro*. Primary endothelial cells can not be harvested and expanded in sufficient amounts to serve this purposes. Thus, ndothelial cells generated by stem cells could be used for covering vascular prostheses and for repairing injured vessels and ischemic tissues. Lung epithelial cells produced from stem cells are of interest for *in vitro* transport studies and cytotoxicity testing. So far established cell line models of the lung epithelium are not optimal.

For these purposes human adult stem cells have to be identified, which are able to differentiate reproducibly into the desired lineages. PSC and MSC are two such possible sources with auspicious properties. In 2004 PSC were described for the first time and had a promising start as pluripotent stem cells. They showed their potential by spontaneously differentiating into cell types from all three germ layers [1]. MSC on the other hand are already established in clinical therapies, where they are so far the sole approved source of stem cells. They have shown to differentiate into multiple lineages and also pluripotency has been reported [2]. Hitherto there have been no established methods to differentiate any stem cell type into endothelial or lung-epithelial lineage. Development of adequate approaches was therefore aim of this thesis. Additionally, a purposeful characterization of PSC had to be performed, since no evaluation of immanent endothelial and epithelial properties of these stem cells had been carried out before.

The human colon carcinoma cell line Caco-2 exhibits the particular ability to undergo spontaneous differentiation to small intestinal enterocyte-like cells. Due to extensive morphological and biochemical analogy Caco-2 are widely used as a small intestinal model in toxicity testing and transport studies. Functional differentiation of Caco-2 is usually monitored by increasing activity of brush border enzymes. During preceding experiments of our cooperation partner, the Department of Biochemistry at the University of Pavia, Italy, it was discovered that the amount of ZO-1 isoforms varied considerably during differentiation of the somatic cell line Caco-2. ZO-1 is a protein found mainly in tight junctions of epithelium and endothelium. It combines a structural role as a cell adhesion protein with a regulatory role by modulating cell proliferation. ZO-1 is expressed in two isoforms, α^+ and α^-, whose function could not be clarified yet. Ratio of the two isoforms was so far considered constant and thus characteristic for a certain cell type. A shift in expression of the two ZO-1 isoforms has not been reported before, neither for Caco-2 nor for any other cell type. Aim of this study was, in cooperation with Pavia, to address the question whether this observation was related to any other parameters. Therefore, expression of ZO-1 isoforms was monitored in parallel to cell number, cell density, proliferating activity, TEER value and expression of occludin, a further tight junction protein and to functional differentiation of Caco-2. For determining whether the change in isoform expression was peculiar for Caco-2 6 cell types were investigated: T84 as a further model of an intestinal cell line, A549 and Calu-3 as a lung epithelial model, ECV304 representing endothelium and PSC as primary stem cells.

5 PSC AND MSC - TWO SOURCES OF HUMAN ADULT STEM CELLS AND THEIR EPITHELIAL AND ENDOTHELIAL POTENTIAL

Parts of this section have been published in:

Meier, K., C.M. Lehr, and N. Daum, Differentiation potential of human pancreatic stem cells for epithelial- and endothelial-like cell types. Ann Anat, 2009. 191(1): p. 70-82 [3]

5.1 INTRODUCTION

5.1.1 GENERAL TERMS AND DEFINITIONS

The term “Stem Cell” has been coined for the first time in 1908 by the Russian histologist Alexander Maksimov. At a congress of the Hematologic Society in Berlin he postulated the existence of stem cells on which he built his model of hematopoiesis. The usage of this term lay nearly idle until the 1960s, when the two Canadian researchers McCulloch and Till found self renewing cells in mouse bone marrow [4, 5]. Roughly 20 years later, in 1981 simultaneously two groups of researchers one in San Francisco, USA, around Gail Martin, and one in Cambridge, UK, around Martin Evans and Matthew Kaufmann, discovered techniques for extracting stem cells from mouse blastocysts [6, 7]. And it was Gail Martin to whom the term “Embryonic Stem Cell” was attributed. Thus, the existence of the two broad types of mammalian stem cells has been asserted: adult and embryonic stem cells (for other types of stem cells see Chapter 5.1.4)

Adult stem cells are present in tissues all over the body throughout the whole lifetime of an organism. Embryonic stem cells (ESC), however, are defined by their origin and are therefore only isolated from the inner cell mass of blastocysts. Apart from their origin both types of stem cells differ also considerably in their abilities and their applications. However, both of them display the combination of two properties, which accounts for the definition of a stem cell: self renewal and potency.

Long-term self renewal is the ability to go through numerous cycles of cell division (self replication) while maintaining an undifferentiated state. Stem cells undergo two different types of cell division to ensure this property (Figure 1). A symmetric division yields in two identical daughter cells, which both display stem cell properties. An asymmetric cell division

produces two different daughter cells. One of them is a stem cell and the other a progenitor cell. These cells have a limited self-renewing potential and undergo several rounds of symmetric cell division until they finally differentiate into one defined mature cell type.

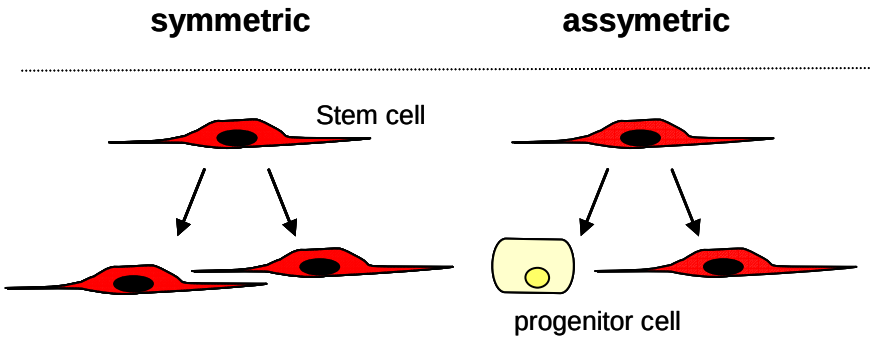

Figure 1: Symmetric and asymmetric division of stem cells.

The ability to give rise to specialized cell types is called potency. Depending on the kind and the number of different mature cell types a stem cell can differentiate into, certain definitions of potency have been established.

Totipotent or omnipotent cells can give rise to embryonic and extraembryonic cell types and can therefore build up a whole, viable organism. Only cells from the first few divisions of a fertilized egg have this ability.

Pluripotent stem cells can give rise to cell types from all three germ layers and thereby construct nearly any tissue of the body. Embryonic and also few types of adult stem cells display this property.

Multipotent cells can differentiate into more than one cell type. The cells they give rise to are usually closely related and originate from the same germ layer. Haematopoietic stem cells are a typical representative for multipotent cells

Oligopotent cells (e.g. lymphoid stem cells) can differentiate into few cell types of a certain tissue.

Unipotent cells can give rise only to one cell type (e.g. fibroblasts).

Defining the different categories of potency also the term plasticity has to be elucidated. Plasticity (also called transdifferentiation) is the ability of tissue-specific stem cells to give rise to cell types of other tissues, even from other germ layers.

5.1.2 EMBRYONIC STEM CELLS

Embryonic stem cells (ESC) are – as their name suggests – derived from embryos and that at the stage of the blastocyst. In human development this stage is reached after 4 to 5 days after fertilization. The blastocyst then includes three structures. The trophoblast is a layer of cells which surrounds the hollow cavity of the blastocoel. The group of cells at one end of the blastocoel, that develops into the actual embryo, is the inner cell mass (ICM). ESC are isolated by transferring cells from the ICM to culture dishes where they are usually grown in the presence of bovine serum and on a feeder layer. This feeder layer consists of growth-arrested mouse fibroblasts, which provide ESC with essential growth factors. Without optimal culture conditions, ESC are prone to rapid differentiation. When they have the possibility to clump together, they easily form embroid bodies (EB) in which they begin to differentiate into cell types from tissues of all three germ layers, thereby demonstrating their pluripotency. As this process is spontaneous there is no possibility to control the way of differentiation. To yield mature specialized cells for application in research and medical treatment, protocols for a directed differentiation of ESC have been established. Amongst others ESC so far have been differentiated to neural [8, 9], cardiac [10, 11], endothelial [12], haematopoietic [13, 14], pancreatic [15, 16], bone [17] and hepatic cell types [18, 19].

Pluripotency and the nearly indefinite capability of self-renewal make ESC a promising tool for regenerative research and medicine. However, there are several arguments opposing the application of ESC.

First of all there is the ethical discussion, as harvesting ESC requires the destruction of a human embryo. Although this happens in the early state of the blastocyst, there is a fervid discussion about the beginning of life and also its sanctity. Stem cell laws all over the world vary significantly by country. In the USA no federal law has ever been adopted, which bans the research with hESC. However, several states prohibit the creation or destruction of a human embryo for medical research. Laws in the European Union vary from the allowance to destroy human embryos for clinical purposes (UK) to the exclusive application of imported stem cell lines (Germany).

Even more drawbacks apply, when ESC or ESC-derived cells are to be injected or transplanted into a human body. As cells and tissues derived from ESC are “foreign” for the host body, the immune system of the host will inevitably try to reject the transplant. Although thorough choice of donor cells can reduce the reaction of the host body, application of immunosuppressive drugs will still be required. Additionally triggered is the immune response by non-human molecules, so called xeno-molecules, which adhere to the implanted

cells. These molecules come in contact with ESC during their isolation, expansion and differentiation, when ESC are in general cultured with mouse feeder-layers and with animal sera in the growth medium. However, during the last years some protocols have been described which allow the cultivation of hESC either without animal serum [20] or without a feeder layer [21]. These protocols may after combination and establishment eliminate the risk coming from contamination with xeno-molecules.

Furthermore to mention is potential cancerogenity of ESC. When ESC are injected or implanted, they are prone to form teratocarcinomas in mice [20, 21]. This is due to a non-complete differentiation into mature cells. In fact there are no protocols, which lead to more than 80% differentiated cells. However, just one undifferentiated cell may after application lead to a cancerous tumour in the host body.

Genetic instability and chromosomal abnormalities after prolonged culture hold further limitations for the usage of hESC.

Due to all these limitations and risks, and despite all the hoped and hyped potential of hESC, there are no approved treatments which utilize ESC, yet. However, one step into this direction has been carried out in January 2009, when the world's first hESC trial was approved by the FDA (United States Food and Drug Administration). This trial now investigates the transplantation of hESC-derived cell populations into spinal cord-injured humans.

5.1.3 ADULT STEM CELLS

Adult stem cells (also called somatic stem cells) are undifferentiated cells, found among differentiated cells in a tissue or organ after embryonic development. Under certain physiologic - or experimental conditions - the can renew themselves and can differentiate to yield some or all of the specialized cell types of the corresponding tissue. They are found in nearly all tissues and also throughout the whole lifetime of an organism. Their primary tasks in organisms are maintenance and repair of their tissue of origin. In some organs, such as gut or bone marrow, stem cells regularly divide to repair or replace worn out or damaged tissue. In other organs, like pancreas or heart, stem cells divide only under special conditions.

The most common source of adult stem cells is the bone marrow, where at least three different types can be distinguished: haematopoietic – forming blood cells- , endothelial – forming the vascular systems- and mesenchymal stem cells –forming bone, cartilage, muscle, fat and fibroblasts. Furthermore stem cells are found in the pancreas [1], the liver [22], the gut [23], somatic muscles [24], the heart [25], the skin [26], in neuronal tissue (brain, olfactory

bulb, spinal cord) [27] and fat tissue [28]. Also findings of corneal [29], mammary [30], salivary [31], dental pulp [32] and thymic [33] stem or progenitor cells have been reported.

Although adult stem cells are found throughout the body, there is only a very small number of stem cells in each tissue. And once removed from the body, their capacity to divide is no longer unlimited, making it essential to develop protocols for growing larger quantities in laboratory.

Corresponding to their natural task most adult stem cells are multi- or oligopotent. Either regularly or due to a trigger -like a damaged tissue- they divide and differentiate into cell types from the tissue they reside in. However, more and more reports are showing that there are adult stem cells, which can cross the lineage-boundaries and differentiate into cell types from another tissue or even germ layer [34-36].

Although the potential of adult stem cells seems to need a bit more help than that of ESC, there are numerous advantages they have over ESC. First of all, there is no ethical concern about their application. Adult stem cells are found throughout the whole human body and no life is harmed by their extraction. Furthermore, their cancerogenity is considered very low compared to ESC. They also offer the unique option of a treatment individually designed for each patient. Individual drug response could be monitored *in vitro* on target cells, which have been generated from the patients own stem cells. Also cells, tissues or organs which have been produced by autogenic stem cells, could be transplanted for replacing their damaged predecessor. Thereby, the risk of a transplant rejection is minimized.

Therapies with adult stem cells have been used for more than 4 decades now. Since the late 1960s bone marrow transplants are applicated for the successful treatment of leukaemia and other bone/blood related cancers.

5.1.4 OTHER TYPES OF STEM CELLS

So the main two types of stem cells have been described: embryonic and adult stem cells. However, there are at least two more sources of stem cells, which are known yet.

Fetal stem cells are derived from the fetus itself or from extra-embryonic structures, which are also of fetal origin. This structures comprise umbilical cord blood, amniotic fluid, Wharton`s jelly, the amniotic membrane and the placenta. Classification of these cells is not finished yet. Based on proliferation rates and plasticity features they seem to be intermediates between embryonic and adult stem cells. And also transcriptomic and proteomic analysis indicate the same conclusion [37, 38].

Another option for the production of stem cells is dedifferentiation. This is a natural phenomenon observed in plants, worms and amphibians, where mature somatic cells loose their properties to reach a state which is comparable to that of stem or progenitor cells. In mammalians, however, this phenomenon does not occur naturally. By genetically reprogramming adult somatic cells, though, it is possible to produce cells of an embryonic-like type. These cells are called induced pluripotent stem cells (iPS). Mouse iPS were first reported in 2006 [39] and human iPSC one year later [40, 41] in late 2007. For producing iPS somatic cells (mostly fibroblasts) are transfected with typical ESC-associated genes. These genes comprise at least Oct-3/4 and Sox2, although transfection of additional factors can enhance the induction efficiency. iPS demonstrate important characteristics of ESC as expression of certain stem cell markers, a similar proliferation rate, embryoid body and also teratoma formation, pluripotency and plasticity. So far transfection has mostly been achieved by viral vectors and therefore cells produced in this way bear the increased potential risk of cancerogenity after implantation. However, in 2009 a protocol was established which renders viruses unnecessary as proteins are directly delivered by means of cell penetrating peptides [42].

5.1.5 APPLIED STEM CELLS

5.1.5.1 PSC

Pancreatic Stem Cells (PSC) are isolated from the exocrine acinary parts of the human pancreas. They were described for the first time in 2004 [1] and raised attention due to their spontaneous pluripotency *in vitro,* which is scarcely found in adult stem cells.
A detailed description of these cells are found in Chapter 5.3.1 and 5.3.3.

5.1.5.2 MSC

Mesenchymal stem cells (MSC) are one of the three groups of adult stem cells, which are found in bone marrow (5.1.3) [43]. Alternatively, they can be isolated from peripheral blood [44], from lung [45], heart [46] or adipose tissue [47]. MSC have a very high capacity for self-renewal, while remaining in an undifferentiated state. Minimal criteria for the identification of MSC are the positive expression of CD 44, CD 73, CD 90 and CD 105 and negative expression of CD 45, CD 34, CD 14 or CD 11b, CD 79a or CD 19 and HLA-DR antigens [48]. Under standard culture conditions they adhere to the surface and they show multipotency by differentiating *in vitro* into adipocytes, chondrocytes and osteoblasts [49]. *In vivo* they are even able to generate endodermal cells like hepatic, intestinal, pancreatic and

lung epithelium [50, 51]. More recent studies suggest even an *in vitro* multi-lineage differentiation and therefore pluripotency of MSC [52].

Apart from their potency MSC are of great interest in research as they exhibit profound immunomodulative abilities *in vitro* and *in vivo* [53]. Firstly, immunosupression is shown by reducing functions of T-, B- and NK-cells comprising proliferation, production of cytokines and cellular cytotoxicity [54]. One apparent effect is the amelioration of acute graft-versus-host disease after administration of MSC [55]. Secondly, MSC have the so called immunoprivilege. This means that autologous and allogeneic MSC are protected from immunological defence mechanisms and are not rejected by the immune system [56]. Furthermore, their conditioned medium has shown to reduce stress induced by cigarette smoke [57] and to inhibit proliferation of lung cancer cell lines [58].

By far, MSC are the adult stem cells most widely used in research all over the world. Several experimental studies have already shown the potential of MSC in the regeneration of infarcted heart muscle by injection of these cells near the infarcted site, and in the treatment of musculo-skeletal disorders, repairing large bone and cartilage defects [59]. Other clinical applications currently being investigated are osteogenesis imperfecta, Alzheimer's disease, spinal cord injury, HIV, liver cirrhosis, aplastic anemia and ALS [60-63].

5.1.6 AIMS AND EXPERIMENTAL OVERVIEW

Directed differentiation is the crucial point when large numbers of differentiated cells are to be generated from stem cells. This is necessary e.g. for usage in *in vitro* screening of pharmaceutical substances or for transplantation in humans. This study was designed to investigate the potential of the human adult stem cells PSC and MSC to give rise to epithelial- and endothelial-like cells.

MSC are known since the 1960s and have been thoroughly characterized (even though not all encompassing) since then. On the contrary PSC were only just discovered to the beginning of this thesis. A systematic characterization regarding their immanent endothelial, epithelial and stem cell properties was therefore one major aim of this thesis. Also the influence of different extracellular matrices (ECM) on expression of the analyzed markers was of interest. Furthermore, the influence of air-interface culture and a method to produce spheroids by means of centrifugation were investigated.

Focus of differentiation approaches were endothelial and epithelial lineages. Especially the lung is target of many drug therapies. Not only topical drugs for asthma, bronchitis, COPD (chronic obstructive pulmonary disease) and other lung diseases are applied but also

inhalational anaesthetics and nicotine for smoking cessation. Individually adapted screening of pharmaceutical agents by means of differentiated autologous stem cells could therefore be good for optimizing drug response rate. Stem cell generated endothelial cells may serve the same purpose for e.g. blood pressure and anti-inflammatory relevant drugs. Furthermore, they may vascularise engineered tissue before and/or after transplantation as primary endothelial cells already did [64]. Detailed information to endothelial end epithelial cell types can be found in Chapter 5.4 and 5.5, respectively.

For endothelial differentiation growth factors VEGF and bFGF were applied as well as starving of stem cells to increase responsiveness to these factors 5.4). To achieve epithelial differentiation of PSC and MSC conditioned medium of the lung epithelial cells A549 and Calu-3 were used and additionally stem cells were cultured upon these fixed epithelial cells (see Chapter 5.5).

5.2 MATERIAL AND METHODS

5.2.1 ROUTINE CELL CULTURE AND BASIC CELL CULTURE DURING EXPERIMENTS

For characterizing and furthermore investigating cells of any kind, the cells need to be subjected to a routine cell culture. This culture has to stick to a strict protocol, for ensuring the data received being due to the experimental set-up and not to changes in the routine cell culture. Naturally, the protocols differ from cell type to cell type to meet the particular needs of each cell.

Cells applied in this study were the stem cells PSC (pancreatic stem cells) and MSC (mesenchymal stem cells), the lung epithelial stem cells A549 and Calu-3, the primary lung epithelial cells hAEpC, the endothelial cells HUVEC (human umbilical vein endothelial cells) and also the colon-derived Caco-2.

The adult human stem cells PSC were a generous gift from Fraunhofer Institut St. Ingbert, Germany. Cell isolation was described by Kruse et al. in 2004 [1]. All experiments were performed with cells from passages 13 to 23 from the clone Cepan 3b. PSC were maintained in a primary medium (PM) consisting of Dulbecco's modified Eagle's medium - High Glucose with L-Glutamin (DMEM, GIBCO Invitrogen; Karlsruhe, Germany) supplemented with 10 % fetal bovine serum gold (FBS GOLD), 100 units/ml penicillin and 100 µg/ml streptomycin (all from PAA; Pasching, Austria). PSC were seeded at a density of 5 x 10^5 in T-75 cell culture flasks and routinely subcultured at 80 to 90 % confluence.

Also the human MSC were a gift from Fraunhofer Institut St. Ingbert, Germany, which isolated the cells from the bone marrow of a 60 year old male Caucasian donor according to a protocol described in 2002 [65]. Before passing the cells on, MSC were routinely tested for positive expression of CD 29, CD 44, CD 73, CD 105 and CD 166 and for chondrogeneic and osteogeneic differentiation. All experiments were performed with cells from passages 7 to 15. MSC were maintained in alpha-minimum essential medium with stabile L-Glutamin and without ribonucleosides and deoxynucleosides (alpha-MEM; Pan-Biotech, Aidenbach, Germany) supplemented with 15% fetal bovine serum (FBS, also Pan-Biotech) and 100 units/ml penicillin and 100 µg/ml streptomycin (PAA). MSC were seeded at a density of 5 x 10^5 in T-75 cell culture flasks and routinely subcultured at 80 to 90 % confluence.

A549 (ATCC-No: CCL-185) were maintained in RPMI-1640 - High Glucose with L-Glutamin (RPMI, GIBCO) supplemented with 10 % FBS (PAA). Subculture ratio was 1:50.

Calu-3 (ATCC-No: HTB-55) were maintained in RPMI-1640 - High Glucose with L-Glutamin and without phenolred (PAA) supplemented with 10 % FBS GOLD and 1 mM

sodium pyruvate (Lonzy, Basel, Switzerland). They were subcultured in a ratio of either 1:5 or 1:10.

HUVEC (Promocell, Heidelberg, Germany) were maintained in Endothelial Growth Medium II (ECGM, Promocell) and subcultured in a ratio of 1:5 once to twice a week (in contrast to all other cells).

Caco-2 were maintained in RPMI-1640 – High Glucose with L-Glutamin (RPMI, GIBCO) and supplemented with 10 % FBS (PAA). They were subcultured in a ratio of 1:10.

hAEpC were isolated and cultured as described by Daum [66].

All cells were routinely incubated at 37°C in 5 % CO_2 and regularly tested for mycoplasm infections. Subculturing was performed once (HUVEC once to twice) and medium was changed three times a week.

During all experiments the medium was replaced also three times a week. For MSC the underlying medium during experiments was the same as described for routine culture. For PSC the experimental medium was a particularly composed differentiation medium (DM). This medium consisted of DMEM supplemented with 20 % FBS GOLD, 2 mM L-Glutamine (Cambrex; Verviers, Belgium), 100 units/ml penicillin, 100 µg/ml streptomycin, 10 µl/ml amino acids non essential (all from PAA) and 0.0007 % β-mercapto ethanol (Sigma, Schnelldorf, Germany). Standard substrate was normal tissue culture plastic (TCP). This information is true as long as not otherwise stated.

5.2.2 CULTURE ON VARIOUS EXTRACELLULAR MATRICES

The development of stem cells is not only influenced by soluble molecules in the medium but also by the substrates they are grown on. This has been shown for various extracellular matrices (ECM) in the past [67, 68].

Therefore, PSC were exposed to the influence of five different matrices: collagen I (C I), collagen IV (C IV), fibronectin (FN), laminin (LN) and poly-D-lysine (PDL) (all Biocoat Cellware, BDBiosciences, Heidelberg, Germany). PSC were seeded in a density of 25,000 cells/cm^2 and were left to grow for 21 days in PM, before they were harvested for further investigations. TCP served as control. All experiments were performed twice (n=2).

5.2.3 GENERATION AND ANALYSIS OF ORGANOID BODIES

Three methods are known in literature for generating spheroids, in which spontaneous stem cell differentiation can occur: centrifugation of cell suspension [69-71], hanging drop culture [1, 72] and continuous spinning or shaking of a cell suspension [73, 74] (Figure 6).

For PSC the hanging drop method is already established [1]. In order to receive bigger spheroids a centrifugation protocol was established. A suspension of 250,000 PSC in 5 ml

was centrifuged for 5 min at 500 g in a 15 ml Falcon tube (BD). Afterwards the supernatant was replaced by fresh medium. Overnight the cell pellet rounded up and was detached from the surface by gently tilting the tube. Medium was replaced every 2 days and spheroids were left to grow until day 23. Samples were histologically investigated every couple of days.

5.2.4 AIR INTERFACE CULTURE

Unilateral access to medium can influence the development of a certain cell type [75]. To achieve such environment, PSC were exposed to an Air Interface Culture (AIF).

For creating this AIF culture 12-well Transwell®s with permeable filter inserts (type 3460, pore size 0.4 µm, 1.13 cm^2, Corning, Wiesbaden, Germany) were applied (Figure 2a). These devices allow a separation of a lower and upper compartment by a water - and electrolyte - permeable membrane (Figure 2b). PSC were seeded in a density of 50,000 cells per well in the upper compartment. The upper compartment was filled with a total volume of 0.5 ml and the lower with 1.5 ml of medium, resulting in a balanced hydrostatic pressure on both sides of the membrane. At day 2 medium of the upper compartment was removed. Volume of medium of the lower compartment was then reduced to 1.2 ml. This was necessary, since medium otherwise would have infiltrated upper compartment through the microporous membrane and could thereby influence adherence of cells on membrane. At day 7 first part of cells were subdued to histological preparation. Remaining cells were allowed to grow until day 21.

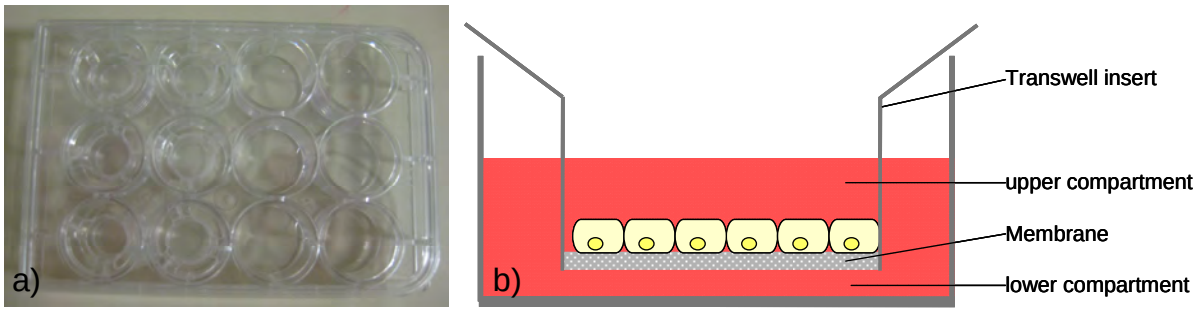

Figure 2: Picture (a) and schematic drawing (b) of a 12 well Transwell®.

5.2.5 HISTOLOGICAL PREPARATION OF ORGANOID BODIES AND AIF CULTURE

Histological preparation was performed as follows: OBs or cells adhering to filter membrane of Transwell®, respectively, were fixed in a 4 % formaldehyde solution at RT for 30 min. Samples were then incubated at RT subsequently with ethanol 70 %, 96 % and 100 % for dehydration and stored temporarily in xylene. For mounting samples were covered with paraffin wax overnight at 60°C and embedded next morning. Samples were cut with a

microtome (Leica Microsystems, Nussloch, Germany) in 4 µm thick sections and subsequently dewaxed with xylene. Rehydration was performed by subsequent incubation for 10 min at a time with ethanol 100 %, 96 % and 70 % at RT and following rinsing with deionised water. Slices were stained with 3 % solution of Alcian Blu (Sigma-Aldrich) for 30 min at RT, rinsed with deionised water and counterstained with fast nuclear red 29 (Sigma-Aldrich) for 5 min at RT. Samples were then again dehydrated by the above mentioned ethanol series and stored in xylene. Sections were mounted with coverslips by using Roti-Histokitt (Roth, Karlsruhe, Germany) and subsequently examined with an Axiovert XY light microscope (Carl Zeiss, Jena, Germany) at 400x magnification.

5.2.6 PROTOCOLS FOR ENDOTHELIAL DIFFERENTIATION

Literature holds different protocols for endothelial differentiation of stem cells ready. These protocols employ mostly the growth factors VEGF (vascular endothelial growth factor) and bFGF (basic fibroblastic growth factor) in various concentrations and orders. Also special media are hold for sale, which are designed for forcing endothelial differentiation, i.e. endothelial growth medium 2 (ECGM; PromoCell, Heidelberg, Germany). Furthermore, culture on FN is reported to enhance endothelial differentiation [76]. Also “starving” of stem cells, by culturing them for a short time in medium containing only little amounts of FBS, is known, to make these cells prone for subsequently added growth factors.

This study applied three basic setups for inducing endothelial differentiation in PSC and MSC (for a schematic overview see Figure 14 in Chapter 5.4.1.2).

For the first approach PSC were seeded at a density of 6,000 and MSC at 3,000 cells/cm^2 in their respective routine culture medium. After three days the medium was replaced with the experimental medium containing VEGF (Chemicon, Hofheim, Germany) in concentrations of (I) 10, (II) 20 and (III) 50 ng/ml, while plain medium served as control. Cells were analyzed after 3, 7 and 14 days (n=2).

For the second setup PSC were seeded at a density of 15,000 and MSC at 7,500 cells/cm^2 either on normal TCP or on FN-coated dishes. The medium consisted either of (I) pure experimental medium or of (II) medium supplemented with 20 ng/ml bFGF (Invitrogen, Karlsruhe, Germany) and 50 ng/ml VEGF or of (III) medium supplemented during the first 7 days with bFGF and for the subsequent 14 days with VEGF. Furthermore, stem cells were cultured in (IV) ECGM 2. The growth factor cocktail in this medium contains 1 µg/ml Ascorbic Acid, 10 ng/ml bFGF, 5 ng/ml epidermal Growth Factor, 22.5 µg/ml Heparin, 0.2 µg/ml Hydrocortisone, 20 ng/ml Insulin-like Growth Factor and VEGF 0.5 µg/ml. All cells were analyzed after 21 days (n=2).

For the third approach PSC were seeded at a half-confluent density of 15,000 and MSC at 7,500 cells/cm^2 in experimental medium containing 0.1 % FBS only. After 48 h the medium was replaced by some which contained 20 % FBS instead. This medium was either pure or supplemented with (I) 50 ng/ml VEGF or with (II) 50 ng/ml VEGF and 20 ng/ml bFGF. Cells were analyzed after 12 days of cultivation (n=2).

5.2.7 *IN VITRO* ANGIOGENESIS

Beside determination of mRNA (Chapter 5.2.9) and protein expression (5.2.11, 5.2.12 and 5.2.13) differentiation of stem cells can be determined by their ”behavior”. For instance, the formation of capillaries in a semisolid medium is considered as a proof for endothelial potential [77].

Therefore, the ability of stem cells to develop such tubes was investigated by applying an *in vitro* angiogenesis assay kit (Chemicon) according to the manufacturer’s instruction. Summarily, a volume of 50 µl of ECMatrix™ solution was filled into one well of a 96-well plate and incubated overnight at 37°C. Prior, PSC and MSC had been incubated with medium containing 50 ng/ml VEGF or with 50 ng/ml VEGF and 20 ng/ml bFGF for 7 days. After trypsinization 10,000 cells were suspended in 50 µl of the corresponding medium onto the solidified ECMatrix™. Stem cells in their routine culture medium (PSC in PM and MSC in MEM) served as controls. Also Caco-2 were applied as a negative control. Cells were incubated on the matrix for up to 8 h, while they were microscopically investigated every two hours (n=3).

5.2.8 PROTOCOLS FOR EPITHELIAL DIFFERENTIATION

Unlike endothelial differentiation there are nearly no established protocols for differentiating stem cells into epithelial-like cells found in literature. Therefore, other promising approaches were applied, which involved the presence of epithelial cells during the differentiation process of stem cells. A549 and Calu-3 were the cells of choice. Stem cells were either incubated with conditioned medium of these cells, cultured on fixed cells or exposed to both stimuli (for schematic overview see Figure 20 in Chapter 5.5.1.2).

As described earlier (5.2.1) during routine cell culture medium of all cells was changed three times, also that of A549 and Calu-3. For receiving conditioned medium, this used medium was not discarded but sterile-filtered and diluted in a ratio of 1:2 with the experimental stem cell medium. Medium consisting of 50% unused epithelial medium and 50% experimental stem cell medium served as control and was named unconditioned medium. Stem cells were then cultured for 21 days with the respective medium (n=4).

For the second approach epithelial cells were seeded in 6-well plates and allowed to grow (A549 at 20,000 cells/cm^2 for 2 days and Calu-3 at 40,000 cells/cm^2 for 4 days). Subsequently, they were washed with sterile PBS (phosphate buffered saline: 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4 x 7 H_2O, 1.3 mM KH_2PO4) and fixed with 70 % isopropanol for 10 min at RT. Finally, fixed cells were washed twice with sterile PBS before stem cells were seeded on top (PSC at a density of 15,000 and MSC at 10,000 cells/$cm^{2)}$. Stem cells were cultured for 12 days either with unconditioned medium before being analyzed.

For the third approach direct and indirect coculture were combined.

5.2.9 REVERSE TRANSCRIPTASE- POLYMER CHAIN REACTION (RT-PCR)

For analyzing the gene expression state of control (A549; Calu-3, HUVEC) and stem cells (PSC, MSC) RT-PCR was applied.

Table 1: Specific primers used for RT-PCR (end: endothelial; ep: epithelial; sc: stem cell).

Primer name	RefSeq Ids	Primer sequences		Amplicon size [bp]	Character
CD°34	NM_001773	F: 5'-gcaagccaccagagctattc-3'	R:5'-aagcagggctgtgagacact-3'	292	end/sc
CD 45	NM_002838	F: 5'-gtacagacgcctcaccttcc-3'	R: 5'-ctgtgatggtggtgttggag-3'	262	sc
CD 71	NM_003234	F: 5'-gaactacaccgaccctcgtg-3'	R: 5'-caggctgaaccgggtatatg-3'	293	sc
CDH 1 (CD 324)	NM_004360	F: 5'-gctggagattaatccggaca-3'	R: 5'-acccacctctaaggccatct-3'	388	ep/sc
CDH 5 (CD 144)	NM_001795	F: 5'-ctgcacatctacggctacga-3'	R: 5'-tggagtttgctatcccaagg-3'	335	end
c-kit (CD 117)	NM_000222	F: 5'-cttgcatccaactccaggat-3'	R: 5'-gcagaattggagaagccttg-3'	234	sc
CTNβ1	NM_001904	F: 5'-tgcagttcgccttcactatg-3'	R: 5'-ctgcacaaacaatggaatgg-3'	355	ep
endoglin (CD 105)	NM_000118	F: 5'-cactagccaggtctcgaagg-3'	R: 5'-gtcattcagctcagcagcag-3'	358	end/sc
EpCAM (CD 326)	NM_002354	F: 5'-cgtcaatgccagtgtacttca-3'	R: 5'-gttcttctgaccccagcagt-3'	251	ep
GAPDH	NM_002046	F: 5'-gtcagtggtggacctgacct-3'	R: 5'-tgctgtagccaaattcgttg-3'	245	
K 7	NM_005556	F: 5'- caggaactcatgagcgtgaa-3'	R: 5'-gggtgggaatcttcttgtga-3'	346	ep
K 8	NM_002273	F: 5'- ccgacgagatcaacttcctc-3'	R: 5'-ggctctgcagctcctcatac-3'	222	ep
K 18	NM_199187	F: 5'- aaggcctacaagcccagatt-3'	R: 5'-gagctgctccatctgtaggg-3'	350	ep
K 19	NM_002276	F: 5'- tttgagacggaacaggctct-3'	R: 5'-aatccacctccacactgacc-3'	211	ep
PECAM 1 (CD 31)	NM_000442	F: 5'-gggagaagtgaccagagcaa-3'	R: 5'-tgagaggtggtgctgacatc-3'	331	end/sc
VCAM 1 (CD 106)	NM_001078	F: 5'-attgacttgcagcaccacag-3'	R: 5'-atctccagcctgtcaaatgg-3'	318	end
VEGFR 1 (FLT-1)	NM_002019	F: 5'-ttgattgaggagctgcactg-3'	R: 5'-ctgggccctcaaatgtagaa-3'	321	end
VEGFR 2 (CD 309, KDR)	NM_002253	F: 5'-tgaagatgggaaggatttgc-3'	R: 5'-agccagagctgcatcatttt-3'	361	end
vWF	NM_000552	F: 5'-tccgaggctgagtttgaagt-3'	R: 5'-cgggatcacaatgaccttct-3'	375	end
ZO-1	NM_003257	F: 5'-aaagggaaagcctcctgaag-3'	R: 5'-cggtttggtggtctgaaagt-3'	270	ep
ZO-1-iso	NM_175610	F: 5'-catagaatagactcccctgg-3'	R: 5'-gcttgaggactcgtatctgt-3'	234	ep
	NM_003257			474	ep

mRNA was isolated by use of RNeasy Mini Kit and cDNA was synthesized using Omniscript RT Kit. Specific PCR was carried out with Taq PCR Master Mix Kit (all by Qiagen, Hilden, Germany) in a final volume of 50 µl with 25 pmol of each nucleotide primer (see Table) and

with a total of 35 cycles. Temperatures applied during amplification were 95°C for denaturation, 52°C for annealing and 72°C for extension. Amplification of GAPDH served as internal control for sample loading. All amplifications were carried out in PTC-200 Peltier Thermal Cycler (MJ Research, GMI, Minnesota, USA) (Figure 3). 1 g of agarose Serva for PCR (Serva, Heidelberg, Germany) was suspended in 100 ml of TAE buffer (40 mMTris base, 20 mM acetic acid, 1 mM EDTA) and microwaved until agarose was completely dissolved. Ethidium bromide was added to a final concentration of 0.5 µm/ml and solution was poured into a gel tray with well comb. After solidification agarose gel was transferred to electrophoresis unit (Mini-sub Cell GT, BioRAD), where it was covered with TBE buffer (45 mM Tris-borate, 1 mM EDTA). All samples were topped off with loading buffer (Serva) and loaded into wells of the gel. First and last lane were loaded with molecular weight marker (either peqGOLD DNA Leiter-Mix 10 000 – 100 bp or peqGOLD 50bp DNA-Leiter 1 000 – 50 bp; PEQLAB Biotechnologie GmbH, Erlangen, Germany). Gels were run for 30 min at 100 mV and subsequently analyzed in UV light (GelDOC, BioRad).,

Figure 3: PCR thermal cycler by MJ Research.

5.2.10 DENSITOMETRIC ANALYSIS OF RT-PCR DATA

For a semi-quantitative analysis PCR gels were examined by creating a density plot profile in ImageJ (National Institute of Health, USA). All data were corrected for background by substracting values for an empty lane of the same gel. Densitometric intensity was defined by integration of the area under the peak corresponding to a band. The graph was designed by means of SigmaPlot 10 (Systat Software, Inc., Germany).

5.2.11 IMMUNOCYTOCHEMISTRY

Beside the analysis of the gene expression state by RT-PCR also the investigation of specific protein expression is pivotal when examining the developmental state of stem cells. In this thesis three methods were applied for visualizing the actual gene expression: immunocytochemistry, fluorescence activated cell sorting (5.2.12) and Westernblot (5.2.13).

For immunocytochemistry cells were incubated in routine cell culture medium on glass slides for 7 days. Cells were washed three times in PBS before they were fixed in 80 % methanol for 5 min at 4°C. Subsequently, they were incubated with primary monoclonal antibodies, these being mouse antibodies against CDH 1 (cadherin 1, Chemicon), endoglin (BD Biosciences, Heidelberg, Germany), VCAM 1 (Dianova, Hamburg, Germany), VEGFR 1 (R&D Systems, Wiesbaden, Germany), vWF (BD Biosciences) and the guinea pig antibody against panKeratin (Progen, Heidelberg, Germany) diluted in PBS / 1 % BSA overnight at 4°C. Subsequently, cells were again rinsed three times with PBS. Mouse antibodies endoglin, VCAM 1, VEGFR 1 and vWF were then incubated with the secondary goat anti-mouse antibody (FITC-conjugated; Dianova). At the same time the guinea pig antibody was incubated with Cy2-conjugated donkey anti-guinea pig (Dianova). Both secondary antibody solutions were supplemented with propidium iodide (500 µg/ml; Sigma, Schnelldorf, Germany) for nuclear counterstaining and cells were left at RT for 30 min in the dark. Agaian, glass slides were rinsed three times with PBS, mounted with Fluosave (CalBiochem, Gibbstown, NJ, USA) and masked with a coverslip.

Fluorescence imaging was performed using a BioRAD MRC-1024 confocal laser scanning microscope equipped with an argon-krypton laser The objective used was an oil immersion objective 40x NA-1.3.

5.2.12 FLUORESCENCE ACTIVATED CELL SORTING (FACS)

While immunocytochemistry primarily enables a qualitative and visual analysis of present proteins, FACS is a method, which allows for exact quantitative measurements. Hence, it was applied for characterizing of immanent characteristics of PSC.

After trypsinating 10^7 PSC were suspended in 1 ml buffer (PBS containing 2 %FBS) and incubated with the same antibodies described in 5.2.11 against endoglin, VCAM 1, VEGFR 1 and panKeratin. After 30 min in ice cells were washed twice with buffer and then incubated with secondary Cy-2 (for panKeratin) or FITC conjugated (for all other proteins) antibodies. After again 30 min on ice in the dark cells were washed in buffer and resuspended in 500 µl FACSFlow Sheath Fluid (Becton Dickinson, Erembodegem, Belgium). PSC were analyzed in channel FL 1 of a FACSCalibur (Figure 4) and by the use of CellQuest Pro software (both

Becton Dickinson). Samples which have been treated with secondary antibody only served as control for detecting unspecific antibody binding.

Figure 4: FacsCalibur® by Becton Dickinson.

5.2.13 WESTERNBLOT

Westernblot is a very established method for a visual and semi quantitative analysis of protein expression. It was applied to supplement immunocytochemistry and FACS. At first, proteins are separated by size by means of SDS- polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins are transferred by electric transfer, the so called blotting, to a nitrocellulose membrane. This membrane is subsequently treated with first and secondary antibody and eventually with a dye to visualize the corresponding proteins.

(Whole sample handling for Westernblot was performed on ice to inactivate proteases)

Sample preparation: for retrieving samples, adherent cells were rinsed twice with ice-cold PBS. PBS was then added and cells removed by means of a cell scraper. Cell suspension was centrifuged for 10 min at 4°C at 3000 rpm. Supernatant was discarded, pellet resuspended in PBS and again centrifuged. Supernatant was again discarded and pellet was stored at -80°C.

Protein was extracted by dissolving frozen pellet in RIPA-buffer (Radio-Immunoprecipitation Assay; 1 ml: 10 µl 1 M Tris pH 7.2, 300 µl 0.5 M NaCl, 100 µl 10 % SDS, 10 µl 10 % sodium deoxycholate, 50 µl 100mM EDTA, 520 µl H_2O, 1 µl 1 µg/µl aprotinin, 1 µl 1 µg/µl leupeptin, 1 µl 1 µg/µl pepstatin, 5 µl 100 mM vanadate, 5 µl 10 mM PEFA-bloc). Solution

was homogenized, centrifuged and supernatant was subjected to protein quantification by means of BCA-Kit (Bichinchonic Acid Kit; Sigma). Samples were incubated with a mixture of solutions A and B of the kit for 30 min at 37°C before absorbance at 550 nm (Infinite 200 M multimode microplate reader; Tecan, Crailsheim, Germany).

was determined. Calibration was performed with a curve of BSA-protein solutions.

Sample volumes corresponding to 30 µg protein were mixed with the same volume of double concentrated reducing buffer ("Lämmli"-buffer: 0.12 M pH 6.8 Tris-HCl, 8 % SDS, 20 % glycerine, 10 % β-mercapto ethanol, 0.1 % bromphenolblue). Samples were heated for 5 min at 95°C and and then topped up to 30 µl with normal concentrated reducing buffer.

Electrophoresis: electrophoresis chamber (Mini Protean II, BioRad, Munich, Germany) was filled with a 10 % separation gel (10 ml contain 0.75 ml glycerol (50 % V/V), 3.42 ml water, 2.5 ml lower-buffer (1.5 M Tris-HCl pH 8.8, 0.4 % SDS (sodium dodecyl sulfate)), 3,33 ml acrylamide (T40 %-C3.7%), 50 µl APS (ammonium persulfate 10 % W/V) and 5 µl TEMED (tetramethylethylenediamine) and subsequently with a 2.8 % collection gel (3.85 ml contain 2.6 ml water, 0.95 l upper-buffer (0.5 M Tris-HCl pH 6.8, 0.4% SDS), 0.3 ml acrylamide (T40 %-C3.7%), 22.5 µl APS (10 % W/V) and 5 µl TEMED) in which a comb was inserted for retrieving sample wells. After solidification of the gels samples were loaded in wells and the chamber was filled with running buffer (0,025 M Tris, 0.192 glycine, 0.1 % SDS) and electrophoresis was started. Voltage was gradually increased up to 100 V and gel was run for up to 2.5 h.

Blotting: Proteins were transferred from gel to membrane (0.45 µm reinforced nitrocellulose BA-S 85; Schleicher & Schuell, Bath, UK) in a blotting chamber (Mini Trans-Blot Electrophoretic Transfer Cell; BioRad)) between layers of Whatman 3MM Chr paper (Sigma-Aldrich) and wash cloths. Blot was run in blotting buffer (0.0.25 M Tris-HCl pH 8.3, 0.192 M glycine, 20 % methanol) and with ice pack for 2 h at 48 V.

Visualisation: For protein staining membrane was incubated for 5 min in Ponceau S staining solution (Sigma) at RT and then decolorized with 5 % acidic acid. Membrane with remaining stained protein bands was scanned and subsequently totally decolorized with blocking buffer (0.02 M Tris-HCl, 0.15 mM NaCl, 5% skimmed milk powder; pH 7.5). For antibody staining the blocking buffer was discarded and primary antibody was added in 12 ml fresh blocking buffer and incubated for 90 min at RT while gently rocking. Primary antibodies used were against EpCAM (C-10) and AQP-5 (H-200) (both Santa Cruz Biotechnology, Dallas, USA) and against CDH 1 and GAPDH (all Chemicon). Membrane was then rinsed twice for 5 min with a solution of PBS and 5 % Triton X and subsequently for 2 min with PBS only. Then

procedure was repeated in the same manner for the secondary antibodys. Membrane was then incubated in a mixture of 200 µl BCIP/NBT stock solution (nitro-blue tetrazolium chloride / 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt; Sigma-Aldrich) and 10 ml buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl2; pH 9.5).When protein bands became sufficiently visible, staining was stopped with water. Membrane was dried between 3MM Chr paper, wrapped in polythene film and finally scanned.

5.3 CHARACTERIZATION OF PSC

5.3.1 INTRODUCTION

PSC are a type of adult stem cells, which are relatively new. During course of this thesis only five publications dealt with this new stem cell type of either human [1, 78] or rat [1, 79, 80] origin. For the first time they were described by Kruse in 2004 [1], where he explicates their isolation, the generation of organoid bodies (OBs) and the spontaneous differentiation within these OBs. Due to their morphologic and immunohistochemical similarity to pancreatic stellate cells, which are located within the interlobular septa and interacinar areas of the pancreas, he termed them PSLC - Pancreatic Stellate Like Cells. It was not before 2007 that they were renamed PSC – Pancreatic Stem Cells [80].

Source of human PSC are pancreatic acini obtained from patients with diseases of the pancreas. The isolation yields cells of a spindle-like or stellate shape, resembling undifferentiated fibroblasts. The obtained human PSC can be grown in their undifferentiated state for at least 20 passages. For inducing spontaneous differentiation a protocol is used established for ESC. 600 cells are cultured in hanging drops below the lid of a Petri dish. After two days cells form aggregates. While these aggregates are named Embryonic Bodies, when originating from ESC, they are termed OBs for PSC. OBs are maintained in suspension culture for six days before they are plated on a gelatin matrix, where they are maintained for a minimum of seven weeks. During this period spontaneous differentiation into cell types from all three germ layers takes place: keratinocytes, nerve and glial cells from ectoderm; chondrocytes and muscle cells from mesoderm; exocrine and endocrine cells of the pancreas from endoderm. As they overcome lineage restrictions during differentiation, PSC are regarded as pluripotent adult stem cells. Establishment of these protocols and observation of spontaneous differentiation by Kruse [1] form the basis for all subsequent studies with PSC.

Guldner was the first to try a directed differentiation of human PSC [78]. Co-culture of PSC with human myocardial biopsies led to a significant higher level of cardiomoycte characteristics as after spontaneous differentiation. Criterions were expression of muscle specific protein MF-20 and cardiac muscle protein troponin I and also occurrence of autonomously contracting cell clusters.

Table 1: Stem cell and other marker expression of rat PSC determined by Kruse [79] and Danner [80].

marker	source / meaning	mRNA		protein		ref.
		primary	clone	primary	clone	
alk.phosphatase	stem cell			pos		[79]
amylase	pancr. enzyme		pos		pos	[79]
Brachyury	stem cell		pos			[79]
CD 9	stem cell	pos	pos			[79, 80]
CD 44	stem cell					[79]
DMC1	meiosis	pos	neg	*(neg)*		[80]
GDF-9	oozyte / ovary	pos/neg	pos			[80]
glucagon	pancr. enzyme		pos			[79]
nestin	stem cell	pos	pos		pos	[79, 80]
neuro filaments	neuron		pos		pos	[79]
Oct-4	stem cell	pos	pos	pos		[79, 80]
Pax 6	stem cell					[79]
SCP3	meiosis	pos/neg	*weak*	*weak*		[80]
SSEA-1	stem cell	pos	pos	pos	pos	[79, 80]
Vasa	germ cell	pos	pos			[80]
α-Fetoprotein	stem cell					[79]
α-SMA	mesenchym	pos		pos	pos	[79]

In 2006 two studies were published addressing differentiation behaviour of PSC originating from rats [79, 80]. Long-term culture of OBs (partially longer than one year) led to complex three-dimensional tissue-like structures with sizes up to 2 cm, termed tissue bodies [79]. While the surface was covered with a multi-layer epithelium the inner mass revealed canal-like structures and an arrangement of cellular and organoid components, both features reminding of different stages of teratoma development. Tissue bodies generated permanently new cells of remarkable size at their surface, which were released into the medium [80]. Closer investigation of these cells revealed an oocytes-like morphology. Also the gene expression pattern showed distinct differences to “normal” PSC and similarities to oocytes.

These cells were therefore named Oocyte-Like Cells. Both studies were the first to deal not only with spontaneous differentiation properties of PSC but also with characterization of undifferentiated PSC. However, these cells were not of human but of rat originating from rat and, moreover, mainly markers typical for stem cells were investigated (Table 1). Further characterization of human PSC was still missing.

Later publications dealt with directed differentiation in certain lineages and also partially with more detailed characterization of human PSC. These studies will be enlarged in the discussion (5.3.3.) of this chapter. Immanent endothelial and epithelial properties in undifferentiated PSC, however, have not been investigated before. As differentiation into these lineages was to be examined in this thesis, a thorough investigation of the immanent characteristics concerning these lineages was pivotal. Otherwise success of differentiation protocol is not discernible.

Also the influence of different substrates on stem cells is regarded as characteristic for the cell type. Certain matrices have therefore been investigated.

Well known is the fact, that epithelial cell, i.e. Calu-3, express a different phenotype and morphology when cultured in AIF culture (Figure 5) compared to a conventional or submerse culture. In an AIF culture cells are grown on a semi-permeable filter membrane. The growth medium levels with this membrane, so that the upper or apical side of the cells is faced by air rather than medium. Also cells originating from either ESC or progenitor cells showed such different behaviour when cultured at AIF [81, 82]. Therefore, PSC were subjected to AIF culture to monitor morphological changes by light-microscopy.

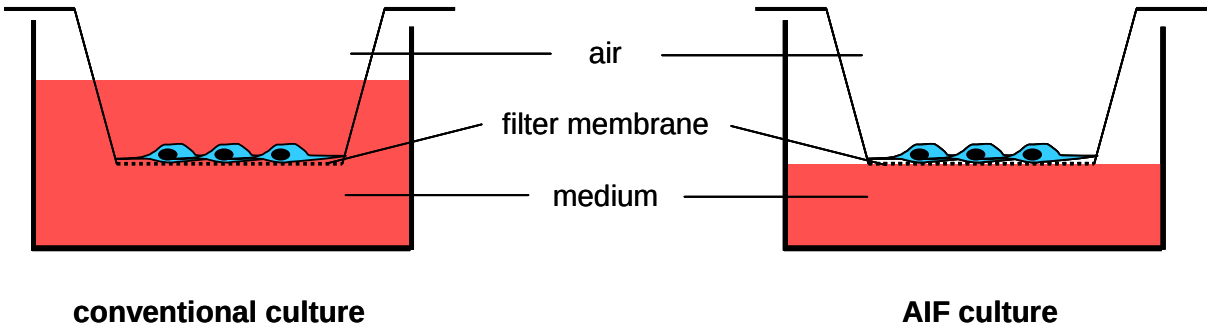

Figure 5: Schematic view of conventional and AIF culture.

As mentioned above also spontaneous differentiation in OBs is typical for PSC [1]. So far the “hanging drops” method (Figure 6b) has been applied on PSC. Generation of OBs with this method is labour intense and the hereby yielded OBs are rather small. Another approach from ESC research is the spinning or shaking of bulk suspension cultures (Figure 6a) in non-

adhesive dishes [83]. This generates large quantities of spheroids but the size of the resulting aggregates differs considerably. A third approach provides the possibility to produce large quantities of spheroids of a definite size by centrifuging down a certain cell number (Figure 6c) in either falcon tubes or in well plates [84]. This method was transferred to PSC in this thesis.

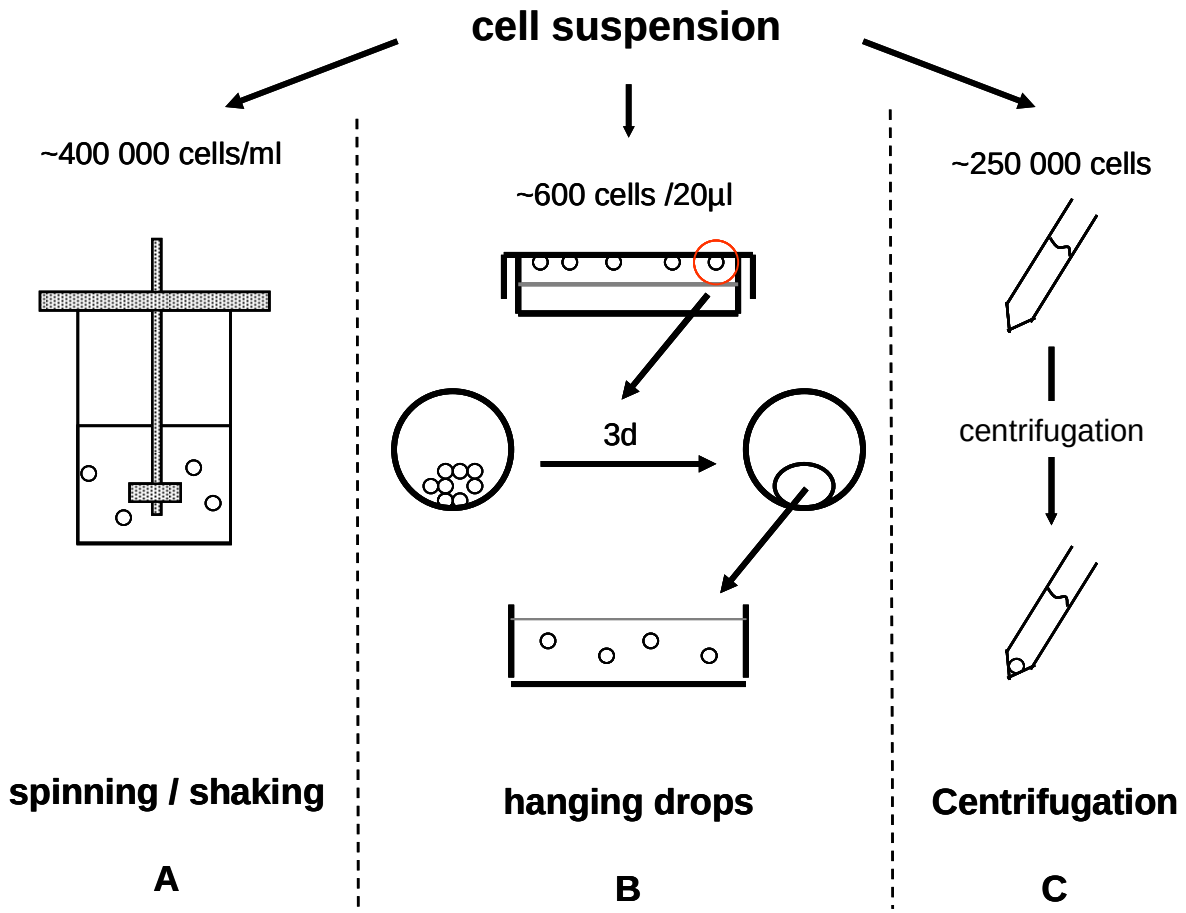

Figure 6: Three methods for generation of stem cell spheroids.

5.3.2 RESULTS

5.3.2.1 IMMANENT ENDOTHELIAL, EPITHELIAL AND STEM CELL CHARACTERISTICS OF PSC

Immanent endothelial, epithelial and stem cell characteristics of PSC were investigated by cultivating cells in plain culture medium for 7 days. Specimens were then analyzed by RT-PCR. Presence of select proteins was additionally determined by immunocytochemistry and FACS analysis.

For investigating stem cell properties of PSC mRNA expression of the general stem cell markers NES, Oct 4 and c-kit and the more specific hematopoetic stem cell markers endoglin,

CD 45 and CD 71 was analyzed (Figure 7). Highest expression was found for NES and endoglin, followed by c-kit and CD 45. Also for Oct 4 a cognizable band was detected. Solely, presence of CD 71 could not be verified.

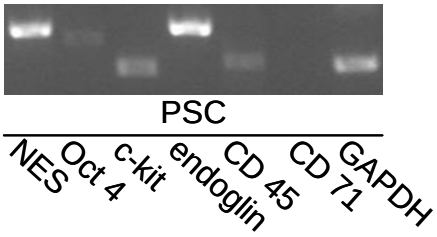

Figure 7: mRNA expression profile of stem cell markers in undifferentiated PSC. Samples were taken after 7 days of culture and analyzed by means of RT-PCR.

Of major interest prior to differentiation experiments were also the immanent endothelial properties of PSC. Endoglin, CDH 5, VCAM 1, VEGFR 1, VEGFR 2 and vWF were chosen as significant markers. First of all occurrence of mRNA was determined by means of RT-PCR (Figure 8a). For classifying the expression of these markers in PSC HUVEC were drawn upon for reference (more to characterization of HUVEC in 5.4.2.1). Most pronounced band was found for endoglin, followed by VCAM 1 (shift of bands is explained in 5.4.1) and VEGFR 1. Also vWF was detected as a weaker, though visible, band. Merely, CDH 5 and VEGFR 2 were not seen. The amount of VCAM 1 in PSC was comparable to that in HUVEC whereas all other bands were weaker than in control.

For determining and also visualizing presence of the mRNA correlating proteins immunocytochemistry was applied. All proteins, whose mRNA were found by RT-PCR, could also be imaged by this method (Figure 8).

For an additional quantification of this data FACS was applied as a third method. The two most pronounced endothelial proteins in PSC, endoglin and VCAM 1, were investigated by such means. Presence of both proteins was determined and statistically evaluated. Compared to control expression of endoglin was at 1600 % and VCAM 1 at 120 % (Figure 9).

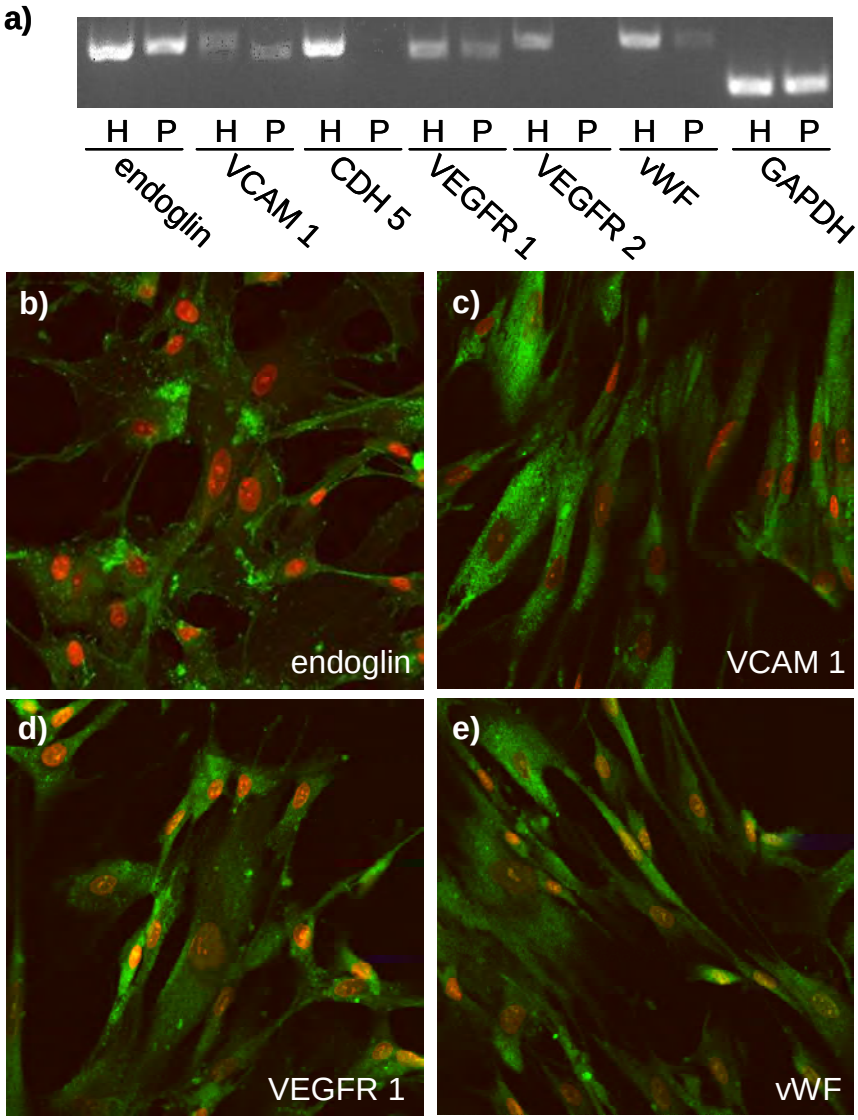

Figure 8: mRNA expression profile of endothelial markers in undifferentiated PSC. Samples were taken after 7 days of culture and analyzed by means of RT-PCR (a) and immunocytochemistry (b-e).

Also the immanent epithelial characteristics of PSC were investigated. Therefore the markers K 7, K 8, K 18, K 19, CTNb1, ZO-1, CDH 1 and EpCAM were analyzed by RT-PCR (Figure 10a). A549 served as an epithelial control. Most pronounced band in PSC was found for K 18, followed by K 7, CTNb1 and ZO-1. Clearly visible bands showed also K 8 and K 19, while CDH 1 and EpCAM were not detected. In PSC bands of K 18, K 19, CTNb1 and ZO-1 were comparable to those in A549, whereas bands of K 7 and K 8 were weaker as in epithelial control.

RFI as median (range)		
endoglin	**control**	**VCAM 1**
108,43 (29-433)	6,67 (1-68)	7,99 (2-95)
Image		

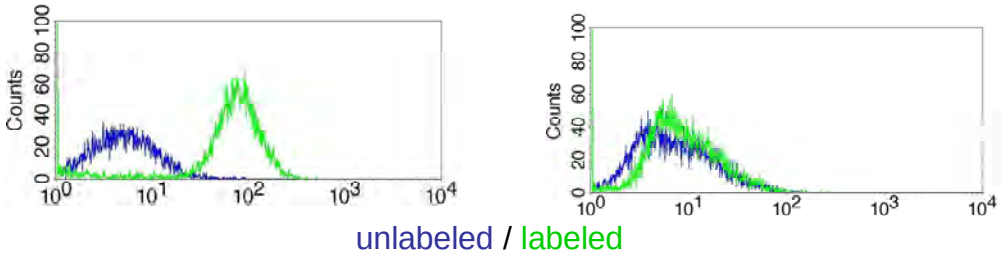

Figure 9: Expression of the endothelial proteins endoglin and VCAM 1 in PSC. Samples were taken after 7 days of culture and analyzed by FACS. For statistical analysis mean fluorescence intensity (MFI), presented as median (range), are displayed.

Since all investigated keratins were found as mRNA, panKeratin was analyzed at protein level. By means of immunocytochemistry all-over expression of this marker was clearly detected (Figure 10b).

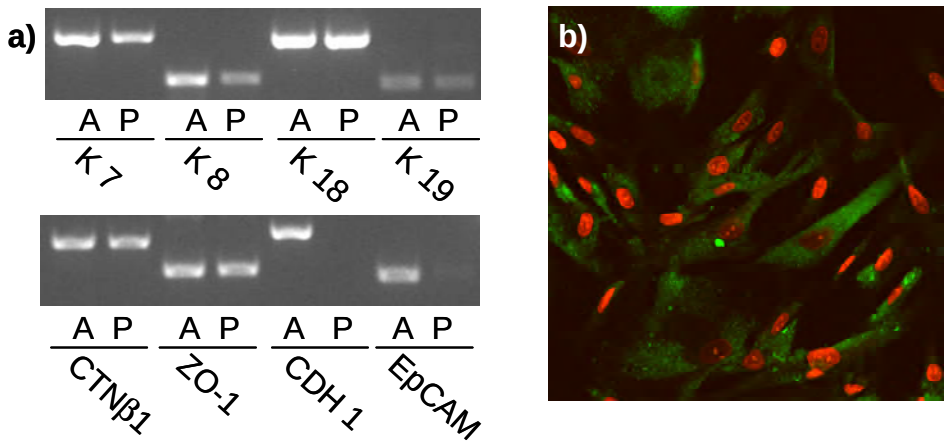

Figure 10: Expression profile of epithelial markers in undifferentiated PSC. Samples were taken after 7 days of culture and determined by means of RT-PCR (a) and for pankeratin also by immunocytochemistry (b).

5.3.2.2 INFLUENCE OF CULTURE ON DIFFERENT ECM ON MARKER EXPRESSION PROFILE OF PSC

The choice of substrate has an impact on the development of the cells cultured upon them. Numerous publications [67, 68] show that depending on the cell type different substrates can trigger a certain direction of differentiation. Therefore, the influence a substrate has on a stem / progenitor cell is supposed to be characteristic of this cell type.

The ECMs C I, C IV, FN, LN and PDL were selected for cultivating PSC upon. After 21 days of culture cells were analyzed for changes in their gene expression profile compared to PSC grown on TCP. Markers for stem cell, endothelial and epithelial qualities, which were prior used for a specific characterization after 7 days of culture, were investigated by means of RT-PCR.

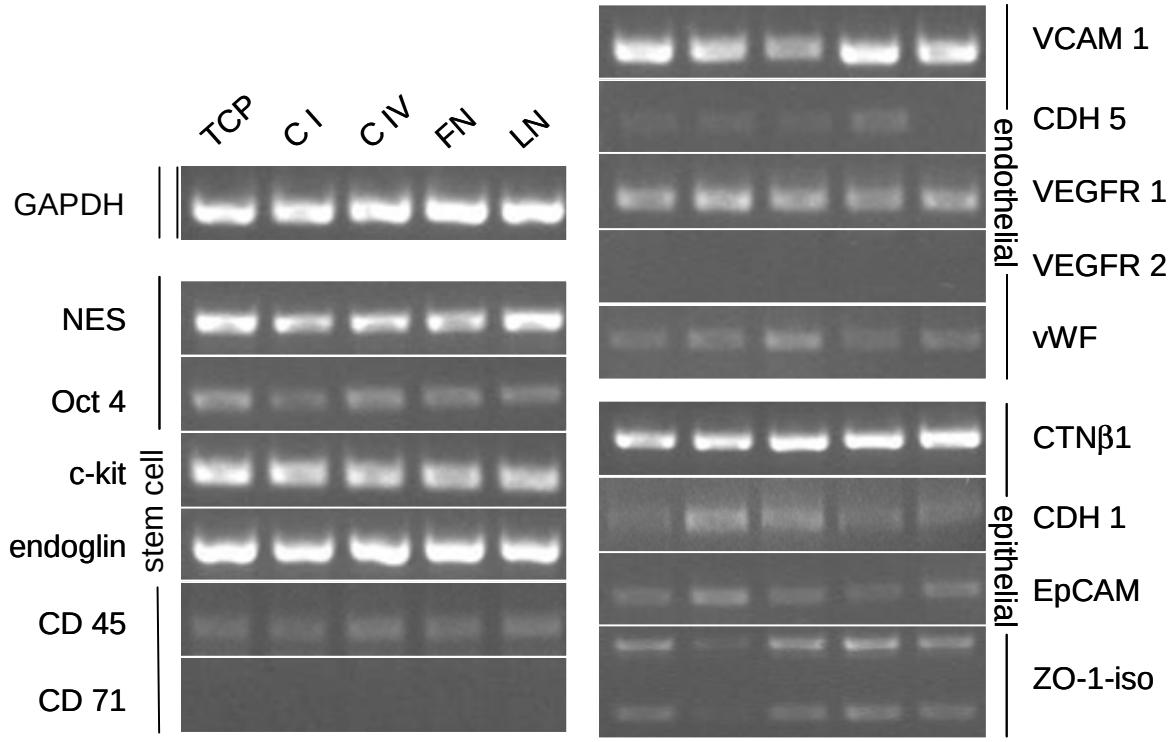

Figure 11: mRNA expression of stem cell, endothelial and epithelial markers of PSC. Samples were taken after 21 days of culture on different ECM (C I, C IV, FN and LN) and analyzed by RT-PCR. PSC grown on TCP served as control.

PSC seeded on PDL grew only insufficiently, which prevented further analysis of these samples. Apart from this singularity only small influence of matrices was observed in an altered expression of CDH 1, CDH 5 and ZO-1 (Figure 11). Whereas CDH 1 and CDH 5 were not detected during characterization of PSC (7 days of growth prior to harvest), slight bands were observed after 21 days. Bands for CDH 1 were found on all substrates, however,

more distinguished on C I and C IV. CDH 5 was not found on LN, but on all other matrices. The primer ZO-1-iso, used for quantification of ZO-1 transcripts, distinguished between the two isoforms α- and α + (see Chapter 6). The ratio of the two isoforms remained constant on all substrates; however, the total amount of ZO-1 was reduced on C I.

5.3.2.3 INFLUENCE OF AIR INTERFACE CULTURE ON PSC

Apart from the substrate also the presence or lack of surrounding cell culture medium has an impact on development of cells. A culture system in which cells have access to the medium only from one direction can direct or maintain a certain way of cell differentiation [75, 85, 86]. This can be achieved by exposing the upper side of the cells to air instead of medium, which is called Air Interface culture (AIF).

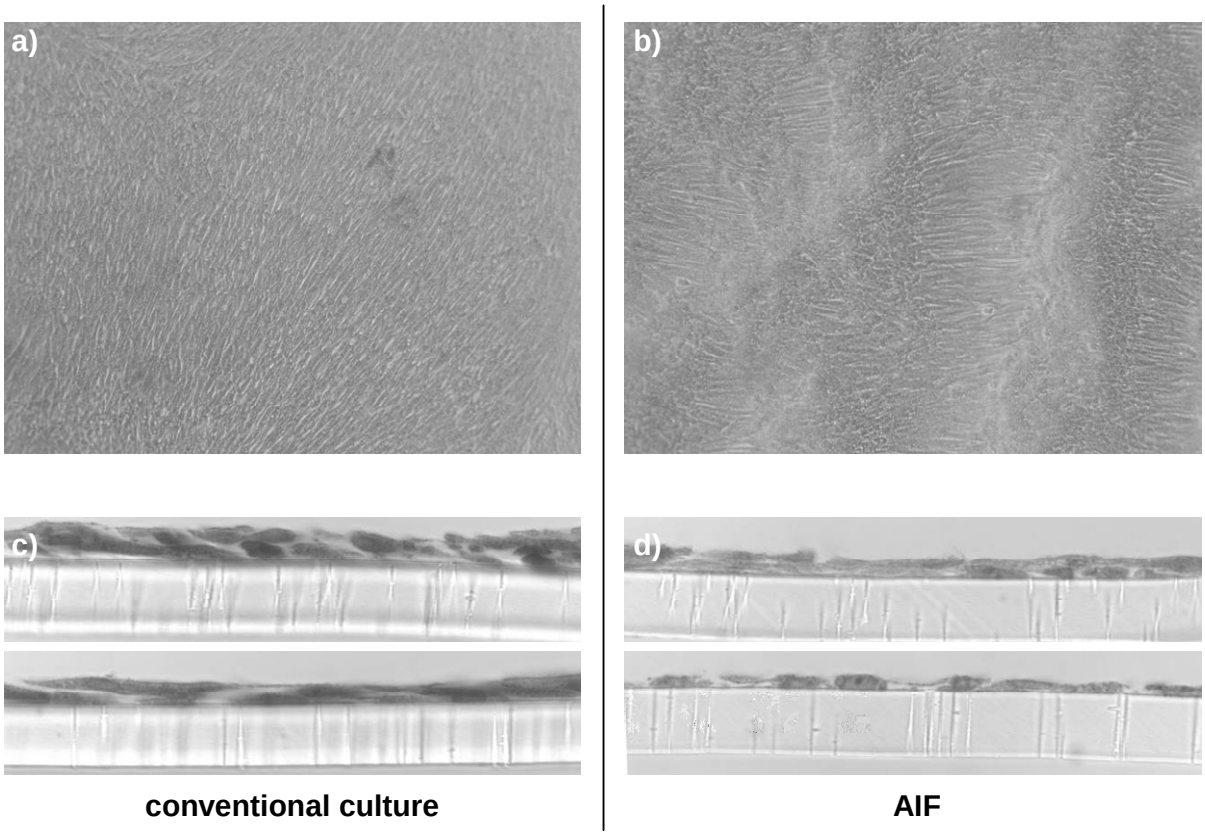

Figure 12: Light microscopic view of PSC morphology in conventional (a, c) and AIF culture (b, d) observed in top view (a, b) and in side view (c, d).

PSC were grown for 7 or 21 days in the apical compartment of a Transwell® system either in a conventional culture, where cells were covered by medium, or in an AIF, where the medium of the upper compartment was removed after 2 days post-seeding. A light microscopic and histological examination was then performed.

After 7 days PSC in the conventional culture showed in top view (Figure 12a) a dense growth and homogenous distribution. In side view (Figure 12c) it was obvious that nearly all cells were not growing in monolayer but in two layers instead. In AIF PSC showed a different morphology. Top view (Figure 12b) revealed a wave-like texture, which consisted of two alternating structures. In the first area PSC had a compact and dense and, as in the conventional culture, a two-layer growth (Figure 12d). In the second area PSC displayed a monolayer and had a distinct stretched morphology. Histological examination after 21 days of culture showed identical results to samples taken after 7 days.

5.3.2.4 SPONTANEOUS DIFFERENTIATION OF PSC IN ORGANOID BODIES

A specific quality of ESC is the spontaneous differentiation within spheroids. The method of hanging drops is by far the most frequently used protocol for generation of spheroids of a certain size [87]. This protocol has already successfully been transferred to PSC [1], which also showed the characteristic of spontaneous differentiation in the thereby generated spheroids, called organoid bodies (OBs).

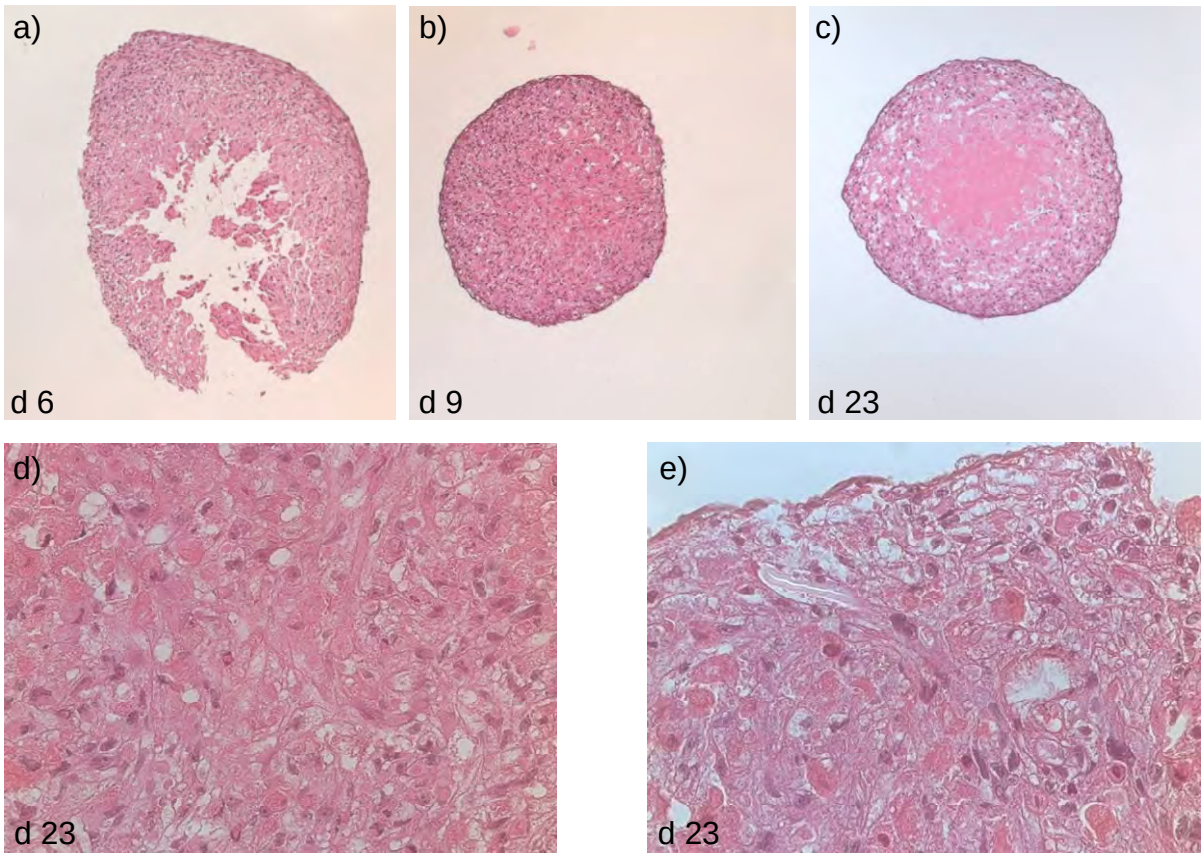

Figure 13: Formation of OBs after centrifugation of PSC. Histological cross section of OBs after 6, 9 and 23 days of culture (a-c) and close ups of day 23 (d, e).

Since the hereby yielded OBs are comparatively small and differentiation therefore limited, an alternative method from ESC research using centrifugation [84] was applied. A suspension containing 250,000 PSC was centrifuged down. The resulting pellet rounded up over night and was detached from the tube by gentle shaking. Spheroids were observed for up to 23 days.

Already after 6 days the former pellet showed a three-layer histology, which could be seen over the whole observation period, consisting of surface, outer core and inner core (Figure 13a). The surface is covered by a one-cell-layer, where the cells display a stretched morphology and a dense coherence. Cells in the core are either stretched or cuboidal. The outer core consists of very densely packed cells, whereas coherence between cells in the inner core is rather loose. Structures at day 9 resemble structures at day 6. After 23 days the inner core is subject of necrosis and no more single cells are detectable. In the outer core long and stretched cells alternate with dense cuboidal cells. Also areas are visible, that look like cross sections of tube-like structures. All in all the outer core has a very heterogeneous appearance. The surface layer consists of coherent stretched cells.

5.3.3 DISCUSSION

PSC are a rather new source of stem cells, described for the first time in 2004 [1]. During the course of this thesis only two publications [1, 78] dealt with human PSC and nearly no knowledge about their endothelial and epithelial properties had been gained. Therefore, a thorough investigation of stem cell, endothelial and epithelial characteristics was pivotal, before any differentiation experiment was carried out. This was necessary on one hand for determining the possible potential of the cells and on the other hand for detecting a successful differentiation.

At mRNA level NES, Oct 4, endoglin, c-kit and CD 45 were detected. NES is regarded as a marker for neural stem cells [88]. Oct-4 is mostly found in the nucleus and is involved in the self-renewal of embryonic and adult stem cells [89]. Endoglin and CD 45 are characteristic for haematopoetic stem cells [90]. C-kit is not only found in haematopoetic stem cells but also in ESC [91]. By expressing these markers PSC display a pattern specific for stem and progenitor cells. Subsequent studies confirmed these findings and added Alkaline Phosphatase, CD 9, CD 29, CD 81, c-Myc, KLF-4, nanog and SOX-2 to the stem cell expression profile [92-94].

Undifferentiated PSC were also positive for endothelial lineage markers. VCAM 1, VEGFR 1 and vWF were found, as well as endoglin, which is also regarded as a marker for stem cells

(see above). These markers are widely used for the identification of endothelial progenitor cells and for detecting successful differentiation of stem cells.

Epithelial commitment of PSC is recognizable by their immanent mRNA expression of CTNβ1 and ZO-1 and of the keratins 7, 8, 18 and 19 and of panKeratin at protein level [95] . The existence of ZO-1 in PSC was unexpected. A former study detected this protein in the pancreas, but only in the pancreatic ducts [96]. In the acinary parts, where PSC are harvested from, existence of this marker could not be verified. ZO-1 is not only characteristic for the epithelium, where it is mainly found in the tight junctions. Furthermore, it modulates cell proliferation in mature cells [97] and also cell proliferation and differentiation during ontogenesis [98, 99] (see also Chapter 6). Therefore, the finding of ZO-1 in PSC underlines once more their stem cell character. Even more unexpected was the presence of panKeratin at protein level in undifferentiated PSC. In 2004 Kruse et al. [1] described an expression of cytokeratin at protein level, which was found at the outer borders of OBs, while the inner mass lacked any presence. Since panKeratin is found in undifferentiated PSC, the protein expression apparently ceases during course of maturation. This demonstrates that differentiation into a certain cell type makes PSC loose their immanent epithelial characteristics. Later studies showed an opposed but not contradictory view of keratin expression. Gorjup et al. [92] did not detect CK 14, 15, 16 or 19 at protein level, whereas Ciba et al. [100] and Petschnik et al. [94] could verify mRNA expression of CK 18 at mRNA and protein level and of panKeratin at protein level.

As it has been reported frequently that cultivation of stem cells on ECM [68, 101-104] can influence differentiation, the matrices C I, C IV, FN, LN and PDL were investigated for their impact on PSC. Most variations in marker expression were small, only CDH 1 and CDH 5 showed distinctive alterations compared to the characterization performed before. PSC analyzed then did not display any of the two cadherins. New expression in this experiment is possibly due to the longer cultivation period. While PSC for characterization were allowed to grow for 7 days only, PSC for the later experiments were cultured for 21 days. During a longer cultivation cells keep on proliferating, density between cells increases and cells have more time and opportunities to build out communication with their neighbours. This direct and close cell-cell contact has often been proofed to be decisive for differentiation of stem and progenitor cells. Exemplary is the production of EBs from ESC in hanging drops [105], which has become a standard procedure for inducing spontaneous differentiation. This cultivation method had also the same effect on PSC [1, 79], which formed OBs, in which tissues from all germ layers were detected. Even in “-D cultures cell-cell contacts have an impact on cell

Table 2: Expression of markers at mRNA and protein level of human PSC.

Marker	source / meaning	mRNA	protein	reference
albumin	endoderm	pos	-	[92, 93]
alk. phosphatase	stem cell	pos	-	[92, 93]
amylase	endoderm / pancr. enzyme	pos	-	[92, 93]
BMP-2	bone / cartilage	pos	-	[92]
CD 9	stem cell	pos	pos	[92-94]
CD 15 (SSEA-1)	stem cell	-	neg	[92-94]
CD 29	stem cell	-	pos	[92, 93]
CD 31 (PECAM-1)	blood / endothelium	-	neg	[92]
CD 34	haematopoetic stem cell	-	neg	[92, 93]
CD 44	T-cell / cancer	-	pos	[92, 93]
CD 81	B cell activation / cancer	-	pos	[92, 93]
CD 90 (Thy-1)	haematopoetic stem cell	-	pos	[92]
CD 105 (endoglin)	haem. stem cell / endothelium	-	pos	[92, 93]
CD133 (PROM-1)	haematopoetic stem cell	-	neg	[92]
CK 14, 15, 16, 19	epithelium	-	neg	[92]
CK18	epithelium	pos	pos	[94, 100]
c-kit	stem cell	pos	neg	[92, 93]
c-Myc	stem cell	pos	-	[94]
GFAP	central nervous system	pos	-	[93]
integrin α6	stem cell	pos	-	[93]
Ki67	proliferation	pos	pos	[94]
KLF-4	stem cell	pos	-	[94]
MEF2D	*Transcription factor*	pos	-	[94]
MHC 1	cell surface molecule	-	pos	[92]
MHC 2	Immune system	-	neg	[92]
nanog	stem cell	*(pos)*	-	[92]
nestin	stem cell	pos	pos	[92-94, 100]
neuro filaments	neuron	pos	pos	[93, 94, 100]
Oct4	stem cell	pos [92, 94], neg [93]	pos	[92-94]
PGP 9.5	neuron	pos	-	[92, 93]
PPARγ	glucose metabolism	pos	-	[94]
S100β	nervous system	pos	-	[94]
Sox2	stem cell	pos	pos	[94]
SPP1	bone	pos	-	[94]
troponin T2	mesenchym	pos	-	[93]
vigilin	protein synthesis	pos	pos	[94]
vWF	endothelium	pos	-	[94]
α-SMA	mesenchym	pos	pos	[92-94, 100, 106]

differentiation [107] and even cell density can influence the fate of stem cells. While in high density cultures cortical stem cells differentiated to neurons, astrocytes and oligodendrocytes, low density cultures led to smooth muscle cells [108]. Therefore, the influence of cultivation period and density is highly probable and might lead to an enhanced expression of CDH 1 and CDH 5 and thereby giving rise to endothelial- and epithelial-like cells.PSC were also subjected to AIF culture. This is a culture method which is i.e. applied in lung epithelial research. Lung epithelial cells differ significantly in morphology, function and phenotypic when cultured in AIF instead of conventional culture. Calu-3 cultured by AIF display a morphology very similar to airway epithelium [109]. Cells grow to a more columnar epithelium with a rugged apical topography and even occasionally cilia-like structures are formed. A changed morphology was also visible for PSC cultured by AIF culture. Instead of a continuous two-layer growth in conventional culture, areas with stretched one-layer PSC alternated with densely packed two-layer cells, resulting in a wave-like morphology. Calu-3 also produce greater amounts of glycoprotein in AIF culture [109]. Further investigations on PSC might also reveal such outcome of the two culture methods on functional differences. Apart from application in original lung and skin epithelial research AIF culture also found successfully its ways into stem cell research. Keratinocytes derived from hESC [81] were cultured on dermal constructs with collagen gels and human dermal fibroblasts. hESC-derived and primary keratinocytes showed a similar induced stratification of surface epithelium in this AIF model.

Formation of the already mentioned OBs was achieved during this thesis by a method applying centrifugation. Thereby generated spheroids, originating from 250,000 cells, were larger than OBs resulting from the established method of hangings drops [1], originating from 600 cells. The spheroids generated by centrifugation displayed a three-layer morphology of surface, outer and inner core. The same structures are known from EBs. There the surface layer consists of epithelial-like cells and a dense ECM [110]. In the inner cell mass also an ECM deposition takes place. Cells at the outside of this basal lamina stay viable, while cells at the interior undergo apoptosis. This results in a fluid-filled cavity at the inner core of the EBs [111]. Cells in the outer core of the EB differentiate into cell types from all three germ layers [112]. Also OBs from PSC generated by centrifugation showed this epithelial-like structure at the surface and the beginning of cell death from day 23 in the inner cell core. The outer cell core showed heterogeneity of structures and morphologies. A closer histological investigation was not performed, but the microscopic insights already suggest the existence of a wide range of cell types and tissues within the so generated OBs.

5.4 ENDOTHELIAL POTENTIAL OF PSC AND MSC

5.4.1 INTRODUCTION

5.4.1.1 BACKGROUND

The endothelium is the innermost single cell layer of blood and lymphatic vessels. It forms an interface between the circulating blood or lymph and the rest of the vessel wall. It is separated from the outer vessel walls by a basal lamina. These two layers build up the intima of the vessel wall, which is present in every single vessel (Figure 14). Depending on the size and function of the vessels their wall can additionally consist of a layer of smooth muscle cells (media) and of connective tissue (adventitia).

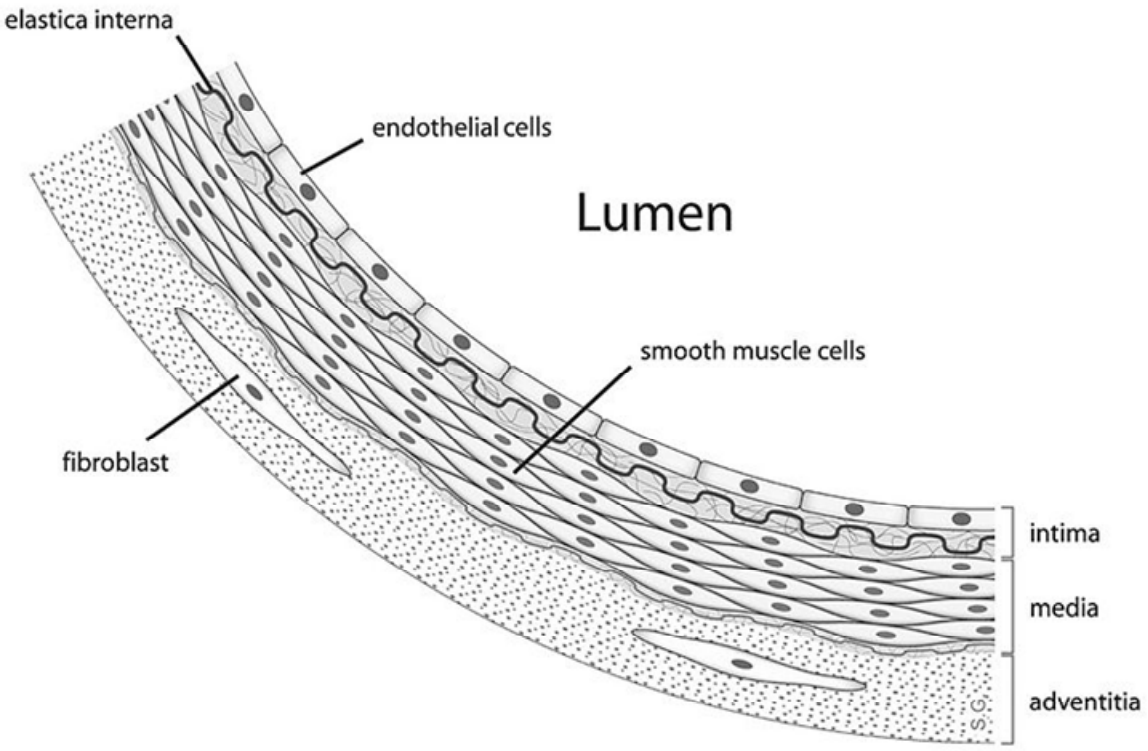

Figure 14: Schematic structure of epithelium. Adopted from [113].

A single endothelial cell has a diameter of approximately 20 µm and a thickness between 0.1 and 1 µm. The entire vascular endothelium of the human body, however, weighs between 1 and 1.5 kg, comparable to other inner organs like liver.

For a long time endothelium has been regarded as the inner lining of vessels only. Today it is known to be involved into numerous physiological processes: regulation of blood pressure,

angiogenesis, blood clotting, oxidation processes, arteriosclerosis and inflammation. Most obvious function remains the barrier function between the vessel lumen and the surrounding tissue. Depending on the location of endothelium its permeability varies considerably. While the endothelium at the blood-brain-barrier is a very “tight” one, using highly selective transport mechanisms, endothelium in the liver is even permeable for cells.

The loss of proper endothelial function is often observed in cardio-vascular diseases like arteriosclerosis, coronary artery disease, diabetes mellitus, hypertension and hypercholesterolemia. Also smokers and patients with rheumatoid arthritis suffer frequently from endothelial dysfunction.

Adult stem cells like MSC and PSC may be an alternative source to endothelial progenitor cells for generating autologous endothelial cells for usage in clinical therapies. They could be the base for the repair of injured blood vessels or for the neovascularisation of ischemic tissues. Also vascular prostheses could be lined with an autologous endothelium.

5.4.1.2 APPROACHES FOR ENDOTHELIAL DIFFERENTIATION OF PSC AND MSC

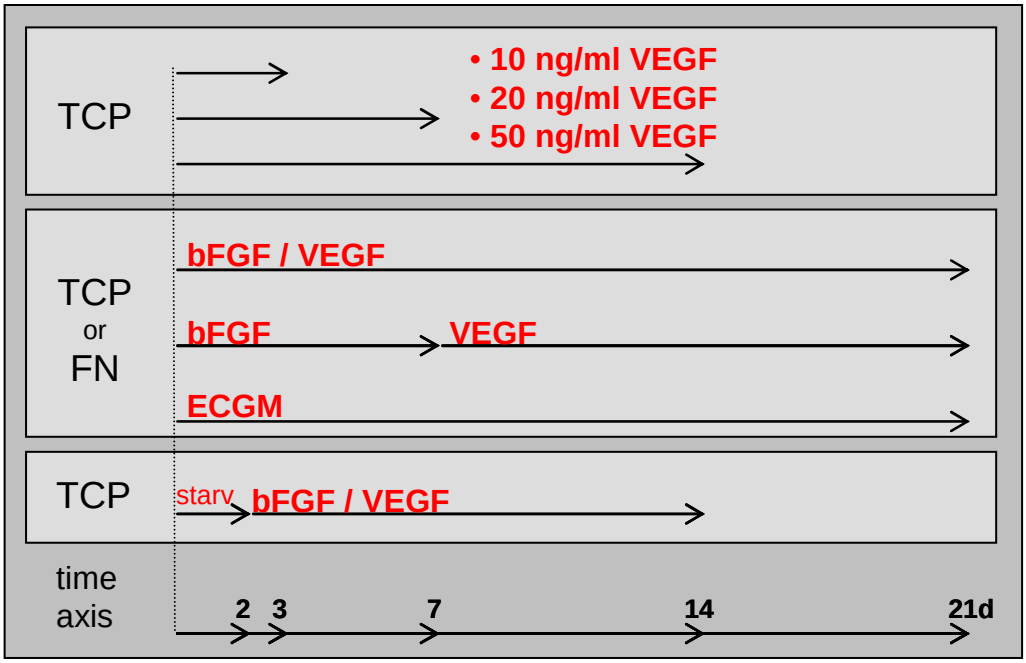

Figure 15: Schematic diagram of the three main protocols for endothelial differentiation of stem cells.

For differentiating adult stem cells into endothelial-like cells, various protocols are found in literature. Most of them apply as a triggering factor either VEGF alone [77, 114] or VEGF in combination with other growth factors [115-118], first of all bFGF. VEGF is a potent agent to induce endothelial differentiation in adult and ESC and bFGF is known to support mesenchymal commitment and also to enhance endothelial differentiation in combination

with VEGF [118-121]. Therefore, several protocols with varying concentrations of VEGF in combination with bFGF were applied.

Stem cells were incubated with 10, 20 and 50 ng/ml VEGF for 3, 7 and 14 d to determine the optimal concentration of the growth factor and also the desired time for differentiation. HUVEC and stem cells, cultured in maintenance medium only, were used as control.

In a further approach 20 ng/ml bFGF and 50 ng/ml VEGF were applied for 21 d either simultaneously or with a temporary offset (7 d bFGF, then 14 d VEGF). Stem cells were also cultured in ECGM, a medium applied for maintenance and proliferation of endothelial cells, which contains an optimized cocktail of growth factors. As known to be of importance, also the influence of ECM was considered. All protocols were applied to cells grown on TCP and on FN.

As a third approach FBS content of medium was reduced prior to incubation of stem cells with growth factors in order to increase responsiveness of stem cells. This ‘‘serum starvation’’ of cells is a common tool e.g. in directed myofibroblastic differentiation [122]. It is known to increase responsiveness to subsequently added differentiating agents. Even a patent is pending, which applies serum starvation of MSC to yield somatic pluripotent cells [2]. Hence, stem cells were cultured for 48 h in DM containing 0.1 % FBS prior to incubation with DM containing 20% FBS supplemented either with VEGF or bFGF / VEGF for 12 d.

All samples were analyzed by RT-PCR for expression of the endothelial markers PECAM 1, CD34, endoglin, VCAM 1, CDH 5, VEGFR 1, VEGFR 2 and vWF.

Beside determination of mRNA and protein expression also a functional assay was performed. The formation of capillaries in semisolid medium is considered as a proof for endothelial potential [77]. Therefore, the ability of stem cells to develop such tubes was investigated by an in vitro angiogenesis assay. Cells were seeded on top of ECMatrix and tube formation was monitored for 8 hours. In order to examine, whether stimulation with growth factors could enhance the potential of forming vessel-like structures, stem cells were cultured for 7 d with VEGF or a combination of bFGF/VEGF before performing an in vitro angiogenesis assay. HUVEC served as positive and Caco-2 as negative control.

5.4.2 RESULTS

5.4.2.1 CHARACTERIZATION OF HUVEC

For determining developing endothelial features in stem cells, a control is needed, which displays all the essential markers distinctively. HUVEC were chosen for this purpose. Two different sources of HUVEC were used. The first one was purchased form PromoCell, the second one had been isolated in the institute (Biophatmaceutics and Pharmaceutical

Technology, Saarland University). For using cells from both sources in parallel, they had to be investigated for comparability of endothelial marker expression. This was performed by using RT-PCR analysis of the markers CD 34, CDH 5, endoglin, PECAM 1, VCAM 1, VEGFR 1, VEGFR 2 and vWF.

Both batches displayed all markers and showed a nearly identical expression profile (Figure 16). HUVEC from both origins had equal bands for PECAM 1, endoglin, CDH 5, VEGFR 1, VEGFR 2 and vWF. HUVEC from PromoCell showed a stronger expression for CD 34. The analysis of VCAM 1 showed two very close bands for the corresponding isoforms of the gene transcript.

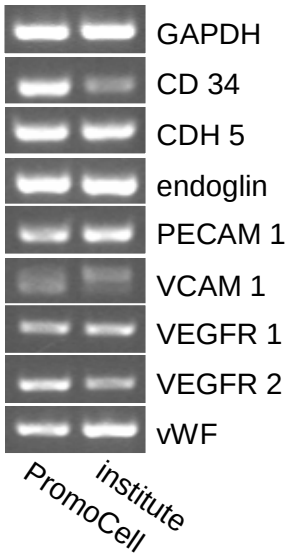

Figure 16: mRNA expression profile of endothelial markers in HUVEC purchased from PromoCell and HUVEC isolated in the institute.

5.4.2.2 ENDOTHELIAL POTENTIAL OF PSC AND MSC

Indifferent of applied differentiation approaches (5.4.1.2) all PSC samples displayed endothelial markers endoglin, VCAM 1, VEGFR 1 and vWF at mRNA level, while PECAM 1, CD 34 and VEGFR 2 were not expressed (Figure 17). No protocol did stimulate a significant stronger expression of any endothelial marker.

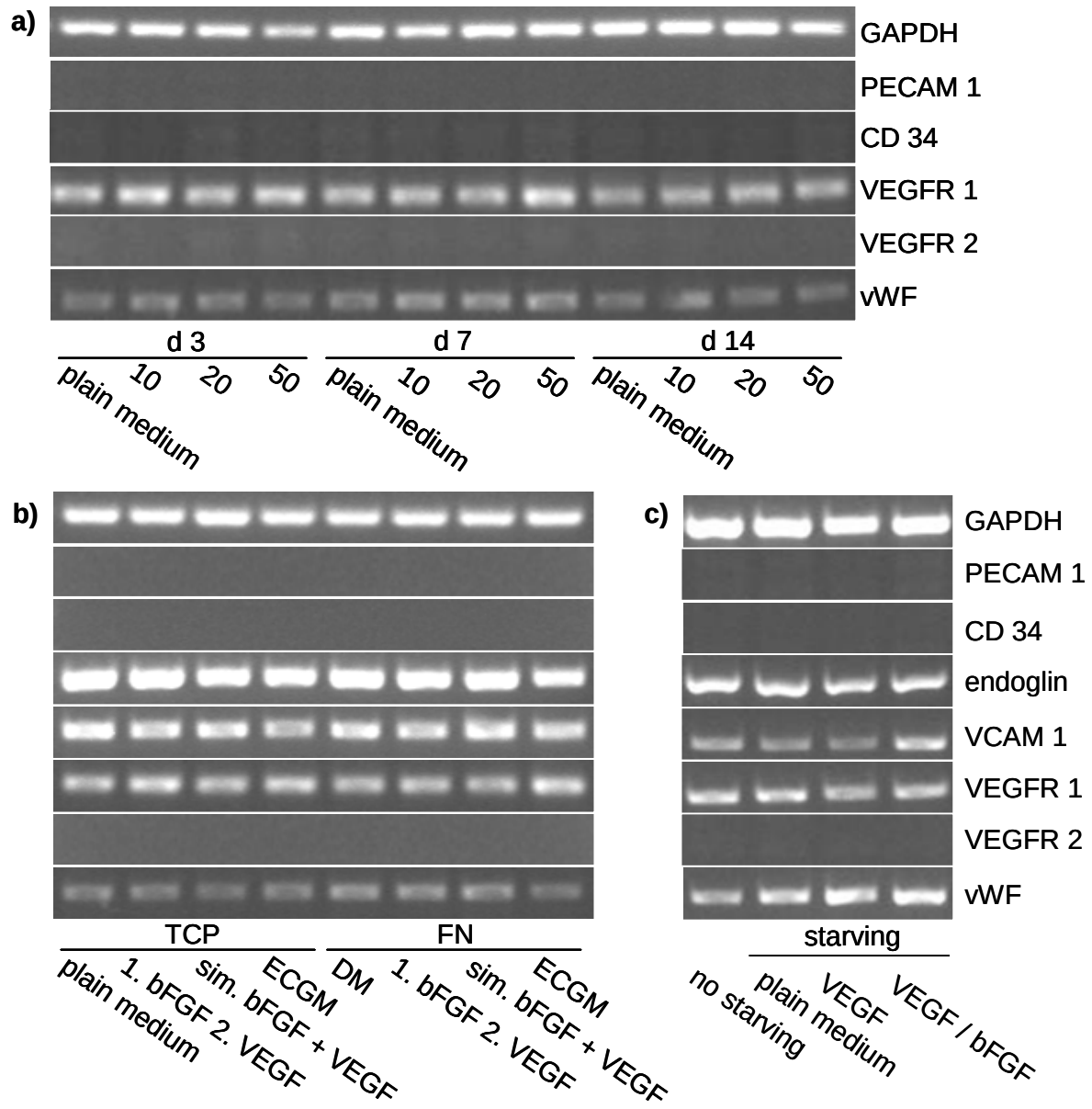

Figure 17: Influence of endothelial differentiation protocols on PSC. Stem cells were cultured with 10, 20 and 50 ng/ml VEGF for 3, 7 and 14 days (a), with 20 ng/ml bFGF and 50 ng/ml VEGF either simultaneously or successively and with ECGM for 21 days on TCP or FN (b), and after starving of PSC with 50 ng/ml VEGF and with 20 ng/ml bFGF and 50 ng/ml VEGF for 12 days (c). mRNA expression was determined by RT-PCR and PSC grown in plain DM and for the last experiment (c) also PSC which had not undergone starving served as controls.

Also MSC showed an unaltered expression of endoglin, VCAM 1 and vWF. VEGFR 2 and PECAM 1 were expressed so weakly, that no alteration was distinguishable. CD 34 was clearly detected for the samples on TCP and FN, which were incubated in plain medium and medium containing VEGF alone or time-delayed bFGF and VEGF. Expression with bFGF and VEGF simultaneously and ECGM was considerably reduced. Sample set of starved MSC

did not show any bands for this marker. Expression of VEGFR 1 was found in all MSC samples. Most specimen displayed an equally strong expression of two alternative transcripts [123], which can be seen best for the starving set of samples. Solely MSC incubated with ECGM showed bands for the shorter transcript only (Figure 18).

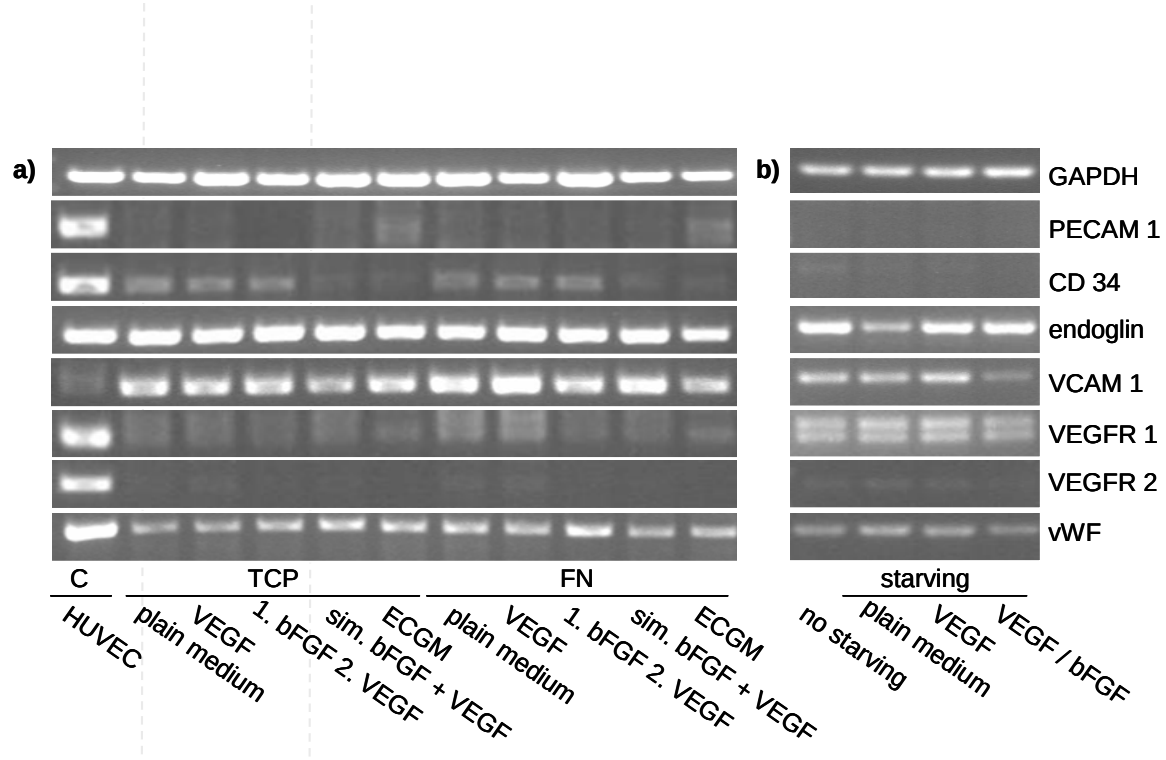

Figure 18: Influence of endothelial differentiation protocols on MSC. Stem cells were cultured with 50 ng/ml VEGF, with 20 ng/ml bFGF and 50 ng/ml VEGF either simultaneously or successively and with ECGM for 21 days on TCP or FN (a), and after starving of MSC with 50 ng/ml VEGF and with 20 ng/ml bFGF and 50 ng/ml VEGF for 12 days (b). mRNA expression was determined by RT-PCR and MSC grown in normal culture medium and for the second experiment (b) also MSC which had not undergone starving served as controls.

The *in vitro* angiogenesis assay showed an extensive tube formation for the positive control HUVEC (Figure 19b) and no such behavior for the negative control Caco-2 (Figure 19a). PSC (Figure 19c) and MSC (Figure 19d) also developed capillaries, which had their maximum extent after 8 hours. Compared to HUVEC fewer stem cells contributed to formation of capillaries, while indifferent cells could be distinguished by their round shape. However, the capillary structures formed by stem cells were fewer but also bigger, especially those formed of PSC; in comparison to those developed by HUVEC. Stem cells additionally treated with growth factors showed a tube formation comparable to that of untreated cells.

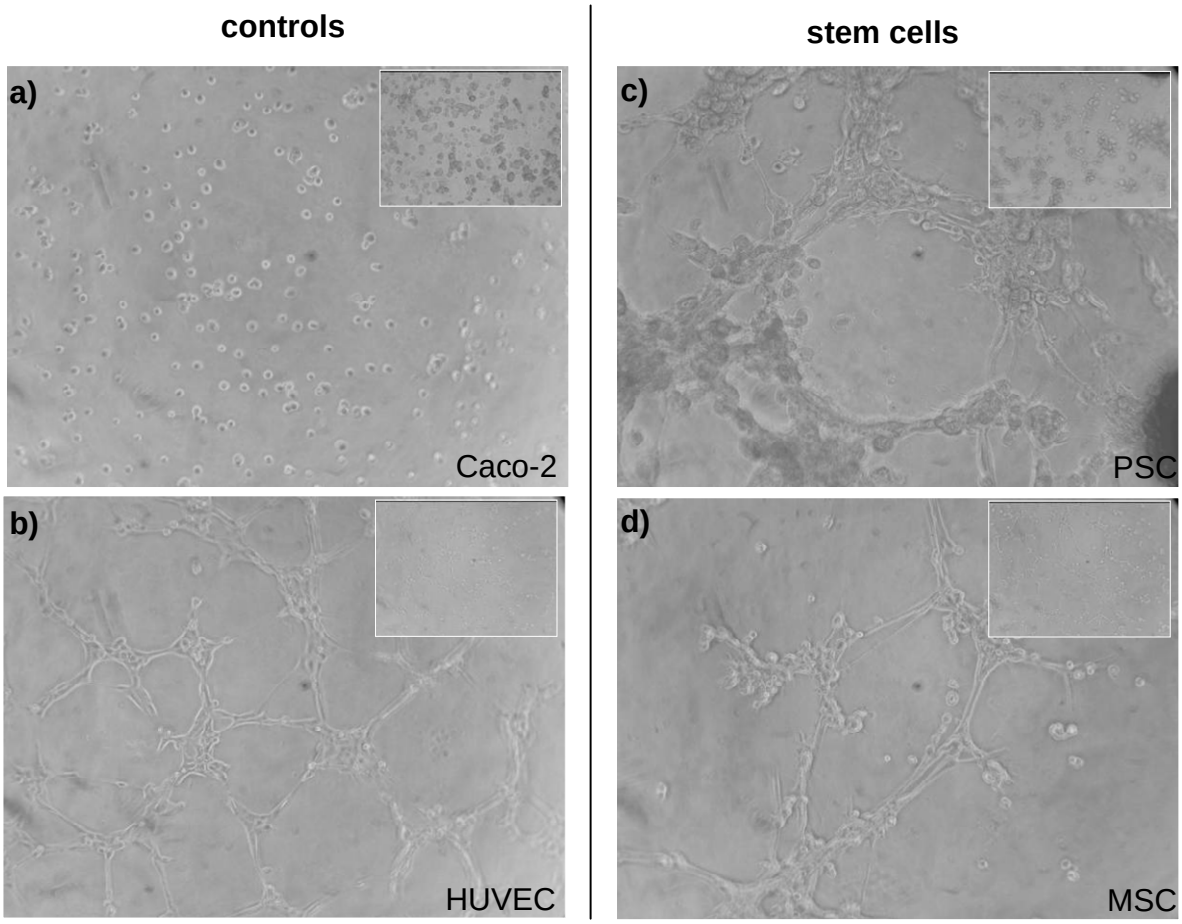

Figure 19: in vitro *angiogenesis assay: PSC (c) and MSC (d) were plated on ECMatrix™ and showed an extensive tube formation with strongest occurrence after 8 h (big pictures; small once show state after 2h). Caco-2 (a) served as negative and HUVEC (b) as positive control.*

5.4.3 DISCUSSION

Endothelial progenitor cells, isolated from human blood, promise a potential for the therapy of myocardial infarction [124] and peripheral arterial disease [125]. However, blood-derived endothelial progenitor cells are difficult to obtain in sufficient amounts. Alternatively, other human stem cells like MSC and PSC may be used for yielding endothelial-like cells for application in medical therapy.

All protocols applied in this study had shown in literature to successfully differentiate stem and progenitor cells and in one case even MSC [77] to endothelial-like cells. An exact transfer of a protocol from one cell type to another is nearly never effective. Minor or even major adaptations are mostly required to optimize the protocol accordingly. In this case PSC were in the beginning treated accordingly to a protocol for MSC by Oswald et al. [77] applying VEGF alone. As no differentiation was achieved with different VEGF concentrations the medium was topped off with bFGF. Both growth factors were applied either simultaneously or with a

time shift. PSC were also incubated in ECGM, which contains additionally to VEGF and bFGF Ascorbic Acid, Epidermal Growth Factor, Heparin, Hydrocortisone and Insulin-like Growth Factor. Still no differentiation was achieved. Furthermore, PSC were grown upon FN as an endothelium-supporting substrate. The last approach applied a method known as 'serum starvation', where responsiveness of stem cells to differentiating factors is increased by incubation in a low serum medium for two days. However, no differentiation at mRNA level was recognizable. Although PSC showed before their ability to spontaneously differentiate into cell types from all three germ layers [1] endothelial cells in particular were not detected then and since. Even established differentiation approaches could not successfully be transferred to PSC. No differentiation was detectable. However, the apparently obvious conclusion, that PSC are utterly not able to generate endothelial cells, is weakened by the also performed functional assay. There PSC displayed an ability, which is considered as an endothelial characteristic [77, 126, 127]. On a semi-solid matrix they form tube-like structures. This is typical for endothelial cells, whose one task among others is to build up new vessels by angiogenesis. This process is essential during embryogenesis, growth and development phases and in wound healing and granulation processes [128]. PSC displayed this ability indifferent of incubation with VEGF but as an immanent feasibility. Additionally, PSC display already in their undifferentiated state a range of endothelial markers, namely endoglin, VCAM 1, VEGFR 1 and vWF at mRNA (see Figure 8 and Figure 9 in Chapter 5.3.2.1). mRNA expression profile and functionality, therefore, suggest the possibility, that PSC may be differentiated to endothelial cells by the right protocol.

Some time after differentiation experiments with PSC had started, MSC were brought in. Endogenously MSC showed the endothelial markers endoglin, VCAM 1, vWF, and also weaker VEGFR 1 and VEGFR 2 after 14 and 21 days of culture in plain stem cell medium without any differentiating agents. CD 34 was not or nearly not detectable for MSC samples, which were starved and for samples which were incubated in ECGM or with bFGF and VEGF simultaneously. Starvation samples were cultured in total for 14 and all other samples for 21 days. Apparently the longer cultivation period enhances expression of this marker, even though ECGM and the simultaneous presence of bFGF and VEGF minimize this effect. This observation is in accordance to findings, which report a commitment to endothelial lineage, when MSC are cultured in high density (see below) [126]. MSC displayed like PSC the ability to build vessel-like structures in an *in vitro* angiogenesis assay. Again, marker expression profile and functionality are indicating a high endothelial commitment.

The same protocols, which had been used for PSC, were also applied for MSC. Also for these stem cells no differentiation was observed. This was surprising, since not all of the protocols had been transferred from other stem cell types on MSC. The first approach had been identical to a publication by Oswald, where he claims to differentiate human adult MSC to endothelial-like cells by means of VEGF. He was the first one to describe such an apparently successful differentiation. This raises the question, why the transfer to our MSC was not successful. Three options are to be considered. (1) Failures during carrying out of experiments or in handling of cells. Mistakes in handling are always possible, but risk was minimized by sticking to standard routines and to the described protocol. (2) Marker expression and also potential of MSC can differ considerably depending on the source of origin [129]. Although both sorts of MSC were isolated from bone marrow, age of donor and also passage numbers are known to be of influence. (3) Original protocol was not accurate, either protocol was incomplete or differentiation was not achieved. Oswald was and is the only one who reported a differentiation of human adult MSC to endothelial-like cells by incubating the confluent stem cells for 7 days with 50 ng/ml VEGF. In the meantime more papers dealt with this subject and all applied much more complex protocols. They describe a combination of VEGF and bFGF [116, 118, 130], of VEGF, Epidermal Growth Factor and Hydrocortisone [131], complex growth cocktails [132], substrates like FN or Matrigel^{TM} [117], co-culture systems [133, 134] or even *in vivo* conditions [130]. Furthermore only some of them are dealing with human MSC [118, 133, 135], while the others apply rat [132, 134] or mouse [130] cells, some of them even of fetal origin [116]. So, how managed Oswald his differentiation and did there remain any ambiguity? Oswald differentiated MSC by incubating confluent MSC for 7 days with VEGF. Apparently he did not run a negative control of MSC in plain medium treated otherwise identically. Instead he compared differentiated cells with undifferentiated MSC, which never reached confluence during cell culture routine. He failed to ensure by his experimental design that not again density of cells plays the main role for the commitment of MSC (see below) [126]. Oswald also reported the detection of Weibel-Palade bodies in differentiated MSC. These organelles are found only in endothelial cells, where they store vWF and P-selectin. In the immunofluorescence, he takes as a proof for existence of Weibel-Palade bodies, vWF is entirely distributed within the cytoplasm of the cell, while the actual storage organelle is empty. Even if the body membrane was disrupted by staining procedure, some of the vWF must have remained within the organelle. Whether the detected structure was a Weibel-Palade body is therefore questionable. All in all the line of evidence Oswald presented is not immaculate. However, it is undeniable that treated MSC displayed

endothelial markers at protein level and functional characteristics by forming tube-like structures on semisolid matrix.

Since 2000 publications also dealt with cell-cell contact and its influence on behavior of human MSC. Conditioned medium, which belongs to methods of indirect coculture (see Chapter 5.5.1.2), of HUVEC stimulated proliferation of MSC [136]. The same effect was seen for indirect and direct coculture of the same cell types, while effect of direct coculture was significantly stronger [137]. In 2005 Wu et al. [133] even demonstrated in an *ex vivo* model a differentiation to endothelial-like cells, when human MSC were simultaneously cocultured with HUVEC directly and with smooth muscle cells indirectly. An endothelial maturation without any coculture was reported by Liu [135]. After 3 weeks of culture in an endothelial growth medium, MSC displayed endothelial markers and also formed tube-like structures upon Matrigel^{TM}. However, expression profile at mRNA and protein level as well as morphology did not comply with cord blood derived endothelial progenitor cells. In 2009Chen was able to differentiate umbilical blood and also bone marrow derived MSC to endothelial-like cells by means of VEGF, Epidermal Growth Factor and Hydrocortisone [131]. However, in 2012 culture of MSC at high density was the first approach to report not only expression of endothelial markers and tube-formation, but also an altered morphology of human MSC [126]. This happened without any other stimulating agents like differentiation factors or a second cell type. Solely close and long contact of MSC to their neighbors triggered this maturation. This development was traced back to Notch signaling, which is known to be involved in many processes of cell-cell communication and also in angiogenesis [138]. High density MSC displayed high levels of Notch. They also secreted VEGF, which was suppressed after incubation with a Notch signaling inhibitor. Furthermore, siRNA knockdown of Notch receptors 1, 2 or 3 decreased VEGF secretion and VEGFR1 expression. Thus, the significance of Notch signaling in the differentiation of MSC to endothelial progenitor like-cells was demonstrated.

Still, the complex process during differentiation of progenitor or stem cells to endothelial cells has not been decoded yet. For each type of cell a particular protocol has to be developed before a differentiation can be achieved. In this study neither PSC nor MSC could be differentiated along the endothelial lineage. However, both cell types express endogenously endothelial markers at mRNA level and also display the typical endothelial characteristic of tube formation on a semi-solid matrix. Both properties suggest a high endothelial differentiation potential. So far just the right protocol is missing.

5.5 EPITHELIAL POTENTIAL OF PSC AND MSC

5.5.1 INTRODUCTION

5.5.1.1 BACKGROUND

Epithelia are tissues consisting of closely packed cells, which line all free surfaces of the body and also the internal body cavities. Epithelia have no vessels but exist on an underlying layer of vascular connective tissue, from which they are separated by a basal lamina existing of ECM. Epithelia are classified by their number of cell layers and the shape of cells in their surface layer. Simple epithelia consist of only one layer of cells, stratified epithelia of two or more layers. According to their height and shape cells are either squamous (scale- or plate-like), cuboidal or columnar. All epithelial cells are polar, meaning that there are two sides to be discriminated. The apical side faces the lumen, the basolateral side the basal lamina and thereby the underlying tissue. Both sides differ considerably in structure and functionality depending on the epithelial cell type. The two primary functions of epithelium are protection and regulation. Underlying tissues are protected against physical trauma, radiation, dehydration and toxins, and the transport of substances between the underlying tissues and the body cavity is regulated. Depending on the type of epithelium it also has a secretory function of either delivering hormones into the blood stream or secretion of enzymes, mucus or sweat by epithelial glands.

The lung epithelium can roughly be discriminated into bronchial and alveolar epithelium. The bronchial epithelium comprises seven different cell types: goblet cells, basal cells, ciliated cells, brush cells, serous cells, Clara cells, and neuroendocrine cells [139]. The alveolar epithelium consists of mainly two cell types. Alveolar cell type I is squamous and covers approximately 95% of the alveolar surface accomplishing the gas transport. Alveolar cell type II is cuboidal and secrets surface active substances [140].

There is a big need of adequate models of lung epithelial tissue for *in vitro* screening of pharmaceutical substances. Usage of cell lines is widely spread but they are neither optimal for cytotoxicity testing nor transport studies. Experiments with human primary cells resemble *in vivo* conditions much better. Unfortunately, sources are scarce and only few laboratories perform isolation of primary lung epithelial cells. Moreover, these cells are complex in maintenance and suffer from a very limited life span [75, 86, 141]. Human adult stem cells may be a promising tool for generating epithelial cells in bigger scale for these needs. This study, therefore, focused on the human stem cells PSC and MSC and their ability to give rise to lung epithelial-like cell types for future application in *in vitro* testing.

5.5.1.2 APPROACHES FOR EPITHELIAL DIFFERENTIATION OF PSC AND MSC

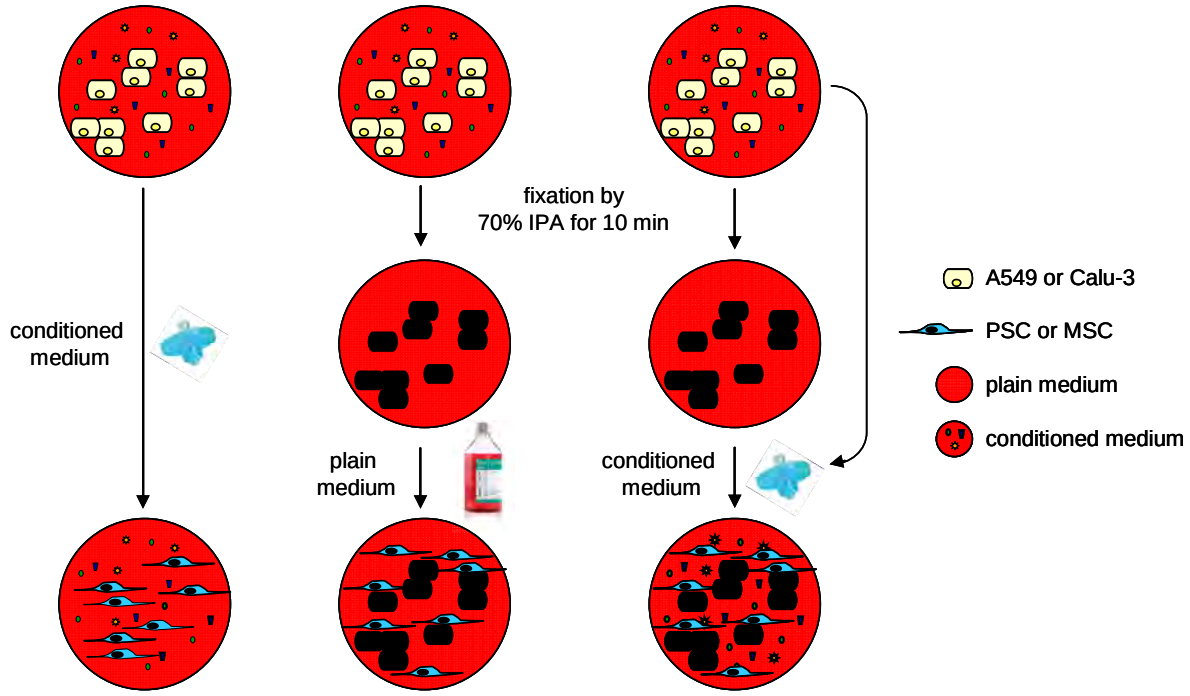

Figure 20: PSC or MSC were either cocultured with the epithelial cell lines A549 or Calu-3 indirectly (in conditioned medium), directly (upon fixed cells) or with a combination of both methods.

Unlike for endothelial differentiation there are so far no established protocols found in literature for differentiating stem cells into lung epithelial like cells by means of soluble factors. A further commonly used approach for influencing differentiation into a certain cell type is the cocultivation of stem and progenitor cells with fully differentiated cells [142-146]. Up to now no such model for epithelial cells has been established. Therefore, several approaches were tried in this thesis comprising different coculture systems. In indirect coculture both cell types are physically separated and communication is mediated by soluble factors only [147]. For example the application of conditioned medium is counted to indirect co-cultures [146]. Conditioned medium is generated by culturing the mature cell type in its usual growth medium. After a certain time the medium is removed, filtered and blended with fresh medium, which is typically the culture medium of the respective stem cells. Stem cells are then allowed to differentiate within this conditioned medium. In direct cocultures stem cells are cultivated with a second cell type [143, 144, 148], which allows for direct contact with the proteins and structures of the cell membrane. Cells of this mature cell type can either be fully viable, hindered in proliferation or even fixed.

In this study PSC were cocultured with the human lung epithelial cell lines A549 and Calu-3, which are both derived from carcinomas. A549 originate from an adenocarcinoma of a 58-year old Caucasian male [149, 150]. They are widely used as an *in vitro* model of alveolar cell type II for investigating drug metabolism. Calu-3 are an adenocarcinoma cell line derived from a 25-year old Caucasian male. They are commonly applied as a model for bronchial epithelial cells in drug metabolism and transport studies [151, 152].

Three different approaches were applied: firstly, the influence of medium conditioned by epithelial cells, secondly, the impact of the surface of fixed epithelial cells and thirdly, a combination of both methods. Stem cells cultivated on tissue culture plates (TCP) and in normal cell culture medium served as control.

5.5.2 RESULTS

5.5.2.1 CHARACTERIZATION OF EPITHELIAL CELL LINES AND EPITHELIAL PRIMARY CELLS

Due to the lack of established protocols with soluble factors the lung epithelial cell lines A549 and Calu-3 were used for triggering epithelial differentiation in stem cells either by using their secreted growth factors or their cell surface (Chapter 5.5.1.2).

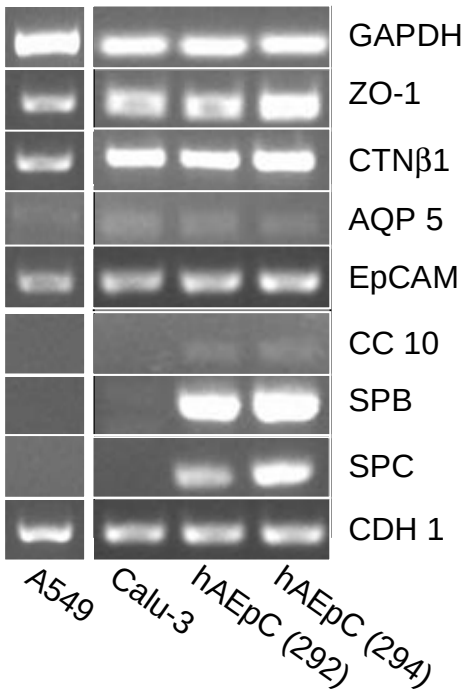

Figure 21: mRNA expression of epithelial markers in the lung epithelial cell lines A549 and Calu-3 and in the primary lung epithelial cells hAEpC (isolation 292 and 294). Samples were analyzed by means of RT-PCR.

For analyzing how far these cell lines resemble primary cells, they were compared to cells freshly isolated from human lung, called hAEpC (human alveolar epithelial cells)
[75]. Primary cells from two different isolations (hL 292 and 294) were used for detecting possible inter-batch variations. All cells were investigated for the expression of the lung epithelial markers ZO-1, CTNβ1, AQP 1, EpCAM, CC 10, SPB, SPC and CDH 1 at mRNA level.

All cell types showed similar strong bands for ZO-1, CTNβ1, EpCAM and CDH 1. AQP 5 was still clearly detectable in Calu-3 and hAEpC, while the corresponding band in A549 was nearly not visible. SPB, SPC and CC 10 were only found in hAEPC. While SPB and SPC were strongly expressed in the primary cells, CC 10 showed only weak bands.

5.5.2.2 EPITHELIAL POTENTIAL OF PSC

As shown earlier [146] soluble factors secreted by fully differentiated cells can have an impact on the fate of stem cells or progenitor cells. Therefore, the influence of conditioned medium of A549 and Calu-3 on the expression of the epithelial markers CDH 1, EpCAM, AQP 5, CTNβ1 and ZO-1 at mRNA and protein level within PSC was investigated (Figure 22a). After 21 days of culture ZO-1, CTNβ1 and EpCAM showed a similar expression for all samples, while AQP 5 was not detected (bands visible in the gel result from unspecific binding. If the amount of AQP5 was sufficient, only one clear band was visible, as can be seen in Figure 22b). However, for CDH 1 an influence was obvious. Whereas PSC cultivated in unconditioned medium showed a distinct band for CDH 1 PSC in conditioned medium displayed no expression.

In order to investigate the trigger of the surface of dead epithelial cells on the differentiation of stem cells, A549 and Calu-3 were fixed before stem cells were seeded on top of them. One part of stem cells was additionally cultured in conditioned medium, while the second part was incubated in unconditioned medium. Stem cells in normal culture medium on TCP and also fixed and unfixed epithelial cells served as controls. After 12 days of culture epithelial cells were no longer visible but were removed by stem cells overgrowing them. mRNA and protein content in the samples of fixed epithelial cells was not detectable, which confirms that analyzed mRNA and protein originated from stem cells only. All investigated markers were expressed by controls of unfixed epithelial cells, although the band of A549 for AQP 5 was only weak (Figure 22b). PSC showed a comparable expression of ZO-1, CTNβ1 and EpCAM for all samples, while AQP 5 was not visible. PSC in normal DM on TCP did not display CDH 1. The same behavior was shown by PSC cultivated on fixed Calu-3. However, CDH 1

expression in PSC grown on fixed A549 was increased independent on incubation with conditioned or unconditioned medium.

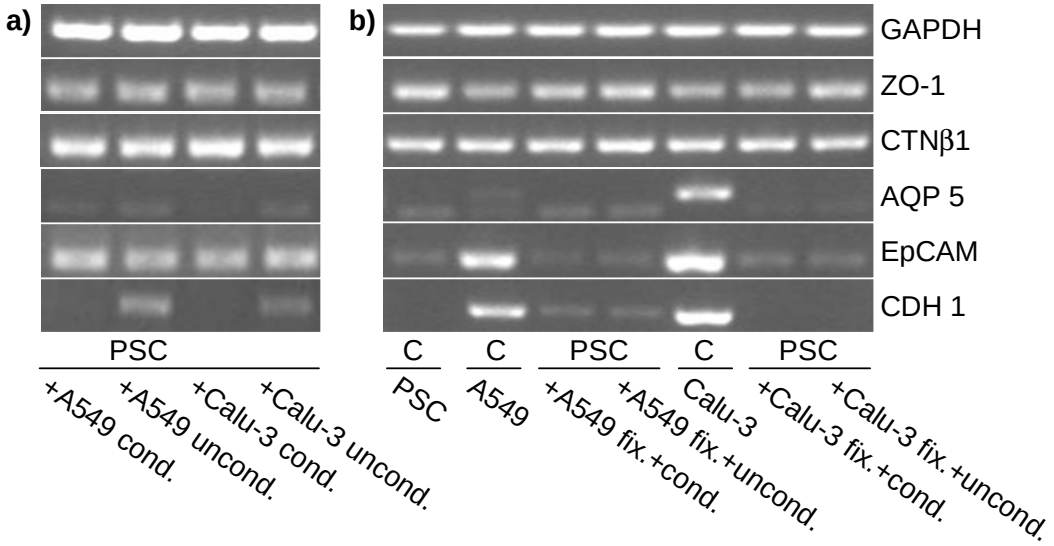

Figure 22: mRNA expression of epithelial markers in PSC after application of epithelial differentiation protocols. Samples were taken after PSC were cultured on TCP (a) and on fixed A549 and Calu-3 (b) with conditioned or unconditioned medium of A549 and Calu-3 for 12 days and were analyzed by means of RT-PCR. Untreated PSC, A549 and Calu-3 served as control (C).

5.5.2.3 EPITHELIAL POTENTIAL OF MSC

Firstly, the influence of conditioned medium was investigated. At mRNA level (Figure 23a) MSC control showed bands for all markers, although expression of AQP5 was very weak. Expression of CDH 1 stayed constant for MSC cultured in unconditioned medium (+ A549 uncond / + Calu-3 uncond), but vanished for MSC incubated in conditioned medium of either A549 or Calu-3 (+ A549 cond / + Calu-3 cond). Bands for ZO-1, CTNβ1, EpCAM and AQP 5 stayed mainly constant. Only the expression of EpCAM in MSC + Calu-3 cond became weaker and the expression of AQP 5 in MSC + A549 uncond grew more intense. At protein level (Figure 23c) MSC control expressed also AQP5 and EpCAM, however, the band for CDH 1 was very weak. As already seen at mRNA level the expression of CDH 1 in MSC + uncond was very intense, while bands for MSC + cond were very weak. Expression of EpCAM and AQP 5 was nearly invariable.

Secondly, differentiation triggered by the surface of fixed epithelial cells was investigated. At mRNA level (Figure 23b) MSC control displayed CDH 1 and EpCAM but nearly no AQP 5. For CDH 1 MSC + A549 fix + cond showed a distinctively triggered expression and MSC + Calu-3 fix + cond and MSC + Calu-3 fix + uncond displayed nearly no expression at all. In

MSC + Calu-3 fix + cond the band for EpCAM was increased, while the other bands did not vary. Nearly all MSC samples showed a higher expression of AQP 5 compared to control apart from MSC + Calu-3 fix + cond. At protein level (Figure 23d) MSC control displayed neither CDH 1 nor AQP 5, but EpCAM in very small amounts. Apart from the Calu-3 control other samples showed solely hardly visible bands for EpCAM. Expression of AQP 5 was weak but visible for all MSC samples in particular for those + A549 fix. However, both samples of MSC + A549 fix + cond, MSC + A549 fix + uncond showed an intelligible expression of CDH 1. MSC samples cocultured with Calu-3 showed also an expression even though very weak.

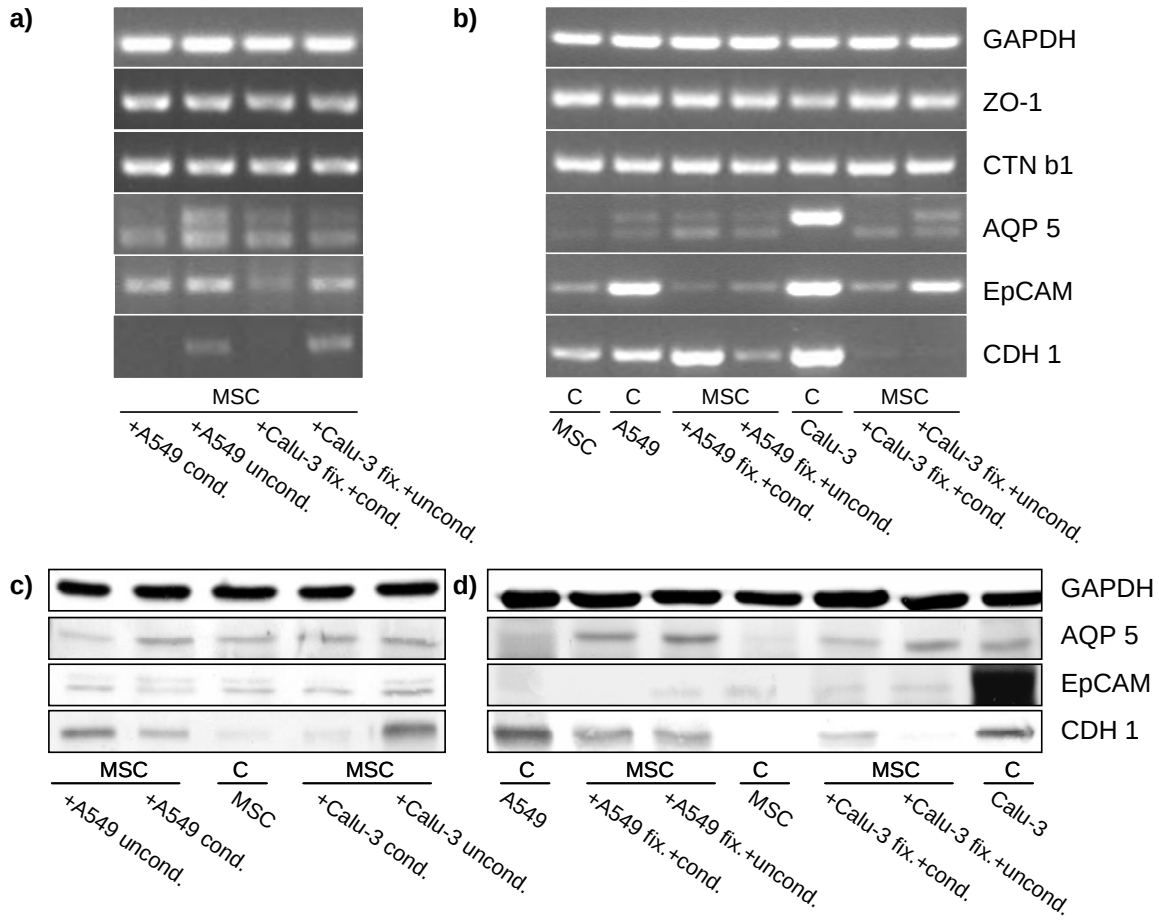

Figure 23: mRNA (a, b) and protein expression (c, d) of epithelial markers in MSC after application of protocols for epithelial differentiation. mRNA samples were analyzed by means of RT-PCR and protein samples by means of Westernblot. MSC were cultured on TCP (a, c) and on fixed A549 and Calu-3 (b, d) with conditioned or unconditioned medium of A549 and Calu-3 for 12 days. Untreated PSC, A549 and Calu-3 served as control (C).

Finally, an immunocytochemical investigation for CDH 1 was performed in order to evaluate the localization of this protein in the native cells. Exemplary the results for Calu-3 and MSC control and for MSC + Calu-3 cond and +Calu-3 uncond are shown (Figure 24). Calu-3 control showed a distinct accumulation of CDH 1 at the cell membrane. The same could be shown for all MSC samples, which had already an intelligible protein expression determined by Westernblot. The MSC control displayed no immunofluorescence for CDH 1 at all. And also the samples of MSC +Calu-3 cond and MSC + A549 fix + uncond showed only a diffuse binding of the antibody. MSC control displayed no expression at all. All samples, which had already a distinct protein expression determined by Westernblot, showed a similar accumulation of CDH 1 at their membrane.

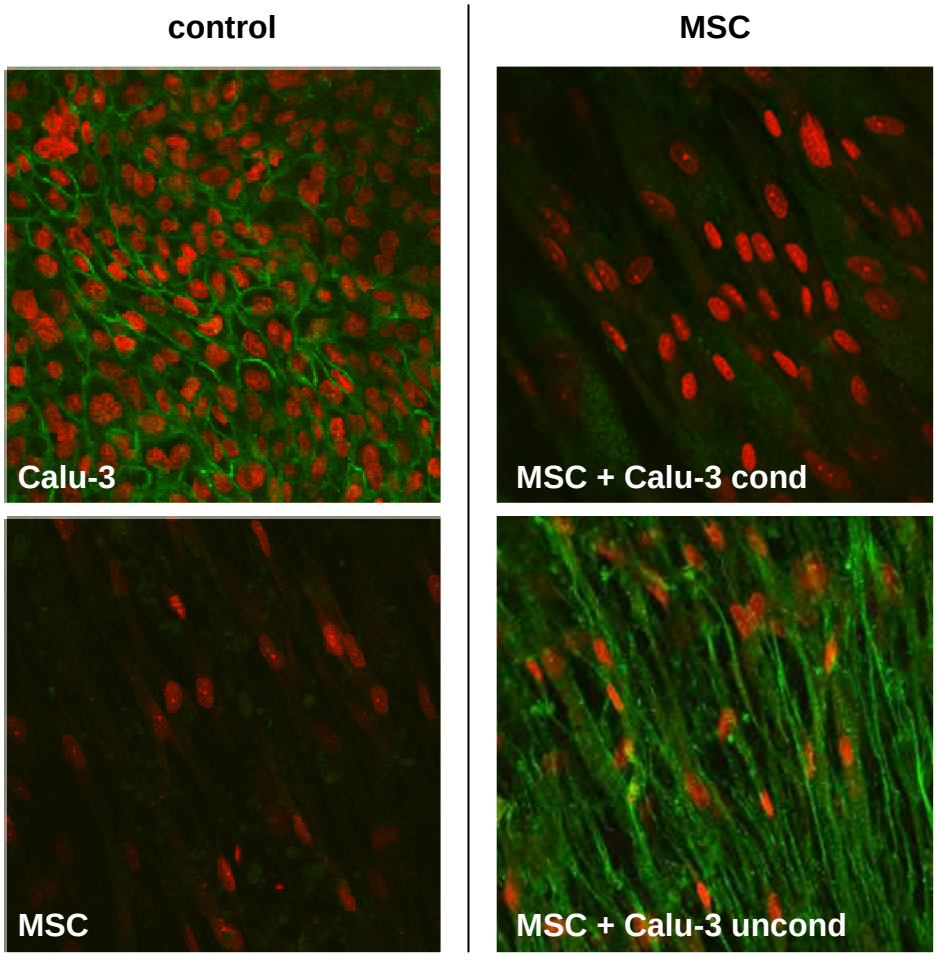

Figure 24: immunocytochemical determination of the expression of CDH 1 at protein level in MSC, which were cultured with conditioned and unconditioned medium of Calu-3. Calu-3 and MSC in normal culture medium served as control.

5.5.3 DISCUSSION

So far there is no established protocol to differentiate human adult stem cells into lung-epithelial cells. Such cells could be of use for cytotoxicity testing and for transport studies, since sources for adequate models are scarce. One possible approach is cocultivation of stem cells with a fully differentiated second cell type. These protocols have often proved to influence direction of differentiation [142-146] and even coculture with lung cells has been investigated [146, 153, 154]. Hematopoetic stem / progenitor cells, for example, express lung-epithelial cell specific mRNA and protein when either cultured in lung tissue conditioned medium or in coculture with lung tissue [155]. For MSC many publications show an impact of coculture on the course of differentiation. When indirectly cocultured with hepatocytes, they differentiate to hepatocytes[156]. In indirect coculture with injured cortical tubular renal epithelial cells, the developed a tubular epithelial-like phenotype [157]. Also PSC showed to be prone to influence of coculture. They were successfully differentiated to cardiomyocytes when cocultivated with biopsies of human myocardium. [148]. All things considered coculture seemed to be a promising approach for differentiation of human adult stem cells to lung epithelial-like cells. Therefore, MSC and PSC were subjected to direct and indirect coculture with the lung-epithelial cells lines A549 and Calu-3.

PSC and MSC displayed nearly no variations in the expression of ZO-1, CTNβ1, AQP 5 and EpCAM. Only the bands for AQP 5 in MSC + A549 uncond became slightly stronger and the expression of EpCAM in MSC + Calu-3 cond was marginally weaker. Within the expression of CDH 1, however, there were distinctive alterations, which were nearly identical for PSC and MSC. One difference was the expression of CDH 1 in the stem cell control after 12 days of culture. MSC showed a band for this marker at mRNA but not at protein level, whereas PSC displayed no expression. The earlier performed characterization (see Chapter 5.3.2.1) and the incubation of PSC on ECM (see Chapter 5.3.2.2) showed an increasing expression of CDH 1 depending on incubation time. This was possibly due to the growing density of cells and the thereby increasing cell-cell contacts (see Chapter 5.3.3).

Neighborhood of cells of the same cell type, though, is obviously supplemented or even outweighed by other factors, namely the presence of a second cell type, either by direct contact or just by its conditioned medium. Cultivation of PSC on fixed A549 triggered synthesis of CDH 1 after 12 days, while culture on fixed Calu-3 had no comparable effect. An influence of the conditioned medium was not observed. Nearly the same expression profile was seen for MSC, merely cells on fixed Calu-3 displayed hardly visible bands for this

marker. Heterotypic cell contact, therefore, obviously modifies expression of CDH 1 in these stem cells.

Also the type of medium was pivotal when stem cells were cultured in conditioned medium of epithelial cells for 21 days on TCP. Only stem cells incubated in unconditioned medium displayed bands for CDH 1. As shown before, PSC (see Chapter 5.3.2.2) express CDH 1 after 21 days of culture and MSC already after 12 days (5.5.2.3). Conditioned medium obviously suppressed its synthesis.

For interpreting these results it is important to focus on the role of CDH 1. The family of cadherins belongs to cell adhesion molecules which are important for the dynamic regulation of adhesive contacts associated with morphogenetic processes. CDH 1 (also named epithelial or E-cadherin) is primarily expressed in epithelial cells, where it is associated with the zonula adherens of the epithelial junctional complex. [158]. The extracellular region of CDH 1 connects the cell surface with cadherins on adjacent cells [159]. The intracellular region interacts with catenins and other regulatory proteins [160]. CDH 1 is essential to form a tight, polarized epithelial cell layer by Ca^{2+}-dependent cell adhesion [161, 162], which can perform barrier and transport functions.

Another function of CDH 1 has to be taken in consideration. It is involved in epithelial to mesenchymal transitions (EMT), where differentiated epithelial cells undergo a phenotypic conversion that gives rise to the matrix-producing fibroblasts and myofibroblasts. This process plays a crucial part in ontogenesis, tissue fibrosis and cancer progression. Loss of CDH 1 facilitates EMT, whereas induced expression of CDH 1 can reverse the transformed phenotype [163, 164]. In regards to cancer, CDH 1 restricts cell movement by promoting tight cell-cell adhesions and therefore reduces metastatic activity [165]. A reduced expression of CDH 1 leads to a looser contact to adjacent cells and therefore increases the motility of these cells supporting a more invasive cell type [166]. Tumor cell lines and biopsies show lower CDH 1 levels compared to normal tissues [167, 168]. Furthermore expression of CDH-1 is opposed to presence of plasminogen activating inhibitor I, whose finding correlates with poor cancer prognosis. In short CDH 1 is regarded as a tumor suppressor gene [158].

Having these properties of CDH 1 in mind, it is obvious, that cell surface and conditioned medium of epithelial cancer cells have a reverse influence on the investigated stem cells. Conditioned medium of A549 and Calu-3 suppresses synthesis of CDH 1. This may lead to an increased motility and even to a more invasive cell type. Which factors are responsible for this effect has not been investigated, yet. However, isolation of these mediators could reveal

mechanisms of altered signal transduction during cancer development. Further studies should therefore try for identification.

The contrary effect was provoked by the surface of A549. CDH 1 expression was enhanced in PSC and maintained in MSC. This could imply an accelerated differentiation into an epithelial-like cell type. Prior studies presented influence of A549 on stem cells. ESC differentiated to a higher scale to endoderm, when cultured in A549 conditioned medium containing activin than with activin alone [153]. And even MSC developed epithelial-like characteristics when indirectly cocultured with heat-shocked A549 [169] and likewise in direct coculture with CFBE41o- [170]. However, this is the first study to report an influence of coculture type on the differentiation: influence of substrate obviously outweighs the impact of conditioned medium during the first 12 days. Further experiments could reveal whether this dominance remains during longer cultivation periods.

One remaining question is whether the CDH 1 suppressing effect of A549 and Calu-3 conditioned medium is due to the malignant origin of the tumor cell lines. A coculture with nontumor-derived primary cells, e.g. HAEpC [66], could help clarification.

5.6 RÉSUMÉ

This part of the thesis explored the potential of PSC and MSC to give rise to endothelial and epithelial-like cells. While MSC have already been applied in research for several decades, PSC had just been discovered at begin of this thesis. However, none of this cell types had before been differentiated to lung epithelial-like cells and only one rather "shaky" protocol had been described for the differentiation of MSC into endothelial cells.

Prior to any differentiation experiment, characterization of PSC revealed a spectrum of immanent endothelial, epithelial and stem cell marker expression. Culture on different ECM led to minor changes only. Culture by means of Air Interface Culture, however, induced significant morphological alterations. Spinning of PSC resulted in three-layered spheroids, resembling EBs, that showed signs of spontaneous differentiation.

None of the multiple approaches for inducing endothelial differentiation were adequate for increasing endothelial characteristics of PSC or MSC. However, endothelial potential of PSC was already present in untreated cells and was not limited to presence of endothelial markers at mRNA and protein level. Additionally, both stem cell types formed spontaneously capillary-like structures, which is considered a substantial characteristic of endothelial cells. Hence, for triggering the endothelial potential of these stem cells more adequate protocols have to be developed.

Coculture with the epithelial cell lines A549 and Calu-3 led not to a complete epithelial differentiation, but to varying expressions of CDH 1. This epithelial marker is also known to be decreased in metastatic cells. Only contact to fixed A549 increased expression of CDH 1. Incubation with fixed Calu-3 did not provoke any changes, while conditioned medium of both lung cancer cell lines suppressed expression of this marker. However, it can be concluded that type of coculture and choice of applied epithelial cells influence the direction of differentiation of PSC and MSC.

Both types of stem cells display epithelial- and endothelial potential by expressing corresponding lineage markers already endogenously. The endothelial appearance is even increased by displaying the endothelial characteristic of building tube-like structures. However, protocols tested in this thesis have to be developed further to lead to optimal results.

6 ZO-1 ISOFORM EXPRESSION IN EPITHELIAL CELL LINES AND STEM CELLS.

Parts of this section have been published in:

Ciana, A.*, Meier, K.*, Daum, N. Gerbes, S., Veith, M., Lehr, C.-M., Minettei, G., A dynamic ratio of the alpha+ and alpha- isoforms of the tight junction protein ZO-1 is characteristic of Caco-2 cells and correlates with their degree of differentiation. Cell Biol Int, 2010. 34(6): p. 669-78.[171]

*equally contributing authors

6.1 INTRODUCTION

The animal body consists of four major tissue types: muscle, nerve, connective and epithelial tissue. In human 60% of the approximately 200 different cell types are epithelial. All but one of these epithelial cells are derived form ectoderm and endoderm during ontogenesis. Solely, the endothelium descends from the mesoderm. However, all epithelia have at least one function in common. They separate fluids of different composition on either side, acting as selective permeable barriers. To achieve this performance epithelial cells need to be sealed closely together, which in vertebrates is performed by tight junctions (TJ). These junctions combine two functions. First, they create the primary barrier to the diffusion of molecules through the paracellular pathway, and, second, maintain cell polarity as a boundary between apical and basolateral plasma membrane domains [172, 173]. TJ are located at the apical side, are embedded in each of the two adjacent plasma membranes and consist of more than 40 different protein types. The two major transmembrane proteins in TJ are claudins and occludin [174, 175]. They associate with the intracellular peripheral membrane proteins, known as *zona occludens* (ZO) proteins. ZO proteins belong to a family of membrane-associated guanylate kinase (MAGUK) homologs, which are found in membrane-associated proteins acting as adapters and molecular organizers in epithelial and endothelial junctions. The three closely related ZO-1, ZO-2 and ZO-3 have so far been identified as members of the ZO protein family [176]. For ZO-1 the following is known. It is a ~220 kDa protein found in the centre of macromolecular assembly of TJ [177]. Apparently it combines a structural and a regulatory role. First, it binds to integral membrane proteins of adjoining cells (occludin,

claudins and junction adhesion molecules) and to the cytoskeletal actin [97]. Second, it modulates cell proliferation through association with the transcription factor ZONAB [178]. The gene encoding for ZO-1 (TJP1) is found in the human genome at chromosome 15 at 15q13 [179]. Its transcript exists in two alternatively sliced isoforms differing by an internal 80 amino acids domain known as the α domain [180, 181]. Relative abundance of the two isoforms seems to be tissue specific. ZO-1 α^- is found as the only or prevailing form in TJ of endothelial cells and in highly specialized cells like Sertoli cells or in podoyctes of the renal glomeruli. ZO-1 α^+ is the dominant isoform in epithelial cells, irrespective of the displayed transepithelial electrical resistance (TEER) [180]. However, a relation to junctional plasticity was postulated. This is the ability of a junction to open and reseal in response to certain stimuli. It was proposed, that α^+ isoform was expressed in TJ of high and α^- in TJ of low plasticity. However, up to present there is no information on possible different functions of the two isoforms.

Since the 1980s the human intestinal cell line Caco-2 has been extensively used as a model of the intestinal barrier. The parental cell line was obtained in the 1970s from an adenocarcinoma of a 72 year old male Caucasian [182]. The peculiar property of Caco-2 is their ability to undergo spontaneous differentiation, after which they display several morphological and biochemical characteristics of small intestinal enterocytes [183]. They grow in monolayer and display a cylindrical polarized morphology with microvilli on the apical side and TJ between adjacent cells. Moreover, they express enzyme activities of the small intestine brush boarder (i.e. sucrose-isomaltase, lactase, aminopeptidase N, dipeptidylpeptidase IV, alkaline phosphatase). Due to all these properties Caco-2 are widely used for biopharmaceutical purposes such as toxicity, transport and proliferation studies and are furthermore recognized by the US FDA as a part of bioequivalence waiver process [184]. One of the most recent extensive applications of Caco-2 as a test system for biocompatibility was during the CellPROM project within the 6th Framework Programme of the European Commission [185], during which also this thesis was generated. CellPROM – short for “Cell Programming by nanosclaed devices – tried to reprogram cells by non-invasive means on an industrial scale. Thereby magnetic carriers have been developed, which allowed the handling of cells in a microfluidic system. Several generations of these carriers were tested with Caco-2 for their biocompatibility before the final model was identified. Not only morphology, cell number and proliferation of Caco-2 were investigated but also functional differentiation. This was determined by means of activity of the brush border enzyme aminopeptidase N and by

expression of the proteins integrin ß1, villin and also ZO-1. No differential expression of the two ZO-1 isoforms was analyzed, though.

So far, the expression of the two ZO-1 isoforms was mainly investigated for a certain cell type or tissue at a given time point at usually quite progressed growth and differentiation stages. This static representation of data led to a proposition of an α^+/α^- ratio, which is invariant and even characteristic for the respective cell type [181]. While investigating a different issue in the Department of Biochemistry at the University of Pavia, Italy, it was found that during cultivation of Caco-2 the amount of expressed ZO-1 isoforms differed considerably. This observation was contrary to all other reports published previously. Therefore, the institute of Pavia and the Institute of Biopharmaceutics and Pharmaceutical Technology, Saarland University, Germany designed a cooperative study. Aim of this study was to answer the questions, where this behaviour resulted from, what other parameters it correlated with and whether it was peculiar for Caco-2. Dynamic expression of ZO-1 isoforms was investigated at mRNA and protein level during *in vitro* proliferation of cells. Not only Caco-2 were analyzed but in total 6 cell types in order to elucidate, whether only Caco-2 showed this property. Three other epithelial cell lines were chosen, T84 as a further model of an intestinal cell line and A549 and Calu-3 representing the lung epithelium. ECV304 were a model of an endothelial cell line and PSC (pancreatic stem cells) as primary stem cells completed selection of cell types. In Saarbrücken all epithelial cell lines and also PSC were investigated for a relationship between ZO-1 isoform expression and cell number / density, proliferating activity, TEER value and expression of another TJ protein, namely occludin, over a period of 16 culture days. In Pavia additional experiments were performed comprising the expression of ZO-1 in ECV304 and the correlation between isoform expression in Caco-2 and their grade of functional differentiation. Experiments carried out in Saarbrücken are explicated extensively in the following Chapter 6.2 Material and Methods and the Results (6.3) part. Results from Pavia are summarized and implicated in the Discussion (6.4).

6.2 MATERIALS AND METHODS

6.2.1 ROUTINE CELL CULTURE AND BASIC CELL CULTURE DURING EXPERIMENTS

Cells applied in this study were the colon derived cell lines Caco-2 and T84, the lung derived cell lines A549 and Calu-3 and the pancreas derived primary stem cells PSC.

Caco-2 (ATCC-No: HTB-37) were maintained in RPMI-1640 – High Glucose with L-Glutamin (RPMI, GIBCO) and supplemented with 10 % FBS (PAA). They were subcultured in a ratio of 1:10.

T84 (ATCC-No: CCL-248) were maintained in Dulbecco's modified Eagle's medium / F12 (DMEM / F12) supplemented with 5 % FBS (PAA). They were subcultured in a ratio of 1:5.
A549 (ATCC-No: CCL-185) were maintained in RPMI-1640 - High Glucose with L-Glutamin (RPMI, GIBCO) supplemented with 10 % FBS (PAA). Subculture ratio was 1:50.
Calu-3 (ATCC-No: HTB-55) were maintained in RPMI-1640 - High Glucose with L-Glutamin and without phenolred (PAA) supplemented with 10 % FBS GOLD and 1 mM sodium pyruvate (Lonzy, Basel, Switzerland). They were subcultured in a ratio of 1:7.5.
The adult human stem cells PSC were a generous gift from Fraunhofer Institut St. Ingbert, Germany. Cell isolation was described by Kruse et al. in 2004 [1]. All experiments were performed with cells from passages 13 to 23 from the clone Cepan 3b. PSC were maintained in a primary medium consisting of Dulbecco's modified Eagle's medium - High Glucose with L-Glutamin (DMEM, GIBCO Invitrogen; Karlsruhe, Germany) supplemented with 10 % fetal bovine serum gold (FBS GOLD), 100 units/ml penicillin and 100 µg/ml streptomycin (all from PAA; Pasching, Austria). PSC were seeded at a density of 5 x 10^5 in T-75 cell culture flasks and routinely subcultured at 80 to 90 % confluence.
All cells were routinely incubated at 37°C in 5 % CO_2 and regularly tested for mycoplasm infections. Subculturing was performed once and medium was changed three times a week.
For setup of experiments all cells were seeded at a starting density of 3 x 10^4 cells/cm^2 in 12-well plates or, for determination of TEER, in Transwell® 12-well permeable supports (all from Corning, Schiphol-Rijk, Netherlands).

6.2.2 CELL NUMBER (CYQUANT AND HOECHST ASSAY)

A determination of cell number was required for two reasons. First, one possible criterion for the expression of different ZO-1 isoforms was the density of cells. Second, cell number was needed for the volume calculation of samples for Westernblot (see Chapter 6.2.7).
This happened by measuring at each time point of cell growth by an assay based on dye fluorescence enhancement upon binding to cellular nucleic acids (Figure 25). For all cells but Caco-2 (quantification was performed in Pavia by HOECHST assay, see below) CyQuant assay (CyQuant Cell Proliferation Assay Kit, Invitrogen, Karlsruhe, Germany) was applied. At the first step of cell lysis the protocol was slightly adapted from the manufacturer's manual. Instead of applying repeated freeze and thaw circles cell lysis was induced after rinsing with PBS by incubating cells at 37°C for 45 min with 50 % (v/v) CelLytic™-M (Sigma-Aldrich, Schnelldorf, Germany) solution in water. Subsequently, provided dye solution was added and cells were incubated for 20 min in the dark at RT. Supernatant was

then transferred to a 96-well plate and fluorescence was measured at 485 / 535 nm in a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany).
Calibration was performed by including standard DNA in the same microplate as the samples to be measured. Already before a conversion factor between DNA amount and cell number of the respective cell type had been determined. This was performed by comparing the fluorescence of the DNA standard with that of cell lysates obtained from cell suspensions, which cell number had been accurately counted.

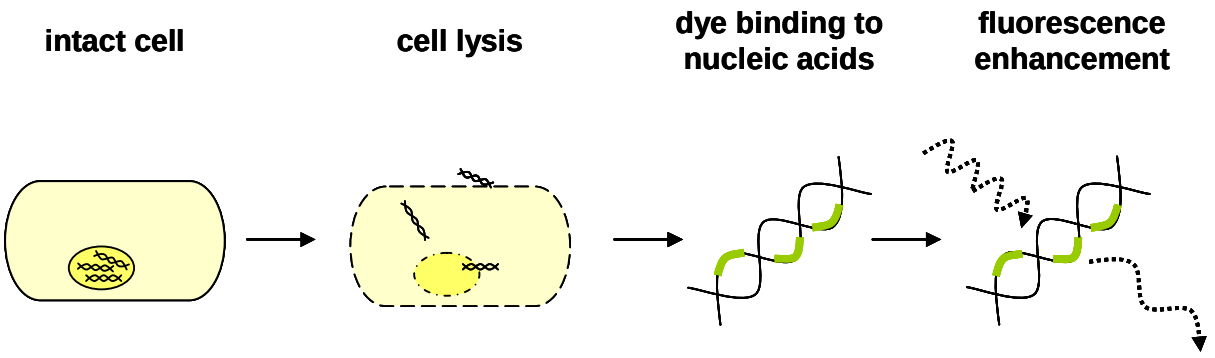

Figure 25: Schematic drawing of principle of CyQuant assay adopted from the manufacturers manual.

For the samples of Caco-2 cells the HOECHST assay, which is also based on fluorescence enhancement after binding to cellular nucleic acids, was performed in Pavia. Adherent cells were rinsed with PBS and lysed in the same lysis buffer as used for CyQuant assay at 37°C for 45 min. A solution of 5 µg/ml Hoechst 33258 dye (Sigma-Aldrich, Milan, Italy) was added, cell lysate resuspended and fluorescence read within 25 min at 360 / 465 nm in a microplate reader (GENios Plus, Tecan, Milan, Italy). The dye solution was prepared by step-wise addition of 1 mg/mL dye stock solution to pre-warmed TNE 2X (20 mM Tris/HCl, 4 M NaCl, 2 mM EDTA, pH 7.4), and subsequently diluting TNE with water to the final 1X concentration

6.2.3 CELL PROLIFERATION (BRDU COLORIMETRIC ASSAY))

Apart from cell number also the state of proliferation was of possible influence to the expression of ZO-1 isoforms.
For following the proliferation of cells an assay was performed, which exploits the incorporation of 5'-Bromo-2'-deoxy-uridine (BrdU) into newly synthesized DNA of replicating cells (**Fehler! Verweisquelle konnte nicht gefunden werden.**). This Cell Proliferation ELISA, BrdU (colorimetric) assay (Roche, Basel, Switzerland) was applied according to the manufacturer's manual. In short, a BrdU labeling solution was added to cells

for 24 h, before fixing and incubating the cells with anti-BrdU-peroxidase solution. Finally, cells were incubated for 15 min with a substrate solution until reaction was terminated by adding a stopping solution. Absorbance was then measured at 450 nm (Infinite 200 M multimode microplate reader; Tecan, Crailsheim, Germany).

6.2.4 TEER (TRANSEPITHELIAL ELECTRICAL RESISTANCE)

A previous publication [180] reported no correlation between the ratio of ZO-1 isoform expression and transepithelial electrical resistance (TEER). In order to reexamine this circumstance for cells applied in this study, values for TEER were measured with an epithelial volt-ohmmeter (EVOM, World Precision Instruments, Berlin, Germany). TEER of plain filters was subtracted as background.

6.2.5 RT-PCR (REVERSE TRANSCRIPTASE- POLYMER CHAIN REACTION)

For analyzing the gene expression status of the two ZO-1 isoforms in the different cell types RT-PCR was performed.

For all samples but Caco-2 mRNA isolation was performed by means of RNeasy Mini Kit (Qiagen, Hilden, Germany). For synthesis of first strand cDNA Omniscript RT Kit (Qiagen) was applied. Both protocols were performed according to the manufacturer's manual. For samples of Caco-2 mRNA isolation and CDNA synthesis were performed in Pavia (see below). PCR was carried out with Taq PCR Master Mix Kit (Qiagen) in a final volume of 50 µl containing 25 pmol of each nucleotide primer. For specific amplification of ZO-1, β-actin (ACTB) and occludin (OCL), Taq PCR Master Mix was supplemented with 25 mM $MgCl_2$ to yield a final concentration of 2.5 mM $MgCl_2$ in reaction mixture. PCR of ZO-1 isoforms was performed with primers flanking the α motif [181] (Table 3), so that amplification products of 474 and 234 bp correspond to ZO-1 α+ and ZO-1 α– mRNA, respectively. PCR of ACTB resulted in products of 315 bp and for OCL in products of 294 bp. One PCR cycle was: 94°C for denaturation, 60°C for annealing and 72°C for extension, with a total of 40 cycles for ZO-1 and 30 cycles for β-actin. For occludin, the PCR cycle was: 95°C for denaturation, 52°C for annealing and 72°C for extension in a total of 35 cycles. All amplifications were carried out in PTC-200 Peltier Thermal Cycler (MJ Research, GMI, Minnesota, USA) (Figure 3).

Table 3: Specific primers used for RT-PCR

Primer name	RefSeq Ids	Primer sequences		Amplicon size [bp]
ACTB	NM_001101	F: 5'- tcctgtggcatccacgaaact-3'	R: 5'- gaagcatttgcggtggcaga-3'	315
OCL	NM_002538	F: 5'- ccgacacaccacacctacac-3'	R: 5'- tcgaacatgcatctctccac-3'	294
ZO-1 (named ZO-1-iso in	NM_175610	F: 5'-catagaatagactcccctgg-3'	R: 5'-gcttgaggactcgtatctgt-3'	234
Charter 5)	NM_003257			474

Isolation of mRNA from Caco-2 samples was performed in Pavia with 1 ml of TRIzol® (Invitrogen, Milan, Italy) for a maximum of 2 x 10^6 cells. First-strand cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Milan, Italy) according to the manufacturer's manual.

6.2.6 DENSITOMETRIC ANALYSIS OF RT-PCR DATA

PCR gels were examined by creating a density plot profile in ImageJ (National Institute of Health, USA). All data were corrected for background by subtracting values for an empty lane of the same gel. The graph was designed by means of SigmaPlot 10 (Systat Software, Inc., Germany) and densitometric intensity was defined by integration of the area under a peak corresponding to a band. Since mRNA bands were not for all cell types on the same gel, the detection intensity was not constant. A comparison or even ranking of the absolute values between the different cell types is therefore not possible (Figure 28). In order to enable at least a partial comparison among the cell types all values were related to a reference. This reference was calculated for each cell type by adding the values of both isoforms for every time point and creating a mean over the total period. This mean was then referred to as 100 % (Figure 29).

6.2.7 WESTERNBLOT

Since not only the gene expression of the ZO-1 motif is important but even more the presence of the protein a Westernblot was performed.

Sample taking and denaturating of protein was performed for all cells but Caco-2. SDS page and the actual blotting were conducted in Pavia (see below). For retrieving samples, adherent cells were incubated with 5 mM di-isopropylfluorophosphate in PBS (0.3 ml/cm^2) for 5 min and subsequently dissolved in 0.5 vol. of SDS-PAGE sample buffer (50 mM Tris/HCl, 5 % (w/v) SDS, 35 % (w/v) sucrose, 5 mM EDTA, 200 mM dithiothreitol and 0.01 % bromophenol blue, pH 6.8) by repeated pipetting. Sample taking was concluded be denturating proteins at 60°C for 15 min.

In Pavia sample volumes corresponding to 2.0 x 10^4 cells (for quantification see Chapter 6.2.2) were subjected to SDS-PAGE [186] in 8 % acrylamide gels. Sample volumes

corresponding to were loaded in each lane. The electrophoretic run was prolonged for 1 h after the front dye had reached the bottom of the gel in order to improve the separation of the two ZO-1 isoforms. Samples were electrotransferred to a 0.2 µm PVDF membrane, either taking the gels as a whole [187] or performing a multistrip Western blotting [188]. PVDF membranes were blocked with 20 mM Tris/HCl, 150 mM NaCl, 0.05 % Tween-20, and 5 % skimmed milk (w/v), pH 7.4, incubated overnight at 4°C with primary antibody. These primary antibodies used were mouse monoclonal anti-ZO-1 (Becton Dickinson, Milan, Italy) and rabbit polyclonal anti-occludin (Zymed Laboratories, Invitrogen, Milan, Italy). After incubation with the corresponding secondary antibody protein bands were visualized by means of ECL kit (Amersham, GE Healthcare, Milan, Italy). ZO-1 isoform ratio was calculated after densitometric quantification of protein bands on a digital image of films, using the software Scion Image (Scion Corporation, Frederick, MD, USA).

6.3 RESULTS

For investigating whether the shifting expression of ZO-1 isoforms is specific only for Caco-2 or whether it occurs also in other cell lines Caco-2 were compared with the following cell types: the epithelial tumor cell lines T84 (colon origin like Caco-2), A549, Calu-3 (both lung origin) and the stem cells PSC (primary cells derived from pancreas) (Figure 26). These cells types were analyzed in respect to shift of isoform expression at mRNA (Figure 27a, RT-PCR) and protein level (Figure 27b, Westernblot) in correlation to cell density (Figure 30a, CyQuant), proliferation (Figure 30b and c, BrdU) and TEER value (Figure 30d). In order to investigate whether other TJ proteins showed similar changes in expression levels during epithelial differentiation mRNA for occludin was also examined (Figure 27a). Culture of cells, sample taking for RT-PCR and Westernblot, performance of RT-PCR, CyQuant (cell number), BrdU (proliferation) and measurement of TEER were performed in Saarbrücken. Pavia accomplished the Westernblots and provided the Caco-2 samples for RT-PCR.

As shown in Figure 27 amounts of ZO-1 isoforms varied for all cell types during the observation period of 16 days. Changes at mRNA level (Figure 27b) were paralleled by corresponding shifts at protein level (Figure 27a).

Since visual judgment of the gels is quite imprecise, methods for a better illustration of mRNA results were applied. First, the bands of the gels were transcribed to a densitometric plot (Figure 28). Intensification and decrease of a signal is much easier to judge in this depiction. However, important to consider is, that mRNA samples for all cell types were not run on one identical gel. It is not guaranteed that the used intensification of signal was constant. The height of peaks can therefore be compared within the particular cell type but not

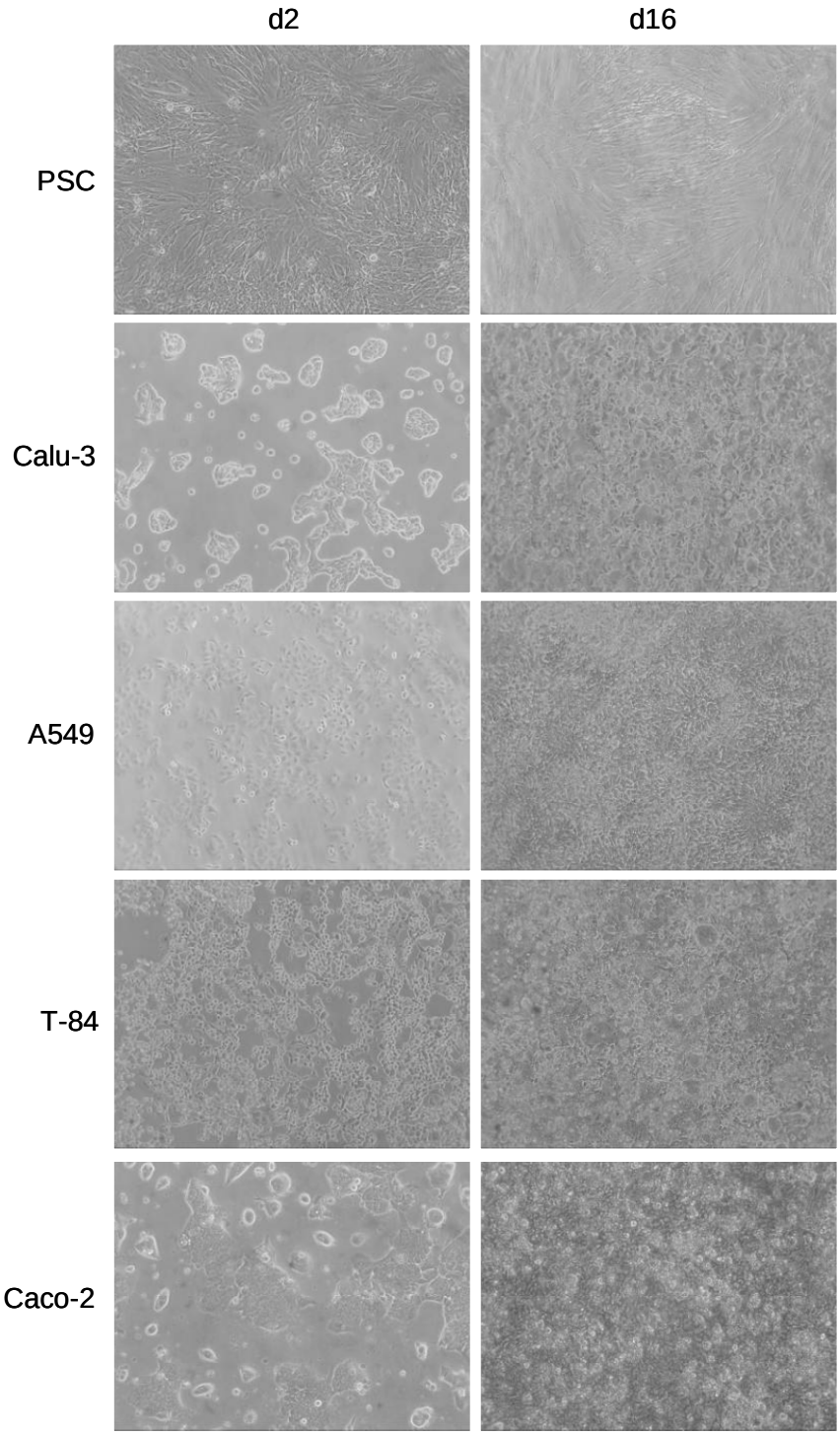

Figure 26: Morphology of Caco-2, T84, A549, Calu-3 and PSC 2 and 16 days postseeding determined by lightmicroscopy.

between the different cell types. For a better quantification and also comparability of data, the relative amount of mRNA was defined by calculating the area under the curve of the corresponding peak. This value was then related to a mean (see 6.2.6) and displayed as a percental value (Figure 29 a-e). Also the α^+ / α^- ratio was calculated and shown in Figure 29f.

From these three visualizations of results several conclusions could be drawn. First of all Caco-2 showed the most noticeable change during the observation period. While the total amount of ZO-1 was nearly constant, the expression of isoforms underwent an extreme shift. The dominating α^- isoform of day 2 and 4 (α^+/α^- = 0.56 and 0.60) decreased during the course of time and the initially only marginally expressed α^+ isoform increased continuously (α^+/α^- at day 16 = 2.09). The change point in prevalence was on day 7 when both isoforms were expressed at nearly identical levels (α^+/α^- = 0.95). All other epithelial cell lines showed a permanent prevailing of α^+ over the whole observation period. However, also T84 and Calu-3 show a similar development in expression of isoforms as Caco-2. In both cell lines α^+ isoform increased at least slightly (with a major recess at day 4), while α^- decreased simultaneously. These opposed developments resulted in a slight decrease of total ZO-1 (with a major recess at day 4 as seen for the α^+ isoform). The α^+/α^- ratio for T84 changed from 1.38 to 2.34 (day 4 = 1.07) and for Calu-3 from 2.04 to 3.44 (day 4 = 2.02). A549 showed a constant increase of both isoforms and therefore also of total ZO-1. The α^+/α^- ratio varied inconsistently between 3.68 and 5.40 (day 2 = 4.41, day 4 = 5.40, day 7 = 4.59, day 11 = 3.78, day 16 = 3.68). PSC, the only not epithelial cell type, showed no major changes in isoform and total ZO-1 expression nor in α^+/α^- ratio (variation between day 4 = 0.9 and day 16 = 1.3).

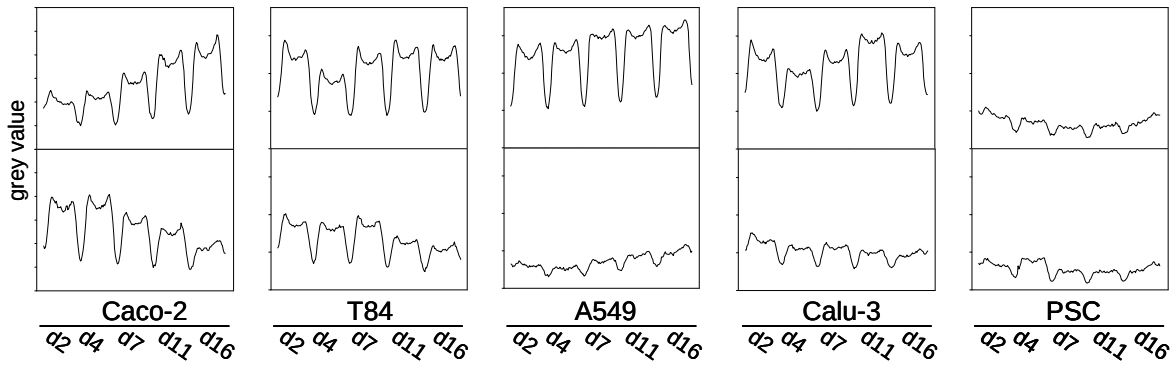

Figure 27: Expression of ZO-1 isoforms and occludin in Caco-2, T84, A549, Calu-3 and PSC over a period of 16 days. Amount of ZO-1 was determined at protein level by means of WesternBlot (a). At mRNA level expression of ZO-1 and also of OCL was analyzed by means of RT-PCR.

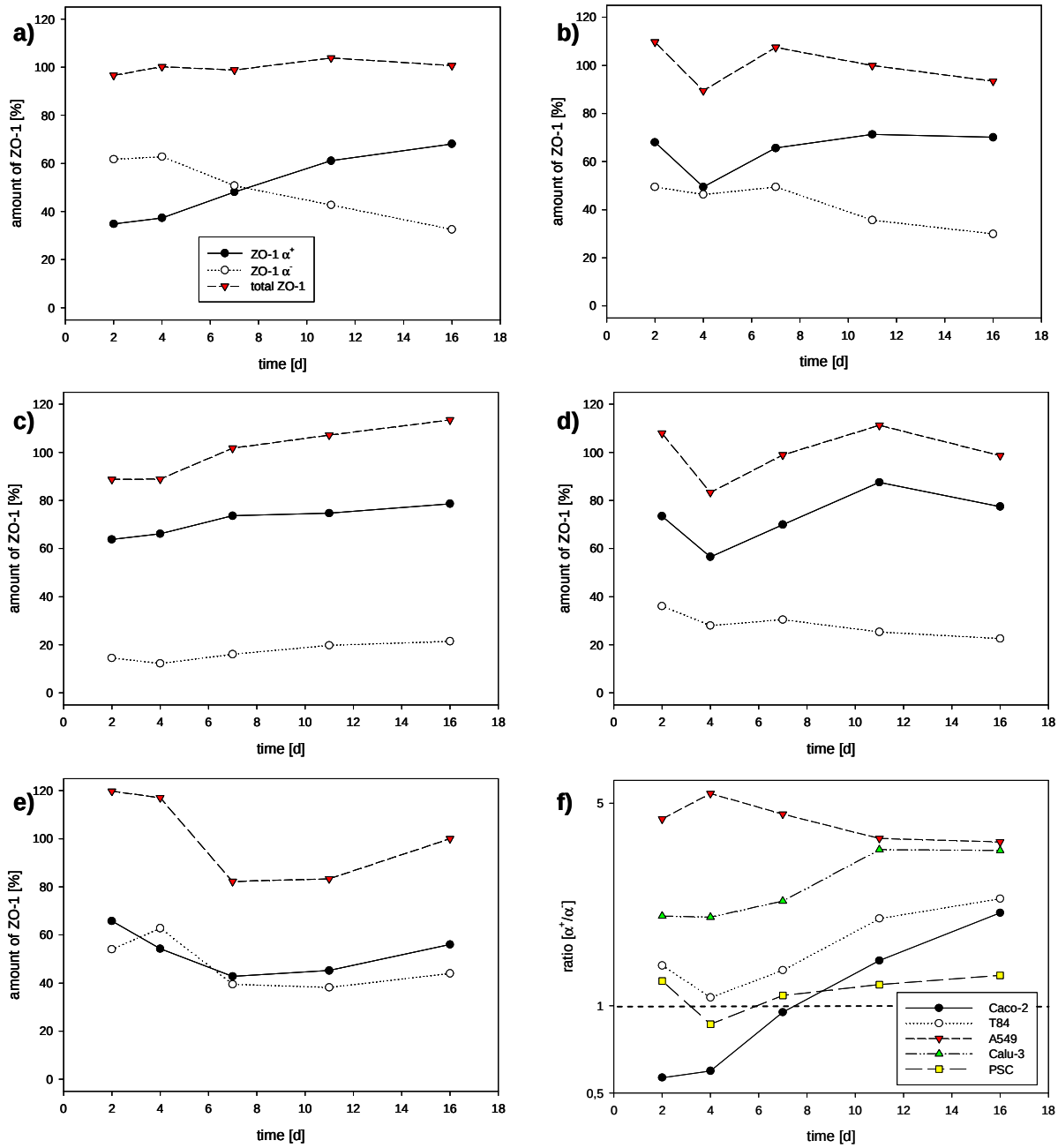

Figure 28: Densitometric plots of ZO-1 isoform RT-PCR bands in Caco-2, T84, A549, Calu-3 and PSC. The upper part refers to α+ isoform, the lower to α-. Grey value of y-axis is given nondimensional by ImageJ.

Thereby, Caco-2 is the only cell type where an inversion of the isoform ratio was observed. Even though T84 and Calu-3 showed a similar development of isoforms no ratio turnover took place, since amount of α^+ was already initially above amount of α^-. All other investigated epithelial cell lines had this prevailing of α^+ isoform in common.

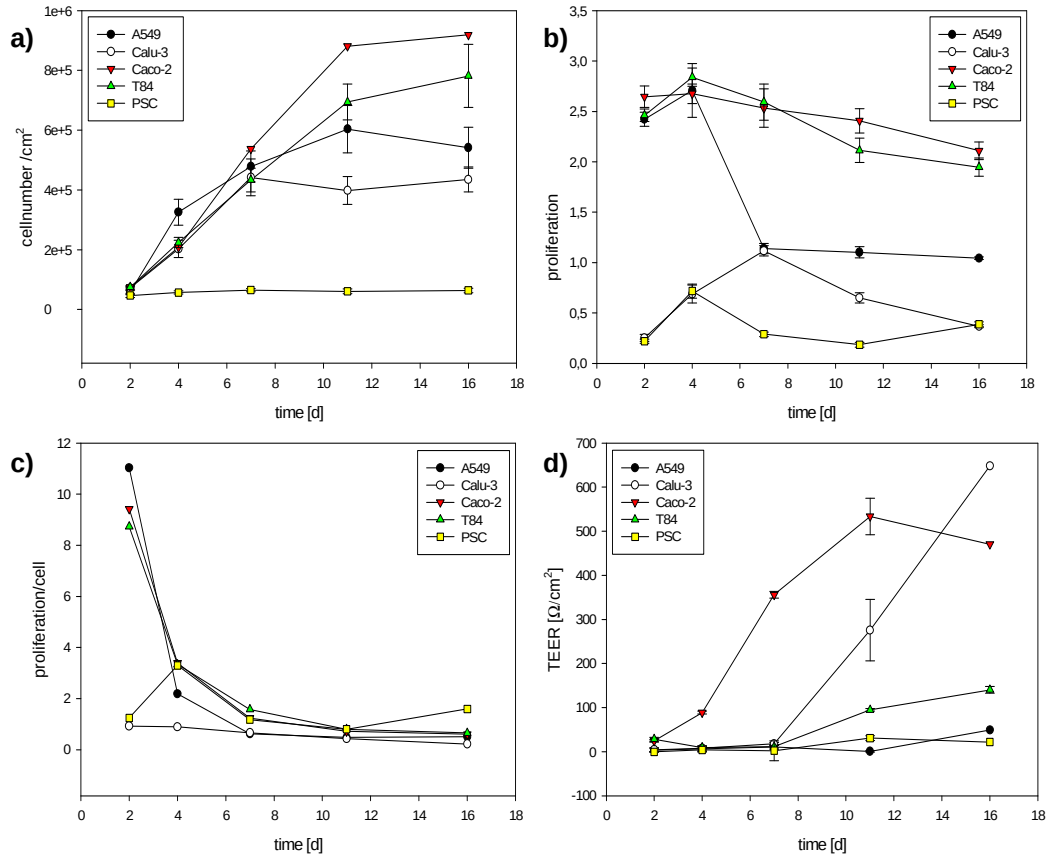

Figure 29: Graphic depiction of the change in expression for both isoforms and the total amount of ZO-1 for Caco-2 (a), T84 (b), A549 (c), Calu-3 (d) and PSC (e). Figure (f) displays a survey of the ratio of $\alpha+/\alpha$ for all cell types in a logarithmic scaling. All values relate to the densitometric intensity as defined by calculating area under the curve of the corresponding peaks in Figure 28.

Such distinct changes as seen for ZO-1 were not observed for the other tight junction protein investigated, occludin (Figure 27). All epithelial cell lines expressed occludin at mRNA level over the whole period of 16 days. A clear increase was shown for Calu-3 only, while all other cell lines displayed a constant expression. For PSC no band was detected at all. Therefore, no apparent relationship between amount of occludin and ZO-1 isoforms was found.

After the development in ZO-1 isoform expression for all cell types had been analyzed, the question arose whether any correlation to cell number / density, proliferating activity, density of cell layer, determined by means of TEER value, was detectable. The observations corresponding to these criteria were the following.

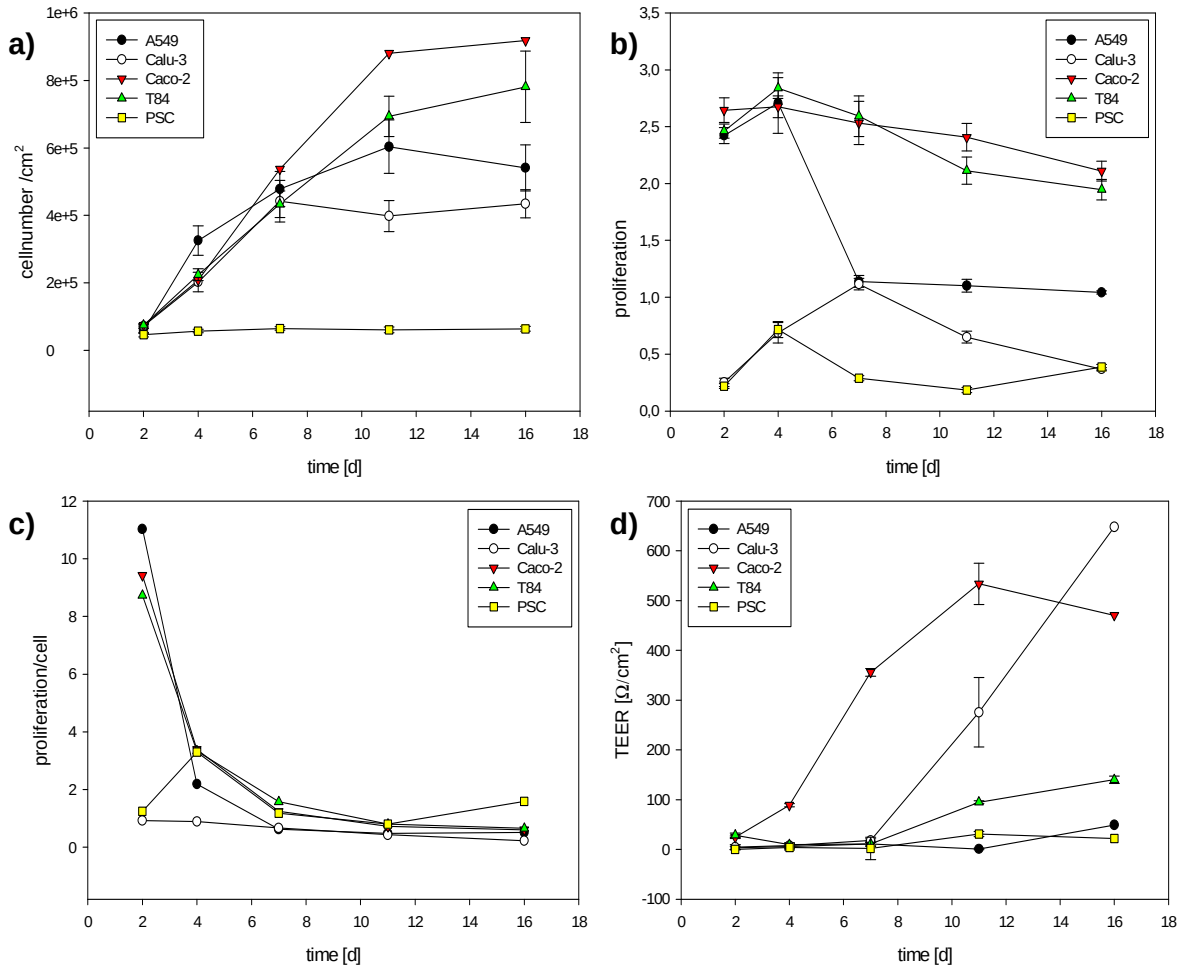

Figure 30: Over a period of 16 days Caco-2, T84, A549,Calu-3 and PSC were investigated for cell number/cm2 (a, CyQuant), total cell proliferation (b, BrdU) relative cell proliferation (c) and TEER (d). Graphs show the average of three independent experiments and error bars indicate SD.

The number of cells increased strongly for all epithelial cell lines in the beginning of the growth period and leveled off from day 7 for A549 and Calu-3 and from day 11 for Caco-2 and T84 (Figure 30a). Although total proliferation (Figure 30b) increased permanently throughout growth of cells, proliferation related to number of cells (Figure 30c) decreased drastically after day 2. TEER values for Caco-2 increased up to day 11 and weakened slightly thereafter, while Calu-3 displayed a very strong and constant enhancement from day 7 on (Figure 30d). A549 and T84 displayed no significant increase in TEER value at all. PSC showed no indicative changes in neither cell number nor proliferation nor TEER value (Figure 30a-d). Therefore, no obvious correlation between any of these criteria and expression of ZO-1 isoforms could be determined.

6.4 DISCUSSION

Previous experiments at the University of Pavia suggested that Caco-2 display a shifting ratio of ZO-1 isoforms. This, however, contradicts the accepted opinion in literature, where the ratio of ZO-1 isoforms is described as invariant and characteristic for the according cell type [181]. In order to investigate whether this observation was reproducible, whether it was particular for Caco-2 and what other parameter it correlated to, a cooperative study between Pavia and Saarbrücken was designed. In Saarbrücken not only Caco-2 but also the epithelial cell lines T84, A549 and Calu-3 and additionally the human primary stem cells PSC were investigated for the expression of ZO-1 isoforms over a period of 16 days. Caco-2 were the only cell type to show a complete turnover in the ratio of ZO-1 α+/α-. The initially prevailing α- diminished over time, while α+ increased to become the dominant isoform. All other epithelial cell lines showed a continuously prevailing α+. However, even here the ratio was not constant but varied considerably up to a factor of 2. PSC, as the only cells of a non-epithelial origin, displayed a balanced ratio of the two isoforms varying around 1. For all cells the ZO-1 protein expression was paralleled by corresponding bands at mRNA level. Hence, the protein expression was already initiated at the transcriptional stage and was not altered in the course of translational events. For none of the cell types a correlation was found between appearance of ZO-1 isoforms and any of the in parallel investigated factors, cell number / density, proliferation, TEER or occludin expression.

Major parts of these results have been published in 2010 together with the University of Pavia [171]. Only the densitometric analysis of mRNA data has been performed later resulting in a more precise view of amounts and ratio of ZO-1 isoforms. While the paper assumed that the isoform expression for all cells but Caco-2 was rather constant, densitometric analysis showed that all epithelial cell lines displayed a varying ratio of α^+/α^- up to the factor 2. Furthermore, the paper suggested a prevailing of α^- in PSC. Densitometric analysis revealed that both isoforms are found at similar levels.

However, the publication of Ciana, Meier et al [171] comprised more data on expression of ZO-1 isoforms. In addition to the already presented four epithelial cell lines and the primary stem cells, also ECV304 as a model for endothelial cells was investigated. The exclusive and constant high expression of α^- was in accordance to previous publications, which report of a solely or predominant occurrence of α^- in endothelia [180, 189]. Moreover, the matter was pursued, whether different growth surfaces had not only an effect on differentiation level of Caco-2, as previously reported [185, 190, 191], but also on isoform ratio shift. In a first experiment cells cultured upon permeable polyethylene therephtalate (PET) inserts displayed

an isoform expression comparable to cells cultured on polystyrene or glass. In a second experiment modified surfaces with nanostructured patterns reduced differentiation, as determined by aminopeptidase (APN) activity. This was mirrored by lower α^+/α^- ratios. This proves that indeed there is a correlation between differentiation level and ZO-1 α^+/α^- ratio in Caco-2. Not only the correlation between APN activity and α^+/α^- ratio during culture on nanostructured substrates hints in this direction but also an earlier report, that found little or no effect on expression of efflux pump P-gp and carrier mediated transporters for Caco-2 grown on permeable substrates [192]. This is concordant to the unaltered α^+/α^- ratio in Caco-2 upon permeable PET inserts.

Although both, Caco-2 and also expression of ZO-1 isoforms in various cell lines and tissues, have been investigated thoroughly in the past, up to date no time-dependant shift in the α^+/α^- ratio was described. Solely, the predominant or exclusive expression of either isoform has been reported. A basic rule said that α^+ was the dominant isoform in most epithelia, while α^- was found prevailing in endothelium [180, 181]. Their difference in function, however, could not be identified, yet. The function of ZO-1 in TJ in general is considered as that of a junctional organizer. It builds up a connection between the adhesion molecules (occludin and claudins) and the cytoskeleton and plasma proteins. It seems not to be essential for the construction of a belt-like zona occludens, though, as reported recently [193]. When ZO-1 biosynthesis in mouse epithelial cell line Eph4 was suppressed, TJ showed a normal organization of occludin and claudins and were formed without visible morphological changes. However, levels of ZO-2 were up-regulated and levels of cingulin, another scaffold protein of TJ, were down-regulated. In a further study Eph4 cells lacking ZO-1, -2 and -3 did not build up any TJ [194]. When expressed exogenously, though, both, ZO-1 and ZO-2 were able to determine independently whether and where claudins polymerized in epithelial cells. Moreover, Pulimeno et al [195] showed, that ZO-1 is essential for recruiting paracingulin to TJ. ZO-2 was of no relevance for this process. These data demonstrate that the two MAGUK proteins ZO-1 and ZO-2 show a functional redundancy up to some extent, even though each protein has its pivotal function.

ZO-1 is not only found in TJ of epithelial and endothelial cells, but frequently also at other places. First of all, it is localized in adherens junctions (AJ), where it is always associated with cadherin-mediated cell adhesion. In AJ it is found in non-epithelial cells like cardiac muscle cells and fibroblasts but also in epithelial cells, which do not express well developed TJ, i.e. hepatocytes [196, 197]. During maturation of epithelial cells ZO-1 is also pivotal for the conversion from loose “fibroblastic” AJ to belt like polarized epithelial cells [198]. Other

publications report findings of ZO-1 in the nucleus [181]. Here it binds to the ZO-1 associated nucleic-acid-binding protein (ZONAB), a Y-box transcription factor [199]. These factors interact with DNA and RNA and are involved in transcription, RNA stability and translation [200]. And indeed a regulation of proliferation by means of this pathway was found for MDCK cells [178].

When concentrating on the differential expression of the two isoforms, it becomes clear that so far no real evidence of the ZO-1 α motif has been found. In epithelial cells both isoforms are found to co-localize at membrane level [181], and there are also reports on a co-localization of α^+ with F-actin and of α^- with G-actin in testes of guinea pigs [201]. The prevailing expression of α^- in endothelial cell types was correlated to the junctional plasticity of the junction [180], which was defined as "the capacity to actively open and reseal the intercellular space or to move within the plane of the plasma membrane during normal physiological activity". A correlation to electrical resistance could not be educed, neither for epithelial nor endothelial cell types, since wide ranges for TEER are found in both endothelia and epithelia, which express predominantly either isoform. This was also underlined by another study, which examined the influence of vascular endothelial growth factor on ZO-1 isoform expression [189]. The two investigated cell types, vascular endothelial cells and retinal pigmented epithelial cells, responded conversely to VEGF, by increasing or decreasing expression of always both isoforms. An up-regulation was paralleled by an increase in TEER, a down-regulation by a decrease. Since both isoforms were regulated always in parallel, no indication of the differential role of the two isoforms was obtained. A similar observation was found in the present study. Four different epithelial cell lines and also PSC were investigated for the expression of ZO-1 isoforms and a parallel to TEER. No correlation was found, however. Neither a correlation to cell density, cell proliferation or occludin expression could be detected.

While the function of the two ZO-1 isoforms in mature cells is thus still open, an important role for ZO-1 and its isoforms was also found during ontogenesis. Its function was investigated thoroughly for the early mouse embryo [99], where temporal and spatial isoform expression was examined at different stages. ZO-1 α^+ was detected in oocytes and in all preimplantation stages. Transcripts of α^- were found not before the late morula phase, but always co-localizing with occludin. Hence, expression of α^+ was understood as a rather late and pivotal step during maturation of functional TJ, ensuring a tight paracellular seal [202]. Findings in this thesis of balanced levels of both isoforms in PSC indicate a level of maturation, which is to be classified between preimplantation and morula stages.

Furthermore, the expression of both isoforms in PSC are in concordance with earlier chapters of this thesis (5.3, 5.4 and 5.5) [3]. Already in their undifferentiated state PSC expressed epithelial and endothelial markers and even showed spontaneous tube formation on a semisolid matrix. This is considered as a typical property of endothelial cells [77]. Presence of α^- therefore underlines the endothelial properties, while findings of α^+ underline the epithelial predisposition.

One aim of this study was to investigate whether a shift in isoform expression was unique for Caco-2 cells. And although all investigated cell types showed variations of the isoform ratio, Caco-2 were the only to display a complete turnover. In comparison to other colon carcinoma cell lines Caco-2 exhibit a better morphological and functional enterocytic phenotype. After spontaneous differentiation they display microvilli, a polarized barrier and also markers of adult enterocytes. It was postulated, that this process is due to a shift from a tumor/adult colonic to a fetal phenotype that expresses functions of the small intestine [203, 204]. The distinction to the mature enterocyte is explained by an incomplete conversion from the proliferative (crypt) to the differentiated (villous) phenotype. This is underlined by analysis of gene expression patterns by means of microarrays [113, 205]. Upon formation of cell-cell contacts a switch in gene expression programs was observed. Proliferating, non-polarized Caco-2 underwent a development to postmitotic polarized cells. While the proliferating cells showed a gene pattern similar to that in human colon cancer *in vivo*, postmitotic Caco-2 displayed a pattern closely to that of a normal colon tissue. In the course of this transition, genes responsible for the evolution of structural and functional characteristics of polarized epithelial cells - like the formation of the apical junctional complex and of the brush border including expression of the corresponding hydrolases - were up-regulated. APN activity for example increased during this transition. This was also found in the course of the extended present study. Here this development was also paralleled with a shift in ZO-1 isoform ratio. Obviously, the switch from a prevailing α^- to a dominant α^+ isoform is a further indication of the switch in gene expression program, whose timing seems to be innate for Caco-2 [205, 206].

In conclusion, this is the first study to report a shift in ZO-1 isoform expression in Caco-2 cells. Although other investigated epithelial cell lines and also stem cells displayed a varying ratio of the two isoforms, Caco-2 were the only cell type to display a complete turnover in isoform amounts. No correlation could be found to origin of cells, cell density, proliferating activity, TEER value or occludin expression. However, a parallel to the spontaneous

differentiation taking place in Caco-2 could be described. Thus, Caco-2 may evolve to a useful *in vitro* model for investigating the function of the ZO-1 α motif.

7 SUMMARY AND PERSPECTIVE

First part of this thesis dealt with directed differentiation of two types of human adult stem cells to endothelial and epithelial-like cell types. Adult stem cells offer definite advantages over ESC. There is no ethical discussion involved, they display nearly no cancerogenity and therapies can be matched by harvesting stem cells directly from the patient. Main application areas are *in vitro* screening of pharmaceutical substances and generation of tissues or organs for transplantation. PSC and MSC were the sources of human adult stem cells, which were applied in this study. PSC had just been discovered at beginning of this thesis, therefore a use-oriented characterization was also part of this study. On the contrary, MSC have been established in research over the last decades.

Before differentiation experiments were started, characterization of PSC showed a broad immanent expression of endothelial, epithelial and stem cell markers. Cultivation on different ECM led to minor differences in marker expression only, AIF provoked obvious morphological changes. The influence of AIF on marker expression profile is to be investigated in further experiments, for determining whether an epithelial differentiation is induced. PSC displayed also the ability to form three-dimensional aggregates after centrifugation, which developed a histology of different layers and tissues similar to structures known from embryoid bodies. Also here a thorough investigation of the three layers would be interesting. Immunocytochemical staining not only for endothelial and epithelial markers, but for further lineages, could give insight in structure of these spheroids and their spontaneous inner differentiation.

Protocols for inducing endothelial differentiation comprised combinations of application of VEGF and bFGF, incubation in ECGM, growth on FN and serum starvation of stem cells. None of the approaches was efficient for increasing endothelial characteristics neither in PSC nor in MSC. However, both types of stem cells showed their endothelial potential not only by marker expression at mRNA and protein level but also by spontaneous formation of capillary-like structures, a substantial endothelial property. Protocols could be improved by application of further growth factors, like Insulin-like Growth Factor, coculture with endothelial cells and also three-dimensional scaffolds.

Approaches for inducing epithelial differentiation based on either direct or indirect coculture with lung-epithelial cell lines or a combination of both. This cocultivation led to a nearly identically altered expression of CDH 1 in both types of stem cells. This protein is not only known as an epithelial marker but is also decreased in metastatic cells. In both cell types,

direct contact to fixed A549 cells increased expression of CDH 1, while incubation with fixed Calu-3 did not. On the other hand, conditioned medium of both lung cancer cell lines suppressed expression of this marker. Which mechanisms are involved in this effect has to be investigated in further studies. However, it is obvious that type of coculture and choice of applied epithelial cells influences the direction of differentiation of PSC and MSC. Possibly, a direct coculture with untreated or heat-shocked living cells could increase the endothelial specialization. Also type of epithelial cells could be switched and primary cells like hAEpC could be applied. Furthermore, combination of AIF and coculture could be efficient.
Both, PSC and MSC, are possible sources for differentiated cells for usage in *in vitro* drug screening and transplant medicine. They display already endogenously endothelial and epithelial markers, which suggests high differentiation ability. Protocols tested in this thesis have to be further developed to lead to optimal results.

Second part of this study focused on varying expression of two isoforms α^+ and α^- of the tight junction protein ZO-1. Isoform ratios of this marker were so far considered as constant for a certain cell type. However, foregone experiments have shown that during culture of Caco-2 the ratio of isoform expression varied considerably. Caco-2 are a colon carcinoma cell line that exhibits after spontaneous differentiation morphological and biochemical features of small intestine and is therefore used as an popular *in vitro* model of this organ.
In order to investigate the observation of changing ZO-1 isoform expressions more closely not only Caco-2 were examined but also T84, a further intestinal cell model, A549 and Calu-3, two lung epithelial cell lines, and PSC, as primary stem cells. For all these cell types expression of ZO-1 isoforms was related to parameters of cell number, cell density, proliferating activity, TEER value and expression of occludin, a further tight junction protein for a period of 16 days.
Caco-2 were the only cell type to show a complete turnover in the ratio of ZO-1 α+/α-. While in the beginning expression of α^-, the longer isoform prevailed, at later time points expression of the short variant α^+ came to the fore. During culture of all other epithelial cell lines α^+ remained the prevailing isoform, even though the ratio here varied also up to factor 2. The only non-epithelial cell type, PSC, showed a rather constant isoform ratio varying around 1.
No correlation of the expressed ZO-1 isoform ratio and any of the other examined parameters was found. However, the ratio turnover in Caco-2 was paralleled by their spontaneous differentiation, which is peculiar for this cell line. Subsequent studies should investigate

isoform expression in further cell types. Examination during differentiation in stem cells could shed light on whether a ratio shift is paralleled by a specialization of cells.

8 REFERENCES

1. Kruse, C., et al., *Pluripotency of adult stem cells derived from human and rat pancreas.* Appl. Phys. A-Mater., 2004. **79**(7): p. 1617-1624.
2. Hwang, S.-M., *SOMATIC PLURIPOTENT CELLS*. 2008: United States of America.
3. Meier, K., C.M. Lehr, and N. Daum, *Differentiation potential of human pancreatic stem cells for epithelial- and endothelial-like cell types.* Ann Anat, 2009. **191**(1): p. 70-82.
4. Becker, A.J., E.A. McCulloch, and J.E. Till, *Cytological Demonstration of the Clonal Nature of Spleen Colonies Derived from Transplanted Mouse Marrow Cells.* Nature, 1963. **197**(4866): p. 452-454.
5. Siminovitch, L., E.A. McCulloch, and J.E. Till, *The Distribution of Colony-Forming Cells among Spleen Colonies.* J Cell Physiol, 1963. **62**: p. 327-36.
6. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells*. 1981. p. 7634-7638.
7. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-156.
8. Schuldiner, M., et al., *Induced neuronal differentiation of human embryonic stem cells.* Brain Res, 2001. **913**(2): p. 201-5.
9. Reubinoff, B.E., et al., *Neural progenitors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1134-40.
10. Kehat, I., et al., *Development of cardiomyocytes from human ES cells.* Methods Enzymol, 2003. **365**: p. 461-73.
11. Mummery, C., et al., *Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells.* Circulation, 2003. **107**(21): p. 2733-40.
12. Levenberg, S., et al., *Endothelial cells derived from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4391-6.
13. Zhan, X., et al., *Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro.* Lancet, 2004. **364**(9429): p. 163-71.
14. Vodyanik, M.A., et al., *Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential.* Blood, 2005. **105**(2): p. 617-26.
15. Segev, H., et al., *Differentiation of human embryonic stem cells into insulin-producing clusters.* Stem Cells, 2004. **22**(3): p. 265-74.
16. Assady, S., et al., *Insulin production by human embryonic stem cells.* Diabetes, 2001. **50**(8): p. 1691-7.
17. Sottile, V., A. Thomson, and J. McWhir, *In vitro osteogenic differentiation of human ES cells.* Cloning Stem Cells, 2003. **5**(2): p. 149-55.
18. Lavon, N., O. Yanuka, and N. Benvenisty, *Differentiation and isolation of hepatic-like cells from human embryonic stem cells.* Differentiation, 2004. **72**(5): p. 230-8.
19. Shirahashi, H., et al., *Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage.* Cell Transplant, 2004. **13**(3): p. 197-211.
20. Schuldiner, M., et al., *Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11307-12.
21. Klimanskaya, I., et al., *Human embryonic stem cells derived without feeder cells.* The Lancet, 2005. **365**(9471): p. 1636-1641.
22. Shafritz, D.A. and M.D. Dabeva, *Liver stem cells and model systems for liver repopulation.* Journal of Hepatology, 2002. **36**(4): p. 552-564.

23. Metzger, M., et al., *Enteric Nervous System Stem Cells Derived From Human Gut Mucosa for the Treatment of Aganglionic Gut Disorders.* Gastroenterology, 2009. **136**(7).
24. Sacco, A., et al., *Self-renewal and expansion of single transplanted muscle stem cells.* Nature, 2008. **456**(7221): p. 502-506.
25. Gorbunov, N., et al., *Regeneration of infarcted myocardium with resveratrol-modified cardiac stem cells.* Journal of Cellular and Molecular Medicine, 2012. **16**(1): p. 174-184.
26. Vishnubalaji, R., et al., *Skin-derived multipotent stromal cells - An archrival for mesenchymal stem cells.* Cell and Tissue Research, 2012. **350**(1): p. 1-12.
27. Wu, Q. and X. Wang, *Neuronal stem cells in the central nervous system and in human diseases.* Protein and Cell, 2012. **3**(4): p. 262-270.
28. Fraser, J.K., et al., *Plasticity of human adipose stem cells toward endothelial cells and cardiomyocytes.* Nature Clinical Practice Cardiovascular Medicine, 2006. **3**(SUPPL. 1): p. S33-S37.
29. Lu, R., et al., *Transcription factor TCF4 maintains the properties of human corneal epithelial stem cells.* Stem Cells, 2012. **30**(4): p. 753-761.
30. Bandyopadhyay, A., Q. Dong, and L.Z. Sun, *Stem/progenitor cells in murine mammary gland: Isolation and functional characterization*, in *Methods in Molecular Biology*. 2012. p. 179-193.
31. Schwarz, S. and N. Rotter, *Human salivary gland stem cells: Isolation, propagation, and characterization*, in *Methods in Molecular Biology*. 2012. p. 403-442.
32. D'Aquino, R., et al., *Dental pulp stem cells: A promising tool for bone regeneration.* Stem Cell Reviews, 2008. **4**(1): p. 21-26.
33. Rzhaninova, A.A., S.N. Gornostaeva, and D.V. Goldshtein, *Isolation and phenotypical characterization of mesenchymal stem cells from human fetal thymus.* Bulletin of experimental biology and medicine, 2005. **139**(1): p. 134-140.
34. Meindl, S., et al., *Characterization, isolation, and differentiation of murine skin cells expressing hematopoietic stem cell markers.* J. Leukoc. Biol., 2006. **80**(4): p. 816-826.
35. Jiang, Y., et al., *Pluripotency of mesenchymal stem cells derived from adult marrow.* Nature, 2002. **418**(6893): p. 41-9.
36. Lakshmipathy, U. and C. Verfaillie, *Stem cell plasticity.* Blood Reviews, 2005. **19**(1): p. 29-38.
37. Guillot, P.V., et al., *Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC.* Stem Cells, 2007. **25**(3): p. 646-54.
38. Roubelakis, M.G., et al., *Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells.* Stem Cells Dev, 2007. **16**(6): p. 931-52.
39. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-76.
40. Yu, J., et al., *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. **318**(5858): p. 1917-20.
41. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-72.
42. Kim, D., et al., *Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins.* Cell Stem Cell, 2009. **4**(6): p. 472-6.
43. Friedenstein, A.J., J.F. Gorskaja, and N.N. Kulagina, *Fibroblast precursors in normal and irradiated mouse hematopoietic organs.* Exp Hematol, 1976. **4**(5): p. 267-74.

44. Cao, C., Y. Dong, and Y. Dong, *[Study on culture and in vitro osteogenesis of blood-derived human mesenchymal stem cells]*. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi, 2005. **19**(8): p. 642-7.
45. Badri, L. and V.N. Lama, *Lysophosphatidic acid induces migration of human lung-resident mesenchymal stem cells through the β-catenin pathway.* Stem Cells, 2012. **30**(9): p. 2010-2019.
46. Beltrami, A.P., et al., *Adult cardiac stem cells are multipotent and support myocardial regeneration.* Cell, 2003. **114**(6): p. 763-76.
47. Fraser, J.K., et al., *Fat tissue: an underappreciated source of stem cells for biotechnology.* Trends Biotechnol, 2006. **24**(4): p. 150-4.
48. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-7.
49. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-7.
50. Shu, S.N., et al., *Hepatic differentiation capability of rat bone marrow-derived mesenchymal stem cells and hematopoietic stem cells.* World J Gastroenterol, 2004. **10**(19): p. 2818-22.
51. Lagasse, E., et al., *Purified hematopoietic stem cells can differentiate into hepatocytes in vivo.* Nat Med, 2000. **6**(11): p. 1229-34.
52. Battula, V.L., et al., *Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation.* Differentiation, 2007. **75**(4): p. 279-291.
53. Le Blanc, K. and O. Ringdén, *Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation.* Biology of Blood and Marrow Transplantation, 2005. **11**(5): p. 321-334.
54. Ren, G., et al., *Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression.* Stem Cells, 2009. **27**(8): p. 1954-1962.
55. Tolar, J., P. Villeneuve, and A. Keating, *Mesenchymal stromal cells for graft-versus-host disease.* Human Gene Therapy, 2011. **22**(3): p. 257-262.
56. Uccelli, A., L. Moretta, and V. Pistoia, *Immunoregulatory function of mesenchymal stem cells.* European Journal of Immunology, 2006. **36**(10): p. 2566-2573.
57. Kim, S.Y., et al., *Mesenchymal stem cell-conditioned media recovers lung fibroblasts from cigarette smoke-induced damage.* American Journal of Physiology - Lung Cellular and Molecular Physiology, 2012. **302**(9): p. L891-L908.
58. Li, L., et al., *Inhibition of lung cancer cell proliferation mediated by human mesenchymal stem cells.* Acta Biochimica et Biophysica Sinica, 2011. **43**(2): p. 143-148.
59. Wakitani, S., et al., *Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees.* J Tissue Eng Regen Med, 2007. **1**(1): p. 74-9.
60. Sykova, E. and S. Forostyak, *Stem cells in regenerative medicine.* Laser Therapy, 2013. **22**(2): p. 87-92.
61. Hilfiker, A., et al., *Mesenchymal stem cells and progenitor cells in connective tissue engineering and regenerative medicine: Is there a future for transplantation?* Langenbeck's Archives of Surgery, 2011. **396**(4): p. 489-497.
62. Morita, E., et al., *A novel cell transplantation protocol and its application to an ALS mouse model.* Experimental Neurology, 2008. **213**(2): p. 431-438.
63. Zhang, Z., et al., *Safety and immunological responses to human mesenchymal stem cell therapy in difficult-to-treat HIV-1-infected patients.* AIDS, 2013. **27**(8): p. 1283-1293.

64. Nagamori, E., et al., *Network formation through active migration of human vascular endothelial cells in a multilayered skeletal myoblast sheet.* Biomaterials, 2013. **34**(3): p. 662-668.
65. Sekiya, I., et al., *Expansion of Human Adult Stem Cells from Bone Marrow Stroma: Conditions that Maximize the Yields of Early Progenitors and Evaluate Their Quality.* Stem Cells, 2002. **20**(6): p. 530-541.
66. Daum, N., et al., *Isolation, cultivation, and application of human alveolar epithelial cells*, in *Methods in Molecular Biology*. 2012. p. 31-42.
67. Schenke-Layland, K., et al., *Collagen IV Induces Trophoectoderm Differentiation of Mouse Embryonic Stem Cells.* Stem Cells, 2007. **25**(6): p. 1529-1538.
68. Takano, N., et al., *Fibronectin Combined with Stem Cell Factor Plays an Important Role in Melanocyte Proliferation, Differentiation and Migration in Cultured Mouse Neural Crest Cells.* Pigm. Cell Res., 2002. **15**(3): p. 192-200.
69. Ong, S.-Y., H. Dai, and K.W. Leong, *Inducing hepatic differentiation of human mesenchymal stem cells in pellet culture.* Biomaterials, 2006. **27**(22): p. 4087-4097.
70. Kim, S.G., et al., *P-glycoprotein expression in extracellular matrix formation of chondrogenic differentiation of human adult stem cells.* Cell Biology International, 2007. **31**(9): p. 1042-1048.
71. Bosnakovski, D., et al., *Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system.* Experimental Hematology, 2004. **32**(5): p. 502-509.
72. Pfannkuche, K., et al., *Fibroblasts facilitate the engraftment of embryonic stem cell-derived cardiomyocytes on three-dimensional collagen matrices and aggregation in hanging drops.* Stem Cells and Development, 2010. **19**(10): p. 1589-1599.
73. Ota, H. and N. Miki, *Microtechnology-based three-dimensional spheroid formation.* Frontiers in bioscience (Elite edition), 2013. **5**: p. 37-48.
74. Joo, D.J., et al., *Manufacturing of insulin-secreting spheroids with the RIN-5F cell line using a shaking culture method.* Transplantation Proceedings, 2011. **42**(10): p. 4225-4227.
75. Fuchs, S., et al., *Differentiation of human alveolar epithelial cells in primary culture: morphological characterization and synthesis of caveolin-1 and surfactant protein-C.* Cell Tissue Res., 2003. **311**(1): p. 31-45.
76. Li, B., A. Pozzi, and P.P. Young, *TNFÎ± accelerates monocyte to endothelial transdifferentiation in tumors by the induction of integrin Î±5 expression and adhesion to fibronectin.* Molecular Cancer Research, 2011. **9**(6): p. 702-711.
77. Oswald, J., et al., *Mesenchymal stem cells can be differentiated into endothelial cells in vitro.* Stem Cells, 2004. **22**(3): p. 377-84.
78. Guldner, N.W., et al., *Autonomously contracting human cardiomyocytes generated from adult pancreatic stem cells and enhance in co-cultures with myocardial biopsies.* International Journal of Artificial Organs, 2006. **29**(12): p. 1158-1166.
79. Kruse, C., et al., *Adult pancreatic stem/progenitor cells spontaneously differentiate in vitro into multiple cell lineages and form teratoma-like structures.* Ann. Anat., 2006. **188**(6): p. 503-17.
80. Danner, S., et al., *Derivation of oocyte-like cells from a clonal pancreatic stem cell line.* Molecular Human Reproduction, 2007. **13**(1): p. 11-20.
81. Metallo, C.M., et al., *Human embryonic stem cell-derived keratinocytes exhibit an epidermal transcription program and undergo epithelial morphogenesis in engineered tissue constructs.* Tissue Engineering - Part A, 2010. **16**(1): p. 213-223.
82. Prytherch, Z., et al., *Tissue-Specific Stem Cell Differentiation in an in vitro Airway Model.* Macromolecular Bioscience, 2011. **11**(11): p. 1467-1477.

83. Carpenedo, R.L., C.Y. Sargent, and T.C. McDevitt, *Rotary Suspension Culture Enhances the Efficiency, Yield, and Homogeneity of Embryoid Body Differentiation.* STEM CELLS, 2007. **25**(9): p. 2224-2234.
84. Ng, E.S., et al., *Directed Differentiation of Human Embryonic Stem Cells as Spin Embryoid Bodies and a Description of the Hematopoietic Blast Colony Forming Assay*, in *Current Protocols in Stem Cell Biology*. 2007, John Wiley & Sons, Inc.
85. Dobbs, L.G., et al., *Maintenance of the differentiated type II cell phenotype by culture with an apical air surface.* Am J Physiol, 1997. **273**(2 Pt 1): p. L347-54.
86. Elbert, K.J., et al., *Monolayers of human alveolar epithelial cells in primary culture for pulmonary absorption and transport studies.* Pharm Res, 1999. **16**(5): p. 601-8.
87. Yoon, B.S., et al., *Enhanced differentiation of human embryonic stem cells into cardiomyocytes by combining hanging drop culture and 5-azacytidine treatment.* Differentiation, 2006. **74**(4): p. 149-159.
88. Hoffman, R.M., *The potential of nestin-expressing hair follicle stem cells in regenerative medicine*. 2007. p. 289-291.
89. Wang, T., et al., *Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell.* International Journal of Cardiology, 2006. **109**(1): p. 74-81.
90. Mancini, M.L., et al., *Endoglin is required for myogenic differentiation potential of neural crest stem cells.* Dev. Biol., 2007. **308**(2): p. 520-533.
91. Klimanskaya, I., et al., *Human embryonic stem cell lines derived from single blastomeres.* Nature, 2006. **444**(7118): p. 481-5.
92. Gorjup, E., et al., *Glandular tissue from human pancreas and salivary gland yields similar stem cell populations.* European Journal of Cell Biology, 2009. **88**(7): p. 409-421.
93. Kajahn, J., et al., *Skin-derived human adult stem cells surprisingly share many features with human pancreatic stem cells.* European Journal of Cell Biology, 2008. **87**(1): p. 39-46.
94. Petschnik, A.E., et al., *A novel xenogeneic co-culture system to examine neuronal differentiation capability of various adult human stem cells.* PLoS ONE, 2011. **6**(9).
95. Chu, P.G. and L.M. Weiss, *Keratin expression in human tissues and neoplasms.* Histopathology, 2002. **40**(5): p. 403-439.
96. Kleeff, J., et al., *Altered expression and localization of the tight junction protein ZO-1 in primary and metastatic pancreatic cancer.* Pancreas, 2001. **23**(3): p. 259-65.
97. Fanning, A.S., et al., *The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton.* J Biol Chem, 1998. **273**(45): p. 29745-53.
98. Takahashi, K., et al., *Direct binding between two PDZ domain proteins Canoe and ZO-1 and their roles in regulation of the jun N-terminal kinase pathway in Drosophila morphogenesis.* Mech. Dev., 1998. **78**(1-2): p. 97-111.
99. Eckert, J.J. and T.P. Fleming, *Tight junction biogenesis during early development.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2008. **1778**(3): p. 717-728.
100. Ciba, P., et al., *In vitro cultures of human pancreatic stem cells: Gene and protein expression of designated markers varies with passage.* Annals of Anatomy, 2009. **191**(1): p. 94-103.
101. Schenke-Layland, K., et al., *Collagen IV Induces Trophoectoderm Differentiation of Mouse Embryonic Stem Cells*

10.1634/stemcells.2006-0729. Stem Cells, 2007. **25**(6): p. 1529-1538.
102. Cool, S.M. and V. Nurcombe, *Substrate induction of osteogenesis from marrow-derived mesenchymal precursors.* Stem Cells Dev, 2005. **14**(6): p. 632-42.

103. Wijelath, E.S., et al., *Fibronectin promotes VEGF-induced CD34+ cell differentiation into endothelial cells.* Journal of Vascular Surgery, 2004. **39**(3): p. 655-660.
104. Takano, N., et al., *Fibronectin Combined with Stem Cell Factor Plays an Important Role in Melanocyte Proliferation, Differentiation and Migration in Cultured Mouse Neural Crest Cells*
doi:10.1034/j.1600-0749.2002.01079.x. Pigment Cell Research, 2002. **15**(3): p. 192-200.
105. Kurosawa, H., *Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells.* J. Biosci. Bioeng., 2007. **103**(5): p. 389-98.
106. Petschnik, A.E., et al., *Controlling Î±-SMA expression in adult human pancreatic stem cells by soluble factors.* Annals of Anatomy, 2009. **191**(1): p. 116-125.
107. Krauss, R.S., et al., *Close encounters: regulation of vertebrate skeletal myogenesis by cell-cell contact.* J. Cell. Sci., 2005. **118**(11): p. 2355-2362.
108. Tsai, R.Y.L. and R.D.G. McKay, *Cell Contact Regulates Fate Choice by Cortical Stem Cells.* J. Neurosci., 2000. **20**(10): p. 3725-3735.
109. Grainger, C., et al., *Culture of Calu-3 Cells at the Air Interface Provides a Representative Model of the Airway Epithelial Barrier.* Pharmaceutical Research, 2006. **23**(7): p. 1482-1490.
110. Carpenedo, R.L., S.A. Seaman, and T.C. McDevitt, *Microsphere size effects on embryoid body incorporation and embryonic stem cell differentiation.* J Biomed Mater Res A, 2010. **94**(2): p. 466-75.
111. Boyd, S.M., M.L. Hooper, and A.H. Wyllie, *The mode of cell death associated with cavitation in teratocarcinoma-derived embryoid bodies.* J Embryol Exp Morphol, 1984. **80**: p. 63-74.
112. Itskovitz-Eldor, J., et al., *Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers.* Mol Med, 2000. **6**(2): p. 88-95.
113. Ghesquiere, S. *Anatomy Artery*. 2005 [cited; Available from: http://en.wikipedia.org/wiki/File:Anatomy_artery.png.
114. Zhang, G., et al., *Arterialâ€“venous endothelial cell fate is related to vascular endothelial growth factor and Notch status during human bone mesenchymal stem cell differentiation.* FEBS Letters, 2008. **582**(19): p. 2957-2964.
115. Wosnitza, M., et al., *Plasticity of human adipose stem cells to perform adipogenic and endothelial differentiation.* Differentiation, 2007. **75**(1): p. 12-23.
116. Wang, M., et al., *Induced endothelial differentiation of cells from a murine embryonic mesenchymal cell line C3H/10T1/2 by angiogenic factors in vitro.* Differentiation, 2010. **79**(1): p. 21-30.
117. Wijelath, E.S., et al., *Fibronectin promotes VEGF-induced CD34+ cell differentiation into endothelial cells.* J. Vasc. Surg., 2004. **39**(3): p. 655-660.
118. Wu, K.H., et al., *In vitro and in vivo differentiation of human umbilical cord derived stem cells into endothelial cells.* J. Cell. Biochem., 2007. **100**(3): p. 608-616.
119. Friedman, M.S. and M.W. Long, *Stem cell plasticity: CD34+ hematopoietic cells and mesenchymal stem cells undergo regulated differentiation into endothelial cells.* Blood, 2001. **98**(11 PART I): p. 550a.
120. Hackanson, B., et al., *Phenotype of bone marrow derived mesenchymal stem cells (msc) in vivo and their endothelial differentiation capacity.* Blood, 2001. **96**(11 PART II): p. 120b.
121. Festag, M., et al., *An in vitro embryotoxicity assay based on the disturbance of the differentiation of murine embryonic stem cells into endothelial cells. I: Establishment of the differentiation protocol.* Toxicology in Vitro, 2007. **21**(8): p. 1619-1630.

122. Thannickal, V.J., et al., *Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase.* J. Biol. Chem., 2003. **278**(14): p. 12384-9.
123. [cited; Available from: http://www.ensembl.org/Homo_sapiens/Transcript/Summary?g=ENSG00000102755;r=13:28973181-29069265;t=ENST00000539099.
124. Schächinger, V., et al., *Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: Final one-year results of the TOPCARE-AMI trial.* Journal of the American College of Cardiology, 2004. **44**(8): p. 1690-1699.
125. Tateishi-Yuyama, E., et al., *Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: A pilot study and a randomised controlled trial.* Lancet, 2002. **360**(9331): p. 427-435.
126. Whyte, J.L., et al., *Density of human bone marrow stromal cells regulates commitment to vascular lineages.* Stem Cell Research, 2011. **6**(3): p. 238-250.
127. Goodwin, A.M., *In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents.* Microvasc. Res., 2007. **74**(2-3): p. 172-183.
128. Flamme, I., T. Frolich, and W. Risau, *Molecular mechanisms of vasculogenesis and embryonic angiogenesis.* J Cell Physiol, 1997. **173**(2): p. 206-10.
129. Hass, R., et al., *Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC.* Cell Commun Signal, 2011. **9**: p. 12.
130. Al-Khaldi, A., et al., *Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response in vivo.* Gene Therapy, 2003. **10**(8): p. 621-629.
131. Chen, M.Y., et al., *Endothelial differentiation of Wharton's jelly-derived mesenchymal stem cells in comparison with bone marrow-derived mesenchymal stem cells.* Experimental Hematology, 2009. **37**(5): p. 629-640.
132. Valarmathi, M.T., et al., *A three-dimensional model of vasculogenesis.* Biomaterials, 2009. **30**(6): p. 1098-1112.
133. Wu, X., et al., *Mesenchymal stem cells participating in ex vivo endothelium repair and its effect on vascular smooth muscle cells growth.* International Journal of Cardiology, 2005. **105**(3): p. 274-282.
134. Valarmathi, M.T., et al., *A three-dimensional tubular scaffold that modulates the osteogenic and vasculogenic differentiation of rat bone marrow stromal cells.* Tissue Engineering - Part A., 2008. **14**(4): p. 491-504.
135. Liu, J.W., et al., *Characterization of endothelial-like cells derived from human mesenchymal stem cells.* Journal of Thrombosis and Haemostasis, 2007. **5**(4): p. 826-834.
136. Villars, F., et al., *Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF?* J Cell Biochem, 2000. **79**(4): p. 672-85.
137. Steiner, D., et al., *Effects of endothelial cells on proliferation and survival of human mesenchymal stem cells and primary osteoblasts.* Journal of Orthopaedic Research, 2012. **30**(10): p. 1682-1689.
138. Liu, Z.J., et al., *Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis.* Mol Cell Biol, 2003. **23**(1): p. 14-25.
139. Nakajima, M., et al., *Immunohistochemical and ultrastructural studies of basal cells, Clara cells and bronchiolar cuboidal cells in normal human airways.* Pathology International, 1998. **48**(12): p. 944-953.
140. Fehrenbach, H., *Alveolar epithelial type II cell: defender of the alveolus revisited.* Respir Res, 2001. **2**(1): p. 33-46.

141. Alcorn, J.L., et al., *Primary cell culture of human type II pneumonocytes: maintenance of a differentiated phenotype and transfection with recombinant adenoviruses.* Am J Respir Cell Mol Biol, 1997. **17**(6): p. 672-82.
142. Formigli, L., et al., *Morphofunctional integration between skeletal myoblasts and adult cardiomyocytes in coculture is favored by direct cell-cell contacts and relaxin treatment.* Am. J. Physiol. Cell Physiol., 2005. **288**(4): p. C795-804.
143. Wurmser, A.E., et al., *Cell fusion-independent differentiation of neural stem cells to the endothelial lineage.* Nature, 2004. **430**(6997): p. 350-6.
144. Yamazaki, S., et al., *Sera from liver failure patients and a demethylating agent stimulate transdifferentiation of murine bone marrow cells into hepatocytes in coculture with nonparenchymal liver cells.* J. Hepatol., 2003. **39**(1): p. 17-23.
145. Yoon, J., et al., *Transdifferentiation of mesenchymal stem cells into cardiomyocytes by direct cell-to-cell contact with neonatal cardiomyocyte but not adult cardiomyocytes.* Ann. Hematol., 2005. **84**(11): p. 715-21.
146. Pompen, M., et al., *Lung epithelial H292 cells induce differentiation of immature human HMC-1 mast cells by interleukin-6 and stem cell factor.* Clin. Exp. Allergy, 2000. **30**(8): p. 1104-1112.
147. Van Vranken, B.E., et al., *Coculture of embryonic stem cells with pulmonary mesenchyme: a microenvironment that promotes differentiation of pulmonary epithelium.* Tissue Eng., 2005. **11**(7-8): p. 1177-87.
148. Guldner, N.W., et al., *Autonomously contracting human cardiomyocytes generated from adult pancreatic stem cells and enhanced in co-cultures with myocardial biopsies.* Int. J. Artif. Organs, 2006. **29**(12): p. 1158-66.
149. Carterson, A.J., et al., *A549 Lung Epithelial Cells Grown as Three-Dimensional Aggregates: Alternative Tissue Culture Model for Pseudomonas aeruginosa Pathogenesis.* Infect. Immun., 2005. **73**(2): p. 1129-1140.
150. Abraham, G., et al., *Expression of functional [beta]2-adrenergic receptors in the lung epithelial cell lines 16HBE14o-, Calu-3 and A549.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2004. **1691**(2-3): p. 169-179.
151. Shen, B.Q., et al., *Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl- secretion.* Am. J. Physiol., 1994. **266**(5 Pt 1): p. L493-501.
152. Foster, K.A., et al., *Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery.* Int. J. Pharm., 2000. **208**(1-2): p. 1-11.
153. Roszell, B., et al., *Efficient derivation of alveolar type ii cells from embryonic stem cells for in vivo application.* Tissue Engineering - Part A, 2009. **15**(11): p. 3351-3365.
154. Young, M.R. and M. Cigal, *Tumor skewing of CD34+ cell differentiation from a dendritic cell pathway into endothelial cells.* Cancer immunology, immunotherapy : CII, 2006. **55**(5): p. 558-568.
155. Aliotta, J.M., et al., *Progenitor/stem cell fate determination: Interactive dynamics of cell cycle and microvesicles.* Stem Cells and Development, 2012. **21**(10): p. 1627-1638.
156. Li, T.Z., et al., *Therapeutic potential of bone-marrow-derived mesenchymal stem cells differentiated with growth-factor-free coculture method in liver-injured rats.* Tissue Engineering - Part A, 2011. **16**(8): p. 2649-2659.
157. Singaravelu, K. and B.J. Padanilam, *In vitro differentiation of MSC into cells with a renal tubular epithelial-like phenotype.* Renal Failure, 2009. **31**(6): p. 492-502.
158. Gumbiner, B.M., *Regulation of cadherin-mediated adhesion in morphogenesis.* Nat Rev Mol Cell Biol, 2005. **6**(8): p. 622-34.
159. Shapiro, L. and W.I. Weis, *Structure and biochemistry of cadherins and catenins.* Cold Spring Harb Perspect Biol, 2009. **1**(3): p. a003053.

160. Perez-Moreno, M. and E. Fuchs, *Catenins: keeping cells from getting their signals crossed.* Dev Cell, 2006. **11**(5): p. 601-12.
161. Gumbiner, B.M., *Regulation of cadherin-mediated adhesion in morphogenesis.* 2005. **6**(8): p. 622-634.
162. Wheelock, M.J. and P.J. Jensen, *Regulation of keratinocyte intercellular junction organization and epidermal morphogenesis by E-cadherin.* J Cell Biol, 1992. **117**(2): p. 415-25.
163. Cano, A., et al., *The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression.* Nat Cell Biol, 2000. **2**(2): p. 76-83.
164. Vleminckx, K., et al., *Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role.* Cell, 1991. **66**(1): p. 107-19.
165. Berx, G., F. Nollet, and F. van Roy, *Dysregulation of the E-cadherin/catenin complex by irreversible mutations in human carcinomas.* Cell. Adhes. Commun., 1998. **6**(2-3): p. 171-84.
166. Andersen, H., et al., *Immediate and Delayed Effects of E-Cadherin Inhibition on Gene Regulation and Cell Motility in Human Epidermoid Carcinoma Cells.* Mol. Cell. Biol., 2005. **25**(20): p. 9138-9150.
167. Windus, L.C.E., et al., *In vivo biomarker expression patterns are preserved in 3D cultures of Prostate Cancer.* Experimental Cell Research, 2012. **318**(19): p. 2507-2519.
168. Gu, S. and J. Qin, *The expression and clinical significance of HIF-1Î±, COX-2 and E-cadherin in patients with lung adenocarcinoma.* Chinese Journal of Lung Cancer, 2011. **14**(3): p. 219-226.
169. Popov, B.V., et al., *Lung Epithelial Cells Induce Endodermal Differentiation in Mouse Mesenchymal Bone Marrow Stem Cells by Paracrine Mechanism.* Tissue Engineering, 2007. **13**(10): p. 2441-2450.
170. Wang, G., et al., *Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: Potential therapy for cystic fibrosis.* Proceedings of the National Academy of Sciences, 2005. **102**(1): p. 186-191.
171. Ciana, A., et al., *A dynamic ratio of the alpha+ and alpha- isoforms of the tight junction protein ZO-1 is characteristic of Caco-2 cells and correlates with their degree of differentiation.* Cell Biol Int, 2010. **34**(6): p. 669-78.
172. Balda, M.S. and K. Matter, *Tight junctions.* J Cell Sci, 1998. **111 (Pt 5)**: p. 541-7.
173. Anderson, J.M., C.M. Van Itallie, and A.S. Fanning, *Setting up a selective barrier at the apical junction complex.* Curr Opin Cell Biol, 2004. **16**(2): p. 140-5.
174. Tsukita, S. and M. Furuse, *Occludin and claudins in tight-junction strands: leading or supporting players?* Trends Cell Biol, 1999. **9**(7): p. 268-73.
175. Feldman, G.J., J.M. Mullin, and M.P. Ryan, *Occludin: structure, function and regulation.* Adv Drug Deliv Rev, 2005. **57**(6): p. 883-917.
176. Mitic, L.L. and J.M. Anderson, *Molecular architecture of tight junctions.* Annu Rev Physiol, 1998. **60**: p. 121-42.
177. Stevenson, B.R., et al., *Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia.* J Cell Biol, 1986. **103**(3): p. 755-66.
178. Balda, M.S., M.D. Garrett, and K. Matter, *The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density.* J Cell Biol, 2003. **160**(3): p. 423-32.
179. Mohandas, T.K., et al., *Localization of the Tight Junction Protein Gene TJP1 to Human Chromosome 15q13, Distal to the Prader-Willi/Angelman Region, and to Mouse Chromosome 7.* Genomics, 1995. **30**(3): p. 594-597.

180. Balda, M.S. and J.M. Anderson, *Two classes of tight junctions are revealed by ZO-1 isoforms.* Am J Physiol, 1993. **264**(4 Pt 1): p. C918-24.
181. Willott, E., et al., *Localization and differential expression of two isoforms of the tight junction protein ZO-1.* Am J Physiol, 1992. **262**(5 Pt 1): p. C1119-24.
182. Fogh, J., J.M. Fogh, and T. Orfeo, *One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice.* J Natl Cancer Inst, 1977. **59**(1): p. 221-6.
183. Pinto M, R.-L.S., Appay M-D, *Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture.* Biol Cell, 1983. **47**: p. 323-330.
184. Kim, J.S., et al., *The suitability of an in situ perfusion model for permeability determinations: utility for BCS class I biowaiver requests.* Mol Pharm, 2006. **3**(6): p. 686-94.
185. Krejci, I., et al., *Process optimization and biocompatibility of cell carriers suitable for automated magnetic manipulation.* Acta Biomater, 2012. **8**(3): p. 1239-47.
186. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
187. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.* Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4350-4.
188. Aksamitiene, E., et al., *Multistrip Western blotting to increase quantitative data output.* Electrophoresis, 2007. **28**(18): p. 3163-73.
189. Ghassemifar, R., C.M. Lai, and P.E. Rakoczy, *VEGF differentially regulates transcription and translation of ZO-1Î±+ and ZO-1Î±- and mediates trans-epithelial resistance in cultured endothelial and epithelial cells.* Cell and Tissue Research, 2006. **323**(1): p. 117-125.
190. Piana, C., et al., *Influence of surface modification on vitality and differentiation of Caco-2 cells.* Differentiation, 2007. **75**(4): p. 308-317.
191. Piana, C., et al., *Growth surface-induced gene and protein expression patterns in Caco-2 cells.* Acta Biomaterialia, 2008. **4**(6): p. 1819-1826.
192. Behrens, I. and T. Kissel, *Do cell culture conditions influence the carrier-mediated transport of peptides in Caco-2 cell monolayers?* Eur J Pharm Sci, 2003. **19**(5): p. 433-42.
193. Umeda, K., et al., *Establishment and characterization of cultured epithelial cells lacking expression of ZO-1.* J Biol Chem, 2004. **279**(43): p. 44785-94.
194. Umeda, K., et al., *ZO-1 and ZO-2 Independently Determine Where Claudins Are Polymerized in Tight-Junction Strand Formation.* Cell, 2006. **126**(4): p. 741-754.
195. Pulimeno, P., S. Paschoud, and S. Citi, *A role for ZO-1 and PLEKHA7 in recruiting paracingulin to tight and adherens junctions of epithelial cells.* J Biol Chem, 2011. **286**(19): p. 16743-50.
196. Itoh, M., et al., *The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy.* Journal of Cell Biology, 1993. **121**(3): p. 491-502.
197. Itoh, M., et al., *Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins.* Journal of Cell Biology, 1999. **147**(6): p. 1351-1363.
198. Ikenouchi, J., et al., *Requirement of ZO-1 for the formation of belt-like adherens junctions during epithelial cell polarization.* J Cell Biol, 2007. **176**(6): p. 779-86.
199. Balda, M.S. and K. Matter, *The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression.* Embo J, 2000. **19**(9): p. 2024-33.

200. Matsumoto, K. and A.P. Wolffe, *Gene regulation by Y-box proteins: coupling control of transcription and translation.* Trends Cell Biol, 1998. **8**(8): p. 318-23.
201. Pelletier, R.M., et al., *Differential distribution of the tight-junction-associated protein ZO-1 isoforms alpha+ and alpha- in guinea pig Sertoli cells: a possible association with F-actin and G-actin.* Biol Reprod, 1997. **57**(2): p. 367-76.
202. Sheth, B., et al., *Tight junction assembly during mouse blastocyst formation is regulated by late expression of ZO-1 Î±+ isoform.* Development, 1997. **124** (10): p. 2027-2037.
203. Sambuy, Y., et al., *The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics.* Cell Biol Toxicol, 2005. **21**(1): p. 1-26.
204. Engle, M.J., G.S. Goetz, and D.H. Alpers, *Caco-2 cells express a combination of colonocyte and enterocyte phenotypes.* J Cell Physiol, 1998. **174**(3): p. 362-9.
205. Halbleib, J.M., et al., *Transcriptional modulation of genes encoding structural characteristics of differentiating enterocytes during development of a polarized epithelium in vitro.* Mol Biol Cell, 2007. **18**(11): p. 4261-78.
206. Saaf, A.M., et al., *Parallels between global transcriptional programs of polarizing Caco-2 intestinal epithelial cells in vitro and gene expression programs in normal colon and colon cancer.* Mol Biol Cell, 2007. **18**(11): p. 4245-60.

9 PUBLICATION LIST

Paper

Meier, K., C.M. Lehr, and N. Daum, Differentiation potential of human pancreatic stem cells for epithelial- and endothelial-like cell types. Ann Anat, 2009. 191(1): p. 70-82.

Ciana, A.*, K. Meier.*, N. Daum, S. Gerbes, M. Veith, C.-M. Lehr, G. Minetti, A dynamic ratio of the alpha+ and alpha- isoforms of the tight junction protein ZO-1 is characteristic of Caco-2 cells and correlates with their degree of differentiation. Cell Biol Int, 2010. 34(6): p. 669-78. *equally contributing authors

Krejci, I., C. Piana, S. Howitz, T. Wegener, S. Fiedler, M. Zwanzig, D. Schmitt, N. Daum, K. Meier, C.-M. Lehr, U. Batista, S. Zemljic, J. Messerschmidt, J. Franzke, M. Wirth, F. Gabor, Process optimization and biocompatibility of cell carriers suitable for automated magnetic manipulation. Acta Biomater, 2012. 8(3): p. 1239-47.

Review

Meier, K. and C.-M. Lehr, Rui L. Reis, Editors, Biodegradable Systems in Tissue Engineering and Regenerative Medicine (2005) CRC Press,Boca Raton 568 pp., $149, ISBN 0-8493-1936-6. European Journal of Pharmaceutics and Biopharmaceutics, 2005. 61(1&2): p. 111-112.

Poster

Meier, K., N. Daum, C.-M. Lehr, Artificial and biological nanoscapes – influence on biocompatibility and differentiation, Nanotech, 14.-15.11.2005, Montreux, Switzerland

Meier, K., N. Daum, C.-M. Lehr **,** USAAR-2 (WP 5): Artificial and biological NanoScapes, 25.4.2006, CellPROM Annual Assessment Meeting, St. Ingbert, Germany

Meier, K., N. Daum, C.-M. Lehr, Characterization and directed differentiation of PSLC, 5.-6.3.2007, CellPROM Annual Assessment Meeting, Lissabon, Portugal

Meier, K., N. Daum, C.-M. Lehr, Pancreatic stellate-like cells possess high differentiation potential for epithelial and endothelial cell types, CellPROM Stem Cell Meeting, 3.-4.9. 2007, Lübeck, Germany

Meier, K., N. Daum, C.-M. Lehr, Differentiation Potential of Human Pancreatic Stem Cells for epithelial- and endothelial-like cell types, CellPROM Concluding Meeting, 29.2.2008, St. Ingbert, Germany

10 CURRICULUM VITAE

11 ACKNOWLEDGMENTS - DANKSAGUNG

This dissertation was partly generated in the context of the CellPROM project, funded under the 6th Framework Programme of the European Community (Contract N° NMP4-CT-2004-500039). The author is solely responsible for its content, it does not represent the opinion of the European Community and the Community is not responsible for any use that might be made of the information contained therein.

An erster Stelle möchte ich mich bei Prof. Claus-Michael Lehr für die Bereitstellung des Themas und die ideenreiche Unterstützung bedanken.

Ein großes Danke geht an Prof. Ulrich Schäfer und Prof Marc Schneider. Obwohl Ihr thematisch an ganz anderer Stelle angesiedelt ward, habt Ihr gerade durch andere Blickwinkel und Eure wissenschaftliche Denkweise immer wieder Unterstützung gegeben und neue Ansatzpunkte gefunden.

Frau Dr. Nicole Daum kann ich gar nicht genug danken für ihren großen Einsatz. Nicole, Du hattest immer Zeit, ein offenes Ohr und Schokocappuccino. Abgesehen von all dem fachlichen Input den Du geleistet hast, bist Du mir eine wirkliche Freundin geworden.

Cornelia Hildebrand und Erwin Gorjup vom Fraunhofer Institut St. Ingbert sei gedankt für den stetigen Nachschub an Stammzellen.

Mit Annarita Ciana und Prof. Giampaolo Minetti gab es nicht nur eine tolle Zusammenarbeit während des ZO-1 Projekt sondern auch unterhaltsame Stunden beim Ausprobieren lokaler Delikatessen von Norditalien und Portugal.

Ein großer Dank geht an die gesamte Arbeitsgruppe. Es herrschte – was keine Selbstverständlichkeit ist – ein tolles Arbeitsklima. Ganz besonders bedanken möchte ich mich bei:

Andi, Barbara, Birgit, Christine, Frank, Hakon, Sebastian, Stephan und Uli. Ihr wart tolle Bürokollegen.

Petra für die viele, viele gewissenhafte Routine in der Zellkultur, das gemeinsame an der Bank sitzen und erzählen.

Eva und Michael für das häufige “Zell-sitting” über die Feiertage.

Leon für die Hilfe bei der histologischen Präparation.

Ein Danke geht auch an Prof. Karl Westhoff. Für die Zeit, die Du zum Korrekturlesen geopfert hast und die stetige Ermunterung nicht aufzugeben.

Ein kleines Danke geht an Lina, die so zuverlässig Mittagsschlaf gemacht hat, dass ich die Zeit zum Schreiben nutzen konnte.

Mein großes Danke gilt Till. Du hast mir immer wieder die Zeit und die Mittel zur Verfügung gestellt, mich ermuntert, gedrängt und aufgefordert weiter zu machen. Ohne Dich wär es schwer gewesen.

Meiner Familie und insbesondere meinen Eltern möchte ich danken für all die Liebe, moralische und auch finanzielle Unterstützung in dieser Zeit. Ihr habt immer an mich geglaubt und mir das auch immer wieder gesagt.