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(19) (CA) **CANADIAN PATENT** (12)

(54) In Vitro Propagation of Embryonic Stem Cells

(72) WILLIAMS, ROBERT LINDSAY, AUSTRALIA
GOUCH, NICHOLAS MARTIN, AUSTRALIA
HILTON, DOUGLAS JAMES, AUSTRALIA

(73) AMRAD CORPORATION LIMITED, AUSTRALIA

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ABSTRACT

10

The present invention relates generally to the use of leukaemia inhibitory factor (LIF) in the maintenance and derivation of embryonic stem (ES) cells in culture. The ES cells are maintained and/or derived from animal embryos by culturing said cells or embryos in a culture medium containing an effective amount of LIF for a time and under conditions sufficient to maintain and/or derive said ES cells. The ES cells may be passaged in LIF and used to make chimaeric animals.

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IN VITRO PROPAGATION OF EMBRYONIC STEM CELLS

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This invention relates to the use of a previously discovered and characterised molecule, leukaemia inhibitory factor (LIF), in the isolation and propagation of embryonic stem cells in vitro.

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Embryonic stem (ES) cells, the pluripotent outgrowths of blastocysts, can be cultured and manipulated in vitro and then returned to the embryonic environment to contribute normally to all tissues including the germline (for review see Robertson, E.J. (1986) Trends in Genetics 2:9-13). Not only can ES cells propagated in vitro contribute efficiently to the formation of chimaeras, including germline chimaeras, but in addition, these cells can be manipulated in vitro without losing their capacity to generate germ-line chimaeras (Robertson, E.J. et.al. 30 (1986) Nature 323:445-447).

ES cells thus provide a route for the generation of transgenic animals such as transgenic mice, a route which has a number of important advantages compared with more conventional techniques, such as zygote injection and

viral infection (Wagner and Stewart (1986) in *Experimental Approaches to Embryonic Development*. J. Rossant and A. Pedersen eds. Cambridge: Cambridge University Press), for introducing new genetic material into such animals.

5 First, the gene of interest can be introduced and its integration and expression characterised in vitro. Secondly, the effect of the introduced gene on the ES cell growth can be studied in vitro. Thirdly, the characterised ES cells having a novel introduced gene can
10 be efficiently introduced into embryos by blastocyst injection or embryo aggregation and the consequences of the introduced gene on the development of the resulting transgenic chimaeras monitored during pre- or post-natal life. Fourthly, the site in the ES cell genome at which
15 the introduced gene integrates can be manipulated, leaving the way open for gene targeting and gene replacement (Thomas, K.R. and Capecchi, M.R. (1987) *Cell* 51:503-512).

However, it is known that ES cells and certain EC (embryonal carcinoma) cell lines will only retain the stem
20 cell phenotype in vitro when cultured on a feeder layer of fibroblasts (such as murine STO cells, e.g. Martin, G.R. and Evans, M.J. (1975) *Proc. Natl. Acad. Sci. USA* 72:1441-1445) or when cultured in medium conditioned by certain cells (e.g. Koopman, P. and Cotton, R.G.H. (1984)
25 *Exp. Cell Res.* 154:233-242; Smith, A.G. and Hooper, M.L. (1987) *Devel.Biol.* 121:1-91). In the absence of feeder cells or conditioned medium, the ES cells spontaneously differentiate into a wide variety of cell types, resembling those found during embryogenesis and in the
30 adult animal. The factors responsible for maintaining the pluripotency of ES cells have, however, remained poorly characterised.

In work leading to the present invention, it has been found that LIF has the capacity to substitute for, or be added to, feeder layers (or conditioned medium) in supporting the maintenance of pluripotential ES cells in vitro.

LIF is a protein that has previously been purified, cloned and produced in large quantities in purified recombinant form from both Escherichia coli and yeast cells. (International Patent Application No. PCT/AU88/00093, filed March 31, 1988 and published October 6, 1988.) LIF has been defined as a factor, the
10 properties of which include:

1. it has the ability to suppress the proliferation of myeloid leukaemic cells such as M1 cells, with associated differentiation of the leukaemic cells; and

2. it will compete with a molecule having the defined sequence of murine LIF or human LIF (defined in International Patent Application No. PCT/AU88/00093) for binding to specific cellular receptors on M1 cells or murine or human macrophages. In addition to the biological properties previously disclosed for murine and human LIF, LIF has now been found to have the following
20 properties:

(a) it allows the derivation and maintenance in the absence of feeder cells of the pluripotential phenotype in vitro of ES cells;

(b) it allows the aforementioned ES cells, after passage in vitro in the presence of LIF, to contribute to somatic and germline cell tissues of chimaeric animals such as mice when re-introduced into the embryonic environment;

(c) it demonstrates selective binding to high affinity receptors on murine ES (EKcs-1 (previously known as CS1) and D3) and EC (PCC3-3A and F9) cells; and

(d) specific binding of ^{125}I -LIF to high affinity
5 receptors is not in competition with insulin, IGF-I, IGF-II, acidic and basic FGF, $\text{TGF}\beta$, $\text{TNF}\alpha$, $\text{TNF}\beta$, NGF, PDGF, EGF, IL-1, IL-2, IL-4, GM-CSF, G-CSF, Multi-CSF nor erythropoietin, but is in competition with murine and human LIF.

Accordingly, a first aspect of the present
10 invention relates to a method for the isolation of embryonic stem (ES) cells from animal embryos in vitro which method comprises deriving ES cells from said embryos in culture medium, said culture medium containing an effective amount of leukaemia inhibitory factor (LIF), for a time and under
15 conditions sufficient for the development of said ES cells. The embryos used may be isolated from animals including, but not limited to, humans and a number of other animal species such as mice, sheep, pigs, cattle, goats, and birds (eg. chickens).

20 A second aspect of the present invention, contemplates a process for maintaining animal embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype, which process comprises culturing said cells in a culture medium containing an effective amount of leukaemia
25 inhibitory factor (LIF) under conditions sufficient to maintain said cells. The ES cells in accordance with this aspect of the invention include cells from humans, mice, sheep, pigs, cattle, goats, and birds (eg. chickens).

The LIF used in the culture medium is preferably

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recombinant LIF produced, by way of example, in accordance with the methods described in International Patent Application No. PCT/AU88/00093. In accordance with the present invention, it has been found that recombinant LIF and in particular recombinant human and murine LIF are effective substitutes for, or additives to, feeder layers or conditioned medium in maintaining ES cells in vitro. For the purposes of the present description recombinant LIF is produced in E. coli and yeast using the methods described in International Patent Application No. PCT/AU88/00093, however, it is within the scope of the present invention to include recombinant LIF produced in other hosts including mammalian and insect cells and to synthetic LIF.

In another aspect, the present invention extends to ES cells derived from animal embryos by passage in a culture medium containing LIF, to such ES cells having additional genetic material inserted therein, and to chimaeric animals such as chimaeric mice or transgenic progeny of said animals generated by known techniques using ES cells which have been maintained in vitro in a LIF-containing culture medium.

In another aspect the invention extends to a substantially homogeneous population of embryonic stem (ES) cells derived from mammalian embryos in vitro, wherein said ES cells have been isolated from embryos cultured in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF), and characterized in that said ES cells maintain a pluripotential phenotype.

According to another aspect of the present invention, there is provided the use described herein wherein LIF is added to a culture medium comprising ES cells in an effective amount and under conditions sufficient to maintain
5 said pluripotential phenotype.

Thus, the invention extends to the generation and maintenance of ES cells from humans, mice, sheep, pigs, cattle, goats and birds (eg. chickens) and to the generation of transgenic chimaeric animals and their transgenic progeny
10 using the ES cells isolated from animal species such as mice, sheep, pigs, cattle, goats and birds (eg. chickens). This invention also includes the use of LIF in culture media to modulate the survival and growth of human and other animal species such as cattle germ cells and embryonic cells, for
15 example, for use in in vitro fertilization and other procedures.

According to another aspect of the present invention, there is provided a method for the isolation of embryonic stem (ES) cells from avian embryos in vitro which
20 method comprises deriving and maintaining said embryos in culture medium containing an effective amount of leukaemia inhibitory factor (LIF) for a time and under conditions sufficient for the development of said ES cells.

According to still another aspect of the present invention, there is provided a method for maintaining avian embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype which process comprises culturing
25 said cells in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF) under conditions
30 sufficient to maintain said cells.

According to yet another aspect of the present invention, there is provided a composition containing a substantially homogeneous population of embryonic stem (ES) cells in a medium comprising isolated or purified leukaemia inhibitory factor (LIF), wherein said ES cells have been isolated from avian embryos cultured in a culture medium containing an effective amount of LIF, and wherein said ES cells maintain a pluripotential phenotype.

According to a further aspect of the present invention, there is provided use of leukaemia inhibitory factor (LIF) for maintaining the pluripotential phenotype of avian embryonic stem (ES) cells.

The present invention may also be described by reference to the following figures:

Figure 1 is a graphical representation showing the effect on ES cells of different concentrations of LIF;

Figure 2 is a pictorial representation showing ES cell morphology in the presence and absence of LIF;

Figure 3 is a graphical (A and C) and pictorial (B) representation showing the binding of ^{125}I -LIF to ES cells (EKcs-1) and EC cells (F9 and PCC3-A).

The present invention is directed to a method for the isolation and maintenance of embryonic stem (ES) cells from animal embryos in vitro which method comprises deriving and/or maintaining said ES cells from said embryos in culture medium containing an effective amount of leukaemia inhibitory factor (LIF), for a time and under conditions sufficient for the derivation and/or maintenance of said ES cells. The animal embryos may be isolated from a number of animal species such as humans, mice, sheep, pigs, cattle, goats and

birds (eg. chickens). Reference herein to "animal embryos" includes reference to "animal blastocysts". Furthermore, the present invention is exemplified using human LIF with murine ES cells (heterologous system) and murine LIF with murine ES
5 cells (homologous system). This is done with the understanding that the present invention contemplates LIF from any animal species in heterologous or homologous systems with animal embryos from animal species such as humans, mice, sheep, pigs, cattle, goats and birds (eg. chickens).
10 Although in certain circumstances,

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a heterologous system will work effectively, it may be preferable to use homologous systems. Given the teachings herein, it will be routine for the skilled technician to ascertain whether a homologous or heterologous system is required in order to isolate or maintain particular animal ES cells.

By "culture medium" is meant a suitable medium capable of supporting growth of ES cells. Examples of suitable culture media useful in practising the present invention are Eagle's medium or modifications or equivalents thereof such as Dulbecco's or Glasgows modified Eagle's medium with supplements such as 5% to 30% (v/v) foetal calf serum and where necessary 0.01 to 1.0 mM β -mercaptoethanol but preferably about 0.1 mM β -mercaptoethanol. The culture medium may or may not contain feeder cells and LIF may be used to substitute for, or add to, said feeder cells. When required, LIF, or more particularly synthetic or recombinant LIF, is added to the medium at a concentration of about 100 to 1,000,000 units/ml and preferably about 100 to 100,000 units/ml and even more preferably 500 to 10,000 units/ml where 50 units are defined as the amount of LIF which in one millilitre induces a 50% reduction in clone formation by murine M1 myeloid cells. By "recombinant LIF" is meant the LIF prepared by genetic engineering means such as, for example, according to International Patent Application No. PCT/AU88/00093 where a number of hosts such as bacteria (e.g. E. coli) or yeast cells may be employed. In accordance with the present invention, the effective derivation time is from 1 day to 20 weeks and particularly from 1 to 8 weeks.

Another aspect of the present invention contemplates a

process for maintaining animal ES cells in vitro while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of LIF under conditions sufficient to
5 maintain said cells. The ES cells in accordance with this aspect of the invention include cells derived from humans, mice, sheep, pigs, cattle, goats and birds (eg. chickens). As with the isolation of ES cells from animal embryos, the LIF used in the aforementioned process is preferably
10 recombinant LIF. The culture medium may or may not contain feeder cells.

In accordance with the present invention, "pluripotential cells" and "embryonic stem cells" are those which retain the developmental potential to differentiate into all somatic and germ cell lineages.

5 The ability of recombinant LIF to maintain the stem cell phenotype of ES cells is demonstrated by transferring ES cells D3 and HD5 into normal cell culture medium in the presence of varying concentrations of purified yeast-derived recombinant human LIF (rY-HLIF) or
10 E. coli - derived recombinant mouse LIF (rE-MLIF). At concentrations of 1000 - 5000 units/ml of rY-HLIF or rE-MLIF more than 90% of the D3 and HD5 ES cells retained their stem cell phenotype. In contrast, the ES cells maintained in normal culture medium differentiated over a
15 period of 3 - 6 days. The proportion of colonies having the stem cell phenotype was related to the concentration of LIF in the culture medium. In addition to maintaining established ES cell lines, six new ES cell lines (MBL-1,2,3,4,5 & 6) were isolated from blastocysts in the
20 absence of feeder cells when the media was supplemented with 1000 units/ml rE-HLIF. Long term maintenance of the ES cell lines D3, HD5 and MBL-1 to 6 in LIF for up to 22 passages (approximately 100 cell generations or 10 weeks) did not noticeably alter the growth characteristics of
25 these ES cells or their dose dependency on LIF. The ability of these ES cells to differentiate into all somatic and germ cell lineages was confirmed by reintroduction of D3 and MBL-1 cells into blastocysts. Approximately 50% of the progeny analysed contained
30 tissues derived from the injected ES cells with levels of overt chimaerism as high as 90% in individual mice. To test for germline transmission of ES derived cells male chimaeras were mated to C57BL/6J mice. Three D3 and two MBL-1 C57BL/6J chimaeras gave rise to agouti progeny
35 confirming that these ES cells can contribute to the formation of germ cells.

The present invention also relates to chimaeric animals generated by known techniques using the ES cells contemplated herein. These ES cells may be isolated from animal embryos and/or maintained in vitro according to the
5 subject invention. Furthermore, genetically manipulated ES cells may be passaged in LIF and used to make chimaeric animals. For example, genetically manipulated ES cells containing a retrovirus vector (N-TK527; derived from pXT1; C.A. Boulter and E.F. Wagner, (1987) Nucl. Acids
10 Res. 15:7194) encoding genes for neomycin resistance and c-src⁵²⁷ were propagated in the presence of LIF but in the absence of feeder cells for over 20 passages. These cells still retained the ability to differentiate as judged by the formation of normal chimaeras following introduction
15 of these cells into preimplantation embryos by blastocyst injection.

Further details of the use of LIF in accordance with the present invention will be apparent from the following Examples.

20

EXAMPLE 1

This example sets out the steps used to maintain ES cells in vitro in LIF, and to generate chimaeric mice using ES cells so passaged.

25

Step 1: Propagation in vitro:

The ES cells used were the D3 (Doetschman, T.C. et.al. (1985) J.Embryol.Exp.Morphol. 87:27-45) the EKcs-1 (previously known as CS1) (Wagner, E.F. et.al. (1985) Cold
30 Spring Harbor Symp.Quant.Biol. 50:691-700) and the HD5 (C. Stewart, unpublished) ES cell lines isolated from 129 SV He blastocysts and the CBL63 (R.Kemler, unpublished) ES cells isolated from C57BL/6J blastocysts. Prior to

culture in LIF, the D3 and CBL63 cells were maintained in Dulbecco modified Eagles medium with 15% (v/v) foetal calf serum on a feeder layer of primary embryo fibroblasts, and the EKcs-1 and HD5 ES cells were maintained in Eagle's 5 medium with 15% (v/v) foetal calf serum and 0.1 mM β -mecraptoethanol, in the presence of medium conditioned by the bladder carcinoma cell line 5637 (ATCC No.HTB9).

The ability of recombinant LIF to maintain the stem cell phenotype of ES cells was demonstrated by 10 transferring ES cells of the lines D3 and HD5 into normal cell culture medium in the presence of varying concentrations of purified yeast-derived recombinant human LIF (hereafter referred to as rY-HLIF), or E.coli derived recombinant mouse LIF (rE-MLIF) (previously disclosed in 15 International Patent Application No. PCT/AU88/00093). The results are shown in Figures 1 and 2. In Figure 1A, HD5 cells previously maintained in 80% 5637 conditioned medium for eight passages were transferred to culture media containing 0-5,000 units ml⁻¹ of purified, recombinant 20 yeast-derived human LIF (H-LIF; see below) (■-■) or purified, recombinant E. coli-derived mouse LIF (M-LIF; see below) (○-○). HD5 cells maintained in medium containig 1,000 units ml⁻¹ H-LIF for a further 13 passages were then transferred to 0-1,000 units ml⁻¹ M-LIF (●-●). 25 In Figure 1B, D3 cells maintined on mouse embryo fibroblasts for 10 passages were transferred to media containing 1,000-5,000 units ml⁻¹ H-LIF and after a further 7 or 15 passages the cells were transferred into media containing 0-5,000 units ml⁻¹ of H-LIF (■-■) or 30 0-1,000 units ml⁻¹ M-LIF (●-●) respectively. Figure 2 shows ES cell morphology in the presence of recombinant LIF. HD5 ES cells cultured in the presence of 80% 5637 conditioned medium were assayed for the ability of purified recombinant LIF to maintain the stem-cell

phenotype by transfer to media containing 1,000 units ml⁻¹ M-LIF (A), or to normal culture media (B). After seven days, the colonies were stained with Giemsa. Compact stem-cell colonies could be distinguished from diffuse differentiated colonies. D3 cells maintained in H-LIF for 15 passages were assayed for the ability to differentiate by transfer into media containing 1,000 units ml⁻¹ M-LIF (C) or normal culture media (D). Immunofluorescence of the cells in the two D3 colony types was carried out using the ECMA-7 monoclonal antibody which recognizes a stem cell-specific cell-surface antigen. Cell-surface-specific immunofluorescence was detected on over 90% of the cells maintained in media containing 1,000 units ml⁻¹ recombinant LIF (E) but on less than 1% of the cells maintained in normal culture media (F). The field of view shown in (F) contains 21 cells.

Figures 1 and 2 indicate that over 90% of the ES cells maintained in 1000-5000 units/ml rY-HLIF or rE-MLIF retained their stem cell phenotype. In contrast, ES cells maintained in normal culture medium differentiated over a period of 3-6 days. The different concentrations of rY-HLIF or rE-MLIF used did not result in any noticeable change in cell number after 6 days in culture, indicating that there is no selection for a specific subpopulation able to grow in LIF. Similar results have been obtained using yeast-derived rMLIF also disclosed in International Patent Application No. PCT/AU88/00093. The data in Figure I indicate that human LIF acts on mouse ES cells, as previously described for the action of human LIF on M1 myeloid leukaemic cells (Gough, N.M. et.al. (1988) Proc.Natl.Acad.Sci.USA 85: 2623-2627). The data in Figure I also indicate that the action of LIF on ES cells is independent of glycosylation, as previously described for the action of LIF on M1 myeloid leukaemic cells.

Four ES cell lines, D3, EKcs-1, CBL63 and HD5, were maintained in medium containing 1000-5000 u/ml rY-HLIF for up to 22 passages (10 weeks or approximately 100 generations). Long-term maintenance of the ES cells in rY-HLIF did not noticeably alter the growth characteristics of the cells. Furthermore, reduction or removal of the LIF from the culture medium resulted in the differentiation of the ES cells with similar kinetics to those explanted directly from bladder carcinoma 5637 conditioned medium or a feeder layer of mouse fibroblasts (for example, see Figures 1 and 2). The stem cell phenotype of ES cells cultured for multiple passages in the presence of LIF was confirmed by immunofluorescence with the ECMA-7 antibody which recognises a cell-surface stem-cell-specific antigen (Kemler, R. in Progress in Developmental Biology Band 26 Sauer, H. W.ed page 175; Fisher, Stuttgart, 1980); ES cells cultured in the presence of LIF expressed the stem cell marker, whereas in the absence of LIF less than 1% did so (Figure 2).

20

Step 2: Isolation of ES cell lines:

Murine blastocysts were isolated from 129 Sv He mice at day 4 of development (day 1 = day of plug) into either Dulbecco's or Glasgows modified Eagle's medium with 15% (v/v) foetal calf serum, 0.1mM β -mercaptoethanol and 1000 units/ml of purified rE-HLIF. ES cell lines were then isolated by two different methodologies.

In the first method the blastocysts were allowed to attach to the culture dish and approximately 7 days later the outgrowing inner cell mass picked, trypsinised and transferred to another culture dish in the same culture media. ES cell colonies appeared 2 - 3 weeks later with between 5 - 7 individual colonies arising from each explanted inner cell mass. The ES cell lines were then

expanded for further analysis. The second method for isolation of ES cell lines used the immunosurgery technique (described in Martin, G. R. (1981) Proc. Natl. Acad. Sci. USA 78:7634-7638) where the trophectoderm cells
5 are destroyed using anti-mouse antibodies prior to explanting the inner cell mass. The efficiency of ES cell lines isolation is shown in Table 1.

Step 3: Generation of Chimaeric Mice:

10 All the ES cell lines cultured in the absence of feeder cells but in the presence of LIF (referred to in step 1) or directly isolated with the aid of culture medium containing LIF (referred to in step 2) retained the ability to differentiate into multiple cell types
15 following the removal of LIF indicating that these cells have retained their pluripotential phenotype. To confirm their developmental potential, D3 ES cells maintained in LIF for 7-22 passages and MBL-1 ES cells maintained in LIF for 14-17 passages were reintroduced into the embryonic
20 environment by blastocyst injection (as described in Williams et al., (1988) Cell 52:121-131). Blastocysts were isolated from the outbred ICR mouse strain or inbred C57BL/6J mice. The expanded blastocysts were maintained in oil-drop cultures at 4°C for 10 min prior to culture.
25 The ES cells were prepared by picking individual colonies, which were then incubated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension was prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum for a further 5 min at
30 4°C. Five to twenty ES cells (in Dulbecco's modified Eagle's Medium with 10% (v/v) foetal calf serum and 3,000 units/ml DNAase 1 buffered in 20 mM HEPES [pH 8]) were injected into each blastocyst. Blastocysts were transferred into pseudopregnant recipients and allowed to

develop normally. Chimaeric mice were identified by coat markers (Hogan et al., (1986) Manipulating the Mouse Embryo, Cold Spring Harbor, New York). Analysis of the subsequent chimaeric mice revealed that up to
5 approximately 50% of the progeny contained tissues derived from the injected cells (Table 2), with levels of overt chimaerism as high as 90% in individual mice. Furthermore analysis of the organs of four D3-chimaeras confirmed that the ES cells maintained in LIF could contribute
10 extensively to the development of all of the somatic tissues (Table 3).

The male chimaeras were tested for germline transmission of ES derived cells by mating to ICR or C57BL/6J females. Three out of four of the D3-C57BL/6J
15 chimaeras and two out of six of the MBL-1-C57BL/6J chimaeras gave rise to agouti offspring derived from the ES cells cultured in LIF (Table 4).

To test whether genetically altered ES cells could be maintained in culture medium containing LIF, D3
20 ES cells were infected with a retrovirus vector (N-TK527) expressing the neomycin resistance gene and a c-src gene mutant (c-src⁵²⁷) (protocol for infection is described in Williams et al., (1988) Cell 52: 121-131). The ES cell clones isolated were maintained in culture medium
25 containing LIF for over 20 passages. These genetically modified ES cells retained the ability to form chimaeric mice following reintroduction into the embryonic environment by blastocyst injection (Table 2)

TABLE 1: Isolation of 129 Sv He ES cell lines in media containing rE-HLIF

Methodology	Blastocyst	ICM outgrowing	Number of ES cell lines derived
5			
Explanted	9	9	4
10 Immunosurgery	11	3	0
Immunosurgery	7	5	2

15 Murine blastocysts were isolated from 129 Sv He mice at day 4 of development (day 1 = day of plug) into either Dulbecco's or Glasgows modified Eagle's medium with 15% (v/v) foetal calf serum, 0.1mM β -mercaptoethanol and 1000 units/ml of purified rE-HLIF. The blastocysts were
 20 then explanted into the same media and left to attach to the culture dish and the inner cell mass picked dissociated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension was prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum
 25 and the cells replated in the cell culture medium described above. The characteristic ES cell colonies appeared within 1 - 3 weeks.

Other blastocysts were treated by immunosurgery (as described in Martin, G. R. (1981) Proc. Natl. Acad. Sci. USA 78:7634-7638). The blastocysts were allowed to
 30 hatch from the zona pelucida, and then treated with anti-mouse antibodies and destroyed by the addition of complement. The exposed inner cell mass was then left to

attach to a tissue culture dish and again treated with anti-mouse antibodies and complement. Within a few days pluripotential stem cell colonies appeared and were dissociated and trypsinised as described above.

Table 2 Chimaeric mice derived from ES cells cultured in LIF

ES cells	Blastocysts transferred	Pups born	Chimaeras
D3	142	60 (42%)	33 (55%)
MBL-1	51	33 (65%)	16 (48%)
D3 N-TK527	42	22 (52%)	12 (54%)

Table 3

Percentage tissue contributions in Individual D3 chimaeric mice														
Chimaera	Necropsy age	C	Bl	Sp	P	Li	T	H	Lu	G	K	M	B	Sa
D3-1	13d	35	0	35	20	10	20	40	30	10	35	30	35	20
D3-2	14d	40	15	35	30	45	30	50	35	20	30	50	50	25
D3-3	11d	90	50	50	35	50	40	60	45	50	50	70	50	55
D3-4	11d	50	50	50	30	40	40	50	50	35	50	50	20	30

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In Table 3, the following abbreviations are used:

C = Coat, Bl = Blood, Sp = Spleen, P = Pancreas, Li = Liver, T = Thymus, H = Heart,
Lu = Lung, G = Gonad, K = Kidney, M = Muscle, B = Brain, Sa = Salivary Gland.

Table 4 Chimaeras demonstrating germline transmission of ES derived cells.

Mice	Chimaerism	Passage No. of D3 cells on feeders	in LIF	129 Sv He	Offspring C57
775-3	75%	10	16	9	24
778-1	70%	10	22	5	33
778-2	50%	10	22	2	36
778-3	55%	10	22	0	0

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The following relates to Tables 2, 3 and 4:

5 D3 and MB1-1 ES cells are derived from 129 Sv He mice (inbred, agouti, homozygous for the glucose phosphate isomerase 1^a allele). The D3 ES cells were originally cultured on primary embryo fibroblasts for 10 passages and then transferred to 1,000-5,000 units/ml recombinant-LIF
10 for 7-22 passages. The MB1-1 ES cells were isolated in the absence of feeder cells but in the presence of rE-HLIF these cells were cultured for 14-17 passages. The ES cells were then injected into ICR (outbred, albino) or C57BL/6J (inbred, black) blastocysts which were then
15 transferred into pseudo-pregnant foster mothers. Both the ICR and C57BL/6J mice are homozygous for the glucose phosphate isomerase 1^b allele. Chimaeric pups were identified by coat pigmentation (only foster mothers which became pregnant were counted in estimating the number of
20 progeny). Tissue chimaerism was estimated using glucose phosphate isomerase strain differences. The extent of tissue chimaerism was determined in two D3-ICR (numbers 1 and 2) and two D3-C57BL/6J chimaeras (numbers 3 and 4). Tissues analysed: C, coat; Bl, blood; Sp, spleen; P,
25 pancreas; Li, liver; T, thymus; H, heart; Lu, lungs; G, gonads; K, kidneys; M, muscle; B, brain; Sa, salivary gland. Male chimaeras were mated to ICR or C57BL/6J mice and offspring identified by coat pigmentation.

EXAMPLE 2

This example sets out the steps used to document specific high affinity receptors on ES and EC cells. Accompanying Figure 3 shows binding of ^{125}I -LIF to ES cells EKcs-1 and EC cells F9 and PCC3-A (Jakob, J. et.al. (1973) Ann.Microbiol.Inst.Pasteur, 124B: 269-282). In relation to Figure 3, (A), Scatchard analysis of ^{125}I -labelled LIF binding to F9 ($\square$), EKcs-1 ($\bullet$), PCC3A-1 ($\blacksquare$) and M1 ($\circ$) cells. Saturation curves for binding were analysed by the method of Scatchard by plotting the amount of LIF specifically bound (defined as the difference between binding observed in the absence and presence of excess unlabelled LIF) versus the ratio of bound to free LIF. Free LIF values were adjusted for the percent of ^{125}I -labelled LIF capable of binding specifically to LIF receptors, in this experiment determined to be 75%. The apparent dissociation constant for the interaction of LIF with its receptor was determined from the slopes of the curves and the receptor number from their intercepts with the ordinate. Results in (A) were standardized to 5×10^6 cells per point and the mean of duplicate points are shown and curves were fitted using the Ligand program. (B), Autoradiography of F9 EC cells labelled with ^{125}I -labelled LIF. (C), Quantitation of silver grains on F9 EC cells after binding of ^{125}I -labelled LIF.

Purified recombinant (yeast-derived) human LIF (rY-HLIF) was radioactively labelled on tyrosine residues as described previously (Hilton, D.J.et.al.(1988) Proc.Natl.Acad.Sci. USA, 85:5971-5975) producing ^{125}I -LIF with a specific radioactivity of approximately 1.2×10^7 cpm/pmole. ^{125}I -LIF ($2 \times 10^3 - 5 \times 10^5$ cpm) was incubated with $1-4 \times 10^6$ target cells with or without at least 100-fold molar excess of unlabelled LIF, in a total volume of 100 μl for 4 hours on ice. Cell-associated and free ^{125}I -LIF were separated by centrifugation through foetal

calf serum (Nicola, N.A. and Metcalf, (1986) D. J. Cell Physiol. 128:160-188). Specific cell-associated ^{125}I -LIF was determined by cold competition.

Figure 3 illustrates the specific saturable and high affinity binding of ^{125}I -LIF to the ES cells EKcs-1 and the EC cells PCC3-A and F9. The number of LIF receptors per cell derived from these Scatchard plots were 295, 190 and 330, respectively, with apparent dissociation constants at 4°C of approximately 90 pM. This compares with the M1 cell line, a LIF-responsive monocytic leukaemia, which displays 50-200 LIF receptors/cell with an apparent dissociation constant of 50-150 pM. All other ES and EC cells tested - D3, NG2, PC13 and P19 - bound similar levels of LIF (data not shown).

The binding of ^{125}I -LIF to M1 cells, EKcs-1 and PCC3-A was also found to be in competition with unlabelled recombinant and native murine and human LIF, but not with the range of other hormones and factors, (including several which act on embryonic cells): insulin, IGF-I, IGF-II, acidic and basic FGF, $\text{TGF}\beta$, $\text{TNF}\alpha$, $\text{TNF}\beta$, NGF, PDGF, EGF, IL-1, IL-4, GM-CSF, G-CSF, Multi-CSF and erythropoietin.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for the isolation of embryonic stem (ES) cells from mammalian embryos in vitro which method comprises deriving and maintaining said embryos in culture medium containing an effective amount of leukaemia inhibitory factor (LIF) for a time and under conditions sufficient for the development of said ES cells.
2. The method according to claim 1 wherein the culture medium is free of feeder cells.
3. The method according to claim 1 wherein the mammalian embryos are derived from humans, mice, sheep, pigs, cattle or goats.
4. The method according to claim 2 wherein the mammalian embryos are derived from humans, mice, sheep, pigs, cattle or goats.
5. The method according to claim 3 wherein the mammalian embryos are derived from mice.
6. The method according to claim 1 wherein the culture medium is Eagle's medium or modifications thereof or equivalents thereto.

7. The method according to claim 2 wherein the culture medium is Eagle's medium or modifications thereof or equivalents thereto.
8. The method according to any one of claims 1 to 7 wherein the LIF is recombinant LIF.
9. The method according to claim 8 wherein the LIF is recombinant human or murine LIF.
10. The method according to claim 9 wherein LIF is added to the culture medium at a concentration of from 10 to 1,000,000 units/ml.
11. The method according to claim 10 wherein the LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.
12. The method according to claim 10 wherein the LIF is added to the culture medium at a concentration of from 500 to 10,000 units/ml.
13. A method according to any one of claims 1 to 7 and 9 to 12 wherein the embryos are cultured in a medium from 1 day to 20 weeks.
14. The method according to claim 13 wherein the embryos are

cultured in a medium from 1 to 8 weeks.

15. A method for maintaining mammalian embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF) under conditions sufficient to maintain said cells.

16. The method according to claim 15 wherein the culture medium is free of feeder cells.

17. The method according to claim 15 wherein the mammalian ES cells are derived from humans, mice, sheep, pigs, cattle or goats.

18. The method according to claim 16 wherein the mammalian ES cells are derived from humans, mice, sheep, pigs, cattle or goats.

19. The method according to claim 17 wherein the mammalian ES cells are derived from mice.

20. The method according to claim 15 wherein the culture medium comprises Eagle's medium or modifications thereof or equivalents thereto.

21. The method according to claim 16 wherein the culture

medium comprises Eagle's medium or modifications thereof or equivalents thereto.

22. The method according to any one of claims 15 to 21 wherein the LIF is recombinant LIF.

5 23. The method according to claim 22 wherein the LIF is recombinant murine or human LIF.

24. The method according to claim 23 wherein the recombinant LIF is added to the culture medium at a concentration of from 10 to 1,000,000 units/ml.

10 25. The method according to claim 24 wherein the recombinant LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.

26. The method according to claim 25 wherein LIF is added to the culture medium at a concentration of from 500 to 10,000
15 units/ml.

27. A composition containing a substantially homogeneous population of embryonic stem (ES) cells in a medium comprising isolated or purified leukaemia inhibitory factor (LIF), wherein said ES cells have been isolated from mammalian embryos
20 cultured in a culture medium containing an effective amount of LIF, and wherein said ES cells maintain a pluripotential phenotype.

28. The composition of claim 27 wherein the culture medium is free of feeder cells.

25 29. The composition of claim 27 or 28 wherein the mammalian embryos are derived from humans, mice, sheep, pigs, cattle or goats.

30. The composition of claim 29 wherein the mammalian embryos are derived from mice.

31. Use of leukaemia inhibitory factor (LIF) for maintaining the pluripotential phenotype of embryonic stem (ES) cells.
32. The use according to claim 31 wherein LIF is added to
5 a culture medium comprising ES cells in an effective amount and under conditions sufficient to maintain said pluripotential phenotype.
33. The use according to claim 32 wherein the culture medium is free of feeder cells.
- 10 34. The use according to any one of claims 31 to 33 wherein the ES cells are derived from humans, mice, sheep, pigs, cattle or goats.
35. The use according to claim 34 wherein the ES cells are derived from mice.
- 15 36. The use according to claim 32 or 33 wherein the culture medium comprises Eagle's medium or modifications thereof or equivalents thereto.
37. The use according to any one of claims 31 to 36 wherein the LIF is recombinant LIF.
- 20 38. The use according to claim 37 wherein the LIF is recombinant murine or human LIF.
39. The use according to claim 37 or 38 wherein the recombinant LIF is added to the culture medium at a concentration of from 10 to 1,000,000 units/ml.
- 25 40. The use according to claim 37 or 38 wherein the recombinant LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.

41. The use according to claim 37 or 38 wherein LIF is added to the culture medium at a concentration of from 500 to 10,000 units/ml.
42. A method for the isolation of embryonic stem (ES) cells from avian embryos in vitro which method comprises deriving and maintaining said embryos in culture medium containing an effective amount of leukaemia inhibitory factor (LIF) for a time and under conditions sufficient for the development of said ES cells.
43. The method according to claim 42 wherein the culture medium is free of feeder cells.
44. The method according to claim 42 or claim 43 wherein the avian embryos are derived from chickens.
45. A method for maintaining avian embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF) under conditions sufficient to maintain said cells.
46. The method according to claim 45 wherein the culture medium is free of feeder cells.
47. The method according to claim 45 or claim 46 wherein the avian ES cells are derived from chickens.
48. The method according to any one of claims 42 to 47 wherein the culture medium is Eagle's medium or modifications thereof or equivalents thereto.
49. The method according to any one of claims 42 to 48 wherein the LIF is recombinant LIF.

50. The method according to any one of claims 42 to 49 wherein the LIF is recombinant human or murine LIF.
51. The method according to claim 49 or claim 50 wherein LIF is added to the culture medium at a concentration
5 of from 10 to 1,000,000 units/ml.
52. The method according to claim 51 wherein the LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.
53. The method according to claim 51 wherein the LIF is
10 added to the culture medium at a concentration of from 500 to 10,000 units/ml.
54. The method according to any one of claims 42 to 53 wherein the embryos are cultured in a medium from 1 day to 20 weeks.
- 15 55. The method according to claim 54 wherein the embryos are cultured in a medium from 1 to 8 weeks.
56. A composition containing a substantially homogeneous population of embryonic stem (ES) cells in a medium comprising isolated or purified leukaemia inhibitory
20 factor (LIF), wherein said ES cells have been isolated from avian embryos cultured in a culture medium containing an effective amount of LIF, and wherein said ES cells maintain a pluripotential phenotype.
57. The composition of claim 56 wherein the culture
25 medium is free of feeder cells.
58. The composition of claim 56 or claim 57 wherein the avian embryos are derived from chickens.

59. Use of leukaemia inhibitory factor (LIF) for maintaining the pluripotential phenotype of avian embryonic stem (ES) cells.

60. The use according to claim 59 wherein the LIF is
5 added to a culture medium comprising avian ES cells in an effective amount and under conditions sufficient to maintain said pluripotential phenotype.

61. The use according to claim 60 wherein the culture medium is free of feeder cells.

10 62. The use according to claim 60 or claim 61 wherein the culture medium comprises Eagle's medium or modifications thereof or equivalents thereto.

63. The use according to any one of claims 60 to 62 wherein the LIF is added to the culture medium at a
15 concentration of from 10 to 1,000,000 units/ml.

64. The use according to claim 63 wherein the LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.

65. The use according to claim 63 wherein LIF is added
20 to the culture medium at a concentration of from 500 to 10,000 units/ml.

66. The use according to any one of claims 59 to 65 wherein the ES cells are derived from chickens.

67. The use according to any one of claims 59 to 66
25 wherein the LIF is recombinant LIF.

68. The use according to claim 67 wherein the LIF is recombinant murine or human LIF.

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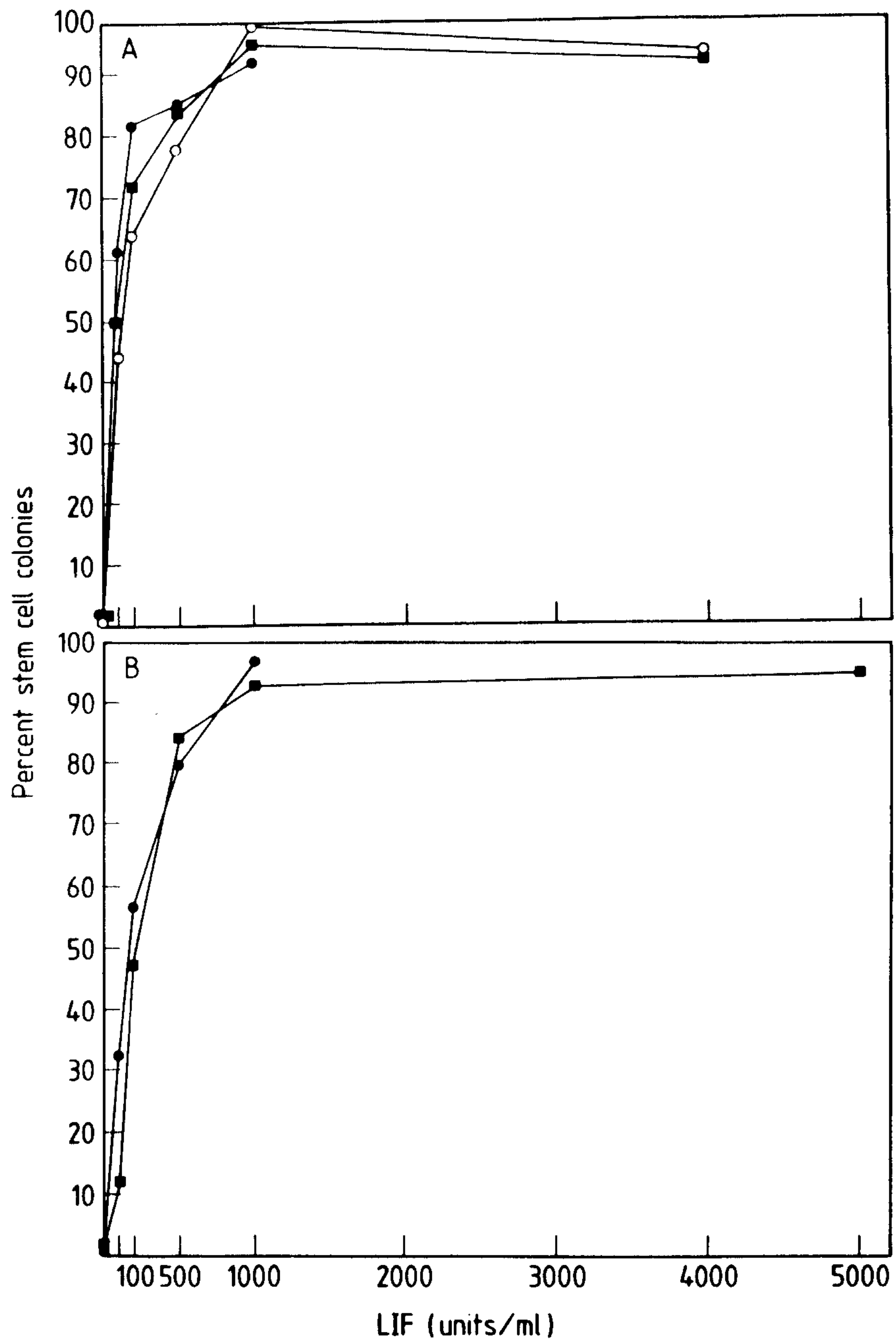


Fig.1.

Application number / numéro de demande: 1341469

Figures: 2, 3

Pages: _____

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received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au
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