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Dedifferentiated, programmable stem cells of monocytic origin, and their production and use

D E S C R I P T I O N

The invention relates to adult dedifferentiated programmable stem cells derived from human monocytes, as well as their production and use for the production of body cells and tissues. According to a particularly preferred embodiment of the invention these cells are autologous human stem cells, i.e. the cell of monocytic origin comes from the patient who is to be treated with the stem cell produced from the original cell and/or with the body cells produced from this stem cell.

In the state of the art, the term "stem cells" designates cells which (a) have the capability of self-renewal and (b) the capability to form at least one and often a number of specialised cell types due to their asymmetrical division capability (cf. Donovan, P.J., Gearhart, J., Nature **414**: 92-97 (2001)). The term "pluripotent" designates stem cells, which can essentially be differentiated into all possible cell types of the human and animal body. Such stem cells have hitherto only been obtainable from embryonic tissue or embryonic carcinoma (testicular tumour) (cf. Donovan, P.J., Gearhart, J., loc cit). The use of embryonic stem cells has been the subject of extensive public discussion, especially in Germany, and is regarded as extremely problematical. Besides the ethical and legal problems connected with embryonic stem cells, the therapeutic use of such cells also comes up against difficulties. By nature, embryonic stem cells are obtained from donor organisms, which are heterologous vis-à-vis the potential recipients of differentiated cells or tissue (hereafter referred to as somatic target cells or target tissue) developed from these cells. It is therefore to be expected, that such target cells will trigger an immediate immunological response in the potential recipients in the form of rejection.

Stem cells can be also isolated from different tissues of adult, i.e. from differentiated individuals. Such stem cells are referred to in the state of the art as "multipotent adult stem cells". In the body they play a role in tissue regeneration and homoeostasis. The essential difference between embryonic pluripotent stem cells and adult multipotent stem cells lies in the number of differentiated tissues, which can be obtained from the respective cells. Presumably, this is due to the fact that pluripotent stem cells come from sperm

cells, or from cells which can produce sperm, whilst adult multipotent stem cells come from the body or soma of adult individuals (cf. Donovan, P.J., Gearhart, J., loc cit, Page 94), which are not capable of sperm production.

The actual problems relating to the obtaining and use of adult stem cells however lie in the rarity of these cells. Thus, in the bone marrow, stem cells are present only in the ratio of 1:10,000, in the peripheral blood of 1:250,000 and in the liver in the ratio of 1:100,000. Obtaining such stem cells is therefore very expensive and stressful for the patient. In addition the generation of large cell quantities, as required for clinical therapy, has scarcely been possible hitherto at reasonable expense.

This is contrasted by a constantly increasing need for possibilities for treatment of destroyed tissue in the form of "tissue engineering" or as cell therapy, within the framework of which skin-, muscle-, heart muscle-, liver-, islet-, nerve-, neurone-, bone-, cartilage-, endothelium- and fat cells etc. are to be replaced.

In this connection, the foreseeable development of the age and disease profile of the population in the western world is decisive, leading to the expectation of a drastic turning point in the next 10 years in the health and care sector of the western European population, including the USA and Canada. In the Federal Republic of Germany alone, the demographic development suggests a 21%-growth in population in the 45-64 year-old age group by 2015, and a 26%-growth in the over-65 age group. This is bound to result in a change in patient structure and in the spectrum of diseases requiring treatment. Predictably, diseases of the cardio-circulatory system (high

pressure, myocardial infarction), vascular diseases due to arteriosclerosis and metabolic diseases, metabolic diseases such an diabetes mellitus, diseases at liver metabolism, kidney diseases as well as diseases of the skeletal system caused by age-related degeneration, and degenerative diseases of the cerebrum caused by neuronal and glial cell losses will increase and require innovative treatment concepts.

These facts explain the immense national and international research and development efforts by the specialists involved, to obtain stem cells which can be programmed into differentiated cells typical of tissue (liver, bone, cartilage, muscle, skin etc.).

The problem underlying the invention therefore resides in making available adult stem cells, the generation of which gives rise to no ethical and/or legal problems, which are rapidly available for the planned therapeutic use in the quantities required for this, and at justifiable production costs, and which, when used as "cellular therapeutics" give rise to no side effects - or none worth mentioning - in terms of cellular rejection and induction of tumours, particularly malignant tumours, in the patient in question.

According to the invention this problem is solved by the production of dedifferentiated programmable cells from human monocytes which, for the purposes of the invention, are referred to hereafter as "stem cells". The term "dedifferentiation" is familiar to the person skilled in the relevant art, cf. for Weissman I.L., Cell **100**: 157-168, Fig.4, (2000). It signifies the regression of an adult, already specialised (differentiated) body cell to the status of a stem cell, i.e. of a cell, which in turn can be transferred

(programmed) into a number of cell types. Surprisingly, it has been demonstrated that the process according to the invention leads to the dedifferentiation of monocytes. The stem cells produced in this way can be transformed (programmed) into a large number of different target cells/target tissue, cf. examples. The stem cells according to the invention express, in addition to the CD14 surface antigen characteristic of differentiated monocytes, at least one, preferably two or three, of the typical pluripotency markers CD90, CD117, CD123 and CD135. In a particularly preferred manner, the stem cells produced according to the invention express the CD14 surface antigen as well as the four pluripotency markers CD90, CD117, CD123 and CD135, cf. Example 2, Table 1. Preferably, the stem cells of the invention express the membrane associated monocyte-specific surface antigen CD 14 and at least one pluripotency markers selected from the group consisting of CD117, CD123 and CD135. More preferably, the stem cells of the invention carry the CD14 antigen in combination with at least the pluripotency marker CD123 and/or CD135. Less than 3%, preferably less than 1% of the stem cells according to the invention express the CD34 antigen. Most preferably, none of the stem cells of the invention express the CD34 antigen. In this way, for the first time adult stem cells are made available, which can within a short time be reprogrammed into preferably autologous tissues.

The generation of the stem cells according to the invention is completely harmless to the patient and - in the case of autologous use - comparable to own blood donation. The quantity of stem cells (10^8 to 10^9 cells) required for the usual therapy options (see above) can be made available cost-effectively within 10 to 14 days after the blood is taken. In addition the cell product provided for the therapy, in the

case of autologous use, does not give rise to any immunological problem in terms of cell rejection, as cells and recipient are preferably genetically identical.

The stem cells according to the invention have also proved to be risk-free in animal experimentation and in culture with regard to giving rise to malignancy, a result which is only to be expected due to the cell of monocytic origin, from which the stem cells according to the invention derive.
The essential steps of the process according to the invention for the production of dedifferentiated programmable stem cells of human monocytic origin comprise:

(a) Isolation of monocytes from human blood;
(b) Propagating the monocytes in a suitable culture vessel containing cell culture medium, which contains the macrophage-colony-stimulating factor (hereafter referred to as M-CSF); and
(c) Cultivating the monocytes in the presence of interleukin-3 (IL-3); and
(d) Obtaining the human dedifferentiated programmable stem cells, by separating the cells from the culture medium.

According to a particularly preferred embodiment of the process, M-CSF and IL-3 are simultaneously added to the cell culture medium in Step b).

It is however also possible, initially only to add M-CSF to the cell culture medium in Step b) in order to cause the monocytes to propagate, and to add IL-3 to the cell culture medium subsequently.

Finally the process in Step b) can also be carried out in such

a way that the monocytes are initially propagated in a cell culture medium containing only M-CSF, then the medium is separated from the cells and a second cell culture medium is then used, which contains IL-3.

According to a preferred embodiment of the invention the culture medium of Step b) is separated from the cells attached to the bottom of the culture vessel and the human, dedifferentiated, programmable stem cells are obtained by detaching the cells from the bottom and by isolating the cells.

According to a preferred embodiment of the invention the cells are further cultivated in the presence of a sulphur compound. The cultivation can be carried out in a separate process step which follows the cultivation Step b) described above. It can however also be carried out in Step b), by further adding the sulphur compound to the culture medium, preferably already at the start of the cultivation.

The process according to the invention surprisingly leads to the dedifferentiation of the monocytes, wherein the adult stem cells resulting from the dedifferentiation, besides the CD14 surface antigen typical of the differentiated monocytes, also express at least one or more, preferably all of the pluripotency markers CD90, CD117, CD123 and CD135 (cf. Table 1). Preferably, the stem cells of the invention express the membrane associated monocyte-specific surface antigen CD 14 and at least one pluripotency markers selected from the group consisting of CD117, CD123 and CD135. More preferably, the stem cells of the invention carry the CD14 antigen in combination with at least the pluripotency marker CD123 and/or CD135. Less than 3%, preferably less than 1% of the stem cells

according to the invention express the CD34 antigen. Most preferably, none of the stem cells of the invention express the CD34 antigen. The expression of the respective markers (surface antigens) can be proved by means of commercially available antibodies with specificity against the respective antigens to be detected, using standard immuno assay procedures, cf. Example 2.

As the cells, during the propagation and dedifferentiation process, adhere to the bottom of the respective culture vessel, it is necessary to separate the cells from the culture medium from Step b) and to detach them from the bottom after completion of the dedifferentiation. According to a preferred embodiment of the invention the cell culture supernatant is discarded before the detaching of the cells adhering to the bottom and subsequently, the adhering cells are preferably rinsed with fresh culture medium. Following the rinsing, fresh culture medium is again added to the cells adhering to the bottom, and the step of releasing the cells from the bottom then follows (cf. Example 13).

According to a preferred embodiment the cells are brought into contact with a biologically well-tolerated organic solvent, at the end of Step c) and before Step d). The biologically well-tolerated organic solvent can be an alcohol with 1-4 carbon atoms, the use of ethanol being preferred.

In a further embodiment, at the end of Step c) and before Step d) the cells are brought into contact with the vapour phase of the biologically well-tolerated organic solvent.

The detaching can moreover also be carried out mechanically, however, an enzymatic detaching process is preferred, for

example with trypsin.

The dedifferentiated programmable stem cells obtained in this way, floating freely in the medium, can either be directly transferred to the reprogramming process, or kept in the culture medium for a few days; in the latter case, a cytokine or LIF (leukaemia inhibitory factor) is preferably added to the medium, in order to avoid premature loss of the programmability (cf. Donovan, P.J., Gearhart, J., loc. cit., Page 94). Finally the cells can be deep-frozen for storage purposes without loss of programmability.

The stem cells according to the invention differ from the pluripotent stem cells of embryonic origin known hitherto and from the known adult stem cells from different tissues, in that besides the membrane-associated monocyte-specific CD14 surface antigen, they carry at least one pluripotency marker from the group consisting of CD90, CD117, CD123 and CD135 on their surface. Preferably, the stem cells of the invention carry the membrane associated monocyte-specific surface antigen CD 14 and at least one pluripotency markers selected from the group consisting of CD117, CD123 and CD135. More preferably, the stem cells of the invention carry the CD14 antigen in combination with at least the pluripotency marker CD123 and/or CD135. Less than 3%, preferably less than 1% of the stem cells according to the invention express the CD34 antigen. Most preferably, none of the stem cells of the invention express the CD34 antigen.

The stem cells produced using the process according to the invention can be reprogrammed into any body cells. Processes for reprogramming stem cells are known in the state of the art, cf. for example Weissman I.L., Science **287**: 1442-1446

(2000) and Insight Review Articles Nature **414**: 92-131 (2001), and the handbook "Methods of Tissue Engineering", Eds. Atala, A., Lanza, R.P., Academic Press, ISBN 0-12-436636-8; Library of Congress Catalog Card No. 200188747.

The differentiated isolated somatic target cells and/or the target tissue obtained by reprogramming of the stem cells according to the invention moreover carry the membrane-associated CD14 differentiation marker of the monocytes. Additionally, less than 3%, preferably less than 1% of these somatic target cells and/or these target tissues according to the invention express the CD34 antigen. Most preferably, none of these cells or tissues express the CD34 antigen. As shown in Example 11, hepatocytes which are derived from the stem cells according to the invention, express the CD14 surface marker which is typical of monocytes, whilst at the same time they produce the protein albumin, which is typical of hepatocytes. The hepatocytes derived from the stem cells according to the invention can therefore be distinguished from natural hepatocytes. In the same way, the membrane-associated CD14 surface marker was detected on insulin-producing cells, which were derived from the stem cells according to the invention (Example 9).

In one embodiment of the invention the dedifferentiated, programmable stem cells are used for the *in-vitro* production of target cells and target tissue (cf. Examples). Therefore, differentiated, isolated tissue cells, which are obtained by differentiation (reprogramming) of the stem cells according to the invention, and which carry the membrane-associated CD14 surface antigen, are also subject of the present invention.

The stem cells according to the invention are preferably

simply and reliably differentiated *in vitro* into desired target cells, such as for example adipocytes (cf. Example 6), neurons and glia cells (cf. Example 3), endothelial cells (cf. Example 5), keratinocytes (cf. Example 8), hepatocytes (cf. Example 7) and islet cells (islet of Langerhans, cf. Example 9), by growing the stem cells in a medium which contains the supernatant of the culture medium, in which the respective target cells and/or fragments thereof have been incubated (cf. Examples 6 to 8). This supernatant is referred to hereafter as "target-cell-conditioned medium".

For the differentiation (reprogramming) of the dedifferentiated stem cells according to the invention the following procedure can therefore be followed, in which:

a) tissue which contains or consists of the desired target cells is crushed;
b) the tissue cells (target cells) and/or fragments of these are obtained;
c) the target cells and/or fragments of these are incubated in a suitable culture medium;
d) the culture medium supernatant is collected during and after the incubation as target-cell-conditioned medium; and
e) for the reprogramming/differentiation of dedifferentiated stem cells into the desired target cells or target tissue, the stem cells are grown in the presence of the target-cell-conditioned medium.

Standard cell culture media can be used as culture medium (cf. Examples). The media preferably contain growth factors, such as for example the epidermal growth factor.

The incubation of the target cells and/or fragments of these

("cell pellet") can be carried out over 5 to 15, preferably 10 days. The supernatant, i.e. the target-cell-conditioned medium is preferably removed in each case after 2 to 4 days and replaced by fresh medium. The supernatants thus obtained can be filtered under sterile conditions separately or pooled and stored at approximately -20°C or used directly for the programming of stem cells. As shown above, the programming of the stem cells into the desired target cells is carried out by growing stem cells in the presence of the medium conditioned with the respective target cells (cf. Examples). The growth medium preferably additionally contains a target-cell-specific growth factor, such as for example the "hepatocyte growth factor" or the "keratinocyte growth factor" (cf. Examples).

In one embodiment of the invention the dedifferentiated, programmable stem cells according to the invention are used per se for the production of a pharmaceutical composition for the *in-vivo* production of target cells and target tissue.

Such pharmaceutical preparations can contain the stem cells according to the invention suspended in a physiologically well-tolerated medium. Suitable media are for example PBS (phosphate buffered saline) or physiological saline with 20 % human albumin solution and the like.

These pharmaceutical preparations contain vital dedifferentiated, programmable stem cells according to the invention, which have on their surface the CD14 surface marker and at least one more of the multipotent stem cell markers CD90, CD117, CD123 and/or CD135, in a quantity of at least 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or

50 %, preferably 60 or 70 %, particularly preferably 80 or 90 % and extremely preferably 100 %, relative to the total number of the cells present in the preparation, and optionally further pharmaceutically well-tolerated adjuvants and/or carrier substances.

Stem cell preparations can contain vital dedifferentiated, programmable stem cells according to the invention, which have on their surface the CD14 surface marker and at least one more of the pluripotent stem cell markers CD90, CD117, CD123 and/or CD135, in a quantity of at least 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58 or 59 %, preferably at least 60 %, relative to the total number of the cells present in the preparation; cell suspensions in a cell culture- or transport medium well-tolerated by cells, such as e.g. PBS or RPMI etc., or deep-frozen cell preparations in a suitable storage medium, such as e.g. RPMI with 50% human albumin solution and 10% DMSO are preferred.

The number of vital cells and hence the proportion of these in the compositions referred to above, can be determined optically by use of the "Trypan blue dye exclusion technique", as vital cells can be optically distinguished from non-vital cells, using this dye.

As a rule, it will be irrelevant for clinical use, if some of the cells present in the pharmaceutical preparation do not fulfil the criteria of dedifferentiated, programmable stem cells according to the invention, provided that a sufficient number of functional stem cells is present. It is however also

possible to eliminate non-dedifferentiated cells by means of processes known in the state of the art on the basis of surface markers typical of the dedifferentiated cells according to the invention in such preparations, so that these contain the desired cells in essentially pure form. One example of a suitable process is "Immuno magnetic bead sorting", cf. Romani et al., J. Immunol. Methods **196**: 137-151 (1996).

Stem cells further have the capability, of spontaneously differentiating *in vivo* by direct contact with a cell group of a specific cell type into cells of this type. Processes for tissue production using cells which can be redifferentiated ("tissue engineering") are known in the state of the art. For example Wang, X. et al. ("Liver repopulation and correction of metabolic liver disease by transplanted adult mouse pancreatic cells" Am. J. Pathol. **158** (2): 571-579 (2001)), have shown that even certain adult cells of the pancreas in mice are able to transform, in FAH- (fumaroylacetoacetate hydrolase)-deficient mice, into hepatocytes, which can fully compensate for the metabolic defect in these animals. A further example is the experiments of Lagasse et al., "Purified hematopoietic stem cells can differentiate into hepatocytes in vivo", Nature Medicine, **6** (11): 1229-1234 (2000). The authors have shown that hematopoietic stem cells from bone marrow were able, after *in- vivo* transfer into FAH-deficient mice, to transform into hepatocytes, which could then compensate for the metabolic defect; see also the review by Grompe M., "Therapeutic Liver Repopulation for the Treatment of Metabolic Liver Diseases" Hum. Cell, **12**: 171-180 (1999).

Particularly preferable forms of application for the *in-vivo* differentiation of the dedifferentiated stem cells according

to the invention are injection, infusion or implantation of the stem cells into one specific cell association in the body, in order to allow for the stem cells to differentiate there, by direct contact with the cell association, into cells of this cell type. For injection or infusion the cells can be administered in PBS (phosphate buffered saline).

Preferred examples of the relevant indications in this connection are: cirrhosis of the liver, pancreatic insufficiency, acute or chronic kidney failure, hormonal under-functioning, cardiac infarction, pulmonary embolism, stroke and skin damage.

Therefore preferred embodiments of the invention are the use of the dedifferentiated, programmable stem cells for the production of different pharmaceutical compositions for the treatment of cirrhosis of the liver, pancreatic insufficiency, acute or chronic kidney failure, hormonal under-functioning, cardiac infarction, pulmonary embolism, stroke and skin damage.

For the therapeutic use of the target cells obtainable from the stem cells according to the invention, a number of concepts are available (see above Science **287**: 1442-1446 (2000) and Nature **414**: 92-131 (2001)).

A further preferred application concerns the injection of the dedifferentiated stem cells according to the invention into the peritoneum, so that they differentiate there, due to the influence of the cells surrounding them, into peritoneal cells. In the case of peritoneal dialysis of patients with kidney insufficiency, these cells can take over a kidney function via their semi-permeable membrane and give off kidney

dependent waste substances into the peritoneum from where these are removed via the dialysate.

Therefore, also the differentiated, isolated, somatic target cells and/or target tissue, which are obtained by reprogramming of the stem cells and are characterised by the membrane-associated CD14 antigen are subject of the invention. These somatic target cells and/or target tissue preferably contain adipocytes, neurons and glia cells, endothelial cells, keratinocytes, hepatocytes and islet cells.

However the cells can also be introduced directly into the organ to be reconstituted. The introduction can be carried out via matrix constructions which are coated with corresponding differentiated cells or cells capable of differentiation. The matrix constructions are as a rule biodegradable, so that they disappear out of the body while the newly introduced cells grow together with the cells present. From this point of view, for example cellular, preferably autologous transplants in the form of islet cells, hepatocytes, fat cells, skin cells, muscles, cardiac muscles, nerves, bones, endocrine cells etc. come under consideration for restitution for example after partial surgical resection of an organ, for repair for example after trauma or for supportive use, for example in the case of lacking or insufficient organ function.

The stem cells according to the invention and target cells obtained from them can further be used to coat implantable materials, in order to increase biocompatibility. Therefore, also implantable materials, which are coated with the dedifferentiated, programmable stem cells or the somatic target cells and/or target tissue are subject of the invention. According to one embodiment of the invention these

implantable materials are prostheses. In particularly preferred embodiments these prostheses are cardiac valves, vessel prostheses, bone- and joint prostheses.

The implantable materials can also be artificial and/or biological carrier materials, which contain the de-differentiated, programmable stem cells or target cells. In this regard, the carrier materials can be bags or chambers for insertion into the human body.

In one embodiment of the invention such a bag, containing islet cells, which are differentiated somatic cells according to the invention, is used for the production of a pharmaceutical construct for use as an artificial islet cell port chamber for the supply of insulin.

According to a further embodiment of the invention, a bag or chamber containing adipocytes, which are differentiated somatic cells according to the invention, is used for the production of an artificial polymer filled with adipocytes as a pharmaceutical construct for breast construction after surgery and in the case of further indications of plastic and/or cosmetic correction.

Moreover, semi-permeable port chamber systems, containing endocrine cells of very widely varying provenance, can be used *in vivo* for the treatment of endocrine, metabolic or haemostatic disorders. Examples of such endocrine cells are cells which produce thyroxine, steroids, ADH, aldosterone, melatonin, serotonin, adrenalin, noradrenalin, TSH, LH, FSH, leptin, cholecystokinin, gastrin, insulin, glucagon, or clotting factors.

Therefore, also implantable materials, which are semi-

permeable port chamber systems, containing differentiated isolated somatic target cells are subject of the invention. These semi-permeable chamber systems are used in different embodiments of the invention for the production of a pharmaceutical construct for the *in-vivo* treatment of endocrine, metabolic or haemostatic disorders.

The target cells obtained from the stem cells according to the invention can in addition be used as cell cultures in bioreactors outside the body, for example in order to carry out detoxification reactions. This form of use is particularly relevant in the case of acute conditions, for example in the case of acute liver failure as a hepatocyte-bioreactor.

The production of the constructs described above and conducting the corresponding therapeutic process have already been described many times in the state of the art, compare for example the review by Lalan, S., et al. "Tissue engineering and its potential impact on surgery" World J. Surg. **25**: 1458-1466 (2001); Nasseri, B.A., et al. "Tissue engineering: an evolving 21st-century science to provide replacement for reconstruction and transplantation" Surgery **130**: 781-784 (2001) and Fuchs, J.R., et al., "Tissue engineering: a 21st century solution to surgical reconstruction" Ann. Thorac. Surg. **72**: 577-591 (2001).

Finally, the pluripotent stem cells according to the invention open up a broad field for transgenic modification and therapy. According to a preferred embodiment of the invention the dedifferentiated programmable stem cells per se or somatic target cells and/or target tissue finally differentiated from these, are transfected with one or more genes. In this way, one or more genes which are required to maintain the

metabolism of certain organs, such as for example livers or kidneys, are restored and/or supported or reintroduced. For example, stem cells or hepatocytes derived from these can be transfected with the FAH (fumaroylacetoacetate hydrolase) gene. In the FAH-deficient mouse model the intrasplenic injection of 1000 FAH-positive donor hepatocytes was sufficient to completely repopularise the liver after 6 to 8 weeks and fully compensate for the metabolic defect leading to cirrhosis of the liver (cf. Grompe, M., et al., Nat. Genet. **12**: 266 ff. (1996)).

Correspondingly, by transfection of the stem cells or the respective target cells obtained from the stem cells by programming (for example hematopoietic cells, hepatocytes, ovary cells, muscle cells, nerve cells, neurons, glia cells, cartilage or bones cells, etc.) with "Multi-Drug-Resistance-genes" extended radical chemotherapy can be made possible in the case of malignant diseases by corresponding hematopoietic reconstitution or radiation resistance can be produced.

The invention is explained in detail as follows:

The starting material for the process according to the invention is monocytes from human blood. These are preferably autologous monocytes, i.e. monocytes, which originate from the blood of the patient to be treated with the stem cells according to the invention or the target cells produced from these.

To obtain the monocytes the blood can first, after standard treatment with an anticoagulant in a known manner, preferably by centrifugation, be separated into plasma and into white and red blood cells. After the centrifugation the plasma is to be

found in the supernatant; below this lies a layer which contains the totality of the white blood cells. This layer is also referred to as "buffy coat". Below this lies the phase containing red blood cells (haematocrit).

The "buffy coat" layer is then isolated and separated to obtain the monocytes for example by centrifuging using a known process. According to a preferred process variant the "buffy coat" layer is coated onto a lymphocyte separation medium (e.g. Ficoll Hypaque) and centrifuged. By further centrifuging and rinsing, the monocyte fraction is obtained from the blood (cf. Example 1).

Examples of alternative processes for obtaining the monocytes from complete blood are "Fluorescence-Activated Cell Sorting" (FACS), "Immunomagnetic Bead Sorting" (cf. Romani et al., J. Immunol. Methods **196**: 137-151 (1996)) and "Magnetic-Activated Cell Sorting" (MACS) or the so called "Rosetting process" (cf. Gmelig-Meyling, F., et al., "Simplified procedure for the separation of human T and non-T cells" Vox Sang. **33**: 5-8 (1977)).

According to the invention, monocytes can be obtained from any isolated human blood, and the blood can also originate from organs such as the spleen, lymph nodes or bone marrow. Obtaining monocytes from organs is considered especially when the separation of the monocytes from human blood, e.g. in the case of anaemia or leukaemia, is not possible, or not in sufficient quantities, and in the case of allogenic use, if, within the framework of multi-organ removal, the spleen is available as a source for isolation of monocytes.

For the production of a sufficient quantity of stem cells

according to the invention it is first necessary to propagate the monocytes. For this purpose, growth media suitable for monocytes can be used, wherein, according to the invention said medium containes M-CSF (macrophage colony stimulating factor). M-CSF (also referred to as CSF-1) is produced by monocytes, fibroblasts and endothelial cells. The concentration of M-CSF in the culture medium can amount to 2 to 20 µg/l medium, preferably 4 to 6 µg/l and in a particularly prefered manner 5 µg/l.

On the monocytes M-CSF binds to the specific c-Fms receptor (also referred to as CSF-1R), which is exclusively present on the surface of monocytes and which only binds M-CSF (Sherr C.J., et al., Cell **41** (3): 665-676 (1985)). As the specific interaction between M-CSF and the receptor induces the division of the monocytes, the medium, in which the monocytes are cultivated contains M-CSF or an analogue thereof, which can bind to the receptor and activate it. Other growth factors such as GM-CSF (granulocyte-monocyte colony stimulating factor) and G-CSF (granulocyte colony stimulating factor) are unsuitable, as, due to the lack of affinity to the c-Fms receptor, they are not capable of inducing monocyte division.

In a particularly preferred embodiment of the process M-CSF and IL-3 are simultaneously added to the cell culture medium in Step b) of the process. The concentration of IL-3 in the medium may amount to 0,2 to 1 µg/l, preferably 0,3 to 0,5 µg/l and in a particularly preferred manner 0,4 µg IL-3/l.

It is however also possible, to add initially only M-CSF to the cell culture medium in Step b) and add IL-3 only thereafter.

In a further embodiment the culture vessel initially contains

cell culture medium which contains only M-CSF, which after the separation of the cells is then replaced by a second cell culture medium, which contains IL-3.

According to a preferred embodiment of the invention the cells in Step b) of the process are additionally cultivated in the presence of a sulphur compound, e.g. a mercapto compound, in which at least one hydrocarbon group is bonded to the sulphur, and said hydrocarbon group(s) may be substituted with one or more functional groups. Mercapto compounds are defined as compounds which have at least one mercapto group (-SH), which is bonded to a hydrocarbon group. By the additional use of such a sulphur compound, the number of the stem cells obtained by dedifferentiation of the cells of monocytic origin, which express one or more of the stem cell markers CD90, CD117, CD123 and CD135, can be increaed.

The functional group(s) is/are preferably hydroxyl- and/or amine groups. In a particularly preferred embodiment, the sulphur compound is 2-mercaptoethanol. According to a further preferred embodiment the sulphur compound is dimethylsulfoxide (DMSO).

The quantity of the sulphur compound used can range from approximately 4 to approximately 200 µmol/l relative to the sulphur. Approximately 100 µmol/l is preferred.

When 2-mercaptoethanol is used, the culture medium should contain approximately 3 µl to approximately 13 µl, preferably approximately 7 µl 2-mercaptoethanol/l.

The treatment with IL-3 and optionally with the sulphur compound can be carried out simultaneously with or following

the propagation of the monocytes by cultivation with M-CSF, simultaneous propagation and treatment with IL-3 and optionally a sulphur compound being prefered. Propagation and dedifferentiation should, taken together, last no more than 10 days, and the treatment with IL-3 and optionally with the sulphur compound should be carried out over at least 3 and at most 10 days, preferably 6 days.

Therefore, according to the invention, in the case of cultivation of the monocytes in a culture medium, which simultaneously contains M-CSF, IL-3 and preferably a mercapto compound, the duration of cultivation until the detaching of the cells from the bottom of the culture vessel amounts to at least 3 and at most 10 days, preferably 5 to 8 days and particularly preferably 6 days.

If in a preferred embodiment the process according to the invention is carried out in such a way that the monocytes in Step b) are initially propagated in a medium containing only M-CSF, the propagation in such a culture medium can take place over a period of at least 2, preferably 3 and particularly preferably 4 days with a maximum duration of 7 days, and a subsequent cultivation in the presence of IL-3 and optionally of a mercapto compound can take place over a further 3 days. Preferably in such a case the cultivation in a medium containing only M-CSF will however only last a maximum of 4 days, followed by a cultivation in the presence of IL-3 and optionally of a mercapto compound over a period of 3, 4, 5 or 6 days.

To carry out the propagation and dedifferentiation jointly, as described in Examples 2 and 13, the monocytes are after isolation transferred into a medium, which contains both M-

CSF, and IL-3 as well as preferably the sulphur compound, in particular mercaptoethanol or DMSO.

Due to their adhesive properties the monocytes and the stem cells produced from them during the process adhere to the bottom of the respective culture vessel. According to a preferred embodiment of the invention, the culture medium is after Step c) separated from the cells adhering to the bottom of the culture vessel and is discarded. This is preferably followed by rinsing of the cells adhering to the bottom with culture medium, and the cells are then covered with fresh culture medium (cf. Example 13).

In this step the propagation and dedifferentiation medium described above can be used as culture medium, as well as a standard cell culture medium, for example RPMI.

According to a further prefered embodiment of the invention, the cells are brought into contact with a biologically well-tolerated organic solvent at the end of Step c) and before Step d), in order to increase the number of stem cells floating freely in the medium at the end of the process. The quantity of the solvent can range from 10 µl to 1 ml. This is preferably an alcohol with 1-4 carbon atoms, the addition of ethanol being particularly prefered. According to a particularly preferred embodiment the cells are brought into contact with the vapour phase of the previously defined biologically well-tolerated organic solvent, preferably with ethanol vapour (cf. Example 2). The time for exposure to the organic solvent, particularly preferably to ethanol vapour, should amount to 4 - 12 hours, preferably 8 - 10 hours.

The process according to the invention is preferably carried

out in culture vessels, the surface of which has previously been coated with foetal calf serum (FCS) (cf. Example 2). Alternatively human AB-Serum from male donors can be also be used. The coating with FCS can be carried out by covering the surface of culture vessels with FCS before use, and after an exposure time of a few, in particular 2 to 12 hours, and in a particularly preferable manner 7 hours, and by removing the FCS not adhering to the surface in a suitable manner.

If treatment with organic solvent take place after Step c) optionally after exchange of the culture medium, the cells already become detached from the bottom to a certain extent in this process step. The (further) detaching can be carried out mechanically, for example with a fine cell scraper, spatula or tip of a pipette (cf. Example 13).

According to a preferred embodiment of the process, complete detaching is carried out by treatment with a suitable enzyme, for example with trypsin (cf. Example 2). The cells may be exposed to the trypsin solution (0,1 to 0,025 g/l, preferably 0,05 g/l) for 2-10 minutes at 35°C to 39°C, preferably at 37°C, in the presence of CO_2.

The trypsin activity is then blocked by a standard method, and the now freely floating dedifferentiated programmable stem cells can be obtained by a standard method, for example by centrifuging and in one embodiment by suspended in a suitable cell culture at the end of Step d). They are now available, suspended in a suitable medium, for example in RPMI 1640 or DMEM, for immediate differentiation into the desired target cells. They can however also be stored in the medium for a few days. In a preferred embodiment the medium contains a cytokine or LIF factor (leukemia inhibitory factor), cf. Nature **414**: 94

(2001, Donovan, P.J., Gearhardt, J., loc. cit.), if the cells are to be stored in culture for longer than approximately 48 hours as dedifferentiated programmable stem cells. In a medium containing such factors stem cells can be kept for at least 10 days as dedifferentiated programmable stem cells.

In a preferred embodiment the cells are suspended for longer storage in a liquid medium and then deep-frozen. Protocols for the deep freezing of living cells are known in the state of the art, cf. Griffith M., et al. "Epithelial Cell Culture, Cornea, in Methods of Tissue Engineering", Atala A., Lanza R.P., Academic Press 2002, Chapter 4, Pages 131 to 140. A preferred suspension medium for the deep freezing of the stem cells according to the invention is FCS-containing DMEM, cf. Example 2.

The invention is further exemplified and described below with reference to examples.

If not defined within the examples, the composition of the media and substances used are as follows:

1. **Penicillin/streptomycin solution:**
   10,000 units of penicillin as sodium salt of penicillin G and 1000 µg streptomycin as streptomycin sulphate per ml physiological sodium chloride solution (NaCl 0,9 %).

2. **Trypsin-EDTA**
   0.5 g trypsin and 0.2 g EDTA (4 Na)/l

3. **Insulin**
   human, recombinant, produced in E.coli, approximately 28 units/mg

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4. RPMI 1640 (1x, liquid (11875)) contains L-Glutamine

RPMI (Roswell Park Memorial Institute) Media 1640 are enriched formulations, which can be used extensively for mammalian cells.

Components	Mol.-weight	Conc. (mg/l)	Molarity (nM)
Anorganic salts			
Calcium nitrate ($Ca(NO_3)_2\ 4H_2O$)	236	100.00	0.424
Potassium chloride (KCl)	75	400.00	5.30
Magnesium sulphate ($MgSO_4$)	120	48.84	0.407
Sodium chloride (NaCl)	58	6000.00	103.44
Sodium bicarbonate ($NaHCO_3$)	84	2000.00	23.800
Sodium phosphate (Na_2HPO_4)	142	800.00	5.63
Further components			
Glucose	180	2000.00	11.10
Glutathione, reduced	307	1.50	0.0032
Phenol red	398	5.00	0.0125
Amino acids			
L-Arginine	174	200.00	1.10
L-Asparagine	132	50.00	0.379
L-Asparaginic acid	133	20.00	0.150
L-Cysteine dihydrochloride	313	65.00	0.206
L-Glutamininc acid	147	20.00	0.136
L-Glutamine	146	300.00	2.05
Glycine	75	10.00	0.133
L-Histidine	155	15.00	0.0967
L-Hydroxyproline	131	20.00	0.153
L-Isoleucine	131	50.00	0.382
L-Leucine	131	50.00	0.382
L-Lysine hydrochloride	146	40.00	0.219
L-Methionine	149	15.00	0.101
L-Phenylalanine	165	15.00	0.0909
L-Proline	115	20.00	0.174
L-Serine	105	30.00	0.286
L-Threonine	119	20.00	0.168
L-Tryptophan	204	5.00	0.0245
L-Tyrosine disodium, dihydrate	261	29.00	0.110
L-Valine	117	20.00	0.171
Vitamins			
Biotin	244	0.20	0.008
D-calcium pantothenate	477	0.25	0.0005
Choline chloride	140	3.00	0.0214
Folic acid	441	1.00	0.0022
i-Inositol	180	35.00	0.194
Niacinamide	122	1.00	0.0081
p-aminobenzoic acid (PABA)	137	1.00	0.0072
Pyridoxine HCl	206	1.00	0.0048
Riboflavin	376	0.20	0.0005
Thiamin HCl	337	1.00	0.0029
Vitamin B12	1355	0.005	0.00000369

Reference: Moore G.E., et al., J.A.M.A. 199: 519 (1967)

5. PBS (Dulbecco's phosphate buffered saline) cf. J. Exp. Med. **98**:167 (1954):

Components	g/l
KCl	0.2
KH_2PO_4	0.2
NaCl	8.00
Na_2PHO_4	1.15

6. 2-Mercaptoethanol

Quality for synthesis; Content > 98%, Density 1.115 to 1.116, cf. e.g. Momo J., et al., J. Am. Chem. Soc. **73**: 4961 (1951).

7. Ficoll-Hypaque:

Lymphocyte separation medium (saccharose/epichlorohydrin-copolymerisate Mg 400,000; Density 1.077, adjusted with Sodium diatrizoate).

8. Retinic acid:

Vitamin A acid ($C_{20}H_{28}O_2$), 300 µl in 1.5 ml PBS corresponding to 1 mM. As medium for programming of neurons and glia cells use 150 µl on 10 ml medium (corresponding to 10^{-6} M).

9. DMEM

Dulbecco's modified Eagle medium (high glucose)
cf. Dulbecco, R. et al., Virology **8**: 396 (1959); Smith, J.D. et al., Virology **12**: 158 (1960); Tissue Culture Standards Committee, In Vitro **6**: 2 (1993)

10. L-Glutamine

Liquid: 29.2 mg/ml

11. Collagenase Type II:

Cf. Rodbell, M. et al., J. Biol. Chem. **239**: 375 (1964).

12. Interleukin-3 (IL-3):

Recombinant human IL-3 from E. coli (Yang Y.C. et al., Cell **47**: 10 (1986)); contains the 133 amino acid residues including mature IL-3 and the 134 amino acid residues including the methionyl form in a ratio of approximately 1:2; calculated mol. mass approximately 17.5 kD; specific activity 1 x 10^3 U/µg; (R&D Catalogue No. 203-IL)

13. Macrophage-colony stimulating factor (M-CSF)

Recombinant human M-CSF from E. coli; contains as monomer (18.5 kD) 135 amino acid residues including the N-terminal methionine; is present as a homodimer with a molar mass of 37 kD; (SIGMA Catalogue No. M 6518)

14. Antibodies:

The antibodies used in the examples against the antigens CD14, CD31, CD90, CD117, CD123, CD135 are commercially available. They were obtained from the following sources:

CD14: DAKO, Monoclonal Mouse Anti-Human CD14, Monocyte, Clone TÜK4, Code No. M 0825, Lot 036 Edition 02.02.01;

CD31: PharMingen International, Monoclonal Mouse Anti-Rat CD31 (PECAM-1), Clone TLD-3A12, Catalogue No. 22711D, 0.5mg;

CD90: Biozol Diagnostica, Serotec, Mouse Anti-Human CDw90,

Clone No. F15-42-1, MCAP90, Batch No. 0699;

CD117: DAKO, Monoclonal Mouse Anti-Human CD117, c-kit, Clone No. 104D2, Code No. M 7140, Lot 016, Edition 04.05-.00;

CD123: Research Diagnostics Inc., Mouse Anti-human CD123 antibodies, Clone 9F5, Catalogue No. RDI-CD123-9F5;

CD135: Serotec, Mouse Anti-Human CD135, MCA1843, Clone No. BV10A4H2.

Example 1

Separation of monocytes from whole blood

To avoid blood clotting and to feed the cells, 450 ml of whole blood in a 3-chamber bag set was mixed with 63 ml of a stabilising solution, which contained for each litre of H_2O, 3.27 g citric acid, 26.3 g trisodium citrate, 25.5 g dextrose and 22.22 g sodium dihydroxyphosphate. The pH-value of the solution amounted to 5.6-5.8.

"Sharp centrifugation" of this mixture was then carried out to separate the blood components at 4000 rpm for 7 minutes at 20°C. This resulted in a 3-fold stratification of the corpuscular and non-corpuscular components. By inserting the set of bags into a pressing machine provided for this purpose, the erythrocytes were then pressed into the lower bag, the plasma was pressed into the upper bag, and the "Buffy-coat" remained in the middle bag, and it contained approximately 50 ml in volume.

The quantity of 50 ml freshly obtained "Buffy-coat" was then divided into 2 portions of 25 ml each, each of which was then coated with 25 ml Ficoll-Hypaque separation medium, which had been introduced into two 50 ml Falcon tubes beforehand.

This mixture was centrifuged without brake for 30 minutes at 2500 rpm. Thereafter, erythrocytes and dead cells still present in the "Buffy coat" lay below the Ficoll phase whilst the white blood cells including the monocytes are separated as a white interphase on the Ficoll.

The white interphase of the monocytes was then carefully pipetted off and was mixed with 10 ml of phosphate buffered physiological saline (PBS).

This mixture was then centrifuged with brake three times for 10 minutes at 1800 rpm; the supernatant was pipetted off after each centrifugation and fresh PBS was filled up.

The cell sediment collected on the base of the centrifugation vessel (Falcon tube) contained the mononuclear cell fraction, i.e. the monocytes.

Example 2

Propagation and dedifferentiation of the monocytes

The cultivation and propagation of the monocytes on the one hand and the dedifferentiation of the cells on the other hand were carried out in one step in nutrient medium of the following composition:

RPMI 1640 medium	440 ml
Foetal calf serum (FCS)	50 ml
Penicillin/Streptomycin solution	5 ml
2-Mercaptoethanol (Stock solution)	5 ml
Total volume	500 ml

The nutrient medium further contained 2,5 µg/500 ml of M-CSF and 0,2 µg/500 ml interleukin-3 (IL-3).

The monocytes isolated in Example 1 were transferred into 5 chambers of a 6-chamber well plate (30 mm diameter per well) in a quantity of approximately 10^5 cells per chamber in each case, and filled up in each case with 2 ml of the above-mentioned nutrient medium. The 6-well plate was previously filled with pure, inactivated FCS and the FCS was decanted after approximately 7 hours, in order to obtain an FCS-coated plate in this way. The cell number for the exact dose per well was determined according to a known process, cf. Hay R.J., "Cell Quantification and Characterisation" in Methods of Tissue Engineering, Academic Press 2002, Chapter 4, Pages 55-84.

The 6-well plate was covered with its lid and stored for 6 days in an incubator at 37°C. The cells settled to the bottom of the chambers after 24 hours. Every second day the supernatant was pipetted off and the chambers of the 6-well plate were again each filled up with 2 ml of fresh nutrient medium.

On the 6th day 2 ml of 70% ethanol was introduced into the 6-well plate's 6th chamber which had remained free, the plate was again closed and was stored for a further 10 hours at 37°C in the incubator.

Subsequently, 1 ml of a trypsin solution diluted 1:10 with PBS were pipetted into each of the chambers of the well plate which contained cells. The closed well plate was placed for 5 minutes at 37°C under 5% CO_2 in the incubator.

The trypsin activity was subsequently blocked by the addition of 2 ml of RPMI 1640 medium to each of the wells. The total supernatant in each of the chambers (1 ml trypsin + 2 ml medium) was pipetted off, pooled in a 15 ml Falcon tube and centrifuged for 10 minutes at 1800 rpm. The supernatant was then discarded and the precipitate was mixed with fresh RPMI 1640 medium (2 ml/10^5 cells).

This cell suspension could be directly used for differentiation into different target cells.

Alternatively, after centrifugation and discarding of the trypsin-containing supernatant the cells were mixed with DMSO/FCS as a freezing medium and deep-frozen at a concentration of 10^6/ml.

The freezing medium contained 95% FCS and 5% DMSO. In each case approximately 10^6 cells were taken up in 1 ml of the medium and cooled down in the following steps:

30 minutes on ice;
2 hours at -20°C in pre-cooled Styropor boxes;
24 hours at -80°C in Styropor;

Storage in tubes in liquid nitrogen (N_2) at -180°C.

For immune-histochemical phenotyping of the cell population of dedifferentiated programmable stem cells of monocytic origin, generated according to the above process, in each case 10^5 cells were taken and fixed as a cytospin preparation on slides for further histochemical staining (Watson, P. "A slide centrifuge; an apparatus for concentrating cells in suspension on a microscope slide." J. Lab. Clin. Med., **68**: 494-501 (1966)). After this the cells could be stained using the technique described by Cordell, J.L., et al., (Literature, see below) with APAAP red complex. If not indicated otherwise, the added primary antibody was diluted 1:100 with PBS, and in each case 200 µl of this concentration of antibodies was used. Monoclonal antibodies were used as primary antibodies against the cell antigen epitopes listed in Table 1. Figure 6 shows stained cytospin preparations and the corresponding proof of the stem cell markers CD90, CD117, CD123 and CD135.

Literature relating to staining technique:

Cordell J.L., et al. "Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes)." J. Histochem. Cytochem. **32**: 219-229 (1984).

Literature relating to the markers:

CD14

Ferrero E., Goyert S.M. "Nucleotide sequence of the gene encoding the monocyte differentiation antigen, CD14" Nucleic Acids Res. **16**: 4173-4173 (1988).

CD31
Newman P.J., Berndt M.C., Gorski J., White J.C. II, Lyman S., Paddock C., Muller W.A. "PECAM-1(CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily" Science **247**: 1219-1222 (1990).

CD90
Seki T., Spurr N., Obata F., Goyert S., Goodfellow P., Silver J. "The human thy-1 gene: structure and chromosomal location" Proc. Natl. Acad. Sci. USA **82**: 6657-6661 (1985).

CD117
Yarden Y., Kuang W.-J., Yang-Feng T., Coussels L., Munemitsu S., Dull T.J., Chen E., Schlessinger J., Francke U., Ullrich A. "Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand." EMBO J. **6**: 3341-3351 (1987).

CD123
Kitamura T., Sato N., Arai K., Miyajima A. "expression cloning of the human IL-3 receptor cDNA reveals a shared beta subunit for the human IL-3 and GM-CSF receptors." Cell **66**: 165-1174 (1991).

CD135
Small D., Levenstein M., Kim E., Carow C., Amn S., Rockwell P., Witte L., Burrow C., Ratajazak M.Z., Gewirtz A.M., Civin C.I.
"STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34+ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells." Proc. Natl. Acad. Sci. USA **91**: 459-463 (1994).

Table 1

Antigen expression of the stem cells according to the invention

Antigen	Colour reaction
Stem cell marker	
CD90	++
CD117	+
CD123	++
CD135	+(+)
Differentiation marker	
CD14 (monocytes)	+

The graduation indicated corresponds to the detected antigen positivity, which becomes apparent from Day 4 to Day 9 after cultivation of the monocytes in the correspondingly specified media and was carried out via microscopic comparison of the respective cytospin colorations with the negative control (coloration observed without primary antibodies).

+ clear colour reaction of the cells with the primary antibody;
++ strong colour reaction of the cells with the primary antibody.

Only cytospin preparations which had more than 70% vital cells with typical stem cell morphology (cf. Figure 6) were evaluated. Less than 1% of these cells expressed the CD34

antigen.

Example 3

Production of neurons and glia cells from adult stem cells

The production of neurons and glia cells was carried out in petri dishes with a diameter of 100 mm. To prepare the petri dishes, 5 ml of pure inactivated foetal calf serum (FCS) was introduced into each dish, so that the bottom was covered. After 7 hours, the proportion of FCS not adhering to the bottom of the petri dish was pipetted off. Approximately 10^6 of the cells produced in accordance with Example 2 were introduced into one of the prepared petri dishes and 10 ml of nutrient medium of the following composition was added:

DMEM solution	440 ml
Fetal calf serum (FCS)	50 ml
l-Glutamine	5 ml
Penicillin (100 U/l)/Strepto-mycin (100 µg/l) solution	5 ml
Total volume	500 ml

The nutrient medium further contained retinic acid in a quantity of 1×10^{-6} M/500 ml.

The reprogramming/differentiation of the stem cells used into neurons and glia cells took place within 10 days, the medium being changed at intervals of approximately 3 days. After this period, the cells were mostly adhering to the bottom of the

chamber and could be detached by brief trypsinization from the bottom of the plate in a manner analogous to that previously described for the stem cells.

Example 4

Evidence of neuronal precursor cells, neurons and glia cells

For the later immunohistochemical characterisation of the target cells induced by the dedifferentiated programmable stem cells, the stem cells generated from monocytes (10^5 cells / glass lid) were applied to glass lids (20mm x 20mm), which were placed on the bottom of the 6-well plates (30 mm diameter per chamber) and cultivated with the nutrient medium (2 ml) per well plate. After the respective target cells were differentiated, these were fixed as follows: After removal of the nutrient medium (supernatant) the cultivated target cells were fixed by the addition of 2 ml Methanol, which took effect over 10 minutes. Subsequently the ethanol was pipetted off, and the well plates were washed twice with PBS (2 ml in each case). After this, the cells could be stained with APAAP red complex using the technique described by Cordell, J.L., et al., "Immunoenzymatic labeling monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes)." J. Histochem. Cytochem. **32**: 219-229 (1994). Unless otherwise specified, the added primary antibody was diluted 1:100 with PBS, in each case 200 µl of this concentration of antibodies were pipetted into each of the 6 wells.

Neuronal precursor cells were detected by staining the cells

with the antibody against the S100-antigen, cf. middle picture of Figure 1 (x200).

Neurons were detected by specific expression of synaptophysin MAP2 (microtubular associated protein 2) or neurofilament 68 with the corresponding specific antibodies (primary antibody diluted 1:300 with PBS), right-hand picture of Figure 1, x200.

Glia cells, such as for example astrocytes, were identified by detection of GFAP (glial fibrillary associated protein) (primary antibody diluted 1:200 with PBS), left-hand picture of Figure 1, x200.

The separation of neurons and glia cells was carried out using antibodies specific against MAP2 (neurons) or GFAP (glia cells), by means of MACS (Magnetic Activated Cell Sorting) according to the process as described for example in Carmiol S., "Cell Isolation and Selection" Methods of Tissue Engineering, Academic Press 2002, Chapter 2, Pages 19-35.

The cell types made visible by staining are shown in Figure 1.

Example 5

Production of endothelial cells from dedifferentiated programmable adult stem cells of monocytic origin

For the cultivation of endothelial cells, Matrigel® (Beckton and Dickinson, Heidelberg, DE) was used as matrix. This matrix consists of fibronectin, laminin and collagens I and IV.

The frozen matrix was slowly thawed at 4°C in a refrigerator

over a period of 12 hours. During this period its state changed, i.e. the originally solid matrix became spongy/liquid. In this state it was introduced into a 48-well plate (10 mm diameter per well) in such a manner, that the bottom of each of the wells was covered.

After application, the plate was kept for 30 minutes at room temperature, until the gel had solidified at the bottom as an adherent layer.

Subsequently approximately 1×10^2 cells per well were incubated on Matrigel® with addition of the nutrient medium (as described in Example 2).

After 4-5 days the first tubular cell strands appeared, which developed after 6-8 days into three-dimensional cell networks. On the cells, the endothelial markers CD31 and factor VIII could be identified with the respective specific primary antibodies (200 µl, in each case diluted to 1:100 with PBS).

In an alternative process the liquefied matrix was applied to a vessel-prosthesis, which was then coated with the dedifferentiated programmable adult stem cells according to Example 2. After approximately 6 days a lawn of endothelial cells could be identified, which coated the prosthesis in a circular manner.

The endothelial cells made visible by staining with corresponding endothelium-specific antibodies (see above) are shown in Figure 2. In the middle picture, the cells are shown after 5 days' incubation on Matrigel®. First tubular strands combine individual cell aggregates. The dark-brown marked cells express CD31 antigen (x200 with yellow filter). After 8

days there is an increasing formation of three-dimensional network structures takes place (anti-CD31-antigen staining, x200 with yellow filter). After 12 days the newly differentiated $CD31^+$ cells, which had been cultivated on Matrigel®, form a vessel-like three-dimensional tube with multi-layer wall structures, which is already morphologically reminiscent of a vessel. It is recognised, that now almost all the cells express the CD31 antigen (CD31 coloration, x400, blue filter), right-hand picture.

Example 6

Production of fat cells (adipocytes)

A: For the programming/differentiation of the adult stem cells according to Example 2 into fat cells, a conditioned medium was first generated. For this purpose 20 g of an autologous fat tissue, i.e. fat tissue from the same human donor, from the blood of whom the monocytes also originated, was processed as follows:

At first, the fat tissue was crushed in a petri dish and the crushed tissue pieces were passed through a sieve (diameter of holes 100 µm).

The suspension thus obtained was then transferred into a petri dish with a diameter of 100 mm and 10 ml DMEM-medium with a content of 30 mg collagenase type II were added. The mixture was left for approximately 60 minutes at room temperature (22°C±2°C) to allow the collagenase to take effect on the fat cells.

Subsequently the mixture was transferred to 50-ml Falcon tubes, and the tubes were centrifuged for 10 minutes at 1800 rpm.

After centrifugation the supernatant was discarded and the cell pellet consisting of adipocytes and precursor cells was taken up in 8 ml of a medium of the following composition and incubated in petri dishes (diameter 100 mm) for 10 days at 37°C in an incubator:

DMEM solution	444,5 ml
Foetal calf serum (FCS)	50 ml
Insulin solution	0,5 ml
Penicillin (100 U/l)/Strepto-mycin (100 µg/l) solution	5 ml
Total volume	500 ml

The insulin solution contained 18 mg insulin (Sigma 1-0259) dissolved in 2 ml of acetic water (consisting of 40 ml of H_2O and 0.4 ml of glacial acetic acid). The solution is diluted 1:10 with acetic water.

During the incubation over 10 days, the fat-cell-conditioned medium (FCCM) formed a supernatant. The supernatant was replaced with fresh nutrient medium after 2 to 4 days in each case. The FCCM obtained during each change of medium was subjected to sterile filtration and stored at -20°C. Subsequently 10 ml of the FCCM described above were introduced into a petri dish (diameter 100 mm) together with approximately 10^6 stem cells according to Example 2. The first precursor cells containing fat

vacuoles became visible after 4 days (Figure 3A). After 6 days, single adipocytes appeared, which could be stained with Sudan red (Figures 3B and C). After 10 days there was typical aggregation and cluster formation of these cells, which at this step could already be observed macroscopically as fat tissue (Figure 3D).
The fat cells made visible by staining in Figures 3A-3D thus differ quite considerably from the controls 3E and 3F: Figure 3E shows the cells of monocytic origin, which were cultivated in the nutrient medium (as indicated in Example 2) for 6 days, but without the addition of IL-3 and 2-mercaptoethanol to the nutrient medium. This was followed by the addition of the FCCM. These cells were not capable of differentiating into fat cells. Figure F. shows cells, which were cultivated for 6 days with complete medium (according to Example 2), and which were then treated for a further 6 days with nutrient medium instead of with FCCM (according to Example 2). The FCCM thus contains components which are required to provide the signal for differentiation into fat cells.

The staining of the cells with Sudan red in Figures 3A, B, C and D took place according to the method described by Patrick Jr., C.W., et al. "Epithelial Cell Culture: Breast", in Methods of Tissue Engineering, Academic Press 2002, Chapter 4, Pages 141-149.

B: In addition to the phenotyping of the fat cells by staining with Sudan red, molecular-biological characterisation of the fat cells was carried out at the mRNA level, in order to check whether the genetic programme of the fat cells, after corresponding programming with the fat-cell-conditioning medium used, undergoes a corresponding

alteration, and typical messenger-ribonucleic acid (mRNA) transcripts, described for fat cells can be identified in the fat cells programmed from programmable monocytes. Two mRNA sequences typical of fat cell metabolism were amplified by means of polymerase chain reaction (PCR) from isolated RNA samples from dedifferentiated programmable stem cells of monocytic origin and, in a parallel test mixture, amplified from the programmed fat cells, namely "peroxisome proliferative activated receptor gamma" (PPARG)-mRNA, (Tontonoz, P., et al. "Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor." Cell **79:** 1147-1156 (1994), gene bank access code number; NM_005037) and "leptin (obesity homolog, mouse)"-mRNA, (Zhang Y., et al. "Positional cloning of the mouse obese gene and its human homologue." Nature **372:** 425-432 (1994), gene bank, access code number: NM_000320).

The RNA-isolation needed for this purpose, the reverse transcription method and the conditions of the PCR amplification of the desired mRNA sequences were carried out as described in detail in the state of the art, see Ungefroren H., et al., "Human pancreatic adenocarcinomas express Fas and Fas ligand yet are resistant to Fas-mediated apoptosis", Cancer Res. **58:** 1741-1749 (1998).

For this purpose the respective primers produced for the PCR amplification were selected so that the forward- and reverse primers bind to mRNA sequences, whose homologous regions in the chromosomal gene lie in two different exons and are separated from one another by a large intron. It could thereby be ensured that the amplification fragment obtained originates from the mRNA contained in the cell and

not from the sequence present in the chromosomal DNA. In particular the following primer sequences were selected for PPAR-γ and for leptin:

PPAR-γ: forward-primer; 265-288 (corresponding gene sequence in exon 1), reverse-primer: 487-465 (corresponding gene sequence in exon 2), this results in an amplification fragment of 487-265 bp = 223 bp, see Figure 3G. As further shown by Figure 3G traces of transcribed PPAR-γ-specific mRNA can already be identified in the programmable stem cell and in the tumor cell line HL-60 (of a human promyeloic leukaemia cell line), although with significantly narrower signal bands than in the fat cell itself. In contrast, the fat-cell-specific protein leptin can only be detected in the fat cells derived from the programmable stem cells at mRNA level by reverse-transcriptase PCR.

The programmable stem cells (progr. stem cell) used as a control and the human tumour cell lines HL-60, Panc-1 and WI-38 transcribe no leptin. As negative controls all the samples without the addition of the reverse transcriptase (fat cell/-RT) and H_2O-samples were simultaneously co-determined. By identification of the GAPDH "house-keeping" gene in the positive controls, it is ensured that the respective PCR amplification steps were properly carried out in the individual mixtures.

Example 7

Production of liver cells (hepatocytes)

A: For the programming of the dedifferentiated programmable stem cells of monocytic origin according to Example 2 into liver cells, a conditioned medium was first generated. For this purpose 40 g of human liver tissue was processed as follows.

First the liver tissue was rinsed several times in PBS, to essentially remove erythrocytes. The tissue was then crushed in a petri dish and incubated with a dissociation solution for approximately 45 minutes at room temperature. The dissociation solution consisted of 40 ml PBS (phosphate buffered saline), 10 ml of a trypsin solution diluted 1:10 with PBS and 30 mg collagenase type II (Rodbel M., et al. J. Biol. Chem. **239**: 375 (1964)). After 45-minutes' incubation the tissue pieces were passed through a sieve (see Example 6).

The mixture was then transferred into 50-ml Falcon tubes, filled up to 50 ml with PBS and centrifuged for 10 minutes at 1800 rpm.

After centrifugation the supernatant was discarded and the cell pellet containing the liver cells was again washed with 50 ml PBS and centrifuged. The supernatant thus produced was again discarded and the cell pellet taken up in 25 ml of a medium of the following composition and incubated in cell culture flasks (250 ml volume) for 10 days at 37°C in an incubator:

Liver cell growth medium

Liver cell growth medium, LCGM	
RPMI 1640 medium	445 ml
Foetal calf serum (FCS)	50 ml
Insulin solution	0.5 ml
Penicillin (100 U/l)/Streptomycin (100 µg/l) solution	5 ml
Total volume	500 ml

The nutrient medium contained in addition 5 µg (10 ng/ml) of epidermal growth factor (Pascall, I.C. et al., J. Mol. Endocrinol. **12**: 313 (1994)). The composition of the Insulin solution was as described in Example 6.

During the incubation lasting 10 days the liver cell conditioned medium (LCCM) formed as a supernatant. The supernatant was replaced by fresh nutrient medium after 2 to 4 days respectively. The respective LCCM obtained during the change of medium in each case was subjected to sterile filtration (filter with 0.2 µm pore size) and stored at -20°C.

1×10^6 dedifferentiated stem cells were then cultivated with 10 ml of a medium of the following composition in a petri dish (Ø 100 mm) or a culture flask.

Liver cell differentiation medium

(Liver cell differentiation medium, LCDM):	
LCCM	100 ml
Insulin solution (cf. Example 6)	0.1 ml
epidermal growth factor	1 µg
hepatocyte growth factor	2 µg

Hepatocyte growth factor (Kobayashi, Y. et al., Biochem. Biophys. Res. Commun. **220**: 7 (1996)) was used in the concentration of 40 ng/ml. After a few days morphological changes towards flat, polygonal mono- or diploid cells could be observed (Figure 4A). After 10-12 days hepatocytes arising from dedifferentiated stem cells could be identified by immune-histochemical detection of the liver-specific antigen alpha-fetoprotein (Jacobsen, G.K. et al., Am.J. Surg.Pathol. **5**: 257-66 (1981)), as shown in Figures 4B and 4C.

B: In addition to the phenotyping of the hepatocytes by immune-histochemical identification of the alpha-fetoprotein, a molecular-biological characterisation of the hepatocytes at mRNA level was carried out, in order to check whether the genetic programme of the stem cells, after corresponding programming with the liver-cell-conditioning medium used undergoes a corresponding alteration, and whether messenger-ribonucleic acid (mRNA) transcripts, described as typical of liver cells in the hepatocytes arising from the stem cells according to the invention can be identified. For this purpose, the presence of five different mRNA sequences typical of hepatocytes was

examined by means of polymerase chain reaction (PCR) in isolated RNA samples from dedifferentiated programmable stem cells of monocytic origin and, in a parallel test sample, from the liver cells obtained by programming of the stem cells. In particular, this is the Homo sapiens albumin-mRNA (Lawn, R.M., et al. "The sequence of human serum albumin cDNA and its expression in E.coli." Nucleic Acids Res. **9**: 6103-6114, (1981), gene bank access code number: NM-000477), alpha-fetoprotein-mRNA (Morinaga T., et al. "Primary structures of human alpha-fetoprotein and its mRNA." Proc. Natl. Acad. Sci. USA **80**: 4604-4608 (1983), gene bank access code number: V01514), Human carbamyl phosphate synthetase I mRNA (Haraguchi, Y., et al. "Cloning and sequence of a cDNA encoding human carbamyl phosphate synthetase I: molecular analysis of hyperammonemia" Gene **107**: 335-340 (1991), gene bank access code number D90282), Homo sapiens coagulation factor II (Thrombin, F2) mRNA (Degen, S.J. et al. "Characterization of the complementary deoxyribonucleic acid and gene coding for human prothrombin" Biochemistry **22**: 2087-2097 (1983), gene bank access code number NM-000506), Homo sapiens coagulation factor VII (serum prothrombin conversion accelerator, F7) mRNA (NCBI Annotation Project. Direct Submission, 06-Feb-2002, National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA, gene bank access code number XM-027508).

The RNA-isolation necessary for this reverse transcriptase method and the conditions of the PCR amplification of the desired mRNA sequences was carried out as described in detail in the state of the art, see Ungefroren H., et al., "Human pancreatic adenocarcinomas express Fas and Fas ligand yet are resistant to Fas-mediated apoptosis" Cancer

Res. **58**: 1741-1749 (1998).

The respective primers for the PCR amplification were selected so that the forward- and reverse primers bind to mRNA sequences whose homologous regions in the chromosomal gene lie in two different exons and are separated from one another by a large intron. In this way it could be ensured that the amplification fragment obtained originates from the mRNA contained in the cell and not from the sequence present in the chromosomal DNA.

The primer sequences indicated below were selected; the results of the respective PCR analyses are reproduced in Figure 4D. The dedifferentiated programmable stem cells according to the invention, are designated there as "progr. stem cell" and the hepatocytes derived by programming of these as "progr. hepatocyte".

- Alpha-fetoprotein: forward primer: 1458-1478 (corresponding gene sequence in Exon 1), reverse primer: 1758-1735 (corresponding gene sequence in Exon 2), this results in an amplification fragment of 1758-1458 bp = 391 bp, see Figure 4D.

As shown in Figure 4, the programmable stem cell (progr. stem cell), which itself contains no identifiable specific mRNA transcripts for alpha-fetoprotein, can be programmed into a hepatocyte (progr. hepatocyte), which contains this mRNA transcript (positive band with a molecular weight of 301 bp). This also explains the immune-histochemical detectability of the alpha-fetoprotein, as shown in Figures 4B and 4C. The positive controls, namely human liver tissue and the liver tumour cell line HepG2 also transcribe alpha-

fetoprotein-specific mRNA, as the 301 bp bands confirm.

- Albumin: forward primer: 1450-1473 (corresponding gene sequence in exon 1), reverse primer: 1868-1844 (corresponding gene sequence in Exon 2), this resulted in an amplification fragment of 1868-1450 bp = 419 bp, see Figure 4D.

  Figure 4D shows traces of transcribed albumin-specific mRNA already in the programmable stem cell, whilst the hepatocytes obtained by programming of the stem cells and normal liver tissue as well as the tumour cell line HepG2, which were both used as positive controls, strongly express the mRNA, as can be recognized by clear bands.

- The carbamyl phosphatase synthetase I: forward primer: 3135-3157 (corresponding gene sequence in exon 1), reverse primer: 4635-4613 (corresponding gene sequence in exon 2), this results in an amplification fragment of 4635-3135 = 1500 bp, see Figure 4D.

  The carbamyl phosphate synthetase I represents an enzyme specific to the hepatocytes, which plays an important role in the metabolisation of urea in the "urea cycle". This detoxification function is guaranteed by functioning hepatocytes. As Figure 4D shows, both in the hepatocytes generated from programmable stem cells and also in the positive controls (human liver tissue and the HepG2-tumour cell line), the mRNA bands (1500 bp) specific to carbamyl phosphate synthetase I can be identified. The somewhat weaker expression of the mRNA bands for the programmed hepatocytes (progr. hepatocyte) is due to the lack of substrate available in the culture dish.

- Clotting factor II: forward primer: 1458-1481 (corresponding gene sequence in exon 1), reverse primer: 1901-1877 (corresponding gene sequence in exon 2), this results in an amplification fragment of 1901-1458 = 444 bp, see Figure 4D.

  This likewise hepatocyte-specific protein can only be detected in the programmed hepatocyte (progr. hepatocyte) and in the positive control from human liver tissue at mRNA level by 444 bp band expression, whereas the programmable stem cell (progr. stem cell) does not show this band, i.e. the gene is not transcribed there, as can be seen in Figure 4D.

- Clotting factor VII: forward primer: 725-747 (corresponding gene sequence in exon 1), reverse primer: 1289-1268 (corresponding gene sequence in exon 2), this results in an amplification fragment of 1289-725 = 565 bp, see Figure 4D.

  As in the case of clotting factor II, also this protein is only transcribed in programmed hepatocytes (progr. hepatocyte) and in the positive control (human liver tissue) (see bands at 656 bp), although weaker than clotting factor II. Neither the programmable stem cell nor the negative control (H_2O) show this specific mRNA band.

- Glycerine aldehyde dehydrogenase: This gene, also referred to as a "house-keeping gene" can be detected in every eukaryotic cell and serves as a control whether PCR amplification was properly carried out in all samples; it is co-determined in parallel and results from the addition of a definite quantity of RNA from the respective cell

samples.

- As negative control H_2O samples were simultaneously co-determined in all tests. If the H_2O is not contaminated with RNA, no amplificate is produced during the PCR and no band is detectable (thus serves as counter-control).

Example 8

Production of skin cells (keratinocytes)

For the programming of dedifferentiated programmable stem cells of monocytic origin according to Example 2 in skin cells a conditioned medium was first generated. For this purpose, 1-2 cm^2 of complete human skin was processed as follows.
The skin material was first freed from the subcutis under sterile conditions. The tissue was then washed in total 10× with PBS in a sterile container by vigorous shaking. After the 2nd washing, the tissue was again freed from demarked connective tissue residues.

The skin material was then placed in a petri dish with a diameter of 60 mm, mixed with 3 ml of a trypsin solution diluted 1:10 with PBS and cut into small pieces (approximately 0.5 to 1 mm^3). After this, 3 ml of the trypsin solution diluted 1:100 with PBS was again added to the mixture and the mixture was incubated at 37°C for 60 minutes with intermittent shaking.

The larger particles were then allowed to settle and the supernatant containing the keratinocytes was poured off and centrifuged at 800 rpm for 5 minutes. The supernatant now produced was pipetted off and the cell pellet was taken up in

3 ml of a medium of the following composition and incubated in petri dishes (Ø 100 mm) for 15 days in an incubator at 37°C.

Keratinocyte growth medium

(Keratinocyte growth medium, KGM):	
DMEM	333.5 ml
Foetal Calf serum (FCS)	50 ml
Ham's F12 medium	111 ml
Penicillin (100 U/l)/Streptomycin (100 µg/l) solution	5 ml
Insulin solution (cf. Example 6)	0.5 ml
Total volume	500 ml

The nutrient medium contained 5 µg of epidermal growth factor (for exact specification see Example 7) and 5 mg of hydrocortisone (Ref. Merck Index: 12, 4828).

During the 15 days' incubation period, the keratinocyte-cell-conditioned medium KCCM formed as supernatant. The supernatant was replaced with fresh nutrient medium after 2-4 days in each case. The KCCM obtained during each change of medium was subjected to sterile filtration and stored at -20°C.

1×10^6 dedifferentiated stem cells were then cultivated with 10 ml of a mediums of the following composition in a petri dish (Ø 100 mm) or a culture flask.

Keratinocyte differentiation medium

(Keratinocyte differentiation medium, KDM)	
KCCM	100 ml
Insulin solution (cf. Example 6)	0.5 ml
epidermal growth factor (EGF)	1 µg
Hydrocortisone	1 mg
keratinocyte growth factor (KGF)	2.5 µg

Keratinocyte growth factor was used in a concentration of 25 ng/ml, as described by Finch et al., Gastroenterology **110:** 441 (1996).

After a few days a morphological change in the cells could be observed. After 6 days the keratinocyte-specific antigens, cytokeratin 5 and 6, which are both bound by the primary antibody used, (Exp. Cell. Res. **162:** 114 (1986)) could be detected (Figure 5A). After 10 days a cell adherence of the clearly larger individual cells already took place in culture, which made it possible to identify a visible cell tissue combination of confluent cells (Figure 5B).

Example 9

Production of insulin-producing cells from differentiated programmed stem cells

The production of insulin-producing cells was conducted in culture flasks with a volume of approximately 250 ml and flat walls (T75 cell culture flasks). Approximately 5×10^6 of the cells produced according to Example 13 were suspended in approximately 5 ml of the culture medium indicated below

(differentiation medium for insulin producing cells) and after being introduced into the flasks, mixed with a further 15 ml of culture medium. For the differentiation of the cells, the flasks were incubated in a horizontal position in an incubator at 37°C and 5% CO_2.

Culture medium (modified according to Rameya V.K. et al., Nature Medicine, **6** (3), 278-282 (2000)):

RPMI 1640	445 ml
Foetal calf serum (FCS)	50 ml
Penicillin (100 U/l)/Streptomycin (100µg/l) solution	5 ml
Nicotinamide	620 mg
Glucose	360 mg
Total volume	500 ml

The nutrient medium further contained the epidermal growth factor in a quantity of 10 ng/ml and the hepatocyte growth factor in a quantity of 20 ng/ml.
Within the first hour the cells adhere to the bottom of the culture vessel. The differentiation of the stem cells was monitored by reference to insulin production. For this purpose the culture medium was changed at intervals of approximately 2 to 3 days, the cell supernatant was collected each time, and frozen at -20°C. The cells adhering to the bottom of the culture flask could be detached by tryptinisation as described in Example 2.

The insulin content of the supernatant collected at the different times was measured by means of ELISA (Enzyme-linked-immunosorbent-assay) against human insulin (Bruhn H.D., Fölsch U.R. (Eds.), Lehrbuch der Labormedizin: Grundlagen,

Diagnostik, Klinik Pathobiochemie *[Textbook of Laboratory Medicine, Principles, Diagnosis, Clinical Pathobiochemistry]* (1999), Page 189) and compared with the blank reading of the medium. The results reproduced in Figure 8 show that the cells have reached the maximum level of insulin production after 14 days in culture. The insulin quantities produced by the cells treated in the course of the differentiation increased after 14 days to 3 µU/ml, whilst no human insulin was detectable in the control medium. The bars in Figure 8 each represent three separate values each determined from three independent individual experiments.

Next to the determination of the insulin production in the deprogrammed stem cells, which were differentiated into insulin producing cells according to the invention, the portion of insulin-producing cells were determined which still expressed the monocyte-specific surface antigen CD14 also 3 weeks after conducting the dedifferentiation. It was found that on a great portion of these cells (about 30 to 40 %) the monocyte-specific antigen CD14 was detectable also after 3 weeks.

Example 10

Alternative method for the production of hepatocytes from dedifferentiated programmable stem cells

As an alternative to the use of hepatocyte-conditioned medium (LCCM), as described in Example 7, the differentiation of the stem cells into hepatocytes was induced by the nutrient medium (Ha) indicated below. The production of hepatocytes from stem cells in turn took place in culture flasks with a volume of

approximately 250 ml and flat walls (T75-cell culture flasks). Approximately 5×10^6 of the cells produced according to Example 13 were introduced into approximately 5 ml of the improved culture medium indicated below (Ha, differentiation medium for hepatocytes) and after being introduced into the flasks, mixed with a further 15 ml of culture medium. For the differentiation of the cells, the flasks were incubated in a horizontal position in an incubator at 37°C and 5% CO_2.

Differentiation medium for hepatocytes (Ha) (modified according to Schwarz et al., "Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells", J. Clin. Invest. **10** (109), 1291-1302 (2002)):

RPMI 1640	445 ml
Foetal calf serum (FCS)	50 ml
Penicillin (100 U/l)/Streptomycin (100µg/l) solution	5 ml
Total volume	500 ml

The nutrient medium also contained fibroblast growth factor-4 (FGF-4) in a quantity of 3 ng/ ml.

Within the first hour the cells adhere to the bottom of the culture vessel. The differentiation of the stem cells was monitored with regard to albumin production. For this purpose the culture medium was changed at intervals of approximately 2 to 3 days, the cell supernatant collected each time, and frozen at -20°C. The cells adhering to the base of the culture flask could be detached by tryptinisation as described in Example 2.

The albumin content of the supernatant collected at the

different times was measured by means of ELISA (Enzyme-linked-immunosorbent-assay) for human albumin (according to the protocol of Bethyl Laboratories Inc. and according to Schwarz et al., loc. cit.) and compared with the blank reading of the medium. The results presented in Figure 9 show that the albumin production of the cells during the period of 14 to 28 days in culture remained approximately constant. The measurements were carried out on days 0 (blank reading of the medium), 14, 21, 28 and 30 relative to the time of addition of the Ha medium. The values determined in each case amounted to ca. 5 ng/ml, 450 ng/ml, 425 ng/ml, 440 ng/ml and 165 ng/ml. The bars in Figure 9 each represent three separate values each determined from three independent individual experiments.

Example 11

Detetermination of the co-expression of albumin and of the monocyte-specific antigen CD14 in hepatocytes derived from dedifferentiated stem cells

The determination of the co-expression of albumin and of the monocyte-specific antigen CD14 in hepatocytes derived from dedifferentiated stem cells was carried out on the one hand by double-staining (A) and on the other hand by FACS analysis (B).

A) Stem cells according to the invention differentiated into hepatocytes according to Example 10 were cultivated on cover glasses in a 6-well plate and fixed with methanol as described in Example 4. A double-staining was then carried out, in order to detect the simultaneous expression of the antigen CD14 (phenotype marker of monocytes) on the one hand and of albumin

(liver-specific marker) on the other hand.

For this purpose the cells were first incubated as described in Example 4 with a primary antibody against human albumin (guinea pig vs. human albumin) in a 1:50 dilution in PBS. Following a washing step, the cells were then incubated for 45 minutes with a secondary antibody mouse anti-rat, which binds the guinea pig antibodies, also in a 1:50 dilution in PBS. The staining process was then carried out according to Example 4 using the method of Cordell J.L., et al. (loc. cit.) with APAAP red complex.

For the second staining step, the cells were then incubated with the primary antibody, mouse anti-human-CD14, and following a washing step according to Example 4 stained with the ABC Streptavidin KIT of Vectastain (Vector) using the method of Hsu, S.M., et al. "The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics" Am. J. Clin. Pathol. **75** (6): 816-821 (1981) with dem DAB-Complex (brown) (Vector Laboratories).

Nucleus counter-staining with haemalaun was then carried out as described in Example 4, followed by embedding in Kaiser's glycerol gelatin.

The results are shown in Figure 10. The figure shows the expression of the antigen CD14 as brown color, which slowly decreases parallel to the morphological transformation of the cells into hepatocytes, whilst the albumin expression as red color increases with the increasing maturation of the hepatocytes. Picture No.4 in Figure 10 shows the cells after three weeks' stimulation with the hepatocyte-

conditioned medium.

B) Parallel with the double marking, the stem cells differentiated into hepatocytes according to the invention were subjected to FACS (fluorescence-activated cell sorting) analysis.

The stem cells differentiated into hepatocytes according to the invention according to Example 10 were first harvested by mechanical detachment of the cells from the culture flask using a cell scraper. The cells were carefully rinsed from the flask with PBS and washed twice, each time in 10 ml of PBS-solution. For this purpose the cell suspensions in the PBS solution were introduced into a 15-ml centrifuge tube and precipitated at 1600 rpm. The resultant cell sediment was diluted with PBS, such that exactly 1 x 10^5 cells were present in 100 µl PBS.

10 µl of each of FITC-marked anti-CD14 antibodies (BD Pharmingen) or FITC-marked anti-albumin antibodies (Beckmann) and FITC-marked non-specific IgG1 mouse anti-human antibodies were then added to this cell suspension. After an incubation period of 20 minutes the cells were resuspended twice in 500 µl PBS and each precipitated for 5 minutes at 1600 rpm and then finally taken up in 200 µl PBS. After resuspension of the cells, fluorescence was measured with a BD FACScalibur flow cytometer from the company BD Biosciences (Franklin Lakes, NJ) (cf. Bruhn H.D., Fölsch U.R. (Eds.), Lehrbuch der Labormedizin: Grundlagen, Diagnostik, Klinik Pathobiochemie *[Textbook of Laboratory Medicine, Principles, Diagnosis, Clinical Pathobiochemistry]*, 395-403 (1999); and Holzer U. et al., "Differential antigen sensitivity and costimulatory

requirements in human Th1 and Th2 antigen-specific CD4(+) cells with similar TCR avidity" J. Immunol. **170** (3): 1218-1223 (2003)). The evaluation of the results was carried out using the Microsoft WinMDI program with reference to Marquez M.G., et al. "Flow cytometric analysis of intestinal intra-epithelial lymphocytes in a model of immunodeficiency in Wistar rats." Cytometry **41** (2): 115-122 (2000).

The results of the FACS-Analyse are reproduced in Figure 11. The figure shows the expression of the CD14 (top row) and of the albumin antigen (bottom row), which was measured in dedifferentiated monocytes (left-hand column) and in the stem cells differentiated into hepatocytes according to the invention (right-hand column). In dedifferentiated monocytes a strong expression of CD14, but no expression of albumin could be detected, whilst in the hepatocytes developed from dedifferentiated monocytes a weaker expression of the CD14 and a very strong expression of the albumin was detectable.

Example 12

In vivo use of dedifferentiated programmed stem cells of monocytic origin

In order to clarify, to what extent the programmable stem cells *in vivo* after injection via the portal vein into the liver of a genetically identical recipient animal undergo a specific differentiation via the signal-providers present in the liver, livers of female LEW rats were first treated with retrorsine, in order to inhibit the hepatocytes present in the

liver (liver parenchyma cells) regarding their proliferation activity (Ref. Lacone, E., et al. "Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine" Am. J. Path. **153**: 319-329 (1998)).

For this purpose the LEW rats received 30 mg of the pyrrolizidine alkaloid retrorsine, injected intraperitoneally twice within 14 days. Subsequently an 80% resection of the livers treated in this way was carried out, followed by the administration of 5×10^5 of the programmable stem cells in 1ml PBS into the portal vein of the remaining residual liver. The stem cells had been obtained, as described in Example 2, from monocytes of male LEW rats. Five days after administration of the stem cells a punch biopsy of the liver was carried out for histological assessment of the liver and to detect the cell types differentiated from the stem cells by means of fluorescence-in-situ-hybridisation (FISH) with Y-chromosome-specific probes, as described in detail in Hoebee, B. et al. "Isolation of rat chromosome-specific paint probes by bivariate flow sorting followed by degenerate oligonucleotide primed-PCR." Cytogenet. Cell Genet. **66**: 277-282 (1994).

Figure 7A shows the Y-chromosome-positive (red points in the cell nucleus) hepatocytes derived from the male LEW stem cells on the 5th day after intraportal injection into retrorsine-pretreated 80%-resectioned livers of female recipient animals. The selective removal of the same liver on day 25 after stem cell injection shows the differentiation of the stem cells into hepatocytes, endothelial cells and bile duct epithelia (Figure 7B). At this point in time, the liver has already reached its normal size, and >90% of the cells have a Y-chromosome. From this, it can be concluded, that the injected syngenic programmable stem cells of monocytic origin are

capable *in vivo*, of effecting a complete restoration of the liver with normal metabolic function. Figure 7C shows in this connection the Kaplan-Meier survival curves (n=4 per group) of stem-cell-treated versus untreated recipient rats following administration of retrorsine and 80% liver resection.

The function parameters bilirubin and ammonia (NH_3) prove the complete metabolic functionality of the long-term surviving stem-cell-treated animals (Figures 7D and 7E).

Example 13

Propagation and dedifferentiation of monocytes in cell culture flasks

Cultivation and propagation of the monocytes on the one hand and the dedifferentiation of the cells of the other side on a larger scale were conducted in culture flasks in the same nutrient medium, which was also used for the cultivation in well-plates (cf. Example 2). The nutrient medium containt 2.5 µg/500 ml M-CSF and 0.2 µg/500 ml interleukine 3 (IL-3).

The monocytes isolated in Example 1 were transferred to the bottom of culture flasks having a volume of 250 ml and flat walls (T75-cell culture flasks). About 10 times x 10^6 cells were transferred into each flasks and were each filled up with 20 ml of the above indicated nutrient medium. The determination of this cell number for the exact dosing per flask was carried out according to known procedures, cf. Hay R.J., "Cell Quantification and Characterization" in Methods of Tissue Engineering, Academic Press (2002), Chapter 4, pages 55-84.

The cell culture flasks were incubated in an incubator at 37°C for 6 days. After 24 hours, the cells settled at the bottom of the flasks. The supernatant was removed every second day and the flasks were each filled with 20 ml fresh nutrient medium.

On day 6, the flasks were rinsed twice with 10 ml PBS each, after the nutrient medium had previously been pipetted off from the flasks. Hereby, all cells were removed, which did not adhere to the bottom of the flasks. The cells growing adhere to the bottom of the flasks were subsequently removed from the bottom of the flasks with a steril cell scraper. The separated cells were now removed from the flasks by rinsing with PBS and were pooled in a 50 ml Falcon tube and were centrifuged at 1800 rpm for 10 minutes. Thereafter, the supernatant was discarded and the sediment was resuspended in fresh RPMI 1640 medium (2 ml/10^5 cells).

This cell suspension could be used directly for differentiating into various target cells.

Alternatively, the cells were mixed with DMSO/FCS as freezing medium after centrifugation and were deep-frozen at a concentration of 10^6/ml.

The freezing medium contained 95% FCS and 5% DMSO. About 10^6 cells were taken up in 1 ml of the medium and were cooled following the subsequent steps:

30 minutes on ice;
2 hours at -20°C in precooled styropor box;
24 hours at -80°C in styropor;
stored in tubes in liquid nitrogen (N_2) at -180°C.

Claims

1. Process for the production of dedifferentiated, programmable stem cells of human monocytic origin, **characterized in that**

a) monocytes from isolated human blood are propagated in a suitable culture medium, which contains the cellular growth factor M-CSF;

b) the monocytes are cultivated simultaneously with or subsequently to step a) in a culture medium containing IL-3; and

c) the human adult dedifferentiated programmable stem cells are obtained by separating the cells from culture medium.

2. Process according to claim 1, **characterised in that** a mercapto compound is further added to the culture medium in step b).

3. Process according to claim 2, **characterised in that** a mercapto compound is used, in which at least one carbon group is bonded to the sulphur, and wherein the hydrocarbon group(s) may be substituted with one or more further functional groups.

4. Process according to claims 2 or 3, **characterised in that** the mercapto compound is 2-mercaptoethanol or Dimethylsulfoxide.

5. Process according to claims 1 to 4, **characterised in that** subsequent to step b) and before step c) the cells are contacted with a biologically acceptable organic solvent.

6. Process according to claim 5, **characterised in that** the biologically acceptable organic solvent is an alcohol with 1-4 carbon atoms.

7. Process according to claim 6, **characterised in that** the alcohol is ethanol.

8. Process according to claims 5 to 7, **characterised in that** the cells are brought into contact with the vapour phase of the biologically acceptable organic solvent.

9. Process according to claims 1 to 8, **characterised in that** the cells are suspended in a suitable cell culture medium subsequent to step c).

10. Process according to claim 9, **characterised in that** the medium is RPMI or DMEM.

11. Process according to claims 9 or 10, **characterised in that** the medium contains a cytokine or LIF.

12. Process according to claims 9 to 11, **characterised in that** the cells are suspended in a liquid medium and subsequently deep frozen.

13. Process according to claim 12, **characterised in that** the medium is a cell culture medium.

14. Dedifferentiated, programmable stem cells of human monocytic origin, **characterised by** the membrane associated monocyte-specific surface antigen CD 14 and at least one pluripotency marker selected from the group consisting of CD117, CD123 and CD135.

15. Pharmaceutical composition, containing the dedifferentiated, programmable stem cells according to claim 14.

16. Use of the dedifferentiated, programmable stem cells according to claims 14 for producing target cells and target tissue *in vitro.*

17. Use according to claim 16, **characterised in that**

a) the tissue containing the desired target cells is crushed;

b) the target cells and/or fragments thereof are obtained from the crushed tissue;

c) the target cells and/or fragments thereof are incubated in a suitable culture medium;

d) the supernatant of the culture medium is collected during and after incubation as target-cell-conditioned medium; and

e) for the reprogramming/differentiation of the stem cells into the desired target cells, the stem cells are allowed to grow in the presence of the target-cell-conditioned medium.

18. Use according to claims 16 or 17, for the production of adipocytes, of neurons and glial cells, of endothelial cells, of keratinocytes, of hepatocytes or of islet cells.

19. Process for the production of transgenically modified dedifferentiated programmable stem cells of human monocytic origin, **characterised in that** cells produced according to claims 1 to 13 are transfected with one or more

genes.

20. Stem cells according to claim 14, **characterised in that** the dedifferentiated, programmable stem cells are transgenically modified by transfection with one or more genes.

21. Composition containing dedifferentiated, programmable stem cells according to claims 14 or 20 in a suitable medium.

22. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition for the treatment of liver cirrhosis.

23. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition for the treatment of pancreatic insufficiency.

24. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition for the treatment of acute or chronic kidney failure.

25. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition for the treatment of hormonal under-functioning.

26. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition for the treatment of cardiac infarction.

27. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition for the treatment of pulmonary embolisms.

28. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition for the treatment of stroke.

29. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition for the treatment of skin damage.

30. Use of the dedifferentiated, programmable stem cells according to claims 14 or 20 for the preparation of a pharmaceutical composition suitable for the *in vivo* production of target cells and target tissue.

31. Differentiated, isolated, somatic target tissue and/or cells from somatic target tissue, obtained by reprogramming the stem cells according to claims 14 or 20, **characterised by** the membrane-associated surface antigen CD14, wherein human monocytes are excluded.

32. Somatic target cells and/or target tissue according to claim 31, selected from the group consisting of adipocytes, neurons and glial cells, endothelial cells, keratinocytes, hepatocytes and islet cells.

33. Somatic target cells and/or target tissue according to claims 31 or 32, **characterised in that** they are transfected with one or more genes.

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34. Implantable materials coated with the dedifferentiated, programmable stem cells according to claims 14 or 20 or the somatic target cells and/or target tissue according to 31 to 33.

35. Implanted materials according to claim 34, **characterised in that** the materials are prostheses.

36. Implantable materials according to claim 35, **characterised in that** the prostheses are selected from the group consisting of cardiac valves, vessel prostheses, bone- and joint prostheses.

37. Implantable materials, **characterised in that** they are artificial and/or biological carrier materials, which contain the dedifferentiated, programmable stem cells according to claims 14 or 20 or the target cells according to claims 31 to 33.

38. Implantable materials according to claim 37, **characterised in that** the carrier materials are bags or chambers for introduction into the human body.

39. Use of a bag or chamber according to claim 38, which contains islet cells according to claim 32, for the production of a pharmaceutical construct suitable for use as an artificial islet cell portchamber for the supply of insulin.

40. Use of a bag or chamber according to claim 38, which contains adipocytes according to claim 32, for the production of a pharmaceutical construct, which contains artificial polymers filled with adipocytes, suitable for breast construction after surgery and for use in the case of plastic and/or cosmetic correction.

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41. Implantable materials according to claims 34 or 38, **characterised in that** they are semi-permeable port chamber systems, which contain differentiated isolated somatic target cells according to claims 31 or 32.

42. Use of the semi-permeable port chamber system according to claim 41 for the production of a pharmaceutical construct suitable for *in vivo* treatment of endocrine, metabolic or haemostatic diseases.

43. Use of M-CSF and IL-3 for the production of dedifferentiated, programmable stem cells of human monocytic origin in a culture medium.