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(54) Title: EXPANSION OF STEM AND PROGENITOR CELLS BY BETA-CATENIN

(57) Abstract: Mammalian progenitor or stem cells are expanded *in vitro* by increasing the levels of  $\beta$ -catenin in the cell. The expanded cells substantially maintain their original phenotype including the ability to give rise to multiple types of differentiated cells. The intracellular levels of  $\beta$ -catenin may be manipulated by providing exogenous  $\beta$ -catenin protein to the cell, or by introduction into the cell of a genetic construct encoding  $\beta$ -catenin. The  $\beta$ -catenin may be a wild-type or stabilized mutant form of the protein. Preferably the long term cell culture medium substantially lacks stromal cells and cytokines.

## EXPANSION OF STEM AND PROGENITOR CELLS BY BETA-CATENIN

## INTRODUCTION

Beta-catenin is a pivotal player in the signaling pathway initiated by Wnt proteins, which are mediators of several developmental processes. Beta-catenin activity is controlled  
5 by a large number of binding partners that affect the stability and the localization of beta-catenin, and it is thereby able to participate in such varying processes as gene expression and cell adhesion. Activating mutations in beta-catenin and in components regulating its stability have been found to contribute to upregulation of cell proliferation.

The  $\beta$ -catenin protein becomes stabilized in response to Wnt/Wg, moves to the  
10 nucleus and forms complexes with the LEF1/TCF transcription factors to regulate gene expression. The level of cytosolic  $\beta$ -catenin is determined by its interaction with a number of proteins including those in a multiprotein complex of Axin, GSK-3 $\beta$ , APC and other proteins. The mechanism by which the Wnt signal is transmitted to this complex is unclear but it involves interaction of Wnt with its receptors, which are members of Frizzled family of seven  
15 transmembrane proteins. The stabilization of  $\beta$ -catenin stimulates the expression of genes including c-myc, c-jun, fra-1, and cyclin D1. This pathway is negatively regulated by Axin.

Beta-catenin is also an adherens junction protein. Adherens junctions are critical for the establishment and maintenance of epithelial layers, such as those lining organ surfaces. AJs mediate adhesion between cells, communicate a signal that neighboring cells are  
20 present, and anchor the actin cytoskeleton. In serving these roles, AJs regulate normal cell growth and behavior. At several stages of embryogenesis, wound healing, and tumor cell metastasis, cells form and leave epithelia. This process, which involves the disruption and reestablishment of epithelial cell-cell contacts, may be regulated by the disassembly and assembly of AJs. AJs may also function in the transmission of the 'contact inhibition' signal,  
25 which instructs cells to stop dividing once an epithelial sheet is complete.

For many purposes, there is an interest in being able to expand stem and progenitor cells in culture. However, it is not simply a matter of maintaining cell viability for the stem cells, but also of ensuring that the stem cells increase in numbers without losing their distinctive phenotype. Current protocols for the *in vitro* culture of hematopoietic stem cells  
30 generally require one or a cocktail of cytokines, such as c-kit ligand (stem cell growth factor), flt-3, thrombopoietin, IL-6, etc. While a substantial increase in cell number can be obtained with such cultures, they do not provide for expanded number of cells that retain a capacity for long term repopulation of all hematopoietic lineages. See Domen and Weissman (1999) Mol Med Today 5(5):201-8; or Ziegler and Kanz (1998) Curr Opin Hematol 5(6):434-40.

Stem cells have also been grown in co-culture with stromal cells. However, it is particularly desirable to expand stem cells in a culture of known composition, rather than relying upon the presence of other cells for their maintenance.

There continues to be a strong demand for improvements in the *in vitro* culture of stem cells and progenitor cells. The present invention addresses this need.

#### SUMMARY OF THE INVENTION

Methods are provided for the expansion of progenitor or stem cells *in vitro*, whereby the cells retain their pluripotential phenotype after expansion. The intracellular level of  $\beta$ -catenin is increased in the cells in culture, either by providing exogenous  $\beta$ -catenin protein to the cell, or by introduction into the cell of a genetic construct encoding  $\beta$ -catenin. The  $\beta$ -catenin may be a wild type protein appropriate for the species from which the cells are derived, or preferably, a stabilized mutant form of the protein. The alteration in cellular levels of  $\beta$ -catenin provide for increased number of cells in cycle, and leads to cultures that containing proliferating cells that maintain an undifferentiated phenotype *in vitro*. The expanded cell populations are useful as a source of stem cells, *e.g.* to reconstitute function in a host that is deficient in a particular cell lineage or lineages. In one embodiment of the invention, the target cells are hematopoietic stem cells, which may be used in transplantation to restore hematopoietic function to autologous or allogeneic recipients.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Activated beta-catenin retrovirus induces increased growth of stem cells. Stem cells infected with control or beta-catenin-GFP retrovirus were sorted and cultured on 96 well plates for two days in the presence or absence of steel factor, and cell numbers were counted at the end of the culture period.

Figure 2. Stem cells infected with beta-catenin retain many stem cell markers in long term culture. Beta-catenin infected stem cell spheres were harvested from long term cultures at 5 weeks, trypsinized and allowed to express their surface proteins for 12 hours. Subsequently they were harvested and stained with antibodies to Thy1.1, Sca1, c-kit, and lineage antigens (B220, Mac-1, Gr-1, Ter119, CD5, CD3, CD8/4).

Figure 3. Stem cells infected with beta-catenin have the ability to give rise to multiple lineages when transplanted. 100,000 beta-catenin infected stem cells were harvested from long term cultures at 7 weeks, trypsinized and injected into lethally irradiated (950 Rads) allotype marked recipients along with 300,000 rescuing bone marrow cells from the host. Analysis of reconstitution along various lineages was carried out at 4 weeks after transplantation.

## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Mammalian progenitor or stem cells are expanded *in vitro* by increasing the levels of  $\beta$ -catenin in the cell. The intracellular levels of  $\beta$ -catenin may be manipulated by providing exogenous  $\beta$ -catenin protein to the cell, or by introduction into the cell of a genetic construct  
5 encoding  $\beta$ -catenin. The  $\beta$ -catenin may be a wild-type or stabilized mutant form of the protein. Preferably the long term cell culture medium substantially lacks stromal cells and cytokines. Cultures that provide stem cell activity can be obtained for at least three weeks, frequently six weeks and can be eight weeks or more. The culture media that are employed are conventional media for the growth of mammalian cells, optionally in the absence of  
10 serum using only defined protein factors. In the absence of the  $\beta$ -catenin, the medium is inefficient at maintaining growth of the undifferentiated cells.

In the first few days of culture, the expansion of stem/progenitor cells is limited, usually the number of phenotypic stem/progenitor cells is maintained, or slightly increased. After 2 to 3 weeks in the subject culture conditions, there is a substantial proliferation of cells  
15 having the desired phenotype, where the number of cells having a functional stem/progenitor cell phenotype is expanded.

## DEFINITIONS

It is to be understood that this invention is not limited to the particular methodology,  
20 protocols, cell lines, animal species or genera, and reagents described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

As used herein the singular forms "a", "and", and "the" include plural referents unless  
25 the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the culture" includes reference to one or more cultures and equivalents thereof known to those skilled in the art, and so forth. All technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs unless clearly indicated otherwise.

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*$\beta$ -catenin*: The term  $\beta$ -catenin, as used herein, is intended to refer to both wild-type and stabilized forms of the  $\beta$ -catenin protein, and to fusion proteins and derivatives thereof. Usually the protein will be of mammalian origin, although the protein from other species may find use. The protein is conserved between species, for example the human sequence is  
35 active in mouse cells. The sequences of many  $\beta$ -catenin proteins are publicly known. For convenience, the sequences of the human and mouse homologs of this protein are provided

in the sequence listing, as SEQ ID NO:1; and SEQ ID NO:2, respectively. In one embodiment of the invention, a stabilized form of beta-catenin is used.

The ubiquitin-dependent proteolysis system is involved in the regulation of beta-catenin turnover. Beta-catenin becomes stabilized when proteasome-mediated proteolysis is inhibited and this leads to the accumulation of multi-ubiquitinated forms of beta-catenin (Aberle et al. (1997) EMBO J 16(13):3797-804). Substitution of the serine residues in the glycogen synthase kinase 3 $\beta$  (GSK3beta) phosphorylation consensus motif of beta-catenin inhibits ubiquitination and results in stabilization of the protein. Examples of stabilized  $\beta$ -catenins include those with the amino acid changes D32Y; D32G; S33F; S33Y; G34E; S37C; S37F; T41I; S45Y; and deletion of AA 1-173. A number of publications describe stabilized  $\beta$ -catenin mutations. For example, see Morin et al. (1997) Science 275(5307):1787-90; Palacios et al. (1998) Cancer Res 58(7):1344-7; Muller et al. (1998) Genes Chromosomes Cancer 22(1):37-41; Miyoshi et al. (1998) Cancer Res 58(12):2524-7; Zurawel et al. (1998) Cancer Res. 58, 896-899; Voeller et al. (1998) Cancer Res. 58, 2520-2526; etc.

The sequence of the beta-catenin polypeptide may be altered in various ways known in the art to generate targeted changes in sequence. The polypeptide will usually be substantially similar to the sequences provided herein, i.e. will differ by at least one amino acid, and may differ by at least two but not more than about ten amino acids. Deletions may further include larger changes, such as deletions of a domain or exon, providing for active peptide fragments of the protein. Other modifications of interest include tagging, e.g. with the FLAG system, HA, green fluorescent protein, etc. Such alterations may be used to alter properties of the protein, by affecting the stability, specificity, etc. The protein may be joined to a wide variety of other oligopeptides or proteins for a variety of purposes, particular for facilitating transport across membranes.

Techniques for *in vitro* mutagenesis of cloned genes are known. Examples of protocols for scanning mutations may be found in Gustin et al., Biotechniques 14:22 (1993); Barany, Gene 37:111-23 (1985); Colicelli et al., Mol Gen Genet 199:537-9 (1985); and Prentki et al., Gene 29:303-13 (1984). Methods for site specific mutagenesis can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, CSH Press 1989, pp. 15.3-15.108; Weiner et al., Gene 126:35-41 (1993); Sayers et al., Biotechniques 13:592-6 (1992); Jones and Winistorfer, Biotechniques 12:528-30 (1992); Barton et al., Nucleic Acids Res 18:7349-55 (1990); Marotti and Tomich, Gene Anal Tech 6:67-70 (1989); and Zhu Anal Biochem 177:120-4 (1989).

*Expression construct:* In one embodiment of the invention, the beta-catenin is delivered to the targeted stem or progenitor cells by introduction of an exogenous nucleic acid expression vector into the cells. Many vectors useful for transferring exogenous genes

into target mammalian cells are available. The vectors may be episomal, e.g. plasmids, virus derived vectors such as cytomegalovirus, adenovirus, etc., or may be integrated into the target cell genome, through homologous recombination or random integration, e.g. retrovirus derived vectors such as MMLV, HIV-1, ALV, etc.

5 Retrovirus based vectors have been shown to be particularly useful when the target cells are hematopoietic stem cells. For example, see Baum *et al.* (1996) *J Hematother* 5(4):323-9; Schwarzenberger *et al.* (1996) *Blood* 87:472-478; Nolta *et al.* (1996) *P.N.A.S.* 93:2414-2419; and Maze *et al.* (1996) *P.N.A.S.* 93:206-210. Lentivirus vectors have also been described for use with hematopoietic stem cells, for example see Mochizuki *et al.*  
10 (1998) *J Virol* 72(11):8873-83. The use of adenovirus based vectors with hematopoietic cells has also been published, see Ogniben and Haas (1998) Recent Results Cancer Res 144:86-92.

Various techniques known in the art may be used to transfect the target cells, e.g. electroporation, calcium precipitated DNA, fusion, transfection, lipofection and the like. The  
15 particular manner in which the DNA is introduced is not critical to the practice of the invention.

Combinations of retroviruses and an appropriate packaging line may be used, where the capsid proteins will be functional for infecting the target cells. Usually, the cells and virus will be incubated for at least about 24 hours in the culture medium. Commonly used  
20 retroviral vectors are "defective", i.e. unable to produce viral proteins required for productive infection. Replication of the vector requires growth in the packaging cell line.

The host cell specificity of the retrovirus is determined by the envelope protein, env (p120). The envelope protein is provided by the packaging cell line. Envelope proteins are of at least three types, ecotropic, amphotropic and xenotropic. Retroviruses packaged with  
25 ecotropic envelope protein, e.g. MMLV, are capable of infecting most murine and rat cell types. Ecotropic packaging cell lines include BOSC23 (Pear *et al.* (1993) *P.N.A.S.* 90:8392-8396). Retroviruses bearing amphotropic envelope protein, e.g. 4070A (Danos *et al.*, *supra.*), are capable of infecting most mammalian cell types, including human, dog and mouse. Amphotropic packaging cell lines include PA12 (Miller *et al.* (1985) *Mol. Cell. Biol.* 5:431-  
30 437); PA317 (Miller *et al.* (1986) *Mol. Cell. Biol.* 6:2895-2902) GRIP (Danos *et al.* (1988) *PNAS* 85:6460-6464). Retroviruses packaged with xenotropic envelope protein, e.g. AKR env, are capable of infecting most mammalian cell types, except murine cells.

The sequences at the 5' and 3' termini of the retrovirus are long terminal repeats (LTR). A number of LTR sequences are known in the art and may be used, including the  
35 MMLV-LTR; HIV-LTR; AKR-LTR; FIV-LTR; ALV-LTR; etc. Specific sequences may be accessed through public databases. Various modifications of the native LTR sequences are also known. The 5' LTR acts as a strong promoter, driving transcription of the  $\beta$ -catenin

gene after integration into a target cell genome. For some uses, however, it is desirable to have a regulatable promoter driving expression. Where such a promoter is included, the promoter function of the LTR will be inactivated. This is accomplished by a deletion of the U3 region in the 3' LTR, including the enhancer repeats and promoter, that is sufficient to inactivate the promoter function. After integration into a target cell genome, there is a rearrangement of the 5' and 3' LTR, resulting in a transcriptionally defective provirus, termed a "self-inactivating vector".

Suitable inducible promoters are activated in a desired target cell type, either the transfected cell, or progeny thereof. By transcriptional activation, it is intended that transcription will be increased above basal levels in the target cell by at least about 100 fold, more usually by at least about 1000 fold. Various promoters are known that are induced in hematopoietic cell types, e.g. IL-2 promoter in T cells, immunoglobulin promoter in B cells, etc.

Preferred genetic constructs are those that can be removed from the target cells after expansion. This can be accomplished by the use of a transient vector system, or by including a heterologous recombination site that flanks the beta-catenin coding sequence. In this manner, after expansion the construct can be removed prior to use of the expanded cell population. Preferably a detectable marker, e.g. green fluorescent protein, luciferase, cell surface proteins suitable for antibody selection methods, etc. is included in the expression vector, such that after deletion of the construct the cells can be readily isolated that lack the exogenous beta-catenin.

The term "heterologous recombination site" is meant to encompass any introduced genetic sequence that facilitates site-specific recombination. In general, such sites facilitate recombination by interaction of a specific enzyme with two such sites. Exemplary heterologous recombination sites include, but are not necessarily limited to, *lox* sequences with recombination mediated by Cre enzyme; *frt* sequences (Golic et al. (1989) *Cell* 59:499-509; O'Gorman et al. (1991) *Science* 251:1351-5; recombination mediated by the FLP recombinase), the recognition sequences for the pSR1 recombinase of *Zygosaccharomyces rouxii* (Matsuzaki et al. (1990) *J. Bacteriol.* 172:610-8), and the like.

Sequences encoding *lox* sites are of particular interest for use in the present invention. A *lox* site is a nucleotide sequence at which the gene product of the *cre* gene, referred to herein as "Cre," catalyzes site-specific recombination. A particularly preferred *lox* site is a *loxP* site. The sequence of *loxP*, which is 34 bp in length, is known and can be produced synthetically or can be isolated from bacteriophage P1 by methods known in the art (see, e.g. Hoess et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:3398). The *loxP* site is composed of two 13 bp inverted repeats separated by an 8 bp spacer region. Other suitable

*lox* sites include *loxB*, *loxL*, and *loxR*, which can be isolated from *E. coli* (Hoess et al. (1982) *Proc. Natl. Acad. Sci. USA* 22:3398).

In an alternative method, expression vectors that provide for the transient expression in mammalian cells may be used. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient short term expansion of cells, but do not affect the long term genotype of the cell.

*Translocation modified  $\beta$ -catenin*: In some cases it is desirable to provide exogenous  $\beta$ -catenin protein, rather than transducing the cells with an expression construct. The beta-catenin may be added to the culture medium at high levels. Preferably the beta-catenin is modified so as to increase its transport into the cells.

In one embodiment of the invention, tat protein is used to deliver beta-catenin. The preferred transport polypeptides are characterized by the presence of the tat basic region amino acid sequence (amino acids 49-57 of naturally-occurring tat protein); the absence of the tat cysteine-rich region amino acid sequence (amino acids 22-36 of naturally-occurring tat protein) and the absence of the tat exon 2-encoded carboxy-terminal domain (amino acids 73-86 of naturally-occurring tat protein). Transport polypeptides are attached to beta-catenin by chemical cross-linking or by genetic fusion, where the beta-catenin moiety may be a wild-type or stabilized form. A unique terminal cysteine residue is a preferred means of chemical cross-linking.

*Stem cell*: The term stem cell is used herein to refer to a mammalian cell that has the ability both to self-renew, and to generate differentiated progeny (see Morrison *et al.* (1997) *Cell* 88:287-298). Generally, stem cells also have one or more of the following properties: an ability to undergo asynchronous, or symmetric replication, that is where the two daughter cells after division can have different phenotypes; extensive self-renewal capacity; capacity for existence in a mitotically quiescent form; and clonal regeneration of all the tissue in which they exist, for example the ability of hematopoietic stem cells to reconstitute all hematopoietic lineages. "Progenitor cells" differ from stem cells in that they typically do not have the extensive self-renewal capacity, and often can only regenerate a subset of the lineages in the tissue from which they derive, for example only lymphoid, or erythroid lineages in a hematopoietic setting.

Stem cells may be characterized by both the presence of markers associated with specific epitopes identified by antibodies and the absence of certain markers as identified by

the lack of binding of specific antibodies. Stem cells may also be identified by functional assays both *in vitro* and *in vivo*, particularly assays relating to the ability of stem cells to give rise to multiple differentiated progeny.

5 Stem cells of interest include hematopoietic stem cells and progenitor cells derived therefrom (U.S. Pat. No. 5,061,620); neural crest stem cells (see Morrison *et al.* (1999) Cell 96:737-749); embryonic stem cells; mesenchymal stem cells; mesodermal stem cells; *etc.*

Other hematopoietic "progenitor" cells of interest include cells dedicated to lymphoid lineages, e.g. immature T cell and B cell populations. The methods of the present invention are useful in expanding selected populations of these cells.

10 Purified populations of stem or progenitor cells may be used to initiate the cultures. For example, human hematopoietic stem cells may be positively selected using antibodies specific for CD34, thy-1; or negatively selected using lineage specific markers which may include glycophorin A, CD3, CD24, CD16, CD14, CD38, CD45RA, CD36, CD2, CD19, CD56, CD66a, and CD66b; T cell specific markers, tumor specific markers, *etc.* Markers useful for  
15 the separation of mesodermal stem cells include Fc $\gamma$ RII, Fc $\gamma$ RIII, Thy-1, CD44, VLA-4 $\alpha$ , LFA-1 $\beta$ , HSA, ICAM-1, CD45, Aa4.1, Sca-1, *etc.* Neural crest stem cells may be positively selected with antibodies specific for low-affinity nerve growth factor receptor (LNGFR), and negatively selected for the markers sulfatide, glial fibrillary acidic protein (GFAP), myelin protein P<sub>0</sub>, peripherin and neurofilament. Human mesenchymal stem cells may be positively  
20 separated using the markers SH2, SH3 and SH4.

The cells of interest are typically mammalian, where the term refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, laboratory, sports, or pet animals, such as dogs, horses, cats, cows, mice, rats, rabbits, *etc.* Preferably, the mammal is human.

25 The cells which are employed may be fresh, frozen, or have been subject to prior culture. They may be fetal, neonate, adult. Hematopoietic cells may be obtained from fetal liver, bone marrow, blood, particularly G-CSF or GM-CSF mobilized peripheral blood, or any other conventional source. The manner in which the stem cells are separated from other cells of the hematopoietic or other lineage is not critical to this invention. As described  
30 above, a substantially homogeneous population of stem or progenitor cells may be obtained by selective isolation of cells free of markers associated with differentiated cells, while displaying epitopic characteristics associated with the stem cells.

*Culture medium:* The stem or progenitor cells are grown *in vitro* in an appropriate  
35 liquid nutrient medium. Generally, the seeding level will be at least about 10 cells/ml, more usually at least about 100 cells/ml and generally not more than about 10<sup>5</sup> cells/ml, usually not more than about 10<sup>4</sup> cells/ml.

Various media are commercially available and may be used, including Ex vivo serum free medium; Dulbecco's Modified Eagle Medium (DMEM), RPMI, Iscove's medium, etc. The medium may be supplemented with serum or with defined additives. Appropriate antibiotics to prevent bacterial growth and other additives, such as pyruvate (0.1-5 mM), glutamine (0.5-5 mM), 2-mercaptoethanol ( $1-10 \times 10^{-5}$  M) may also be included.

Culture in serum-free medium is of particular interest. The medium may be any conventional culture medium, generally supplemented with additives such as iron-saturated transferrin, human serum albumin, soy bean lipids, linoleic acid, cholesterol, alpha thioglycerol, crystalline bovine hemin, etc., that allow for the growth of hematopoietic cells.

Preferably the expansion medium is free of cytokines, particularly cytokines that induce cellular differentiation. The term cytokine may include lymphokines, monokines and growth factors. Included among the cytokines are thrombopoietin (TPO); nerve growth factors such as NGF- $\beta$ ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- $\alpha$  and TGF- $\beta$ ; erythropoietin (EPO); interferons such as interferon- $\alpha$ , - $\beta$ , and - $\gamma$ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; etc. In some circumstances, proliferative factors that do not induce cellular differentiation may be included in the cultures, e.g. c-kit ligand, LIF, and the like.

#### EXPANSION OF STEM/PROGENITOR CELLS

A population of cells comprising progenitor and/or stem cells is cultured *in vitro* in the presence of enhanced levels of  $\beta$ -catenin, either by genetically altering the cells, or by providing exogenous  $\beta$ -catenin, as described above. The upregulation in  $\beta$ -catenin is sufficient to maintain or increase the number of assayable progenitor cells in the culture. The number of assayable progenitor cells may be demonstrated by a number of assays. After one week the progenitor cell cloning efficiency will usually be at least about 75% that of the starting cell population, more usually 100% that of the starting cell population, and may be as high as 200% that of the starting cell population.

Following the initial period, there is an increased expansion, where the number of assayable cells having the functional phenotype of the initial cell population can increase from about 5 to about 100 fold or more. After this time, the cells can remain in cycle, and expansion is limited primarily by considerations of space. The cells can be frozen using conventional methods at any time, usually after the first week of culture.

Frequently stem cells are isolated from biological sources in a quiescent state. Certain expression vectors, particularly retroviral vectors, do not effectively infect non-cycling cells. Cultures established with these vectors as a source of beta-catenin sequences are

induced to enter the cell cycle by a short period of time in culture with growth factors. For example, hematopoietic stem cells are induced to divide by culture with c-kit ligand, which may be combined with LIF, IL-11 and thrombopoietin. After 24 to 72 hours in culture with cytokines, the medium is changed, and the cells are contacted with the retroviral culture, using culture conditions as described above.

After seeding the culture medium, the culture medium is maintained under conventional conditions for growth of mammalian cells, generally about 37° C and 5% CO<sub>2</sub> in 100% humidified atmosphere. Fresh media may be conveniently replaced, in part, by removing a portion of the media and replacing it with fresh media. Various commercially available systems have been developed for the growth of mammalian cells to provide for removal of adverse metabolic products, replenishment of nutrients, and maintenance of oxygen. By employing these systems, the medium may be maintained as a continuous medium, so that the concentrations of the various ingredients are maintained relatively constant or within a predescribed range. Such systems can provide for enhanced maintenance and growth of the subject cells using the designated media and additives.

These cells may find various applications for a wide variety of purposes. The cell populations may be used for screening various additives for their effect on growth and the mature differentiation of the cells. In this manner, compounds which are complementary, agonistic, antagonistic or inactive may be screened, determining the effect of the compound in relationship with one or more of the different cytokines.

The populations may be employed as grafts for transplantation. For example, hematopoietic cells are used to treat malignancies, bone marrow failure states and congenital metabolic, immunologic and hematologic disorders. Marrow samples may be taken from patients with cancer, and enriched populations of hematopoietic stem cells isolated by means of density centrifugation, counterflow centrifugal elutriation, monoclonal antibody labeling and fluorescence activated cell sorting. The stem cells in this cell population are then expanded *in vitro* and can serve as a graft for autologous marrow transplantation. The graft will be infused after the patient has received curative chemotherapy.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is

weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

#### EXPERIMENTAL

Bone marrow cells from BCl2 transgenic mice were isolated, enriched for c-kit over a magnetic column, and then stained with antibodies to sort the Sca1<sup>+</sup> Thy1.1<sup>lo</sup> c-kit<sup>+</sup> lin<sup>-lo</sup> population on a cell sorter. The cells were double sorted to ensure a high level of purity.

The cells were cultured to initiate cell cycle with Steel factor 100ng/ml with 5% serum in X-Vivo 15 containing the retrovirus. At 3 days 50% of media was replaced with only X-vivo 15, and this dilution was repeated every 2 days. The cells were then cultured with supernatant containing retrovirus encoding activated beta-catenin and recombinant steel factor. The increased growth of the stem cells is shown in Figure 1.

The retroviral supernatant had been generated in commercially available X-vivo 15 media using phoenix cells and a MSCV retroviral construct containing beta-catenin driven by the LTR. The retroviral construct is called MSCV and contains an IRES-GFP, in order to label infected cells. The activating beta catenin mutation is a mutation at the amino terminus that prevents phosphorylation and subsequent degradation by proteosomes. The accumulation of beta catenin in the cytosol allows it to translocate to the nucleus where it associates with the LEF/TCF family of transcription factors to turn on gene expression.

50% of the culture supernatant was replaced every day for 3 days. At the end of this culture period the media was replaced with X-vivo 15. Clusters of cells grew out of this culture, and were analyzed at 5 weeks. By May-Gruenwald-Geimsa staining, these cells appeared to have an immature phenotype with large nuclei and small cytoplasm. By FACS staining a majority of cells are Thy1<sup>lo</sup> Sca-1<sup>+</sup>Lin<sup>lo</sup>/kit<sup>lo</sup>, a phenotype resembling that of stem

cells. About 50% of the cells are Lin<sup>-</sup> (LT-HSC phenotype), and 50% Lin<sup>lo</sup> (ST-HSC phenotype). The analysis is shown in Figure 2.

5 These cells give rise to lineage positive cells at 4 weeks when transplanted into lethally irradiated mice suggesting that they are able to differentiate to various lineages *in vivo*, while remaining immature *in vitro*.

10 Lethally irradiated mice were injected with 300, 000 host bone marrow and 100,000 cultured cells. Peripheral blood was take at a later time, at 2 weeks, 3 weeks and 4 weeks so far. Donor type was marked with Ly5.1+ cells. Level of differentiation was determined by using antibodies to mature lineage markers. The results are shown in Figure 3, demonstrating that stem cells over-expressing  $\beta$ -catenin have the ability to give rise to multiple lineages when transplanted.

## WHAT IS CLAIMED IS:

1. A method for *in vitro* expansion of mammalian stem or progenitor cells, the method comprising:

5 increasing the intracellular concentration of  $\beta$ -catenin in a progenitor or stem cell in an *in vitro* culture medium for a period of time sufficient for said progenitor or stem cell to divide;

wherein the number of cells having the functional phenotype of said stem or progenitor cells is expanded.

10 2. The method of Claim 1, wherein said stem or progenitor cell is a stem cell.

3. The method of Claim 2, wherein said stem cell is a hematopoietic stem cell.

4. The method of Claim 2, wherein said stem cell is a neural crest stem cell.

15

5. The method of Claim 2, wherein said stem cell is a mesenchymal stem cell.

6. The method of Claim 2, wherein said stem cell is an embryonic stem cell.

20

7. The method of Claim 3, wherein said hematopoietic stem cell is a human cell.

8. The method of Claim 1, wherein said step of increasing the intracellular concentration of  $\beta$ -catenin comprises:

25 introduction of an exogenous nucleic acid comprising beta-catenin coding sequences operably linked to a promoter.

9. The method of Claim 8, wherein said beta-catenin is a wild-type beta-catenin.

30 10. The method of Claim 8, wherein said beta-catenin is a stabilized mutant beta-catenin.

11. The method of Claim 8, wherein said exogenous nucleic acid is a retroviral vector.

35 12. The method of Claim 11, wherein said retroviral vector comprises sites for recombination, flanking said beta-catenin coding sequences.

13. The method of Claim 8, wherein said exogenous nucleic acid is an episomal vector.

14. The method of Claim 1, wherein said step of increasing the intracellular concentration of  $\beta$ -catenin comprises:  
5 addition of exogenous beta-catenin to said culture medium.

15. The method of Claim 14, wherein said beta-catenin is a wild-type beta-catenin.  
10

16. The method of Claim 14, wherein said beta-catenin is a stabilized mutant beta-catenin.

17. The method of Claim 14, wherein said beta-catenin is genetically fused to a transport moiety.  
15

18. The method of Claim 17, wherein said transport moiety is a fragment of HIV tat protein.

19. The method of Claim 1, wherein said stem or progenitor cell is a progenitor cell.  
20

20. The method of Claim 19, wherein said progenitor cell is a hematopoietic progenitor cell.  
25

21. The method of Claim 20, wherein said hematopoietic progenitor cell is a lymphoid cell.

22. The method of Claim 21, wherein said lymphoid cell is a B cell.  
30

23. The method of Claim 21, wherein said lymphoid cell is a T cell.

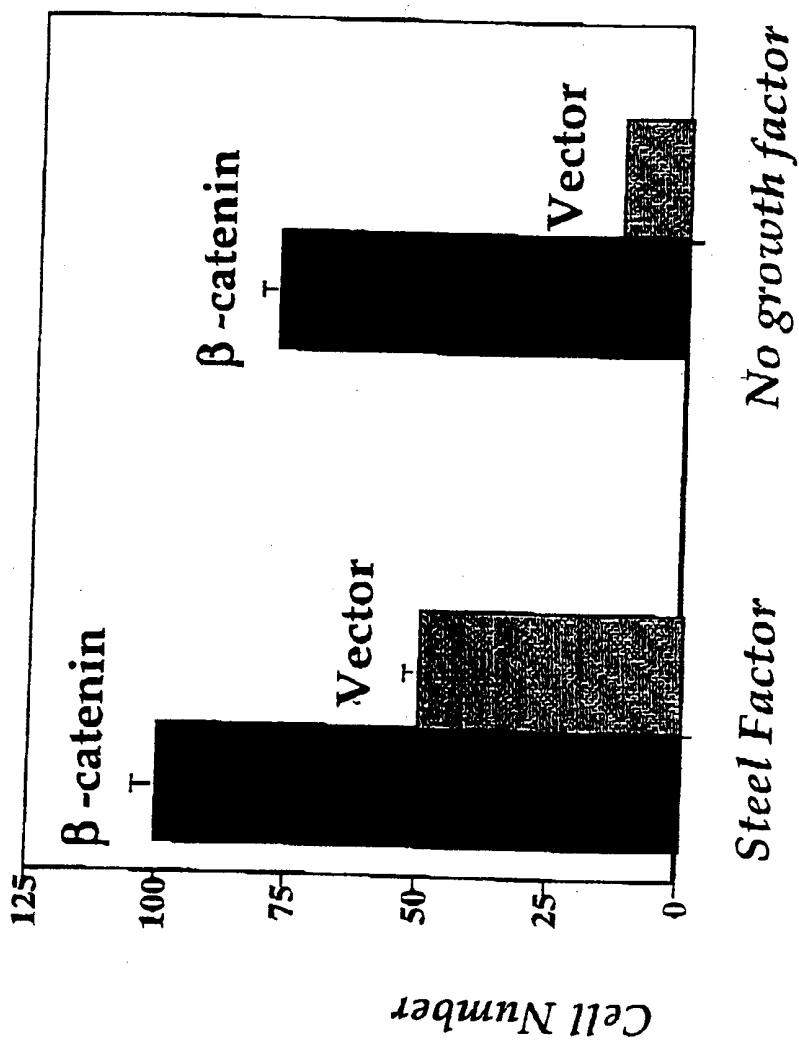


Figure 1

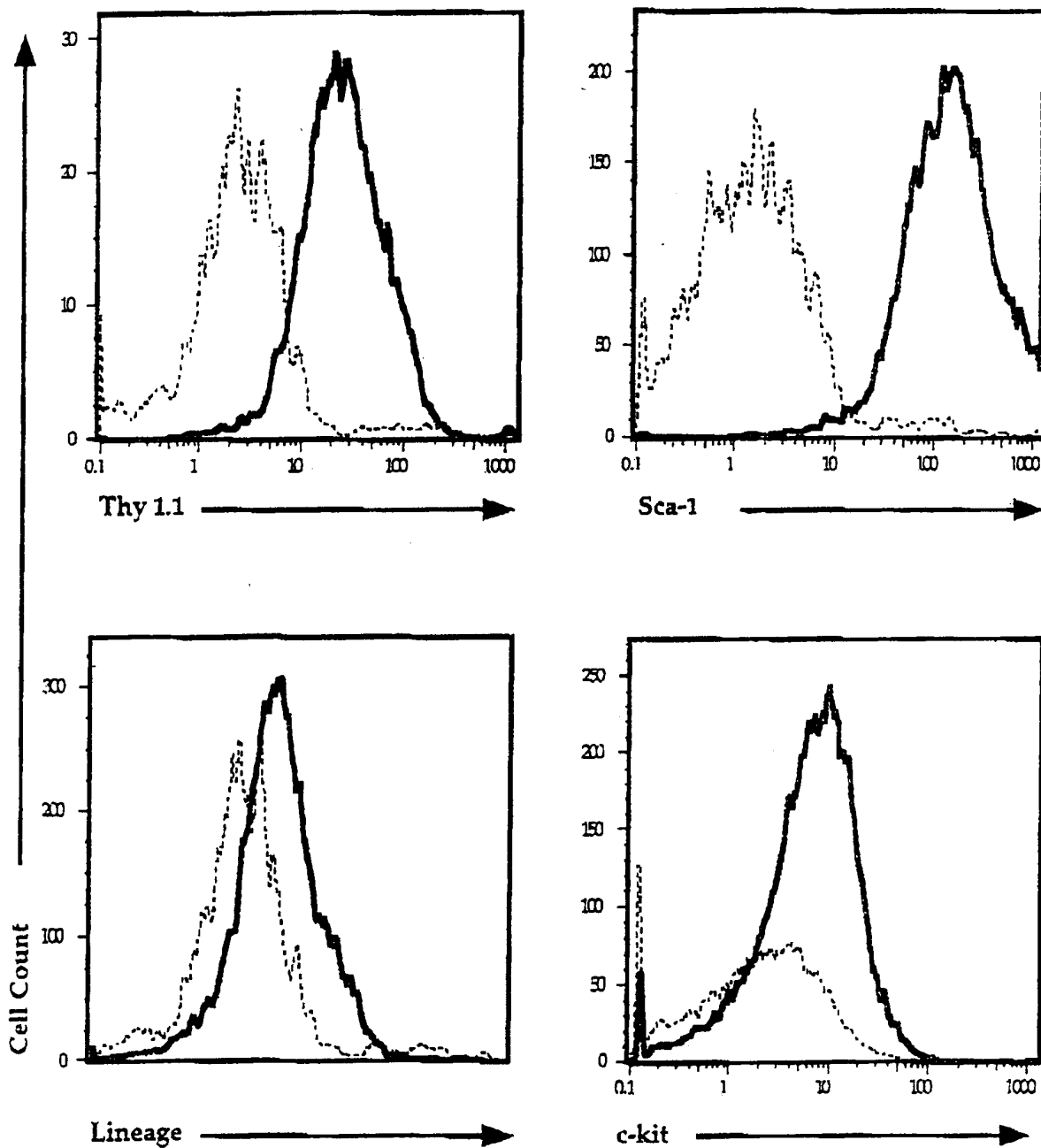


Figure 2

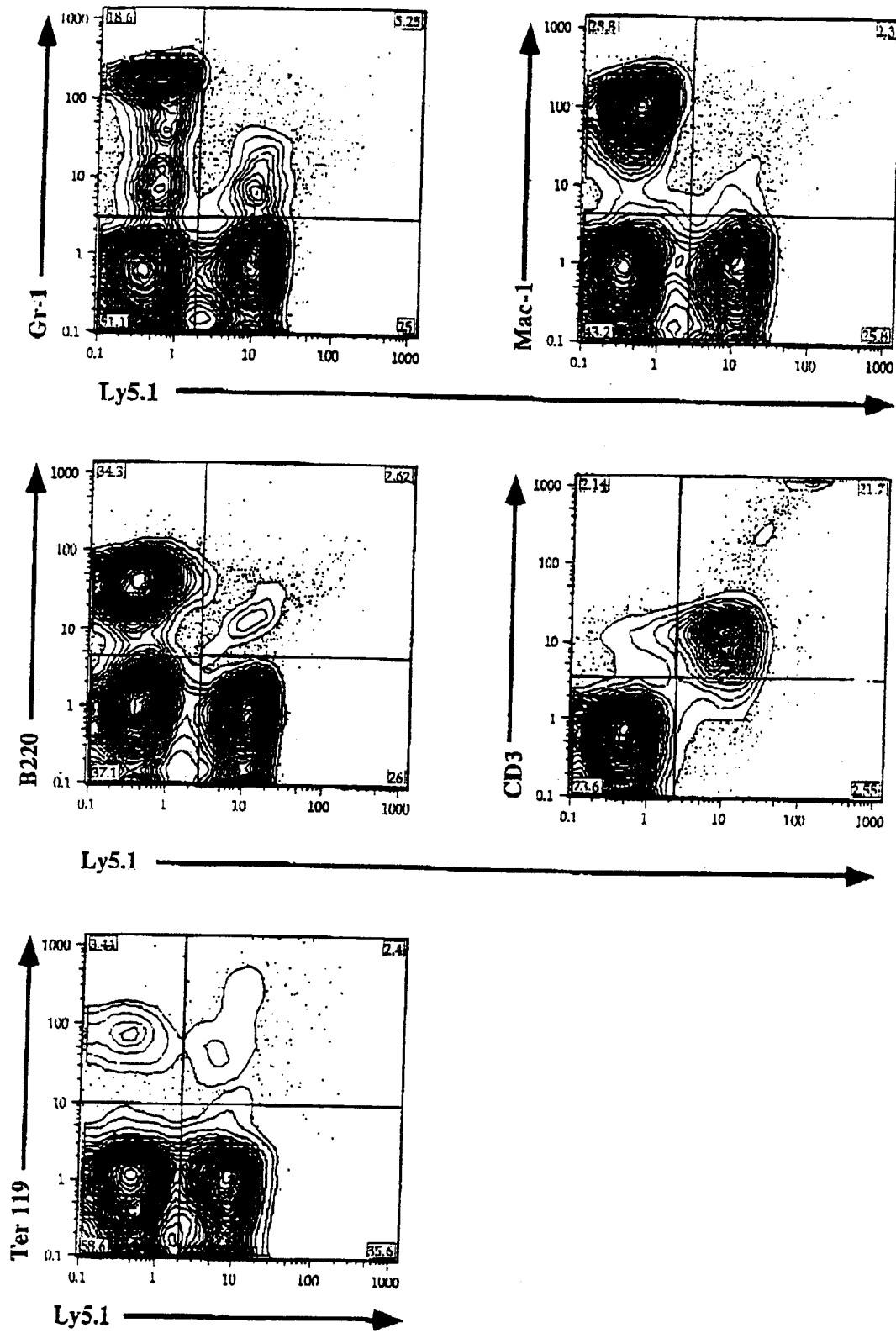


Figure 3

SEQUENCE LISTING

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	215		220		225	230

atc ttt aag tct ggt ggc atc cca gcg ctg gtg aaa atg ctt ggg tca	835
Ile Phe Lys Ser Gly Gly Ile Pro Ala Leu Val Lys Met Leu Gly Ser	
235 240 245	
cca gtg gat tct gta ctg ttc tac gcc atc acg aca ctg cat aat ctc	883
Pro Val Asp Ser Val Leu Phe Tyr Ala Ile Thr Thr Leu His Asn Leu	
250 255 260	
ctg ctc cat cag gaa gga gct aaa atg gca gtg cgc cta gct ggt gga	931
Leu Leu His Gln Glu Gly Ala Lys Met Ala Val Arg Leu Ala Gly Gly	
265 270 275	
ctg cag aaa atg gtt gct ttg ctc aac aaa aca aac gtg aaa ttc ttg	979
Leu Gln Lys Met Val Ala Leu Leu Asn Lys Thr Asn Val Lys Phe Leu	
280 285 290	
gct att aca aca gac tgc ctt cag atc tta gct tat ggc aat caa gag	1027
Ala Ile Thr Thr Asp Cys Leu Gln Ile Leu Ala Tyr Gly Asn Gln Glu	
295 300 305 310	
agc aag ctc atc att ctg gcc agt ggt gga ccc caa gcc tta gta aac	1075
Ser Lys Leu Ile Ile Leu Ala Ser Gly Gly Pro Gln Ala Leu Val Asn	
315 320 325	
ata atg agg acc tac act tat gag aag ctt ctg tgg acc aca agc aga	1123
Ile Met Arg Thr Tyr Thr Tyr Glu Lys Leu Leu Trp Thr Thr Ser Arg	
330 335 340	
gtg ctg aaa gtg ctg tct gtc tgc tct agc aac aag ccg gcc att gta	1171
Val Leu Lys Val Leu Ser Val Cys Ser Ser Asn Lys Pro Ala Ile Val	
345 350 355	
gaa gct ggt ggg atg cag gca ctg ggg ctt cat ctg aca gac cca agt	1219
Glu Ala Gly Gly Met Gln Ala Leu Gly Leu His Leu Thr Asp Pro Ser	
360 365 370	
cag cga ctt gtt caa aac tgt ctt tgg act ctc aga aac ctt tca gat	1267
Gln Arg Leu Val Gln Asn Cys Leu Trp Thr Leu Arg Asn Leu Ser Asp	
375 380 385 390	
gca gcg act aag cag gaa ggg atg gaa ggc ctc ctt ggg act cta gtg	1315
Ala Ala Thr Lys Gln Glu Gly Met Glu Gly Leu Leu Gly Thr Leu Val	
395 400 405	
cag ctt ctg ggt tcc gat gat ata aat gtg gtc acc tgt gca gct gga	1363
Gln Leu Leu Gly Ser Asp Asp Ile Asn Val Val Thr Cys Ala Ala Gly	
410 415 420	
att ctc tct aac ctc act tgc aat aat tac aaa aac aag atg atg gtg	1411
Ile Leu Ser Asn Leu Thr Cys Asn Asn Tyr Lys Asn Lys Met Met Val	
425 430 435	
tgc caa gtg ggt ggc ata gag gct ctt gta cgc acc gtc ctt cgt gct	1459
Cys Gln Val Gly Gly Ile Glu Ala Leu Val Arg Thr Val Leu Arg Ala	
440 445 450	
ggt gac agg gaa gac atc act gag cct gcc atc tgt gct ctt cgt cat	1507
Gly Asp Arg Glu Asp Ile Thr Glu Pro Ala Ile Cys Ala Leu Arg His	
455 460 465 470	

ctg acc agc cgg cat cag gaa gcc gag atg gcc cag aat gcc gtt cgc	1555
Leu Thr Ser Arg His Gln Glu Ala Glu Met Ala Gln Asn Ala Val Arg	
475 480 485	
ctt cat tat gga ctg cct gtt gtg gtt aaa ctc ctg cac cca cca tcc	1603
Leu His Tyr Gly Leu Pro Val Val Val Lys Leu Leu His Pro Pro Ser	
490 495 500	
cac tgg cct ctg ata aag gca act gtt gga ttg att cga aac ctt gcc	1651
His Trp Pro Leu Ile Lys Ala Thr Val Gly Leu Ile Arg Asn Leu Ala	
505 510 515	
ctt tgc cca gca aat cat gcg cct ttg cgg gaa cag ggt gct att cca	1699
Leu Cys Pro Ala Asn His Ala Pro Leu Arg Glu Gln Gly Ala Ile Pro	
520 525 530	
cga cta gtt cag ctg ctt gta cga gca cat cag gac acc caa cgg cgc	1747
Arg Leu Val Gln Leu Leu Val Arg Ala His Gln Asp Thr Gln Arg Arg	
535 540 545 550	
acc tcc atg ggt gga acg cag cag cag ttt gtg gag ggc gtg cgc atg	1795
Thr Ser Met Gly Gly Thr Gln Gln Gln Phe Val Glu Gly Val Arg Met	
555 560 565	
gag gaa ata gtc gaa ggg tgt act gga gct ctc cac atc ctt gct cgg	1843
Glu Glu Ile Val Glu Gly Cys Thr Gly Ala Leu His Ile Leu Ala Arg	
570 575 580	
gac gtt cac aac cgg att gta atc cga gga ctc aat acc att cca ttg	1891
Asp Val His Asn Arg Ile Val Ile Arg Gly Leu Asn Thr Ile Pro Leu	
585 590 595	
ttt gtg cag ttg ctt tat tct ccc att gaa aat atc caa aga gta gct	1939
Phe Val Gln Leu Leu Tyr Ser Pro Ile Glu Asn Ile Gln Arg Val Ala	
600 605 610	
gca ggg gtc ctc tgt gaa ctt gct cag gac aag gag gct gca gag gcc	1987
Ala Gly Val Leu Cys Glu Leu Ala Gln Asp Lys Glu Ala Ala Glu Ala	
615 620 625 630	
att gaa gct gag gga gcc aca gct ccc ctg aca gag tta ctc cac tcc	2035
Ile Glu Ala Glu Gly Ala Thr Ala Pro Leu Thr Glu Leu Leu His Ser	
635 640 645	
agg aat gaa ggc gtg gca aca tac gca gct gct gtc cta ttc cga atg	2083
Arg Asn Glu Gly Val Ala Thr Tyr Ala Ala Ala Val Leu Phe Arg Met	
650 655 660	
tct gag gac aag cca cag gat tac aag aag cgg ctt tca gtc gag ctg	2131
Ser Glu Asp Lys Pro Gln Asp Tyr Lys Lys Arg Leu Ser Val Glu Leu	
665 670 675	
acc agt tcc ctc ttc agg aca gag cca atg gct tgg aat gag act gca	2179
Thr Ser Ser Leu Phe Arg Thr Glu Pro Met Ala Trp Asn Glu Thr Ala	
680 685 690	
gat ctt gga ctg gac att ggt gcc cag gga gaa gcc ctt gga tat cgc	2227
Asp Leu Gly Leu Asp Ile Gly Ala Gln Gly Glu Ala Leu Gly Tyr Arg	
695 700 705 710	



Arg Glu Gly Leu Leu Ala Ile Phe Lys Ser Gly Gly Ile Pro Ala Leu  
 225 230 235 240  
 Val Lys Met Leu Gly Ser Pro Val Asp Ser Val Leu Phe Tyr Ala Ile  
 245 250 255  
 Thr Thr Leu His Asn Leu Leu Leu His Gln Glu Gly Ala Lys Met Ala  
 260 265 270  
 Val Arg Leu Ala Gly Gly Leu Gln Lys Met Val Ala Leu Leu Asn Lys  
 275 280 285  
 Thr Asn Val Lys Phe Leu Ala Ile Thr Thr Asp Cys Leu Gln Ile Leu  
 290 295 300  
 Ala Tyr Gly Asn Gln Glu Ser Lys Leu Ile Ile Leu Ala Ser Gly Gly  
 305 310 315 320  
 Pro Gln Ala Leu Val Asn Ile Met Arg Thr Tyr Thr Tyr Glu Lys Leu  
 325 330 335  
 Leu Trp Thr Thr Ser Arg Val Leu Lys Val Leu Ser Val Cys Ser Ser  
 340 345 350  
 Asn Lys Pro Ala Ile Val Glu Ala Gly Gly Met Gln Ala Leu Gly Leu  
 355 360 365  
 His Leu Thr Asp Pro Ser Gln Arg Leu Val Gln Asn Cys Leu Trp Thr  
 370 375 380  
 Leu Arg Asn Leu Ser Asp Ala Ala Thr Lys Gln Glu Gly Met Glu Gly  
 385 390 395 400  
 Leu Leu Gly Thr Leu Val Gln Leu Leu Gly Ser Asp Asp Ile Asn Val  
 405 410 415  
 Val Thr Cys Ala Ala Gly Ile Leu Ser Asn Leu Thr Cys Asn Asn Tyr  
 420 425 430  
 Lys Asn Lys Met Met Val Cys Gln Val Gly Gly Ile Glu Ala Leu Val  
 435 440 445  
 Arg Thr Val Leu Arg Ala Gly Asp Arg Glu Asp Ile Thr Glu Pro Ala  
 450 455 460  
 Ile Cys Ala Leu Arg His Leu Thr Ser Arg His Gln Glu Ala Glu Met  
 465 470 475 480  
 Ala Gln Asn Ala Val Arg Leu His Tyr Gly Leu Pro Val Val Val Lys  
 485 490 495  
 Leu Leu His Pro Pro Ser His Trp Pro Leu Ile Lys Ala Thr Val Gly  
 500 505 510  
 Leu Ile Arg Asn Leu Ala Leu Cys Pro Ala Asn His Ala Pro Leu Arg  
 515 520 525  
 Glu Gln Gly Ala Ile Pro Arg Leu Val Gln Leu Leu Val Arg Ala His  
 530 535 540  
 Gln Asp Thr Gln Arg Arg Thr Ser Met Gly Gly Thr Gln Gln Gln Phe  
 545 550 555 560  
 Val Glu Gly Val Arg Met Glu Glu Ile Val Glu Gly Cys Thr Gly Ala  
 565 570 575  
 Leu His Ile Leu Ala Arg Asp Val His Asn Arg Ile Val Ile Arg Gly  
 580 585 590  
 Leu Asn Thr Ile Pro Leu Phe Val Gln Leu Leu Tyr Ser Pro Ile Glu  
 595 600 605  
 Asn Ile Gln Arg Val Ala Ala Gly Val Leu Cys Glu Leu Ala Gln Asp  
 610 615 620  
 Lys Glu Ala Ala Glu Ala Ile Glu Ala Glu Gly Ala Thr Ala Pro Leu  
 625 630 635 640  
 Thr Glu Leu Leu His Ser Arg Asn Glu Gly Val Ala Thr Tyr Ala Ala  
 645 650 655  
 Ala Val Leu Phe Arg Met Ser Glu Asp Lys Pro Gln Asp Tyr Lys Lys  
 660 665 670  
 Arg Leu Ser Val Glu Leu Thr Ser Ser Leu Phe Arg Thr Glu Pro Met  
 675 680 685  
 Ala Trp Asn Glu Thr Ala Asp Leu Gly Leu Asp Ile Gly Ala Gln Gly  
 690 695 700

Glu	Ala	Leu	Gly	Tyr	Arg	Gln	Asp	Asp	Pro	Ser	Tyr	Arg	Ser	Phe	His
705					710					715					720
Ser	Gly	Gly	Tyr	Gly	Gln	Asp	Ala	Leu	Gly	Met	Asp	Pro	Met	Met	Glu
			725						730						735
His	Glu	Met	Gly	Gly	His	His	Pro	Gly	Ala	Asp	Tyr	Pro	Val	Asp	Gly
			740					745					750		
Leu	Pro	Asp	Leu	Gly	His	Ala	Gln	Asp	Leu	Met	Asp	Gly	Leu	Pro	Pro
		755					760					765			
Gly	Asp	Ser	Asn	Gln	Leu	Ala	Trp	Phe	Asp	Thr	Asp	Leu			
	770					775					780				

# INTERNATIONAL SEARCH REPORT

International application No.  PCT/US01/01459
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**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC(7) : A01N 1/02; C12N 5/00, 5/02, 7/00, 15/63, 15/86; C12P 21/04, 21/06  
 US CL : 435/2, 69.1, 70.1, 455, 235.1, 325, 375, 377  
 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)  
 U.S. : 435/2, 69.1, 70.1, 455, 235.1, 325, 375, 377

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 practicable, search terms used)  
 MEDLINE, EAST, DIALOG

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DAMALAS et al. Excess beta-catenin promotes accumulation of transcriptionally active p53. EMBO J. 1999, Vol 18. No. 11, pages 3054-3063, see entire document.	1, 8-9, 11-12
Y	CHU et al. Retrovirus-mediated gene transfer into human hematopoietic stem cells. J. Mol. Med. 1998, Vol 76, pages 184-192, see entire document.	1-3, 7-8, 11-12, 19-20
Y	ZARRIN et al. Comparison of CMV, RSV, SV40 viral and V-lambda-1 cellular promoters in B and T lymphoid and non-lymphoid cell lines. Biochimica et Biophysica Acta 1999, Vol. 1446, pages 135-139, see entire document and Figure 2.	1, 19-23
Y	MORIN et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 1997, Vol. 275, pages 1787-1790, especially page 1789.	9-10
Y	SATOH et al. Successful transfer of ADA gene in vitro into human peripheral blood CD34 positive cells by transfecting EBV-based episomal vectors. FEBS Letters 1998, Vol 441. No.1, pages 39-42, see entire document.	1, 8, 13, 19-23
A	FAGOTTO et al. Cell contact-dependent signaling. Dev. Biol. 1996, Vol 180, pages 445-454, especially pages 449 and 451.	1-3, 7-13, 19-23
A	WILLERT et al. Beta-catenin: a key mediator of Wnt signaling. Curr. Biol. 1998, Vol 8, pages 95-102, see entire document.	1-3, 7-13, 19-23

Further documents are listed in the continuation of Box C.  See patent family annex.

Special categories of cited documents:		Description
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent published on or after the international filing date	"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
"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)	"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
"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	"&"	document member of the same patent family

Date of the actual completion of the international search 04 April 2001 (04.04.2001)	Date of mailing of the international search report <b>27 APR 2001</b>
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/Us01/01459

## C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZIEGLER et al. Expansion of stem and progenitor cells. Curr. Opin. Hematol. 1998, Vol 5, pages 434-440, see entire document.	1-3, 7-13, 19-23
A	US 5,851,984A (MATTHEWS et al) 22 December 1998 (22.12.1998), see entire document.	1-3, 7-13, 19-23

# INTERNATIONAL SEARCH REPORT

International application No.

PC1/JS01/01459

**Continuation of Item 4 of the first sheet:** The current title is too long under PCT Rule 4.3. The new title suggestion: "Expansion of Stem and Progenitor Cells by beta-catenin".

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-13 and 19-23, in part, drawn to a method for *in vitro* expansion of mammalian stem or progenitor cells comprising increasing intracellular concentration of  $\beta$ -catenin by introduction of an exogenous nucleic acid comprising  $\beta$ -catenin coding sequences.

Group II, claim(s) 1-7 and 14-23, in part, drawn to a method for *in vitro* expansion of mammalian stem or progenitor cells comprising increasing intracellular concentration of  $\beta$ -catenin by addition of exogenous  $\beta$ -catenin.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-II claim different methods. Group I recites the special technical feature of *in vitro* expansion of stem or progenitor cells comprising introduction of an exogenous beta-catenin nucleic acid molecule which is not required by the method of Groups II. Group II recites the special technical feature of *in vitro* expansion of stem or progenitor cells comprising introduction of an exogenous beta-catenin protein which is not required by the method of Group I.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

- A. hematopoietic stem cell
- B. neural crest stem cell
- C. mesenchymal stem cell
- D. embryonic stem cell

The following claim(s) are generic: 8-23.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The special technical feature of (A) is a hematopoietic stem cell population. This special technical feature is not shared by any of the other species.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

- E. wild-type beta-catenin
- F. stabilized mutant beta-catenin

The following claim(s) are generic: 2-7, 11-13, and 17-23.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The special technical feature of (E) is an isolated beta catenin protein that has not been mutated. This special technical feature is not shared by any of the other species.