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(54) Title: CELL CULTURE SUPPLEMENT

(57) Abstract: The present invention relates to a conditioned medium for use as a cell culture supplement. The medium is obtainable by cultivating mesenchymal cells under conditions they form multicellular spheroids. The medium so obtained may be used in a method for promoting proliferation, growth and/or motility of cells, preferably human fibroblasts.



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Cell culture supplement

The present invention relates to a conditioned medium for use as a cell culture supplement. The medium is obtainable by cultivating mesenchymal cells under conditions they form
5 multicellular aggregates or spheroids. The medium so obtained may be used in a method for promoting proliferation of cells, preferably human fibroblasts.

Many media for growing cells in culture are described in the literature or are commercially available. Deriving an optimal medium for specific cell culture needs can lead to improve-
10 ments in cell growth including increased growth rates, growth to high cell densities, controlling the stage and amount of cell differentiation, increasing protein secretion or increased phenotypic and genetic stability. However, there is a constant need for effective cell culture additives or supplements for cell culturing media.

15 Mesenchymal-epithelial interactions in the skin between fibroblasts and epithelial keratinocytes present a complex interplay consisting of various cytokines and growth factors guiding cell proliferation, migration as well as differentiation. As seen with Rheinwald and Greens method of cultivating keratinocytes on a feeder layer, mesenchymal paracrine signals, such as growth factors and cytokines, remain essential for keratinocyte proliferation
20 and survival (Werner *et al.*, 2007). One versatile dermis derived growth factor implicated in tissue regeneration and wound healing is hepatocyte growth factor/scatter factor (HGF) (Conway *et al.*, 2006). It has been linked to increased migration and proliferation of epithelial cells and its ligand-specific receptor c-Met is expressed in keratinocytes (Chmielowiec
25 *et al.*, 2007).

We previously showed that mesenchymal cell-cell activation in so-called nemosis produces massive amounts of HGF (Bizik *et al.*, 2004), and that stimulation of keratinocytes by nemosis-derived factors enhances their migration utilizing the HGF/c-Met/PI3K pathway (Peura *et al.*, 2009). Nemosis is a novel biological programmed cell response to high-
30 density cell-cell contacts, and can be effectively activated in dermal fibroblasts (Kankuri *et al.*, 2005; Kankuri *et al.*, 2008).

The cell culture supplement according to this invention is a medium obtainable by cultivating mesenchymal cells under conditions that induce the cells to adhere to each other to form multicellular spheroids. The multicellular spheroids as formed are then incubated under conditions mesenchymal cells secrete growth factors to the culture medium. The
5 medium will be ready for use after the cells have been separated therefrom.

The medium so obtained comprises various growth factors, e.g. HGF (hepatocyte growth factor), FGF7 (keratinocyte growth factor) and amphiregulin/epiregulin. As can be seen from Figure 1, the medium according to the invention contains much more amphiregulin
10 than a medium from the same cells grown as a monolayer. The receptors in the target cells (e.g. keratinocytes and fibroblasts) are c-Met for HGF, FGFR2 for FGF7 and EGFR for amphiregulin/epiregulin. These receptors are illustrated in Figure 2.

The medium of the invention was named as "Xenera". We have shown that Xenera supports the proliferation, growth and motility of cells in a culture. It is therefore applicable to,
15 for example, stem cell market and tissue engineering. For instance, the appended Figure 3 shows that Xenera stimulates proliferation of CRL fibroblasts. From Figure 4 it can be seen that even after freezing and thawing Xenera retains its stimulating effect. Xenera can also be used to promote isolation of primary cultures of, for instance, hepatocytes and melanocytes, which are difficult to cultivate.
20

The primary object of the present invention is thus use of a conditioned medium, which comprises factors secreted by mesenchymal cells when cultured under conditions in which said cells form multicellular spheroids, as a cell culture supplement. Preferably, the mesenchymal cells are skin fibroblasts.
25

Another object of the invention is use of said conditioned medium for promoting isolation of primary cultures of hepatocytes or melanocytes, which are difficult to cultivate.

30 A further object of the invention is a method for promoting proliferation of cells, comprising culturing said cells in a growth medium supplemented with a conditioned medium comprising factors secreted by mesenchymal cells when cultured under conditions in which said cells form multicellular spheroids. The cells whose proliferation can be pro-

moted are, for instance, keratinocytes or fibroblasts. Preferably, the cells are human fibroblasts.

A still further object of the invention is an improved cell culture medium for stimulating proliferation of cells, wherein the improvement comprises addition to the cell culture medium of an effective amount of a conditioned medium comprising factors secreted by mesenchymal cells when cultured under conditions in which said cells form multicellular spheroids.

The conditioned medium of the present invention is prepared by growing mesenchymal cells, preferably fibroblasts in such cell culture conditions in which the cells form multicellular spheroids. Preferably, a serum-free, non-supplemented cell culture medium is used. Suitable media are, e.g. DMEM (Dulbecco's Modified Eagle Medium), MEM (Minimum Essential Medium), α -MEM, F12 (F12 Nutrient Mixture), DMEM/F12, RMPI 1640, Keratinocyte defined medium (Gibco) and Endothelial cell growth medium (Lonza). Incubation is preferably carried out at 37 °C in a humidified 5% CO₂ atmosphere for 3 to 5 days, and the conditioned medium so formed is harvested.

Brief Description of Drawings

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Figure 1. The amount of amphiregulin in the conditioned medium of the invention ("Xenera") and the same in a "monolayer conditioned medium".

Figure 2. Figure showing the target for Xenera comprising HGF (hepatocyte growth factor), FGF7 (keratinocyte growth factor) and amphiregulin/epiregulin.

Figure 3. Proliferation curves

Autostimulation of CRL fibroblasts growth by Xenera; - ■ - Xenera, - ♦ - control

Y-axis shows the number of cells (10^3)

30

Figure 4. Proliferation curves

Activity of freeze-thawed Xenera on autostimulation of fibroblasts. Y-axis as in Figure 3.

Experimental

Production of conditioned medium (Xenera)

Multicellular spheroids were formed on U-bottomed 96-well plates (Costar, Cambridge, MA). The plates were treated with low-electroendosmotic agarose (Bio Whittaker, Rockland, ME) prepared in sterile water to form a thin film of non-adhesive surface. CRL-2088 fibroblasts were seeded into individual wells (15000 cells per spheroid) in 80µl of serum free DMEM. Incubation was carried out at 37 °C in a humidified atmosphere containing 5% CO₂. As a control, same amount of BMSCs (Bone Marrow Stromal Cells) were directly grown as a monolayer culture in a similar medium. The conditioned medium from the spheroids, as well as the monolayer medium, were collected after 5 days of incubation.

The entire contents of all the references cited herein are hereby incorporated by reference.

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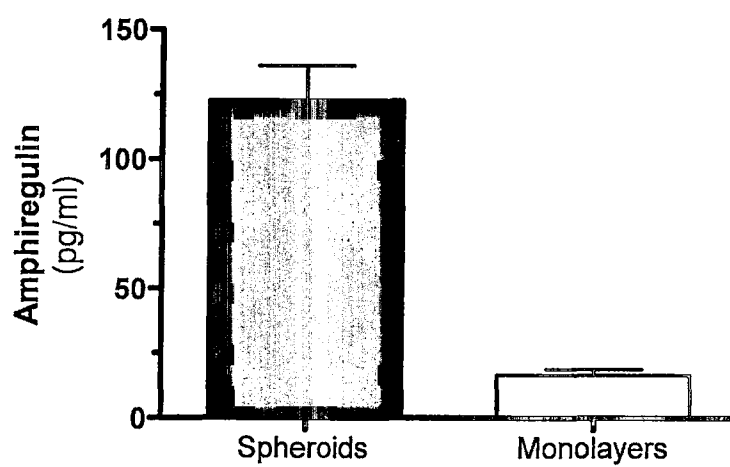
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Claims

1. Use of a conditioned medium, which comprises factors secreted by mesenchymal cells when cultured under conditions in which said cells form multicellular spheroids, as a cell culture supplement.
2. Use according to claim 1, wherein the mesenchymal cells are human skin fibroblasts.
3. Use according to claim 1 for promoting proliferation, growth and/or motility of the cells in a culture.
4. Use according to claim 1 for promoting isolation of primary cultures of hepatocytes or melanocytes.
5. A method for promoting proliferation of human fibroblasts, comprising culturing said fibroblasts in a growth medium supplemented with a conditioned medium comprising factors secreted by mesenchymal cells when cultured under conditions in which said cells form multicellular spheroids.
6. An improved cell culture medium for stimulating proliferation of cells, wherein the improvement comprises addition to the cell culture medium of an effective amount of a conditioned medium comprising factors secreted by mesenchymal cells when cultured under conditions in which said cells form multicellular spheroids.
7. The improved cell culture medium according to claim 6 for stimulating proliferation of keratinocytes or fibroblasts.

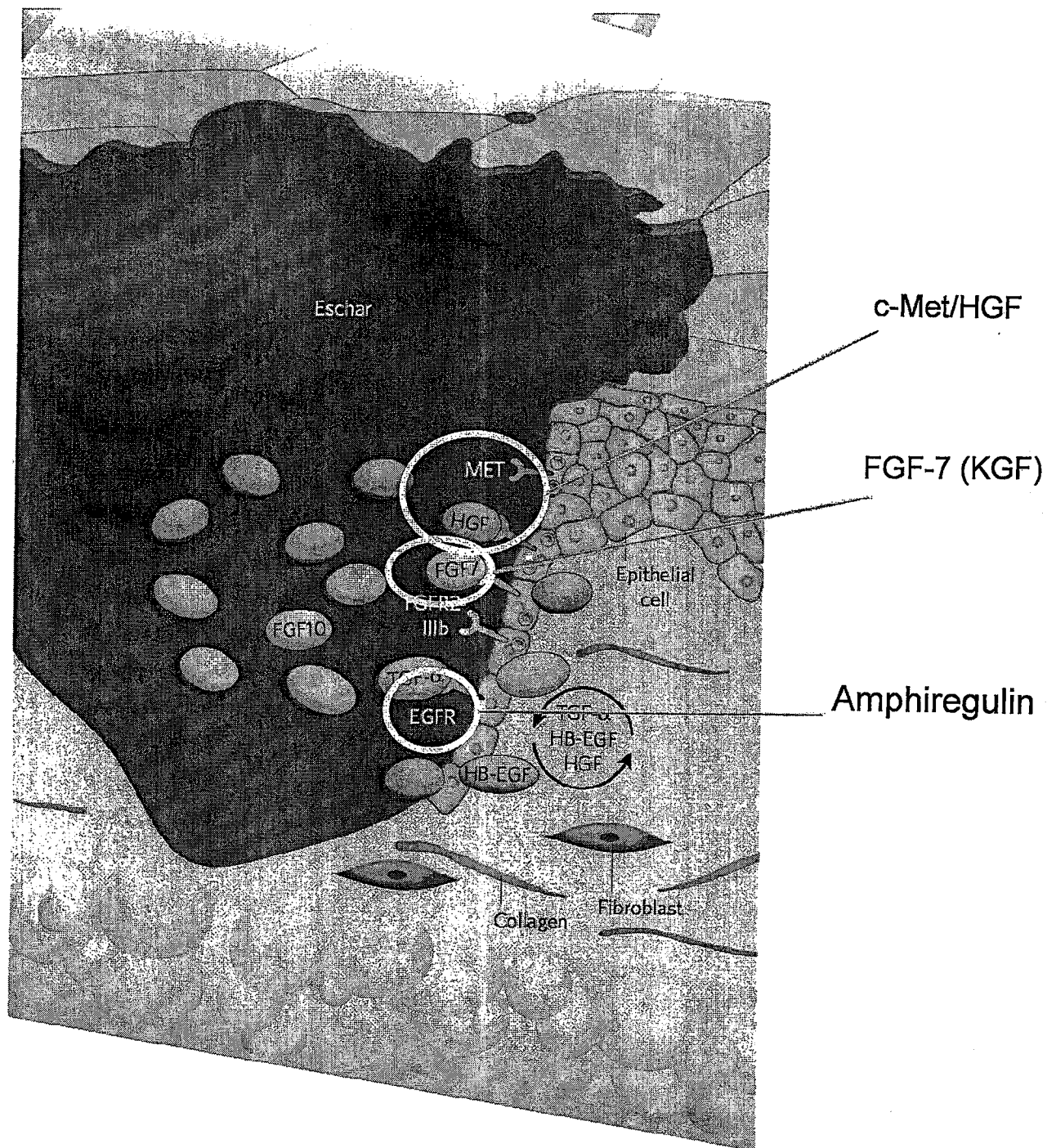
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Figure 1.



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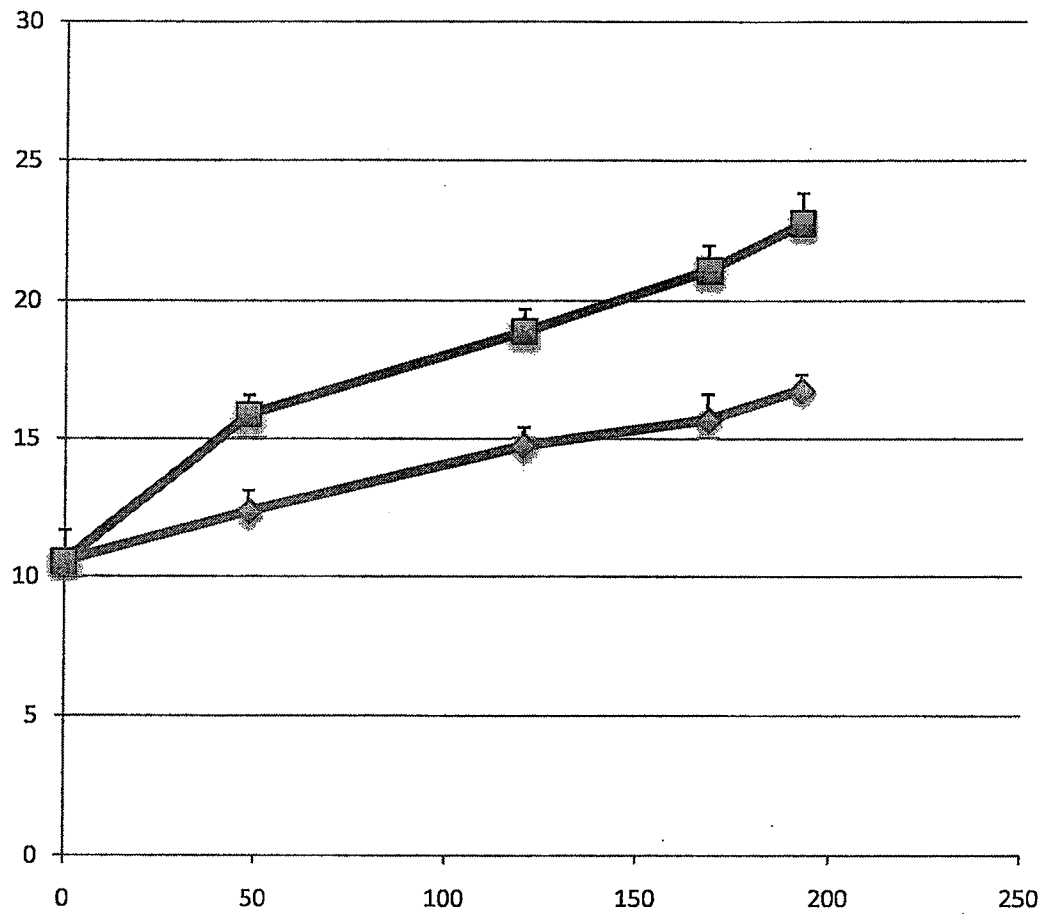
Figure 2.



(Picture from NATURE, Vol. 453 15 May 2008 Doi:10.1038/nature07039)

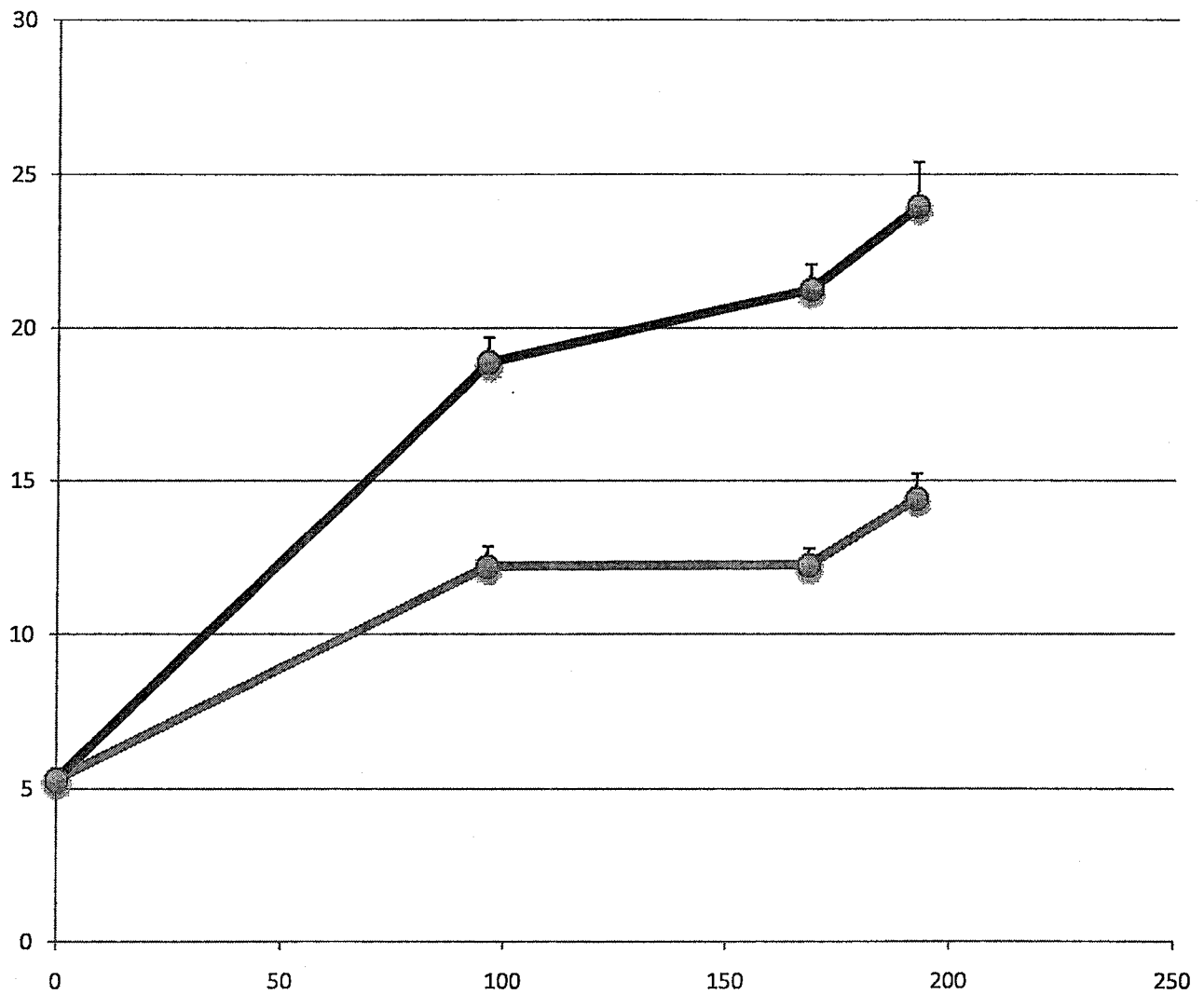
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Figure 3.



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Figure 4.



INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12N

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

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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