

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 March 2006 (16.03.2006)

PCT

(10) International Publication Number
WO 2006/027202 A1

(51) International Patent Classification⁷: **C12N 5/16,**
C07K 14/005

on Biotherapy and Oncology (RUBIO), Hôpital Erasme,
Route de Lennik 808, B-1070 Brussel (BE).

(21) International Application Number:
PCT/EP2005/009541

(74) Agent: **BRANTS, Johan, Philippe, Emi;** De Clercq,
Brants & Partners, E. Gevaertdreef 10a, B-9830
Sint-Martens-Latem (BE).

(22) International Filing Date:
6 September 2005 (06.09.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PCT/EP2004/009920
6 September 2004 (06.09.2004) EP

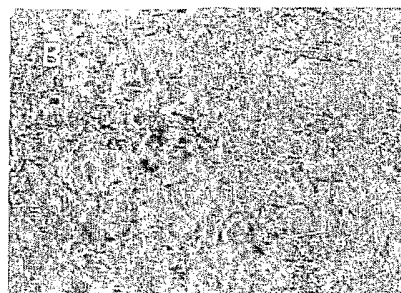
(71) Applicant (for all designated States except US): **UNITE
DE RECHERCHE EN BIOTHERAPIE ET ONCOLO-
GIE (RUBIO)** [BE/BE]; Hôpital Erasme, 808 Route de
Lennik, B-1070 Anderlecht (BE).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA,
MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ,
OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL,
SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC,
VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,
RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA,
GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: GENERATION OF MULTIPARENT CELL HYBRIDS



- dox



+ dox

(57) Abstract: The present invention relates to a cell line, wherein the cells of said line express a fusogenic glycoprotein and are insensible for said fusogenic glycoprotein such that said cell line is suitable as a fusogenic agent. The present invention further relates to a cell line wherein said fusogenic glycoprotein is of viral origin chosen from the group consisting of orthomyxoviruses, rhabdoviruses, retroviruses, paramyxoviruses, and poxviruses. The present invention further relates to the use of the fusogenic cell line as a carrier for added genes that may be of a therapeutic value.

WO 2006/027202 A1



Declaration under Rule 4.17:

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Generation of multiparent cell hybrids

The present invention relates to fusogenic cell line, their use as a fusion agent to generate multiparent cell hybrids, and their preparation.

5

Technical field

The present invention in particular relates to a method to generate multiparent cell hybrids comprising the use of a fusogenic cell line insensitive to the fusogenic polyprotein which it expresses, and at least two partner cells expressing a receptor sensitive to said fusogenic polyprotein. The invention further relates to the fusogenic cell line as such.

10

Background of the invention

Multiparent cell hybrids are made to combine characteristics of different cells onto one cellular vehicle. In the past, different approaches to induce cell fusion have been used, the most frequent being polyethyleneglycol (PEG) and electrofusion.

15

The most classical method to fuse cells applies polyethylene glycol (PEG) as fusion agent. PEG covers and stabilizes the cellular membrane thereby stimulating cell fusion of contacting cells. This technique is rather simple, easy to perform and does not need the necessity of specialized equipment. Cell fusion using PEG is frequently used, for instance to establish antibody-producing hybridomas. However, PEG fusion is hampered by toxicity, inducing a high percentage of dying cells. For certain cells said mortality reaches up to 80% within 48 hours. Therefore said technique may not be implicated to easily generate hybrids with high frequency. Furthermore, said approach lacks reproducibility and difficulties for standardization.

20

Electrofusion is considered as better method to generate cell hybrids compared to PEG fusion because it involves fewer cell manipulations. The electrofusion technique allows cell fusion through the generation of electrical currents. Said technique requires two independent subsequent steps: bringing the cells in immediate contact with each other, and, introducing a reversible rupture of the cellular membranes of the cell to be fused. When the cells are submitted to a direct current of very high voltage during a very short period (order of microseconds), the lipid membrane bilayer is disrupted resulting in an increase of the conductivity and permeability. Under controlled conditions, the membranes spontaneously resolve. As the membranes of different cells are in close contact with each other, the membranes of many of said cells may resolve together forming cell hybrids. Also the use of electrical parameters, duration and strength of the pulse, allows for better standardization compared to the PEG fusion. Furthermore,

25

30

35

electrofusion relies less on individual technical skills for reproducibility. However, the two most critical parameters of electrofusion, which influence the cellular viability, are the intensity and the duration of the electrical pulse produced. Many scientists tried to improve said technique, however, the electrofusion results in a high cellular mortality. Furthermore, said technique limits the development of electrofusion through the fact that specific equipment is needed to generate the electric pulse.

Another approach to fuse cells uses the expression of fusogenic membrane glycoproteins (FMG). Envelope proteins of some viruses, such as the HIV virus and some retroviral viruses type C (type C retroviruses), have the capacity to induce fusion between viral particles and membranes of target cells through the interaction of specific receptors. Different retroviruses use different receptors on the surface of the target cells. Expression of said viral FMG proteins in one of the cells to be fused, allows the fusion with a same neighbouring cell (Fielding et al. (2000) Human Gene Therapy 11:817-826; Bateman et al. (2000); Cancer Research 60:1492-1497; and; WO02/080983).

Phan et al. (2003), Nature Medicine 9 : 1215 to 1219 and US2002/0042127 describe the possibility to produce dendritic cell and tumour cell dihybrids. In the method applied by the authors of this document one cell to be fused was transduced with a recombinant retroviral vector encoding the FMG glycoprotein of VSV (vesicular stomatitis virus). Conditional (pH dependent) FMG expression (activation) was used to prevent cells to fuse prior to the addition of the second fusion partner.

The above described methods need a technically difficult step of transducing or transfecting at least one of the partner cells with a nucleic acid molecule coding for an FMG glycoprotein. Furthermore, transfection or transduction of cells with FMG (except as above with VSV-FMG which can induce fusion conditionally) was found cytotoxic due to syncytia formation. Moreover, transfection or transduction even with transgenes not causing substantial cytotoxicity is typically not reproducible. This makes said approach sensitive to un-reproducibility, laborious and of low efficiency.

US 2002/0042127 described a method to produce diparental hybrids between tumour cells and dendritic cells, in which one of the partner cell types is transiently transfected in order to express an FMG glycoprotein. Therefore, this method requires the technically difficult and time consuming step of transfection of one of the partners. This may be particularly disadvantageous when the partners are freshly recovered primary cells. For example, transfection of primary cells may cause considerable toxicity and may have low efficiency. Moreover, transfection necessitates prolonged culturing of primary cells in order to obtain suitable conditions for transfection and to obtain sufficient expression of the FMG protein. Said conditions must be set up for each individual primary

culture. Even in cell lines that are more efficiently transfected or infected with viral vectors, the number of FMG-expressing cells and the level of FMG expression are too low to produce efficient fusion between neighbouring cells. Disadvantageously, transfection may lead to cytotoxic formation of syncytia. Moreover, the properties of primary cells may be altered during culturing, even during short time culturing.

Another limitation of US 2002/0042127 is that the resulting diparental hybrids will only have properties of the parental cell types (tumour cells and dendritic cells). Additional properties cannot be introduced into the hybrids unless they are first introduced to at least one of the parental cells by transient transfection. However, this increases the number of transient transfections which need to be performed and aggravates the above mentioned problems.

Golding et al. 1993 (J Immunol. 150(6): 2506-16) described a method to fuse CD4-expressing B-cells with CD4⁺ T-cells, in which the latter were transiently transfected with the FMG glycoprotein from HIV virus, gp120/41. However, Golding et al. only studied under what conditions T-cells were able to fuse with B-cells. Therefore, the fusion was only between these two parental cell types and the T-cells had to express the FMG glycoprotein. In view hereof, Golding et al. did not address the problem of improving methods for cell fusion or obtaining hybrids with useful properties.

Brade et al. 2003 (Hum Gene Ther. 14(5):447-61) described a method to specifically kill tumour cells by high level expression of an FMG glycoprotein in these cells. The tumour cells were sensitive to the FMG protein and therefore its expression induced massive syncytia formation and death of the cells. The object of Brade et al. is therefore to obtain the best possible lethal effect of the FMG protein on cells in which it is expressed. Brade et al. did not address the problem of improving methods for cell fusion or providing a fusogenic agent or obtaining hybrids with useful properties.

Therefore, there is a need to find alternative approaches to produce multicellular cell hybrids with high efficiency and high reproducibility using a method which is easy, applicable to different cells, and, inducing low mortality.

Objects of the invention

The present invention is directed towards providing a new method to generate easily, with high frequency and reproducibility multiparent cell hybrids. A particular object of the present invention is directed towards the development of a stable cell line expressing a fusogenic peptide. Furthermore, said method may be applied for different kinds of cells, of same or different origin. In a first instance the application illustrates that hybrid formation is easily obtained for two partner cells (tumour and dendritic cells), this

technique may also be applied to other kinds of cells applying the teaching of the present invention.

A further object of the present invention is directed towards the use of the obtained multiparent cell hybrids. In the application, multiparent cell hybrids are made between DCs and tumour cells, thereby suggesting their use for anti-cancer treatment. However, multiparent cell hybrids may be applied depending on the cell partners used and the additional functional proteins that are introduced and expressed in said multiparent cell hybrids.

These aims have been met by following embodiments.

10

Detailed description of the invention

The solution provided in the present invention may be found in the provision of a new fusogenic agent. Said agent allows the development of an efficient method to produce multi-parent cell hybrids. Through the said agent, there is no need anymore to transfect or transduce one of the partner cells to be fused, making said fusion method easy, reliable and efficient. Furthermore, through said agent, properties not present in the parent cells to be fused may be readily introduced into the resulting multi-parent hybrids.

In a first embodiment the present invention relates to said fusogenic agent which is a cell line, wherein the cells of said line express a fusogenic glycoprotein (FMG) and are insensible for said fusogenic glycoprotein such that said cell line is suitable as a fusogenic agent. Throughout the application a cell line may be interpreted as one separate cell or a group of cells sharing an average of the characteristics of the cell line.

According to the present invention, said cell line is preferably stable. A stable cell line allows to maintain the expression of a gene of interest over a required time in culture. It can preferably be subcloned if necessary, for example when expression of the gene decreases. In our preferred examples, said cell line stably expresses the GaLV-FMG-glycoprotein, expression of other characteristics of said cell line may vary.

As exemplified, a cell line of the present invention may stably express the fusogenic glycoprotein (a stable cell line). Cells of such cell line will produce the fusogenic glycoprotein continuously without the need for an inducer or another regulatory signal (constitutive expression). This may be preferred, because such cell line can be used as a fusogenic agent without first having to induce the expression of the fusogenic protein. This simplifies the use of the cell line. Also, the cell line is more readily available as a fusogenic agent – this is advantageous, e.g., when the time of using the fusogenic agent cannot be precisely planned. Constitutive expression may be achieved by placing the coding region for the fusogenic glycoprotein under the control of a constitutive promoter. Constitutive

promoters are well known in the art and exemplary eukaryotic constitutive promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I.

In another embodiment, a cell line of the present invention may inducibly express the fusogenic glycoprotein (an inducible cell line). Cells of such cell line will not produce the fusogenic glycoprotein continuously, but its expression will depend on the presence of an inducer, such as an exogenously added compound, or another regulatory signal (inducible expression). This may be useful, e.g., when the gene encoding the fusogenic protein is present (and capable of being expressed) in a multi parent hybrid resulting from a fusion involving the fusogenic cell line. In this situation, it may be advantageous to down regulate the expression of the fusogenic protein in the hybrid (e.g., in order not to express the fusogenic protein and its respective receptor in the hybrid). Inducible expression of the fusogenic glycoprotein may be achieved by placing the coding region for the fusogenic glycoprotein under the control of an inducible promoter. Inducible promoters with their corresponding inducers, repressors or regulatory signals are well-known in the art. Exemplary promoters include ones regulated by or sensitive to tetracycline, rapamycin, IPTG, steroid hormone (e.g. ecdysone) or other suitable small molecule inducers.

As exemplified, the cell line of the present invention is preferably a permanent cell line. Permanent cell lines do not manifest considerable senescence in culture and can undergo cell divisions in excess of the Hayflick limit, e.g., more than about 60 cell divisions and usually more than 70, 80, or more than 90 cell divisions. A permanent cell line stably expressing a fusogenic protein (a stable fusogenic cell line) will allow to maintain the expression of a gene of interest over a required time in culture.

In addition said cell line is preferably easy to grow at large scale (including serum-free conditions), at high densities (preferably in a bioreactor) and/or at clinical grade (GMP) conditions. These characteristics will be demonstrated hereunder.

All cells of said cell line preferably express the FMG glycoprotein (100%), however, the present invention also relates to a cell line of which only 80% of the cells may express the FMG glycoprotein. Furthermore said cell line may also comprise cells of which at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95% expresses said fusogenic glycoprotein. In the examples of the present application, evidence is given that the cell line should preferably comprise at least 40% and preferably 95% of FMG-expressing cells for a very effective use as fusogenic agent. Lower percentages can also function as fusogenic agents, but would likely be, self-evidently, less effective. Using said cell line multiparent cell hybrids are detected using flow cytometry (FACS). However when using more sensible detection techniques

compared to FACS, cell lines can be used comprising even lower percentages of FMG-expressing cells.

The coding region for said fusogenic glycoprotein in said cell line may be present extra-chromosomally (i.e. on a plasmid introduced using transfection or by infection with a non-integrating viral vector) or intra-chromosomally (i.e. introduced as a transposon, as viral vector, or introduced via homologous recombination or random insertion or other techniques known by a person skilled in the art). The coding region for the fusogenic glycoprotein is preferably stably maintained in the fusogenic cell line during cell proliferation. This allows to maintain the expression of a gene of interest over a required time in culture. Typically, intra-chromosomal integration of the coding region for the fusogenic glycoprotein in the cells may be preferred, because it results in such stable maintenance of the coding region during proliferation of the cell line. Extra-chromosomal vectors with stable propagation in cells may also be used to carry the coding region for the fusogenic glycoprotein.

In addition, said cell line may be of mammalian, bacterial, yeast, fungal, arthropod (including insect) or plant origin. According to the invention, said cell line is preferably of mammalian origin.

A "fusogenic membrane glycoprotein" as used herein has the ability to mediate or induce fusion between a cell expressing the fusogenic membrane glycoprotein and cells expressing a receptor for the fusogenic membrane glycoprotein. Examples of fusogenic membrane proteins include, but are not limited to fertilin β , and fusogenic membrane glycoproteins, including viral fusogenic membrane glycoproteins or recombinant forms thereof modified to be selective for a given cell surface receptor or to have enhanced fusogenicity.

A "viral fusogenic membrane glycoprotein" is a virally-derived fusogenic membrane protein that, in nature, mediates membrane fusion of a virus to its host target cell. The viral fusogenic membrane glycoprotein subset of the fusogenic membrane proteins includes, but is not limited to: type G glycoproteins in Rabies, Mokola, vesicular stomatitis virus and Togaviruses, Gibbon Ape Leukemia Virus (GaLV) FMG, murine hepatitis virus JHM surface projection protein; porcine respiratory coronavirus spike- and membrane glycoproteins; avian infectious bronchitis spike glycoprotein and its precursor; bovine enteric coronavirus spike protein; the F and H, HN or G genes of Measles virus, canine distemper virus, Newcastle disease virus, human parainfluenza virus 3, simian virus 41, Sendai virus and human respiratory syncytial virus; gH of human herpesvirus 1 and simian varicella virus, with the chaperone protein gL; human, bovine and cercopithecine herpesvirus gB; envelope glycoproteins of Friend murine leukaemia virus and Mason

Pfizer monkey virus; influenza haemagglutinin; mumps virus hemagglutinin neuraminidase, and glycoproteins F1 and F2; and membrane glycoproteins from Venezuelan equine encephalomyelitis.

As used herein, the term "fusogenic membrane protein receptor" refers to a cell surface polypeptide or other molecule that serves as the receptor for a given fusogenic membrane protein. Viral fusogenic membrane glycoprotein receptors are reviewed by Weiss & Tailor (1995, Cell 82: 531-533). Examples of viral fusogenic membrane protein receptors include CD46 (receptor for measles virus F and H fusion proteins), PIT-1 (receptor for Gibbon ape leukaemia virus), CD4 (receptor for HIV), CAT (receptor for MLV (murine leukaemia virus) -E), and Ram-1 (receptor for MLV-A). Receptors for non-viral fusogenic membrane proteins include, for example, alpha -6, beta-1 integrin, the receptor for fertilin β (GenBank Accession No. X69902).

Further viral and non-viral fusogenic membrane glycoproteins which may also be useful in the fusogenic cell line of the present invention, as well as membrane protein receptors recognized by such FMG proteins, are extensively discussed in US 2002/0042127 by Russell et al., which is herein incorporated by reference.

Preferably, the fusogenic glycoprotein which is expressed in the cell line of the present invention is of viral origin chosen from the group consisting of orthomyxoviruses, rhabdoviruses, retroviruses, paramyxoviruses, and poxviruses. Orthomyxoviruses and rhabdoviruses induce fusion at low pH whereby no specific receptor is involved and only require ubiquitous membrane components. Retroviruses, paramyxoviruses, and poxviruses induce fusion at neutral pH via a specific receptor. Example(s) of orthomyxoviruses is for instance Influenza; of rhabdoviruses are VSV and rabies; of retroviruses are GaLV and HIV; of paramyxoviruses are Sendai, measles and Newcastle disease virus; and of poxviruses is Vaccinia.

Preferred examples are given wherein the cell line comprises cells expressing the retroviral GaLV fusogenic glycoprotein GaLV-FMG. As said cells need to be insensitive to said fusogenic glycoprotein, said cells carry no or a low number of receptor for said glycoprotein or carry a receptor insensible for said glycoprotein. Cells are considered insensitive to said fusogenic membrane protein when they do not fuse in the presence of each other, but do fuse with another cell which is sensitive to said glycoprotein. When expressing a fusogenic glycoprotein, said insensitive cell is thus a preferable tool to be used as fusogenic agent.

The GaLV-FMG recognizes the PIT-1 and in some cases the PIT-2 receptor. Therefore, when expressing the GaLV-FMG the cell line has preferably no or a low expression of the PIT-1 receptor or a PIT-1 receptor insensible to the GaLV-

FMG. However, the teaching of the application, illustrated by the GaLV-FMG can be extended to other FMG glycoproteins.

The cell line according to the invention may be originally insensible or has been made insensible for said fusogenic glycoprotein. For instance, said cell may be a human
5 line wherein the human PIT-1 receptor (e.g., on level of the PIT-1 gene) is, or has been made, resistant to GaLV-FMG fusion. In particular said cell line may be preferably the adenocarcinoma cell line HT29 (colon) or SW13 (adrenal) as described for example by Eglitis et al. 1995 (Gene Therapy 2: 486-49).

Alternatively, the resistance to GaLV-FMG fusion is introduced through the
10 delivery of (a) mouse component(s) responsible for the resistance to GaLV infection in mouse cells. Said component may be for instance region A of the mouse PIT-1 receptor as described by Johann et al. (1993) J. Virol. 67:6733-36.

In a preferred embodiment, the cell of the cell line of the present invention, is of a mammalian, more preferably a rodent, and yet more preferably a hamster cell. More
15 precisely, said cell may preferably be a Chinese Hamster cell originating from the ovary (abbreviated as CHO). More in particular, said cell line is preferably the cell line termed GFMG 10M6, as deposited at the BCCM-LMBP culture collection on September 3, 2004 under registered Accession Number LMBP 6327CB.

In an embodiment, the cell of the cell line of the present invention, is of a
20 mammalian origin. For example, the fusogenic cell line may be derived from a human or from a non-human mammal. In some uses, it may be advantageous if the fusogenic cell line is derived from a non-human organism, e.g., a non-human mammal, when it is used to induce fusion between two or more partner cell types derived from a human. In an exemplary embodiment, the fusogenic cell line may facilitate fusion between a human
25 antigen presenting cell and a human cell expressing tumour antigen(s). A fusogenic cell line derived from a non-human mammal will expose on its surface molecules, such as membrane proteins and glycoproteins, which are different from those exposed on the surface of the fusion partners derived from a human. These non-human surface molecules will also be exposed on the multi parental hybrid resulting from the fusion
30 reaction. When such multi parent hybrid is introduced to a body of a patient having a tumour, the non-human surface molecules will expectedly induce a strong xenogeneic rejection immune response. This rejection immune response may be accompanied by increased levels of immuno-stimulatory factors, such as interleukins, e.g., IL-2, that may greatly enhance the specific immune reaction directed against the tumour antigens also
35 presented by the multi parent hybrid. Suitable non-human mammal cell lines may be

preferably derived from a rodent and more preferably from hamster, and may be a CHO cell line, such as the cell line termed GFMG 10M6.

A xenogeneic rejection immune response in a human subject could also be obtained if the cells of a fusogenic cell line of the present invention were modified to (over)express at least one functional molecule, e.g., peptide, polypeptide or protein, derived from a non-human species, e.g., a non-human mammal, such as a rodent, or from a virus. Such molecule may preferably be exposed on cell surface and is capable of inducing a xenogeneic rejection immune response in humans. Such considerations may also apply to other species.

Suitable examples of molecules which when expressed by the fusogenic cell line may cause a xenogeneic rejection immune response in humans or other animal species can be found in the following publications, herein incorporated by reference: Welsh RM, McNally JM, Brehm MA, Selink LK. Consequences of cross-reactive and bystander CTL responses during viral infections. *Virology*. 2000 270:4-8; Nagai A, Zbar B, Terata N, Hovis J. Rejection of retrovirus-infected tumor cells in guinea pigs: effect on bystander tumor cells. *Cancer Res*. 1983 43:5783-8; Korsgren O, Wallgren AC, Satake M, Karlsson-Parra A. Xenograft rejection of fetal porcine islet-like cell clusters in the rat: effects of active and passive immunization. *Xenotransplantation*. 1999 6:271-80.

Another alternative embodiment may be a cell line expressing a HIV-FMG fusogenic glycoprotein. In this case, the cells of said cell line carry no or a low number of CD4 marker and HIV co-receptor such as CCR5 for macrophage tropic HIV strains or carry a CD4 marker and a CCR5 receptor insensible for said glycoprotein. Many cells do not express CD4, notable exceptions being neurons, macrophages and lymphocytes. Possible cells for the cell line of the present invention would be human cells such as HeLa, HEK293, HT29 or non-human such as CHO.

Another embodiment of the present invention is a cell line expressing a murine leukaemia virus (MLV)-10A1-FMG fusogenic glycoprotein. In this case, the cells of said cell line carry no receptors for said glycoprotein or carry a receptor insensible for said glycoprotein. MLV-10A1 fuses through PIT-1 and PIT-2 receptors. Cell lines insensible for said glycoprotein are known (Thomsen S, Vogt B, von Laer D, Heberlein C, Rein A, Ostertag W, Stocking C Lack of functional Pit-1 and Pit-2 expression on hematopoietic stem cell lines. *Acta Haematol*. 1998 99:148-55).

The fusogenic glycoprotein expressed in the cells of the cell line of the present invention may be full length compared to the corresponding wild type glycoprotein, providing that such glycoprotein retains its fusogenic activity in the full-length form; however, derivatives of said glycoproteins may also be suitable. In particular, said

5 fusogenic glycoprotein may have a C-terminal truncation lacking the 16 AA R-peptide of the wild type protein (for example, a C-terminally truncated form of a fusogenic protein, such as of GALV-FMG, may be preferably used which lacks the 16 amino acid R-peptide of the wild-type protein; the R-peptide normally serves to restrict fusion of the viral envelope until it is cleaved by viral protease during virion maturation; this modification renders the protein constitutively highly fusogenic to Pit-1 expressing cells - Green et al. PNAS 78: 6023-3748, 1981), and/or, said fusogenic glycoprotein may have a modified N terminal extracellular part so as to modify the selectivity of fusion. The selectivity of fusion with cells expressing a membrane molecule that interacts with said N terminal
10 extracellular part of the fusion glycoprotein may hereby be increased or decreased. For example, truncation of the cytoplasmic (C-terminal) domains of some retroviral and herpes virus glycoproteins has been shown to increase their fusion activity. In another example, the selectivity of fusion induction by a viral FMG may be modified by fusing targeting moieties, which provide novel binding specificities, to FMG; such fusion proteins are
15 described, e.g., in U.S. Pat. No. 5,723,287, herein incorporated by reference. Such modified FMG may have novel binding specificities to recognize, e.g., a select receptor or antigen on a target cell, thereby targeting the fusogenic activity to specific cell types.

Furthermore, the cells of the cell line of the present invention may be further modified (over)expressing specific functional molecules. According to the present
20 invention, said functional molecules may be chosen from a group consisting of proteins (such as cytokines), chemoattracting molecules, co-stimulatory molecules, antigens, and, molecules with adjuvant properties. Said antigen may be a tumour antigen or is expressed by patient tumour or is of human, mammalian, insect or/and viral and/or bacterial and/or protozoan and/or fungal origin.

25 Such functional molecules may be peptides, polypeptides and/or proteins. Such functional molecules may be of higher eukaryotic, such as mammalian or especially human origin. The cells may be modified to (over)express one or more such specific functional molecules.

In an embodiment, the cells of the fusogenic cell line may over(express) one or
30 more functional molecules which are capable of functioning locally or systemically, e.g., capable of regulating the activity of cells belonging to the immuno-hemopoietic system (e.g., immuno-modulatory polypeptides, immuno-stimulatory polypeptides) and/or capable of affecting the immune regulation or induction of inflammatory or immune responses and/or capable of affecting the viability, growth or differentiation of various normal or
35 neoplastic cells and/or capable of exerting endocrine activities affecting local or whole-body metabolism, etc.

Non-limiting examples of such functional molecules comprise cytokines, such as interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL12, IL-13), granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), stem cell factor (SCF), interferon-alpha (IFN-alpha), interferon-beta (IFN-beta), interferon-gamma (IFN-gamma), or a hybrid interferon, erythropoietin (EPO), leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), growth hormone (GH), pre-B-cell leukaemia transcription factor-1 (PRL), tumour necrosis factor (TNF) family of cytokines (e.g., TNF-alpha and TNF-beta), CD40, CD27 or FAS receptors and ligands, the fibroblast growth factors (FGF) family, the platelet derived growth factors (PDGF) family, the transforming growth factors (TGF) family, the nerve growth factors (NGF) family, the epidermal growth factor (EGF) family, the insulin related cytokines (insulin-like growth factors, e.g., IGF-I and II), mast cell growth factor, chemokines : C chemokines like XCL1 (lymphotactin) and receptor XCR1; CC chemokines like CCL1 (I-309) to CCL28, including MIP1alpha (CCL3) and receptors CCR1 to CCR11 (bonzo), especially CCR7; CXC chemokines such as CXCL1 (GRO alpha) to CXCL14 (BRAK) and receptors CXCR1 (IL8 receptor) to CXCR6; CX3C chemokines such as CX3CL1 (fractalkine) and receptor CX3CR1; chemR23 and its ligand chemerin; antibodies or parts of antibodies such as anti-CD3, and the like.

When a fusogenic cell line is modified to (over)express such functional molecules, these molecules will be present in multi parent hybrids prepared using the modified fusogenic cell line. Such modified fusogenic cell lines can therefore introduce into the multi parent hybrids molecules and corresponding biological properties which were not present or were present at low levels in the parent cell types (other than the fusogenic cell line itself) used to prepare the multi parent hybrids. Introduction of said molecules and biological properties to the resulting multi parent hybrids may be beneficial, e.g., in medical applications involving such hybrids. In a non-limiting example, a fusogenic line may be used to fuse an antigen presenting cell with a cell expressing tumour antigens and the resulting hybrids can be used as tumour vaccines. The fusogenic cell line may be advantageously modified to (over)express specific molecules which can provide an immuno-stimulatory or adjuvant effect to enhance the immune response of the body to the tumour vaccine. For instance, said molecules could comprise cytokines, such as pro-inflammatory interleukins.

A single fusogenic cell line may be modified to (over)express one or more functional molecules such as above. Moreover, one or more fusogenic cell lines, each modified to (over)express one or more functional molecules may be used together to

facilitate fusion between suitable cell partners. Hereby, a desired number of functional molecules and corresponding biological properties can be introduced to the multi parent hybrids which were not present or were present at low levels in the parent cell types (other than the fusogenic cell lines themselves).

5 Fusogenic cell line of the present invention may be modified to (over)express the functional molecules by methods well-known in the art. For example, a fusogenic cell line may be transfected with appropriate nucleic acid constructs to obtain transient (over)expression of the functional molecule(s). Alternatively, a fusogenic cell line may stably maintain the coding region for such functional molecule(s). The transcription of the
10 coding region for the functional molecule(s) may be controlled by suitable constitutive or inducible promoters.

Functional molecules from a group consisting of regulatory molecules involved in the regulation of the expression of target molecules of other cells in such a way that these target molecules will be either overexpressed or downregulated when said regulatory
15 molecules are introduced in said other cells. Said target molecules may be used to diagnose a cell fusion event (e.g., see Phan et al. 2003, Nature Medicine 9 : 1215 -1219) and /or may be used to purify the resulting hybrid.

For example, a fusogenic cell line may be modified to (over)express one or more transcriptional regulators, e.g., transcription factors or transcriptional repressors, capable
20 of inducing or repressing transcription from specific promoters. Such specific promoters may control the transcription of one or more genes or coding regions in one or more of parent cell types. Accordingly, when the modified fusogenic cell line induces fusion of the parent cell types, the transcriptional regulator(s) expressed by the fusogenic cell line will be introduced into the resulting multi parent hybrids and will alter transcription of gene(s)
25 or coding region(s) controlled by the corresponding promoters. This may lead to the expression of gene(s) or coding region(s) which were not expressed or expressed at low levels in the parent cell type(s) before the fusion. Alternatively, this may inhibit the expression of gene(s) or coding region(s) which were expressed in the parent cell type(s) before the fusion.

30 In an illustrative example, a fusogenic cell line may be modified to (over)express tetracycline repressor (*tetR*). In the absence of the inducer anhydro-tetracycline, *tetR* binds to a specific DNA sequence in *tetR*-responsive promoters and inhibits transcription of downstream genes. This inhibition is lifted by anhydro-tetracycline which causes dissociation of *tetR* from the promoters. Accordingly, if specific gene(s) in one or more
35 parent cell types (other than the fusogenic cell line itself) are controlled by *tetR*-responsive

promoters, expression of such genes in the resulting multi parent hybrid will be inhibited in the absence of anhydro-tetracycline by *tetR* derived from the fusogenic cell line.

In another example, expression of select gene(s) in the multi parent hybrid may be down-regulated by specific antisense nucleic acids (e.g., antisense RNA) or short interfering nucleic acids (e.g., short interfering RNA (siRNA) or short hairpin RNA (shRNA) capable of mediating RNA interference) expressed by a suitably modified fusogenic cell line. In yet another example, the function of select protein(s) in the multi parent hybrid may be diminished by dominant negative form of such protein(s) expressed by a suitably modified fusogenic cell line.

It should be noted that throughout the present invention cell line and cell(s) of said cell line i.e. having average characteristics of said cell line are used interchangeable.

Blocking replication for example by irradiation or other means such as mitomycin treatment may be advisable if the use of the cells must be controlled (such as for commercial purposes).

The cell lines of the present invention and products thereof, may for instance be used in vivo. Therefore, the present invention also relates to an irradiated cell line according to the present invention. This illustrates the advantage of using a fusion agent. Indeed, tumour cells may not be irradiated if we need to use (propagate) these to produce viable hybrids. The irradiation of the fusion agent of the present invention stops the replication of the DNA, however it does not change the short term expression of protein due to the presence of RNA molecules in said cells. Said irradiation may be performed using 20, 40, 80, 160, 200 Gy. According to the present invention said irradiation is preferably performed using 20 Gy.

In an embodiment, the present invention provides a stable CHO cell line wherein 5%-95% of the cells of said line express the GaLV-FMG fusogenic glycoprotein. Said CHO cell line may be the GFMG 10M6 cell line. Multi parent hybrids made using such fusogenic CHO cell line or membranes thereof may expose non-human (rodent) surface molecules. Such multi parent hybrids can therefore induce a xenogeneic rejection immune response in human subjects. As mentioned, xenogeneic rejection immune response may be advantageous as it can enhance specific immune reaction against antigens. Such antigens, e.g., tumour antigens, may be presented by the multi parent hybrids.

The inventors found that the production of a CHO cell line, wherein at least 40% and preferably 95% of the cells express a GaLV-FMG fusogenic glycoprotein, needs an intense selection and purification strategy. After a large number of experiments, the inventors could optimise a method to isolate a cell line carrying said high number of FMG expressing cells. After transfection only a low/undetectable number of cells expressed

said glycoprotein. Moreover, those cells that expressed the FMG did so at 10 fold lower levels than the stable cell line. Furthermore, the application of only one selection and/or purification method did not allow to increase the number of FMG-expressing cells in the cell line. According to the present invention, a cell line wherein at least 40% and preferably 95% of said cell expresses a FMG can be obtained using a method comprising different subsequent selection and/or purification steps such as : 1) transfecting a stable (permanent) cell line with a vector coding for a fusogenic glycoprotein and a selection marker, 2) selecting the transfected cells (stably transfected cells) using said selection marker, and, 3) selecting the cells expressing the fusogenic glycoprotein using a specific FMG-antibody (i.e. magnetic cell sorting), 4) optionally, further purifying the FMG-expressing cells using limiting dilution. In the enclosed experiments the need of such an intensive purification method has been illustrated for a CHO cell line wherein 40% and preferably 95% of the cells express the GaLV-FMG. However, said extensive selection and/or purification will probably also be needed when any cell line, wherein at least 40% and preferably 95% of the cells express the GaLV-FMG, needs to be obtained.

The present invention provides polyclonal antibodies, such as mouse antisera 168B and 167E, and monoclonal antibodies, such as 168B 1F5, 168B 6B3 and 168B 6B5, recognizing specifically the GaLV-FMG fusogenic glycoprotein. These anti-FMG antibody can thus be used for the identification, selection and/or purification of FMG-expressing cells. A skilled person will understand that other available polyclonal or monoclonal anti-FMG antibodies may be used for these and other applications.

The main property of the cells of the invention is that they produce FMG glycoproteins which makes them suitable as a fusogenic agent. The present invention suggests that suspensions of membranes from highly FMG-expressing cells may therefore be sufficient to induce cell fusion between two other cells. Therefore, the present invention also relates to a membrane preparation of said cell line according to the present invention.

The present invention further contemplates a composition comprising a cell line of the present invention, a cell derived from said cell line, or a membrane preparation thereof.

Another embodiment of the present invention, is an *in vitro* use of a cell line of the present invention, a cell derived from said cell line, a membrane preparation thereof, or a composition thereof as a fusing agent for the production of multiparent cell hybrids.

Throughout the whole text, the term 'multiparent cell hybrid' needs to be interpreted as triparent cell hybrid, four-parent cell hybrid, five-parent, six-parent cell hybrid, seven-parent cell hybrid etc. The present application gives proof through the

experiments that tri-hybrids may easily and efficiently be made using a fusogenic cell line of the present invention. It is clear for a skilled person that the proposed concept may also be envisaged for the production of multiparent cell hybrids other than triparent cell hybrids.

Another embodiment of the present invention relates to a triparental cell hybrid
5 carrying at least : a) one characteristic or specific marker of a cell line according to any of the embodiments described (a fusogenic cell line of the present invention), which is different from a second and a third cell of b) and c), b) one characteristic or specific marker of a second cell different from the first cell and the third cell and, c) one characteristic or specific marker of a third cell different from the first and the second cell.

10 The cell line of a) may be modified to (over)express functional molecules as described.

According to the present invention said triparental cell hybrid may preferably also carry at least: a) one characteristic or marker, which is heterologous or xenogeneic to a second and a third cell of b) and c), b) one characteristic or marker of a second cell allogeneic or autologous to the third cell of c), and, c) one characteristic or marker of a
15 third cell allogeneic or autologous to the second cell of b). Also here, said descriptions of tri-hybrid may be broadened for a multi-parental cell hybrid carrying at least one characteristic for each cell partner fused. Said characteristic or marker a) may be chosen from the group consisting of mammalian-, bacterial-, fungal, viral and plant-marker; and, wherein characteristics or markers b) and c) are mammalian, and preferably human,
20 characteristics or markers. The specific marker can be genetically or chemically added to the parental cell lines before the cell fusion process. For instance, cells carrying characteristic or marker a) and some tumour cell lines can express the heterologous fluorescent protein 'GFP' (genetic modification). In addition, all cells can be labelled (e.g., membrane labelled) with different fluorescent chemicals (for example N-
25 hydroxysuccinimide esters of fluorescent molecules) for the time of the experiments.

The term "autologous" means that cells or materials are derived from same individual. The term "allogeneic" means that cells or materials are derived from the same species. The term "xenogeneic" means that cells or materials are derived from a different species. The term "heterologous" means that cells or materials are derived from another
30 individual of the same or another species.

When the cell line of the present invention used to make a triparental hybrid is of CHO origin, the triparent cell hybrids of the present invention carry a characteristic or marker a) which is a CHO characteristic or marker, which is chosen from the group consisting of the membrane antigens defined by relevant antibodies obtained by
35 immunizing mice or other animals against CHO cells. A triparental cell hybrid carrying at least the CHO marker may be recognized by antibody CHO 105C 8C7, 257D 6B10, 281E

3D4 or a polyclonal anti-CHO antibody, the added tumour cell marker Fluorescein NHS, CFSE or SNARF (or other) and the DC marker HLA-DR , CFSE or SNARF (or other). The cell line and the second and third cell may come from the same species (human); usually b) and c) come from the same species (human, rat for model etc).

5 When in the triparent cell hybrid the second cell is a tumour cell, the characteristic or marker b) is a tumour characteristic or marker. Tumour antigen or a marker (e.g., membrane marker) chemically or genetically added for the purpose of identification and purification of said cells or known by a skilled person. We hereby refer to : "A Listing of tumour antigens recognized by T cells" N Renkvist et al Cancer Immunol Immunother.
10 2001 Mar;50(1):3-15.

 The term "tumour cell" refers to a transformed cell of an individual or to a cell from a transformed cell line. The term encompasses a cell of a "tumour cell vaccine line", which is a tumour cell line that expresses one or more tumour cell antigens and is capable of eliciting an anti-tumour immune response following administration of irradiated cells of
15 the tumour cell line to a patient. Characteristics of tumour cells include anomalous behaviour in tissue culture (for example, growth factor independence, loss of contact inhibition, capacity for anchorage-independent growth, growth to higher density than non-tumour cells, and failure to reach senescence after multiple passages), the ability to invade tissues or metastasise to distant sites, the ability to form tumours when injected
20 into nude mice, and the ability to stimulate angiogenesis.

 The term "antigen" refers to a peptide or polypeptide that elicits an immune response in a mammal (i.e., at least a T cell response). An antigen may be "self" (i.e., a polypeptide that is made by the mammal and present in a healthy individual, or "non-self", that is, a polypeptide that is not normally present in a healthy individual (i.e., a foreign
25 polypeptide).

 The term "tumour cell marker" ("tumour antigen" or "tumour associated antigen") refers to a class of protein markers, or antigens that tend to be expressed to a greater extent in transformed tumour cells than in non-transformed cells. As such, tumour antigens may be expressed by non-tumour cells, although usually at lower concentrations
30 or during an earlier developmental stage of a tissue or organism. Exemplary tumour cell markers are disclosed in the reference "A Listing of tumour antigens recognized by T cells" N Renkvist et al. Cancer Immunol Immunother. 2001 Mar;50(1):3-15.

 When in the triparent cell hybrid the third cell is a DC cell, the characteristic or marker c) is a DC characteristic or marker, which is chosen from the group consisting of
35 HLA-DR, CD83, CD80, CD86, CHEMR23, CD40, CD1a. According to the present invention, the third cell may be any antigen presenting cell.

The term "antigen presenting cell" refers to a cell that has the cellular mechanisms necessary to present on its surface an antigen (that is, a non-APC polypeptide, one which is not normally present on the surface of an APC) and one or more major histocompatibility complex Class II antigens. A cell with the cellular mechanisms necessary to present antigen can ingest antigen (such as in a receptor-mediated manner) or produce it from exogenous DNA and represent on its surface peptides derived from the antigen in combination with MHC Class I or II antigens. Examples of naturally-occurring professional APCs include macrophages, dendritic cells and B lymphocytes. Macrophages express at least the following combination of cell surface "markers": CD11a, -b and -c, CD16, CD17, CD63, CD64, CD68 and CD71. Dendritic cells express at least the following combination of cell surface "markers": CMRF-44, CD83 and CMRF-56. B lymphocytes express at least the following combination of cell surface "markers": CD19, CD20, CD21, CD22, CD40, CD72 and CD78.

The term "surface markers of an antigen presenting cell" refers to MHC class I and II markers, F_c receptors, and adhesion molecules. MHC Class I markers include HLA (human leukocyte antigen) -A, -B and -C and MHC Class II markers include HLA-DR, -DQ, and -DP. F_c receptors include but are not limited to CD16 (FcRIII), CD23 (FceRIIb), CDw32 (FcRII), and CD64 (FcRI). Adhesion molecules broadly expressed by antigen presenting cells include, but are not limited to CD11a (LFA-1 α), CD18 (LFA-1 β), CD29 (VLA- β), CD54 (ICAM-1), and CD58 (LFA-3). A given APC (macrophage, dendritic cell, B cell or synthetic APC) according to the invention expresses at least one MHC Class II marker on its surface and may express one or more of the MHC Class I, Fc receptor or adhesion molecules.

Accordingly, the present invention also encompasses an exemplary triparental cell hybrid carrying any combination of characteristics or specific markers, said combination made by choosing at least one entry from each respective row I, II and III of the following Table 1:

I	a characteristic or a marker derived from a human cell line
	a characteristic or a marker derived from a non-human mammal cell line
	a characteristic or a marker derived from a rodent cell line, such a CHO cell line
	a characteristic or a marker derived from a CHO cell line, chosen from the group consisting of CHO markers recognized by antibody CHO 105C 8C7, 257D 6B10, 281E 3D4 or by a polyclonal anti-CHO antibody
	a functional molecule which is capable of inducing a xenogeneic rejection immune response in humans
	a functional molecule chosen from a group consisting of cytokines, chemoattracting molecules, co-stimulatory molecules, antigens, and molecules with adjuvant properties
	a functional molecule chosen from a group consisting of cytokines, interleukins IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL12, IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), stem cell factor (SCF), interferon-alpha (IFN-alpha), interferon-beta (IFN-beta), interferon-gamma (IFN-gamma), a hybrid interferon, erythropoietin (EPO), leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), growth hormone (GH), pre-B-cell leukaemia transcription factor-1 (PRL), tumour necrosis factor (TNF) cytokine, TNF-alpha and TNF-beta, CD40, CD27, FAS receptors and ligands, the fibroblast growth factors (FGF) family, the platelet derived growth factors (PDGF) family, the transforming growth factors (TGF) family, the nerve growth factors (NGF) family, the epidermal growth factor (EGF) family, the insulin related cytokines, IGF-I and II, mast cell growth factor, chemokines, C chemokines, like XCL1 (lymphotactin) and receptor XCR1, CC chemokines, CCL1 (I-309) to CCL28 (CCL1, CCL2, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9, CCL10, CCL11, CCL12, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CCL28), including MIP1alpha (CCL3) and receptors CCR1 to CCR11 (bonzo) (CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CCR11), CXC chemokines, CXCL1 (GRO alpha) to CXCL14 (BRAK) (CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14) and receptors CXCR1 (IL8 receptor) to CXCR6 (CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6), CX3C chemokines such as CX3CL1 (fractalkine) and receptor CX3CR1; chemR23 and its ligand chemerin; antibodies or parts of antibodies such as anti-CD3

II	a tumour antigen
	a tumour antigen chosen from 707 alanine proline, alpha (a)-fetoprotein (AFP), adenocarcinoma antigen recognized by T cells 4 (ART-4), B antigen, b-catenin/m, b-catenin/mutated (BAGE); breakpoint cluster region-Abelson (Bcr-abl), CTL-recognized antigen on melanoma (CAMEL), carcinoembryonic antigen peptide - 1 (CAP-1), caspase-8 (CASP-8), cell-division cycle 27 mutated (CDC27m), cycline-dependent kinase 4 mutated (CDK4/m), carcinoembryonic antigen (CEA), cancer/testis antigen (CT), cyclophilin B (Cyp-B), differentiation antigen melanoma (DAM), elongation factor 2 mutated (ELF2M), Ets variant gene 6/acute myeloid leukemia 1 gene ETS (ETV6-AML1), glycoprotein 250 (G250), G antigen (GAGE), N-acetylglucosaminyltransferase V (GnT-V), glycoprotein 100 kD (Gp100), helicase antigen, human epidermal receptor-2/neurological (HER-2/neu), arginine (R) to isoleucine (I) exchange at residue 170 of the a-helix of the a2-domain in the HLA-A2 gene (HLA-A*0201-R170I), human papilloma virus E7 (HPV-E7), heat shock protein 70 - 2 mutated (HSP70-2M), human signet ring tumor - 2 (HST-2), human telomerase reverse transcriptase (hTERT or hTRT), intestinal carboxyl esterase (iCE), KIAA0205, L antigen (LAGE), low density lipid receptor/GDP-L-fucose: b-D-galactosidase 2-a-L-fucosyltransferase (LDLR/FUT) melanoma antigen (MAGE), melanoma antigen recognized by T cells-1/Melanoma antigen A (MART-1/Melan-A), melanocortin 1 receptor (MC1R), myosin mutated, mucin 1 (MUC1), melanoma ubiquitous mutated 1, 2, 3 (MUM-1, -2, -3), NA cDNA clone of patient M88 (NA88-A), New York - esophageous 1 (NY-ESO-1), protein 15 (P15), protein of 190 kD bcr-abl, promyelocytic leukaemia/retinoic acid receptor a (Pml/RARa), preferentially expressed antigen of melanoma (PRAME), prostate-specific antigen (PSA), prostate-specific membrane antigen (PSM), renal antigen (RAGE), (renal ubiquitous 1 or 2 (RU1 or RU2), sarcoma antigen (SAGE), squamous antigen rejecting tumor 1 or 3 (SART-1 or SART-3), translocation Ets-family leukemia/acute myeloid leukemia 1 (TEL/AML1), triosephosphate isomerase mutated (TPI/m), tyrosinase related protein 1 or gp75 (TRP-1), tyrosinase related protein 2 (TRP-2), TRP-2/intron 2 (TRP-2/INT2), Wilms' tumor gene (WT1)
	a chemically added fluorescent marker such as with CFSE or SNARF
	a genetically added marker such as GFP
	a characteristic or a marker of an antigen presenting cell
	a characteristic or a marker of a macrophage and/or a dendritic cell and/or a B lymphocyte
	a DC characteristic or marker, chosen from the group consisting of HLA-DR, CD83, CD80, CD86, CHEMR23, CD40, CD1a
	a combination of cell surface markers: CD11a, -b and -c, CD16, CD17, CD63, CD64, CD68 and CD71
	a combination of cell surface markers: CMRF-44, CD83 and CMRF-56
	a combination of cell surface markers: CD19, CD20, CD21, CD22, CD40, CD72 and CD78
III	a chemically added fluorescent marker such as with CFSE or SNARF
	a genetically added marker such as GFP

Typically, in the above triparental cell hybrids, the characteristics in row I may be derived from the fusogenic cell line according to the invention, the characteristics in row II may be derived from a tumour cell or cell line, and the characteristics in row III may be derived from an antigen presenting cell.

In our preferred examples, a CHO/tumour/DC triparental hybrid is formed carrying at least the CHO marker recognized by antibody CHO 105C 8C7, 257D 6B10 or 281E 3D4, the added tumour cell marker Green Fluorescent Protein (GFP) and the DC marker HLA-DR.

5 The present invention not only relates to a purified cell or population of cells of the invention, but also relates to a composition comprising a triparental cell hybrid of the invention.

As pointed out above, the present invention provides a unique approach of making multiparent cell hybrids through the use of a fusogenic cell line. For the generation of triparent cell hybrids the method comprises the steps of : 1) culturing a stable cell line according to any of the embodiments described (a fusogenic cell line of the present invention) as fusion agent, expressing a fusogenic glycoprotein, 2) culturing a second cell line, the first fusion partner, expressing a receptor for said fusogenic glycoprotein, 3) culturing a third cell line, the second fusion partner, expressing a receptor for said fusogenic glycoprotein, 4) mixing the cells of steps 1), 2) and 3), 5) incubating the mixture of cells obtained in step 4) to allow fusion, obtaining (generating) the triparental cell hybrids. Also here, the method may be applied for the fusion of multiple partner cells, as long as they carry a receptor sensible to the FMG expressed by the fusogenic agent. As described, the fusogenic cell line may be irradiated prior to steps 4) and 5). Also as described, membrane preparation of the fusogenic cell line of step 1) may be used in steps 4) and 5).

If in the method of the present invention said fusogenic glycoprotein is HIV-FMG, the receptor for said fusogenic protein may be CD4 and one of the HIV coreceptors such as CCR5. If in the method of the present invention said fusogenic glycoprotein is GaLV-FMG, the receptor for said fusogenic protein may be PIT-1. If in the method of the present invention said fusogenic glycoprotein is murine leukaemia virus 10A1-FMG, the receptor for said fusogenic protein is PIT-1 or PIT 2. When using for instance the fusogenic glycoprotein of VSV-FMG fusion is activated by low pH treatment.

In the method of the present invention, the second and/or third cell line is/are preferably of mammalian origin, e.g., human origin. Said second and/or third may be stable cell lines, or primary cell lines. An efficient fusion for two primary cell lines has till now never been described before. The concept of the present invention gives also here a perfect solution to fuse easily and fast primary cell lines. Therefore, the present invention relates in particular to a method for the preparation of multiparent cell hybrids, wherein (a) the second and/or (a) the third cell line is/are primary cell line(s). For instance said primary cell lines may be isolated and prepared from the patient to be treated.

Said isolated cell is preferably a cell that is removed from its natural environment and is substantially pure. For example, APCs may be isolated from a sample of the patient's peripheral blood; dendritic cells are isolated from peripheral blood, bone marrow or from spleen tissue (the latter usually only for animal experiments); macrophages may
5 be isolated from peripheral blood, or, alternatively, from alveolar lavage fluid or from peritoneal lavage fluid; tumour cells may be isolated from a tissue biopsy sample from a diseased individual, for example, primary melanoma cells or primary fibroblasts are isolated, for example, from a skin biopsy.

The second cell line used in the method of the present invention may be chosen
10 from the group consisting of a tumour cell, cells freshly recovered from a surgically removed tumour or from a biopsy, or an established tumour cell line or a cell line expressing a tumour antigen. The third cell used in the method of the present invention may be chosen from the group consisting of an antigen presenting cell. (dendritic cell, macrophage, B-cell or a cell derived thereof). According to the present invention, said
15 antigen presenting cell may be an antigen presenting cell precursor, an immature antigen presenting cell or a mature antigen presenting cell. In addition, said antigen presenting cell may be prepared *in vitro* using following steps: 1) inducing the differentiation of monocytes (PBMC, CD14+) present in a blood sample through the incubation of said cells in the presence of GM-CSF and IL-4, Type I IFN (IFN alpha or beta) and IL-3, or,
20 Type I IFN (IFN alpha or beta and GM-CSF, thereby making immature DCs (CD1a+, HLA-DR++, CD86+, CD83-, CD40+), 2) optionally, inducing the maturation of said immature DC through the incubation in the presence of for instance LPS, bacterial DNA, ds RNA, INF, IL-1, IL-6, IL-10, TGF-beta, prostaglandines, and/or T lymphocytes, thereby making mature DCs (CD1a-, HLA-DR+++, CD86++, CD83+, CD40++), The dsRNA may be a
25 synthetic poly I:C.

More specifically, the present invention elaborates on a method to prepare a CHO/tumour/DC triparental hybrid, comprising the steps of : 1) culturing a stable CHO cell line wherein at least 40% and preferably 95% of the cells in said cell line express the GaLV-FMG cell line as fusion agent; 2) culturing a second cell line, the first fusion partner,
30 expressing a PIT-1 receptor for said fusogenic glycoprotein, 3) culturing a third cell line, the second fusion partner, expressing a PIT-1 receptor for said fusogenic glycoprotein; 4) mixing the cells of steps 1) to 3); 5) incubating the mixture of cells obtained in step 4) to allow fusion, making CHO/tumour/DC triparental hybrids. In said specific method the first fusion partner of step 2) and the second fusion partner of step 3-) may be primary cells;
35 the fusion partner of step 2) may be cells directly recovered from surgically removed tumours.

Conditions which allow fusion are these wherein a cell expressing a fusogenic membrane protein and at least two partner cells, either engineered to express or naturally expressing a corresponding fusogenic membrane protein receptor, are mixed together at a concentration and ratio such that contact between the fusogenic membrane protein on one cell and the partner cell occurs, resulting in fusion of these cells, such that typically (but not always necessarily – e.g., the surface proteins of in particular the fusogenic cell line need not be expressed on the resulting fusion hybrid) at least one cell surface protein of each cell is expressed in the resulting fusion hybrid.

In step 4) of the method of the present invention cells are mixed. A ratio used during the mixing of the cells in step 4) between the first fusion partner, the second fusion partner and the fusing agent of 1:1:0.1; 1:1:2.3; 1:1:1.6; 1:1:1; 1:1:2; 1:1:3 is acceptable for conditions that allow cell fusion. Actual ratios used may be re-assessed. However, in the present invention a ratio of 1:1:1 between the first fusion partner, the second fusion partner and the fusing agent is preferably used.

According to the present invention, the triparental hybrids obtained in step 5/ of the proposed method are further analysed and/or purified. Said analysis and/or purification is performed using microscopic techniques (confocal microscopy), Flow cytometry or Magnetic cell sorting. Said analysis or purification may be performed based on the presence of DC- (HLA-DR, CD83, CD80, CD86, CHEMR23, CD40, CD1a), tumour- and/or CHO (an antibody recognizing membrane antigens obtained by immunizing mice or other animals against CHO (ex. CHO105C 8C7, 257D 6B10 or 281E 3D4) markers. Throughout the text the abbreviation of PE of HLA-DR-PE stands for Phycoerythrin (a fluorescent dye).

When needed, the FMG may be detected using a specific FMG antibody. According to the present invention, the antibody is a monoclonal or a polyclonal antibody specific for GaLV-FMG, such as a rabbit or mouse polyclonal antiserum, for example, the antisera from mice 168B or 167E described herein, or the monoclonal antibodies 168B 1F5, 168B 6B3 or 168B 6B5 derived from mouse 168B.

In the method of the present invention the fusion efficiency may be further improved by the addition of the polycation polybrene during the mixing or fusion of the cells in step 4 and/or step 5 of said method.

An embodiment of the invention relates to a triparental cell hybrid obtainable or produced according to a method of the present invention, and to a composition comprising the triparental cell hybrid. Irradiation of the triparental hybrid preparation may also be desirable to protect patients from possible tumorigenic properties of the composition. Accordingly, the triparental cell hybrid may optionally be irradiated.

The invention further relates to a triparental cell hybrid according to the present invention, such as a triparental cell hybrid obtainable or produced using a fusogenic cell line of the present invention and/or a triparental cell hybrid obtainable or produced by the method according to the present invention, as well as compositions comprising the triparental cell hybrid, for use as a medicament.

The invention also encompasses a therapeutic composition comprising a cell described herein, in a mixture with physiologically acceptable carrier. The invention also encompasses a method of preparing a therapeutic composition for the treatment of malignant disease, the method comprising the step of admixing a cell as described above with a physiologically acceptable carrier.

The invention also encompasses a therapeutic composition comprising a triparental cell hybrid according to the present invention, such as a triparental cell hybrid obtainable or produced using a fusogenic cell line of the present invention and/or a triparental cell hybrid obtainable or produced by the method according to the present invention, in a mixture with physiologically acceptable carrier.

The invention also encompasses a method of preparing a therapeutic composition for the treatment of malignant disease, the method comprising the step of admixing a triparental cell hybrid according to the present invention, such as a triparental cell hybrid obtainable or produced using a fusogenic cell line of the present invention and/or a triparental cell hybrid obtainable or produced by the method according to the present invention, with a physiologically acceptable carrier.

Also these aspects may be applied for multiparental cell hybrids of the present invention.

Depending on the partners used for the production of a multiparent cell hybrid it may be evident to derive a possible application thereof. As mentioned before multiparent cell hybrids are made to combine characteristics of different cells onto one cellular vehicle. In the present application proof is given for the production of a CHO/tumour/DC tri-hybrid. However, the teaching of the present application goes beyond this example. The examples given should be seen as a model to proof the general concept.

Another embodiment of the present invention relates to the use of a triparental cell hybrid of the present invention and a composition thereof for the production of a medicine. In particular, they can be used as immune vaccine. According to the present invention, said medicine or vaccine may induce an immune response in vitro and/or in vivo. In addition, when applied in patients, said cells may have antigenic and/or immunomodulatory properties including adjuvant and/or therapeutic properties. In addition the immune response obtained in vitro can be used in vivo for therapy.

The medicine of the present invention may be used to treat diseases or disorders resulting from the proliferation of oncogenically transformed cells. Symptoms of malignant disease vary depending upon the nature of the transformed cell type and the particular location(s) of tumours or transformed cells. For example, bronchogenic carcinoma, or lung cancer, of which there are several forms, including squamous cell carcinoma, adenocarcinoma, small cell carcinoma and large cell carcinoma, has the symptoms of cough, dyspnea, chest pain, hemoptysis and anorexia, in addition to the presence of a tumour mass on X-ray. As another example, acute leukaemia often has the symptoms of weakness, malaise, anorexia, bone and joint pain, fever, petechiae, lymph node swelling, and splenomegaly, in addition to the presence of abnormal cells in the peripheral blood. As another example, brain tumours often have the symptoms of personality changes, intellectual decline, emotional lability, seizures, headaches, nausea. Depending upon the site of the tumour mass, brain tumours may cause visual field defects, hearing loss, loss of or altered olfactory function, motor phenomena and aphasia, among other symptoms.

The medicine of the present invention may be used to reduce symptoms thereby decreasing the severity or extent of the indicators of a disease. Examples of specific symptoms that may be directly quantitated include fever, high (or low) white blood cell count, elevated or decreased blood pressure, or the presence of abnormal cells in a blood or tissue specimen. A change of 1% or more, 2% or more, 5% or more, 10% or more, up to 25%, 50% or 75% or more in these or other quantitative measurements of disease status is considered to be indicative of reduced symptoms. Other symptoms such as pain, lethargy, nausea and restlessness, among others, may be considered reduced if there is a difference noted by the physician or reported by the patient following treatment, and the difference persists over time, for example, for two days, for two weeks, for two months or longer.

The medicine of the present invention may also be used to maintain the disease or disorder, which means that the size or symptoms do not increase or worsen in severity with the passage of time, for example, over weeks or months.

A therapeutic composition can be made effective for the treatment of a disease. The term "amount effective to reduce the symptoms" means that a number of multiparent cell hybrids of the present invention (formed in vitro or ex vivo) is administered to a patient in need of treatment for a tumour such that the outward indicators of the disease caused by the tumour are decreased. Said composition may further comprise a physiologically acceptable carrier or diluent. Said carrier or diluent may be a solution or composition in which cells or hybrid cells of the invention may be suspended to allow administration (e.g., intravenously, intraperitoneally, etc.) of the hybrids or cells to an

individual. A physiologically acceptable carrier or diluent will generally be isotonic and will often be buffered; a large number of acceptable diluents or carriers are known in the art.

The triparental cell hybrids according to the present invention, such as triparental cell hybrids obtainable or produced using the fusogenic cell line of the present invention
5 and/or triparental cell hybrids obtainable or produced by the method according to the present invention, such as the tri-hybrids produced in the examples of the present invention, may be used for the production of a medicine or a vaccine for the treatment of cancer. This also applies for multiparent cells of similar nature. This also applied to cellular preparations of immune responses gained in vitro as described.

10 Alternatively, multiparent cell hybrids or compositions thereof can also be used for the production of a medicine or a vaccine for the treatment of infections (bacterial, viral, fungal, protozoal, parasitic). In this case one of the cell partners of said hybrid carries antigenic components typical for said infection.

Another embodiment relates to the use of a (multi) triparental hybrid, membranes
15 and, a composition thereof, of the present invention for in vitro tests. According to the invention, said test may be used to determine the immunogenic potential of cells and specific antigens. In particular, the cell line of the present invention (a fusogenic cell line) may be used, a membrane preparation of the cell line, a triparental cell hybrid according to the present invention, such as triparental cell hybrids obtainable or produced using a
20 fusogenic cell line of the present invention and/or triparental cell hybrid obtainable or produced by the method according to the present invention, to follow the serologic immune responses and the cellular immune responses elicited by patients against the triparental hybrids and against its parental cells. There are many ways known from the people skilled in the art to carry out such diagnostic tests. For instance triparental hybrids
25 and or its parental cells can be exposed to the serum of the patient and antibody binding to the triparental hybrid and / or to the parental cells present in the patient's serum thereafter evidenced by a fluorescent anti human Ig antibody and flow cytometry and/or confocal microscopy. Similarly triparental hybrids and or its parental cells can be labelled with a label such as a radioactive or a fluorescent substance and exposed to blood cells of
30 the patient, the release in the medium of said label indicating the presence of effector cells.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Exemplary methods and materials are described below, although
35 methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications and other references

mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and do not intend to be limiting. Other features and advantages of the invention will be apparent from the following drawings, detailed description, and from the claims.

Brief description of the figures

Figure 1: Transgene expression by 293T cells transiently transfected with pTet-on eGFPneo (A and C) and pTet-on FMGneo (B and D) could be induced with dox. Four days post-transfection uninduced cells show few cells positive for eGFP (A) or fusion (C). With 1µg/ml dox stimulation, the number of eGFP positive cells (B) was increased and cell fusion (D) was induced.

Figure 2: By flowcytometry the inducibility of the 518Tet-eGFP clones, selected by geneticin, was determined. The fluorescence intensity of eGFP (FL-1) of uninduced population (solid fill) was compared with that of the induced population (grey line). Most of the 36 clones were not inducible by doxycyclin like clone 1 (A). About 5% of the population of two clones, clone 15 (B) and clone 23 were found inducible.

Figure 3: 518Tet-eGFP subclones 15.1 (A) and 15.4 (B) were best inducible. Subclone 15.4 was selected for its high inducibility combined with its low background fluorescence. The fluorescence intensity of eGFP (FL-1) of the induced population (black line) was compared with that of the uninduced population (grey line) and the parental cell line 518.A2 (dotted line).

Figure 4: The percentage of induced 518Tet-eGFP cells over time as determined by flowcytometry. At time 0 induction of cells with different concentrations of dox was started. Fresh medium with dox was supplied three times a week. On day 11 fresh medium without dox was supplied to all cells. As a control the uninduced parental cell line 518.A2 was also included in the experiment.

Figure 5: The level of eGFP expression in the parental 518.A2 cells (A) and 518Tet eGFP (B) followed over 7 days (from light gray to black). For the inducible cell line, 1µg/ml dox induction was started at time 0.

Figure 6: Mean fluorescence of the induced population of 518Tet-eGFP over time as determined by flowcytometry. At time 0 induction with dox in different concentrations was started. On day 11 dox was removed from all cultures. Their level eGFP expression was compared with that of the parental cell line 518.A2 (□), uninduced 518Tet-eGFP (●).

5

Figure 7: Kinetics of Tet-on induction of 518Tet eGFPtTS subclone 1.4 as determined by flow cytometry. At day 0 stimulation with different concentrations of dox was started. The unstimulated parental cell line 518.A2 was included as control. A) Percentage of induced cells. B) Level of gene expression determined as eGFP mean fluorescence in the induced and uninduced populations.

10

Figure 8: Transduction efficiency of 518.A2 cell line with AAV-eGFP vectors of AAV serotype 1(▲), 2(■), and 5(●) as determined by flowcytometry, 2 days post-infection.

15 Figure 9: Transduction efficiency of AAV2 eGFP vector in human melanoma cell lines. Cells were infected at different MOI and the percentage of transduced cells was determined 48h post-infection by flowcytometry.

20 Figure 10: Two human melanoma cell lines 518.A2 (■) and MelZ2 (▲) transduced with AAV2-eGFP at MOI 5 at time 0. The level of gene expression was monitored over time by flowcytometry.

Figure 11: Human melanoma cell line MelZ2 were infected with AAV-GaLV FMG at MOI 1.3. The formation of multinucleated cells was observed 48h post-infection (A). Prolonged fusion resulted in large syncytia, as shown here 6 days post-infection (B). 100 fold magnification.

25

Figure 12: Green PKH pre-stained 518.A2 cells were infected with AAV-FMG (AAV-GaLV) and overlaid with DC next day, after overnight fusion cells were stained with a PE-coupled anti-HLA-DR antibody. The percentage of double-stained cells was determined by flowcytometry (A) and compared with the co-culture control using uninfected 518.A2 cells (B).

30

Figure 13: Percentage of double-stained cells when simply co-culturing two different cell types, as determined by flowcytometry (grey bars).

35

Figure 14: Fluorescence intensity by PKH pre-staining decreases over time. The black line indicates intensity of the red fluorescence by PKH staining of 518.A2 cells compared to the background fluorescence of 518Tet-eGFP cells (dotted line). While 24h after staining the two cell populations are well discriminated, decreased red intensity results in overlap of the two populations.

Figure 15: The stable cell line HeLa-eGFP constitutively expresses intracellular eGFP, which can be read as FL-1 by flowcytometry (A). The stable cell line 518-EGFR could be determined (identified) by staining with a specific anti-EGFR antibody coupled to a red fluorochrome, read as FL-2 (B). Parental cell lines (untransfected) are shown with the dotted lines.

Figure 16: 518-EGFR cells (A) and 518Tet eGFP cells (B) were either fused (C) or co-cultured (D) overnight at a ratio of 1:3. In the fusion sample the 518-EGFR cells were transiently transfected with pCR3.1GalV. Cells were stained with the anti-EGFR-PE antibody before flowcytometry.

Figure 17: Cells were marked with CFSE (I) (5 μ M) or SNARF (II) (50 μ M) and (a) analysed by FACS after 1, 16 and 24 hours for intracellular fluorescence or mixed with non stained cells for 6 hours (b) directly after staining or (c) 16 hours after staining.

Figure 18: Fusion with GALV-FMG-transduced cells. 518-EGFR cells were transfected with pCR3.1GALV (C) or infected with AAV2-GALV at a multiplicity of 1000 total particles/cell (B) or neither transduced nor transfected (A). After mixing at a ratio of 1:1 with HeLa-eGFP and overnight culture, cells were either stained with Diff-Quik for microscopy (bottom panel) or labelled with anti-EGFR antibodies for flow cytometry (top panel).

Figure 19: Flow cytometry to determine fusion between CHO and Hela-GFP cells. CHO transfected with pCR GaLV (A), Hela-GFP (B), co-culture of Hela-GFP with either untransfected CHO (C) or pCR GaLV transfected CHO (D) in a ratio of 1:1. In order to identify CHO cells, samples were stained by a mouse α CHO antibody followed by goat- α mouse Ig secondary antibody coupled to phycoerythrin

Figure 20: Comparison between untransfected CHO cells (dotted line) and CHO-FMG clone 8 (A) and 10 (B). Cells were stained with the putative anti-FMG mouse serum 167E

followed by goat anti-mouse Ig-PE antibody. Clone 8 and 10 show 1.5% and 5.4% positively stained cells respectively.

Figure 21: CHO-FMG clone 8 (A and B) and 10 (C and D) one week after enrichment using 1.5 μ l (A and C) and 4.0 μ l (B and D) of antiserum 167E in comparison with untransfected CHO cells (dotted line). Cells were stained with antiserum 167E followed by goat anti-mouse-PE antibody before analysis by flowcytometry.

Figure 22: CHO-FMG is a CHO cell line stably transfected by pCR3.1 GaLV. Clone 10M6 was retained after selection on neomycin-resistance, MACS selection and re-cloning by limiting dilution, and at least 40% and preferably 95%, of the cells expressed FMG at a high level. Cells were stained with anti-FMG antibody 168B 6B5 followed by goat anti-mouse-PE secondary antibody. CHO cells are shown with the dotted line.

Figure 23: Fusion efficiency of CHO cells transfected or transduced with GALV-FMG compared with the stable CHO-FMG cell line. (I) CHO cells were either untransfected (A), transfected with pCR3.1GALV (C) or infected with AAV5-GALV at a multiplicity of 10 infectious particles per cell (B). Fusion efficiency was compared with the CHO-FMG cell line (D). After overnight co-culture with 518-EGFR, cells were stained with Diff-Quik for microscopy (lower panels) or labelled with anti-CHO followed by PE-coupled anti mouse and anti-EGFR-FITC antibodies for flow cytometry (upper panels). Light microscopy shows that overnight incubation of CHO-FMG cells with 518-EGFR (I, lower panels) or HeLa-eGFP (II) results in the formation of multinucleated cells (23 ID, lower panel or 23II A, respectively), which are absent in the co-culture sample in which CHO cells are incubated with 518-EGFR (23 I A) or HeLa-eGFP cells ((23 II B). Cells were stained with Diff-Quik for better visualization of the cell membrane and the nuclei.

Figure 24: Fusion between CHO-FMG and HeLa-eGFP cells in a ratio of 1:1. (I) Flowcytometry: samples were stained by a mouse α CHO antibody followed by PE conjugated goat- α mouse Ig secondary antibody. The results are representative of 19 independent experiments. (A) CHO-FMG cells, (B) HeLa-eGFP cells, (C) co-culture of CHO cells and HeLa-eGFP cells, (D) fusion of CHO-FMG cells with HeLa-eGFP cells. (II) Confocal microscopy of fusion sample, 400x magnification. CHO-FMG and HeLa-eGFP cells were mixed 1:1 on microscope coverslips and incubated overnight. Next day cells were stained with an anti-CHO antibody followed by a goat anti-mouse Ig-PE secondary antibody. By confocal microscopy cells were acquired for transmission view (a), PE

staining at the cell membrane (b), intracellular eGFP expression (c) and the overlay of the three (d). Double-stained cells as indicated with the arrow, were only found in the fusion sample.

- 5 Figure 25: Fusion between CHO-FMG and HeLa-eGFP cells. CHO or CHO-FMG cells were mixed at a ratio of 1:3, 1:1 or 3:1 with HeLa-eGFP cells and incubated overnight. Double-stained cells (PE+eGFP) in co-culture (CHO, white bars) and fusion (CHO-FMG, black bars) samples were detected as in figure 3. The percentage of hybrids (grey bars) was calculated by subtracting values for co-culture from those for fusion samples. (A)
- 10 Proportion of double-labelled cells among total, (B) Percentage of HeLa-eGFP cells involved in hybrid formation, calculated as follows: $n(\text{PE}+\text{eGFP}) / n(\text{PE}+\text{eGFP}) + n(\text{eGFP})$ where n = number of cells determined by flow cytometry.

- 15 Figure 26: Fusion experiment using a ratio of 1:1 of CHO(-FMG) cells and HeLa-eGFP cells. CHO-FMG cells were diluted with the parental CHO cell line to obtain different percentages of CHO cells that actually express FMG. After overnight incubation the cells were stained with the anti-CHO antibody followed by the goat anti-mouse Ig-PE secondary antibody. The percentage of double-stained cells were determined (n=4).

- 20 Figure 27 FMG expression in CHO-FMG cell line (I) and GALV-FMG-transduced parental CHO (II) or 518.A2 (III) cells. Cells were infected with AAV-GALV (A) or transfected with pCR3.1GALV (B). 24 hours after transduction they were stained with anti-FMG and PE-conjugated anti-mouse antibodies. Results for AAV-infected cells are representative of 4 independent experiments. Transfection of CHO and 518.A2 was done in parallel in one
- 25 experiment. CHO-FMG cell were tested >10 times.

- Figure 28: DNA content distribution of CHO and CHO-FMG cells untreated (A and B) and treated with mitomycin C (C and D) for cell cycle arrest. Untreated cells show peaks in G₀/G₁ phase and G₂ phase while treated cells are blocked in the S and G₂ phase. Cells
- 30 were stained with propidium iodide before analysis by flowcytometry.

Figure 29: Fusion efficiency of untreated and mitomycin C treated CHO and CHO-FMG cells with HeLa-eGFP cells. The percentage of double-stained cells found in fusion samples (black bars) and co-culture samples (white bars) are indicated.

Figure 30: The addition of polybrene does not significantly increase the fusion efficiency between CHO-FMG and HeLa-eGFP cells. Fusion samples are indicated in black and co-culture controls are indicated in white.

- 5 Figure 31: Fusion experiment using CHO-FMG cells and 518Tet-eGFP cells induced by doxycycline during two weeks. CHO-FMG cells (A) were stained with an α CHO antibody followed by a PE-coupled secondary antibody and are located in the upper left quadrant. Only a part of the 518Tet-eGFP cells (B) were induced. These cells are located in the lower right, while the uninduced cells that express less eGFP, are situated more to the
- 10 left. Co-culture of CHO cells with 518Tet-eGFP cells (C) show the three described populations, while fusion between CHO-FMG cells and 518Tet-eGFP cells (D) shows a fourth population of cells, positive for α CHO staining and eGFP expression

- Figure 32: Fusion between CHO-FMG and human immature DCs (iDC) at a ratio of 1:1. (I)
- 15 Flow cytometry: Cells were stained with a PE-conjugated mouse anti-HLA-DR and a biotinylated mouse anti-CHO antibody followed by avidin-FITC. (A) CHO-FMG cells, (B) iDC cells, (C) co-culture of CHO cells and iDC cells, (D) fusion of CHO-FMG cells with iDC cells. While in this experiment co-culturing parental CHO cells with DC at a ratio of 1:1 results in 6.4% of double-stained cells (C), fusion between CHO-FMG cells and DC at
- 20 a ratio of 1:1 gives rise to 21.7% of double-stained cells (D). (II) Confocal microscopy of co-culture and fusion samples as in (I), 400x magnification. After overnight fusion on coverslips, an α CHO antibody was used to detect CHO cells (a) and DC were stained red for HLA-DR (b). The overlay of both colours (c) reveal that hybrid cells were present expressing both markers on their cell membranes.

25

Figure 33: The efficiency of hybrid formation of CHO-FMG cells using different ratios of CHO to DC (hatched bars) was calculated by subtracting the percentage of double-stained cells in the co-culture control (white bars) from that of the fusion sample (grey bars).

30

Figure 34: The efficiency of hybrid formation of CHO-FMG cells using two different DC types at two ratios of CHO to DC. The percentage of hybrids formed (hatched bars) was calculated by subtracting the percentage of double-stained cells in the co-culture control (white bars) from that of the fusion sample (grey bars).

35

Figure 35: The efficiency of hybrid formation of CHO-FMG cells using immature or mature DCs at two ratios of CHO to DC. The percentage of hybrids formed (hatched bars) was calculated by subtracting the percentage of double-stained cells in the co-culture control (white bars) from that of the fusion sample (grey bars). Results from two independent experiments.

Figure 36: Fusion of 518-EGFR and DCs via CHO-FMG cells. Cells were mixed in a 1:1:1 ratio and grown overnight. (I) Flow cytometry: Cells were stained with FITC-conjugated mouse anti-EGFR antibody and PE-conjugated mouse anti HLA-DR antibody. (A) 518-EGFR, (B) iDC, (C) CHO-FMG, all displayed as single cell types, (D) co-culture control of 518-EGFR and DC with CHO and (E) fusion between 518-EGFR and DCs with CHO-FMG. (II) Light microscopy of Diff-Quik stained cells: The results are from one of the experiments summarized in figure 39, (A)-(E) stand for the same as in (I).

Figure 37: Confocal microscopy of co-culture and fusion sample as in figure 36, i.e., CHO cells were co-cultured (left panel) or CHO-FMG cells were fused (right panel) with human DC and 518-EGFR cells. 400x magnification. After overnight incubation on coverslips, 518-EGFR cells were stained green with an α EGFR antibody (b) and DCs were stained red (PE) for HLA-DR (c). The two colours were acquired separately in two channels (b and c). The overlay (d) of the colour channels reveals the presence of hybrid cells that are positively stained for both colours on the cell membrane. The cells were also captured in transmission mode (a).

Figure 38: Confocal microscopy picture of CHO (I) or CHO-FMG (II) cells incubated with human DC and human 518EGFR cells. After overnight incubation on coverslips, 518EGFR cells were stained green with an α EGFR antibody, DCs were stained red for HLA-DR and CHO cells were stained with a biotinylated α CHO antibody followed by a streptavidin-PE-Cy5 conjugate and was read in the blue channel. The colours were acquired separately in three channels (A,B and C). The overlay of the colour channels (D) shows three clearly distinguishable cell types in the co-culture sample (I-D). Double-stained cells were only found in the fusion sample (II-D). No triple-stained cells were detected.

Figure 39: Fusion of 518-EGFR and DCs (iDCs) via CHO-FMG. 518-EGFR and DCs (iDCs) were mixed 10^5 of each in a 24 well dish and between 10^4 (ratio 0.1) and 3×10^5 (ratio 3) of CHO or CHO-FMG cells were added. Double-stained cells (PE+eGFP) in co-

culture (CHO, white bars) and fusion (CHO-FMG, black bars) samples were detected as in figure 3. The percentage of hybrids (fusion yields, grey bars) and percentage of 518-EGFR cells involved in hybrid formation was calculated as in figure 25. Results are an average from 8 experiments.

5

Figure 40: Generation of double-stained cells by cell-free CHO-FMG membranes. 518-EGFR cells and DCs were mixed in a ratio of 1:1. CHO (panels A-D and lozenges in panel I) or CHO-FMG (panels E-H and squares in panel I) membranes were added in ratios of cell equivalents and incubated overnight. DCs were red stained for HLA-DR and 518-EGFR cells were stained green for EGFR. The percentage of double-stained cells were quantified by flowcytometry. No increase of this percentage was observed by the use of CHO-FMG membranes.

Figure 41: Detection (selection) of FMG-specific recognition (antibodies) by flowcytometry. 2×10^5 CHO cells (solid fill) or transiently FMG-transfected CHO cells (black line) were stained with 5 μ l of mouse antiserum, followed by goat α mouse Ig - Alexa488 secondary antibody. Antisera 167E and 168B show positive staining of a part of the FMG-transfected CHO cells.

Figure 42: FMG recognition as determined by signal to noise ratio (S/N ratio). A mixture of CHO-FMG and CHO-GPR7 cells were labelled with 25 μ l of anti-FMG supernatant and PE-coupled secondary antibody. 43 putative anti-FMG hybridoma supernatants were analysed by flow cytometry.

Figure 43 : Triple staining of tri-parental hybrids. Hybrids between DC and 518-EGFR cells (TC) were generated via CHO-FMG cells and analysed for the presence of markers of the three fusion partners: anti-HLA-DR-PE for DC, anti-EGFR-FITC and anti-CHO-alexa 647. Cells were checked for double-staining in different combinations: DC:TC (panels B), CHO-FMG:DC (panels C) and CHO-FMG:TC (panels D). The anti-HLA-DR and anti-EGFR staining for each individual cell type is also shown (A). Cells contained in region R2 (panels B), representing DC:TC hybrids, were checked for the presence of the CHO marker (panels E). Panels F show the anti-CHO staining for each fusion partner.

Table 2:

Table 2: Mean fluorescence and inducibility of 518Tet eGFPtTS clones as determined by flow cytometry after 5-7 days of stimulation with 2µg/ml dox. n.a.= non applicable. *Data obtained from different experiments after 7 days (518Tet eGFP) or 18 days (518Tet eGFPtTS 1.4.3) of dox stimulation. Level of gene expression was compared with that of 518Tet eGFP 15.4 (n.d.= not determined).

Table 3:

Hybrid formation in pellets: CHO-FMG cells were mixed with tumour cells (fusion partner) spun down and resuspended in a small volume of medium. Fusion was allowed to proceed for the indicated time in tubes, in a CO₂ incubator at 37°C.

Table 4:

GaLV-FMG expression 24 and 48 hours after transfection (pCR3.1GaLV) or infection (AAV-GaLV) of CHO or 518.A2 cells. Summary of results from different experiments. In some of the experiments infection with AAV-eGFP was added as a control of transduction efficiency.

Modes for carrying out the invention:

In the present examples the generation of human tumour cell - dendritic cell hybrids has been explored by the expression of the Gibbon ape Leukemia Virus (GaLV) derived fusogenic membrane glycoprotein (FMG). In the present experiments, a C-terminally truncated form of GALV-FMG was used, which lacks the 16 amino acid R-peptide of the wild-type protein (Green et al. Sequence-specific antibodies show that maturation of Moloney leukemia virus envelope polyprotein involves removal of a COOH-terminal peptide. PNAS 78: 6023-3748, 1981). The R-peptide normally serves to restrict fusion of the viral envelope until it is cleaved during viral infection. This modification renders the protein constitutively highly fusogenic to Pit-1 expressing cells.

Example 1: Establishing dendritic cell - tumour cell hybrids via an inducible cell line

Continuous GaLV-FMG expression by human cells leads to lethal syncytia formation. In order to establish a stable cell line, the expression of GaLV-FMG should be inducible. The Tet-on system, in which the promoter is only active when induced with doxycyclin (dox) was used. The pTet-on FMGneo plasmid was constructed. This single plasmid carries the GaLV-FMG gene under the regulation of the Tet-on promoter, the neomycin resistance gene and also all elements required for the Tet-on system. Since the

expression of FMG could not be quantified and results in lethal syncytia, an inducible cell line with the reporter plasmid pTet-on eGFPneo was established in parallel to study the induction kinetics.

Transient transfection of these plasmids with and without dox stimulation showed the inducibility of transgene expression (figure 1). 293T cells transfected with pTet-on eGFPneo without dox showed few cells expressing eGFP (1A) while the number of positive cells was increased after four days of dox stimulation (1C). The increase of eGFP positive cells was also quantified by flowcytometry and was found to be less than 3-fold (from 2.7% to 6.6%). After transfection with pTet-on FMGneo cells did not show fusion activity (1B), while after dox stimulation formation of multinucleated syncytia occurred (1D).

1.1 Tet-on clones

The human melanoma cell line 518.A2 was stably transfected with pTet-on eGFPneo and pTet-on FMGneo. After selection with geneticin, 36 and 55 clones were picked from 518Tet-eGFP and 518Tet-FMG respectively

1.1.1 518-Tet-eGFP

The 518-Tet-eGFP clones were split into two groups: uninduced and induced with 1µg/ml dox. After one week of induction, eGFP expression was determined by flowcytometry. Most clones were not inducible (figure 2A). About 5% of the cell population of two out of the 36 clones, namely clone 15 (figure 2B) and 23 were found to be inducible.

Both 518Tet-eGFP clones 15 and 23 were subcloned by limiting dilution and induced 4 days with dox before analysis by flowcytometry. All inducible subclones showed increased basal fluorescence in comparison with the parental cell line 518.A2 (3.8 arbitrary units (AU)). Out of the 13 subclones of clone 15, subclone 15.4 was selected for its highest inducibility (over 65% of cells) combined with the lowest background fluorescence (44.7 AU; fig. 3B) When fully induced 90% of the 518Tet-eGFP clone 15.4 expressing eGFP and was found stable over time. From clone 23 only two subclones were obtained but the population of inducible cells remained low (below 5% of cells).

1.1.2 518Tet FMG clones

The level of FMG expression could not be quantified. Inducibility of the clones could only be evaluated by detection of fusion activity. The 518Tet FMG clones were split into two, one group was kept in culture and the other group was induced with dox 1µg/ml. Cultures were carefully examined daily by microscopy for fusion activity. Many clones showed atypical growth and tended to form clumps that (partially) come off the culture plate. No obvious differences between uninduced or induced clones could be found. Limiting dilution was performed on three potentially inducible clones (clones 13, 14 and 16) in

order to obtain more inducible subclones. 14, 14 and 9 subclones were isolated from 518Tet FMG clone 13, 14 and 16 respectively. Again, subclones were either uninduced or induced and carefully compared. Some subclones were lost over time, which may be caused by lethal fusion. Clones were either not inducible or showed fusion activity in both groups. No inducible clones could be selected. All clones were stored in N₂ for later analysis.

1.1.3 Kinetics of Tet-on induction

The kinetics of induction was determined by stimulating 518Tet-eGFP cells with different concentrations of dox. By flow cytometry the percentage of induced cells and the level of gene expression were assessed at least three times a week. Silencing of the promoter was also determined after removing dox (figure 4). This experiment was performed twice.

The addition of 2.0 µg dox per ml of culture medium resulted in maximal induction after 7 days of induction. The expression of eGFP induced by lower concentrations of dox was found slower. The highest achievable level of induced cells also seems to depend on the concentration used, although the maximum may not have been obtained yet after 11 days of induction. After the removal of dox on day 11, the percentage of induced cells rapidly dropped for all concentrations. By day 4 after removing dox, the percentage of induced cells had dropped below 1% in all conditions.

The level of gene expression was also evaluated over time. The induction by 1 µg/ml dox is shown in figure 5. The mean fluorescence per cell in the induced populations using different concentrations of dox is shown in figure 6. Within one day of dox induction, regardless of the concentration of dox used, the mean fluorescence (MF) raised over one log. Prolonged dox induction slowly increased the MF another 4-fold. When dox was removed, the MF decreased to background level in four days, this decrease seems to be slower than the increase at the start of induction.

The level of fluorescence in uninduced 518Tet-eGFP cells (34.7 arbitrary units (AU) ±7.4) was found significantly higher than the background fluorescence from the parental 518.A2 cell line (3.2 AU ±0.4). This level of background expression is probably caused by leakiness of the Tet-on promoter.

1.2 *Tet-on tTS clones*

In order to reduce background gene expression in uninduced 518Tet-on clones, new stable transfections were performed, co-transfecting pTet eGFPneo or pTet FMGneo with the silencer construct pTet-tTS. After geneticin selection 36 clones of each transfection were picked. These stably transfected clones were called 518Tet eGFP-tTS and 518Tet FMG-tTS.

In parallel, 518Tet eGFP clone 15.4 was supertransfected with pTet-tTS and pTK-Hyg and selected for hygromycin B-resistance. Four clones were picked and called 518Tet eGFP 15.4 tTS.

1.2.1 *518Tet eGFP tTS*

518Tet eGFP tTS clones were induced with 2µg/ml dox and eGFP expression was determined by flowcytometry after 5-12 days of induction. Three clones, 1, 29 and 34, were found to be partially inducible. Limiting dilution was performed on clone 1, 8 subclones were obtained and tested for inducibility and background level of eGFP expression. Results are displayed in Table 2. The background fluorescence determined in the uninduced cells was found lower than that of 518Tet eGFP without the silencer, but still higher than that of the parental cell line 518.A2. The kinetics of dox induction, started at time 0, were monitored on subclone 1.4 (figure 7A), which was re-stimulated three times a week. The induction of gene expression seems to be delayed: only 12 days after starting dox stimulation the percentage of induced cells was significantly increased. Stimulation with dox was dose-responsive, but 5µg/ml dox stimulated less well than 2µg/ml dox.

Here again, the level of gene expression is independent from the concentration of dox used (figure 7B). Expression was in either an OFF or ON state, reached maximum after 2 days of dox-stimulation. The drop in eGFP expression observed in this experiment between days 3 and 4 is probably an artefact. In parallel, subclone 1.4 was further purified (by another round of limiting dilution) to 85% of inducible cells (subclone 1.4.3, shown in table 2).

1.2.2 *518Tet eGFP 15.4 tTS*

Four clones of the supertransfection of 518Tet eGFP 15.4 with pTet-tTS were picked. One of these clones showed a 42% decrease of the background fluorescence after the addition of the silencer, while the level of eGFP expression in the induced population was 16% decreased.

1.2.3 *518Tet FMG-tTS*

518Tet FMG-tTS clones were frozen and stored at N₂ for later analysis

Example 2: Establishing dendritic cell - tumour cell hybrids after transduction with recombinant AAV

A second approach to achieve GaLV-FMG expression by human cells is transduction with a recombinant virus carrying the GaLV-FMG gene. As viral vector AAV serotypes 2 or 5 were used.

2.1 AAV serotype

The transduction efficiency of AAV serotypes 1, 2 and 5 in the human melanoma cell line 518.A2 was tested using vectors carrying the eGFP reporter gene. Cells were infected at MOI 300 and three-fold dilutions thereof. Two days post-infection, the percentage of transduced cells was determined by flowcytometry (figure 8)

AAV2 showed by far to be the best serotype to transduce the human melanoma cell line, it was about ten-fold and hundredfold more efficient than serotype 1 and 5 respectively. Therefore it was decided to use AAV serotype 2 vectors as vehicle for GaLV-FMG gene transfer into melanoma cells. On the other hand, CHO cells (see below) were best transduced with AAV5 vectors (results not shown).

The transduction efficiency of the AAV serotype 2 AAV2-eGFP vector was tested on other human melanoma cell lines. The percentage of transduced cells were monitored by flowcytometry after 48 hours of infection at different MOI (Figure 9).

2.2 Kinetics of transgene expression after AAV infection

In order to test at what timepoint the transgene is best expressed after AAV infection, two human melanoma cell lines, 518.A2 and MelZ2 were infected by AAV2-eGFP at MOI 5. The percentage of cells expressing eGFP was monitored every 24h by flowcytometry. The percentage of transduced cells was highest two days after infection (figure 10), in 518A2 cells this percentage was almost reached 24h post-infection, while the transduction of MelZ2 cells was found to be slower.

2.3 Construction and production of AAV-GaLV-FMG

In the vector plasmid pTReGFP, the eGFP gene was replaced by GaLV-FMG and the vector size was adjusted to allow packaging. Two variants were made, pTRGaLV-B/E and pTRGaLV-S/E with a size of 93% and 98% of that of wild-type AAV respectively. Both plasmids carried a functional GaLV-FMG gene: after transient transfection into 293T cells, cell fusion and the formation of multinucleated syncytia were observed by light microscopy. Small scale production of AAV-GaLV-FMG using either of the two constructs resulted in comparable virus concentrations as AAV-eGFP production, as determined by *in situ* hybridisation (data not shown).

The purified AAV-GaLV-FMG stocks were tested for their functionality by infecting human melanoma cell lines 518.A2 and MelZ2. Cell fusion was observed in both cell lines

48h post-infection by microscopy. Prolonged fusion resulted in the formation of large syncytia. In figure 11 fusion of MelZ2 after AAV-GaLV-FMG infection at MOI 1.3 is shown.

Larger stocks of AAV2-GaLV-FMG and AAV5-GaLV-FMG were later produced by the Vector Core of the University Hospital in Nantes (France). Titres of these stocks were much lower than those usually obtained by the production centre. Syncytia formation might interfere with vector production depending on the conditions used.

2.4 DC-TC fusion after AAV-GaLV-FMG transduction

In order to fuse tumour cells (TC) with dendritic cells (DC), tumour cells were infected with AAV-GaLV-FMG at MOI 10. For detection, TC membranes had been pre-stained green with PKH. Next day, infected tumour cells were overlaid with a 1:1 ratio of DC. As negative control, uninfected TC were similarly co-cultured with DC. After overnight incubation cells were examined by microscope. No syncytia were detected, but these may have been still too small to discriminate from unfused cells. Cells were detached and stained for the DC marker HLA-DR coupled with the red fluorochrome PE and analysed by flowcytometry for the presence of double-stained (hybrid) cells. Repeated experiments always generated a background of about 7% of double-stained cells in the co-culture controls (figure 12B), while this percentage was similar or only slightly higher in the fusion samples (11%) (figure 12A).

It was thought that the background of double-stained cells could be aggregates or a result of the phagocytosis of TCs or their debris by DCs.

2.5 Conclusion

The transduction of human tumour cells with AAV-GaLV-FMG had resulted in the formation of multinucleated cells. However, formation of hybrids between these cells and DC could not be determined by flowcytometry. Therefore, methods for unambiguous hybrid detection were firstly sought.

Example 3: Towards unambiguous hybrid determination

It was decided to perform cell fusion in a simplified model, optimize the fusion conditions and detection methods before performing fusion between DCs and tumour cells.

3.1 Determination (quantification) of double-positive cells in co-cultures

As described above, in early attempts at fusing human 518.A2 melanoma cells with DC, a high background of double-stained cells were detected in co-culture samples by flowcytometry, in the absence of FMG expression (figure 13A).

Arguing that this high background could be due to phagocytosis of cell debris by DCs, we set up the fusion protocol using two types of tumour cells, 518.A2 or 518-EGFR

on one hand and 518-GFP on the other hand. 518.A2 and 518-EGFR cells were identified by membrane staining with PKH before fusion or anti-EGFR antibodies after fusion, respectively. The other fusion partner, 518Tet eGFP, was detected through the expression of cytoplasmic green fluorescence. Co-culture of these cells for times ranging from 3 to 48 hours again led to a high background of double-stained cells which was very variable from one experiment to the other, ranging from 1% to 9% (average 5%) (figure 13 B). Staining the FMG-expressing cells with PKH before co-culture (518.A2) or anti-EGFR antibody after co-culture (518-EGFR) did not significantly modify the number of double-stained cells (figure 13 A, B). The formation of double-stained false positive cells is apparently a process that requires time since extra controls in which two cell types were mixed and stained (hatched bar in figure 13 B) or stained and mixed (figure 13 vertically striped bar) just before flowcytometry show much lower backgrounds of double-stained cells.

3.2 TC-TC fusion after AAV-GaLV transduction

In order to detect fusion among two different cell lines, the fully induced 518Tet eGFP cell line was either uninfected, infected with AAV-GaLV-FMG or an irrelevant control AAV-GDNF at MOI 10. Next day these cells were overlaid with red PKH pre-stained 518.A2 cells in a ratio of 1:1 or 1:3. Double-stained cells were quantified by flowcytometry after overnight, 2 and 3 days incubation. The percentage of double stained cells was always found similar in both fusion samples and controls, and it decreased over the 3 day period of the experiment. This decrease is due to the dilution of the PKH stain in 518.A2 cells, cell-staining became less intense over time and the percentage of positively stained cells decreased from 65% to 12% (figure 14).

Although hybrid formation between red stained 518.A2 cells and AAV-GaLV-FMG infected 518Tet-eGFP could not be detected by flowcytometry, green syncytia were detected by fluorescence microscopy in the AAV-GALV infected samples after two days of incubation. Some of these syncytia also showed red membrane stain.

To allow more time for hybrid formation without losing PKH staining, the experimental settings were changed to infection of 518.A2 cells with AAV-GaLV-FMG at MOI 10 and to overlay them next day with fully induced 518Tet eGFP cells. The formation of green syncytia would indicate fusion of the two cell types. Although AAV-eGFP infection in parallel showed 48% of transduced cells, no formation of green syncytia was observed by fluorescent microscopy.

In another set of experiments, 518-EGFR cells, a variant of 518.A2 over-expressing the epidermal growth factor receptor (figure 15B), were infected with AAV2-GaLV-FMG or transfected with pCR3.1GaLV and fused with HeLa-eGFP cells (figure 15A)

overnight. Cells were stained with Diff-Quik and observed under the light microscope (figure 18 bottom panel) or labelled with PE-coupled anti-EGFR antibodies and analysed by flow cytometry for the presence of double stained (PE + eGFP) cells (figure 16, figure 18 top panel). Following GaLV-FMG transduction of 518-EGFR cells, syncytia were
5 formed as judged by microscopy but the percentage of double labelled cells did not increase compared to the co-culture control.

3.3 Optimization of cell staining

PKH membrane staining was not optimal for the determination of hybrid formation between DC and TC. Tumour cells were to be pre-stained and any tumour debris that
10 could be taken up by DC phagocytosis will also be PKH stained. Moreover, prolonged cell culture results in dilution of this stain and decrease of the intensity (figure 14), rendering some tumour cells undetectable.

Different methods were chosen to mark cells: firstly intracellular eGFP expression: Beside 518Tet eGFP cells that have to be constantly induced with dox, stably transfected
15 HeLa-eGFP cells, constitutively expressing eGFP under the regulation of a CMV promoter, were established (figure 15A). A second method for identifying cells (staining) was the use of specific antibodies for cellular markers. The 518.A2 cell line was stably transfected to over-express the epithelial growth factor receptor (EGFR), resulting in 518-EGFR cells that could be stained with the anti-EGFR antibody (figure 15B). DCs could be
20 stained with antibodies for HLA-DR expression or other DC specific markers.

To allow identification of cells for which no specific marker is identified, such as primary cells, we tested intracellular staining with CFSE (green) or SNARF (red). These compounds provide stable staining and they do not leak out of cells once complexed to cellular proteins (figure 17). Cells were stained with CFSE or SNARF and analysed at
25 different times by FACS. Mixing non stained cells after 6 hours (SNARF: figure 17b) or 16 hours (CFSE: figure 17c) did not lead to transfer of the dyes.

3.4 Fusion after transient transfection with GaLV-FMG

In comparison to infection with AAV-GaLV-FMG vectors, a (slightly) higher degree of fusion was observed in human cells after transient transfection by the plasmid coding
30 for GaLV-FMG. While fusion was visible by microscope after overnight transfection, only two days post-infection with AAV-GaLV-FMG syncytia had become large enough for detection. For this reason it was decided to transiently transfect tumour cells with the pCR3.1 GaLV plasmid for experiments to set up clear hybrid detection methods. After 4h transfection 518-EGFR cells were detached and mixed at a ratio of 1:3 with 518Tet eGFP
35 cells. Next day cells were examined by fluorescent microscopy. Syncytia were only found in cells that had been transfected with pCR3.1GaLV. In the fusion samples in which these

cells had been mixed with 518Tet eGFP cells, some of the syncytia were also green fluorescent (data not shown). Cells were then stained red for EGFR and analysed by flowcytometry.

As can be seen in figure 16 A and B, the two different cell types could be easily distinguished after staining, however not all 518Tet eGFP cells were induced. Surprisingly the percentage of double-stained in the fusion sample was repetitively found similar to that of the co-culture control (figure 16 C and D). Similar results were found when transfected (pCR3.1GaLV) or infected (AAV2-GaLV-FMG) 518-EGFR cells were fused with HeLa-eGFP cells (figure 18).

Although cell fusion could be shown by light microscopy and hybrids may be detected by fluorescent microscopy, hybrid formation remained undetected by flowcytometry. The hybrids may be masked by the high background of double-stained cells, which are probably cell aggregates since phagocytosis is excluded in this model because no Dcs are involved, or may be too large for the acquisition by the FACScan. The syncytia observed by microscopy were rather large and may therefore be easily detectable, even when they represent only a low percentage of the total. The percentage of hybrids formed in this model may be low since the 518-EGFR cell line transfected by pCR3.1GaLV may as well fuse with itself as with the 518Tet eGFP or HeLa-eGFP cell lines, since they are both human cell lines expressing the PIT-1 receptor which is used for fusion. Fusion among the same cell line may even be more likely since cell proliferation continues and daughter cells are always in the direct vicinity. A considerable part of the expressed FMG may thus be lost due to "self-fusion".

3.5 Fusion with CHO cells

In order to force cell fusion between two different cell types generating hybrids, a fusion model was designed using Chinese Hamster Ovarian (CHO) cells that are not susceptible to GaLV-FMG fusion.

3.5.1 Fusion using transiently transfected CHO cells

In a first approach CHO cells were transiently transfected with pCR3.1 GaLV and overlaid with HeLa-eGFP (figure 19) or 518-EGFR (figure 23 I) cells. Syncytia, which theoretically can only consist of CHO and HeLa-eGFP or 518-EGFR cells, were observed by microscopy after overnight incubation (figure 23 I) but could not be revealed by flowcytometry after staining the CHO cells with an anti-CHO antibody followed by a goat anti-mouse Ig-PE antibody (figure 19 and 23 I).

In this fusion model no FMG is lost by "self-fusion" of the CHO cells. It was therefore likely that the percentage of cells that was actually expressing FMG was not high enough to form hybrids above detection level (see below).

3.5.2 Establishment of a stable CHO-FMG cell line

In parallel with the establishment of a stably transfected CHO-FMG cell line, anti-FMG antibodies were generated for their identification and purification. A high percentage, if not all cells of this cell line should express FMG and should be able to fuse with human
5 tumour cells.

CHO cells were transfected with pCR3.1GaLV and selected for neomycin resistance. Clones were picked and cross-examined for FMG expression by staining (labelling) with nine putative anti-FMG mouse antisera (see section III Generation of anti-FMG antibodies), followed by staining with PE-coupled goat anti-mouse Ig antibody. By
10 flowcytometry most clones were found negative, but two clones showed low percentages of cells that may be positive for FMG expression. As can be seen in figure 20, compared to untransfected CHO cells, 1.5% and 5.4% of the population from CHO-FMG clones 8 and 10 respectively are slightly more positive for staining with the putative anti-FMG serum from mouse 167E.

15 In order to enrich the population of positive cells MACS selection was performed on clones 8 and 10. Cells were stained with 1.5 μ l and 4.0 μ l of the putative anti-FMG serum from mouse 167E in 100 μ l of sterile FS buffer for 1h on ice. After washing, cells were resuspended in 80 μ l of buffer and mixed with 20 μ l of goat anti-mouse-Ig microbeads (Miltenyi Biotec). After another 30min incubation on ice, cells were washed, resuspended
20 in 0.5ml of buffer and applied to MiniMACS columns (Miltenyi Biotec). Columns were washed and cells were eluted according the manufacturer's directions. These cells were called CHO-FMG 8M and 10M.

One week later, these cells were stained with antiserum 167E for analysis by flowcytometry. The percentage of positive cells in CHO-FMG 8M was only increased to
25 $\pm$ 3%, the enrichment of FMG positive cells had been unsuccessful. MACS selection of CHO-FMG clone 10 using 1.5 μ l and 4.0 μ l antiserum resulted in 79% and 61% positive cells respectively (figure 21).

CHO-FMG 10M was further purified by limiting dilution to clone CHO-FMG 10M6 in which about 95% of the cells expressed FMG as determined with the finally obtained anti-FMG antibodies (see example 8 Generation of anti-FMG antibodies) (figure 22).
30

3.5.3 Hybrids generated with the stably transfected CHO-FMG cells

In order to establish hybrids between CHO cells and human cells, CHO-FMG cells were mixed with HeLa-eGFP or 518-EGFR cells and incubated overnight. Next day multinucleated cells could be clearly distinguished in the fusion sample containing CHO-FMG and HeLa-eGFP or 518-EGFR cells by light microscopy (figure 23 IIA arrows and ID
35 lower panel, respectively) while the co-culture containing CHO and HeLa-eGFP or 518-

EGFR cells only showed cells with a single nucleus (figure 23 IIB and IA lower panel, respectively).

Cells were stained with the anti-CHO antibody followed by goat anti-mouse-Ig-PE secondary antibody and FITC-coupled anti-EGFR for analysis by flowcytometry (figure 23, upper panel).

Figure 24 I shows the results of FACS analysis for CHO-FMG fusion with HeLa-eGFP cells. Both CHO and HeLa-eGFP cells are well distinguished by their antibody-staining and intracellular eGFP expression respectively. When untransfected CHO cells and HeLa-eGFP cells were co-cultured in a ratio of 1:1 overnight, 3.5% of double-stained cells were found, which are probably cell aggregates. When stably transfected CHO-FMG cells were mixed with HeLa-eGFP cells for fusion, beside the same double-stained population as found in the co-culture sample, an entirely new population of double-stained cells was found, with decreased eGFP expression. In this sample the percentage of double-stained cells was 24.7%. Taking to account that 3.5% of these cells are aggregates, about 21.2% of the double-stained cells should be hybrid cells. The decrease in eGFP expression in hybrids is probably due to quenching.

The fusion between CHO and HeLa-eGFP was repeated on microscope coverslips and adherent cells were similarly stained for determination by confocal microscopy. Double stained cells detected by flowcytometry could be two different cell types sticking to each other (aggregate) as well as a true hybrid that possesses markers from both cell types. Confocal microscopy is able to discriminate cell aggregates from hybrid cells (Figure 24 II).

Example 4: Optimization of best fusion conditions

The above described experiments had unambiguously shown that hybrids could be generated with the CHO-FMG fusogenic cell line, such as hybrids with the HeLa-eGFP or 518-EGFR cell lines. This CHO-FMG model was now used to study different fusion conditions to optimize the efficiency of hybrid formation.

4.1 Adherence

After overnight incubation of the CHO(-FMG) cells with human tumour cells or DCs, some cells were still non-adherent. Analysis of adherent cells and non-adherent cells separately showed that most double-stained cells were found in the adherent fraction. When DCs are involved, they form the major part of the non-adherent cells (data not shown). In early experiments, culture dishes were rinsed after incubation and only the adherent fraction of cells were examined. Later all cells were recovered.

4.2 Incubation time

Cells were detached for counting and mixing for fusion experiments. Cells were initially incubated overnight for fusion because some time is required to adhere. However, it was observed that cells start to adhere within a couple of hours. Therefore shorter incubation periods were tested. When CHO and HeLa-eGFP cells were mixed and incubated for only 2.5h, less double-stained cells (<1%) were detected in the co-culture sample. The percentage of double-stained cells in fusion of CHO-FMG and HeLa-eGFP was found higher than the co-culture control, but did not exceed 4%.

In order to enhance cell-cell contact during the short incubation, cells were also mixed, spun down in a tube and incubated as a pellet. Overnight incubation as a pellet resulted in about 20% of dead cells as determined by trypan blue exclusion. Shorter incubation periods yielded efficient fusion for some cell types (Table 3)

4.3 Ratio of fusion partners

CHO(FMG) and HeLa-eGFP cells were mixed in different ratios. Preliminary experiments are summarized herein:

#CHO(FMG) : #HeLa-eGFP

		FACS
	- exp VI	1:1 & 3:1 & 10:1 & 1:3
	- exp VII	3:1 & 6:1 & 9:1
20	- exp VIII	1:1 & 3:1
	- exp IX	3:1 & 1:1 & 1:3
		1:1 best, other same

consequences of ratios as determined by microscopy in:

#nuclei/syncytia independent from cell ratio → probably time-dependent

#syncytia/microscope field, 1:3>1:1>3:1 → different from FACS results

Later more detailed experiments are summarized in figure 25. The highest hybrid yield is obtained when equal numbers of both cell types are mixed. Results are expressed as %hybrids among total cells (grey bars, 25A) or proportion of tumour cells engaged in hybrids (grey bars, 25B).

4.4 Cell density

adherent cells

2×10^5 , 3×10^5 or 6×10^5 cells were seeded in 24well dishes. Optimal cell density has to be defined for each combination of cells.

4.5 Percentage of FMG expression

Early fusion experiments using transiently transfected CHO cells with pCR3.1GaLV had always been unsuccessful. Since unambiguous hybrid formation could be determined and quantified in the developed CHO-FMG fusion model (such as in fusion

with HeLa-eGFP or 518-EGFR cells), it was tested what proportion (percentage) of the CHO expressing FMG is actually required to generate a detectable hybrid yield (percentage of hybrids), above background which is mainly given by aggregates.

A fusion experiment was performed using a ratio of 1:1 of CHO-FMG cells and HeLa-eGFP cells. In order to mimic inefficient transfection, the CHO-FMG cells were diluted with CHO cells to obtain different percentages of CHO cells that actually express FMG. After overnight incubation cells were stained with the anti-CHO antibody followed by goat anti-mouse Ig-PE secondary antibody for flowcytometry. The percentage of double-stained cells was determined and plotted in figure 26.

Co-culture of CHO cells with HeLa cells resulted in 3% of double-stained cells. This percentage increased with the percentage of FMG expressing cells. A maximum of 15-20% of double-stained was obtained when using CHO-FMG cells alone.

This experiment shows that it is required that about 40% and preferably 95% of the CHO cells express FMG in order to increase the percentage of double-stained cells sufficiently (i.e., generate sufficient hybrid yields) to be able distinguish hybrid formation from the background of aggregates.

4.6 *Level of FMG expression*

We also tested the level of FMG expression after transduction of cells with GaLV-FMG. CHO cells were transfected with pCR3.1GaLV or infected with AAV5-GaLV (produced by the Vector Core) at an MOI of 10 infectious particles per cell. Transduction efficiencies were variable from one experiment to the other (table 4). Apart from the fact that less than 40% of cells expressed FMG after transduction, the level of expression per cell was lower than that observed for the stable cell line, CHO-FMG (figure 27). This could explain the inefficient hybrid formation in fusion experiments performed in parallel (figures 18 and 23).

4.7 *Cell cycle arrest*

In our fusion experiments hybrid yields (the percentage of double-stained cells) did not exceed in earlier experiments 15-20% and in later experiments 20-30% of total cells. It is unknown why this percentage of double-stained cells cannot be increased. It was hypothesized that the cell cycle may be involved in the process of fusion. Therefore cell cycle arrest was induced in CHO and CHO-FMG cells by mitomycin C. Cell cycle blockage at S-phase was confirmed by flowcytometry (figure 28). (Abbas T et al JBC 2002 277:40513-19).

Fusion experiments with HeLa-eGFP cells were performed. The fusion efficiency of cell cycle arrested CHO-FMG cells with HeLa-eGFP cells was compared with that of

untreated cells. Cell cycle arrest did not significantly affect nor increase the fusion efficiency of CHO-FMG cells as can be seen in figure 29.

4.8 *Cell cycle synchronization of the cell cycle*

Two methods, serum starvation and aphidicolin treatment, were used to
5 synchronize CHO and CHO-FMG cells. Serum starvation by growing cells for 20h in culture medium containing 0.5% FCS should block cells in G₀ phase. The use of normal culture medium in the fusion experiment should release this blockage and result in synchronized cells in G₀ phase. Release from 20h of aphidicolin treatment should result in cells synchronized in G₁/S phase.

10 The DNA content of the cell population was stained with propidium iodide (PI) in order to monitor the cell cycle by flowcytometry but no significant difference in cell cycle of treated cells could be discriminated from untreated cells. Influences of the cell cycle on fusion efficiency could not be assessed by cell synchronization.

4.9 *Addition of polybrene*

15 Polybrene is a small, positively charged molecule that binds to cell surfaces and neutralizes surface charge. It is used to enhance retroviral infection, because it allows viral glycoproteins to bind more efficiently to their receptors. To test whether polybrene can improve the FMG induced fusion efficiency, fusion between CHO-FMG cells and HeLa-eGFP cells was performed in the presence of polybrene in the culture medium. No
20 significant increase in fusion efficiency was observed as can be seen in figure 30.

Example 5 *Hybrid formation between CHO and cell lines other than HeLa-eGFP*

Hybrid formation by CHO-FMG fusion was tested with other cells than HeLa-eGFP. CHO-FMG was fused to the human dox-induced 518Tet-eGFP cell line. Comparable
25 results were obtained with the dox-induced 518Tet-eGFP cell lines in the CHO-FMG fusion with HeLa-eGFP. A new population of cells that are positive for both α CHO staining and eGFP expression was generated (figure 31) when CHO-FMG cells were used. This population of cells was not found in the co-culture controls (see section 3.5.3).

CHO-FMG cells have also been fused with the non-adherent human lymphoma cell
30 line U937. Determination of cell fusion has only been determined by light microscopy since specific markers were not available for these cells. The formation of multinucleated syncytia was only observed after incubation with CHO-FMG cells and never with the parental CHO cells.

CHO-FMG cells fuse very efficiently with the rat glioblastoma 9L. These cells will
35 provide a model for testing the *in vivo* efficiency of tri-parental hybrids.

Example 6: CHO-FMG hybrid formation with human dendritic cells

CHO-FMG fusion has been shown in human cell lines. Fusion was then performed using human immature DCs (iDC) generated from PBMC. Hybrid formation was determined by staining iDCs red for HLA-DR expression and CHO cells green with an α CHO antibody. Double-stained cells were determined by flowcytometry and microscopy (Figure 32).

In contrast to hybrids formed by fusion of CHO-FMG cells with eGFP expressing cells which were differently located from the unfused cells in the dotplots, due to the decreased level of eGFP expression, the hybrids formed by CHO-FMG fusion with DC are only distinguished as an increase in the percentage of double-stained cells, as compared to the co-culture control.

In order to confirm true hybrid formation, CHO-FMG cells and DC were plated on microscopic coverslips and stained with the same specific cell markers for confocal microscopy. Indeed, as can be seen in figure 32 hybrids can be found whose membranes are positively stained for both HLA-DR and the CHO determinant.

6.1 Hybrid formation using different DC ratios

CHO-FMG cells were incubated with different ratios of iDCs to determine the best fusion condition. As control, the CHO cells were co-cultured similarly with the iDCs to determine the background of double-stained cells by cell aggregates. Results are shown in figure 33.

As can be seen in figure 33, higher percentages of double-stained cells were obtained when more DCs than CHO cells were used. The more the ratio of DCs was increased, the higher the percentage of double-stained cells was found in the fusion sample, up to a ratio of 6 DCs to 1 CHO-FMG cell. The percentage of double-stained cells however increased similarly in the co-culture controls. In the flowcytometry analysis it was found that when more DCs were used, the more the DC population shifted from the upper left quadrant to the upper right quadrant in the dotplot. The best efficiency of hybrid formation was found using CHO:DC in a ratio of 1:3 and 1:6.

6.2 Hybrid formation using different DC types

DCs differentiated from CD14⁺ monocytes by GM-CSF and IL4 stimulation were used for the fusion experiments. First experiments were performed with immature DCs. The phenotype of the generated DCs was monitored by flowcytometry to confirm correct differentiation and to exclude T-cell contamination. The GM-CSF/IL4 generated DCs were HLA-DR⁺, CD1a⁺, CD83^{low} and CD14⁻ and were found negative for CD3 expression.

One experiment performed comparing the fusion efficiency of immature DCs generated by GM-CSF/IL4 stimulation and DCs generated by IFN β /IL3 stimulation, which were HLA-DR⁺, CD1a^{low}, CD83^{low}, and CD14⁺. Results are shown in figure 34.

In this experiment it seems that hybrid formation with DCs generated by IFN β /IL3 stimulation is less efficient than with DCs generated with GM-CSF/IL4. The analysis of DC marker expression reveals that the level of HLA-DR expression by IFN β /IL3 DCs is one log higher than that by GM-CSF/IL4 DCs (data not shown), which should facilitate the determination of hybrid formation. Therefore the lower percentage of hybrid formation is probably specific to the DC type.

It was tested whether the grade of maturation could influence the fusion efficiency. DCs were matured by 24h incubation with poly I:C. Maturation was confirmed by an upregulation of HLA-DR, CD83 and CD86 expression. Results are shown in figure 35.

As can be seen in figure 35, hybrid formation is not impaired by DC maturation. Moreover, hybrid formation to CHO-FMG cells seems to be more efficient when mature DCs are used. However, the detection of hybrids by flowcytometry is facilitated by DC maturation since these mature DCs express higher levels of HLA-DR, for which they are stained for the fusion experiment. The upregulation of HLA-DR expression may have positively influenced the outcome of the experiment.

Example 7: The generation of triparental hybrids via CHO-FMG cells

Hybrid formation between CHO-FMG cells and human cells has been unambiguously shown. For the generation of hybrids between human tumour cells and human DC, the transduction of the cells to express FMG remains problematic. First, the transduction (efficiency thereof and transgene expression) has to be sufficiently high (see sections 4.5 and 4.6) in order to be able to monitor true hybrid formation. Secondly, human cells that express FMG will tend to fuse among themselves, which will result in reduced numbers of FMG expressing cells available for fusion with the fusion partner. Therefore, CHO-FMG cells were tested to serve as a fuser-cell line for human tumour cells and human DC, generating triparental hybrids.

In a preliminary experiment HeLa-eGFP cells and DC were mixed in a ratio of 1:1, while different ratios of CHO-FMG or CHO cells were added for fusion or co-culture control respectively. DCs were identified by staining for HLA-DR expression, HeLa-eGFP cells were detected by their intracellular eGFP expression and CHO cells remained unstained. Hybrids were calculated by subtracting the percentage of double-stained cells in the fusion sample by that of the co-culture control. Up to 5.8% of double-stained cells were obtained, indicating that triparental hybrids may be generated (data not shown).

7.1 *The generation of triparental hybrids with human tumour cell and DCs*

CHO-FMG cells were tested to serve as fuser-cell line for the generation of triparental hybrids with the stably transduced human melanoma cell line 518-EGFR and DCs. As co-culture control, CHO cells were used instead of CHO-FMG cells. The two human cell types and CHO cells were co-incubated overnight in a ratio of 1:1:1. For the identification of the fusion products, cells were stained red (FL2) for HLA-DR to determine the DC fusion partner and green (FL1) for EGFR to determine the tumour cell partner. In the first experiments CHO cells remained unstained. The percentage of double-stained cells was assessed by flowcytometry. Figure 36 I shows the three single cell types separately: 518-EGFR cells (A), DC (B) and CHO-FMG cells (C). Co-culture of the two human cell types with the parental CHO cells result in 1.8% of double-stained cells, while incubation of the human cells with CHO-FMG cells leads to 12.4% of double-stained cells in the fusion sample. These results suggest that 10.6% of total cells are hybrid cells. Observation of Diff-Quik-stained cells by microscopy reveals extensive fusion in the presence of CHO-FMG cells (figure 36 II). It is likely that some of the larger hybrids are not detected by FACS analysis thus underestimating the actual efficiency of hybrid formation.

In preliminary experiments, tri-parental hybrids have also been stained for the detection of CHO markers. Hybrids represent 5% of total cells and 20% of tumour cells were involved in the formation of these hybrids (figure 43 panels B). 93% of anti-HLA-DR:anti-EGFR double labelled cells displayed the CHO marker in the fusion sample, against 39% in the co-culture control.

7.2 *Evaluation of triparental hybrid formation by confocal microscopy*

For analysis by confocal microscopy, cells were incubated overnight on microscope coverslips and stained with the same antibodies as for flowcytometry: DCs were stained red, 518-EGFR cells were stained green and CHO cells were unstained. The overlay of the two colour-channels reveals the presence of hybrid cells that are double-stained at the cell membrane (figure 37d). These cells were only observed in the fusion sample and not in the co-culture control (figure 37).

In following experiments, samples that were also stained for CHO cells with the biotinylated α CHO antibody followed by a streptavidin-PE-Cy5 conjugate were prepared for confocal microscopy. At the acquisition this fluorochrome was read in the blue channel. In co-culture samples, the three cell types could be easily identified by their specific staining. In this experiment, no triple-stained cells could be detected in the fusion samples, however double-stained cells in each combination between the three cell types were found. This may be due to a technical problem. (figure 38).

7.3 Optimization of triparental cell hybrid generation

First experiments for the generation of triparental hybrids were performed using the two human cell lines and the CHO-FMG cell line in a ratio of 1:1:1. In order to optimize the efficiency of triparental hybrid formation, the previous experiment was repeated with variations of the CHO-FMG ratio from 0.1 to 3 CHO-FMG cells to each 518-EGFR cell and DC. Co-culture controls using CHO cells in the same ratios were performed in parallel to determine the background percentage of double-stained cells. The percentage of generated hybrids was determined by the subtraction of the percentage found in the co-culture controls from the percentage found in the fusion sample. The proportion of tumour cells involved in hybrids was similarly determined as follows: double-stained cells (d-sC) / d-sC+TC. Results are shown in figure 39.

Co-incubation of 518-EGFR cells with DC give rise to a background of about 2-3% of double-stained cells. The more CHO cells are added, the more this background decreases, likely due to the fact that 518-EGFR and DC then represent a smaller fraction of the total number of cells. The percentage of double-stained cells in the fusion sample however increases with the ratio of CHO-FMG cells added, in this experiment up to the ratio of 1:1:1. In this experiment, the percentage of double-stained cells did not substantially increase at CHO-FMG ratios higher than 1. Best net percentage of hybrids are formed when equal numbers of 518-EGFR, DC and CHO-FMG cells are used (figure 39A). However, the proportion of tumour cells contained in hybrids is optimal for slightly higher ratios of CHO-FMG (figure 39 B)

The proportion of tumour cells involved in hybrid formation is equivalent for direct fusion (CHO-FMG:TC) and fusion via the fusogenic cell line (figures 25 and 39) showing the efficiency of the method.

7.4 Generation of human tumour cell - DC hybrids via CHO-FMG membranes

Cell fusions described here are induced by the expression of the viral FMG expressed by the CHO-FMG cells. Suspensions of membranes from highly FMG expressing cells may therefore be sufficient to induce cell fusion between two human cell types. Cell-free CHO-FMG and CHO membrane suspensions, prepared according to a protocol for binding assays, were obtained from Dr E. Burgeon (Euroscreen, Gosselies). The equivalent of number of cells used for preparation was calculated to express the quantity of cell membranes.

518-EGFR cells and DCs were mixed in equal numbers in a 24-wells dish, 10^5 cells of each. CHO or CHO-FMG membranes were added in a equivalent of ratio 0.1 to 3 and incubated overnight. Next day, cells were stained for EGFR and HLA-DR and the

percentage of double-stained cells was quantified by flowcytometry. Results are shown in figure 40.

No increase of the percentage of double-stained cells over the background percentage as determined in the CHO membranes control was found when using CHO-FMG membranes.

In order to rule out any fusion inhibitory activities by the buffer in which the membranes were resuspended, fusion experiments between CHO-FMG cells and HeLa-eGFP cells were performed with the addition of this buffer. No fusion inhibition was detected (data not shown).

10 Example 8: The generation of anti-GaLV FMG antibodies

8.1 *Genetic immunization and antiserum test*

BALB/C mice that had been genetically immunized with a plasmid carrying the GaLV-FMG gene under the regulation of a strong CMV promoter were bled to obtain antiserum. Eight antisera were tested for specific recognition of FMG on CHO cells transiently transfected with the pCR3.1GaLV plasmid. In later tests, stable CHO transfectants selected for G418 resistance were used.

Per sample, 2×10^5 cells were stained (labelled) with 5 μ l of antiserum for flowcytometry. As secondary antibody cells were stained with a goat α mouse Ig secondary antibody, coupled to either Alexa488 or PE. The percentage of positively stained cells were compared between the parental CHO cell line and the FMG-transfected CHO cells.

Most antisera were found negative for both FMG-transfected and untransfected CHO cells. Antisera from mice 167E and 168B were found to be slightly positive for the staining of FMG-transfected CHO cells over untransfected CHO cells, as indicated by the arrows in figure 41.

In parallel, a stably transfected CHO-FMG cell line has been established (see section 3.5.2). Testing of the CHO-FMG clones confirmed the anti-FMG-specificity of antisera 167E and 168B. Still only a small part of the CHO-FMG cells were clearly positively stained compared to the parental CHO cells.

30 8.2 *Production of anti-FMG monoclonal antibodies*

The proliferation of FMG-specific antibody producing cells was boosted in mice 167E and 168B by intraperitoneal injections of CHO FMG 10M cells. Ten days later these mice were sacrificed, antiserum was again obtained and the splenocytes of these mice were fused to generate antibody-producing hybridomas.

35 Supernatants from immortalized hybridomas were obtained and tested for specific binding to the CHO 10M6 cells. At this early step no supernatant capable of inhibiting the

fusion of the CHO 10M6 cells with the human U937 cell line could be found. Monoclonal antibodies 168B 1F5, 168B 6B3 and 168B 6B5 were produced and could be used throughout the experiments described.

5 Example 9: Fusion blocking assay

In order to screen a great number of α FMG antibody-producing hybridoma clones for their fusion blocking properties, two assays were designed.

In the first fusion assay, CHO-FMG cells were pre-blocked by the addition of hybridoma supernatant to the culture medium. HeLa-eGFP cells were subsequently
10 added at a ratio of 1:1 and fusion was allowed to proceed overnight. Next day the CHO-FMG cells were stained red with an α CHO antibody followed by goat-anti mouse Ig-PE antibody.

The percentage of cells that were both green (eGFP expression) and red (stained with α CHO) was determined by flowcytometry. The % of fusion inhibition was calculated
15 by the following formula.

$$20 \quad 1 \text{ minus } \left(\frac{(\% \text{ double positive cells})_{\text{blocked}}}{(\% \text{ double positive cells})_{\text{unblocked}}} \right) \times 100\%$$

In order to allow automated high-throughput screening of hybridoma supernatants for fusion blocking properties, a second assay was designed using non-adherent U937 cells. CHO-FMG cells were plated in 96 well dishes. Next day CHO-FMG cells were pre-
25 blocked with hybridoma supernatant, then U937 cells were added and spun down on the dish for 5min at 2000rpm at room temperature to speed up fusion. Four hours after centrifugation, non-adherent, unfused U937 cells were recovered and counted to determine the amount of cells that had not undergone fusion.

When using U937-luc cells that constitutively (stably) express luciferase,
30 automated high-throughput screening of hybridoma supernatants for fusion blocking properties could be performed.

References

35 Its is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. All of

the references cited in the description are incorporated by reference. Other aspects, advantages, and modifications are within the scope of the following claims.

Table 2

Clone	Mean fluorescence		% induced
	Uninduced	Induced	
518A2	3.0	n.a.	n.a.
518Tet eGFPtTS 1.1	15	1392	24.1
518Tet eGFPtTS 1.2	n.d	n.d	n.d
518Tet eGFPtTS 1.3	5	410	3.1
518Tet eGFPtTS 1.4	10	1564	18.6
518Tet eGFPtTS 1.4.3	11	1291*	Approx 85*
518Tet eGFPtTS 1.5	7	798	4.9
518Tet eGFPtTS 1.6	n.d	n.d	n.d
518Tet eGFPtTS 1.7	11	1577	8.9
518Tet eGFPtTS 1.8	5	730	1.6
518Tet eGFP 15.4	31	2566*	approx 90*

Table 3

fusion partner	Incubation time	% double-stained cells in co-culture	% double-stained cells in fusion	% hybrids
HeLa-eGFP	2 hours	2.3	4.8	2.5
	Overnight (18h)	3.9	10.7	6.8
9L	0			3.6
	30 min			5.6
	2 hours			13.5
	4 hours			15.1
U937	0			0.7
	30 min			4.5
	2 hours			13.7
	4 hours			18.5

Table 4

5

Cell line	Vector/plasmid	% cells expressing the transgene			
		24 hours		48 hours	
			average		average
518-EGFR	AAV2-GaLV	23	12%	0	3%
		8		0	
		10		0	
		7		12	
CHO	AAV5-GaLV	20	26%	0	36%
		27		47	
		41		28	
		14		69	
518-EGFR	pCR3.1GaLV	12	12%	24	24%
CHO	pCR3.1GaLV	70	44%	24	26%
		17		28	
518-EGFR	AAV2-eGFP	59	63%	70	69%
		56		65	
		73		74	
CHO	AAV5-eGFP	27	27%	26	26%

Claims

1. A stable cell line, wherein the cells of said line stably express a fusogenic glycoprotein and are insensible for said fusogenic glycoprotein such that said cell line is suitable as a
5 fusogenic agent.

2. Cell line according to claim 1, wherein said fusogenic glycoprotein is of viral origin chosen from the group consisting of orthomyxoviruses, rhabdoviruses, retroviruses, paramyxoviruses, and poxviruses.

10

3. Cell line according to claim 1 or 2, wherein the fusogenic glycoprotein is the retroviral GaLV fusogenic glycoprotein GaLV-FMG.

4. Cell line according to any of claims 1 to 3, wherein the receptor for the fusogenic
15 protein is the PIT-1 receptor.

5. Cell line according to any of claims 1 to 4, wherein said cell is a rodent, preferably a hamster cell.

20 6. Cell line according to claim 5, wherein said cell is a Chinese Hamster cell originating from the ovary.

7. Cell line according to claim 6, wherein said cell line is termed GFMG 10M6, as deposited at the BCCM-LMBP culture collection on September 3, 2004, 2004 under
25 registered Accession Number LMBP 6327CB.

8. Cell line according to any of claims 3 to 7, wherein said fusogenic glycoprotein has a C-terminally truncation lacking the 16 AA R-peptide of the wild type protein, and/or, said fusogenic glycoprotein has a modified N terminal extracellular part, so as to modify the
30 selectivity of fusion.

9. Cell line according to any of claims 1 to 8, wherein the cells of said cell line are further modified to (over)express specific functional molecules, chosen from a group consisting of proteins (such as cytokines), chemoattracting molecules, co-stimulatory molecules, antigens, and molecules with adjuvant properties.

35

10. Cell line according to claim 9, wherein said antigen is a tumour antigen or is expressed by patient tumour.

11. Cell line according to claim 9, wherein said antigen is of human, mammalian, insect
5 or/and viral and/or bacterial and/or protozoan and/or fungal origin.

12. Cell line according to any of claims 1 to 8, wherein the cells of said cell line are further modified to express or over express one or more functional molecules chosen from the group comprising transcriptional regulators, such as transcription factors or transcriptional
10 repressors, anti-sense nucleic acids, such as antisense RNA, short interfering nucleic acids, such as siRNA or shRNA, and dominant negative forms of cellular proteins.

13. Cell line according to any of claims 1 to 4, wherein the cells of said cell line are modified to (over)express at least one functional molecule, such as a peptide, polypeptide
15 and/or or protein, derived from a non-human species, such as a non-human mammal or a virus, said molecule being capable of inducing a xenogeneic rejection immune response in humans.

14. A membrane preparation of a cell line according to any of claims 1 to 13.
20

15. A composition comprising a cell line according to any of claims 1 to 13 or a membrane preparation according to claim 14.

16. An *in vitro* use of a cell line according to any of claims 1 to 13, a cell derived from said
25 cell line, a membrane preparation according to claim 14 or a composition according to claim 15, as a fusing agent for the production of multiparent cell hybrids.

17. A triparental cell hybrid having at least three different characteristics:

- a) a first characteristic or specific marker originating from a cell line according to claims 1
30 to 13, a cell derived from said cell line or membrane preparation according to claim 14 or a composition according to claim 15;
- b) a second characteristic or specific marker originating from a second cell different from the first cell and a third cell;
- c) a third characteristic or specific marker originating from the third cell, different from the
35 first and the second cell.

18. A triparental cell hybrid according to claim 17,
a) wherein the first cell is heterologous to the second and the third cell and
b) wherein the second and third cells are allogeneic or autologous cells to each other.
- 5 19. Triparental cell hybrid according to claim 16 or 17, wherein the first characteristic or marker is a CHO characteristic or marker, which is chosen from the group consisting of the membrane antigens defined by relevant antibodies obtained by immunizing mice or other animal against CHO cells.
- 10 20. Triparental cell hybrid carrying at least the CHO marker recognized by antibody CHO 105C 8C7, 257D 6B10, 281E 3D4 or polyclonal antibodies, a tumour cell marker or the added tumour cell marker Fluorescein NHS or CFSE or SNARF and the DC marker HLA-DR or the added marker CFSE or SNARF.
- 15 21. Triparental cell hybrid carrying any combination of characteristics or specific markers, said combination made by choosing at least one entry from each respective row I, II and III of the following table:

I	a characteristic or a marker derived from a human cell line
	a characteristic or a marker derived from a non-human mammal cell line
	a characteristic or a marker derived from a rodent cell line, such a CHO cell line
	a characteristic or a marker derived from a CHO cell line, chosen from the group consisting of CHO markers recognized by antibody CHO 105C 8C7, 257D 6B10, 281E 3D4 or by a polyclonal anti-CHO antibody
	a functional molecule which is capable of inducing a xenogeneic rejection immune response in humans
	a functional molecule chosen from a group consisting of cytokines, chemoattracting molecules, co-stimulatory molecules, antigens, and molecules with adjuvant properties
	a functional molecule chosen from a group consisting of cytokines, interleukins IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL12, IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), stem cell factor (SCF), interferon-alpha (IFN-alpha), interferon-beta (IFN-beta), interferon-gamma (IFN-gamma), a hybrid interferon, erythropoietin (EPO), leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), growth hormone (GH), pre-B-cell leukaemia transcription factor-1 (PRL), tumour necrosis factor (TNF) cytokine, TNF-alpha and TNF-beta, CD40, CD27, FAS receptors and ligands, the fibroblast growth factors (FGF) family, the platelet derived growth factors (PDGF) family, the transforming growth factors (TGF) family, the nerve growth factors (NGF) family, the epidermal growth factor (EGF) family, the insulin related cytokines, IGF-I and II, mast cell growth factor, chemokines, C chemokines, like XCL1 (lymphotactin) and receptor XCR1, CC chemokines, CCL1 (I-309) to CCL28 (CCL1, CCL2, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9, CCL10, CCL11, CCL12, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CCL28), including MIP1alpha (CCL3) and receptors CCR1 to CCR11 (bonzo) (CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CCR11), CXC chemokines, CXCL1 (GRO alpha) to CXCL14 (BRAX) (CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14) and receptors CXCR1 (IL8 receptor) to CXCR6 (CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6), CX3C chemokines such as CX3CL1 (fractalkine) and receptor CX3CR1; chemR23 and its ligand chemerin; antibodies or parts of antibodies such as anti-CD3

II	a tumour antigen
	a tumour antigen chosen from 707 alanine proline, alpha (a)-fetoprotein (AFP), adenocarcinoma antigen recognized by T cells 4 (ART-4), B antigen, b-catenin/m, b-catenin/mutated (BAGE); breakpoint cluster region-Abelson (Bcr-abl), CTL-recognized antigen on melanoma (CAMEL), carcinoembryonic antigen peptide - 1 (CAP-1), caspase-8 (CASP-8), cell-division cycle 27 mutated (CDC27m), cycline-dependent kinase 4 mutated (CDK4/m), carcinoembryonic antigen (CEA), cancer/testis antigen (CT), cyclophilin B (Cyp-B), differentiation antigen melanoma (DAM), elongation factor 2 mutated (ELF2M), Ets variant gene 6/acute myeloid leukemia 1 gene ETS (ETV6-AML1), glycoprotein 250 (G250), G antigen (GAGE), N-acetylglucosaminyltransferase V (GnT-V), glycoprotein 100 kD (Gp100), helicase antigen, human epidermal receptor-2/neurological (HER-2/neu), arginine (R) to isoleucine (I) exchange at residue 170 of the a-helix of the a2-domain in the HLA-A2 gene (HLA-A*0201-R170I), human papilloma virus E7 (HPV-E7), heat shock protein 70 - 2 mutated (HSP70-2M), human signet ring tumor - 2 (HST-2), human telomerase reverse transcriptase (hTERT or hTRT), intestinal carboxyl esterase (ICE), KIAA0205, L antigen (LAGE), low density lipid receptor/GDP-L-fucose: b-D-galactosidase 2-a-Lfucosyltransferase (LDLR/FUT) melanoma antigen (MAGE), melanoma antigen recognized by T cells-1/Melanoma antigen A (MART-1/Melan-A), melanocortin 1 receptor (MC1R), myosin mutated, mucin 1 (MUC1), melanoma ubiquitous mutated 1, 2, 3 (MUM-1, -2, -3), NA cDNA clone of patient M88 (NA88-A), New York - esophageous 1 (NY-ESO-1), protein 15 (P15), protein of 190 kD bcr-abl, promyelocytic leukaemia/retinoic acid receptor a (Pml/RARa), preferentially expressed antigen of melanoma (PRAME), prostate-specific antigen (PSA), prostate-specific membrane antigen (PSM), renal antigen (RAGE), (renal ubiquitous 1 or 2 (RU1 or RU2), sarcoma antigen (SAGE), squamous antigen rejecting tumor 1 or 3 (SART-1 or SART-3), translocation Ets-family leukemia/acute myeloid leukemia 1 (TEL/AML1), triosephosphate isomerase mutated (TPI/m), tyrosinase related protein 1 or gp75 (TRP-1), tyrosinase related protein 2 (TRP-2), TRP-2/intron 2 (TRP-2/INT2), Wilms' tumor gene (WT1)
	a chemically added fluorescent marker such as with CFSE or SNARF
	a genetically added marker such as GFP
III	a characteristic or a marker of an antigen presenting cell
	a characteristic or a marker of a macrophage and/or a dendritic cell and/or a B lymphocyte
	a DC characteristic or marker, chosen from the group consisting of HLA-DR, CD83, CD80, CD86, CHEMR23, CD40, CD1a
	a combination of cell surface markers: CD11a, -b and -c, CD16, CD17, CD63, CD64, CD68 and CD71
	a combination of cell surface markers: CMRF-44, CD83 and CMRF-56
	a combination of cell surface markers: CD19, CD20, CD21, CD22, CD40, CD72 and CD78
	a chemically added fluorescent marker such as with CFSE or SNARF
	a genetically added marker such as GFP

22. A composition comprising a triparental cell hybrid according to any of claims 17 to 21.

5

23. A method to prepare a triparental cell hybrid, comprising the steps of :

- 1) culturing a stable cell line according to any of claims 1 to 13 as fusion agent, expressing a fusogenic glycoprotein;
- 2) culturing a second cell line, the first fusion partner, expressing a receptor for said fusogenic glycoprotein;
- 5 3) culturing a third cell line, the second fusion partner, expressing a receptor for said fusogenic glycoprotein;
- 4) mixing the cells of steps 1 to 3, and
- 5) incubating the mixture of cells obtained in step 4 to allow fusion, obtaining the triparental cell hybrids.

10

24. Method according to claim 23, wherein said fusogenic glycoprotein is GaLV-FMG, and the receptor for said fusogenic protein is PIT-1.

15

25. Method according to claims 23 or 24, wherein the second and/or third cell line is/are of mammalian origin.

26. Method according to any of claims 23 to 25, wherein the second and/or the third cell line is/are primary cell line(s).

20

27. Method according to any of claims 23 to 26, wherein the ratio used during the mixing of the cells in step 4/ between the first fusion partner, the second fusion partner and the fusing agent is 1:1:0.1; 1:1:2.3; 1:1:1.6; 1:1:1; 1:1:2; 1:1:3.

25

28. Method according to any of claims 23 to 27, wherein the cells of step 1) are irradiated prior to steps 4) and 5).

29. A triparental cell hybrid produced or obtainable according to a method of any of claims 16, 23 to 28, said triparental hybrid may optionally be irradiated.

30

30. A composition comprising the triparental cell hybrid of claim 29.

31. A triparental cell hybrid according to any of claims 17 to 21 and 29, or a composition comprising a triparental cell hybrid according to claims 22 or 30, for use as a medicament.

35

32. Use of a triparental cell hybrid according to any of claims 17 to 21 and 29, and, a composition of claims 22 and 30 for the production of a medicine.

33. Use according to claim 32, wherein the medicine is an immune vaccine.
34. Use according to claim 32 or 33, for the production of a medicine or a vaccine to
5 induce an immune response in vivo and/or in vitro.
35. Use according to claim 34, for the production of a medicine or a vaccine for the treatment of cancer.
- 10 36. Use of a cell line according to claims 1 to 13, a triparental cell hybrid according to any of claims 17 to 21 and 29, and, a composition of claims 22 and 30 for the evaluation and diagnostic of serological and/or cellular immune responses.

1/44

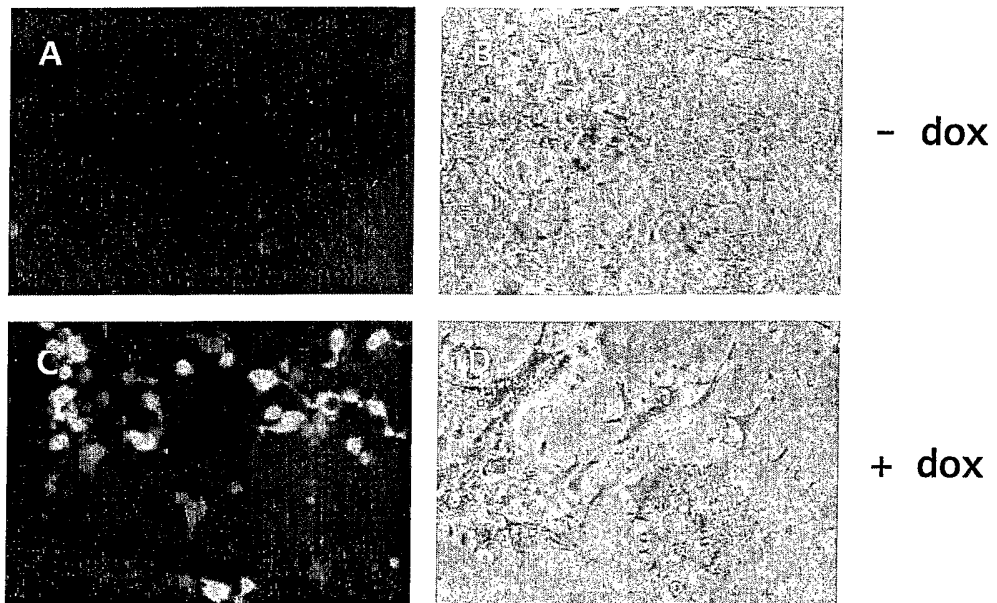


FIGURE 1

2/44

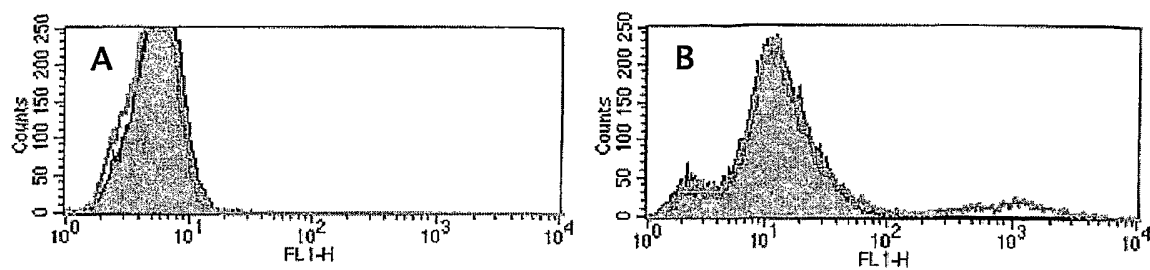


FIGURE 2

3/44

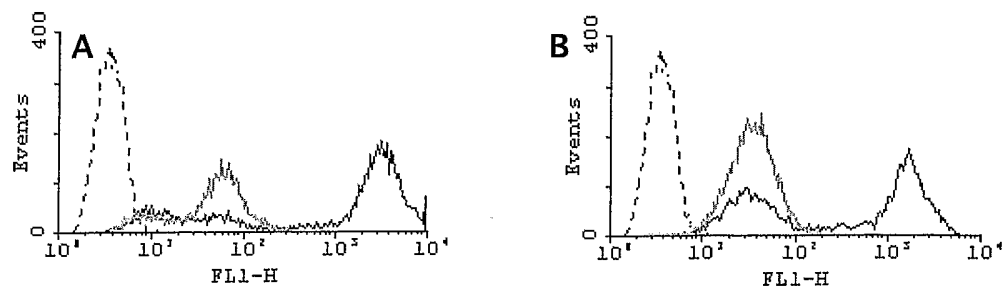


FIGURE 3

4/44

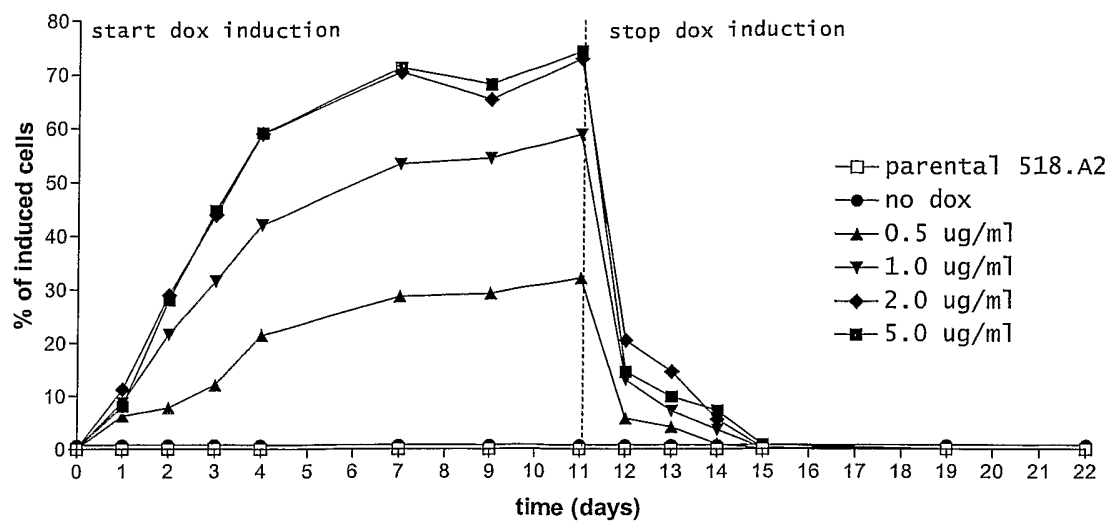


FIGURE 4

5/44

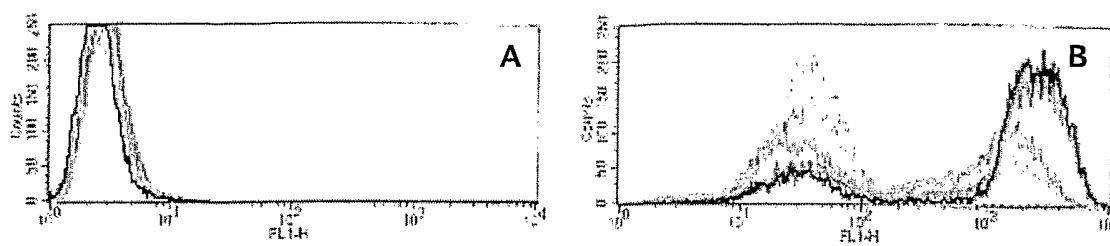


FIGURE 5

6/44

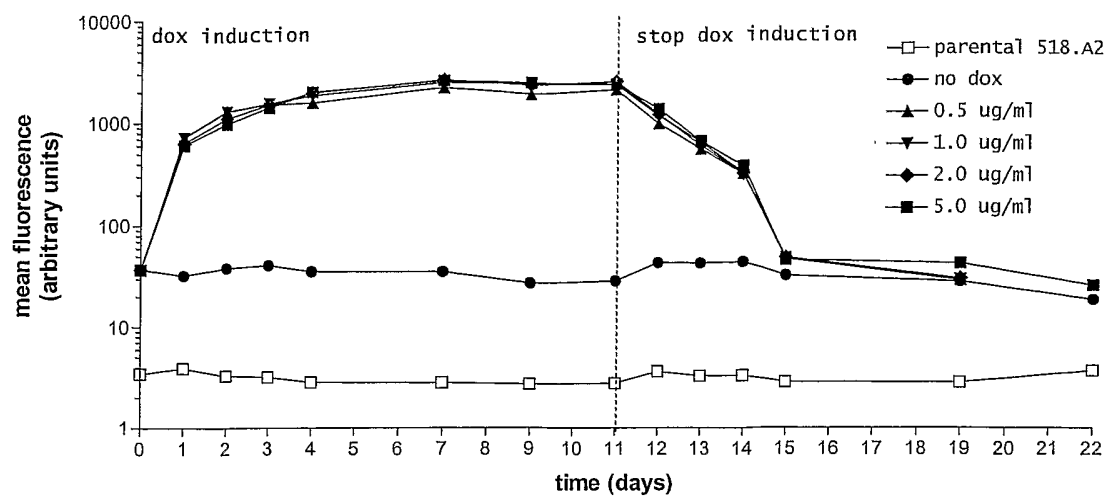


FIGURE 6

7/44

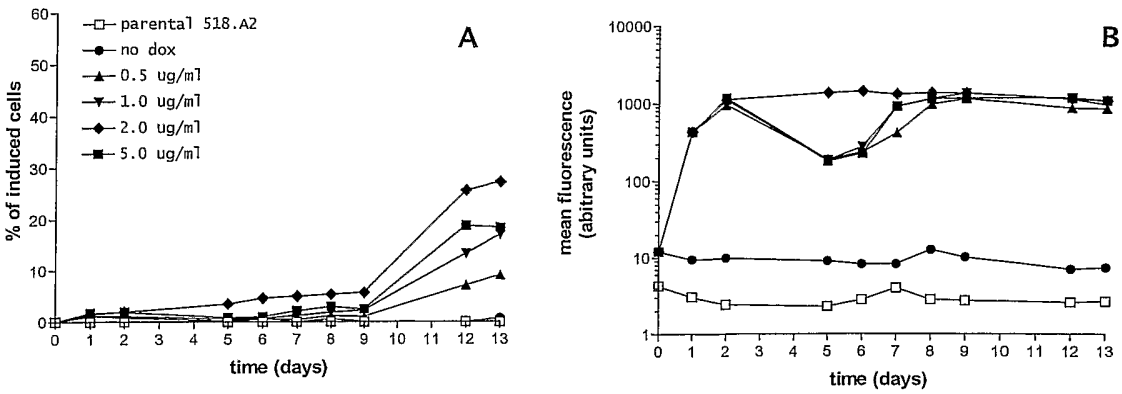


FIGURE 7

8/44

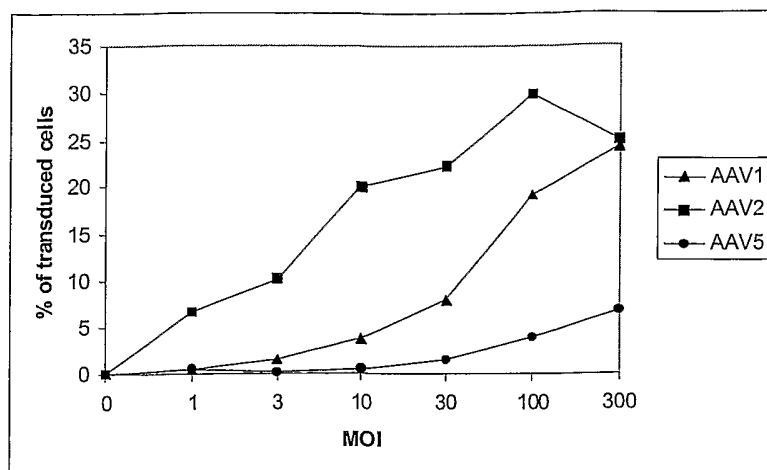


FIGURE 8

9/44

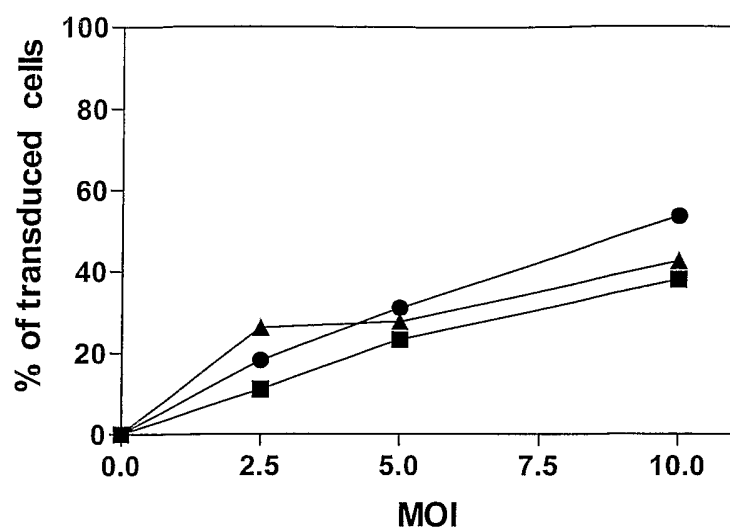


FIGURE 9

10/44

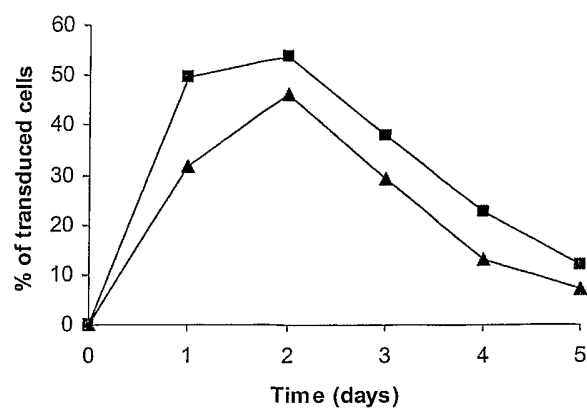


FIGURE 10

11/44

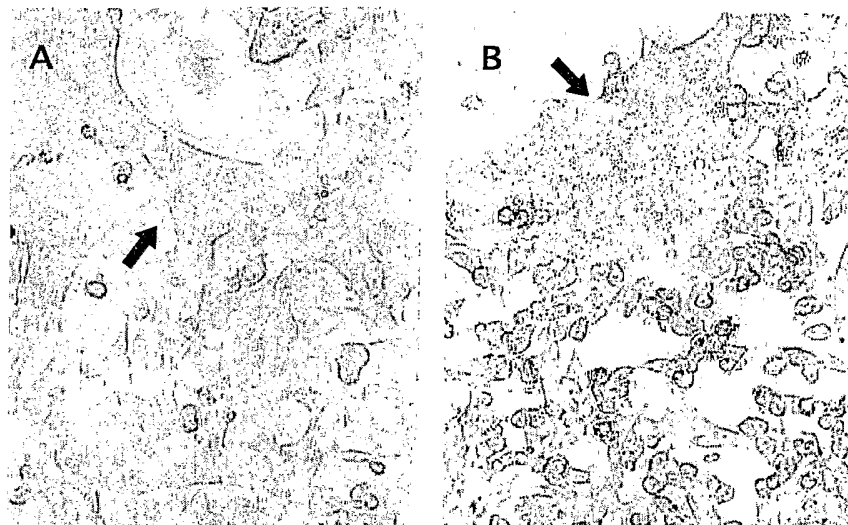


FIGURE 11

12/44

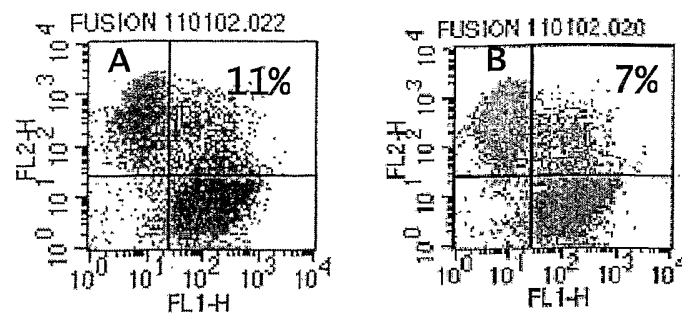


FIGURE 12

13/44

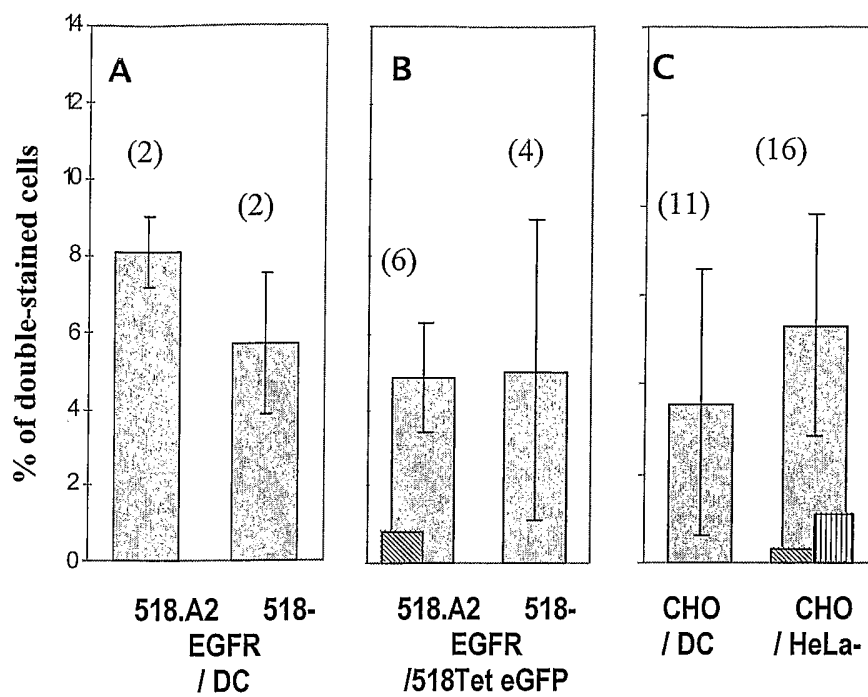


FIGURE 13

14/44

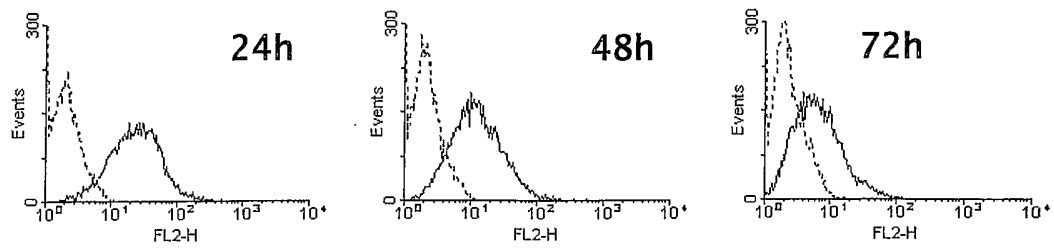


FIGURE 14

15/44

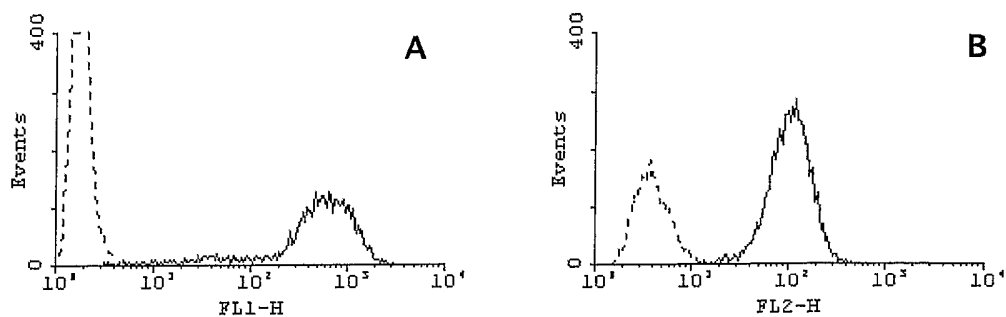


FIGURE 15

16/44

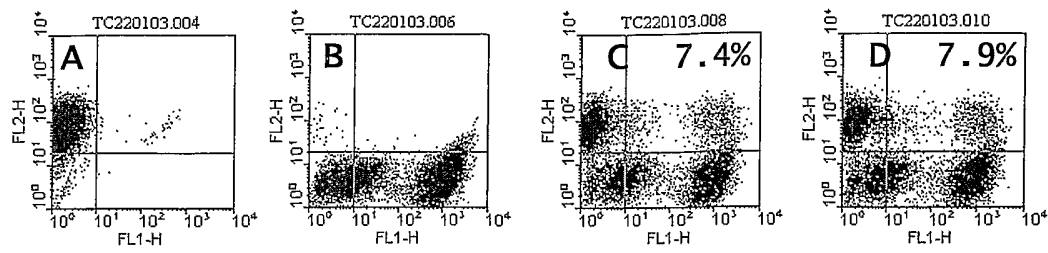


FIGURE 16

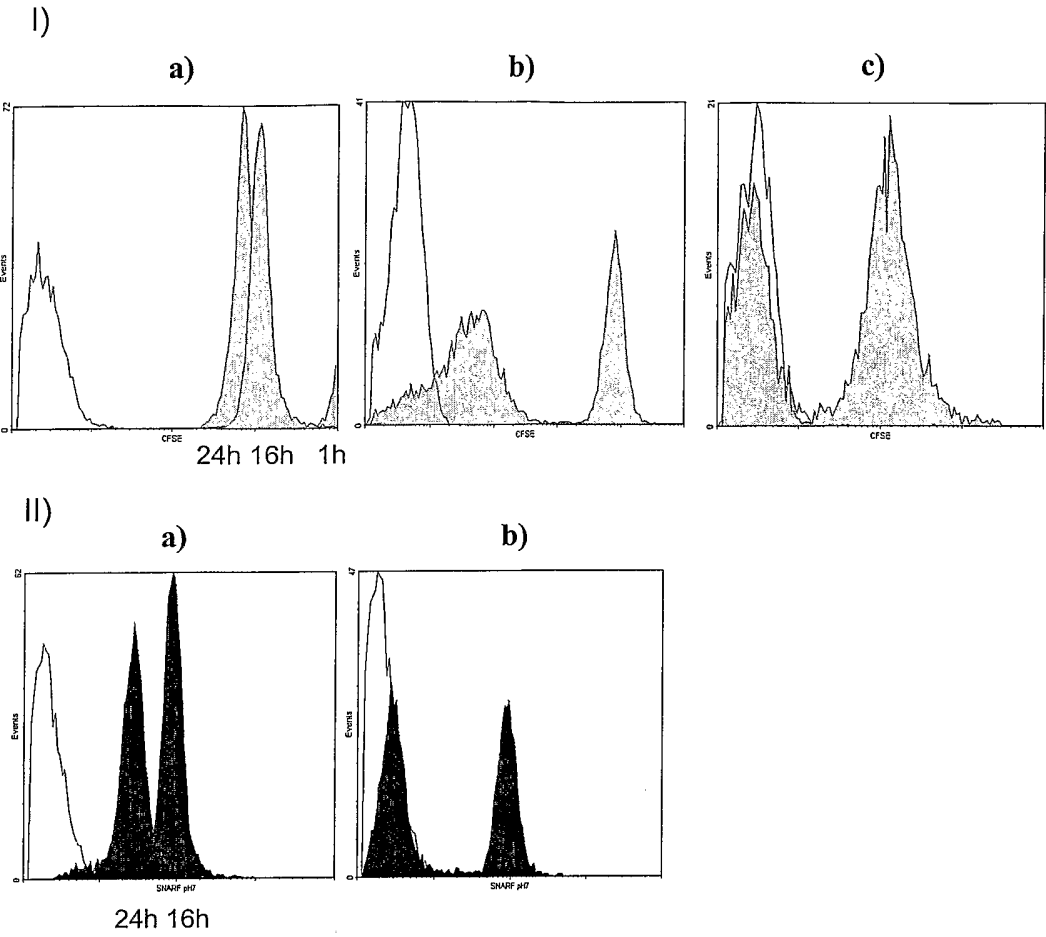


FIGURE 17

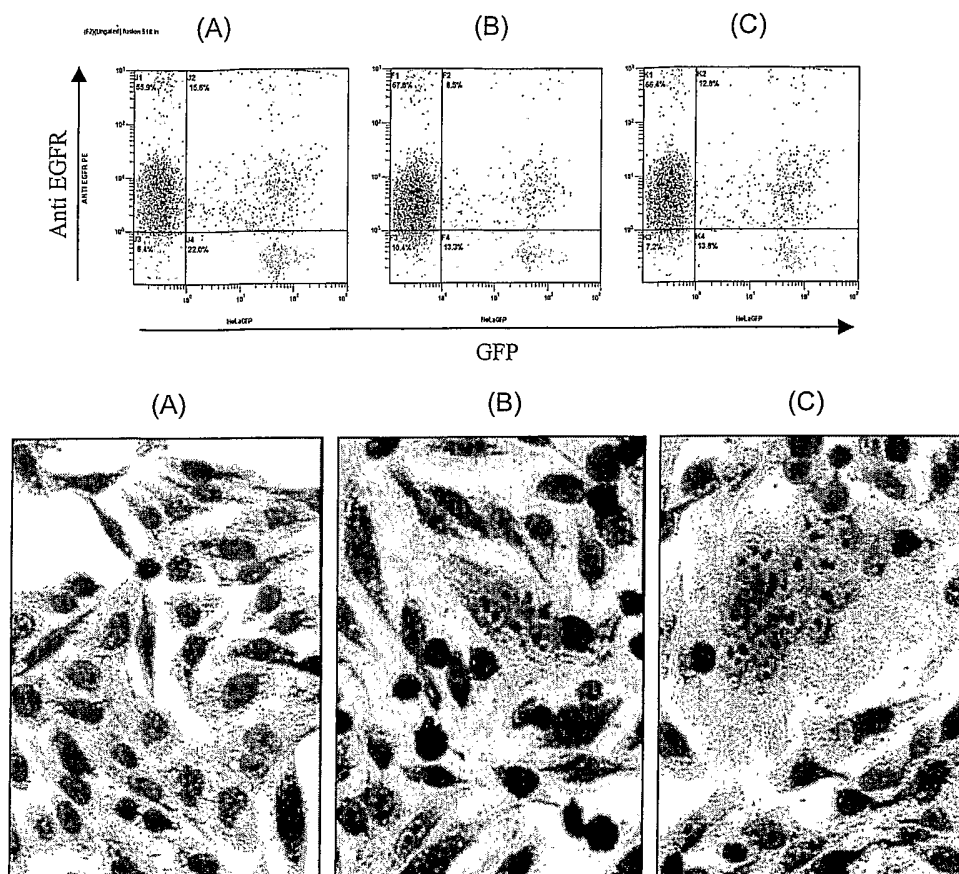


FIGURE 18

19/44

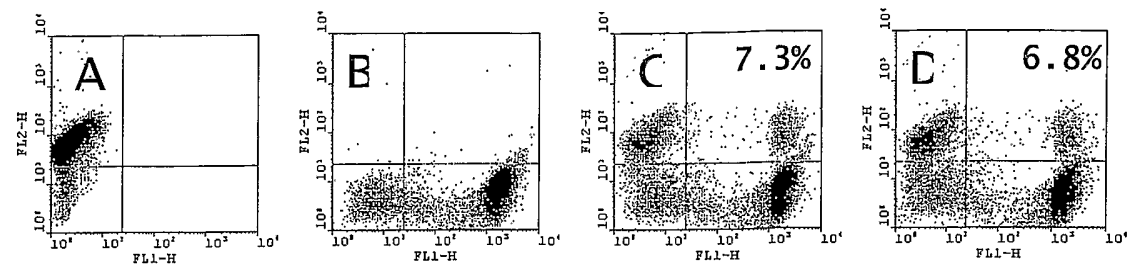


FIGURE 19

20/44

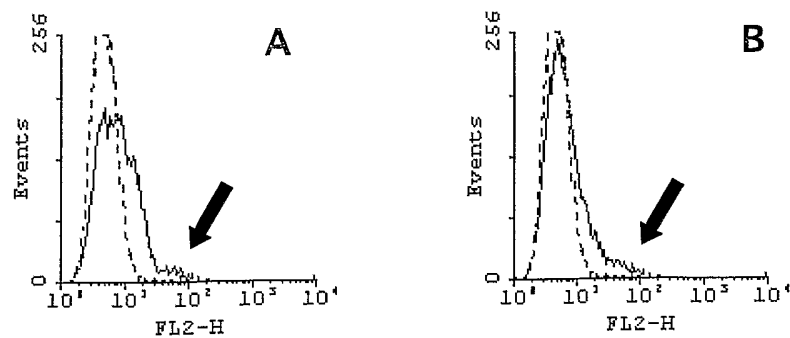


FIGURE 20

21/44

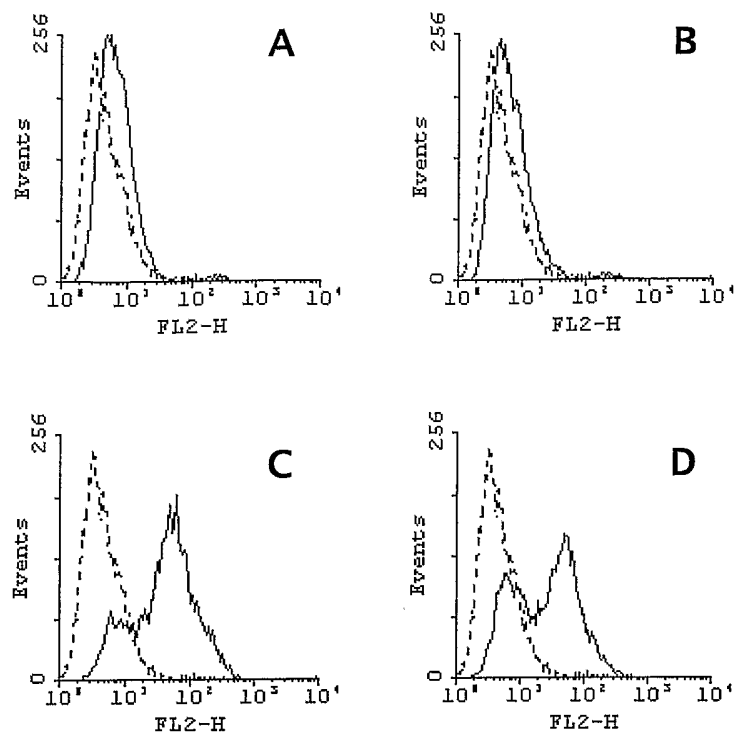


FIGURE 21

22/44

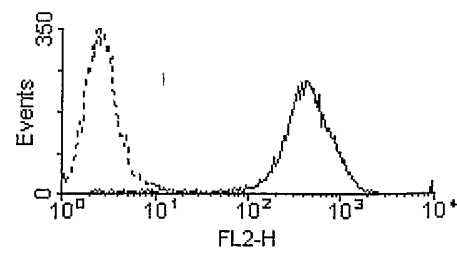


FIGURE 22

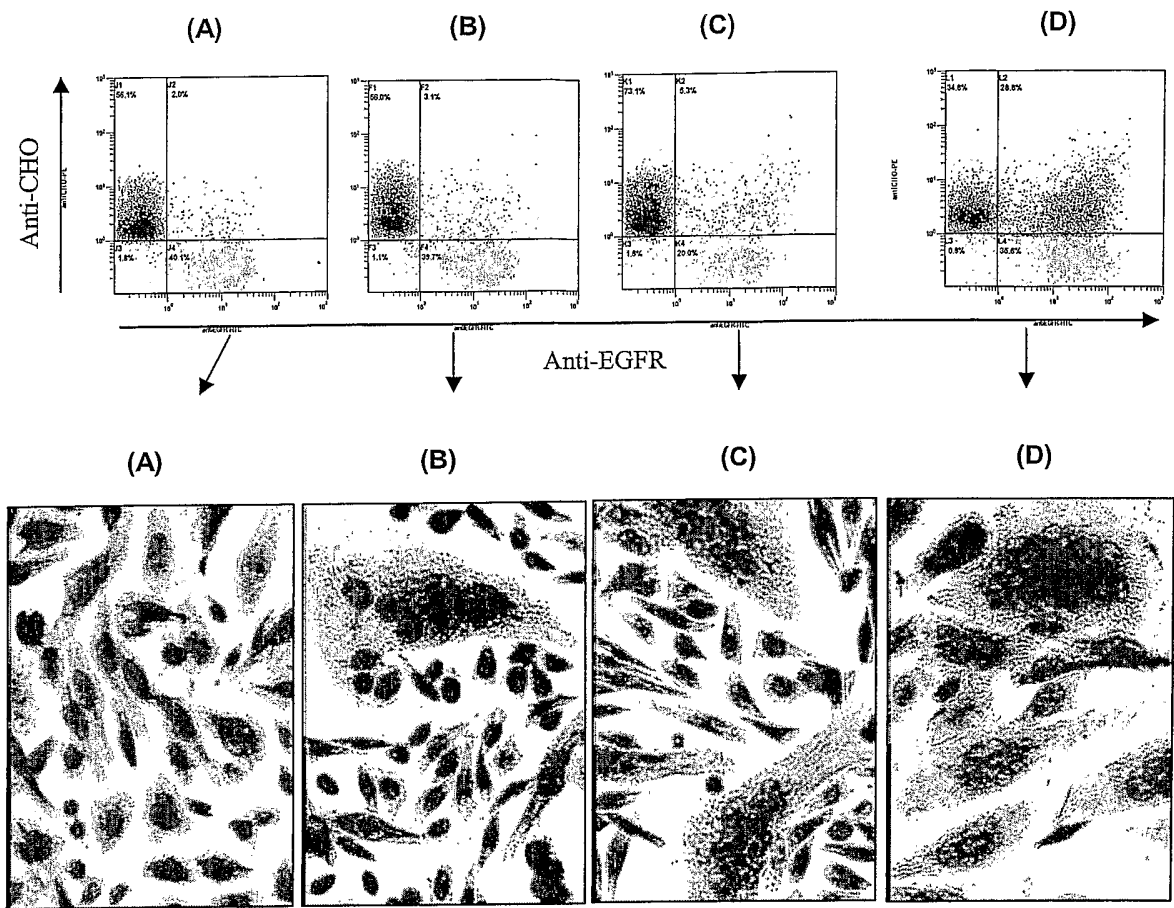


FIGURE 23 (I)

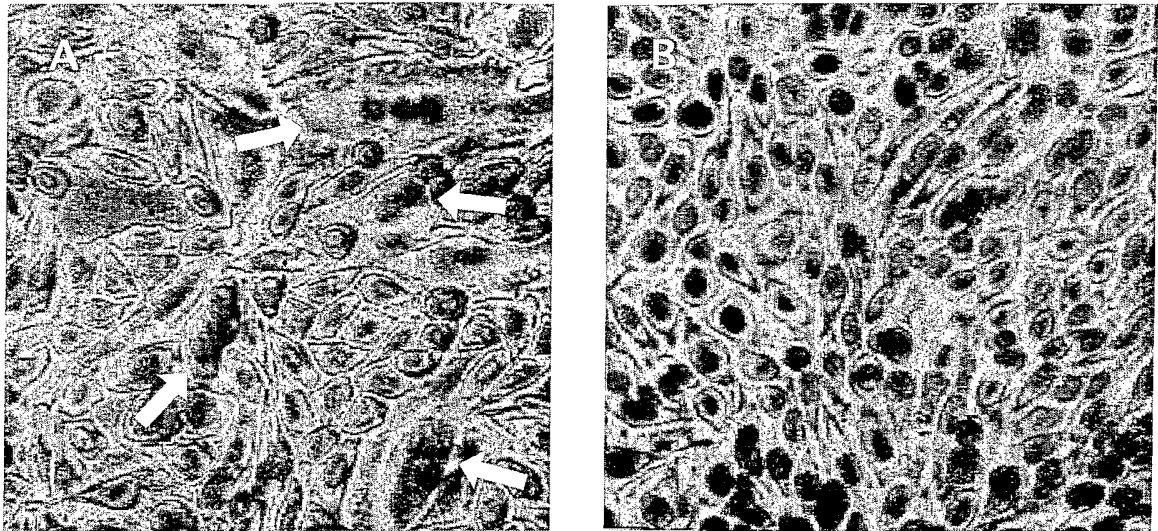


FIGURE 23 (II)

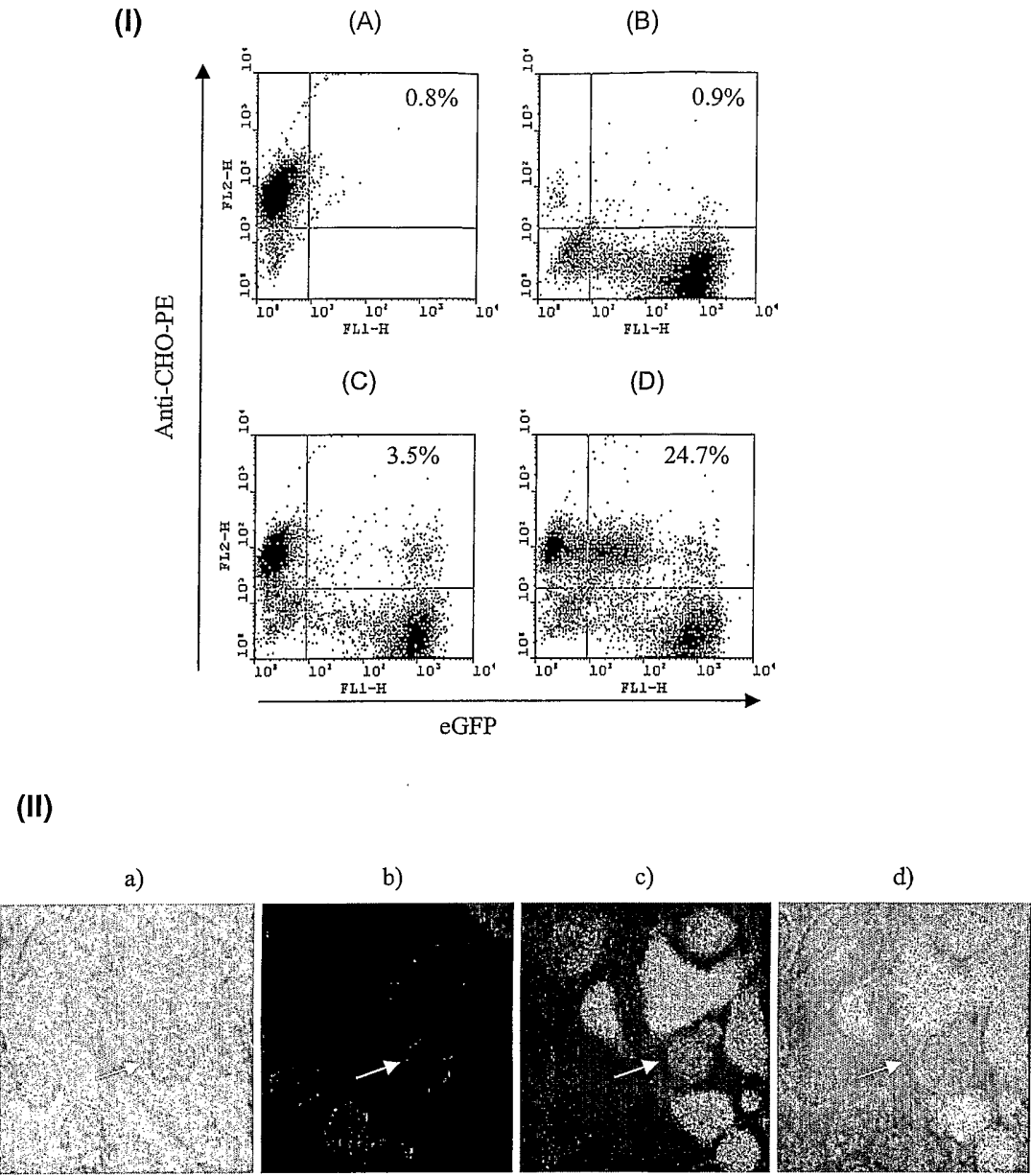


FIGURE 24

26/44

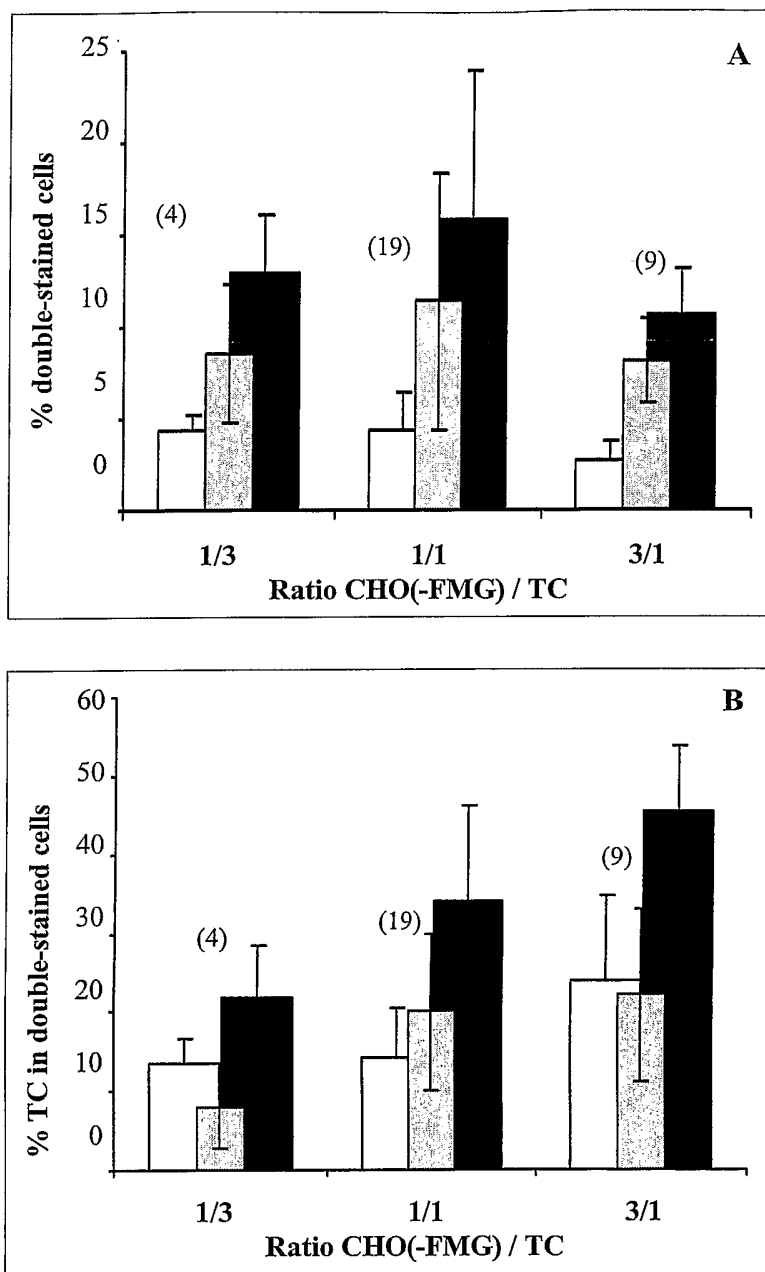


FIGURE 25

27/44

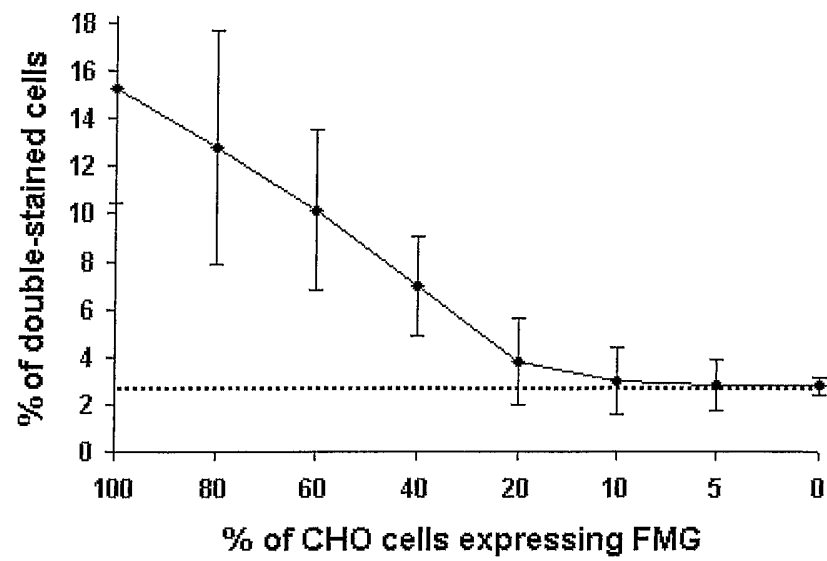


FIGURE 26

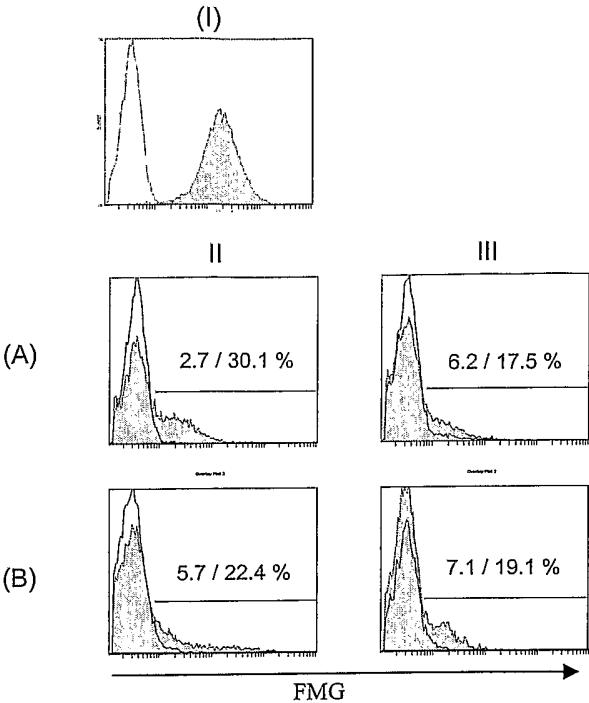


FIGURE 27

29/44

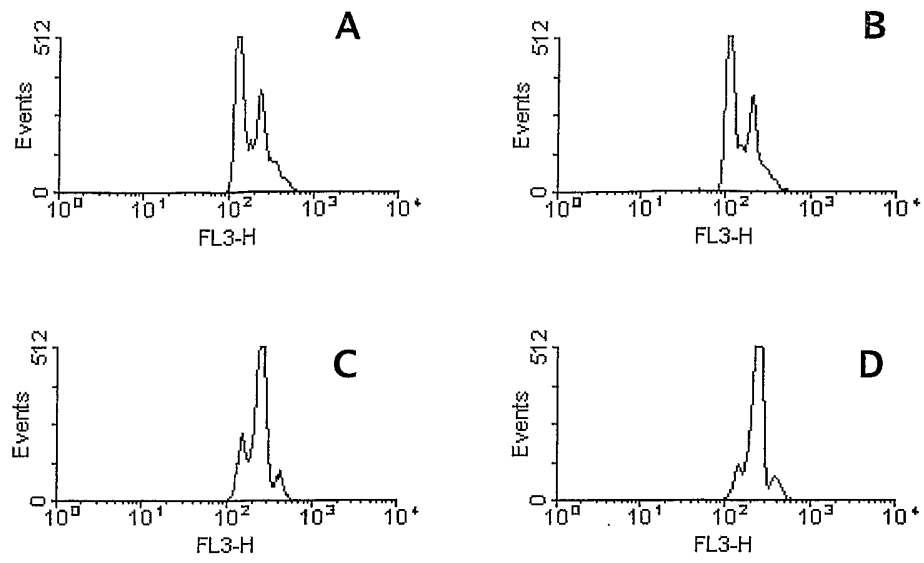


FIGURE 28

30/44

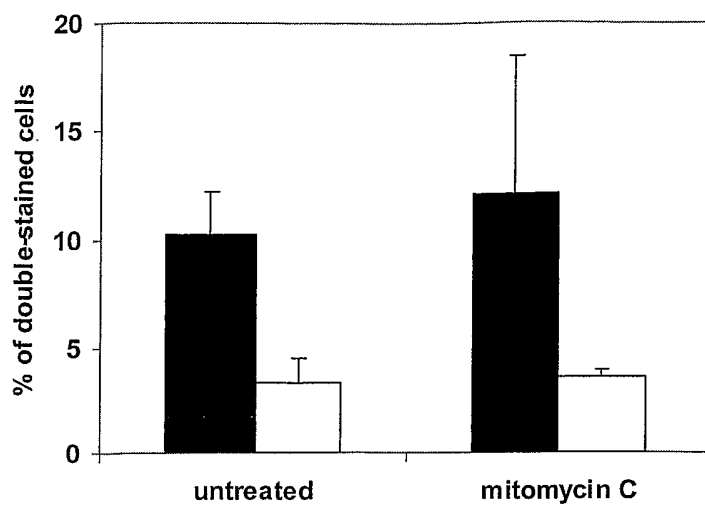


FIGURE 29

31/44

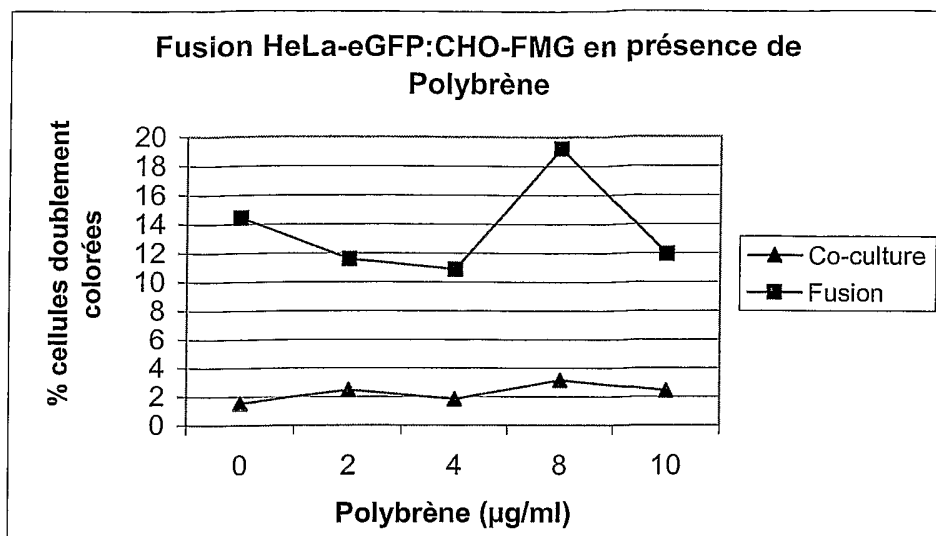


FIGURE 30

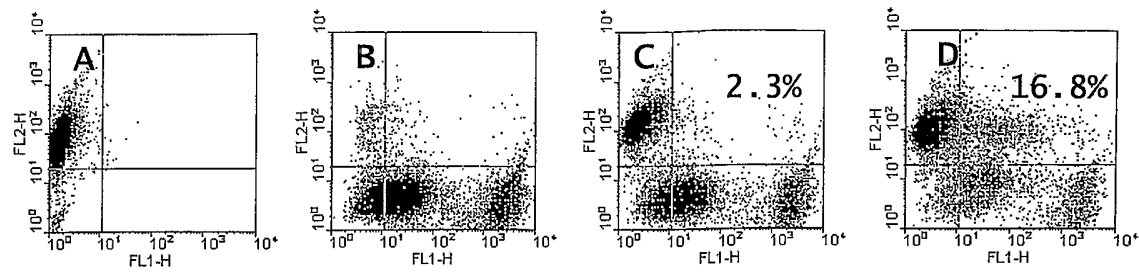


FIGURE 31

33/44

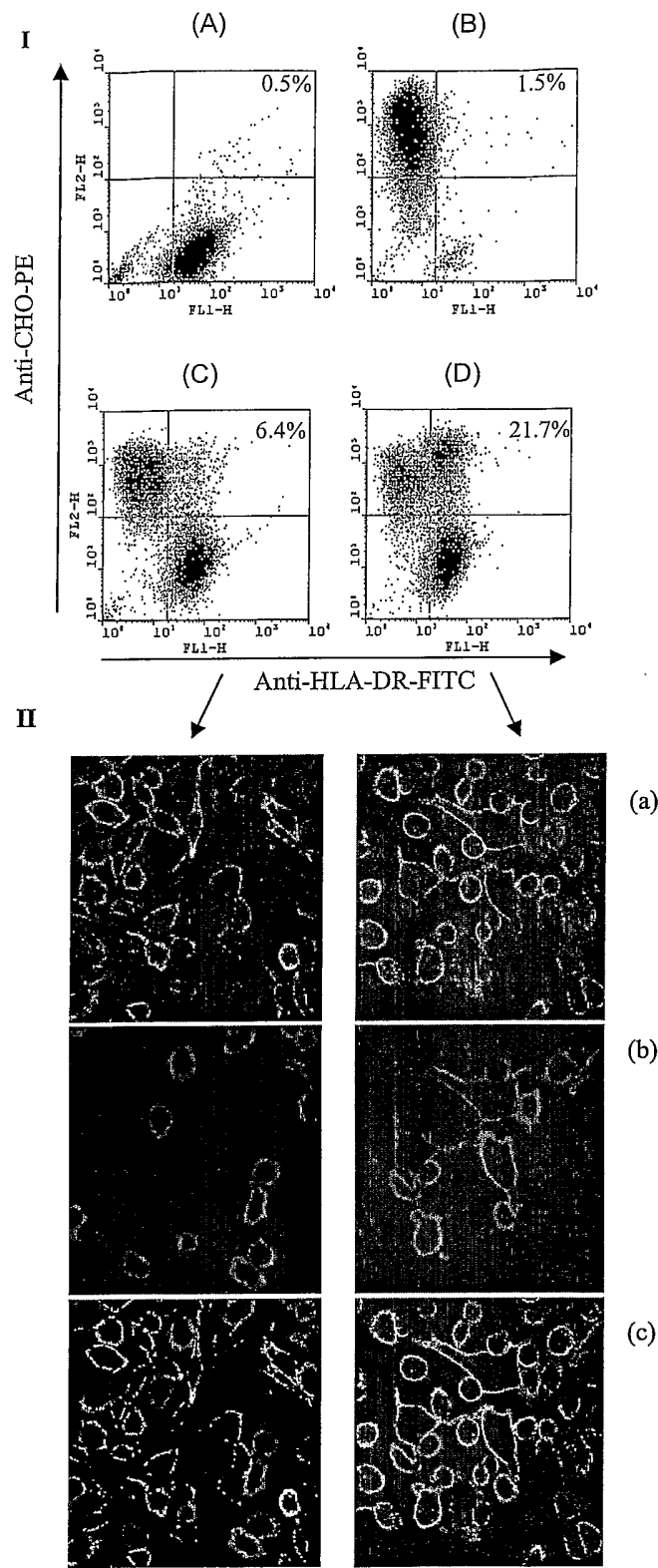


FIGURE 32

34/44

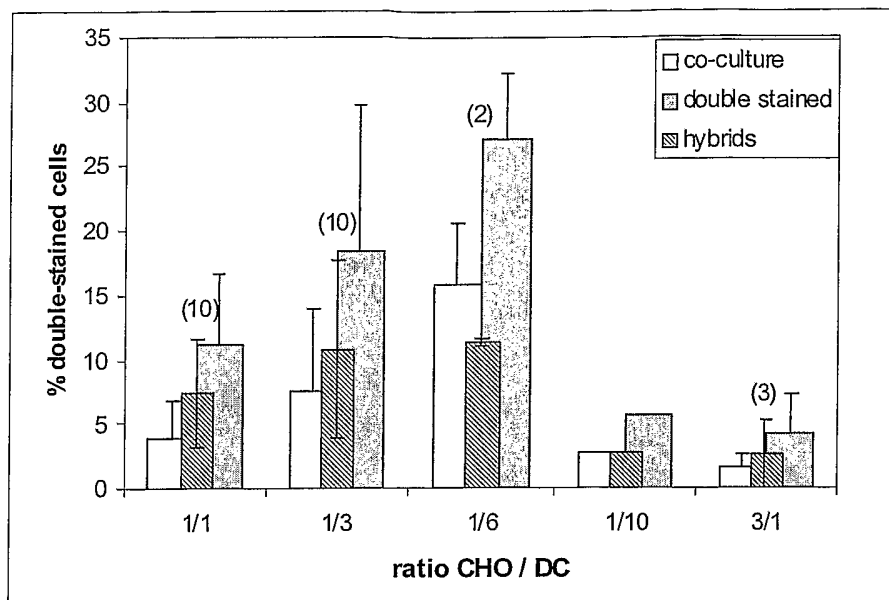


FIGURE 33

35/44

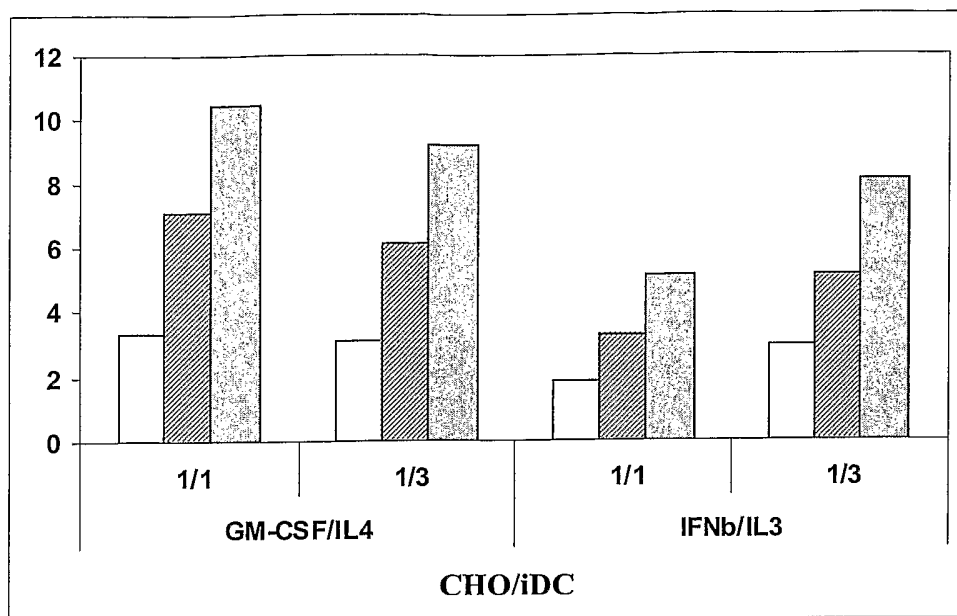


FIGURE 34

36/44

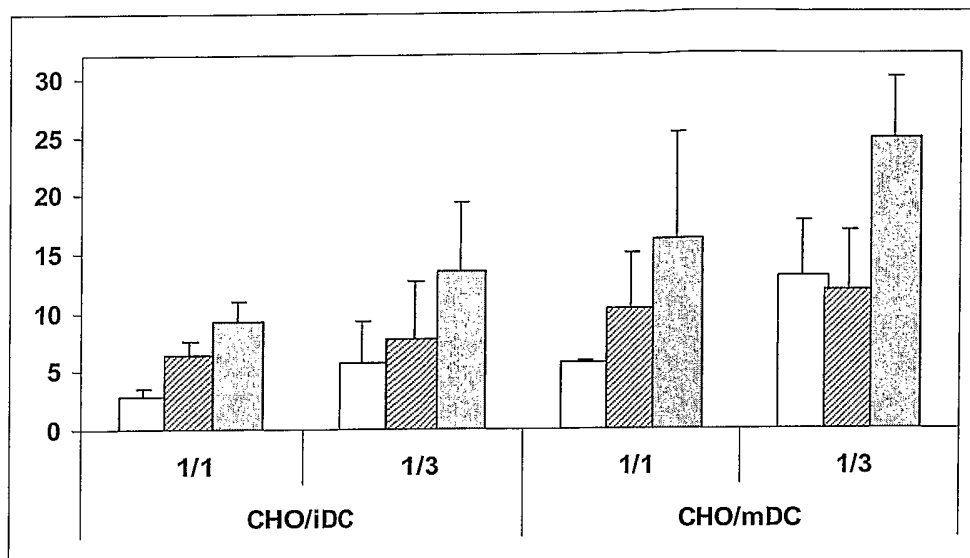


FIGURE 35

37/44

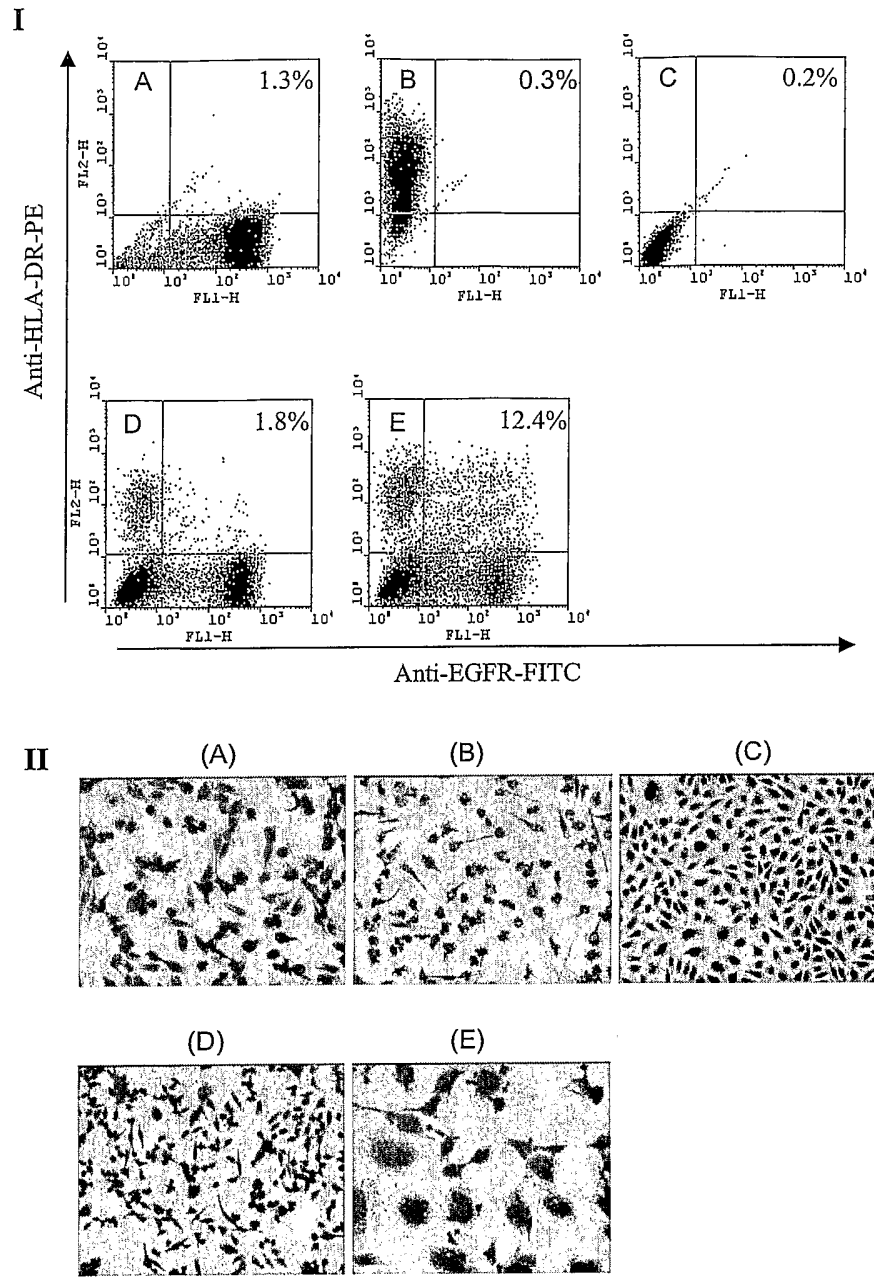


FIGURE 36

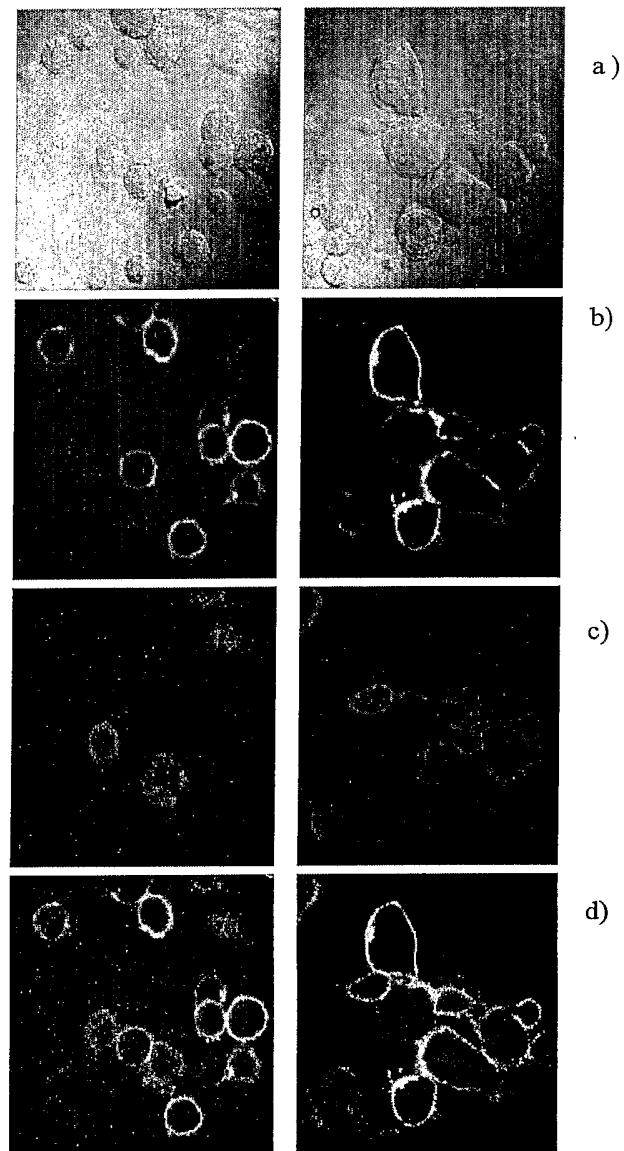


FIGURE 37

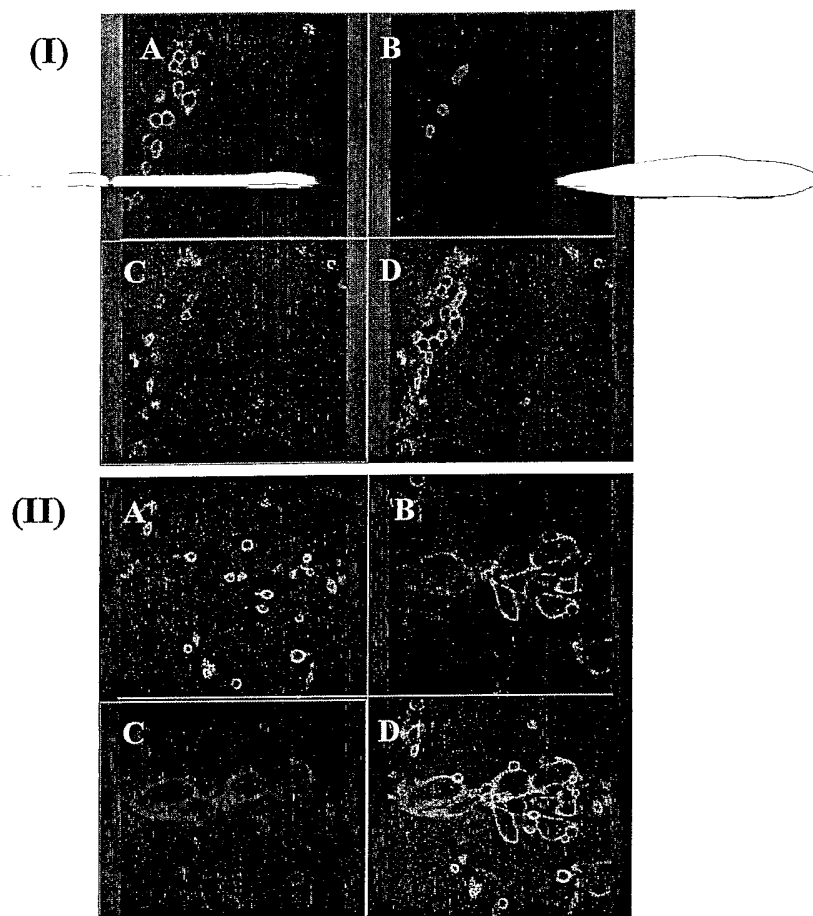


FIGURE 38

40/44

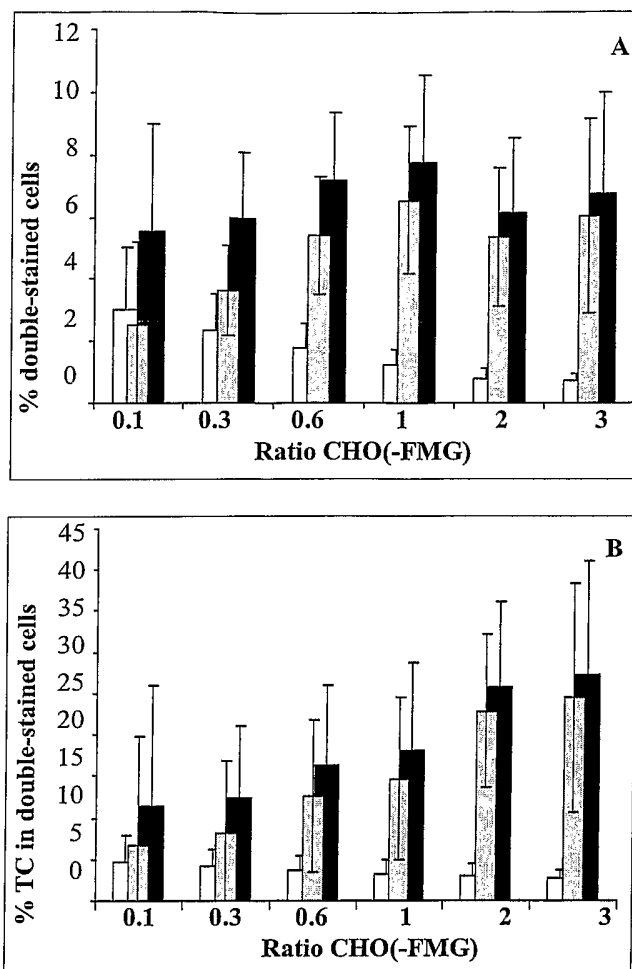


FIGURE 39

Co-culture

41/44

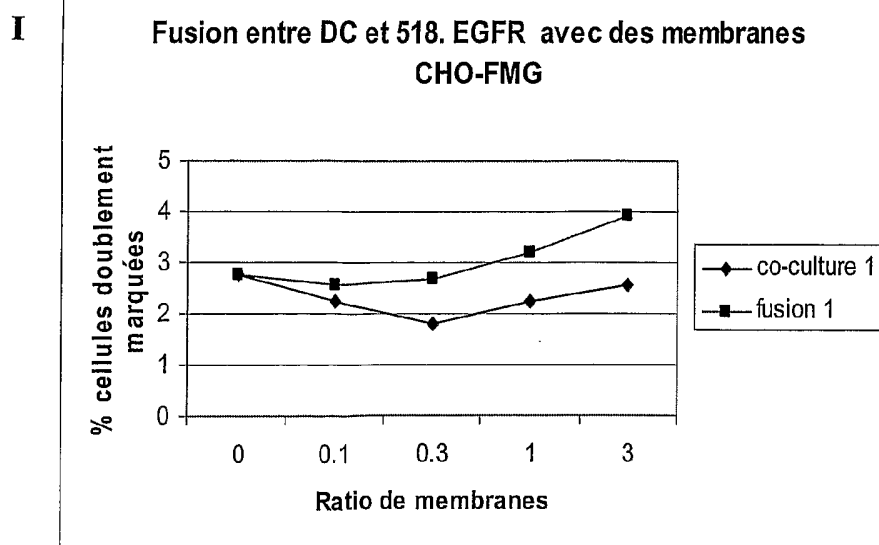
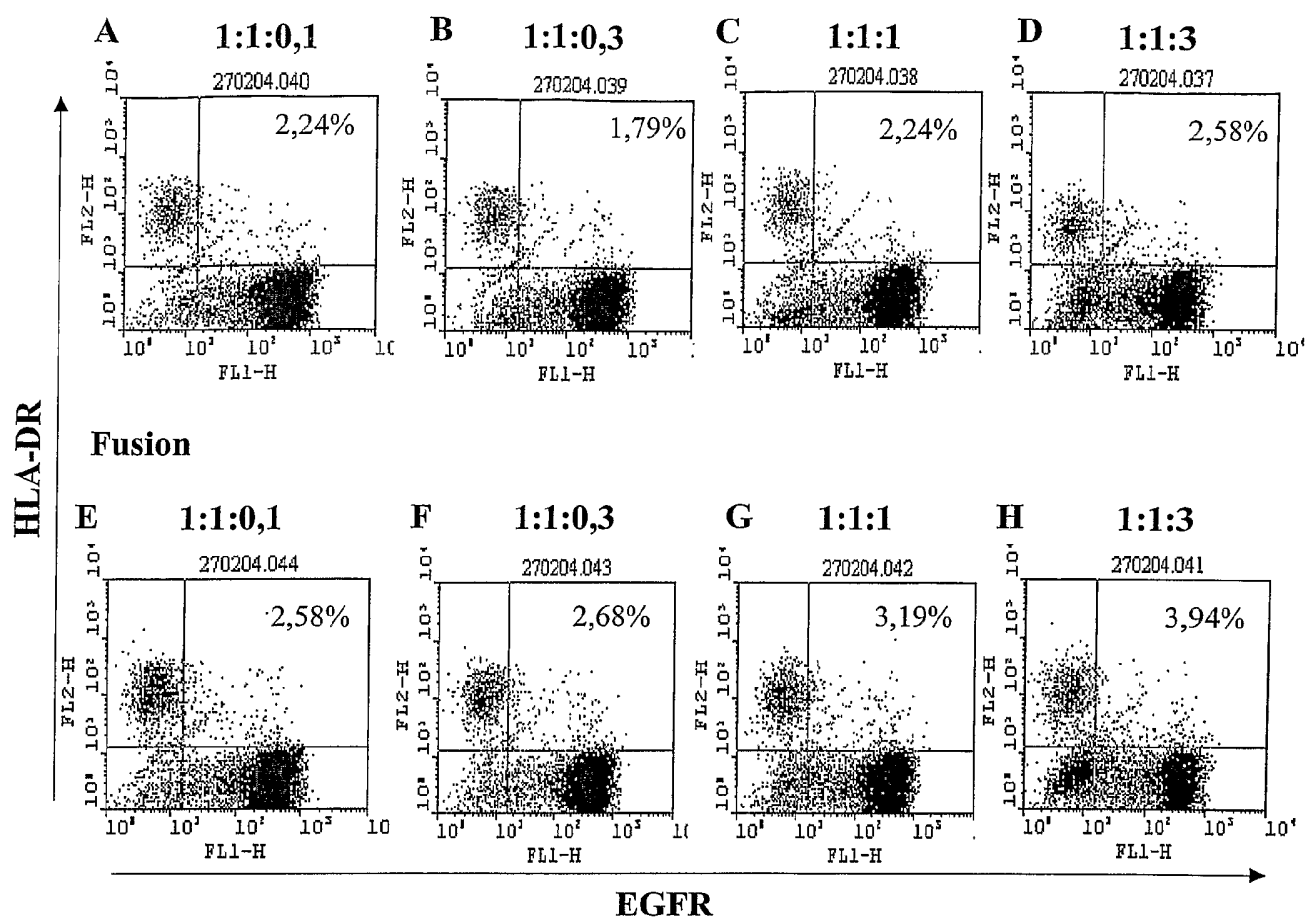


FIGURE 40

42/44

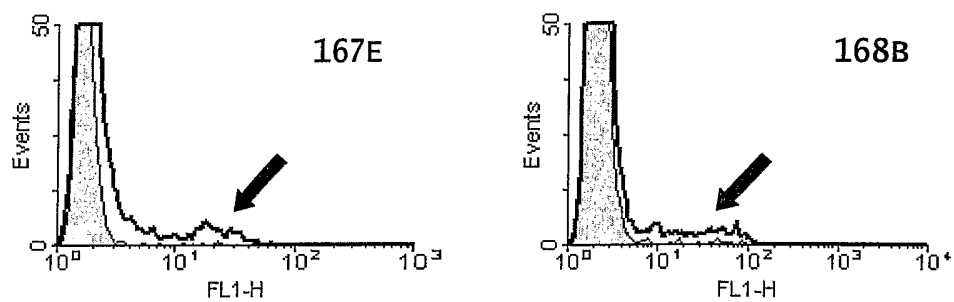


FIGURE 41

43/44

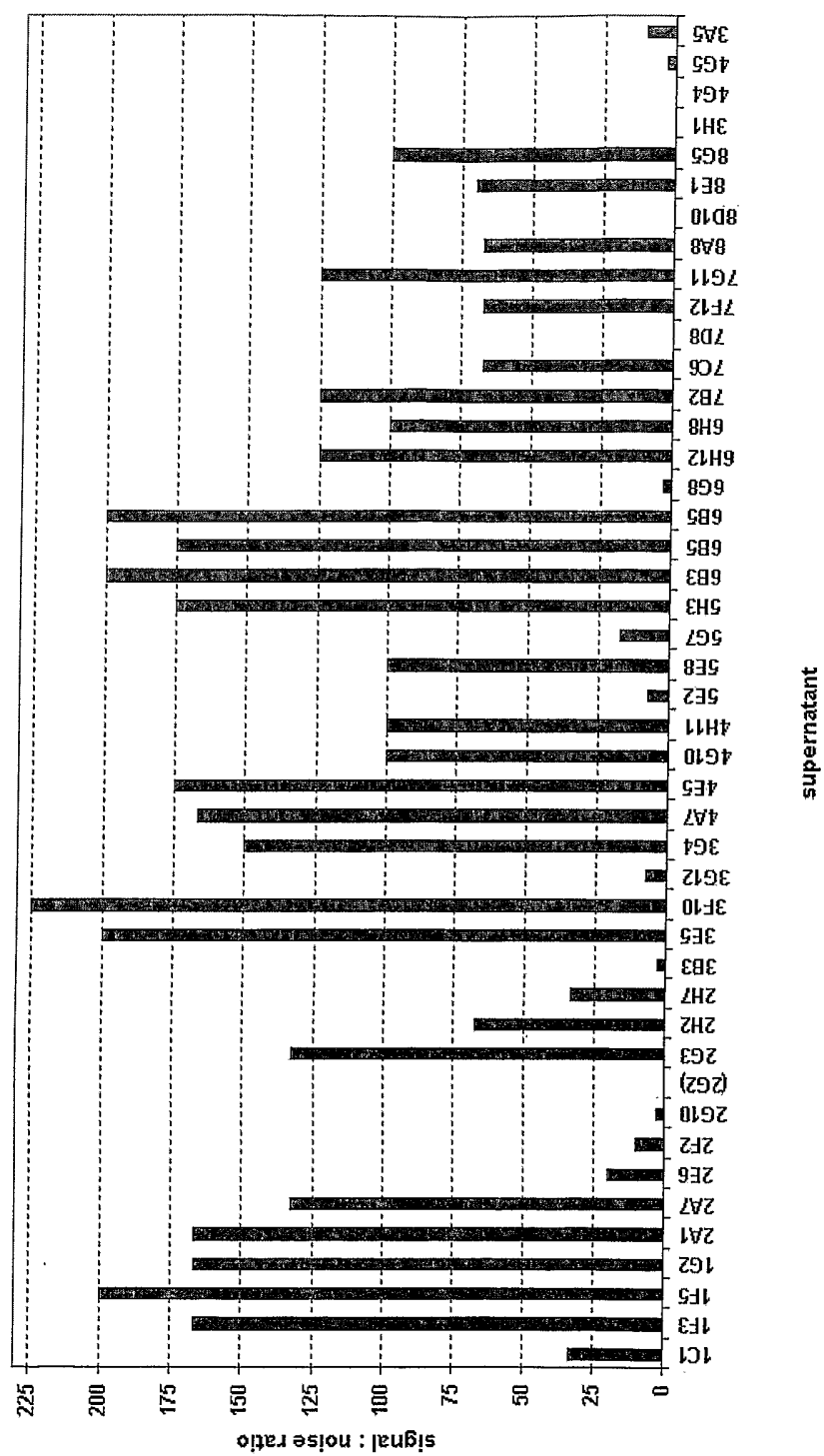


FIGURE 42

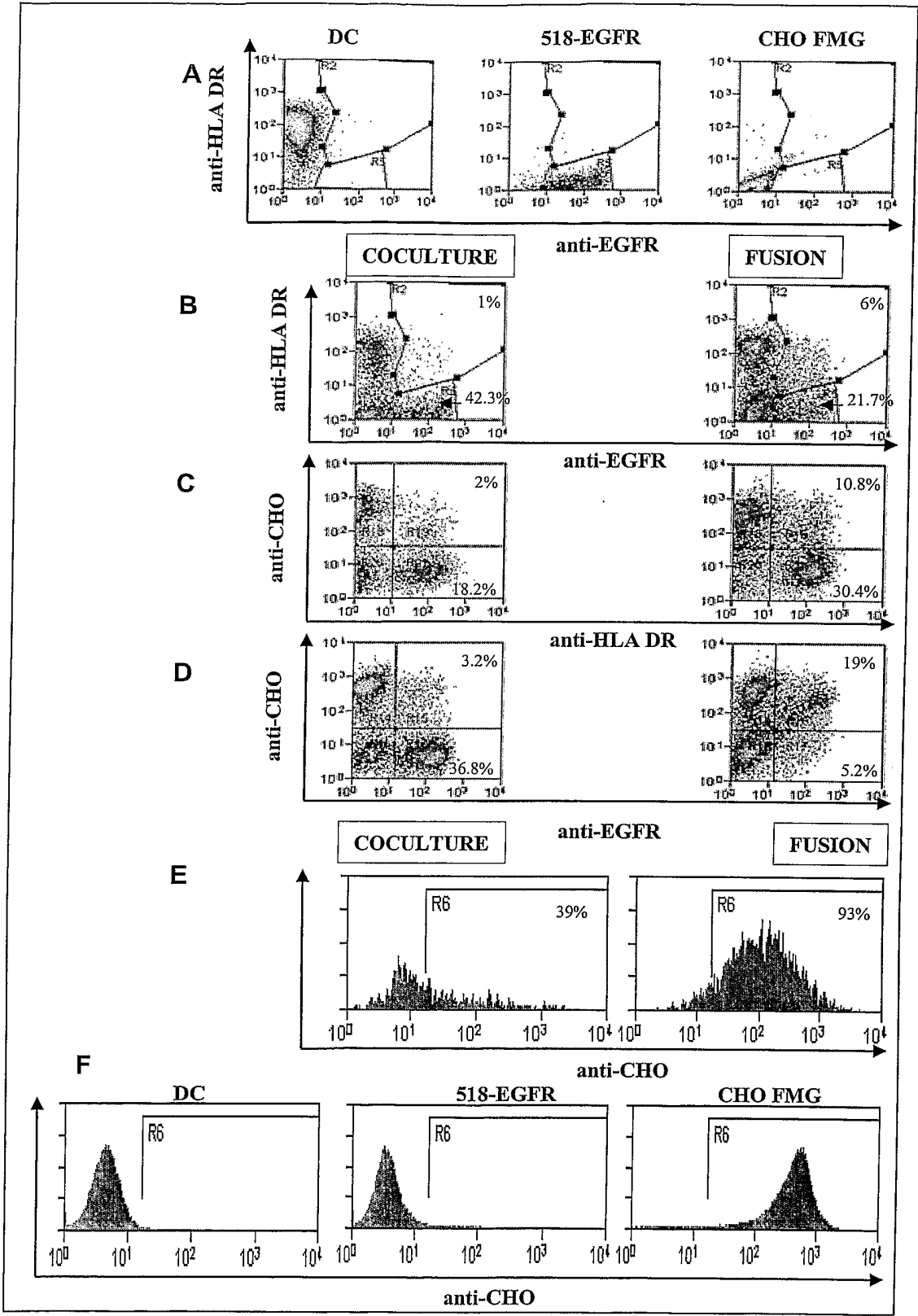


FIGURE 43

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/009541

A. CLASSIFICATION OF SUBJECT MATTER C12N5/16 C07K14/005				
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) C12N C07K				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	US 2002/042127 A1 (RUSSELL STEPHEN JAMES ET AL) 11 April 2002 (2002-04-11)	1-8, 14-27, 30-36		
X	cited in the application paragraphs '0030!, '0031!	1-8, 14-27, 30-36		
X	paragraph '0048!	1-8, 14-27, 30-36		
X	paragraph '0076!	1-8, 14-27, 30-36		
X	paragraph '0107!	1-8, 14-27, 30-36		
----- -/--				
☒ Further documents are listed in the continuation of box C.				
☒ Patent family members are listed in annex.				
° Special categories of cited documents :				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *G* document member of the same patent family </td> </tr> </table>			*A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *G* document member of the same patent family
A document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *G* document member of the same patent family			
Date of the actual completion of the international search 3 November 2005		Date of mailing of the international search report 24/11/2005		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Friedrich, C		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/009541

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GOLDING H ET AL: "FUSION OF HUMAN B CELL LINES WITH HIV-1 ENVELOPE-EXPRESSING T CELLS IS ENHANCED BY ANTIGEN-SPECIFIC IG RECEPTORS POSSIBLE MECHANISM FOR ELIMINATION OF GP120-SPECIFIC B CELLS IN VIVO" JOURNAL OF IMMUNOLOGY, THE WILLIAMS AND WILKINS CO. BALTIMORE, US, vol. 150, no. 6, 15 March 1993 (1993-03-15), pages 2506-2516, XP002925995 ISSN: 0022-1767	1,2
X	page 2507, column 1, paragraph 3 - column 2, paragraph 3	1,2
Y	the whole document	3-28
X	----- JOHNSON K J ET AL: "Targeting the cytotoxicity of fusogenic membrane glycoproteins in gliomas through protease-substrate interaction." GENE THERAPY. MAY 2003, vol. 10, no. 9, May 2003 (2003-05), pages 725-732, XP002352309 ISSN: 0969-7128 figure 1	8
X	----- FIELDING A K ET AL: "A hyperfusogenic gibbon ape leukemia envelope glycoprotein: targeting of a cytotoxic gene by ligand display." HUMAN GENE THERAPY. 10 APR 2000, vol. 11, no. 6, 10 April 2000 (2000-04-10), pages 817-826, XP002352310 ISSN: 1043-0342 figure 1	8
Y	----- SABATINO D E ET AL: "Amphotropic or gibbon ape leukemia virus retrovirus binding and transduction correlates with the level of receptor mRNA in human hematopoietic cell lines." BLOOD CELLS, MOLECULES & DISEASES. DEC 1997, vol. 23, no. 3, December 1997 (1997-12), pages 422-433, XP002305104 ISSN: 1079-9796 page 423, column 1, paragraph 2 ----- -/--	1-36

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/009541

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HIGUCHI H ET AL: "Viral fusogenic membrane glycoprotein expression causes syncytia formation with bioenergetic cell death: implications for gene therapy" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 60, no. 22, 15 November 2000 (2000-11-15), pages 6396-6402, XP002953649 ISSN: 0008-5472	1-36
Y	page 6398, column 2, paragraph 4 - page 6399, column 1, paragraph 1	1-28
Y	page 6396, column 2, paragraph 2	1-28
Y	PHAN VY ET AL: "A new genetic method to generate and isolate small, short-lived but highly potent dendritic cell-tumor cell hybrid vaccines." NATURE MEDICINE. SEP 2003, vol. 9, no. 9, September 2003 (2003-09), pages 1215-1219, XP002305105 ISSN: 1078-8956 cited in the application	1-36
Y	page 1215, column 2, paragraph 2	1-28
Y	page 1219, column 1, paragraph 2	1-28
Y	BRADY ANTHONY M ET AL: "Heat-directed tumor cell fusion." HUMAN GENE THERAPY. 20 MAR 2003, vol. 14, no. 5, 20 March 2003 (2003-03-20), pages 447-461, XP002305106 ISSN: 1043-0342 the whole document	1-36

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP2005/009541

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002042127 A1	11-04-2002	AU 2346800 A WO 0030454 A1	13-06-2000 02-06-2000