Hyaline cartilage of the joints, due to its properties of a high elasticity to pressure and a very even surface, allows for a friction-poor movement of the joints. The main part of this tissue is formed of a plurality of extracellular matrix proteins, whereas the cellular component, the chondrocytes, is embedded sparsely in this matrix either separately or in small groups (chondron). The lack of nerve fibres and blood vessels and the resulting low supply of the tissue with oxygen and nutrients generate a unique micro-environment for the chondrocytes. The cartilage tissue can be classified in multiple layers, wherein, due to the horizontal orientation of the matrix molecules, the uppermost layer can counteract friction and, due to the vertical orientation, the subjacent middle and lower layers can absorb impact force originating from movement. Although chondrocytes emerge early during endochondral ossification of the initial process of mesenchymal condensation and these cells contribute significantly to bone growth during epiphysis, the hyaline cartilage tissue in the joint develops only at a very late point in time during embryogenesis. Fully differentiated, fully functional layers of cartilage are formed only postnatal by beginning mechanical stimulation of the tissue. In the adult organism, homeostasis of cartilage tissue is highly dependent of the kind and strength of mechanic load and of the micro-environment of the embedded chondrocytes.

Numerous attempts have been made to develop and provide *in vitro* models and test systems for bone and cartilage tissue. However, these approaches are directed either to the separated provision of bone or cartilage tissue. Thus, these models do represent the natural environment only partially and insufficiently in which development and homeostasis of bone, bone marrow and cartilage tissue takes place.

It is an object of the present invention to overcome one or more problems of the prior art.

DISCLOSURE OF THE INVENTION:

In a first aspect, the present invention is directed to a three dimensional (3D) in vitro model which takes into consideration the close interaction and cooperation of cartilage and bone tissue. The present invention provides a 3D *in vitro* bi-phasic cartilage-bone organoid comprising:

a) a layer of artificial cartilage tissue; and

b) a layer of artificial bone tissue, wherein the artificial bone tissue comprises a structure-giving 3D zirconium oxide based, hydroxyapatite coated, ceramic scaffold and a bone marrow structure;

wherein the bone marrow structure of the artificial bone tissue is prepared by seeding mesenchymal stem cells on the structure-giving scaffold and by adding hematopoietic stem cells after culturing of mesenchymal stem cell on the structure-giving scaffold for 2 to 10 days; and

;

wherein the layer of artificial cartilage tissue contacts at least one surface of the layer of artificial bone tissue.

The artificial cartilage tissue 3D *in vitro* bi-phasic cartilage-bone organoid of can be prepared using mesenchymal progenitor cells, preferably using mesenchymal progenitor cells comprising at least 50% of or consist of cells that are $CD105^+$, $CD106^+$, $CD44^+$, $CD73^+$, $CD90^+$ and $CD13^+$, more preferably from isolated primary chondrocytes. In particular, the artificial cartilage tissue may be prepared by a method of the invention specified below.

The bone marrow structure of the artificial bone tissue of the 3D *in vitro* bi-phasic cartilage-bone organoid of the invention is prepared by seeding mesenchymal stem cells on the structure-giving scaffold. The bone marrow structure of the artificial bone tissue is prepared preferably by seeding mesenchymal stem cells on the structure-giving scaffold and by adding hematopoietic stem cells after culturing of mesenchymal stem cell on the structure-giving scaffold for 2 to 10 days, preferably for 5 to 8 days, more preferably for 7 days.

The structure-giving scaffold of the artificial bone tissue of the 3D *in vitro* bi-phasic cartilage-bone organoid of the invention comprises or consists of a 3D zirconium oxide based, hydroxyapatite coated ceramic scaffold.

According to another aspect, the present invention is directed to a method of producing the 3D in vitro biphasic cartilage-bone organoid of the invention. Said method comprises or consists of the steps:

- seeding mesenchymal stem cells on a structure-giving 3D zirconium oxide based, hydroxyapatite coated ceramic scaffold;
- culturing said mesenchymal stem cells for 2 to 10 days on said structure-giving scaffold, preferably for 5 to 8 days, more preferably for 7 days;
- adding hematopoietic stem cells to the mesenchymal stem cells cultured on the strucuture giving scaffold and culturing the resulting mixture for at least 5 days, preferably for 5 days to 8 weeks, more preferably for 1 week to 4 weeks, in order to prepare a layer of artificial bone tissue comprising a structure-giving scaffold seeded with a bone marrow structure;

- providing artificial cartilage tissue prepared by a method of preparing chondrogenic cell aggregates, comprising the steps:
    a) providing mesenchymal progenitor cells; and
    b) culturing the mesenchymal progenitor cells under non-adherent conditions for at least two weeks to form chondrogenic cell aggregates and
- placing a layer of said artificial cartilage tissue on at least one surface of said layer of artificial bone tissue.

According to another aspect, the present invention is directed to a transplant comprising a 3D *in vitro* bi-phasic cartilage-bone organoid of the present invention.

Further, the present invention provides a pharmaceutical composition comprising a 3D *in vitro* bi-phasic cartilage-bone organoid of the invention or a transplant of the invention and at least one pharmaceutically acceptable excipient.

The present invention is also directed to a method for screening substances *in vitro*, which modulate properties of cartilage or bone tissue, comprising the steps of:

- providing a sample of a 3D *in vitro* bi-phasic cartilage-bone organoid of the invention;
- dividing the respective sample into portions;
- incubating at least one portion with a substance to be screened; and
- comparing parameters measured for the treated portion with another portion that was not incubated with the substance to be screened.

According to a further aspect of the invention, a self-contained 3D *in vitro* bi-phasic cartilage-bone organoid-on-a-chip device is provided, comprising at least one medium feed reservoir, at least one organ growth section comprising at least one organ cavity housing a 3D *in vitro* bi-phasic cartilage-bone organoid of the invention, wherein the medium feed reservoir is connected to the at least one organ growth section by a microfluidic feed channel.

The present invention includes a method of preparing chondrogenic cell aggregates, comprising the steps:

a) providing mesenchymal progenitor cells; and
b) culturing the mesenchymal progenitor cells under non-adherent conditions for at least two weeks to form chondrogenic cell aggregates.

$CD44^+$, $CD73^+$, $CD90^+$ and $CD13^+$ or may consist of cells that are $CD105^+$, $CD106^+$, $CD44^+$, $CD73^+$, $CD90^+$ and $CD13^+$. Preferably, the mesenchymal progenitor cells comprise or consist of isolated primary chondrocytes like e.g. human primary chondrocytes.

The 3D *in vitro* bi-phasic cartilage-bone organoid of the present invention comprises a layer of artificial bone tissue, wherein the artificial bone tissue comprises a structure-giving 3D zirconium oxide based, hydroxyapatite coated, ceramic scaffold and a bone marrow structure. An artificial bone tissue is a bone tissue that has been prepared and assembled *de novo* and *in vitro*. Usually artificial bone tissue comprises fully or partially differentiated osteoblasts, osteoclasts and hematopoietic stem cells.

There are numerous techniques available to the skilled person to prepare artificial bone tissue *in vitro*. Since bone tissue usually comprises more than a simple 2D layer of a certain cell type, artificial bone tissue generated from 3D culture techniques are preferred. Suitable techniques are summarized in Tortelli and Cancedda ("Three-dimensional cultures of osteogenic and chondrogenic cells: A tissue engineering approach to mimic bone and cartilage in vitro", European Cells and Materials (2009), 17, 1-14). The layer of artificial bone tissue can e.g. be derived from techniques comprising culturing and/or organisation of cells or cell aggregates by micromass culture, and use of biocompatible degradable or non-degradable scaffolds. Suitable culture techniques and scaffolds are known in the art. E.g. bio-degradable or non-degradable scaffolds comprising or consisting of a biological or non-biological material may be used. For instance Tan Jin et al. („Maintenance and expansion of hematopoetic stem/progenitor cells in biomimetic osteoblast niche", Cytotechnology, 2010, vol. 62, no. 5, 439-448) describe a 3D system of a natural cancellous bone scaffold comprising MSCs differentiated to osteoblasts which is colonised by HSCs. With this system the HSCs could be maintained and expanded in long term culture in vitro. The use of synthetic based polymers as scaffold has been described as well as use of collagen-based scaffolds, metal-based scaffolds like e.g. titanium based scaffolds, or ceramic-based scaffolds.

The cells used for preparation of the artificial bone tissue may comprise mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC). Preferably, isolated MSCs and/or isolated HSCs are used. The term "isolated" means that MSCs and/or HSCs are cells that have been isolated from a natural source or represent progeny thereof which e.g. has been derived by cell proliferation. The MSCs and/or HSCs used are preferably derived from suitable tissue of a donor, individual or patient. Preferably, the MSCs and/or HSCs are primary cells which have not been transformed or immortalized. In particular, the MSCs and/or HSCs may comprise or consist of adult MSCs and/or HSCs. Such adult MSCs and/or HSCs are derived from non-

embryonic tissue, preferably from bone or bone marrow tissue of a donor, individual or patient. The MSCs and/or HSCs are preferably human cells. Human MSCs and/or HSCs are preferably derived from bone or bone marrow tissue of human origin.

The method of preparing artificial bone tissue in the sense of the present invention comprises seeding and culturing MSCs on a 3D zirconium oxide based, hydroxyapatite coated, ceramic scaffold. The populated ceramic 3D scaffold is cultured for a predetermined period of time and subsequently HSCs may be added. In a preferred embodiment, the HSCs are added after culturing the MSCs on the ceramic scaffold for 2 to 10 days. The populated ceramic 3D scaffold is then further cultured either under static culture conditions or in a perfused system for continuous nutrient supply.

The techniques for generating artificial bone tissue can be further amended. For example growth factors or other signalling molecules can be added to support bone marrow functions. Hypoxic conditions may be used that are similar to the natural conditions bone marrow has to face. The culture times may have to be optimized as well as the sequence of administration of the various cell types to the scaffold. In addition to MSCs and HSCs further cell types may be used for generation of artificial bone tissue. These additional cells may comprise cell types which normally occur in bone or bone marrow structures e.g. immune cells, endothelial cells and the like.

The 3D *in vitro* bi-phasic cartilage-bone organoid of the invention may be used as a research tool *in vitro* and/or *in vivo*. It may be used to investigate the biology or one or more of its components. Alternatively it may be used in a method of screening for substances *in vitro* and/or *in vivo*, which modulate properties of cartilage or bone tissue.

The present invention is also directed to a transplant comprising a 3D *in vitro* bi-phasic cartilage-bone organoid of the invention and to a pharmaceutical composition comprising a 3D *in vitro* bi-phasic cartilage-bone organoid of the present invention or a transplant of the invention and at least one pharmaceutically acceptable excipient.

In a further aspect, the present invention is directed to a method for screening substances *in vitro*, which modulate properties of cartilage or bone tissue, comprising the steps of:

- providing a sample of a 3D *in vitro* bi-phasic cartilage-bone organoid of the invention;
- dividing the respective sample into portions;
- incubating at least one portion with a substance to be screened; and

testing the activity, pharmacodynamic and pharmacokinetic of compounds or to study self-assembly, homeostasis, damage, regeneration or interaction of organs or organoids and stem cell niches, as well as phenomena of maturation, aging, death and chronobiology. For that purpose, the self-contained 3D *in vitro* bi-phasic cartilage-bone organoid-on-a-chip device of the present invention comprises at least one medium feed reservoir, at least one organ growth section comprising at least one organ cavity housing a 3D *in vitro* bi-phasic cartilage-bone organoid of the invention, wherein the medium feed reservoir is connected to the at least one organ growth section by a microfluidic feed channel.

Subject matter of the present invention allows the establishment of miniaturized in vitro culture of an organoid comprising cartilage and bone tissue. The resulting 3D *in vitro* bi-phasic cartilage-bone organoid of the invention is suitable for long term culture of up to 3 months when cultured e.g. under conditions allowing for continuous supply with oxygen and nutrients like e.g. continuous perfusion conditions.

Subject matter of the present invention allows the integration of mechanical stimuli to mimic e.g. physical forces originating from body movement and the induction of vascular structures within the organoid and, thus, the provision of an environment that comes close to the natural situation. Based on these properties, subject matter of the present invention allows for generation of various scientific findings in the field of tissue engineering, molecular biology of cell and tissue differentiation as well as regenerative medicine. Subject matter of the present invention may also lead to replacement of cost and labour intensive animal experiments by in vitro tests.

At present there has been no attempt to provide a 3D *in vitro* organoid which combines the functionally interrelated tissues bone, bone marrow and cartilage in one single integrated system, which is compatible with prolonged culture techniques like perfusion culture and which allows the induction of vascularisation. By exposure of the system to mechanical forces or other stimuli, the natural environment will be mimicked to an extent that has never been reached before.

The present invention includes a method of preparing *in vitro* a chondrogenic cell aggregate. The method comprises the steps of:

a) providing isolated mesenchymal progenitor cells; and

b) culturing the mesenchymal progenitor cells under non-adherent conditions for at least two weeks to form a chondrogenic cell aggregate.

It has surprisingly been found that isolated mesenchymal progenitor cells are capable of forming a three dimensional cell aggregate which exhibits chondrogenic differentiation without influence of embryonic cells. This effect is achieved by culturing the mesenchymal progenitor cells under non-adherent conditions. It could be shown that under such non-adherent culture conditions the mesenchymal progenitor cells arrange freely to each other and condense to a cell aggregate which exhibits expression of markers specific for chondrogenic differentiation and which is therefore denoted chondrogenic cell aggregate.

In the following, different aspects of the invention are provided in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly stated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being part of the invention or being indicated as being preferred or advantageous.

The present invention includes a method of preparing chondrogenic cell aggregates *in vitro*. For the purpose of the present invention the term “chondrogenic cell aggregate” refers to a functional cell aggregate that exhibits at least one function that is specific for chondrocytes or cartilage tissue. Preferably the chondrogenic cell aggregate of the present invention exhibits the majority or essentially all organ or tissue functions of differentiated chondrocytes or cartilage tissue. In particular the chondrogenic cell aggregate of the present invention will behave like a functional cartilage tissue and/or is capable of inducing cartilage development or differentiation *in vitro* and/or *in vivo*. The chondrogenic cell aggregate of the present invention may be characterized by elevated expression of marker genes or proteins that are associated with development or differentiation of chondrocytes or cartilage tissue. Preferably the chondrogenic cell aggregate of the present invention may be characterized by up regulated expression of collagen II, IX and XI as well as down regulated expression of collagen I and XII compared to expression in the mesenchymal progenitor cells immediately prior to subjecting to non-adherent culturing conditions.

The chondrogenic cell aggregate of the present invention is formed within a method of the present invention. The chondrogenic cell aggregate may exhibit a cell aggregate with a substantially rounded or spherical shape. It may have an average diameter of 0,5mm to 3mm, preferably with an average diameter of 0,5mm to 2mm. The chondrogenic cell aggregate of the invention is free of any artificial biological or non-biological scaffold or carrier which does not originate from the cells used in the production of chondrogenic cell aggregate. The chondrogenic cell aggregate of the invention preferably consists of the cell aggregate formed by a method included in the invention, wherein the method has been conducted without the

use or addition of any biological or non-biological scaffold or carrier which does not directly originate from the cells used in said method.

In the method of the invention, isolated mesenchymal progenitor cells are used. The term "isolated" means that the mesenchymal progenitor cells are cells that have been isolated from a natural source or progeny thereof which e.g. has been derived by cell proliferation. The mesenchymal progenitor cells used are preferably derived from cartilage tissue of a donor, subject or patient. Preferably, the mesenchymal progenitor cells are primary cells which have not been transformed or immortalized. In particular, the mesenchymal progenitor cells may comprise or consist of adult mesenchymal progenitor cells. Such adult mesenchymal progenitor cells are derived from non-embryonic cartilage tissue of a donor, subject or patient. The mesenchymal progenitor cells can be derived from cartilage tissue of any donor tissue that has been differentiated to comprise cartilage tissue. Preferably the mesenchymal progenitor cells are derived from cartilage tissue of a joint, more preferably from cartilage of a knee or elbow of a donor. The mesenchymal progenitor cells used in the method of the invention are preferably human cells. Human mesenchymal progenitor cells are derived from cartilage tissue of human origin. The mesenchymal progenitor cells may comprise at least 50% of cells that are $CD105^+$, $CD106^+$, $CD44^+$, $CD73^+$, $CD90^+$ and $CD13^+$ or may consist of cells that are $CD105^+$, $CD106^+$, $CD44^+$, $CD73^+$, $CD90^+$ and $CD13^+$. Preferably, the mesenchymal progenitor cells comprise or consist of isolated primary chondrocytes like e.g. human primary chondrocytes.

In the method of the invention, the mesenchymal progenitor cells can be subjected to culturing under non-adherent conditions at any stage after isolation. However, the formation of cell aggregates is further enhanced if the mesenchymal progenitor cells have undergone at least some culturing under adherent 2D monolayer conditions. Preferably, the mesenchymal progenitor cells have been cultured in 2D monolayer culture for at least 5 passages prior to subjecting to culturing under non-adherent conditions. In order to ensure a dedifferentiated phenotype, it is desirable to culture the mesenchymal progenitor cells in 2D monolayer adherent conditions for at least 10 passages. A high efficiency in formation of cell aggregates is maintained over a broad spectrum of passages. However, it has been found that best results are achieved if the mesenchymal progenitor cells have been cultured in 2D monolayer culture for at least 5 passages and not more than 20 passages. Preferably the mesenchymal progenitor cells are subjected to non-adherent culture after culture in 2D monolayer for at least 5 passages and not more than 15 passages after isolation.

In the method of the invention, after provision of the isolated mesenchymal progenitor cells, the mesenchymal progenitor cells are subjected to non-adherent culture conditions for at least two weeks to form chondrogenic cell aggregates.

The formation of cell aggregates is particularly effective if the mesenchymal progenitor cells are subjected to non-adherent culture conditions at a certain concentration. If the concentration is too low, cells have only rarely contact to each other and condensation to cell aggregates is less effective. On the other hand, if the concentration of mesenchymal progenitor cells is too high, cells are less flexible or mobile and, thus, the formation of cell aggregates is less effective. Preferably, the mesenchymal progenitor cells are subjected to non-adherent culture conditions at a concentration of $5x10^4$ to $5x10^7$ per ml. Even better results are achieved if the mesenchymal progenitor cells are subjected to non-adherent culture conditions at a concentration of $1x10^5$ to $1x10^7$ per ml, preferably of $5x10^5$ to$5x10^6$ per ml, and most preferably of $9x10^5$ to $1,1x10^6$ per ml.

In the method of the invention, the isolated mesenchymal progenitor cells are cultured under non-adherent culture conditions. This means that mesenchymal progenitor cells are cultured under conditions wherein the cells do not adopt a flattened, spread-out shape indicating strong attachment and adherence to the culture surface. Preferably, the mesenchymal progenitor cells after seeding remain rounded and, if at all, only weakly associated with the culture surface. Appropriate means for non-adherent cell culture are well known in the art. Non-adherent culture conditions may comprise culture of the mesenchymal progenitor cells in culture vessels with a culture surface that does not support adherence of the mesenchymal progenitor cells. E.g. culture vessels with culture surfaces exhibiting ultra-low cell attachment can be used. For that purpose culture vessels with a neutral or positively charged culture surface may be used. Preferably, the culture surface may be coated by a layer of a material which further reduces interaction of the mesenchymal progenitor cells and the culture surface. The culture surface may be covered with a hydrophilic hydrogel.

It appears that under non-adherent culture conditions, the mesenchymal progenitor cells associate and condense to cell aggregates rather quickly. Already after 24 hours of non-adherent cell culture, the mesenchymal progenitor cells aggregated into one large complex. However, in order to prepare cell aggregates that represent chondrogenic cell aggregates with progressed development, differentiation and/or function, it is beneficial to conduct non-adherent culturing for a period of time that exceeds 24 hours. In the method of the invention, the mesenchymal progenitor cells may be cultured under non-adherent conditions for at least 2 weeks, preferably for at least 4 weeks. It is disclosed that the mesenchymal progenitor cells

may be cultured under non-adherent conditions for 48 hours to 8 weeks, preferably for 60 hours to 6 weeks, more preferably for 72 hours to 5 weeks. Upon cultivation in normal culture dishes aggregates adhere the dish surfaces and the cells start to migrate and proliferate resulting in disintegration of the aggregate structure. Therefore the aggregates are preferably kept under non adhered conditions for the entire period of cultivation.

In the method of the invention, the mesenchymal progenitor cells are preferably cultured under non-adherent conditions at least until a cell aggregate is formed which exhibits a rounded shape with an average diameter of 0,5mm to 3mm, more preferably with an average diameter of 0,5mm to 2mm.

The suitability of the chondrogenic cell aggregate generated by the method of the present invention is dependent on its ability to induce or provide differentiated chondrocytes or cartilage tissue. Thus, it is preferred to culture the isolated mesenchymal progenitor cells under non-adherent conditions until the resulting cell aggregate starts to exhibit properties and/or functions of partially or fully differentiated chondrocytes or cartilage tissue. The progression in differentiation can be monitored by the relative expression of respective marker genes, proteins or structures. Preferably, the isolated mesenchymal progenitor cells are cultured under non-adherent conditions at least until the cell aggregate formed exhibits up regulated expression of collagen II, IX and XI as well as down regulated expression of collagen I and XII compared to expression in the mesenchymal progenitor cells immediately prior to subjecting to non-adherent culturing conditions. Since expression analysis for stage specific marker molecules revealed maintained expression of N-Cadherin and elevated levels of Collagen type II but no expression of Collagen type X, a differentiated phenotype between chondrogenic precursor and columnar chondrocyte, but no further differentiation to the prehypertrophic stage is suggested. This phenotype reflects the arrested phenotype of chondrocytes in articular cartilage tissues. Relative expression levels of genes can easily be determined by well known methods like e.g. quantitative or semi-quantitative RT-PCR or Northern blotting. Relative expression levels of proteins can also be determined by well known methods like e.g. Western blotting and ELISA techniques. In addition to the analysis of the expression pattern on mRNA and protein level further detailed characterization of the aggregate structure by immune histochemical staining as well as pressure resistance determination will be included to assign the quality of the aggregate.

One of the drawbacks encountered with most of the methods of the prior art is that in these methods, the presence of an artificial biological or non-biological scaffold or carrier is needed on or within which cells are cultured to form cartilage tissue. A biological or non-biological

scaffold or carrier is regarded as artificial or added if said scaffold or carrier is provided from the outside and is not directly formed by the cells used in the method of the invention during formation of cell aggregates. In the method of the present invention for preparing chondrogenic cell aggregates, the use or presence of such an artificial biological or non-biological scaffold or carrier is not needed. The method of the invention yields an chondrogenic cell aggregate according to the invention without the use of any such artificial biological or non-biological scaffold or carrier which does not originate from the cells used. In a preferred embodiment, the method of the present invention is performed such that no addition of biological or non-biological scaffold is performed in the formation of chondrogenic cell aggregates.

In the method of the invention, the mesenchymal progenitor cells are cultured in non-adherent conditions with standard medium. There is no specialized culture medium necessary to induce proper cell aggregation under non-adherent conditions. The skilled person is well aware of suitable media. Typically standard DMEM is used with a certain content of fetal calf serum (FCS), preferably FCS is present in a concentration of 5% to 15%, more preferably in a concentration of 10% FCS.

Despite the fact, that proper chondrogenic differentiation is induced upon aggregate formation without additional treatments, a further optimization may be beneficial. Therefore, in the method of the invention once the aggregation process is completed further optimization steps regarding growth factor administration or cultivation under hypoxic conditions will further improve development and differentiation.

The present invention is also directed to a transplant comprising or consisting of an artificial chondrogenic cell aggregate of the present invention or of a tissue or structure derived therefrom.

In another aspect of the invention a pharmaceutical composition is provided comprising an 3D *in vitro* bi-phasic cartilage-bone organoid or a transplant of the invention and at least one pharmaceutically acceptable excipient.

The artificial chondrogenic cell aggregate of the invention, the transplant of the invention or the pharmaceutical composition of the invention may be used in the treatment of cartilage impairment and/or destruction or loss of cartilage.

Since the method of the invention works with isolated mesenchymal progenitor cells derived from non-embryonic sources, the method allows the production of artificial chondrogenic cell

aggregate starting from cells derived from a particular donor or patient. Thus, it is possible to provide artificial chondrogenic cell aggregates that have been derived from the cells of the person to be treated with the artificial chondrogenic cell aggregates, pharmaceutical composition or transplant of the invention. Thus, it appears possible to provide a transplant that is mainly, substantially or completely derived from cells of the recipient of the transplant himself so that rejection reactions will be reduced to a minimum or will be completely absent.

It is disclosed that the artificial chondrogenic cell aggregate prepared by the method of the invention may be used for the *in vitro* or *in vivo* generation of differentiated chondrocytes or cartilage tissue.

It is disclosed that the artificial chondrogenic cell aggregate prepared by the method of the invention, the transplant of the invention or the pharmaceutical composition of the invention may be used as a research tool that can be used *in vitro* and *in vivo*.

It is disclosed that the artificial chondrogenic cell aggregate prepared by the method of the invention, the transplant of the invention or the pharmaceutical composition of the invention may be used in a method of screening for substances *in vitro* or *in vivo*, which modulate properties of chondrocytes or cartilage tissue.

The present invention additionally teaches a method for screening substances *in vitro*, which modulate properties of chondrocytes or cartilage tissue, comprising the steps of:

- providing a sample of an artificial chondrogenic cell aggregate of the invention or a cell aggregate prepared by a method of the invention or a tissue derived therefrom;
- dividing the respective sample into portions;
- incubating at least one portion with a substance to be screened; and
- comparing parameters measured for the treated portion with another portion that was not incubated with the substance to be screened.

In a preferred embodiment, the portion is subjected to a self-contained organ-on-a chip device prior to incubating the portion with a substance to be screened.

Briefly, the inventive method makes the identification and analysis of substances possible, which exert an influence on chondrocytes or cartilage tissue via the artificial chondrogenic cell aggregate of the invention. The sample, which shall be understood to comprise a certain number of product subjects according to the invention, is divided into multiple portions. At least two subsets are provided; one is used for screening while the other one serves as negative

control. Preferably, the number of screening parts exceeds the number of control parts. Usually, numerous portions are subjected to a high-throughput screening. The substances to be screened in the inventive method are not restricted anyway. In an embodiment of the invention, the substances are selected from the group of nucleic acids including RNAi, ribozymes, aptamers, antibodies, peptides, carbohydrates, polymers, small molecules having a molecular weight between 50 and 1.000 Da, and proteins, preferably antibodies, cytokines and lipocalins. These substances are often available in libraries. It is preferred to incubate a single compound within a distinct portion of the sample. However, it is also possible to investigate the cooperative effect of substances by incubating at least two substances within one portion. A further subset of subjects is simultaneously incubated in the absence of the substances. The incubation process depends on various parameters, e.g. the cell types and the sensitivity of detection, which optimization follows routine procedures known to those skilled in the art. The identification of effective substances in the meaning of the invention is preferably indirectly performed, e.g. by determining the expression patterns and/or the cell viability, which are altered. The determination may be performed at a specified moment and correlated to the signal strength at the beginning of the experiment and the negative control. Suitable tests are known to those skilled in the art or can be easily designed as a matter of routine.

Since the artificial chondrogenic cell aggregate of the invention can be regarded as an organ, organoid or a precursor of an organ or part thereof, it may be particularly beneficial to use a test system wherein the artificial chondrogenic cell aggregate can be prepared and/or cultured for a prolonged time under conditions which mimic natural perfusion. It appears particularly suitable to use the method of the present invention preparing the artificial chondrogenic cell aggregate of the present invention in an assay system based on the self-contained organ-on-a-chip device system described in the European patent application with the filing number EP 10 008 244 or in the PCT application with the publication number WO2009/146911.

The present invention also discloses a self-contained 3D *in vitro* bi-phasic cartilage-bone organoid-on-a-chip device comprising a self-contained organ-on-a-chip device as described in WO 2009/146911 and an artificial chondrogenic cell aggregate of the invention.

The self-contained 3D in vitro bi-phasic cartilage-bone organoid-on-a-chip device of the present invention is formed to allow establishing or maintaining the artificial chondrogenic cell aggregate of the invention in a miniaturized chip format, suitable for online observation by live cell imaging and for example two photon microscopy and their use for, e.g. testing the activity, pharmacodynamic and pharmacokinetic of compounds or to study self-assembly,

homeostasis, damage, regeneration or interaction of organs or organoids and stem cell niches, as well as phenomena of maturation, aging, death and chronobiology. For that purpose, the self-contained 3D *in vitro* bi-phasic cartilage-bone organoid-on-a-chip device of the present invention comprises at least one medium feed reservoir, at least one organ growth section comprising at least one organ cavity housing an 3D *in vitro* bi-phasic cartilage-bone organoid of the invention, wherein the medium feed reservoir is connected to the at least one organ growth section by a microfluidic feed channel.

It is disclosed for the first time an artificial chondrogenic cell aggregate and a method of producing the same, which is characterized by non-forced cell assembly, the lack of requirement of any artificial biological or non-biological scaffold or carrier and which is produced from adult cells so that no embryonic cells or tissues are needed.

The artificial chondrogenic cell aggregate of the present invention represents a functional inductive aggregate which is capable of inducing or initiating cartilage tissue development *in vitro* and *in vivo*. The resulting cartilage tissue is characterized by the verifiable presence of structures typically forming part of cartilage tissue which are arranged in the physiological order.

FIGURES:

Fig.1 shows that dedifferentiated chondrocytes display mesenchymal stem cell character. [A] Dedifferentiated chondrocytes were stained for the mesenchymal surface marker panel. The cells were positive for CD105, CD106, CD44, CD73, CD90 and CD13 and negative for the hematopoietic markers CD34 and CD45 (one representative experiment is shown, n=6). Adipogenic differentiation. [B-D] Dedifferentiated chondrocytes were treated with adipogenic medium for 28 days. Adipocyte differentiation was evaluated by light microscopy and Oil-Red-O-staining. B) negative control of monolayer cells, unstained; C) negative control of monolayer cells, Oil-Red-O-stained ; D) adipocyte differentiation after 28 days is evidenced by formation of lipid filled droplet in the cytoplasm ; Oil-Red-O-staining of lipid filled droplets in differentiated adipocytes ; (one representative experiment is shown, n=6); Osteoblast differentiation. [F-G] Dedifferentiated chondrocytes were treated with osteogenic medium for 28 days. Successful differentiation was evaluated by von Kossa staining. E) stained negative

CLAIMS:

1. 3D *in vitro* bi-phasic cartilage-bone organoid comprising:
   a) a layer of artificial cartilage tissue; and
   b) a layer of artificial bone tissue, wherein the artificial bone tissue comprises a structure-giving 3D zirconium oxide based, hydroxyapatite coated, ceramic scaffold and a bone marrow structure;

   wherein the bone marrow structure of the artificial bone tissue is prepared by seeding mesenchymal stem cells on the structure-giving scaffold and by adding hematopoietic stem cells after culturing of mesenchymal stem cell on the structure-giving scaffold for 2 to 10 days; and

   wherein the layer of artificial cartilage tissue contacts at least one surface of the layer of artificial bone tissue.

2. 3D *in vitro* bi-phasic cartilage-bone organoid of claim 1, wherein the artificial cartilage tissue is prepared using mesenchymal progenitor cells preferably using mesenchymal progenitor cells comprising at least 50% of or consist of cells that are $CD105^+$, $CD106^+$, $CD44^+$, $CD73^+$, $CD90^+$ and $CD13^+$, more preferably from isolated primary chondrocytes.

3. 3D *in vitro* bi-phasic cartilage-bone organoid of claim 1 or 2, wherein the artificial cartilage tissue is prepared by a method of preparing chondrogenic cell aggregates, comprising the steps:
   a) providing mesenchymal progenitor cells; and
   b) culturing the mesenchymal progenitor cells under non-adherent conditions for at least two weeks to form chondrogenic cell aggregates.

4. 3D *in vitro* bi-phasic cartilage-bone organoid of claim 3, wherein the mesenchymal progenitor cells comprise at least 50% of or consist of cells that are $CD105^+$, $CD106^+$, $CD44^+$, $CD73^+$, $CD90^+$ and $CD13^+$.

5. 3D *in vitro* bi-phasic cartilage-bone organoid of claim 3 or 4, wherein the mesenchymal progenitor cells comprise or consist of isolated dedifferentiated primary chondrocytes.

6. Method of producing a 3D in vitro biphasic cartilage-bone organoid of one of claims 1 to 5, the method comprising the steps:
   - seeding mesenchymal stem cells on a structure-giving 3D zirconium oxide based, hydroxyapatite coated, ceramic scaffold;

- culturing said mesenchymal stem cells for 2 to 10 days on said structure-giving scaffold;
- adding hematopoietic stem cells to the mesenchymal stem cells cultured on the structure giving scaffold and culturing the resulting mixture for at least 5 days in order to prepare a layer of artificial bone tissue comprising a structure-giving scaffold seeded with a bone marrow structure;
- providing artificial cartilage tissue prepared by a method of preparing chondrogenic cell aggregates, comprising the steps:
  a) providing mesenchymal progenitor cells; and
  b) culturing the mesenchymal progenitor cells under non-adherent conditions for at least two weeks to form chondrogenic cell aggregates; and
- placing a layer of said artificial cartilage tissue on at least one surface of said layer of artificial bone tissue.

7. Method of claim 6, wherein the mesenchymal progenitor cells comprise at least 50% of or consist of cells that are $CD105^+$, $CD106^+$, $CD44^+$, $CD73^+$, $CD90^+$ and $CD13^+$.

8. Method of claim 6 or 7, wherein the mesenchymal progenitor cells comprise or consist of isolated dedifferentiated primary chondrocytes.

9. Transplant comprising a 3D *in vitro* bi-phasic cartilage-bone organoid of one of claim 1 to 5.

10. Pharmaceutical composition comprising a 3D *in vitro* bi-phasic cartilage-bone organoid of one of claims 1 to 5 or a transplant of claim 8 and at least one pharmaceutically acceptable excipient.

11. A method for screening substances *in vitro*, which modulate properties of cartilage or bone tissue, comprising the steps of:
- providing a sample of a 3D *in vitro* bi-phasic cartilage-bone organoid of one of claims 1 to 5;
- dividing the respective sample into portions;
- incubating at least one portion with a substance to be screened; and
- comparing parameters measured for the treated portion with another portion that was not incubated with the substance to be screened.

12. Self-contained 3D *in vitro* bi-phasic cartilage-bone organoid-on-a-chip device, comprising at least one medium feed reservoir, at least one organ growth section

comprising at least one organ cavity housing a 3D *in vitro* bi-phasic cartilage-bone organoid of one of claims 1 to 5, wherein the medium feed reservoir is connected to the at least one organ growth section by a microfluidic feed channel.