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(54) Title: CELL CULTURE

(57) Abstract: There is provided a method of retarding differentiation of a biological cell, the method comprising culturing the cell in the presence of an inhibitor of E-cadherin activity. The method is particularly advantageous in retarding the differentiation of stem or progenitor cells, and allows suspension culture of such cells in a manner that enables large scale expansion of cell populations. There is also provided a stem or progenitor cell comprising a construct encoding an inhibitor of E-cadherin activity; and a cell culture medium, for use in the retardation of biological cell differentiation, comprising an inhibitor of E-cadherin activity.

CELL CULTURE.

The present invention provides a method of retarding the differentiation of a biological cell. The biological cell may preferably comprise a stem or progenitor cell. The invention further provides cell culture media that may be used to retard differentiation of cultured cells, biological cells comprising constructs useful in the retardation of cell differentiation, and methods for the therapeutic manipulation of biological cells.

The production, maintenance and use of stem and progenitor cells is currently the subject of much scientific interest and research. Stem and progenitor cells constitute a highly valuable system for studying aspects of development and have the potential to revolutionise the treatment of injury and disease as the basis of cellular therapies.

The therapeutic use of stem cells offers a powerful new therapeutic approach as compared to existing drug-based therapies. This new approach may have applications in degenerative illnesses (e.g. Alzheimer), cardiovascular diseases, cancer and diseases of the nervous system (e.g. Multiple sclerosis). Many such diseases are currently untreatable.

Disease management is currently achieved through the use of disease modifying drugs, often whose activities the body often poorly tolerates. In addition, these drugs are limited in their ability to control only the symptoms of a disease and are unable to offer cures. Poor disease coverage coupled with the failings in current drug based therapies is driving the quest for new disease management methods and treatments.

The most promising development towards such goals is in the development of regenerative tissue engineering stem cell based therapies. In fact, the potential of stem cells as therapeutic cures is well known; with over 10,000 individuals undergoing successful bone marrow stem cell transplantations yearly in the UK.

Estimations currently predict an explosion in the stem cell market, reaching a value in the region of $10 billion by 2010.

The therapeutic use of stem cells relies on the ability of these cells to give rise to multiple tissue types. Accordingly such cells are able to generate replacement tissue in subjects to which they are administered.

Stem cells such as human embryonic stem (hES) cells are wildly believed to have the capability to revolutionize disease therapeutics with the potential of meeting many of the unmet medical needs. Embryonic stem cells are unique in their ability to develop and differentiate into all the cells and tissues of the body. As such, they are a potential source of replacement cells and tissues for organ repair in chronic diseases. The unique characteristics that commend embryonic stem cells to therapeutic use are:

i) They are unspecialised cells capable of proliferation and self-renewal.

ii) Under specific physiological conditions they can be induced to become cells with specific functions, such as beating cells of the heart.

Current protocols for the culture and growth of stem cells (such as from stem cells from mouse and human sources) requires the involvement of skilled technicians, as well as the use of specialist cell culture media intended to maximise the yield of pluripotent or multipotent cells within such cultures.

However, current techniques for the culture of stem or progenitor cells are subject to spontaneous differentiation of the cultured cells which gives rise to the development of various differentiated cell types. Such spontaneous differentiation severely decreases the yield of pluripotent or multipotent cells over extended passages, a decrease that is particularly notable in human cell cultures. If cells to be used for therapy undergo uncontrolled differentiation during culture the number of possible lineages into which they may develop, and hence their ultimate therapeutic potential, is reduced. Therefore, for ES cells to realise their potential in cellular therapy applications it is essential that increased yields of pluripotent cells are achievable using cost-effective medium, absence of animal products (such as serum) and minimum technical requirements.

Stem cells offer the promise of treatment, and possibly, the cure of a broad array of human diseases, benefiting patients, family members, physicians and society in general.

Current approaches to maintaining the undifferentiated phenotype of ES cells are focused on the identification of exogenous and endogenous factors able to maintain the pluripotent state (the ability of the cells to differentiate into all cell types). For example, addition of leukaemia inhibitory factor (LIF) to mouse ES cells can promote the undifferentiated growth of these cells. However, even LIF, the "gold standard" factor for undifferentiated mouse ES cell culture, cannot maintain homogeneous undifferentiated ES cell populations. Neither can it prevent spontaneous differentiation of the cells in suspension culture, a technique essential for obtaining sufficient quantities of pluripotent cells to allow transfer of ES cell therapies into the clinic.

The market readiness of stem cell therapy awaits the development of technologies capable of imparting control and direction on hES cell growth.

Current methods for the derivation and maintenance of stem cells such as embryonic stem cells are technically demanding and inefficient, with a success rate generally less than 30%.

Current laboratory methods used to maintain cultures of proliferative hES cells are also unable to produce such cells fast enough to respond to increasing demand. Current methods rely on recapitulation of the cell-cell and cell-matrix environment and are limited to production in inefficient monolayer culture.

To date pluripotent hES cells have proven difficult to expand *in vitro* and significant spontaneous differentiation occurs under current "optimal" growth conditions. Leukaemia inhibitory factor (LIF) is the current "gold standard" for undifferentiated mouse ES cell culture. However LIF cannot maintain homogeneous ES cell populations neither can it prevent spontaneous differentiation of the cells.

The growth factor fibroblast growth factor 2 (FGF-2) is also commonly used as a supplement in the culture of human stem cells, such as human embryonic stem cells. This growth factor helps human stem cells to remain undifferentiated and capable of proliferation in culture.

Currently, hES cells are generally grown in direct contact with mouse feeder cells or in media pre-conditioned by nutrient components derived from such cells. Such cells carry the risk of passing microbes and infectious agents to the recipient. As such the FDA has stated that it will demand extensive testing and long term follow up studies on therapies using such technologies. Mechanisms aimed at removing such dangers have thus far focused on the use of expensive growth factor supplements.

To date, none of the prior art techniques have been consistently successful enough to allow their widespread clinical use in biological cell-based therapies. There therefore remains a need to develop improved methods for preparing biological cells for therapeutic use, and improved methods of therapy utilizing biological cells.

Furthermore, it will also be appreciated in the light of the above that there exists a need to develop new or improved cell culture methods, conditions and media capable of promoting biological cell growth without maturation and/or differentiation. Such new or improved cell culture resources may be of use not only in the therapeutic adaptation of stem and/or progenitor cells, but also in the culture of biological cells (and particularly stem and/or progenitor cells) for research and/or development purposes. Although it is desirable to be able to culture such cells (for example to allow expansion of cell numbers without maturation or differentiation) there is a general lack of suitable resources available to the skilled person in the prior art.

In a first aspect of the present invention there is provided a method of retarding differentiation of a biological cell, the method comprising culturing the cell in the presence of an inhibitor of E-cadherin activity.

In a second aspect of the invention there is provided the use of an inhibitor of E-cadherin activity to maintain undifferentiated biological cells in culture.

In a third aspect the present invention also provides the use of an inhibitor of E-cadherin activity in the manufacture of a culture medium for retarding the differentiation of biological cells.

In a fourth aspect the invention provides a cell culture medium suitable for the retardation of biological cell differentiation, the cell culture medium comprising an inhibitor of E-cadherin activity.

The present invention is based upon the inventors' new and surprising finding that the inhibition of E-cadherin activity is able to retard the differentiation of biological cells. The cadherins are a family of integral membrane proteins which are involved in calcium-dependent cell adhesion. E-cadherin is so called because of its association with the epithelium. Cadherins comprise an extracellular domain of approximately 600 amino acid residues, a transmembrane domain, and an intracellular domain of 150 amino acid residues. The extracellular domain comprises four repeated sequences that are believed to be associated with calcium ion binding. The gene encoding E-cadherin is known as cdh1.

The amino acid sequence of human E-cadherin is shown in Sequence ID No. 2, and the sequence of DNA encoding this protein is shown in Sequence ID No. 1. The amino acid sequence of murine E-cadherin is shown in Sequence ID No. 4, and the sequence of DNA encoding this protein shown in Sequence ID No. 3. The amino acid sequence of rat E-cadherin is shown in Sequence ID No. 6 (with the sequence of DNA encoding this protein shown in Sequence ID No. 5), and the amino acid sequences of canine and bovine E-cadherin are shown in Sequence ID No. 8 and Sequence ID No. 10 respectively (with the sequences of DNA molecules encoding these proteins shown in Sequence ID Nos. 7 and 9 respectively).

It is known that E-cadherin molecules may bind one another by an extracellular domain designated "CAD-HAV". The CAD-HAV domain of human E-cadherin is represented by

amino acid residues 233 to 235 of Sequence ID No. 2, and is shown in Sequence ID No. 11. The CAD-HAV domain may represent a preferred region through which activity of E-cadherin activity may be inhibited, as considered further below.

Another region that may be targeted in the inhibition of E-cadherin activity is the area around the tryptophan residue found at position 156 (Trp156) of Sequence ID No. 2. This residue has been shown to be crucial in the dimerisation of E-cadherin EC1 domain (Laur et al., Archives of Biochemistry and Biophysics, 2002: 400;141-147). Suitable inhibitors of E-cadherin activity targeting this domain may include antibodies capable of binding to epitopes incorporating Trp156, as well as fragments or derivatives of E-cadherin comprising this residue (for example soluble fragments incorporating Trp156).

It will be appreciated that the biological cell differentiation of which is to retarded be may be any of a wide range of biological cell types. However, this activity is particularly pronounced in the case of progenitor and stem cells, given the undifferentiated nature of such cells, and so stem and progenitor cells constitute preferred biological cells in the context of the present invention. As set out in the introduction, the development of techniques by which differentiation of stem or progenitor cells in culture may be avoided or retarded is much needed in association with the use of such cells for therapeutic or research purposes. Without wishing to be bound by any hypothesis, the inventors believe that inhibitors of E-cadherin activity function with a dual role, both as survival factors for cultured cells, and also as agents capable of preventing cell differentiation and maturation during culture.

For the purposes of the present invention stem cells are taken to comprise nullipotent, totipotent or pluripotent cells, and progenitor cells (or precursor cells) to comprise multipotent cells. Totipotent cells are those cells capable of giving rise to all extraembryonic, embryonic and adult cells of the embryo. Accordingly it can be seen that totipotent cells may ultimately give rise any type of differentiated cell found in an embryo or adult. By comparison, pluripotent cells are cells capable of giving rise to some extraembryonic and all embryonic and adult cells. Thus it can be seen that pluripotent cells are able to give rise to a more limited range of cell types than are totipotent cells.

Nullipotent cells are those that will not undergo differentiation without the action of an exogenous cue to differentiation. Multipotent cells are cells able to give rise to diverse cell types in response to appropriate environmental cues (such as action of soluble growth factors or the substrate on which the cell, or its progeny, is located), but are more restricted in their potential lineage formation than are pluripotent, nullipotent or totipotent cells.

One suitable source of pluripotent stem cells that may be used in accordance with the present invention is those derived from the inner cell mass/epiblast (or other cells, such as blastomeres) of pre-implantation embryos. Such embryonic stem (ES) cells are readily obtainable and are capable of giving rise to all possible embryonic and adult cell lineages. Accordingly ES cells represent preferred cells for use in the invention.

The skilled person will appreciate that, although the methods and media of the invention are suitable for use with stem cells of all types and derived from all species (including human cells such as human embryonic stem cells), it may be preferred for the purposes of the present invention that any stem cell is other than a human embryonic stem cell.

Although they do not wish to be bound by any hypothesis, the inventors believe that the beneficial effects observed on culturing biological cells in the presence of inhibitors of E-cadherin activity arise as a result of the inhibitor's action as a survival factor, an extracellular signal required in order to induce a cell to divide, and without which a cell may undergo apoptosis. Accordingly, in a further aspect the present invention also provides the use of an inhibitor of E-cadherin activity as a survival factor for biological cells.

The action of inhibitors of E-cadherin activity as survival factors that are able to prevent cell differentiation and maturation is of great value in the culture of stem and progenitor cells, since it has previously been difficult to induce such cells to divide without inducing their maturation and differentiation at the same time. It will be appreciated that the use of inhibitors of E-cadherin activity in the culture of biological cells, such as stem or progenitor cells, has use in both therapeutic and non-therapeutic applications. Clearly, the

ability to retard differentiation of biological cells (such as stem and progenitor cells) in culture has utility in cell culture undertaken for a wide range of non-therapeutic purposes, including (but not limited to) research and development uses. Therapeutic applications making use of the newly identified anti-differentiation properties of inhibitors of E-cadherin activity are considered elsewhere in the specification.

Conventional prior art techniques for promoting the expansion of stem or progenitor cell populations *ex vivo* rely on the use of "cocktails" of multiple cytokines. The cytokines are typically provided either as part of, or in addition to, serum supplementation. Commercially available media intended for use in the expansion of stem cell populations include factors such as leukaemia inhibitory factor (LIF), interleukin-3 and interleukin-11 (IL-3 and IL-11), stem cell factor, FGF-2 and Flt-3 ligand. In such an embodiment the inventors believe that inhibitors of E-cadherin activity may serve to enhance the cultured cells' proliferation in response to the activity of the supplementing cytokines (i.e. the inhibitors of E-cadherin activity may be used to augment proliferation in response to known cytokine supplementation regimes). The inventors have found that the use in cell culture of inhibitors of E-cadherin activity in combination with one or more of the factors listed above is beneficial in that it allows greater expansion of cell populations than may be achieved using the prior art techniques.

The inventors have found that supplementation with inhibitors of E-cadherin activity allows stem cells populations to be expanded in culture using simpler cytokine cocktails than have previously been utilised. Such relatively simpler cytokine cocktails may be expected to provide reduced differentiation stimuli to cells so cultured. Of particular benefit is the combination of inhibitors of E-cadherin activity with LIF, which may be provided in combination with other factors, but is preferably provided alone.

Although the use of the factors listed above is of use in the *ex vivo* expansion of biological cells such as stem or progenitor cells, it is not without certain drawbacks. The presence of such cytokines, while helping to promote cell division, causes maturation and differentiation of the cultured cells. This maturation is outside the control of the practitioner, and may represent a major disadvantage, since it decreases the number of

different cell lineages to which the cells may ultimately give rise and may prevent controlled differentiation of the cells into preferred cell types.

In contrast to prior art techniques, the inventors have found that supplementation of cell culture medium with an inhibitor of E-cadherin activity promotes cell survival without differentiation. This effect has been observed in all cell types investigated thus far. Inhibitors of E-cadherin activity may be used to retard cell differentiation, and/or promote cell survival, even in the absence of other growth factor supplements. For example, the inventors have found that inhibitors of E-cadherin activity may be used in accordance with the invention in order to retard cell differentiation, and/or promote cell survival, in the absence of LIF. Alternatively or additionally, inhibitors of E-cadherin activity may be used in accordance with the invention in order to retard cell differentiation, and/or promote cell survival, in the absence of FGF-2.

Multipotent or nullipotent cells cultured in the presence of inhibitors of E-cadherin activity are particularly useful in both therapeutic and non-therapeutic applications since they retain their capability to give rise to a wide range of cell types (i.e. retain their multipotent or nullipotent characteristics). In accordance with this finding it will be appreciated that in preferred embodiments of the invention biological cells may be cultured in the presence of inhibitors of E-cadherin activity whilst in media that are devoid of other cytokines or serum. In the context of use of inhibitors of E-cadherin activity in serum or cytokine free conditions a major function of the inhibitor may be to provide a survival signal for the cultured cells.

Accordingly, in a further aspect, the present invention also provides a cell culture medium, for use in the retardation of biological cell differentiation, comprising an inhibitor of E-cadherin activity, wherein the medium is serum free. The invention also encompasses inhibitors of E-cadherin activity that are formulated for use as a supplement for a serum free culture medium. The use of serum-free media as described herein is particularly advantageous in the context of the inhibition of differentiation of cells intended for therapeutic applications, since this reduces the risk of contamination by agents (such as infectious or otherwise deleterious agents) that may be present in serum.

It will be appreciated that synthetic media and artificial serum may be used without the risk of potential contamination with animal agents that may occur via use of "natural" serum. Serum free media in accordance with the invention may advantageously also be free of supplements such as LIF and/or FGF-2.

In an additional and notable advantage over the prior art, the inventors have found that culture of stem or progenitor cells in the presence of inhibitors of E-cadherin activity allows the three-dimensional liquid culture ("suspension culture") of such cells. It will be appreciated that culture in accordance with this embodiment of the invention may preferably be undertaken using fermenters. In a preferred application, culture in accordance with this embodiment of the invention may utilise a stirred bioreactor system in which the cultured cells are either free-floating in the medium or grown on an inert surface (e.g. glass beads). Optimised conditions for the growth of cells cultured in accordance with this aspect of the invention, including preferred rates of propeller rotation, preferred oxygen tension and suitable culture media for use, may be derived by the practitioner using normal culture optimisation techniques.

The inventors have found that, in the event it is wished that cell culture in accordance with the invention be suspension culture, E-cadherin activity should preferably be inhibited totally (or substantially totally). Total (or substantially total) inhibition of E-cadherin activity helps to prevent the generation of cell to cell interactions between cultured cells. Such interactions may otherwise contribute to formation of embryoid bodies in which cultured cells may differentiate.

In the case that it is wished that cell culture in accordance with the invention be effected on a substrate, partial or total inhibition may be used. Indeed it may be preferred that partial inhibition (either by use of an inhibitor that is unable to totally inhibit E-cadherin activity, or by use of an amount of an inhibitor that is not sufficient to totally inhibit E-cadherin activity) is used, since this may advantageously allow cells cultured in this manner to retain functional cell to cell interactions. Suitable substrates may include solid substrates (such as conventional tissue plastics) or suitable gels. It may be preferred that an inhibitor of E-cadherin activity be incorporated in a cell culture substrate. Suitable

inhibitors of E-cadherin activity that may be incorporated in such a cell culture substrate include peptide inhibitors (such as those incorporating the CAD-HAV domain or Trp156) or their derivatives, or antibodies such as DECMA-1 or SHE78.7. Means by which such inhibitors may be incorporated in cell culture substrates in such a way that they are able to inhibit E-cadherin activity of cultured cells will be apparent to those of skin in the art. Cell culture substrates in accordance with this aspect of the invention may be provided in the form of tissue culture dishes or flasks, or as beads for use in suspension culture.

Except for where the context requires otherwise, references to "cell culture in accordance with the invention" should be taken to encompass cell culture techniques making use of the methods or uses of the invention; and/or cell culture techniques making use of cells of the invention; and/or cell culture techniques making using of cell culture media of the invention.

Inhibitors of E-cadherin activity suitable for use in accordance with the preceding aspects of the invention may be selected from the entire range of inhibitors described within the present specification. It will be appreciated that the preceding aspects of the invention may employ a single inhibitor of E-cadherin activity, or they may employ a combination of two or more inhibitors of E-cadherin activity.

For the purposes of the present invention an inhibitor of E-cadherin activity may be any substance, compound or molecule capable of decreasing, blocking or otherwise abrogating the biological activity of E-cadherin. It will be appreciated that in the present context the "biological activity" of E-cadherin referred to above is the capacity of E-cadherin to contribute to the differentiation of biological cells.

An example of an inhibitor of E-cadherin activity suitable for use in accordance with the present invention may comprise E-cadherin neutralising antibodies. Suitable neutralising antibodies are those that, when bound to an epitope present on E-cadherin, prevent E-cadherin's contribution to cell differentiation.

For example, the anti-E-cadherin antibody DECMA-1 (available from Sigma, Dorset, UK under the catalogue number U3254) may be used as an inhibitor of E-cadherin activity suitable for use in accordance with the invention. Alternatively, a preferred inhibitor of E-cadherin activity may be an antibody other than DECMA-1. One example of a further E-cadherin neutralising antibody that may be used in accordance with the present invention is SHE78-7 (also referred to as SHE78.7), which is commercially available from Zymed Labs, Inc., S. San Francisco, CA (Cat. No. 13-5700). DECMA-1 antibody was raised against mouse embryonal carcinoma cell line PCC4 Aza RI and SHE78.7 was raised against human placenta, therefore. In the light of this, it will be appreciated that DECMA-1 may be more effective at inhibition of E-cadherin activity in mouse (including mouse stem cells such as mouse embryonic stem cells) and SHE78.7 more effective for inhibition of E-cadherin activity in human cells (including human stem cells such as human embryonic stem cells).

In particular, it may be preferred that SHE78.7 be used as an inhibitor of E-cadherin activity when it is wished to inhibit E-cadherin activity associated with human cells. The inventors have found that DECMA-1 be used as a preferred inhibitor of E-cadherin activity when it is wished to inhibit E-cadherin activity associated with murine cells.

Antibodies suitable for use as inhibitors of E-cadherin activity in accordance with the present invention include monoclonal activity-neutralizing antibodies and polyclonal activity-neutralizing antibodies, as well as fragments of such antibodies that retain the neutralizing activity. Suitable examples of fragments that may be used include, but are not limited to, Fab or F(ab')hd 2, and Fv fragments.

Methods suitable for the generation and/or identification of antibodies capable of binding specifically to a target such as E-cadherin are well known to those skilled in the art. In general suitable antibodies may be generated by the use of isolated E-cadherin as an immunogen. E-cadherin may be administered to a mammalian organism, such as a rat, rabbit or mouse and antibodies elicited as part of the immune response. Suitable immunogens may include the full-length E-cadherin or an antigenic peptide fragment thereof (such as a preferred epitope associated with E-cadherin's biological function).

Monoclonal antibodies capable of neutralizing E-cadherin activity can be produced by hybridomas, immortalized cell lines capable of secreting a specific monoclonal antibody. Suitable immortalized cell lines can be created *in vitro* by fusing two different cell types, usually lymphocytes, one of which is a tumour cell.

However, not withstanding the above, it may be preferred that the inhibitors to be used are inhibitors other than neutralising antibodies. In particular, it may be preferred that inhibitors for use in accordance with these aspects of the invention are other than the antibody DECMA-1 or SHE78.7. Examples of suitable inhibitors of E-cadherin activity that may be used in accordance with the invention are considered further below.

Further examples of suitable inhibitors of E-cadherin activity that may be used in accordance with the present invention may comprise proteins (or protein derivatives) able to bind to E-cadherin and thereby prevent its biological activity. Such proteins or derivatives include naturally occurring proteins able to inhibit E-cadherin activity, as well as derivatives based on such naturally occurring proteins, and novel proteins or derivatives possessing suitable activity.

For example, it is well known that E-cadherin binds to other E-cadherin molecules via the most terminal CAD extracellular domain (CAD-HAV). Similarly, it has been shown that tryptophan residue Trp156 is linked to dimerisation of E-cadherin. Accordingly, suitable inhibitors of E-cadherin activity for use in accordance with the present invention may include protein or other binding molecules capable of binding the CAD-HAV sequence (Sequence ID No. 6) or a sequence incorporating residue Trp156. Preferred inhibitors of E-cadherin activity may comprise the CAD-HAV sequence, and a particularly preferred example of a suitable inhibitor of E-cadherin activity consists of the CAD-HAV sequence. Preferred inhibitors may comprise soluble E-cadherin fragments incorporating CAD-HAV and/or Trp156. Alternatively suitable protein or other binding molecules for use as inhibitors of E-cadherin activity in accordance with the present invention may be based on modified forms of the CAD-HAV sequence, or a sequence incorporating Trp156. Such modified forms may include derivatives that are modified in order to

increase their biological activity, increase their resistance to protein degradation, increase their half-life, or otherwise increase their availability.

Suitable peptide inhibitors comprising the CAD-HAV sequence or Trp156 may comprise three or more contiguous amino acids from the sequence of E-cadherin shown in Sequence ID No. 2, or may comprise five, ten, twenty or more contiguous amino acid residues from Sequence ID No. 2 including the CAD-HAV sequence or Trp156.

Peptide inhibitors (such as those comprising the CAD-HAV sequence and/or sequences incorporating Trp156) may constitute preferred inhibitors of E-cadherin activity for use in accordance with the invention. Other suitable inhibitors of E-cadherin activity may be derived from such peptide inhibitors. Derivatives of this sort, such as peptoid derivatives, may have greater resistance to degradation, and may thus have improved shelf-lives compared to the peptides from which they are derived.

Suitable inhibitors of E-cadherin activity may also be conjugated with polyvalent/monovalent synthetic polymers, thereby increasing avidity of the inhibitors to their target protein. For example, in one preferred embodiment multiple forms of inhibitors suitable for use in accordance with the invention may be conjugated to a single polymer. Alternatively or additionally a suitable inhibitor may be conjugated to a suitable polymer in combination with one or more other factors required to maintaining pluripotency (e.g. suitable oligosaccharides).

Inhibitors of E-cadherin activity suitable for use in accordance with the invention may alternatively, or additionally, be capable of binding to the membrane proximal region of E-cadherin.

Further inhibitors of E-cadherin activity suitable for use in accordance with the present invention include the $\alpha_E\beta_7$ integrin, which is a naturally occurring binding partner of E-cadherin. Other suitable inhibitors may include E-cadherin-binding fragments of $\alpha_E\beta_7$ integrin, or derivatives of this integrin or its fragments. Suitable fragments may be

selected in the light of the disclosure of Shiraishi et al, (J Immunol. 2005 Jul 15;175(2):1014-21).

Small molecule inhibitors of E-cadherin may represent preferred inhibitors for use in accordance with the present invention.

In a preferred embodiment of the invention cells may be induced to over-express naturally occurring inhibitors of E-cadherin activity. It may be preferred that such over expression of naturally occurring inhibitors by a cultured cell is achieved transiently (for instance such that expression occurs only during *ex vivo* culture and ceases on administration of cells to the subject requiring therapy, or such that expression occurs only during *ex vivo* expansion of cell populations and ceases in order to allow differentiation of the expanded population into cells having a desired phenotype).

One example of such a naturally occurring inhibitor of E-cadherin activity is “Slug” (which is also known as “Snai2” and “snail homolog 2”). The amino acid sequence of the human form of Slug (NCBI reference number NP_003059) is shown in Sequence ID No. 12, and the amino acid sequence of the mouse form of Slug (NCBI reference number NP_035545) is shown in Sequence ID No. 13.

Another example of a suitable naturally occurring inhibitor of E-cadherin activity is “Snail”.. The amino acid sequence of the human form of Snail (NCBI reference number NP_005976) is shown in Sequence ID No. 14, and the amino acid sequence of the murine form of snail (NCBI reference number NP_035557) is shown in Sequence ID No. 15.

A further naturally occurring inhibitor of E-cadherin activity suitable for use in accordance with the present invention comprises SMAD interacting protein 1 “SIP1”. The amino acid sequence of the human form of SIP1 (NCBI reference number BAB40819) is shown in Sequence ID No. 16, and the amino acid sequence of the mouse form of SIP1 (NCBI reference number AAD56590) is shown in Sequence ID No. 17.

E2A comprises a further naturally occurring inhibitor of E-cadherin activity suitable for use in accordance with the present invention. The human form of E2A is also known as "Homo sapiens transcription factor 3", "E2A immunoglobulin enhancer binding factors E12/E47" and "TCF3". The human form of E2A has been given NCBI reference number NM_003200. The amino acid sequence of human E2A is shown in Sequence ID No. 18, and DNA encoding the human form of E2A is shown in Sequence ID No. 19. The murine form of E2A is also known as "Mus musculus transcription factor E2a" and has NCBI reference number BC006860. The amino acid sequence of murine E2A is shown in Sequence ID No. 20, and the sequence of DNA encoding the murine form of E2A is shown in Sequence ID No. 21.

It will be appreciated that the naturally occurring inhibitors of E-cadherin described above merely represent examples of the range of naturally occurring inhibitors that may be used in accordance with the invention. These (and other) inhibitors may be used singly or in combination with other inhibitors (including combinations of naturally occurring and artificial inhibitors).

The inventors believe that Snail, Slug, SIP1 and E2A inhibiting E-cadherin expression by methylation/hypermethylation of the E-cadherin promoter, thus preventing or reducing gene transcription. Accordingly, agents capable of causing methylation or hypermethylation of the E-cadherin promoter represent preferred inhibitors of E-cadherin suitable for use in accordance with all aspects of the present invention. It will be appreciated that once such agents have caused methylation or hypermethylation of the E-cadherin promoter they need no longer be provided to cells the differentiation of which it is wished to retard.

Aptamers comprise a further example of preferred inhibitors of E-cadherin activity suitable for use in accordance with the present invention. Aptamers are nucleic acid molecules that that assume a specific, sequence-dependent shape and bind to specific target ligands based on a lock-and-key fit between the aptamer and ligand. Accordingly suitable aptamers may be designed to interact with E-cadherin protein or with nucleic acids encoding E-cadherin. Typically, aptamers may comprise either single- or double-

stranded DNA molecules (ssDNA or dsDNA) or single-stranded RNA molecules (ssRNA).

As indicated above, aptamers may be used to bind (and thereby inhibit) E-cadherin protein and/or nucleic acids encoding E-cadherin protein. ssDNA aptamers may be preferred for use in the investigation of nucleic acids encoding E-cadherin.

Suitable aptamers may be selected from random sequence pools, from which specific aptamers may be identified which have suitably high affinity for E-cadherin protein or nucleic acid targets. Methods for the production and selection of aptamers having desired specificity are well known to those skilled in the art, and include the SELEX (systematic evolution of ligands by exponential enrichment) process. Briefly, large libraries of oligonucleotides are produced, allowing the isolation of large amounts of functional nucleic acids by an iterative process of *in vitro* selection and subsequent amplification through polymerase chain reaction.

The use of aptamers as inhibitors of E-cadherin activity in accordance with the present invention may be advantageous, since aptamers have relatively stable shelf lives. This may be particularly preferred in association with cell culture media of the invention Preferably aptamers suitable for use in accordance with the invention may be stabilized by chemical modifications (for example 2′-NH_2 and 2′-F modifications).

Although the inventors do not wish to be bound by any hypothesis, it is believed that certain inhibitors, such as the antibody DECMA-1 mentioned above, achieve their effect through the internalisation of E-cadherin. Such internalised protein cannot achieve its normal biological function, and so biological activity is thereby inhibited. Accordingly agents capable of causing the internalisation of E-cadherin represent preferred inhibitors for use in accordance with the invention.

The preceding examples have concentrated primarily on inhibitors able to prevent biological activity that may otherwise be associated with E-cadherin that has already been expressed. It will be appreciated that other suitable inhibitors may include agents capable

of preventing the expression of E-cadherin. Such inhibitors may prevent or reduce transcription of the E-cadherin gene, or may prevent or reduce translation of E-cadherin gene transcripts.

Examples of such inhibitors capable of preventing the expression of E-cadherin include aptamers (as considered above), antisense oligonucleotides and ribozymes. Suitable inhibitors will also encompass agents that can disrupt the E-cadherin gene.

The skilled person will realise that many of the inhibitors of E-cadherin activity described in the present specification, and particularly protein or nucleic acid agents as described herein, are suitable for cellular production (using the mechanism of gene transcription and expression). The skilled person will recognise that preferably such agents may be produced by the cells differentiation of which is to be retarded. Accordingly, in a further aspect, the invention also provides a biological cell comprising a construct encoding an inhibitor of E-cadherin activity. Such a biological cell is preferably a stem or progenitor cell, since these cells, which exhibit little or no differentiation, may advantageously be grown in culture to produce expanded populations of such stem or progenitor cells. Populations of this type may be beneficially used in therapeutic or non-therapeutic applications. It may be preferred that a biological cell in accordance with this aspect of the invention is one in which the construct is transiently incorporated, or in which the construct is transiently expressed. The inhibitor of E-cadherin activity encoded by the construct may preferably comprise an siRNA molecule, such as those set out in Sequence ID Nos. 22 to 29.

In a still further aspect of the invention, there is provided a method of retarding differentiation of a biological cell, the method comprising:

i) introducing a construct encoding an inhibitor of E-cadherin activity into a biological cell the differentiation of which is to be retarded; and
ii) expressing the construct such that differentiation of the cell is retarded.

Suitable constructs for use in accordance with the invention may be designed with reference to the nature of the inhibitor of E-cadherin that it is desired to use. Illustrative examples are described further below.

Suitable constructs may be extra-genomic, or may be incorporated into the biological cell's genomic DNA. In the case of constructs integrated into the DNA of a biological cell it may be a preferred feature that at least part of the construct encoding the RNAi product may be flanked by sequences (such as *lox* P sites) allowing the functional excision of the construct (i.e. allowing sufficient of the construct to be excised that the construct is substantially unable to further inhibit E-cadherin activity). For instance, in the case that the sequence is flanked by *lox* P sites excision may be effected using the site specific DNA recombinase Cre. The use of sequences allowing excision of constructs encoding inhibitors of E-cadherin activity may be preferred in situations where it is wished to transiently express the inhibitor before resuming E-cadherin activity in the cells ("return of function"). Examples of such situations include those in which it is wished to expand undifferentiated populations of cells, such as stem or progenitor cells, *ex vivo* before effecting the controlled differentiation of the expanded cell population into cells having a desired phenotype (or phenotypes). The differentiated cells may, for example, be used therapeutically or experimentally once either expression of the inhibitory construct has ceased or the desired differentiation has taken place.

Expression of constructs may be constitutive, in the event that it is desired to inhibit cell differentiation permanently, or to retard cell differentiation for protracted periods of time. Alternatively, it may more often be preferred that expression of the construct be inducible in accordance with the requirements of the practitioner. Appropriate techniques by which the expression of constructs encoding inhibitors of E-cadherin activity may be induced as required will be well known to those skilled in the art, and include the use of vectors inducible by agents such as tetracycline.

In a preferred embodiment a construct encoding an inhibitor of E-cadherin activity may comprise a construct encoding an RNA interference (RNAi) product capable of inhibiting E-cadherin activity.

Preferably an RNAi inhibitor of E-cadherin activity may comprise a so-called "hairpin loop" RNAi inhibitor. Such inhibitors are preferred in situations in which long-term inhibition of E-cadherin activity is required. Indeed, use of hairpin loop RNAi inhibitors may be able to effect total long-term inhibition of E-cadherin activity (referred to as "knocked down" gene activity) without the need to remove the naturally occurring E-cadherin gene.

Particularly preferred constructs for use in accordance with the invention may be based upon pRNAtin-H1.2neo/hygro tetracycline inducible vectors that are commercially available from Genscript Corporation. However, any RNAi vector may suffice, whether a plasmid or virus.

Preferred RNAi constructs which may be used to inhibit E-cadherin activity in accordance with the present invention are illustrated in Sequence ID No. 22 to Sequence ID No. 29. These sequences have been designed to achieve optimal inhibition of mRNA encoding E-cadherin, and thereby optimal inhibition of E-cadherin activity. However, it may be preferred that two or more of the RNAi constructs illustrated in Sequence ID No. 22 to Sequence ID No. 29 be used in combination to bring about a desired inhibition of E-cadherin activity (and thereby retardation of cell differentiation). As will be appreciated, such combinations should preferably be selected with reference to the species in which the desired inhibition is to be effected, so that to retard differentiation of human cells it may be preferred to combine constructs selected from the group comprising Sequence ID No. 22 to Sequence ID No. 26, whereas in the case of murine cells it may be preferred to combine constructs selected from the group comprising Sequence ID No. 27 to Sequence ID No. 29.

The genetic stability of cells into which constructs encoding inhibitors of E-cadherin activity have been introduced may be investigated using known techniques such as karyotype analysis. By way of example, a protocol suitable for effecting karyotype analysis in a manner suitable to investigate genetic stability of cells is described in the accompanying Experimental Protocols section.

Ribozymes complementary to mRNA encoding E-cadherin represent a further suitable class of inhibitors of E-cadherin activity that may be used in accordance with the present invention. Sequences encoding ribozymes are also eminently suitable for incorporation in constructs of the types described above. It is well recognised that RNA molecules comprising the well-categorised catalytic centre of ribozymes in combination with sequences complementary to an mRNA of interest may be used to cleave the selected mRNA and thereby prevent its translation to protein. Suitable oligonucleotides comprising the ribozyme catalytic centre in combination with sequences complementary to mRNA encoding E-cadherin (human form shown in Sequence ID No. 30 and murine form shown in Sequence ID No. 31) may thus be used as the template for generation of E-cadherin neutralising ribozymes.

It will be appreciated that a nucleic acid encoding an inhibitor of E-cadherin activity may be delivered to a biological cell as part of a vector of the type outlined above. Preferred vectors may additionally comprise selection elements allowing selection of those cells into which the vectors have been successfully introduced. Preferred methods for the introduction of vectors into cells may include uptake through electroporation.

The skilled person will recognise that a suitable nucleic acid encoding an inhibitor of E-cadherin activity may be introduced into a cell without the nucleic acid being incorporated in a vector. For instance, a nucleic acid encoding an inhibitor of E-cadherin activity may be incorporated within a liposome or virus particle. Alternatively a "naked" DNA molecule encoding such an inhibitor may be inserted into a biological cell by a suitable means e.g. direct endocytotic uptake.

A nucleic acid encoding an inhibitor of E-cadherin may be transferred to the biological cells by transfection, infection, electroporation, microinjection, cell fusion, protoplast fusion or ballistic bombardment. For example, transfer may be by ballistic transfection with coated gold particles, liposomes containing the exogenous gene, and means of providing direct DNA uptake (e.g. endocytosis).

Electroporation (for example using the methodology made commercially available by Amaxa) may represent a preferred method by which vectors encoding inhibitors such as RNAi inhibitors (for example of the types described above) may be introduced into cells in which the vectors are to be expressed. It will be appreciated that the cells into which such vectors are introduced and expressed may preferably be cells that are to have their differentiation retarded in accordance with the invention.

The suitability of a putative inhibitor of E-cadherin activity for use in accordance with the present invention may be readily investigated by the skilled person using well-known techniques. A preferred technique that may be used to determine or assess the ability of a substance to inhibit E-cadherin activity is described in the Experimental results section. By way of example, a suitable technique capable of determining or assessing whether or not a test substance has E-cadherin inhibiting activity that makes it suitable for use in accordance with the invention may involve culturing cells in the presence of the test substance and LIF (or FGF-2 for human), and assessing the ability of cells so cultured to divide once LIF (or FGF-2 for human) is removed from the culture conditions. Such a technique makes use of cell counts to assess the efficacy of the test substance. Alternatively (or additionally) suitable techniques for the determination or assessment of E-cadherin inhibition by a test substance may compare the morphology of cells cultured in the presence or absence of the test compound, suitable inhibitory activity being indicated by a loss of cell-cell contact in ES, or suitable epithelial cell line, cultured in the presence of the test substance.

Alternative techniques that may be used to investigate suitable inhibitors of E-cadherin activity will be apparent to the skilled person.

Preferably an inhibitor of E-cadherin activity suitable for use in accordance with the present invention may be able to achieve at least 50% reduction in E-cadherin activity as assessed using the techniques described above. More preferably a suitable inhibitor of E-cadherin activity may be able to achieve at least 60%, 70% or 80% reduction. Even more preferably a suitable inhibitor may be able to achieve at least 85%, 90% or 95% reduction in E-cadherin activity. A most preferred inhibitor of E-cadherin activity may be able to

totally prevent E-cadherin's biological activity (i.e. 100% reduction of E-cadherin activity as measured using the above techniques).

Accordingly it will be recognised that a preferred inhibitor of E-cadherin activity suitable for use in accordance with the present invention may be able to achieve at least 50% reduction in E-cadherin activity. A more preferred inhibitor of E-cadherin activity may be able to achieve at least 60%, 70% or 80% reduction. An even more preferred inhibitor may be able to achieve at least 85%, 90% or 95% reduction in E-cadherin activity, and a most preferred inhibitor of E-cadherin activity may be able to totally prevent E-cadherin's biological activity (i.e. 100% reduction of E-cadherin activity as measured using the above techniques).

Optimisation of the amount of an inhibitor of E-cadherin activity that may be required in order to bring about a desired level of retardation of cell differentiation may be achieved using techniques well known to the person skilled in the art. Suitable techniques may be applicable to both externally administered inhibitors and constructs encoding suitable inhibitors, and may include dose response studies using cultured cells. Optimisation may be undertaken with reference to the efficacy of the inhibitor of E-cadherin activity assessed using the criteria and methods set out above.

For example, using the methods described in the accompanying protocols and Experimental Results sections, the inventors have identified that it is not necessary to completely inhibit E-cadherin activity in order to culture human stem cells in accordance with the invention. Partial inhibition of E-cadherin activity may, in fact, be preferable to total inhibition in the event that it is wished to culture human stem cells in monolayers. However, these studies reported elsewhere in the specification have also identified that total inhibition of E-cadherin activity is optimal for suspension culture of human stem cells.

By retardation of differentiation of a biological cell is meant the prevention of expression of markers associated with differentiation and/or the loss of totipotent, multipotent, nullipotent, or pluripotent capabilities. For example, a pluripotent cell may be expected to

express the stem cell markers Oct-4 and Nanog. As pluripotent cells undergo differentiation expression of Oct-4 and/or Nanog will decrease (even to the extent that such markers are no longer expressed), whilst expression of markers such as transthyretin (an endoderm marker), Sox-1 (a neuroectoderm marker), brachyury (a mesoderm marker) and/or zeta globin (a further mesoderm marker) may increase (depending on the differentiating cell types formed). PCR analysis represents a suitable method by which the absence or presence of transcripts for these may be investigated, and so by which retardation of differentiation may be assessed.

The use of inhibitors of E-cadherin activity to inhibit differentiation of stem and progenitor cells has specific utility in the culture of such cells from animal species that are normally difficult to culture. The rat constitutes an example of such a species.

By improving the viability of cultured stem and progenitor cells derived from such "difficult" species (which in addition to rat include cats and dogs, as well as the African clawed frog Xenopus laevis) it is consequently possible to utilise the cultured cells in the preparation of transgenic animals. The generation of transgenic animals is a standard laboratory procedure in respect of species such as mice, but has, until now, been impossible in respect of species such as rat, cat, dog or Xenopus.

In producing transgenic animals in this manner, fertilised zygotes may be isolated from a subject animal and E-cadherin activity inhibited in the cells of the inner cell mass of the embryo, thereby retarding the differentiation of these cells. The time at which inhibitors of E-cadherin are administered should be selected in order to allow proper formation of the inner cell mass (an activity known to require function of E-cadherin), without allowing further differentiation of the cells. Suitable timings may be developed with reference to published studies which further describe the times at which E-cadherin activity required for inner cell mass formation takes place.

Embryos prepared as described above may then be cultured in vitro to isolate the ES cells and inhibition of E-cadherin stopped (either by ending the administration or expression of inhibitors) when differentiation is required. It is the recognition that inhibitors of E-

cadherin activity may be used to retard cellular differentiation that provides the inventive contribution to the production of transgenic animals in accordance with this aspect of the invention. The techniques that may be used for embryonic culture are (in so far as they do not relate to the inhibition of E-cadherin activity) not inventive themselves, and may be based on those known to the skilled person. For example, suitable references that may be used when selecting protocols for embryo culture include (but are not limited to) Thomson et al, Science. 1998 Nov 6;282(5391):1145-7 (Human); Thomson et al, Proc Natl Acad Sci U S A. 1995 Aug 15;92(17):7844-8 (Primate); Buehr et al Biol Reprod. 2003 Jan;68(1):222-9 (Rat); Dattena et al, Mol Reprod Dev. 2006 Jan;73(1):31-9 (Sheep); Brook and Gardner, Proc Natl Acad Sci U S A. 1997 May 27;94(11):5709-12 (Mouse).

The finding that inhibitors of E-cadherin activity may be used to retard differentiation of biological cells and act as a survival factor for stem and progenitor cells lends itself to a further aspect of the present invention, which is the use of an inhibitor of E-cadherin activity in the isolation of embryonic stem (ES) cells. A major application of this is in the isolation of rat ES cells for use in genetic models of human disease. To date, a rat ES cell has not been isolated and maintained in vitro, and, to date, knockout/knockin technologies that have proven very useful in mouse cannot be performed in rat. The present invention provides a means by which rat ES cells may be isolated (using the methods described above), these cells used according to known procedures to create a knockout ES cell line, and E-cadherin inhibition then ceased or reversed to allow the formation of a rat with a specific genotype, in the same way that is presently done for transgenic mice.

The action of inhibitors of E-cadherin activity as survival factors that are able to prevent cell differentiation and maturation, readily lends itself to the culture of cells that are to be adapted for use in therapeutic applications, since cells cultured in the presence of E-cadherin inhibitors retain the greatest possible therapeutic effectiveness.

According to a further aspect of the present invention there is provided a method of preparing a biological cell for therapeutic use, the method comprising the consecutive or concurrent steps of:

i) culturing the biological cell in the presence of an inhibitor of E-cadherin activity; and
ii) adapting the biological cell for therapeutic use.

According to a still further aspect of the present invention there is provided a method of therapy, the method comprising the consecutive or concurrent steps of:

i) obtaining a biological cell;
ii) culturing the biological cell in the presence of an inhibitor of E-cadherin activity; and
iii) adapting the biological cell for therapeutic use

and further comprising administering the adapted biological cell to a subject in need of such therapy.

Biological cells prepared or adapted for therapeutic use in accordance with the preceding aspects of the invention may preferably be stem or progenitor cells.

Stem cell therapy represents a therapeutic method by which degenerative diseases (such as those caused by premature death or malfunction of cell types that the body is unable to replace) may be treated. It is hoped that addition of stem cells to a patient may help and promote the development of functional cells and/or tissues to replace those lost, thereby restoring normal healthy activity. Stem cells provided to a patient may be able to undergo differentiation under the control of suitable stimuli (either naturally occurring or artificially induced) and thereby replace or augment damaged, dysfunctional or diseased tissues. Ultimately it may be possible to regenerate new functional tissues *ex vivo* which may then be administered to subjects requiring therapy.

The adaptation of biological cells for use in stem cell therapy may typically involve *ex vivo* expansion of stem cell or progenitor cell numbers in order to produce an increased stem cell population, the cells of which are suitable for administration to a subject requiring such therapy. In order to have therapeutic effectiveness, cells to be used in stem cell therapy (which may either be true stem cells or certain types of progenitor cells) must retain their ability to differentiate into multiple cell lineages when administered to a

subject. Currently the application of stem cell therapy is limited by the lack of suitable methods by which stem cells may be propagated without undergoing differentiation and maturation.

Cells cultured in the presence of inhibitors of E-cadherin activity are useful in methods of stem cell therapy since they promote stem cell survival in culture, and hence aid the expansion of stem cell numbers, but do not induce differentiation of the cultured cells. Suspension culture in accordance with the invention may be particularly advantageous in expansion of stem cell numbers. The inventors have found that suspension culture in accordance with the invention is able to achieve a 268,000,000 fold expansion of total stem cell numbers.

It is believed that stem cell therapy may have wide applications across a broad range of diseases. For example stem cell therapy may be used in the treatment of blood disorders (such as leukaemia and sickle-cell anaemia), diseases of the brain and nervous systems (such as Parkinson's disease and Alzheimer's disease), musculo-skeletal disorders (such as muscular dystrophy, arthritis and osteoporosis), liver diseases (such as cirrhosis and hepatitis), spinal injuries, heart disease and diabetes.

Stem cell therapy may also be used to replace damaged tissue lost as a result of injury, trauma or cytotoxic insult. For example, such therapies may be used in neurodegenerative conditions, where central nervous system (CNS)-derived stem cells may be utilised to replace or augment damaged somatic cells, such as those located in the brain or spinal cord. Stem cells may be used therapeutically in contexts in which the circulatory system has been injured, such as ischemic tissue damage after vascular occlusion. In such contexts suitable stems cells may be administered to cause formation of new blood vessels, or to replace other damaged tissues. Expanded populations of stem cells may also be utilised in conditions in which the liver has been damaged, in order to induce regeneration of the injured tissue.

It will be appreciated that biological cells cultured, prepared and/or adapted in accordance with the invention are also suitable for use in the preparation and manufacture of

medicaments. Therefore according to a still further aspect of the invention there is provided the use of a biological cell, cultured in the presence of an inhibitor of E-cadherin activity and adapted for therapeutic use, as a medicament. Medicaments in accordance with this aspect of the invention are suitable for use in the treatment of the diseases, disorders and injuries considered above.

Medicaments in accordance with the invention may be formulated according to protocols well known in the art. Suitable formulations may be determined based on the preferred route by which the medicament is to be administered. Preferably medicaments according to the invention may be prepared in forms suitable for administration by inhalation, by injection, or by implantation.

Preferably formulations for inhalation may preferably comprise biological cells provided in a suitable liquid carrier. Such a liquid carrier is preferably non-immunogenic, and may comprise a saline solution, cell culture medium, or distilled water. Formulations for injection may be as described above, or may also be provided in the form of a gel, which may preferably be capable of resolution by the body of the subject treated. Formulations suitable for implantation may take the forms described for injection or inhalation, and may also comprise biological cells provided in a scaffold or matrix capable of providing a foundation for new tissue development.

In both methods of therapy according to the present invention, and in the use of medicaments according to the invention, a therapeutically effective amount of biological cells (such as those adapted for therapeutic use) should be administered to the subject requiring therapy. A "therapeutically effective amount" in the context of the present invention is considered to be any amount of suitable biological cells (such as therapeutically adapted cells) which, when administered to a subject suffering from a disease against which the biological cells are effective, causes reduction, remission, or regression of the disease. A "subject" may be a human being, or any other animal, particularly a domestic or agricultural mammal.

In a further aspect, the invention provides A method of isolating biological cells deficient in E-cadherin, the method comprising:

i) culturing the cells in the absence of LIF and/or FGF-2;

ii) allowing biological cells that express E-cadherin to die or differentiate; and maintaining the cultured cells until biological cells deficient in E-cadherin proliferate.

The invention will now be illustrated, by way of further example, with reference to the following Experimental Protocols, Experimental Results, and Figures in which:

Figure 1A shows a bar chart illustrating the percentage of wild type and E-cadherin knockout cells expressing the stem cell marker protein OCT-4;

Figure 1B shows immunofluorescence detection of OCT-4 protein in wild type and Ecad-/- cells;

Figure 2 shows the results of cell counts of populations of wild-type and Ecad-/- cells grown under various culture conditions;

Figure 3 shows expression of transcripts of a number of markers by wild type cells, Ecad -/- cells and Ecad +/- cells;

Figure 4 shows expression of transcripts from sample cells induced to differentiate by overgrowing in culture;

Figure 5 shows changes in cell numbers of wild type D3 and Ecad-/- ES cells cultured in the presence of LIF and assessed at each passage

Figure 6 the results of studies investigating the effect on cell numbers of culture in the presence of an E-cadherin function neutralising antibody, with or without LIF;

Figure 7 shows Sequence ID No. 1 and Sequence ID No. 2;

Figure 8 shows Sequence ID No. 3 and Sequence ID No. 4;

Figure 9 shows Sequence ID No. 5 and Sequence ID No. 6;

Figure 10 shows Sequence ID No. 7 and Sequence ID No. 8;

Figure 11 shows Sequence ID No. 9 and Sequence ID No. 10;

Figure 12 shows Sequence ID No. 11;

Figure 13 shows Sequence ID No. 12 to Sequence ID No. 21;

Figure 14 shows Sequence ID No. 22 to Sequence ID No. 29;

Figure 15 shows Sequence ID No. 30;

Figure 16 shows Sequence ID No. 31;

Figure 17 shows the results described in the study set out under heading 12 of the Experimental Results section;

Figure 18 shows the results described in the study set out under heading 13 of the Experimental Results section;

Figure 19 shows the results described in the study set out under heading 14 of the Experimental Results section;

Figure 20 shows the results described in the study set out under heading 15 of the Experimental Results section;

Figure 21 shows the results described in the study set out under heading 16 of the Experimental Results section;

Figure 22 shows the results described in the study set out under heading 17 of the Experimental Results section;

Figure 23 shows the results described in the study set out under heading 18 of the Experimental Results section;

Figure 24 shows the results described in the study set out under heading 19 of the Experimental Results section;

Figure 25 shows the results described in the study set out under heading 20 of the Experimental Results section;

Figure 26 shows the results described in the study set out under heading 21 of the Experimental Results section;

Figure 27 shows the results described in the study set out under heading 22 of the Experimental Results section;

Figure 28 shows the results described in the study set out under heading 23 of the Experimental Results section;

Figure 29 shows the results described in the study set out under heading 24 of the Experimental Results section;

Figure 30 shows the results described in the study set out under heading 25 of the Experimental Results section;

Figure 31 shows the results described in the study set out under heading 26 of the Experimental Results section;

Figure 32 shows the results described in the study set out under heading 27 of the Experimental Results section; and

Figure 33 shows the results described in the study set out under heading 28 of the Experimental Results section.

EXPERIMENTAL RESULTS.

The inventor has found that mouse ES cell lacking the *Cdh1* (E-cadherin) gene exhibit decreased spontaneous differentiation in the presence of LIF and show similar properties when cultured in the absence of LIF. This is a new and surprising finding, and has led to the development of a valuable method by which the culture and manipulation of homogeneous populations of pluripotent stem cells may be effected.

The inventors have cultured E-cadherin null (Ecad-/-) ES cells in the laboratory for upwards of 42 passages in the absence of LIF and have found that these cultured cells maintain expression of the pluripotent transcript markers Oct-4 and Nanog and expression of OCT-4 protein. This pattern of expression clearly identifies that cells cultured such that E-cadherin activity is not present are able to maintain an undifferentiated phenotype. Furthermore, the division of such cells is not altered compared to Ecad-/- ES cells grown in the presence of LIF, illustrating that inhibition of E-cadherin activity does not adversely effect the ability of cells so cultured to divide. In comparison, wild type ES cells cultured in the same conditions (absence of LIF) lose pluripotency through differentiation and fail to divide at passage numbers 6-8 (when cultured under identical conditions to E-cadherin null ES cells).

1. Loss of E-cadherin gene from mouse ES cells allows the continuous culture of undifferentiated cells in the absence LIF

Wild type (wt) mouse ES cells can be maintained in an undifferentiated (pluripotent) state in the absence of an embryonic fibroblast feeder layer by culture in the presence of LIF and foetal bovine serum. Upon removal of LIF from wt ES cells they differentiate to various lineages and lose expression of the pluripotent marker OCT-4.

The inventors have illustrated that removal of E-cadherin activity enables stem cells to be cultured without undergoing differentiation, even when LIF is removed from the culture conditions.

E-cadherin null (Ecad-/-) ES cells maintain OCT-4 protein expression in the absence of LIF.

In this study Ecad-/- and wt D3 ES cells were cultured in the presence or absence of LIF for 12 days in a gelatin-treated 6-well plate. The prior art would suggest that stem cells cultured in the absence of LIF would undergo differentiation or apoptosis, since these outcomes represent a common response of cultured stem cells to the stress of LIF removal. The results of this study are shown in Figure 1.

Figure 1A shows a bar chart illustrating the percentage of cells expressing the stem cell marker protein OCT-4. OCT-4 expression in both wild-type and E-cad -/- cells is shown, and comparison is made between cell populations cultured in the presence and absence of LIF. OCT-4 protein expression was determined using immunofluorescence and quantified by counting the number of OCT-4 positive and negative cells in 5 fields of view. Error bars show the SD.

Surprisingly the inventors found that Ecad-/- ES cells expressed the pluripotent marker OCT-4 in >99% of the cultured cell population, irrespective of LIF supplementation. Furthermore, Ecad-/- cells cultured in the absence of LIF maintained a phenotype identical to cells cultured in the presence of LIF, and apoptosis did not appear to be increased in cells cultured in the absence of LIF compared to those cultured in LIF. In contrast, wild type ES cells cultured under identical conditions exhibited 95.2% (±2.3) OCT-4 positive cells in the presence of LIF but only 8.3% (±4.2) OCT-4 positive cells in the absence of LIF.

The results clearly indicate that cultured stem cells in which E-cadherin activity is inhibited are able to maintain a pluripotent phenotype, and this indicates that differentiation among the cultured cells is retarded.

Figure 1B shows immunofluorescence detection of OCT-4 protein and DAPI (cell nucleus) in Ecad-/-and wt ES cells cultured for 12 days in the absence of LIF. The images compare expression of the pluripotent marker in populations of cells that have

been cultured for 12 days in the absence of LIF. It can be seen that OCT-4 protein levels were significantly decreased in wild-type cells compared to Ecad-/- ES cells.

Figure 2 shows the results of cell counts of populations of wild-type and Ecad-/- cells cultured in various culture medium in the presence or absence of LIF over 6 days. Ecad-/- and wt D3 ES cells were cultured in a gelatin-treated 6-well plate in the presence or absence of LIF in ES cell medium containing either serum (FCS+) or synthetic serum (FCS-). In addition, the cells were cultured in DMEM +FCS/+L-glutamine. Cell numbers are shown as a percentage of the cell numbers obtained in optimal ES cell medium (FCS+/LIF+). Cell number was assessed at each passage by trypan blue exclusion method.

As can be seen, Ecad -/- ES cells cultured in the absence of LIF with serum (LIF- FCS+) exhibit crisis for two days following removal of LIF. However, these cells recover to exhibit doubling times similar to cells cultured in the presence of LIF (LIF+ FCS+) and express OCT-4 in >99% of the cell population. It will be appreciated that such crisis events are common upon transfer of ES cells to different culture media (Chambers et al, 2003 Cell. 2003 May 30;113(5):643-55), and that this crisis in no way detracts from the utility of the invention, illustrated by the subsequent recovery of the cells.

In contrast, wt ES cells exhibit decreased cell numbers, and these populations of cells do not recover over time (when cultured under identical conditions to E-cadherin null ES cells). Furthermore, such cells lack OCT-4 expression in the majority of the cell population indicating that the cultured cells have differentiated and thereby lost their pluripotent phenotype.

Figure 2 also shows the results of cell counts of Ecad-/- and wild-type ES cell populations cultured in synthetic serum in the absence of LIF (LIF- FCS-). Under these conditions Ecad-/- ES cells exhibited crisis and subsequent increase in cell numbers (as found during culture in the presence of serum) whereas the wild-type ES cells were dead by day 6.

This study indicates that cells in which E-cadherin activity is inhibited (illustrated by Ecad-/- ES cells) do not differentiate in the absence of LIF and, by definition, are nullipotent. In an extension of this study Ecad-/- ES cells have now been cultured for 42 passages (approximately 90 days) in the absence of LIF and maintain OCT-4 protein expression in >99% of the cells.

The inventors believe that the inhibition of E-cadherin activity during cell culture provides a simple system allowing culture of ES cells in non-specialised medium without the need for experienced technicians. Furthermore, such culture may allow three-dimensional liquid culture of stem cells in fermenters, thereby markedly increasing the number of undifferentiated stem cells that may be produced. In summary, inhibition of E-cadherin activity in ES cells is an efficient method of inhibiting spontaneous differentiation and apoptosis of these cells while maintaining a homogeneous population of nullipotent cells.

2. *Investigation of transcript expression in Ecad-/- cells cultured in the presence or absence of LIF*

E-cad-/-, Ecad+/- and wild type ES cells were cultured for 12 passages in a gelatin-treated 6-well plate in serum-containing medium in either the presence or absence of LIF. RNA representative of gene expression in the cultured cells was extracted and cDNA formed according to known protocols. Transcripts for pluripotent and differentiated markers were assessed by RT-PCR RNA was extracted and cDNA formed as described in the materials and methods. PCR was performed for 35 cycles (Ecad-/-)
or 45 cycles (wt and Ecad+/-).

The results of this study are shown in Figure 3. As can be seen, Ecad-/- ES cells cultured in the presence of LIF express the pluripotent markers Oct-4 and Nanog and the neuroectoderm marker NF68k. Of the transcript markers assessed, Ecad-/- cells exhibited the same expression profile as wild type D3 ES cells when grown in the presence of LIF. Detection of the neuroectoderm marker NF68k is consistent with published data (Ward et al, 2004 Exp Cell Res. Feb 15;293(2):229-38.), and is consistent with the maintenance of

a pluripotent phenotype. The mesodermal marker brachyury is also consistently detected in Ecad+/- cells cultured in the presence of LIF, and not in Ecad-/- cells. This pattern of brachury expression is in contrast to patterns of expression that have been reported in the prior art.

Ecad-/- ES cells cultured for 17 passages (approximately 34 days) in the absence of LIF do not exhibit an altered transcript expression profile compared to Ecad-/- ES cells cultured in the presence of LIF (results indicated "-LIF"). For example, cells cultured in the absence of LIF maintain expression of the pluripotent markers Oct-4 and Nanog and the neuroectoderm marker NF68k.

When wild-type cells or Ecad +/- cells (i.e. cells in which E-cadherin activity is not inhibited) are cultured in the absence of LIF, no cells remain at passage 17 (they fail to maintain sufficient cell numbers between passages 6-8). The results indicate that this is due to the differentiation of the cells leading to significant cell death and increasing cell cycle times.

In order to assess the transcript expression in differentiating wild-type and Ecad +/- ES cells the inventors assessed transcript marker expression after removal of LIF for 12 days. Results of this study are also shown in Figure 3 (in which 1is b tubulin; 2 is oct4; 3 is nanog; 4 is fgf; 5 is bmp2; 6 is bmp4; 7 is TBra; 8 is zg; 9 is TTR; and 10 is NF68). In wild-type ES cells cultured in the absence of LIF for 12 days upregulation of the transcripts for the endodermal marker transthyretin and maintenance of the neuroectoderm marker NF68k was noted, and this change in transcription indicates the differentiation of the cells. After 12 days in the absence of LIF, Ecad+/- ES cells exhibit upregulation of transcripts encoding brachyury (mesoderm), ζ-globin (mesoderm) and transthyretin (endodermal), as well as maintenance of NF68k expression (neuroectoderm). Detection of Oct-4 and Nanog in differentiating cultures reflects the heterogeneity of the population and is consistent with published data (Ward et al, 2003, 2004).

The results of this study demonstrate that cells in which E-cadherin activity is inhibited (such as Ecad-/- ES cells) may be cultured and passaged in the absence of LIF without differentiation, and cells so cultured maintain a transcript expression profile consistent with an undifferentiated ES cell phenotype.

3. *Overgrowth of Ecad-/- ES cells induces differentiation to the three primary germ layers.*

Wild-type, Ecad+/- and Ecad-/- ES cells were plated out in a well of a 6-well culture plate in the presence of LIF and the medium replaced daily for 12 days without passaging of the cells. Overgrowth of ES cells is a very efficient method of inducing differentiation, since it induces toxic stress (such as oxygen tension etc.) that leads to the differentiation of the cultured cells. Results of this study are shown in Figure 4. Ecad-/-, Ecad+/- and wt ES cells were cultured in a gelatin-treated 6-well plate in the presence of LIF and the medium replaced daily for 12 days without passaging of the cells. RNA was extracted and cDNA formed as described in the materials and methods. PCR was performed for 35 cycles

Ecad-/- ES cells cultured under the conditions referred to above exhibited upregulation of transcripts for brachyury (mesoderm), ζ-globin (mesoderm) and transthyretin (endodermal) as well as maintenance of NF68k transcript expression (neuroectoderm). This demonstrates that at least a proportion of the cells within the Ecad-/- ES cell population were differentiating into the three primary germ layers. Wild-type cells exhibited a similar transcript profile to Ecad-/- ES cells, whereas Ecad+/- lacked expression of ζ-globin transcripts (probably reflecting the transient expression nature of this transcript during ES cell differentiation; Ward et al, 2004).

The skilled person will appreciate that the results of this study indicate that, in cultured cells in which E-cadherin activity is inhibited, differentiation of the cells is retarded to give rise to a phenotype that is effectively pluripotent rather than nullipotent. This in turn clearly indicates the suitability of inhibition of E-cadherin activity to inhibit stem cell differentiation while preserving the full range of therapeutic lineages that may be produced on subsequent differentiation of the stem cell in question.

Embryonic stem cells entirely lacking E-cadherin are unlikely to be able to form epithelium (as they have no E-cadherin) and should therefore be considered to be multipotent, rather than pluripotent, but, as shown elsewhere in the Experimental Results, stem cells in which E-cadherin activity is only transiently inhibited regain the ability to differentiate and form cell types representative of all tissues, on cessation of E-cadherin inhibition.

4. Ecad-/- ES cells divide faster than wt ES cells

Wild type D3 and Ecad-/- ES cells were cultured in wells of a gelatin-treated 6-well plate (3×10^6 cells/well) in the presence of LIF and cell numbers assessed at each passage. The results of this study are shown in Figure 5.

Cells were passaged at days 2, 4 and 5, and cell numbers calculated at each passage. On average, Ecad-/- ES cells exhibited 1.67-fold increased cell numbers compared to wild-type ES cells (designated D3 in Figure 5). E-cadherin null (Ecad-/-) ES cells exhibit increased cell numbers compared to wild-type (wt) D3 ES cells over 5 days in culture. Ecad-/- and wt D3 ES cells were plated at 3,000,000 cells/well in a gelatin-treated 6-well plate and cultured in the presence of LIF for 5 days. Cells were passaged as described in the materials and methods and viable cell numbers assessed using trypan blue exclusion.

Repetition of this experiment on three separate occasions has shown that a similar trend of increased proliferation of cells in which E-cadherin activity is inhibited is consistently demonstrated (data not shown).

The results of this study illustrate that inhibition of E-cadherin activity represents a powerful means by which cell proliferation may be promoted. This indicates that inhibitors of E-cadherin activity may be used to expand biological cell populations *in vitro*, while retarding differentiation of the proliferating cultured cells.

5. DECMA-1 treatment of Oct4-GFP ES cells in the absence of LIF delays differentiation

The inventors have investigated whether inhibition of the activity of cell surface E-cadherin protein is able to prevent differentiation of ES cells in culture. To do so, the inventors cultured biological cells from a number of cell lines in the presence of the neutralising antibody DECMA-1 (Sigma, Dorset, UK; Cat. No. U3254), an inhibitor of E-cadherin activity. OCT4-GFP ES cells were cultured in either DECMA-1 or control antibody in the presence or absence of LIF for 12 passages and assessed for GFP (Oct-4) expression by fluorescent flow cytometry. (Figure 6A) At passage 8 all control cells cultured in the absence of LIF were dead. Right hand profiles show the proportion of DECMA-1 treated OCT4-GFP ES cells that are undifferentiated (GFP+; shifted to the right) or differentiated (GFP-;shifted to the left) cells in the +LIF (top) and –LIF (bottom) cell populations. Left hand FACS profile shows an overlay of the +LIF and –LIF cell populations show in the right hand profiles. (Figure 6B) DECMA-1 treated cells at passage 10; profiles as described above. (Figure 6C) DECMA-1 treated cells at passage 11, profiles as described above. (Figure 6D) DECMA-1 treated cells at passage 12; profiles as described above. (Figure 6E) Total cell numbers accumulating over 12 passages in OCT4-GFP ES cells treated with DECMA-1 and cultured in the presence or absence of LIF.

DECMA-1 appears to induce the internalisation of cell surface E-cadherin protein, thereby inhibiting the normal biological function of the molecule. The concentrations of the neutralising antibody required to induce a phenotype similar to that observed in Ecad-/- ES cells was dependent on the ES cell line investigated. For example, MESC ES cells required 2.9μg/ml of antibody, D3 required 5.8μg/ml and E14TG2a required 11.6μg/ml DECMA-1 to induce loss of cell-cell contacts. An anti-tenascin antibody (Sigma; Cat. No. T3413) was used as a control antibody.

To study the effect of DECMA-1 on ES cell pluripotency/differentiation (i.e. the ability of the inhibitor of E-cadherin activity to induce cellular proliferation without differentiation) the inventors utilised E14TG2a ES cells expressing GFP under the regulatory elements of Oct-4 (Oct4-GFP ES cells). Loss or retention of pluripotency was assessed using fluorescent flow cytometry. The results of this study are shown in Figure 6.

11.6μg/ml of DECMA-1 or control antibody was added to the cells in a six well plate and the cells cultured for 12 passages in the presence or absence of LIF (fresh antibody was added at each passage). Addition of either DECMA-1 or control Ab to Oct4-GFP ES cells cultured in the presence of LIF for 12 passages did not affect Oct-4 expression, as defined by GFP expression.

At passage 8 (results shown in Figure 6A) Oct4-GFP ES cells cultured in the absence of LIF in the presence of control Ab were unable to maintain cell proliferation (when cultured under identical conditions to nAb (DECMA-1)-treated ES cells) whereas Oct4-GFP ES cells cultured in the presence of LIF and the inhibitor of E-cadherin activity DECMA-1 exhibited 93.45% GFP-positive cells and Oct4-GFP ES cells cultured in the absence of LIF and presence of DECMA-1 exhibited 62.21% GFP-positive cells.

At passage 10 (results shown in Figure 6B) Oct4-GFP ES cells cultured in the presence of LIF and DECMA-1 exhibited 93.74% GFP-positive cells and Oct4-GFP ES cells cultured in the absence of LIF and presence of DECMA-1 exhibited 63.32% GFP-positive cells (a slight increase on passage 8).

At passage 11 (results shown in Figure 6C), Oct4-GFP ES cells cultured in the presence of LIF and DECMA-1 exhibited 93.34% GFP-positive cells and Oct4-GFP ES cells cultured in the absence of LIF and presence of DECMA-1 exhibited 55.65% GFP-positive cells.

At passage 12 (results shown in Figure 6D), Oct4-GFP ES cells cultured in the presence of LIF and DECMA-1 exhibited 93.34% GFP-positive cells and Oct4-GFP ES cells cultured in the absence of LIF and presence of DECMA-1 exhibited 55.65% GFP-positive cells (no change from passage 11). These results clearly show that addition of the E-cadherin neutralising antibody DECMA-1 to Oct4-GFP ES cells can delay differentiation in the absence of LIF compared to control Ab treated cells.

However, the total cell numbers obtained from the Oct4-GFP cells +DECMA-1 in the absence of LIF were somewhat reduced compared to cells cultured in the presence of LIF (as shown in Figure 6E), although the inventors believe that this decrease may be overcome on optimisation of the dosage of the inhibitor of E-cadherin activity employed.

6. Inhibition of E-cadherin activity by transient expression of RNAi.

Vectors (pRNATin-H1.2 from Genscript) encoding the RNAi of Sequence ID No. 22 to Sequence ID No. 26 are introduced into human embryonic stem cells by means of electroporation using the Amaxa protocol. Vectors (pRNATin-H1.2 from Genscript) encoding the RNAi of Sequence ID No. 27 to Sequence ID No. 29 are introduced into murine embryonic stem cells by means of the same protocol.

Vectors encoding suitable inhibitors of E-cadherin activity may preferably also comprise selection elements allowing selection of cells into which the vectors have been successfully introduced. Examples of such selection elements are well known to those skilled in the art and include antibiotic resistance genes. For example, it may be preferred that vectors encoding inhibitors of E-cadherin activity also comprise selection elements comprising a neomycin resistance gene. In accordance with this example, cells into which the vectors have been successfully introduced (for instance by means of electroporation) may be identified by virtue of their resistance to the antibiotic neomycin, this resistance being conferred by the neomycin resistance elements incorporated in the vectors encoding the RNAi inhibitors.

7. Assessment of E-cadherin "knockdown".

7.1 In human stem cells.

Human stem cells transfected with vectors encoding inhibitors of E-cadherin activity as described elsewhere in the specification may be further investigated in order to confirm that E-cadherin activity in the cells is functionally inhibited. Such inhibition may be brought about through combined activity of one or more RNAi inhibitor (for example those encoded by Sequence ID No. 22 to Sequence ID No. 26). Functional inhibition of E-cadherin activity may be brought about by either partial inhibition or total inhibition of E-cadherin, so long as sufficient inhibition of E-cadherin activity occurs to give rise to the

beneficial effects of retarding biological cell differentiation. Such functional inhibition of E-cadherin activity may be termed E-cadherin "knockdown".

The knockdown effectiveness of the individual RNAi inhibitors encoded by Sequence ID No. 22 to Sequence ID No. 26 may be investigated through the introduction of vectors encoding individual inhibitors selected from this group into human cells such as stem cells. Preferably each inhibitor may be individually tested, and combinations of individual inhibitors may also be investigated in order to identify whether such combinations are able to give rise to additive (or synergistic) inhibitory effects that may be of particular benefit in accordance with the present invention. It will be appreciated that when investigating the effects of vectors in this manner the vectors may be introduced into human cells by means of Amaxa electroporation, as described elsewhere.

7.2 In murine stem cells.

Murine stem cells may be transfected (as described above with reference to human cells) and studied to confirm that E-cadherin activity in the cells is inhibited, either partially or entirely, through the combined activity of the RNAi inhibitors. Such inhibition of E-cadherin activity may be termed E-cadherin "knockdown"

The knockdown effectiveness of the individual RNAi inhibitors may be investigated through the introduction of vectors encoding individual inhibitors selected from Sequence ID No. 27 to Sequence ID No. 29 into murine stem cells. Each inhibitor is individually tested. Vectors may be introduced by means of Amaxa electroporation, as before.

8. Generation of human and murine cell lines in which E-cadherin knockdown has been efficiently achieved.

Clonal populations may be derived by culture of cells in which E-cadherin knockdown has been efficiently achieved (for instance by means of the methods described above). Such clonal populations may be generated by methods such as ring cloning, and then expanded to generate cell lines in which E-cadherin activity is functionally inhibited through the action of RNAi inhibitors.

9. Evaluation of reversible RNA inhibition of E-cadherin in hES cells as a tool for inhibition of differentiation.

The use of transient RNAi inhibition (for example using RNAi inhibitors as considered elsewhere encoded for by vectors such as Genscript pRNATin-H1.2) to block cellular differentiation may be investigated by visualisation of gross cellular morphology and by monitoring the expression of pluripotent markers (investigation of both marker proteins and mRNA encoding such proteins). Previous evaluation of this technology by repression of Oct-4 in mES cells has demonstrated its utility.

A preferred protocol by which transient expression of inhibitors of E-cadherin activity may be assessed will use Amaxa electroporation to allow incorporation of vectors encoding the RNAi inhibitors of Sequence ID No. 22 to Sequence ID No. 26. The study will initially investigate the effects achieved using combinations of RNAi inhibitors (including the introduction of all vectors into experimental cells by electroporation) to confirm E-cadherin knockdown and identify preferred inhibitor combinations. Thereafter, individual vectors encoding specific RNAi inhibitors will be assessed individually to identify preferred inhibitors suitable for use to bring about E-cadherin knockdown. As described previously, vectors used in this investigation will include a neomycin resistance gene, and cells into which the vectors have been successfully introduced (either singly or in combination) will be isolated by virtue of their neomycin resistance. Clonal populations of cells in which E-cadherin activity is inhibited will be isolated and used to derive efficient E-cadherin knockdown cell lines.

Genetic stability of the cells into which vectors encoding RNAi inhibitors have been introduced will be analysed by karyotype analysis in accordance with the protocols set out elsewhere in the specification.

10. Reversal of E-cadherin repression in mouse and human ES cells.

It will clearly be appreciated that inhibition of E-cadherin activity allows differentiation of biological cells to be inhibited or retarded. Subsequent reversal of this inhibition (a reversal herein designated EcadR) allows the differentiation of biological cells to resume thereby giving rise to useful differentiated cell types.

Inhibition of E-cadherin activity in human and mouse ES cells (achieved using either RNAi inhibitors or function neutralising antibodies, although it will be appreciated that any suitable method of inhibition may be used) will be reversed. The ability of cells in which EcadR has been effected to differentiate into cells of the primary germ layers may then be assessed by RT-PCR and immunofluorescent analysis of various lineage markers. The ability of EcadR cells to differentiate into various cell lineages may additionally or alternatively be investigated via overgrowth of such cells (as described elsewhere in the specification) followed by suitable analysis to investigate whether markers of differentiation are expressed.

In vivo confirmation of the ability of the EcadR cells to differentiate and give rise to the three germ layers will be achieved by sub-cutaneous injection of the cells into Severe Combined Immunodeficiency Disease (SCID) mice. Tissue sections derived from the sites where EcadR cells have grown will then be processed for histology and the differentiation of the cells assessed. Such assessment may be carried out using standard histological analysis of suitably stained sections (for example stained with haemotoxylin and eosin) from the cell growths.

Mouse EcadR cells constitutively expressing β-galactosidase (ROSA26 cell line) will be injected into pre-implantation mouse blastocysts and their incorporation into the embryo assessed at E9.5 by β-galactosidase expression in embryo sections. These experiments will confirm that inhibition of E-cadherin and subsequent reversal of the inhibition does not affect the pluripotency of the ES cells.

11. To determine whether inhibition of E-cadherin in mouse embryos increases the efficiency of ES cell derivation.

Current techniques for the derivation of ES cell lines from mouse embryos are only 30% efficient. The inventors believe that efficiency of ES derivation may be improved using the methods of the invention. Therefore studies will be undertaken to determine whether E-cadherin repression in mouse embryos improves the derivation of ES cell lines. Briefly, E-cadherin expression will be inhibited in delayed implantation mouse embryos

using either double stranded oligonucleotide RNAi or neutralising Abs (although it will be appreciated that alternative strategies may be used) and isolation efficiency of ES cell lines in which E-cadherin activity is inhibited compared to that achieved using control embryos.

12. Cell surface SSEA-1 expression in wild type D3 (wt) and E-cadherin null (Ecad-/-) ES cells overgrown for 12 days.

Wild type (wt) or E-cadherin null (Ecad-/-) embryonic stem cells were cultured in a gelatin-treated 6-well plate in the presence of LIF. Culture medium was replaced daily for 12 days without passaging of the cells. Cells were trypsinised and assessed for expression of the primitive cell marker stage specific embryonic antigen-1 (SSEA-1) using a phycoerythrin-conjugated antibody recognising SSEA-1. Cell fluorescence was analysed using a Becton Dickinson FACScaliber. Viable cells were gated using forward and side scatter.

Data generated from representative of cells from this population are shown in Figure 17. The level of SSEA-1 expression by wild type and E-cadherin null cells is comparable at both day 0 and day 12. This indicates that, although the differentiation of cells in which E-cadherin activity is inhibited (such as E-cadherin null ES cells) is retarded, such cells are able to differentiate when induced to overgrow. The data shown in Figure 17 corroborate the results of RT-PCR shown in Figure 4.

13. E-cadherin null ES cells remain undifferentiated in suspension culture.

Wild type (wt) or E-cadherin null (Ecad-/-) ES cells were cultured for 30 days in suspension by plating 10^6 cells (in 10mls of medium lacking LIF) in a plastic bacteriological Petri dish. Culture medium was changed every day. Cells were passaged when required (usually every 2 days for E-cadherin null ES cells) by transfer of 2.5mls of cell suspension into 7.5mls of fresh medium.

Cells cultured in this manner were investigated in a number of ways, and the results of these investigations are shown in Figure 18.

Panel A of Figure 18 shows phase contrast microscopy images comparing cultures of wild type and E-cadherin null cells at day 5 in suspension culture. It can be seen that E-cadherin null ES cell suspensions lack adhesion as compared to wild-type ES cells, since the E-cadherin null cells do not form embryoid bodies characteristic of the wild-type ES cells.

Panel B of Figure 18 shows the results of analysis of the RNA content of cultured wild type and E-cadherin null cells. RNA was collected from the cell suspensions at day 4, 12 and 30 and assessed for Oct-4, transthyretin (TTR), fibroblast growth factor-5 (fgf-5), zeta-globin (zg), brachyury (T), neurofilament-68 (NF68) and alpha-foetal protein (AFP) transcripts using RT-PCR. Note that E-cadherin null ES cells fail to express the endoderm markers TTR and AFP and retain Oct-4 expression over 30 days. Detection of Fgf-5 transcripts suggest that a proportion of the E-cadherin null ES cells are of a primitive ectoderm (pluripotent) cell type, equivalent to the epiblast stage of the early embryo.

Panel C of Figure 18 compares labelling for NANOG protein in wild type and E-cadherin null cells. After 30 days in the absence of LIF, cell suspensions of the type described above were cultured in gelatin-treated 6 well plates in the presence of LIF for 2 passages and assessed for NANOG protein expression using fluorescent microscopy. It can be seen that E-cadherin null ES cells maintain expression of nuclear NANOG, while wild type cells cultured in this manner did not exhibit any NANOG positive cells.

The results shown in panels B and C of Figure 18 clearly illustrated that biological cells in which E-cadherin activity is inhibited do not differentiate in suspension culture. Furthermore, ES cells in which E-cadherin activity is inhibited maintain expression of the pluripotent nuclear Nanog protein. The expression of FGF-5 transcripts by these cells indicates that they may be representative of pluripotent primitive ectoderm cells.

The results shown in panel D of Figure 18 investigate the RNA content of E-cadherin null cells cultured in suspension and then subject to forced expression of E-cadherin. E-cadherin null cells were cultured for 30 days in suspension in the absence of LIF and transfected with either a control vector (pCMV) or a vector expressing full length E-cadherin cDNA. The cells were then cultured for 3 days in the absence of LIF in gelatin treated tissue culture plates and assessed for expression of pluripotency-associated transcripts (Oct-4 and Nanog) and differentiation-associated transcripts brachury (T), zeta-globin (zg) and transthyretin (TTR). Both populations of cells expressed the pluripotency-associated transcripts, but only those cells transfected with full length E-cadherin cDNA expressed the differentiation markers.

These results illustrate that retardation of differentiation that occurs on inhibition of E-cadherin activity can be reversed when such inhibition ceases (in this case by forced expression of full length E-cadherin cDNA). This indicates that suspension culture of cells, such as ES cells, may be carried out while E-cadherin activity is inhibited in order to derive an expanded population of undiffererentiated pluripotent cells, and that differentiation of these cells may then be induced by cessation or reversal of E-cadherin inhibition.

14. Addition of E-cadherin inhibitor DECMA-1 antibody to wild-type ES cells delays their differentiation in suspension culture.

Wild-type MESC ES cells (approx. 10^6 cells in 10mls of medium) were cultured for 10 days in the presence of either control antibody (cAb) or the E-cadherin inhibitor DECMA-1 antibody (nAb) (30μl total antibody) in suspension in the absence of LIF in a plastic bacteriological Petri dish. Culture medium was changed every day. Cells were passaged when required (usually every 2 days for nAb treated ES cells) by transfer of 2.5mls of cell suspension into 7.5mls of fresh medium containing the appropriate antibody concentration.

Panel A of Figure 19 compares phase contrast micrographs of cells cultured in suspension for 10 days in either cAb or nAb. It can be seen that cAb treated cultures include

embryoid bodies, indicative of adhesions between the cultured cells, whereas the incidences of adhesion between nAb treated cell is much reduced.

Panel B of Figure 19 compares phase contrast micrographs of cAb and nAb treated cells cultured as above, and then plated onto gelatin-treated tissue culture plates in the presence of LIF for 1 passage. Assessment of colony morphology illustrates that nAb treated cells exhibited ES cell like colony morphology but that cAb treated cells do not.

cAb and nAb cells were plated onto gelatin-treated tissue culture plates in the presence of LIF for 2 passages and assessed for SSEA-1 expression using fluorescent flow cytometry as described above. The results of this study are shown in panel C of Figure 19. These results show that nAb treated populations contained higher levels of SSEA-1 expressing cells than did their cAb treated counterparts. This indicates that cells cultured in the presence of the E-cadherin inhibitor DECMA-1 retain a less differentiated state than cells cultured in the presence of a control antibody.

nAb treated cells cultured as described in the preceding paragraph were further cultured in the absence of LIF and nAb for 3 days in gelatin treated tissue culture plates and assessed for expression of the pluripotent transcripts (Oct-4 and Nanog) and the differentiation-associated transcripts brachury (T), zeta-globin (zg) and transthyretin (TTR). The results of this assessment are shown in panel D of Figure 19. These clearly indicate that, although nAb treated cells cultured in this manner can be induced to express markers of differentiation, they also retain pluripotency-associated transcripts.

15. Investigation of transcript expression in E-cadherin null (Ecad-/-) cells transfected with full length E-cadherin cDNA and wild type (wt) D3 ES cells cultured in FCS-LIF for 3 days.

E-cadherin null cells were transfected with a vector expressing full length E-cadherin cDNA (EHA) and cultured for 3 days in the absence of LIF in gelatin treated tissue culture plates. RT-PCR was used to assess the expression of the pluripotent transcripts (Oct-4 and Nanog) and the differentiation-associated transcripts brachury (T), zeta-globin

(zg) and transthyretin (TTR). The results of this assessment are shown in Figure 20. Here it can be seen that when E-cadherin null cells are transfected with full length E-cadherin cDNA they express markers indicative of differentiation. Wild-type D3 ES cells represent a positive control. These results demonstrate that when inhibition of E-cadherin activity is ceased (in this case through forced expression of full length E-cadherin cDNA in E-cadherin-/- ES cells) cells are able to undergo differentiation.

16. RNAi inhibition of E-cadherin activity in wild-type MESC ES cells results in inhibition of cell surface E-cadherin and allows culture of the cells, and retardation of differentiation, in the absence of LIF.

MESC20 ES cells were transfected with hairpin loop RNAi contructs as described in the experimental protocols. The cells' expression of E-cadherin and of the primitive cell marker SSEA-1 were investigated using fluorescence flow cytometry. The results of this investigation are shown in Figure 21.

E-cadherin expressed by wild-type (wt) and RNAi treated MESC20 ES cells (clone M1) was labelled using the E-cadherin neutralising antibody DECMA-1, and labelling analysed by flow cytometry. The results are shown in panel A of Figure 21, in which it can be seen that the labelling in M1 cells corresponds almost exactly to control values, while wild type cells exhibit far greater labelling. This indicates that E-cadherin was absent from almost the entire population of M1. This demonstrates that RNAi treatment represents a suitable means by which E-cadherin activity can be inhibited in cells such as stem cells.

MESC20 clone M1 ES cells (prepared as above) were cultured for 5 passages in the absence of LIF. SSEA-1 expression was then determined by fluorescent flow cytometry. The results are shown in panel B of Figure 21, and show that that SSEA-1 expression was not decreased following removal of LIF. These results clearly indicate that inhibition of E-cadherin activity can retard cell differentiation (as evidenced by the high expression of the primitive cell marker SSEA-1) even in the absence of LIF.

17. RNAi inhibition of E-cadherin activity in wild type MESC ES cells allows suspension culture for 30 days in the absence of differentiation.

Wild type (wt) MESC or MESC ES cells transfected with E-cadherin RNAi cells (clone M1 as described above) were cultured for 30 days in suspension by plating 10^6 cells in 10mls of medium lacking LIF in a plastic bacteriological Petri dish. The culture medium was changed every day. Cells were passaged when required (usually every 2 days for E-cadherin RNAi ES cells) by transfer of 2.5mls of cell suspension into 7.5mls of fresh medium.

Culture in this manner was able to achieve a 268,000,000 fold expansion of total cell numbers.

The properties of these cells were investigated in a series of studies, the results of which are shown in Figure 22.

Panel A of Figure 22 compares phase contrast microscopy images of day 5 cultures of M1 ES cells and wild type ES cells. These images clearly show the lack of adhesion in clone M1 ES cell suspensions compared to wild-type ES cells. Clone M1 ES cells (lacking E-cadherin activity) do not form the embryoid bodies observed in wild type cultures.

After 30 days in the absence of LIF, the cell suspensions were cultured in gelatin-treated plates in the presence of LIF for 2 passages and assessed for NANOG protein expression using fluorescent microscopy. Representative fluorescent microscopy images are shown in panel B of Figure 22. Here it can be seen that that E-cadherin null ES cells (shown in the lower two images) maintain expression of nuclear NANOG. Wild-type cells did not exhibit any NANOG positive cells. These results further serve to illustrate that cells in which E-cadherin activity is inhibited (in this case by RNAi) express markers indicative of pluripotency.

Panel C of Figure 22 shows the results of fluorescent flow cytometry undertaken to assess expression SSEA-1 in clone M1 cells cultured for 30 days in suspension in the absence of LIF in gelatin treated plates.

Panel D of Figure 22 shows the results of fluorescent flow cytometry undertaken to assess expression of SSEA-1 by clone M1 cells cultured for 30 days in suspension in the absence of LIF and after 2 passages in the presence of LIF. The expression of SSEA-1 in these cells demonstrates that the majority of the cells are pluripotent (a finding that substantiates the result shown in Figure 22B and E).

Expression of pluripotent transcripts (Oct-4 and Nanog) and the differentiation-associated transcripts brachury (T), zeta-globin (zg) and transthyretin (TTR) by clone M1 after 30 days in suspension culture in the absence of LIF was investigated using RT-PCR analysis, and the results are shown in panel E of Figure 22. These show that the cells expressed the pluripotent markers but not differentiation markers. This provides a further illustration of the fact that inhibition of E-cadherin allows biological cells to divide without undergoing differentiation.

18. Complete inhibition of E-cadherin is not required for undifferentiated monolayer culture of ES cells in the absence of LIF.

MESC20 ES cells were transfected with RNAi contructs as described in the experimental protocols to produce two separate clones, M1 (as described previously) and "clone 2" (cl2).

Panel A of Figure 23 illustrates E-cadherin expression by wild type ES cells (wt), M1 and clone 2, as determined using fluorescently labelled DECMA-1 antibody for fluorescent flow cytometry. It can be seen that (as shown previously) clone M1 exhibits little or no expression of E-cadherin (levels comparable to negative control), whereas wild type cells express E-cadherin protein. Clone 2 exhibits a level of E-cadherin expression that is intermediate between that of wt and M1 cells.

Panel B of Figure 23 shows SSEA-1 expression (determined by fluorescent flow cytometry) of MESC20 clone 2 ES cells cultured for 5 passages in the presence of LIF. In contrast, panel C of Figure 23 shows SSEA-1 expression (determined by fluorescent flow cytometry) in MESC20 clone 2 ES cells cultured for 5 passages in the absence of LIF. Comparison of panels B and C illustrates that expression of the primitive cell marker SSEA-1 was not decreased following removal of LIF from clone 2 ES cells, indicating that inhibition of E-cadherin activity represents a technique by which differentiation of cells in monolayer culture may be retarded (and cell numbers increased) even without the addition of LIF.

Panel D of Figure 23 shows a phase contrast microscopy image illustrating the formation of three dimensional embryoid bodies following culture of clone 2 ES cells in suspension culture in the absence of LIF.

The results show that, although partial inhibition of E-cadherin activity is sufficient to retard differentiation of murine cells when grown in monolayer culture without LIF, it is not sufficient to retard differentiation of murine cells in suspension culture without LIF. Accordingly it will be appreciated that, in the event that it is wished to retard the differentiation of murine cells in suspension culture, it will be preferred that E-cadherin should be substantially totally inhibited. In the event that it is wished to retard the differentiation of murine cells in monolayer culture, this may be achieved using partial or total inhibition of E-cadherin activity. By the same token, it will be recognised that an inhibitor of E-cadherin activity that is only capable of partial inhibition of E-cadherin may still be used to retard the differentiation of murine cells in accordance with the present invention, but that the cells differentiation of which is to be retarded should be grown in monolayer culture.

Monolayer cultures of murine cells grown in the absence of LIF and with only partial inhibition of E-cadherin may exhibit some level of differentiation. As a result, it may generally be preferred that total (or substantially total) inhibition of E-cadherin activity be used in such cultures of cells in order to most effectively retard differentiation.

It should be noted that the results described in Figure 23 were derived in investigations using murine cells. Accordingly, the information gained from these studies may be most applicable to the culture of murine cells. Further investigations of the effects of total or partial inhibition of E-cadherin activity in cultured human cells are described elsewhere in the specification.

19. Inhibition of E-cadherin activity in human HES4 ES cells retards differentiation of the cells and results in increased numbers of undifferentiated ES cell colonies.

Human ES cell line HES4 was cultured in the presence of either control antibody (cAb) or SHE78.7 an E-cadherin neutralising antibody that serves to inhibit E-cadherin activity. 10μl/ml of media of a stock 0.5mg/ml solution of the relevant antibody was added to serum replacement medium containing FGF-2. Cells were grown on a fibroblast feeder layer for 2 days and assessed for colony morphology (phase contrast), actin cytoskeleton arrangement (phalloidin labelling) and expression of E-cadherin and OCT-4 proteins by immunofluorescent microscopy. Location of OCT-4 labelling was compared with that of DAPI staining, which shows cell nuclei. The results of these assessments are shown in panel A of Figure 24.

Images from phase contrast microscopy show that addition of SHE78.7 causes loss of cell-cell contact. Incidences of cell-to cell adhesion were reduced among cells treated with the inhibitor of E-cadherin activity compared to those treated with control antibody which exhibited normal levels of cell to cell adhesion in forming a monolayer.

The images obtained using phalloidin staining illustrate alteration of the actin cytoskeleton in cells treated with the inhibitor of E-cadherin activity, and it can be seen that these cells also exhibit decreased E-cadherin staining. However, expression of the pluripotency-associated marker OCT-4 is not decreased in human cells cultured in the presence of an inhibitor of E-cadherin activity (as shown by labelling of OCT-4).

HES4 colonies cultured in the presence of E-cadherin neutralising antibody or control antibody were passaged mechanically, and at passage 2 the number of undifferentiated colonies was assessed (after removal of nAb for 2 days). The results of this assessment are shown in panel B of Figure 24. These results clearly illustrate that cells treated with a neutralising antibody inhibitor of E-cadherin activity exhibited higher numbers of undifferentiated colonies than did cells treated with a control antibody.

The number of undifferentiated colonies was also assessed at passage 3 (after removal of nAb for 2 days), and these results are shown in panel C of Figure 24. It can be seen that, among cells treated with the inhibitor of E-cadherin activity SHE78.7, almost 90% of colonies exhibited an undifferentiated phenotype colonies compared to only approximately 60% of colonies among cells treated with control antibody.

HES4 cells cultured in the presence of control antibody or neutralising antibody (10μl/ml of media of a stock 0.5mg/ml solution) were assessed for cell numbers over 5 passages. The results are shown in panel D of Figure 24, and illustrate that cell proliferation is decreased among cells treated with neutralising antibody compared to cells treated with control antibody.

20. Removal of inhibitors of E-cadherin activity, such as neutralising antibody SHE78.7, from HES4 ES cells for 7 days restores cell-cell contact.

The results reported above indicated that inhibition of E-cadherin activity was able to reduce E-cadherin expression, and also to reduce the incidence of cell to cell contacts between cultured cells. The following study was undertaken to investigate whether cells cultured in this manner were able restore normal cell to cell contact on cessation of E-cadherin inhibition.

Human ES cells HES4 were cultured in the presence of either control antibody (cAb) or E-cadherin neutralising antibody SHE78.7 (10μl/ml of media of a stock 0.5mg/ml solution) on a fibroblast feeder layer in serum replacement medium containing FGF-2 for 2 days, and then nAb removed for 7 days. Cells were then assessed for colony

morphology (by phase contrast microscopy), actin cytoskeleton arrangement (by phalloidin labelling) and expression of OCT-4 protein (immunolabelling combined with a DAPI counterstain to show cell nuclei).

The results of this assessment are shown in panel A of Figure 25. Here it can be seen that removal of the inhibitor of E-cadherin activity SHE78.7 restores cell-cell contact and cortical actin cytoskeleton arrangement so that these resemble cells treated with a control antibody (for example, compared with "icAb" cells shown in panel A of Figure 24). It can also be seen that, even after cessation of E-cadherin inhibition, the majority of the population of cells treated in this manner maintain expression of OCT-4 protein.

HES4 ES cell colonies treated as described in the preceding paragraph were assessed for expression of transcripts associated with pluripotency (Oct4) and further markers as described in Ward et al, 2006 and in the Methods. The expression profile observed (shown in panel B of Figure 25) is consistent with that that has previously been observed in undifferentiated HES4 ES cells (for example in an expression profile described by Ward et al., 2006).

HES4 ES cell colonies described as described in the preceding passages were allowed to overgrow in the culture plates (i.e. without passaging) for 20 days to induce differentiation of the cells. Expression of various markers of differentiation was then investigated. The results of this investigation are shown in panel C of Figure 25. Markers of differentiation expressed following differentiation of the cells included markers representative of all three germ layers (endoderm – HNF, TF, AMY; mesoderm – FLK, CD34, AC133; ectoderm – NES, NFM, NSE, PAX and PLP) and extra-embryonic visceral endoderm (AFP). This clearly illustrates that inhibition of E-cadherin activity does not prevent subsequent differentiation of cells once inhibition is ceased.

21. Inhibition of E-cadherin activity (such as by the neutralising antibody SHE78.7) allows the culture of hES in the absence of FGF-2.

HES4 and H1 human ES cell lines were cultured in the presence of a minimal fibroblast feeder layer (approximately 1000 cells/dish) in the absence of FGF-2 in serum replacement medium in the presence of either the E-cadherin neutralising antibody SHE78.7 or control antibody (0.5μl/ml of media of a stock 0.5mg/ml solution). The inventors believe that this concentration of SHE78.7 in culture medium is sufficient to partly inhibit E-cadherin activity in cells grown in the medium.

Panel A of Figure 26 shows phase contrast microscopy images of HES4 ES cells cultured in control antibody (cAb) or E-cadherin neutralising antibody SHE78.7 (nAb) after 2 passages in the absence of FGF-2. Analysis of colony morphology shows that cells grown in the presence of the inhibitor of E-cadherin activity retain normal colony morphology (indicating that the cells had not undergone differentiation), whereas cells cultured using control antibody have morphologies indicating that they have undergone differentiation.

Panel B of Figure 26 compares the number of undifferentiated and differentiated HES4 colonies among populations of cells treated with E-cadherin neutralising antibody or with control antibody as described above. It is very surprising to note that all colonies exhibited of cells grown in the presence of the inhibitor of E-cadherin activity SHE78.7, and in the absence of FGF-2, exhibit undifferentiated morphology.

Panel C of Figure 26 shows the results of fluorescent flow cytometry analysis of cell surface expression of the pluripotent marker Tra-1-60 on HES4 ES cells. Expression of Tra-1-60 after 3 passages was compared between:

1. HES4 ES cells treated with control antibody in the absence of FGF-2 (labelled cAb in Panel C);
2. HES4 ES cells treated with E-cadherin neutralising antibody in the absence of FGF-2 (labelled nAb in Panel C); and
3. HES4 ES cells cultured under normal prior art conditions on a fibroblast feeder layer containing FGF-2 (labelled HES4 in Panel C).

The results illustrate that cells cultured in the presence of an inhibitor of E-cadherin activity (nAb cells) exhibited the highest expression of Tra-1-60, even when compared to HES4 cells cultured under normal prior art conditions.

Panel D of Figure 26 shows phase contrast microscopy images of H1 ES cells cultured in control antibody (cAb) or E-cadherin neutralising antibody SHE78.7 (nAb) after 2 passages in the absence of FGF-2. As above, cells grown in the presence of an inhibitor of E-cadherin activity (nAb) exhibited normal colony morphology indicating that they had not undergone differentiation, whereas the morphology of cAb treated cells indicated that they were differentiated.

Panel E of Figure 26 compares the number of undifferentiated and differentiated H1 colonies in cAb or nAb treated cells as shown in panel D above. This clearly shows that all nAb colonies exhibited undifferentiated morphology in the absence of FGF-2, whereas the majority of cAb treated colonies were differentiated.

Panel F shows the results of fluorescence flow cytometry analysis of surface expression of the pluripotent marker Tra-1-60 was on nAb treated HES4 ES cells (all cAb treated cells had died) after 5 passages in the absence of FGF-2. It can be seen that >99% of the nAb treated cells exhibited Tra-1-60 expression.

Taken together, these results shown in Figure 26 illustrate that treatment of hES cells with an inhibitor of E-cadherin activity (such as the E-cadherin neutralising antibody SHE78.7) allows successful culture of the cells, and their maintenance in an undifferentiated state, in the absence of FGF-2.

The inventors believe that the concentration of SHE78.7 utilised in this study is sufficient to bring about partial (as opposed to complete) inhibition of E-cadherin activity (indicated by the fact that cells treated in this manner still exhibit cell-cell contact, indicative of retention of some E-cadherin activity). The partial inhibition of E-cadherin activity achieved by this concentration is sufficient to retard differentiation of treated cells, as compared to differentiation occurring in control treated populations. The partial

inhibition of E-cadherin activity in this manner confers advantages in that human cells (such as stem cells) cultured in this way are able to retain normal cell to cell contacts, while still being subject to retarded differentiation. Accordingly the partial inhibition of E-cadherin activity represents a preferred mode by which differentiation of human cells in monolayer culture may be retarded, in particular when using a mechanical passage technique.

Not only is colony morphology maintained in populations in which E-cadherin activity is inhibited, but so is the cell surface pluripotent marker Tra-1-60. The results reported confirms the utility of this method in two independent hES cell lines; H1 and HES4.

22. Culture of hES cells in E-cadherin neutralising antibody SHE78.7 allows their prolonged culture in the absence of FGF-2.

HES4 and H1 human ES cells were cultured in medium containing E-cadherin neutralising antibody SHE78.7 ("nAb") (0.5µl/ml of media of a stock 0.5mg/ml solution) in the presence of minimal fibroblast feeder layer in the absence of FGF-2 in serum replacement medium for 10 passages (approximately 90 days).

Plate A of Figure 27 shows phase contrast microscopy images of HES4 ES cells cultured in E-cadherin neutralising antibody SHE78.7 (nAb) after 10 passages in the absence of FGF-2 (2 days after transfer). It can be seen that all nAb-treated cells exhibited normal colony morphology (x100 magnification).

Plate B of Figure 27 shows phase contrast microscopy images of H1 ES cells cultured in E-cadherin neutralising antibody SHE78.7 (nAb) after 10 passages in the absence of FGF-2 (2 days after transfer). It can be seen that all nAb-treated cells exhibited normal colony morphology (x100 magnification).

23. Culture of HES4 hES cells in E-cadherin neutralising antibody SHE78.7 allows their prolonged culture in the absence of FGF-2.

HES4 ES cells were cultured in nAb (0.5μl/ml of media of a stock 0.5mg/ml solution) in the presence of minimal fibroblast feeder layer in the absence of FGF-2 in serum replacement medium for 10 passages (approximately 90 days). Figure 28 sets out phase contrast microscopy images showing colony morphology at x100 and x200 magnification. It can be seen that the cells cultured in the presence of an inhibitor of E-cadherin activity exhibit normal colony morphology.

24. Culture of H1 ES cells in E-cadherin neutralising antibody SHE78.7 allows their prolonged culture in the absence of FGF-2.

H1 ES cells were cultured in nAb (0.5μl/ml of media of a stock 0.5mg/ml solution) in the presence of minimal fibroblast feeder layer in the absence of FGF-2 in serum replacement medium for 10 passages (approximately 90 days). Figure 29 sets out phase contrast microscopy images showing colony morphology at x100 and x200 magnification. As before, it can be seen that the cells exhibit normal colony morphology.

Taken as a whole, the results shown in Figures 27 to 29 illustrate that inhibition of E-cadherin activity (such as with the E-cadherin neutralising antibody SHE78.7) allows human stem cells (such as H1 and HES4 hES cells) to be maintained in culture, without significant differentiation, for prolonged periods in the absence of FGF-2 in synthetic serum using minimal feeder layers. Cells maintained in culture in this manner retain their pluripotent nature.

25. HES4 ES cells cultured for 10 passages in E-cadherin neutralising antibody exhibit normal transcript expression and are able to differentiate to produce all three germ layers.

HES4 ES cell colonies were cultured in nAb SHE78.7 (0.5μl/ml of media of a stock 0.5mg/ml solution) in the presence of a minimal fibroblast feeder layer in the absence of FGF-2 in serum replacement medium for 10 passages (approximately 90 days) and assessed for expression of transcripts associated with pluripotency (Oct4) and various lineage markers (as described in Ward et al., 2006). The results of this assessment are

shown in panel A of Figure 30. The transcript profile expression is consistent with that observed for undifferentiated HES4 ES cells (as reported in Ward et al, 2006).

HES4 ES cell colonies described in the preceding paragraph were allowed to overgrow in the culture plates (i.e. by culture without passaging) in normal ES cell culture medium (i.e. +FGF-2) for 20 days in the absence of nAb to induce differentiation of the cells. The cells were then assessed for expression of transcripts associated with pluripotency (Oct4) and various lineage markers (as described in Ward et al., 2006). The results of this assessment are shown in panel B of Figure 30. Note that markers of differentiation expressed following differentiation of the cells included all three germ layers (endoderm – as shown by markers HNF, TF, AMY; mesoderm – as shown by markers FLK, CD34, AC133; ectoderm – as shown by markers NES, NFM, NSE, PAX and PLP) and extra-embryonic visceral endoderm (AFP).

26. H1 ES cells cultured for 10 passages in E-cadherin neutralising antibody exhibit normal transcript expression and differentiate to all three germ layers.

H1 ES cell colonies were cultured in nAb SHE78.7 (0.5µl/ml of media of a stock 0.5mg/ml solution) in the presence of a minimal fibroblast feeder layer in the absence of FGF-2 in serum replacement medium for 10 passages (approximately 90 days). The cells were then assessed for expression of transcripts associated with pluripotency (Oct4) and for various lineage markers (as described in Ward et al., 2006). The results of this assessment are shown in panel A of Figure 31. This demonstrates that the transcript profile expression is consistent with that observed for undifferentiated H1 ES cells (as reported in Ward et al., 2006).

H1 ES cell colonies grown as described in the preceding paragraph were allowed to overgrow in the culture plates (i.e. grown without passaging) in normal ES cell culture medium (i.e. +FGF-2) for 20 days in the absence of nAb to induce differentiation of the cells. The cells were then assessed for expression of transcripts associated with pluripotency (Oct4) and various lineage markers as described in Ward et al., 2006. The results of this assessment are shown in panel B of Figure 31, and illustrate that markers of

differentiation expressed following differentiation of the cells included all three germ layers (endoderm –TF, AMY; mesoderm – FLK, CD34, AC133; ectoderm – NES, NFM, NSE, PAX and PLP) and extra-embryonic visceral endoderm (AFP).

The results set out in Figures 30-31 show that human stem cells (such as H1 and HES4 human embryonic stem cells) can be cultured for prolonged periods in the absence of FGF-2 in synthetic serum in minimal feeders when E-cadherin activity is inhibited (for example by treatment with E-cadherin neutralising antibody SHE78.7). Cells cultured in this manner exhibit transcript expression associated with hES cells cultured under normal conditions known in the prior art (conditions that utilise components such as FGF-2). When inhibition of E-cadherin activity is ceased (for example on removal of nAb) differentiation of the cells may proceed as normal, and is able to give rise to cells of the three germ layers. This clearly shows that, on reversal of inhibition of E-cadherin activity, the retardation of differentiation ends, and normal differentiation (for example to achieve therapeutically useful cell types) can be achieved.

27. Human stem cells (HES4 and H1 ES cells) treated with an inhibitor of E-cadherin activity (neutralising antibody SHE78.7) maintain undifferentiated colony morphology after prolonged passage in the absence of FGF-2.

H1 and HES4 ES cells were cultured in nAb (0.5μl/ml of media of a stock 0.5mg/ml solution) in the presence of a minimal fibroblast feeder layer in the absence of FGF-2 in serum replacement medium for 15 passages (approximately 130 days) and assessed for colony morphology by phase contrast microscopy. All colonies exhibited characteristic undifferentiated morphology, as shown in the results set out in Figure 32. These results clearly indicate that differentiation of human stem cells in culture can be inhibited by inhibition of E-cadherin activity.

28. Prolonged culture of wild type D3 ES cells in the absence of LIF selects for E-cadherin negative/SSEA-1 positive cells.

D3 ES cells were cultured in gelatin-treated 6-well plates in the presence of LIF and assessed for cell surface E-cadherin or SSEA-1 expression by fluorescent flow cytometry. The results of this assessment are shown in panel A of Figure 33, in which it can be seen that cells at the outset expressed high levels of both cell surface E-cadherin and SSEA-1.

D3 ES cells were then cultured for 12 passages without LIF by carefully sub-culturing the cells according to the cell number present. For example, where low cell numbers were observed the entire population of cells was transferred to a fresh 6-well plate (such a transfer was not counted as a passage since all of the cells were transferred). After approximately 30 days (12 passages) a sudden expansion of cells was observed and these cells appeared to lack any cell-cell contacts. The cells were assessed at passage 12 for cell surface E-cadherin or SSEA-1 expression by fluorescent flow cytometry. The results of this assessment are shown in panel B of Figure 33, in which it can be seen that the majority of the cell population lacked E-cadherin but expressed SSEA-1, demonstrating an undifferentiated phenotype.

In both fluorescent flow cytometry experiments cell fluorescence was analysed using a Becton Dickinson FACScaliber. Viable cells were gated using forward and side scatter and the data represent cells from this population.

Panel C of Figure 33 shows phase contrast image of D3 cells cultured in the presence (FCS+LIF) or absence (FCS-LIF) of LIF for 12 passages. Note that the FCS-LIF cells lacked cell-cell contacts, similar to the phenotype observed for E-cadherin null ES cells.

Without wishing to be bound by any hypothesis, the inventors believe that these data indicate that wild type ES cells may naturally down-regulate E-cadherin in response to withdrawal of LIF, thus allowing them to divide in its absence and increase cell division compared to E-cadherin positive cells. Alternatively, it may be that the E-cadherin negative cells identified at passage 12 in FCS-LIF are derived from a small number of E-cadherin negative cells present at the outset that were subsequently expanded. Irrespective of the mechanism, it demonstrates that lack of E-cadherin asserts a distinct advantage to the growth of the cells in the absence of LIF.

Experimental Protocols

1. Karyotype analysis

Cell numbers required for this method range from 5 x 10^6 - 10^7, and at the minimum, should be 50% confluent, ideally 70% confluent.

1. Add colcemid at 0.02-μg/ml in tissue culture medium to the cells.
2. Incubate for 2 hours at 37°C / 7.5% CO_2.
3. Trypsinise the treated cells and collect in 15-ml centrifuge tubes in PBS.
4. Pellet cells by centrifugation.
5. Remove the supernatant and disrupt cell pellet.
6. Using a plastic pasteur pipette, add 1ml of KCl (0.56% w/v) drop by drop, and then an excess up to 6ml. Invert the tube several times to ensure thorough mixing.
7. Leave for 10-min for cells to swell.
8. Pellet by centrifugation.
9. Remove the supernatant and disrupt the pellet.
10. Add ice-cold fixative (3:1 absolute methanol:glacial acetic acid) 1 drop at a time and mix thoroughly to prevent cell clumping.
11. Add the fixative to give a final volume of 6-ml, and leave for 5 min at room temperature.
12. Pellet the cells by centrifugation.
13. Repeat steps 9 – 12 a further 3 times, leaving a final volume of 1-ml of cells in fixative.

Spread cells onto a slide. Prepare 4 – 5 spreads per specimen and stain overnight with Giemsa

Human ES cell culture in normal medium

HES4 hES cells (Reubinoff et al., 2000 *Nature Biotechnology* 18, 399-404.) and H1 hES cells (Thomson et al, 1998 *Science* 282, 1145-1147) were cultured in tissue organ culture dishes (BD Falcon, Bedford MA, USA) coated with 0.1% gelatin and 1x10^5 irradiated 129 mouse embryonic fibroblast feeder cells per dish. Cells were grown in DMEM + F12 mix media supplemented with 20% serum replacement (synthetic serum; Invitrogen

Corp.), L-glutamine (1mM), 2-mercaptoethanol (50 μM), NEAA (100X, 1:100 dilution) and bFGF (FGF-2; 0.2μg/ml in 0.1% BSA) (all Invitrogen), as described by Thomson et al (Thomson et al., 1998) and incubated at 37°C/5% CO_2. The media was changed daily. Cells were passaged after 7-10 days by gently cutting and teasing the morphologically undifferentiated cells using a yellow pipette tip and transferring colony pieces to a fresh culture dish.

Human ES cell culture in the presence of E-cadherin neutralising antibody SHE78.7

HES4 hES cells and H1 hES cells were cultured in tissue organ culture dishes (BD Falcon, Bedford MA, USA) coated with 0.1% gelatin and $1x10^3$ irradiated 129 mouse embryonic fibroblast feeder cells per dish. Cells were grown in DMEM + F12 mix media supplemented with 20% serum replacement (synthetic serum; Invitrogen Corp.), L-glutamine (1mM), 2-mercaptoethanol (50 μM) and NEAA (100X, 1:100 dilution) and and bFGF (FGF-2; 0.2μg/ml in 0.1% BSA) (all Invitrogen) as described by Thomson et al (Thomson et al., 1998) and incubated at 37°C/5% CO_2 in the presence of a control antibody (Mouse IgG_{2a}; Invitrogen Corp) or E-cadherin neutralising antibody SHE78.7 (Invitrogen) (10μl/ml of media of a stock 0.5mg/ml solution). The media was changed daily. Cells were passaged after 7-10 days by gently cutting and teasing the morphologically undifferentiated cells using a yellow pipette tip and transferring undifferentiated colony pieces to a fresh culture dish.

Human ES cell culture in the presence of E-cadherin neutralising antibody SHE78.7 in the absence of FGF-2

HES4 hES cells and H1 hES cells were cultured in tissue organ culture dishes (BD Falcon, Bedford MA, USA) coated with 0.1% gelatin and $1x10^3$ irradiated 129 mouse embryonic fibroblast feeder cells per dish. Cells were grown in DMEM + F12 mix media supplemented with 20% serum replacement (synthetic serum; Invitrogen Corp.), L-glutamine (1mM), 2-mercaptoethanol (50 μM) and NEAA (100X, 1:100 dilution) (all Invitrogen) and incubated at 37°C/5% CO_2 in the presence of a control antibody (Mouse IgG_{2a}; Invitrogen Corp) or E-cadherin neutralising antibody SHE78.7 (0.5μl/ml of media of a stock 0.5mg/ml solution). The media was changed daily. Cells were passaged after 7-10 days by gently cutting and teasing the morphologically undifferentiated cells using a

yellow pipette tip and transferring undifferentiated colony pieces to a fresh culture dish. Some spontaneous differentiation of the cells was observed in the early passage cultures, which is to be expected due to the stress of the altered culture conditions.

Differentiation of hES cells

Control and neutralising antibodies were removed from the cultures and the cells differentiated by overgrowth of the cells in DMEM + F12 mix media supplemented with L-glutamine (1mM), 2-mercaptoethanol (50 µM), NEAA (100X, 1:100 dilution), and bFGF (0.2µg/ml in 0.1% BSA) (all Invitrogen) without removal of the feeder layer.

Mouse ES cell culture

MESC20 (and E-cadherin RNAi cell lines derived from this parental line), D3, OCT4-GFP (E14TG2a parental cell line) and E-cadherin null ES cells were cultured on gelatin-treated plates in Knockout DMEM supplemented with 10% foetal bovine serum, 2mM L-glutamine, non-essential amino acids (100X, 1:100 dilution), 50µM 2-mercaptoethanol (all Invitrogen Corporation, Paisley, UK) and 1000 units/ml leukaemia inhibitory factor (ESGRO; Chemicon Int., Middx., UK) (FCS+LIF) at 37°C/5% CO_2. The media was replenished every 24h and cells passaged before confluence. Alternatively, foetal bovine serum was replaced with serum replacement at the same concentration (synthetic serum; Invitrogen Corporation). Gelatin-treated plates were prepared as described previously (Ward et al., 2003). Cells were also cultured in the medium as described above but in the absence of LIF (culture conditions and cultured cells referred to elsewhere in the specification "FCS-LIF" or "–LIF").

Antibody induced loss of cell-cell E-cadherin contacts in mouse ES cells

E-cadherin mediated cell to cell contacts were abrogated by culture of D3 or MESC20 ES cells in 5.8µg/ml IgG component of rat-anti E-cadherin DECMA-1 ascites solution (Sigma, Dorset, UK) for various times as described elsewhere. Rat-anti Tenascin Ab was used as a control in all experiments at the same concentrations as above (Sigma).

Suspension culture of mES cells in the presence of inhibiting antibody.

MESC20 ES cells were cultured in suspension by plating 10^6 cells in 10mls of medium lacking LIF in a plastic bacteriological Petri dish and the media changed every day. Cells were treated with either control antibody (cAb) or the E-cadherin inhibitory antibody DECMA-1 (nAb) at a concentration of 116μg total IgG component. Cell suspensions were agitated every 24h by pipetting up and down several times in a 10ml pipette. Cells were passaged when required (usually every 2 days for nAb treated cells ES cells) by transfer of 2.5mls of cell suspension into 7.5mls of fresh medium.

Suspension culture of E-cadherin null or E-cadherin RNAi ES cells

E-cadherin null or RNAi E-cadherin ES cells were cultured in suspension by plating 10^6 cells in 10mls of medium lacking LIF in a plastic bacteriological Petri dish and the media changed every day. Cell suspensions were agitated every 24h by pipetting up and down several times in a 10ml pipette. Cells were passaged when required (usually every 2 days for E-cadherin RNAi ES cells) by transfer of 2.5mls of cell suspension into 7.5mls of fresh medium.

Forced expression of E-cadherin cDNA in E-cad-/- ES cells

E-cadherin null ES cells were cultured as described above, trypsinised and washed twice in PBS. Cells were electroporated using an Amaxa Biosystems NucleofectorII and ES cell electroporation kit (Amaxa Biosystems, Germany) as described in the manufacturer's instructions. Briefly, 2 x 10^6 E-cadherin null ES cells were suspended in Amaxa ES cell solution and either pCMVα or pCMVα-E-cadherin vectors (2μg total plasmid) and electroporated using program A-30 on the Amaxa NucleofectorII. Cells were plated out in a single well of a gelatinized 6-well plate in ES cell medium lacking LIF for 3 days (without overgrowth of the cells) and assessed for transcript expression by RT-PCR.

Isolation of mouse ES cells exhibiting E-cadherin repression using hairpin loop RNAi.

MESC20 mouse ES cells were cultured as described above, trypsinised and washed twice in PBS. Cells were electroporated as described in the manufacturer's instructions using an Amaxa Biosystems NucleofectorII and ES cell electroporation kit (Amaxa Biosystems, Germany). Briefly, 2 x 10^6 E-cadherin null ES cells were suspended in Amaxa ES cell

solution containing a mixture of E-cadherin RNAi vectors (see Figure 14 (part 2)) (2μg total plasmid) and electroporated using program A-30 on the Amaxa NucleofectorII. Cells were plated out in a single well of a gelatinized 6-well plate in ES cell medium for 2 days and G418 added to the medium (350μg/ml) for 10 days. Surviving colonies were isolated and transferred to a single well of a gelatin-treated 96-well plate in ES cell medium. Cell numbers were increased by transfer of the entire population to an individual well of a 24-well and then 6-well plates. Clones (designated M1, 2, 5 and 7, as described elsewhere in the specification) were isolated using this method. Alternatively, MESC20 ES cells were transfected with the E-cadherin RNAi vectors as described above and cells lacking E-cadherin isolated by removal of LIF from the medium for 10 days. Clone MM was isolated in this way.

Immunofluorescent imaging of ES cells

Human ES cells were cultured on Nunc plastic slide flask chambers (Nalge, Nunc International, Rochester, NY, USA) coated with feeder layers (of the type described previously) and grown in the stated media for 2-7 days. Mouse ES cells were cultured on gelatin-treated tissue culture grade 6-well plates. Cells were rinsed in PBS and fixed in 4% paraformaldehyde for 15 min, followed by two washes with PBS. Cells were blocked for 30 min in filtered 1% goat serum, 0.1% Triton-X-100 in PBS for prevention of non-specific binding. Primary antibodies used were as follows: NANOG (Chemicon, rabbit anti-mouse), OCT-4 (Santa Cruz; mouse IgG_{2b}), E-cadherin (SHE78.7, Invitrogen Corp. for human or mouse IgG_{2a}, Santa Cruz for mouse). Actin cytoskeleton was detected using phalloidin-Texas Red Conjugate (1:1000 dilution; Sigma). Primary antibodies were diluted in blocking buffer (all 1:100) and incubated for 2h at room temperature. Cells were then washed 4x 5min in PBS prior to secondary antibody labelling. Secondary antibodies that recognised the primary antibody to be detected, and were conjugated with Alexa Fluor 488 or 546 (Molecular Probes, OR, USA), were diluted in blocking buffer (1:500 dilution) and incubated with the cells for 1h at room temperature. Final washes in PBS 2x 5min, 1x 15min and 2x 5min were performed before mounting samples in DAPI Vector shield (Vector, Peterborough, UK). The cells were viewed on an Olympus BX51 fluorescence microscope and/or a Zeiss Laser Scanning Confocal Microscope. Images were overlaid using Adobe Photoshop version 6.0.

Fluorescent flow cytometry analysis of ES cells

Human or mouse ES cells were trypsinised, washed once in 900μl of PBS, and resuspended in 100μl of 0.2% BSA in PBS (FACS buffer) containing a primary antibody from the group listed below. Cells were then incubated in this solution for 1h on ice. Primary antibodies used were as follows: SSEA-1 (Santa Cruz; mouse IgM), Tra-1-60 (phycoerythrin conjugated anti-Tra-1-60; Santa Cruz), E-cadherin (DECMA-1). After incubation, cells were washed once in 900μl of PBS, resuspended in 100μl of FACS buffer containing a phycoerythrin-conjugated secondary antibody that recognised the primary antibody (all 1:100 dilution; Santa Cruz) and incubated for 30min on ice. The cells were washed once in 900μl of PBS and fixed in 400μl of 1% formaldehyde. Cell fluorescence was analysed using a Becton Dickinson FACScaliber. Viable cells were gated using forward and side scatter and the data represent cells from this population.

RT-PCR

Total RNA was extracted from cells using RNAzol B according to the manufacturer's instructions (Biogenesis, Dorset, UK), treated with DNase (Promega, WI, USA) and phenol/chloroform extracted. Synthesis of cDNA from mRNA transcripts was performed using the following method: RNA (10μg), dNTP (250μM), oligo(dT) (5.0μg total), were combined with reverse transcriptase (40 U) in a total volume of 200μl and incubated at 42°C for 1 hour to produce cDNA. RT-PCR was performed using 1μl of the cDNA solution and 35 or 45 cycles. Samples were run on 2% agarose gels containing 400ng/ml ethidium bromide and visualised using an Epi Chemi II Darkroom and Sensicam imager with Labworks 4 software (UVP, CA, USA).

Primers used were as follows (read 5′ to 3′; all 60°C annealing):

β-tubulin (β-Tub): forward primer (Sequence ID No. 32): GGA ACA TAG CCG TAA ACT GC, and reverse primer (Sequence ID No. 33): TCA CTG TGC CTG AAC TTA CC, giving a product of 317bp;

Oct-4 (OCT): forward primer (Sequence ID No. 34): AGA AGG AGC TAG AAC AGT TTG C; and reverse primer (Sequence ID No. 35): CGG TTA CAG AAC CAT ACT CG, giving a product of 415bp;

alpha-foetal protein (AFP): forward primer (Sequence ID No. 36): CCA TGT ACA TGA GCA CTG TTG; and reverse primer (Sequence ID No. 37): CTC CAA TAA CTC CTG GTA TCC, giving a product of 338 bp;

hepatocyte nuclear factor (HNF): forward primer (Sequence ID No. 38): GAG TTT ACA GGC TTG TGG CA; and reverse primer (Sequence ID No. 39): GAG GGC AAT TCC TGA GGA TT, giving a product of 390 bp;

nestin (NES): forward primer (Sequence ID No. 40): GCC CTG ACC ACT CCA GTT TA; and reverse primer (Sequence ID No. 41): GGA GTC CTG GAT TTC CTT CC, giving a product of 199 bp;

neurofilament middle chain (NFM): forward primer (Sequence ID No. 42): GAG CGC AAA GAC TAC CTG AAG A; and reverse primer (Sequence ID No. 43): CAG CGA TTT CTA TAT CCA GAG CC, 430 bp;

neuron-specific enolase (NSE): forward primer (Sequence ID No. 44): CCC ACT GAT CCT TCC CGA TAC AT; and reverse primer (Sequence ID No. 45): CCG ATC TGG TTG ACC TTG AGC A, giving a product of 254 bp;

Pax-6 (PAX): forward primer (Sequence ID No. 46): AAC AGA CAC AGC CCT CAC AAA CA; and reverse primer (Sequence ID No. 47): CGG GAA CTT GAA CTG GAA CTG AC, giving a product of 275 bp;

proteolipid protein (PLP): forward primer (Sequence ID No. 48): CCA TGC CTT CCA GTA TGT CAT C; and reverse primer (Sequence ID No. 49): GTG GTC CAG GTG TTG AAG TAA ATG T, giving products of 354 bp (plp) and 249 bp (dm-20);

amylase (AMY): forward primer (Sequence ID No. 50): GCT GGG CTC AGT ATT CCC CAA ATA C; and reverse primer (Sequence ID No. 51): GAC GAC AAT CTC TGA CCT GAG TAG C, giving a product of 490 bp;

α1-antitrypsin (TRP): forward primer (Sequence ID No. 52): AGA CCC TTT GAA GTC AAG GAC ACC G; and reverse primer (Sequence ID No. 53): CCA TTG CTG AAG ACC TTA GTG ATG C, giving a product of 360 bp;

Flk-1 (Flk): forward primer (Sequence ID No. 54): GGT ATT GGC AGT TGG AGG AA; and reverse primer (Sequence ID No. 55): ACA TTT GCC GCT TGG ATA AC, giving a product of 203 bp;

CD34 (CD34): forward primer (Sequence ID No. 56): TGA AGC CTA GCC TGT CAC CT; and reverse primer (Sequence ID No. 57): CGC ACA GCT GGA GGT CTT AT, giving a product of 200 bp;

AC133 (AC1): forward primer (Sequence ID No. 60): CAG TCT GAC CAG CGT GAA AA; and reverse primer (Sequence ID No. 61): GGC CAT CCA AAT CTG TCC TA, giving a product of 199 bp;

Transferrin (Tf): forward primer (Sequence ID No. 62): CTG ACC TCA CCT GGG ACA AT; and reverse primer (Sequence ID No. 63): R CCA TCA AGG CAC AGC, giving a product of 367 bp;

Transthyretin (TTR): forward primer (Sequence ID No. 64): GGT ATT TGT GTC TGA AGC TGG; and reverse primer (Sequence ID No. 64): GGT TGC TGA CGA CAG CCG TGG giving a product of 392bp.

fibroblast growth factor-5 (FGF-5): forward primer (Sequence ID No. 66): GGC AGA AGT AGC GCG ACG TT; and reverse primer (Sequence ID No. 67): TCC GGT TGC TCG GAC TGC TT, giving products of 537 and ~515bp.

zeta-globin (ZG): forward primer (Sequence ID No. 68): GATGAAGAATGAGAGAGC; and reverse primer (Sequence ID No. 69): AGTCAGGATAGAAGACAGG, giving a product of 406bp.

Neurofilament-68 (NF68): forward primer (Sequence ID No. 70): CCA GGA AGA GCA GAC AGA GGT; and reverse primer (Sequence ID No. 71): GTT GGG AAT AGG GCT CAA TCT, giving a product of 302bp.

Brachyury (T): forward primer (Sequence ID No. 72): CAT TAC ACA CCA CTG ACG; and reverse primer (Sequence ID No. 73): GAT ATA GGA CCC TAC CTA GC, giving a product of 472bp.

α-fetoprotein (AFP): forward primer (Sequence ID No. 74): GAA GAA TTG CAG AAA CAC ATC G; and reverse primer (Sequence ID No. 75): AGCCAAAAGGCTCACACC, giving a product of 699bp.

Isolation of E-cadherin negative/SSEA-1 positive cells from wild-type ES cells cultured in the absence of LIF.

D3 ES cells were cultured on gelatin-treated plates in Knockout DMEM supplemented with 10% foetal bovine serum, 2mM L-glutamine, non-essential amino acids (100X, 1:100 dilution), 50μM 2-mercaptoethanol (all Invitrogen Corporation, Paisley, UK) and 1000 units/ml leukaemia inhibitory factor prior to the experiment (ESGRO; Chemicon Int., Middx., UK) (FCS+LIF) at 37°C/5% CO_2. The media was replenished every 24h and cells passaged before confluence. Gelatin-treated plates were prepared as described previously (Ward et al., 2003). To select for E-cadherin negative ES cells, LIF was removed from the culture medium and the cells carefully expanded over approximately 30 days. It should be noted that significant differentiation and apoptosis was observed in the early passages (passage 3-9), with the cells being transferred in their entirety to fresh 6-well plates on several occasions to encourage cell growth (such a transfer was not counted as a passage since all of the cells were transferred). All passages were carried out prior to confluence by transferring one third of the cell population to a fresh gelatin-treated 6-well plate. After approximately 30 days (passage 12) a sudden increase in cell

number was observed and the cells assessed for cell surface E-cadherin and SSEA-1 to confirm loss of the former but presence of the latter.

CLAIMS

1. A method of retarding differentiation of a biological cell, the method comprising culturing the cell in the presence of an inhibitor of E-cadherin activity.

2. A method according to claim 1, wherein the biological cell is a stem or progenitor cell.

3. A method according to claim 1 or claim 2, wherein the biological cell is a human cell.

4. A method according to claim 1 or claim 2, wherein the biological cell is a murine cell.

5. A method according to claim any preceding claim, wherein the biological cell is cultured in suspension.

6. A method according to any preceding claim, wherein the biological cell is cultured on a substrate.

7. A method according to any preceding claim, wherein the biological cell is cultured in a medium lacking LIF.

8. A method according to any preceding claim, wherein the biological cell is cultured in a medium lacking FGF-2.

9. A method according to any preceding claim, wherein the inhibitor of E-cadherin activity is selected from the group consisting of: an inhibitor comprising the CAD-HAV domain; an inhibitor comprising the Trp156 residue of E-cadherin; slug; snail; SIP1; E2A; and Twist.

10. A method according to any preceding claim, wherein the inhibitor of E-cadherin activity comprises an E-cadherin neutralising antibody.

11. A method according to claim 10, wherein the E-cadherin neutralising antibody is selected from the group consisting of: DECMA-1; and SHE78.7.

12. A method according to any preceding claim, wherein the inhibitor of E-cadherin activity comprises an aptamer specific to E-cadherin or nucleic acids encoding E-cadherin.

13. A method according to any preceding claim, wherein the inhibitor of E-cadherin activity is selected from the group consisting of: an antisense oligonucleotide specific to E-cadherin mRNA; an RNAi molecule specific to E-cadherin mRNA; a ribozyme specific to E-cadherin mRNA; and a molecule that causes methylation of the E-cadherin promoter.

14. A method according to claim 13, wherein the inhibitor of E-cadherin comprises an inhibitor selected from the group consisting of: the siRNA insert set out in Sequence ID No. 22; the siRNA insert set out in Sequence ID No. 23; the siRNA insert set out in Sequence ID No. 24; the siRNA insert set out in Sequence ID No. 25; the siRNA insert set out in Sequence ID No. 26; the siRNA insert set out in Sequence ID No. 27; the siRNA insert set out in Sequence ID No. 28; and the siRNA insert set out in Sequence ID No. 29.

15. A method according to any preceding claim, wherein sufficient of an inhibitor of E-cadherin activity is provided to partially inhibit E-cadherin activity.

16. A method according to any preceding claim, wherein sufficient of an inhibitor of E-cadherin activity is provided to substantially totally inhibit E-cadherin activity.

17. The use of an inhibitor of E-cadherin activity to maintain undifferentiated biological cells in culture.

18. The use of an inhibitor of E-cadherin activity in the manufacture of a culture medium for retarding the differentiation of biological cells.

19. The use according to claim 17 or claim 18, wherein the biological cell is a stem or progenitor cell.

20. The use according to any one of claims 17 to 19, wherein the biological cell is a human cell.

21. The use according to any one of claims 17 to 19, wherein the biological cell is a murine cell.

22. The use according to any of claims 17 or claim 21, wherein the inhibitor of E-cadherin activity is as defined in any of claims 9 to 14.

23. A cell culture medium suitable for the retardation of biological cell differentiation, the cell culture medium comprising an inhibitor of E-cadherin activity.

24. A cell culture medium according to claim 23, wherein the inhibitor of E-cadherin activity is as defined in any of claims 9 to 14.

25. A cell culture medium according to claim 23 or claim 24, wherein the medium lacks LIF.

26. A cell culture medium according to any of claims 23 to 25, wherein the medium lacks FGF-2.

27. A stem or progenitor cell comprising a construct encoding an inhibitor of E-cadherin activity.

28. A stem or progenitor cell according to claim 27, wherein the construct encodes an inhibitor of E-cadherin activity is as defined in any of claims 9 to 14.

29. A stem or progenitor cell according to claim 27 or claim 28 wherein the stem or progenitor cell is a human cell.

30. A stem or progenitor cell according to claim 27 or claim 28 wherein the stem or progenitor cell is a murine cell.

31. A method of retarding differentiation of a biological cell, the method comprising:
    i) introducing a construct encoding an inhibitor of E-cadherin activity into a biological cell the differentiation of which is to be retarded; and
    ii) expressing the construct such that differentiation of the cell is retarded.

32. A method according to claim 31, wherein the biological cell is a stem or progenitor cell.

33. A method according to claim 31 or claim 32, wherein the biological cell is a human cell or a murine cell.

34. A method according to any one of claims 31 to 33, wherein the inhibitor of E-cadherin activity is as defined in any of claims 9 to 14.

35. A method according to claim 34, wherein the inhibitor of E-cadherin comprises an inhibitor selected from the group consisting of: the siRNA insert set out in Sequence ID No. 22; the siRNA insert set out in Sequence ID No. 23; the siRNA insert set out in Sequence ID No. 24; the siRNA insert set out in Sequence ID No. 25; the siRNA insert set out in Sequence ID No. 26; the siRNA insert set out in Sequence ID No. 27; the siRNA insert set out in Sequence ID No. 28; and the siRNA insert set out in Sequence ID No. 29.

36. A method of preparing a biological cell for therapeutic use, the method comprising the consecutive or concurrent steps of:

i) culturing the biological cell in the presence of an inhibitor of E-cadherin activity; and

ii) adapting the biological cell for therapeutic use.

37. A method of therapy, the method comprising the consecutive or concurrent steps of:

i) obtaining a biological cell;

ii) culturing the biological cell in the presence of an inhibitor of E-cadherin activity; and

iii) adapting the biological cell for therapeutic use

and further comprising administering the adapted biological cell to a subject in need of such therapy.

38. A method according to claim 36 or claim 37, wherein the biological cell is a stem or progenitor cell.

39. A method according to any of claims 36 to 37, wherein the biological cell is a human cell.

40. A method according to any one of claims 36 to 39, wherein the biological cell is cultured in suspension.

41. A method according to any one of claims 36 to 39, wherein the biological cell is cultured on a substrate.

42. The use of an inhibitor of E-cadherin activity to maintain undifferentiated biological cells in culture.

43. The use of an inhibitor of E-cadherin activity as a survival factor for biological cells.

44. The use according to claim 42 or claim 43, wherein the biological cell is a stem or progenitor cell.

45. The use according to any of claims 42 to 44, wherein the biological cell is a human cell or a murine cell.

46. The use of an inhibitor of E-cadherin activity in the isolation of embryonic stem (ES) cells.

47. A cell culture medium, for use in the retardation of biological cell differentiation, comprising an inhibitor of E-cadherin activity, characterised in that the medium is serum free.

48. A cell culture medium according to claim 47, wherein the medium is also free from LIF and/or FGF-2.

49. A method of isolating biological cells deficient in E-cadherin, the method comprising:

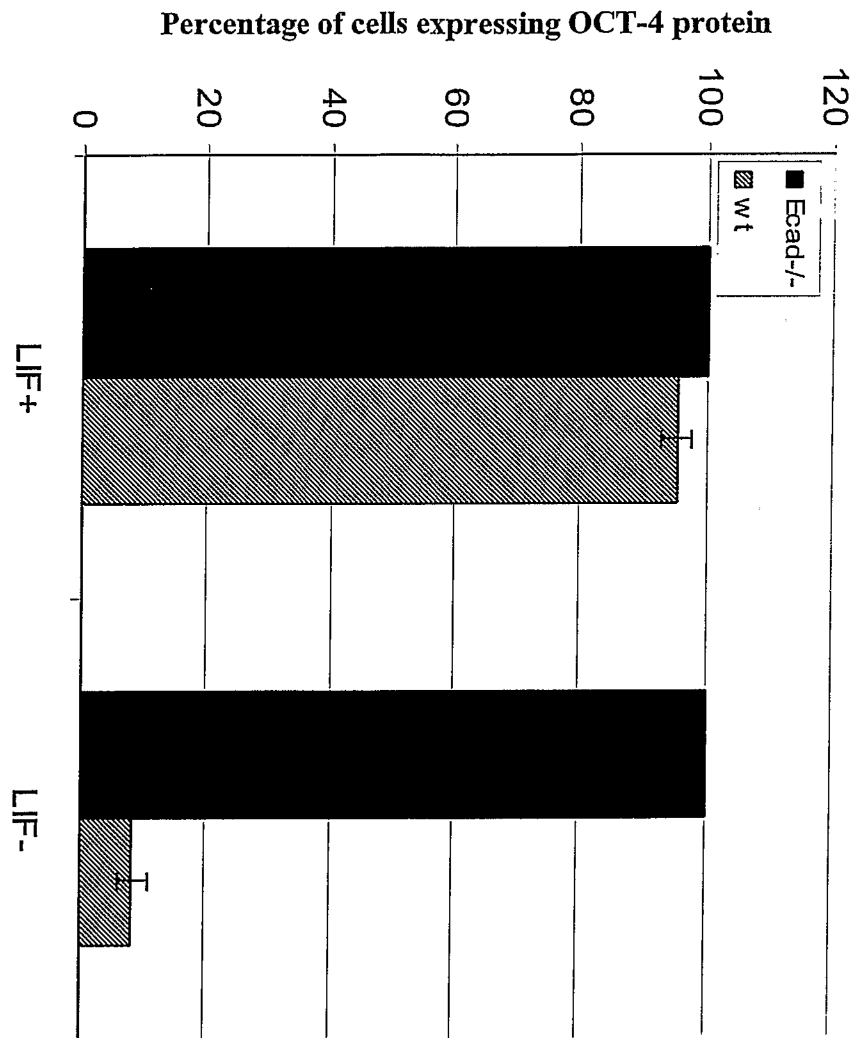
i) culturing the cells in the absence of LIF and/or FGF-2;

ii) allowing biological cells that express E-cadherin to die or differentiate; and

iii) maintaining the cultured cells until biological cells deficient in E-cadherin proliferate.

50. A cell culture substrate incorporating an inhibitor of E-cadherin activity.

Figure 1a



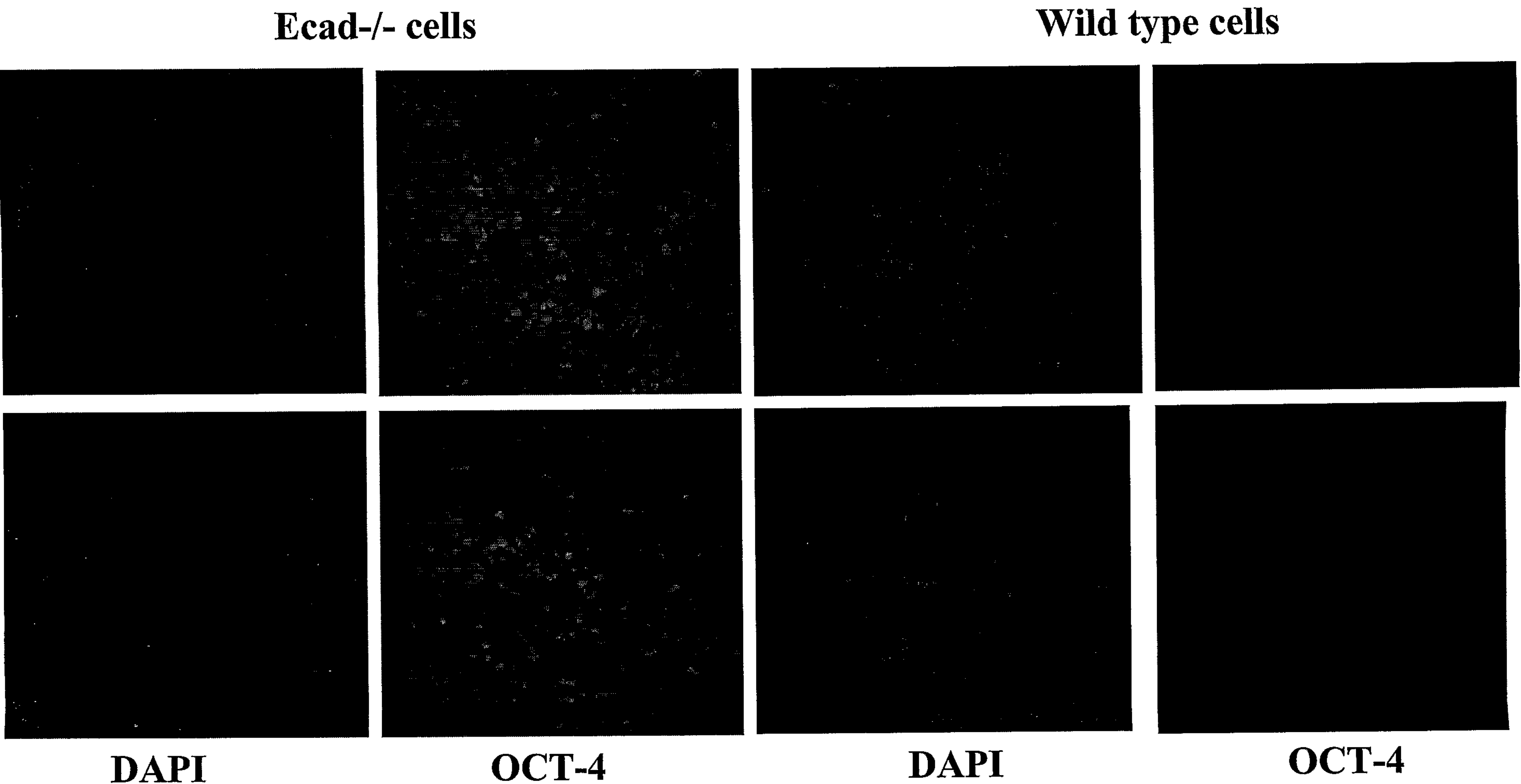


Figure 1.

Cell number as a percentage of the LIF+/FCS+ culture conditions

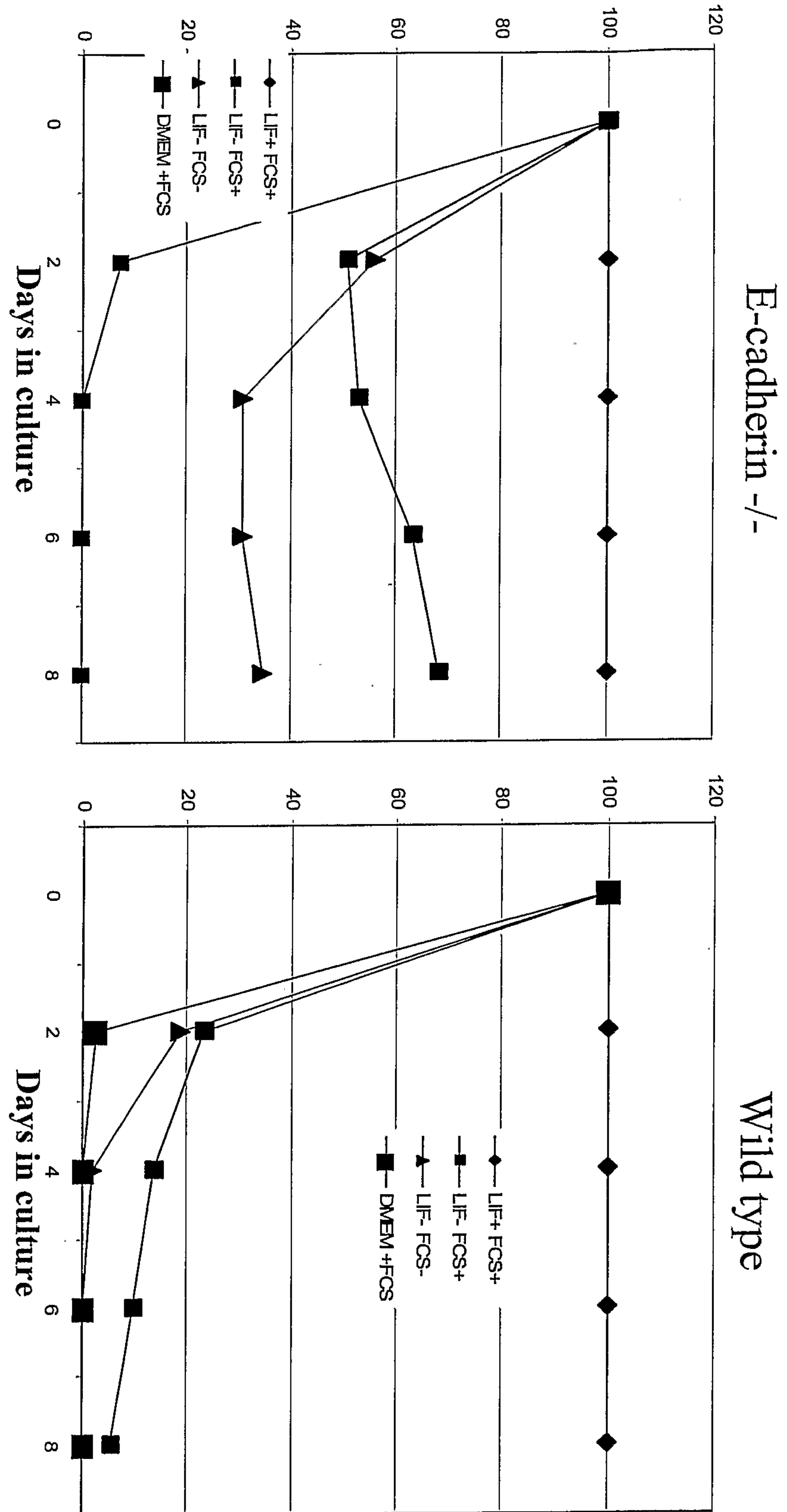


Figure 2.

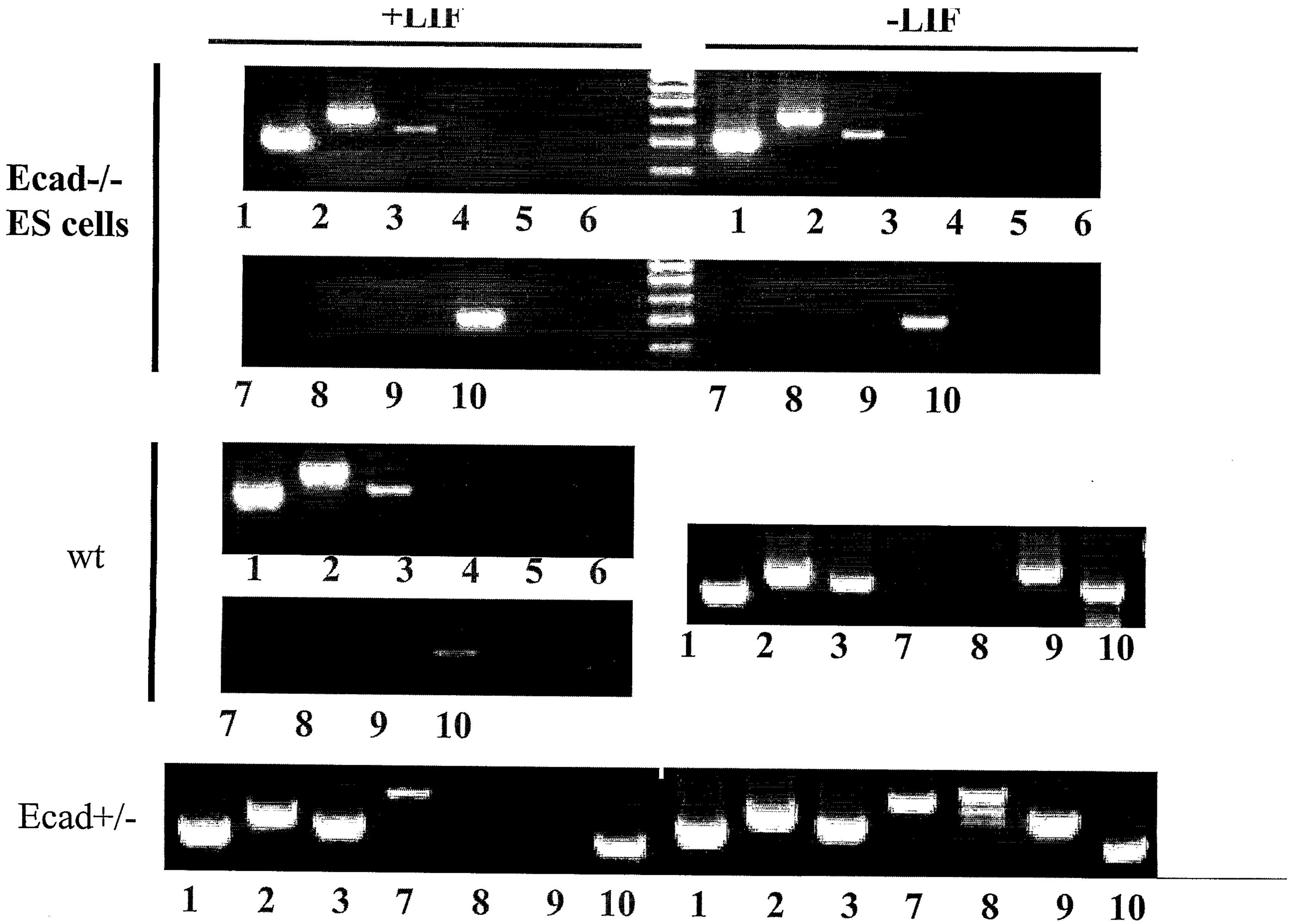


Figure 3.

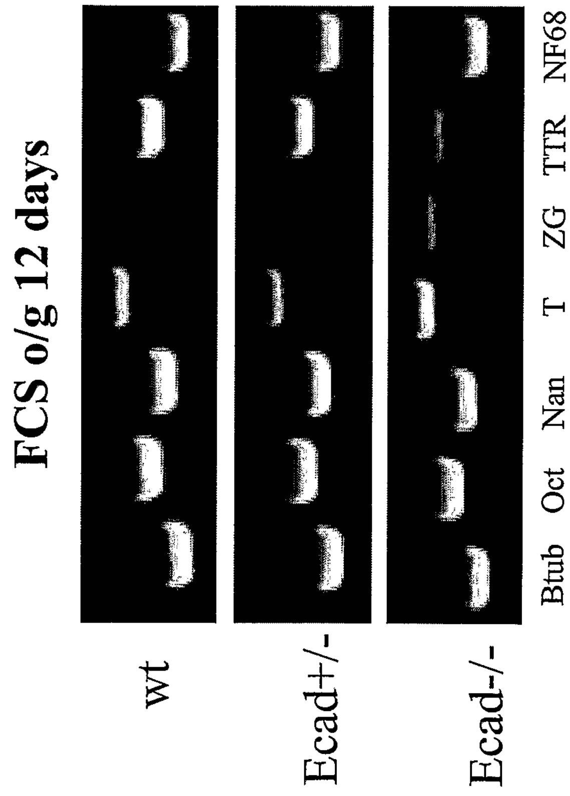


Figure 4..

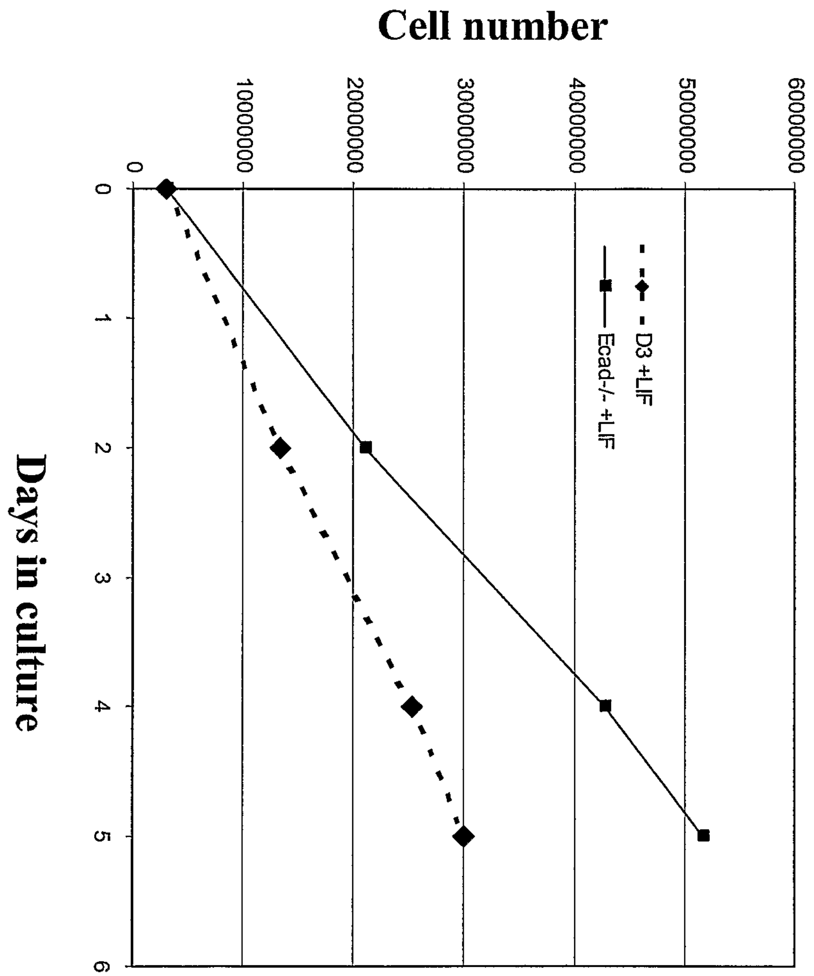


Figure 5.

Figure 6a

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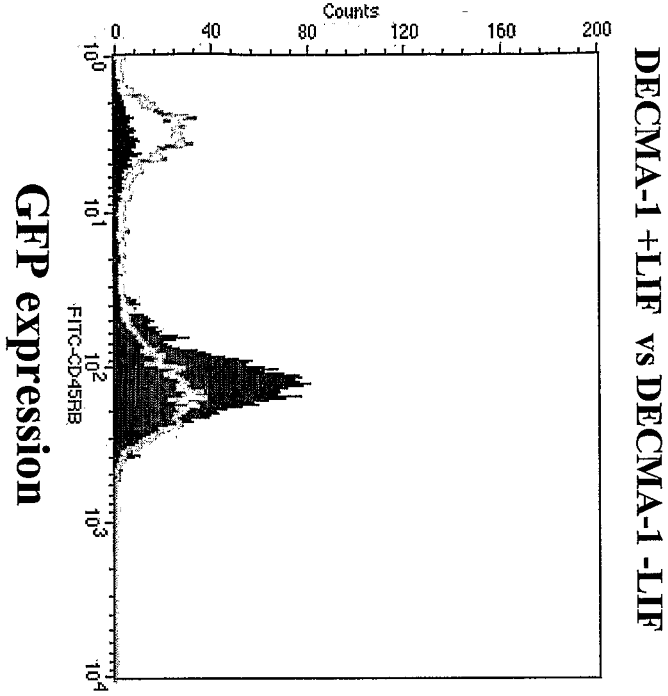


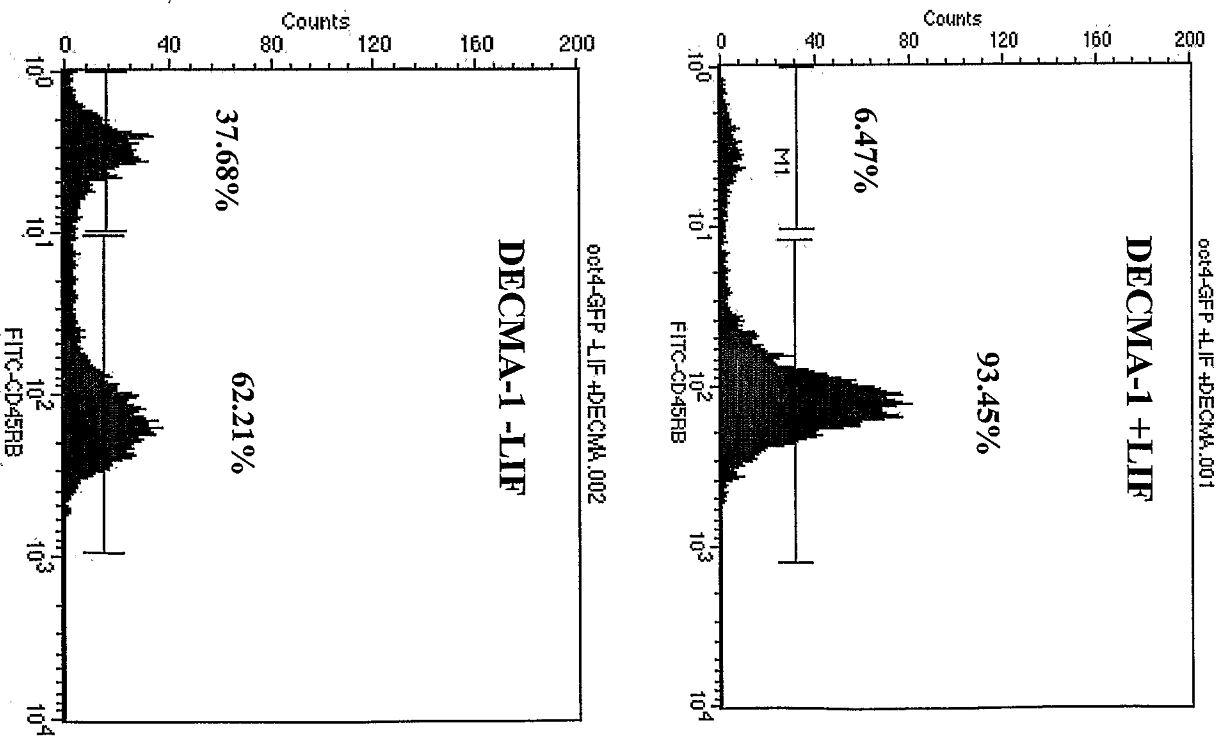
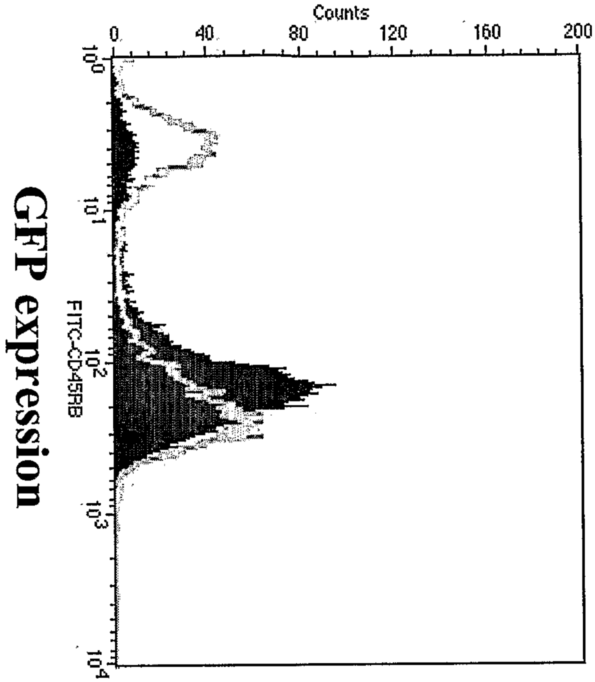
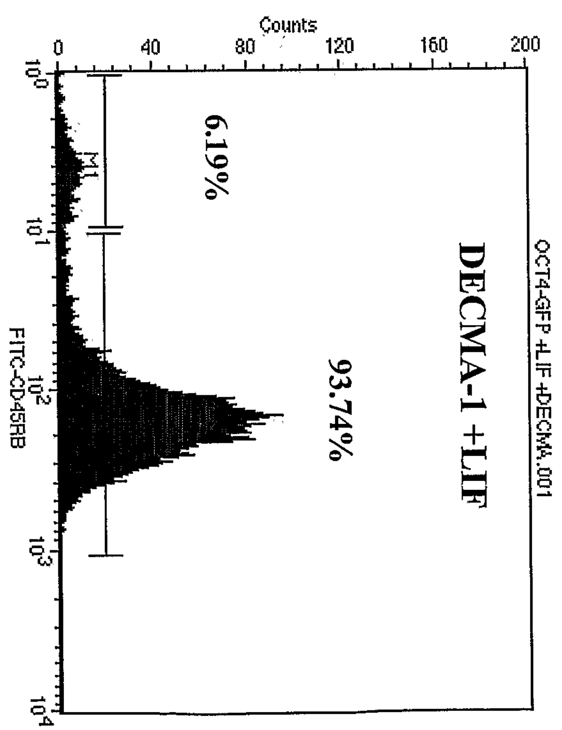
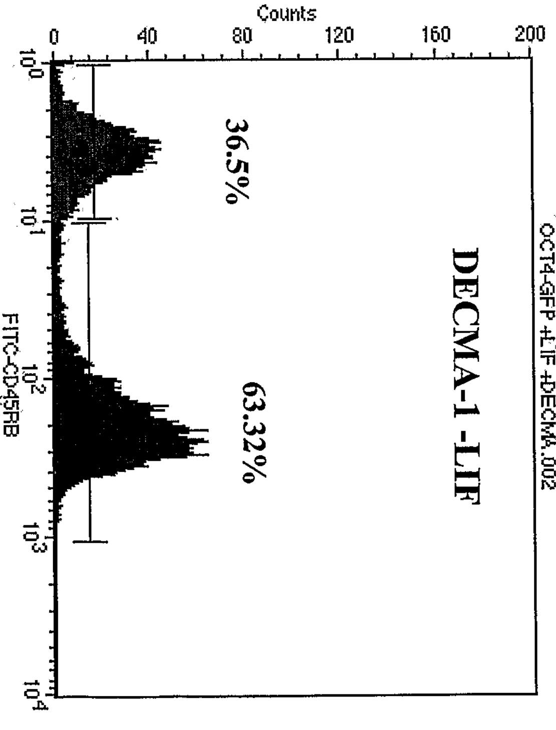


Figure 6b







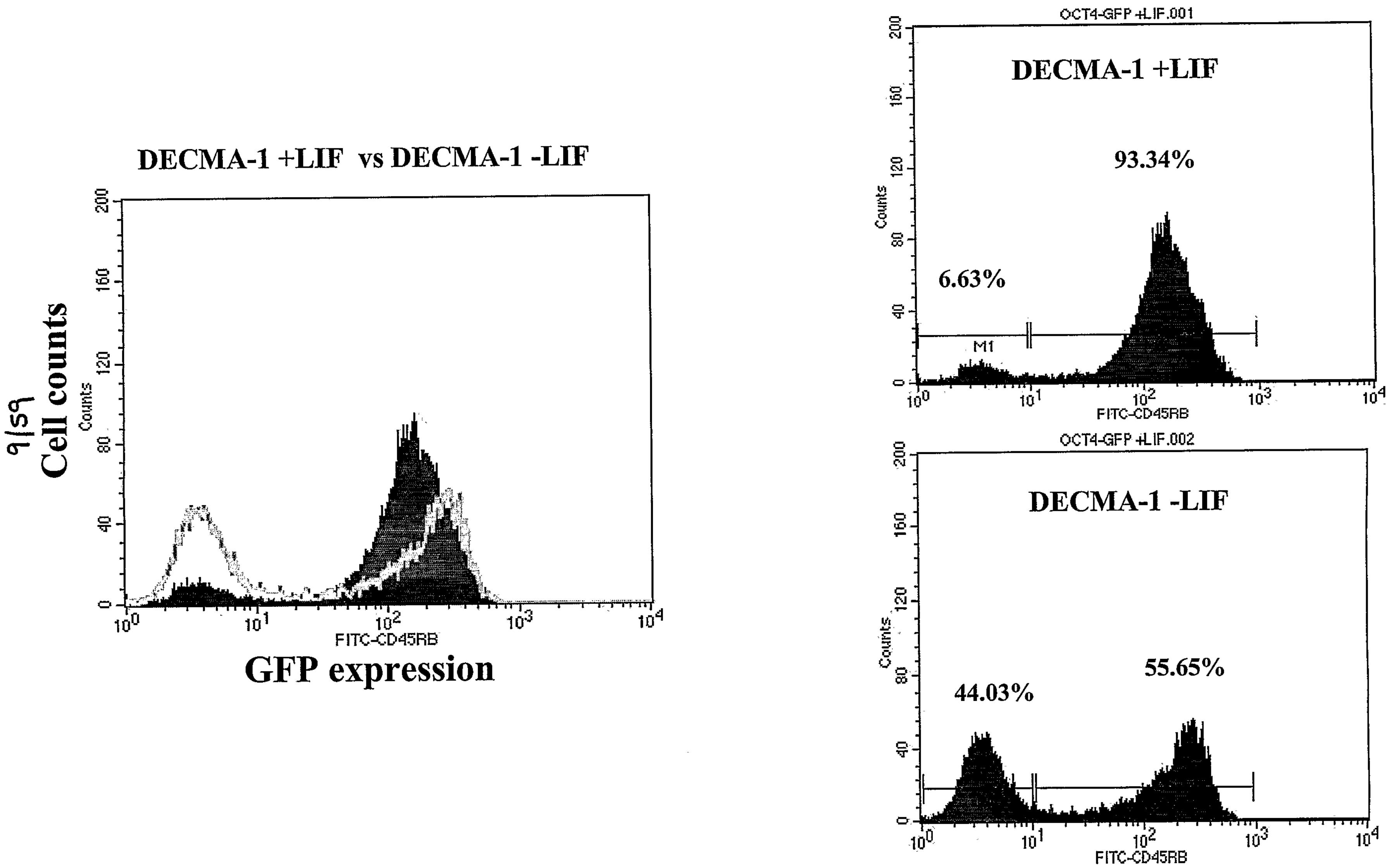


Figure 6c
DECMA-1 +LIF vs DECMA-1 -LIF
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Cell counts
Counts
FITC-CD45RB
GFP expression
OCT4-GFP +LIF.001
DECMA-1 +LIF
93.34%
6.63%
M1
OCT4-GFP +LIF.002
DECMA-1 -LIF
44.03%
55.65%

Figure 6d

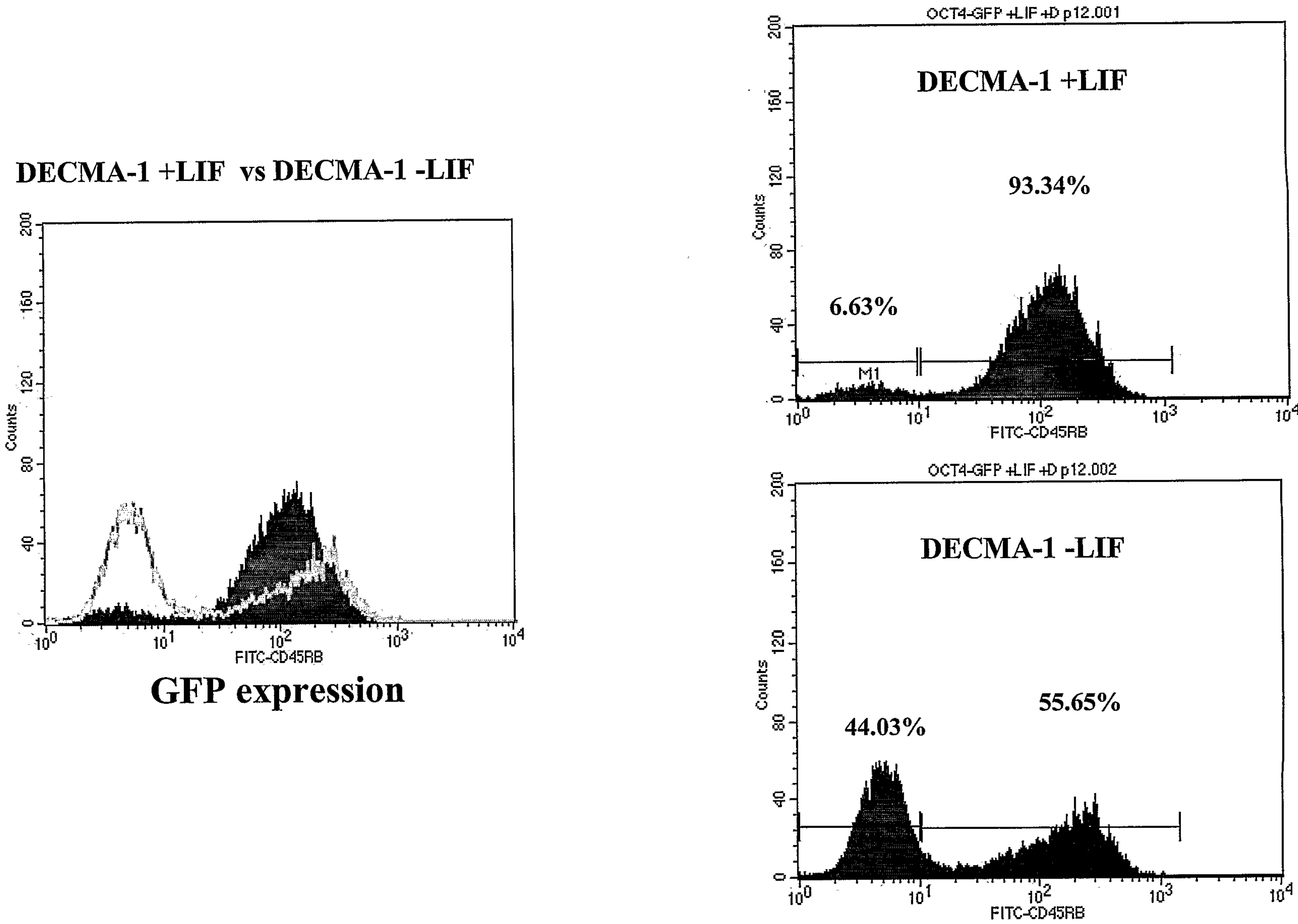
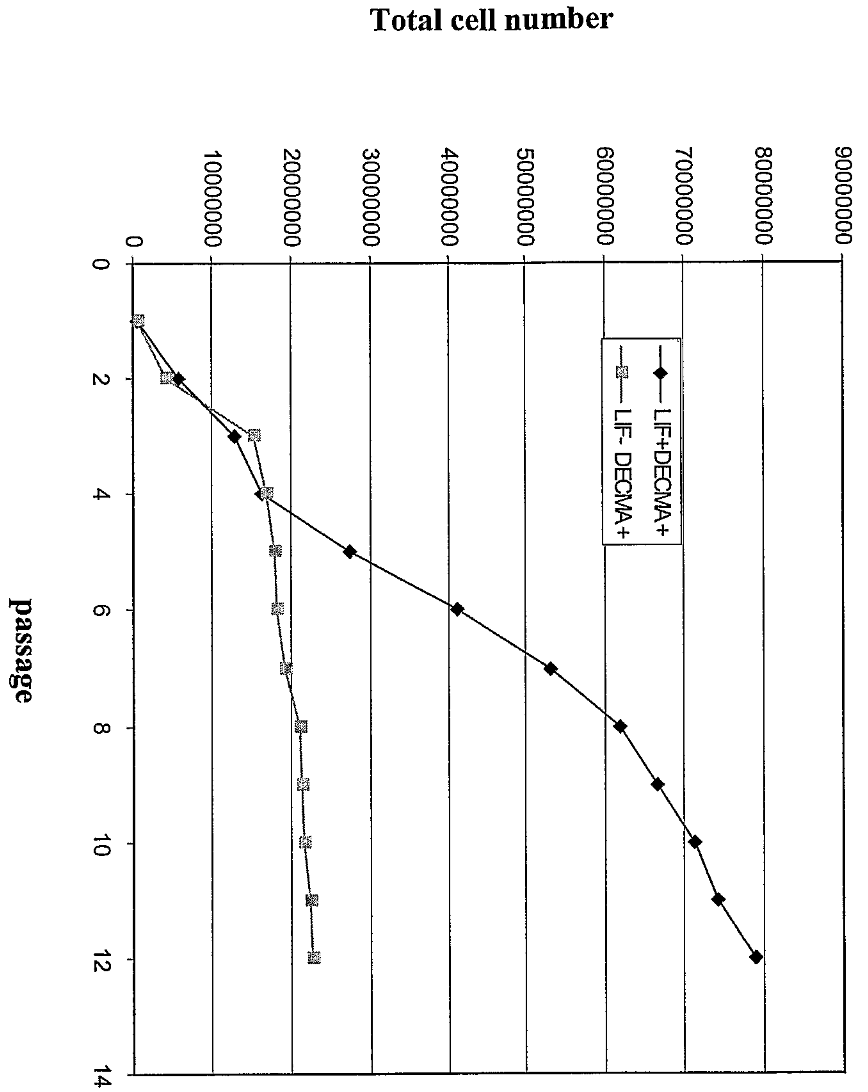


Figure 6e



passage

Figure 6.

Figure 7 – part 1

Human E-cadherin sequences

Sequence ID No. 1 DNA sequence – NCBI NM_004360

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1081 taacaggaac acaggagtca tcagtgtggt caccactggg ctggaccgag agagtttccc
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Sequence ID No. 2 Protein sequence – NCBI AAY68225

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121 hhhrppphqa svsgiqaell tfpnsspglr rqkrdwvipp iscpenekgp fpknlvqiks
181 nkdkegkvfy sitgqgadtp pvgvfiiere tgwlkvtepl dreriatytl fshavssngn
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301 aytilsqdpe lpdknmftin rntgvisvvt tgldresfpt ytlvvqaadl qgeglsttat
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Figure 7 – part 4

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Figure 8 – part 1

Mouse E-cadherin

Sequence ID No. 3 DNA sequence - from NCBI BC098501

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        61 ggccctgccc gaccgcaccc gagctcagtg tttgctcggc gtctgccggg
tccgccatgg
       121 gagcccggtg ccgcagcttt tccgcgctcc tgctcctgct gcaggtctcc
tcatggcttt
       181 gccaggagct ggagcctgag tcctgcagtc ccggcttcag ttccgaggtc
tacaccttcc
       241 cggtgccgga gaggcacctg gagagaggcc atgtcctggg cagagtgaga
tttgaaggat
       301 gcaccggccg gccaaggaca gccttctttt cggaagactc ccgattcaaa
gtggcgacag
       361 acggcaccat cacagtgaag cggcatctaa agctccacaa gctggagacc
agtttcctcg
       421 tccgcgcccg ggactccagt catagggagc tgtctaccaa agtgacgctg
aagtccatgg
       481 ggcaccacca tcaccggcac caccaccgcg accctgcctc tgaatccaac
ccagagctgc
       541 tcatgtttcc cagcgtgtac ccaggtctca gaagacagaa acgagactgg
gtcatccctc
       601 ccatcagctg ccccgaaaat gaaaagggtg aattcccaaa gaacctggtt
cagatcaaat
       661 ccaacaggga caaagaaaca aaggttttct acagcatcac cggccaagga
gctgacaaac
       721 cccccgttgg cgttttcatc attgagaggg agacaggctg gctgaaagtg
acacagcctc
       781 tggatagaga agccattgcc aagtacatcc tctattctca tgccgtgtca
tcaaatgggg
       841 aagcggtgga ggatcccatg gagatagtga tcacagtgac agatcagaat
gacaacaggc
       901 cagagtttac ccaggaggtg tttgagggat ccgttgcaga aggcgctgtt
ccaggaacct
       961 ccgtgatgaa ggtctcagcc accgatgcag acgatgacgt caacacctac
aacgctgcca
      1021 tcgcctacac catcgtcagc caggatcctg agctgcctca caaaaacatg
ttcactgtca
      1081 atagggacac cggggtcatc agtgtgctca cctctgggct ggaccgagag
agttacccta
      1141 catacactct ggtggttcag gctgctgacc ttcaaggcga aggcttgagc
acaacagcca
      1201 aggctgtgat cactgtcaag gatattaatg acaacgctcc tgtcttcaac
ccgagcacgt
      1261 atcagggtca agtgcctgag aatgaggtca atgcccggat cgccacactc
aaagtgaccg
      1321 atgatgatgc ccccaacact ccggcgtgga aagctgtgta caccgtagtc
aacgatcctg
      1381 accagcagtt cgttgtcgtc acagacccca cgaccaatga tggcattttg
aaaacagcca
      1441 agggcttgga ttttgaggcc aagcagcaat acatccttca tgtgagagtg
gagaacgagg
      1501 aaccctttga ggggtctctt gtcccttcca cagccactgt cactgtggac
gtggtagacg
      1561 tgaatgaagc ccccatcttt atgcctgcgg agaggagagt cgaagtgccc
gaagactttg
      1621 gtgtgggtca ggaaatcaca tcttataccg ctcgagagcc ggacacgttc
atggatcaga
```

Figure 8 – part 2

1681 agatcacgta tcggatttgg agggacactg ccaactggct ggagattaac ccagagactg
1741 gtgccatttt cacgcgcgct gagatggaca gagaagacgc tgagcatgtg aagaacagca
1801 catatgtagc tctcatcatc gccacagatg atggttcacc cattgccact ggcacgggca
1861 ctcttctcct ggtcctgtta gacgtcaatg ataacgctcc catcccagaa cctcgaaaca
1921 tgcagttctg ccagaggaac ccacagcctc atatcatcac catcttggat ccagaccttc
1981 cccccaacac gtcccccttt actgctgagc taacccatgg ggccagcgtc aactggacca
2041 ttgagtataa tgacgcagct caagaatctc tcattttgca accaagaaag gacttagaga
2101 ttggcgaata caaaatccat ctcaagctcg cggataacca gaacaaagac caggtgacca
2161 cgttggacgt ccatgtgtgt gactgtgaag ggacggtcaa caactgcatg aaggcgggaa
2221 tcgtggcagc aggattgcaa gttcctgcca tcctcggaat ccttggaggg atcctcgccc
2281 tgctgattct gatcctgctg ctcctactgt ttctacggag gagaacggtg gtcaaagagc
2341 ccctgctgcc accagatgat gatacccggg acaatgtgta ttactatgat gaagaaggag
2401 gtggagaaga agaccaggac tttgatttga gccagctgca caggggcctg gatgcccgac
2461 cggaagtgac tcgaaatgat gtggctccca ccctcatgag cgtgccccag tatcgtcccc
2521 gtcctgccaa tcctgatgaa attggaaact tcatcgatga aaacctgaag gcagccgaca
2581 gcgaccccac ggcacccccct tacgactctc tgttggtgtt cgattacgag ggcagtggtt
2641 ctgaagccgc tagcctgagc tcactgaact cctctgagtc ggatcaggac caggactacg
2701 attatctgaa cgagtggggc aaccgattca agaagctggc ggacatgtac ggcggtggcg
2761 aggacgacta ggggactagc aagtctcccc cgtgtggcac catgggagat gcagaataat
2821 tatatcagtg gtctttcagc tccttccctg agtgtgtaga agagagactg atctgagaag
2881 tgtgcagatt gcatagtggt ctcattctcc ttactggact gtctgtgtta ggatggtttt
2941 cactgattgt tgaaatcttt ttttattttt tatttttaca gtgctgagat ataaactgtg
3001 ccttttttttg tttgtttgtt tctgtttttg ttcttttgag cagaacaaaa aaaagggacc
3061 actatgcatg ctgcacacgt ctcagattct taggtacaca cctgattctt aggtgcatgc
3121 catagtggga tatgttgctt tgatcagaac ctgcagggag gttttcgggc accacttaag
3181 tttcttggcg tttctttcaa accgttctct aagatgcatt tttatgaatt ttattaaaga
3241 gttttgttaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
3301 aaaaaaaaaa aaaa

Figure 8 – part 3

Sequence ID No. 4 Protein sequence – from NCBI NP_033994.

mgarcrsfsa lllllqvssw lcqelepesc spgfssevyt fpvperhler ghvlgrvrfe
gctgrprtaf fsedsrfkva tdgtitvkrh lklhkletsf lvrardsshr elstkvtlks
mghhhhrhhh rdpasesnpe llmfpsvypg lrrqkrdwvi ppiscpenek gefpknlvqi
ksnrdketkv fysitgqgad kppvgvfiie retgwlkvtq pldreaiaky ilyshavssn
geavedpmei vitvtdqndn rpeftqpvfe gfvaegavpg tsvmkvsatd adddvntyna
aiaytivsqd pelphknmft vnrdtgvisv ltsgldresy ptytlvvqaa dlqgeglstt
akavitvkdi ndnapvfnps tyqgqvpene vnariatlkv tdddapntpa wkavytvvnd
pdqqfvvvtd pttndgilkt akgldfeakq qyilhvrven eepfegslvp statvtdvv
dvneapifmp aerrvevped fgvgqeitsy tarepdtfmd qkityriwrd tanwleinpe
tgaiftraem dredaehvkn styvaliiat ddgspiatgt gtlllvlldv ndnapipepr
nmqfcqrnpq phiitildpd lppntspfta elthgasvnw tieyndaaqe slilqprkdl
eigeykihlk ladnqnkdqv ttldvhvcdc egtvnncmka givaaglqvp ailgilggil
allilillll lflrrrtvvk epllppdddt rdnvyyydee gggeedqdfd lsqlhrglda
rpevtrndva ptlmsvpqyr prpanpdeig nfidenlkaa dsdptappyd sllvfdyegs
gseaaslssl nssesdqdqd ydylnewgnr fkkladmygg gedd

Figure 9 – part 1

Rat E-cadherin sequences

Sequence ID No. 5 DNA sequence – NCBI NM_031334

1 ctggtgtggg agccgcggcg cactactgag ttctcaagaa cttctgctag acttcagccc
61 ggcctaaccc ggctctgccc gaccgcaccc gagctcagtg tttgctcggc gtttgcccgg
121 ccagccatgg gagcccggtg ccgcagcttc tccgcgctcc tgctcctgct gcaggtctcc
181 tcgtggcttt gtcagcagcc ggagtcggag tctgactcct gccgtcccgg cttcagttcc
241 gaggtctaca ccttcctggt gccggagagg cacctggaga gaggccacat cctgggcaga
301 gtgaaatttg aaggatgcac cggccgtcca aggacagcct tcttttctga agactcccga
361 ttcaaagtgt ctacagatgg cgtcatcaca gtcaaacggc atctaaagct tcacaagctg
421 gagaccagtt ttctcgtcca tgcctgggac tccagttaca ggaagctttc taccaaagtg
481 acactgaagt ccctgggcca ccaccaccac cggcatcacc acagagaccc tgtctctgaa
541 tccaacccag agctgctcac gtttcccagc tttcaccagg gtctgagaag acagaaacga
601 gactgggtca tccctcccat caactgcccg gaaaatcaaa agggcgaatt cccccagcga
661 ctggttcaga tcaaatccaa cagggacaaa gagacaacgg ttttctacag catcaccggc
721 ccaggagctg acaaaccccc tgttggcgtt ttcatcattg agagggagac aggctggctg
781 aaagtgacgc agcctctgga cagagaagcc attgacaagt accttctcta ctctcatgct
841 gtgtcatcaa atggggaagc cgtggaggat cccatggaga tagtggtcac agtcacagat
901 cagaatgaca acaggccaga gtttatccag gaggtctttg agggatctgt tgcagaaggc
961 gctcttccag gaacctccgt gatgcaggtc tcagccactg atgcagacga tgacataaac
1021 acctacaatg ctgccatcgc ctacaccatc ctcagccaag atcctgagct gcctcacaaa
1081 aacatgttca ctgtcaaccg ggacactggg gtcatcagtg tggtcacctc cggactggac
1141 cgagagagtt accctacata tactctggtg gttcaggctg ccgaccttca aggcgaaggc
1201 ctaagcacaa cagcaaaagc tgtgatcact gtcaaggata ttaatgacaa cgctcccatc
1261 ttcaacccaa gcacgtacca gggtcaagtg cttgagaatg aggtcggtgc ccgtattgcc
1321 acactcaagg tgactgatga tgatgccccc aacactccag cgtggaatgc tgtgtacacc
1381 gtagtcaatg atcctgatca tcagttcact gtcatcacag accccaagac caacgagggc
1441 attctgaaaa cagccaaggg cttggatttt gaggccaagc agcagtacat tctgcacgtg
1501 acagtggaaa atgaggagcc ctttgagggg tctcttgtcc cttccacagc cactgtcacc
1561 gtggatgtgg tagacgtgaa tgaagccccc atttttgtgc ctgcggagaa gagagtcgag
1621 gtgcctgagg actttggtgt gggtctggag atcgcatctt acactgcgcg agagccagac
1681 acattcatgg aacagaagat cacgtatcgg atttggaggg acactgccaa ttggctggag

Figure 9 – part 2

1741 attaacccag agactggggt catttccact cgggctgaga tggacagaga agattcggag
1801 catgtgaaga acagcacgta tacagctctc atcattgcca cagatgatgg ttcacccatt
1861 gccactggca cagggactct tctcctggtc ctgtcagacg tcaacgacaa tgctcccatc
1921 ccagaacctc gaaatatgca gttctgccag agaaacccga agccccatgt catcaccatc
1981 ttggatccag accttccccc aaacacatcc cccttcactg cagagctcac ccatggggcc
2041 agcgtcaact ggaccattga gtacaatgac gcagaacaag aatctctcat tttgcaacca
2101 agaaaggact tagagattgg cgaatacaaa atcaatctca agctctcgga taaccagaat
2161 aaagaccagg tgaccacgtt ggaggtccac gtgtgtgact gtgaagggac cgtcaacaac
2221 tgcatgaagg cgatctccct ggaagcagga ttacaagttc ccgccatcct tggaatcctg
2281 ggagggatcc tggccctcct gattctgatc ctcctgctcc tactgtttct acggaggaga
2341 acggtggtca aagagccctt gctgccacca gatgacgata cccgggacaa tgtgtattac
2401 tatgatgaag agggaggtgg agaagaagac caggactttg atttgagcca gctgcacagg
2461 ggccttgatg ccagaccgga agtgattcga aatgatgtgg ctcccaccct catgagcatg
2521 ccccagtatc gtccccgtcc agccaatcct gatgaaatcg ggaacttcat cgatgaaaac
2581 ctgaaggcag cggacagtga ccccacagcg ccccccttacg actctctgtt ggtgtttgac
2641 tatgagggga gtggttctga agctgcctcc ctgagctcgc tgaactcctc tgagtcagat
2701 caggaccagg actacgatta tctgaacgaa tggggcaacc ggttcaagaa gctggccgat
2761 atgtatggtg gcggcgaaga agactagaga gtcgttcctg tgtggcacca tgggagatgc
2821 agaatcatga tgtcagtggt ctttcagctc cttccctgac tttgtagaag agagactgat
2881 ctgagaagta tgcagattgc atactggtcc cactctacct accagtctgt ctgtgttagg
2941 agggttttca ctggttgttg gaatcttttt ctaaaatgtt tttgttttta cagtgctgtg
3001 atgtgatgaa ctgtaccctc tttttgtttt tgttttgagc tatgttctgc tccggacaca
3061 cagcccccaa gcccttcacc cctcactaat ttttttacat tgtatacttt cactcaatta
3121 ccatgtttat gttctgtatt ctaatagcca ctaagttcct gaattctgtt gcctggccca
3181 ggtgctattc tgtgacacag tagtgcctgg gccctttttat ggtaagagac aggtttcttg
3241 gtgtgggtgc aactgagctg gatagtgtat gtttcaaaca cctttcctgt gttctctccc
3301 cacctccaga gtgtctttac ttattcagct gtgtgtttgg ggcagaacaa aaaaataatg
3361 ggaccactat gcaagctgcg aagattctaa ggtgcacacc tgattcttag gcagatgcca
3421 tagtgagata tgttgctttg gttctctatc caatgctgtg accgggacct gcagcgaggt
3481 tttcggacac cgtggtttct tgcgtttctt tcaaaccagc agtaaaaaat ggttttttct

Figure 9 – part 3

3541 gagagagact ggagtgccac caccaaagat agaggagaga agccaagctt ggggacagca
3601 agcatgccag tgaacctgac cactgtcatg agtcatgtgg gtggccacat gtccgtgaac
3661 ctggccagtt ggcacactga tggtgagggt acaaggaggc tagacctcgt cccacaaaat
3721 ttctggaaga attagggttg tctcagccaa tgtttcctag ctggaatcct gtccatgtat
3781 gtgttcctga agcccaggaa atacacccct ctagtgcctg cttttgatgg tagttataga
3841 aaagaccggc tgatttggac ctgagttgcc caatcttaag tacaaataga aaactgagac
3901 tatgctgggt gtgttggtgc acgcctttaa tcccggcact cgggaggcag agacagtctc
3961 agatctctct gagttcaagg tcagcctggt atagtaagtg aattccagga cagccagggc
4021 tacacagaaa ctctgtcttg gaaaaccaaa aaagaaaact gagaatatta gagattgtgc
4081 atttctcag aaagcaggaa gaaaacacca ctctgatggg aaaagggagg caaggccctt
4141 gagacttttc attgaaattg ctgtactcac ataattttgg aagcaaatga tgactgcaat
4201 caactgtgag aactgttggt ttctctgtag tttaattgtc taatgttgat agcgtgccct
4261 ttgtatgtag tttgagtgta tatgtgtgtg ggtgctgata attttgtatt ttgtggggag
4321 tggaaaaggc aagcaatcgg aactgttctc taagatgcat ttttatgaat tttattaaag
4381 agttttgtta aactgt

Sequence ID No. 6 Protein sequence – NCBI NP_112624

1 mgarcrsfsa lllllqvssw lcqqpesesd scrpgfssev ytflvperhl erghilgrvk
61 fegctgrprt affsedsrfk vstdgvitvk rhlklhklet sflvhawdss yrklstkvtl
121 kslghhhhrh hhrdpvsesn pelltfpsfh qglrrqkrdw vippincpen qkgefpqrlv
181 qiksnrdket tvfysitgpg adkppvgvfi ieretgwlkv tqpldreaid kyllyshavs
241 sngeavedpm eivvtvtdqn dnrpefiqev fegsvaegal pgtsvmqvsa tdadddinty
301 naaiaytils qdpelphknm ftvnrdtgvi svvtsgldre syptytlvvq aadlqgegls
361 ttakavitvk dindnapifn pstyqgqvle nevgariatl kvtdddapnt pawnavytvv
421 ndpdhqftvi tdpktnegil ktakgldfea kqqyilhvtv eneepfegsl vpstatvtvd
481 vvdvneapif vpaekrvevp edfgvgleia sytarepdtf meqkityriw rdtanwlein
541 petgvistra emdredsehv knstytalii atddgspiat gtgtlllvls dvndnapipe
601 prnmqfcqrn pkphvitild pdlppntspf taelthgasv nwtieyndae qeslilqprk
661 dleigeykin lklsdnqnkd qvttlevhvc dcegtvnncm kaisleaglq vpailgilgg
721 ilallilill lllflrrrtv vkepllppdd dtrdnvyyyd eegggeedqd fdlsqlhrgl
781 darpevirnd vaptlmsmpq yrprpanpde ignfidenlk aadsdptapp ydsllvfdye
841 gsgseaasls slnssesdqd qdydylnewg nrfkkladmy gggeed

Figure 10 – part 1

Canine E-cadherin sequences

Sequence ID No. 7 DNA sequence (predicted) – NCBI XM_536807

```
1 atggccttgt ttgggatcaa ggctgcccgc ttccatgtgg aggggtgcag ccgcagccaa
61 tcagcggcgc gggggcgggg gcctcgcggc tcacctggcg gccggacgcg gccccgctca
121 gtggcgtcgg gcgctgcggg cacctgtgat tcgcggaagt cctgccgcct cgcgccgcct
181 cgcgccgcct cgcgccgcct cgcgcccggc tctcgacccc cgcccgccat gggccctcgg
241 tacggcggcg cccccgcgct cctgctcccg ctgctgctgc tgctgcaggt ctcatcgggg
301 ctctgccaag agccggagcc ctgccgccct ggctttggcg ctgacagcta cacgttcacc
361 gtgccccggc gacacttgga gagaggccgt gtcctgggca gggtgagttt tgaaggatgc
421 accggtctac ctaggacagc ctatgtttct gatgacaccc gattcaaagt gggcacagat
481 ggtgtgatta cagtcaagcg gcctctacaa cttcataaac cagagataag ttttcttgtc
541 catgcctggg actccagccg caggaagctc tccaccagag ttaggctgaa ggcagcgacg
601 caccaccacc accaccatca tgatgctccc tctaaaaccc agacagaggt gctcacattt
661 cccagttccc agcatggact cagaagacag aagagagact gggttatccc tcctatcagc
721 tgcccggaaa acgagaaagg cccatttcct aaaaacctgg ttcagatcaa gtctaacagg
781 gacaaagaaa tcaaggtttt ctacagcatc actggccaag gagctgacgc acctcctgtt
841 ggtgtgttta ttattgaaag agaaacagga tggctgaagg tgactgagcc tctggataga
901 gaacaaattg ctaagtacat tctctactct catgccgtat cttctaatgg gaatgcggtt
961 gaagacccaa tggagatcgt gatcacggtg acagatcaga atgacaacaa gcccgagttc
1021 acccaggcag tcttccaagg atctgtcacg gaaggtgccc ttccaggcac ctctgtgatg
1081 caggtgacag ccacagatgc ggatgatgat gtgaatacct acaacgctgc catcgcttac
1141 agcatcctca cacaagaccc cctcctgcct agcagcatga tgttcactat caacaaggac
1201 acaggagtca tcagcgtgct caccactggg ctggaccgag agggtgtccc catgtacacc
1261 ttggtggttc aggctgctga cctgcaaggc gaaggcttaa ctacaactgc aacagctgtg
1321 atcacagtca ctgacatcaa tgataacccc cccatcttca acccaaccac gtaccaggga
1381 cgggtgcctg agaacaaggc taacgtcgaa atcgctgtac tcaaagtgac ggatgctgat
1441 gtccccgata ccccggcctg gagggctgtg tacaccatat tgaacaataa caatgatcaa
1501 tttgttgtca ccacagaccc agtaactaac gacggcattt tgaaaacaac taagggcttg
1561 gattttgagg acaagcagca gtatgtcttg tacgtgactg tggtgaacgt gaccccgttt
1621 gaggtcatcc tctccacctc cacagccact gtcactgtgg acgtggaaga tgtgaatgaa
```

1681 gcccccatct tcatcccttg cccaaaggta gtgtcaatcc ctgaagactt tggtgtgggc
1741 caggaaatca catcctacac cgccgaggat ccagatacat atatggaaca gaggataacg
1801 tatcggattt ggagggatgc tgccggttgg ctggaggtta atccagaatc tggtgccatt
1861 ttcactcggg ctgagctgga cagagaggat tttgagcacg tgaagaatag cacgtatgaa
1921 gccctcatta tagccattga caacggttct ccagttgcta ctggaacggg aactcttcta
1981 ctggtcctct ctgatgtgaa tgacaatggc cccattccag aacctcgaaa tatggacttc
2041 tgccagaaaa acccacagcc tcatgtcatc aacatcattg atccagatct tccccccaac
2101 acatctccct tcacagcaga actaacacac ggcgcaagtg tcaactggac catcgagtac
2161 aatgacccag gtgggaattg gactcgtgaa tctctaattt tgaagccaaa gaaaacttta
2221 gagttgggtg actacaaaat aaatctcaag ctcacagata accagaacaa ggaccaggtg
2281 accaccctag atgtgtttgt gtgcgactgc gaaggtgtcg tcaacagctg caagaggacg
2341 gcgccttacg ccgaagcagg cttgcaggtt cctgccatct tgggcattct cggaggaatc
2401 ctcgctctac taatcctgat tctgctgctt ctgctatttg ttcggaggag aagggtggtc
2461 aaagagccct tacttccccc agaagatgac acccgggaca atgtttatta ctatgatgaa
2521 gaaggaggtg gagaggagga tcaggacttt gacttgagcc agttgcacag gggcctggat
2581 gctcggcctg aagtgactcg caatgatgtg gccccaaccc tcctgagtgt gccccagtat
2641 cggccccgcc ctgccaatcc tgatgaaatt ggaaacttta ttgatgaaaa cctgaaggca
2701 gcggacactg accctactgc tcctccttat gactctctgc tcgtgtttga ctatgaagga
2761 agcggttctg aagctgctag tctgagctcc ttgaactcct cagagtcaga ccaagaccag
2821 gactatgact acctgaatga atggggcaat cgcttcaaga agctggcgga catgtatgga
2881 ggtggcgagg acgactag

DNA sequence (promoter region) – NCBI AF330163 SEQ ID No. 58

1 ccgcccgccg caggtgcagc cgcagccaat cagcggcgcg gggggcgggg cctcgcggct
61 cacctggcgg ccggacgcgg ccccgctcag t

DNA sequence (partial coding sequence) – NCBI AF330162

1 agtggcgtcg ggcgctgcgg gcacctgtga ttcgcggaag tcctgccgcc tcgcgccgcc
61 tcgcgcccgg ctctcgaccc ccgcccgcca tgggccctcg gtacggcggc gcccccgcgc
121 tcctgctccc gctgctgctg ctgctgcagg tctcatcggg gctctgccaa gagccggagc
181 cctgccgccc tggctttggc gctgacagct acacgttcac cgtgccccgg cgacacttgg

241 agagaggccg tgtcctgggc agggtgagtt ttgaaggatg caccggtcta cctaggacag
301 cctatgtttc tgatgacacc cgattcaaag tgggcacaga tggtgtgatt acagtcaagc
361 ggcctctaca acttcataaa ccagagataa gttttcttgt ccatgcctgg gactccagcc
421 gcaggaagct ctccaccaga gttaggctga aggcagcgac gcaccaccac caccaccatc
481 atgatgctcc ctctaaaacc cagacagagg tgctcacatt tcccagttcc cagcatggac
541 tcagaagaca gaagagagac tgggttatcc ctcctatcag ctgcccggaa aacgagaaag
601 gcccatttcc taa

Sequence ID No. 8 Protein sequence (incomplete)– NCBI AAL09464 SEQ ID No. 59

1 mgpryggapa lllplllllq vssglcqepe pcrpgfgads ytftvprrhl ergrvlgrvs
61 fegctglprt ayvsddtrfk vgtdgvitvk rplqlhkpei sflvhawdss rrklstrvrl
121 kaathhhhhh hdapsktqte vltfpssqhg lrrqkrdwvi ppiscpenek gpfp

Figure 11 – part 1

Bovine E-cadherin sequences

Sequence ID No. 9 DNA sequence – NCBI AY508164

```
atgggccctt ggagccgcag cctctctgcg ctctgctgct gctgcaggtg taatccgtgg
      61 ctctgccggg agccggagcc ctgcattcct ggctttggtg ctgagagtta
cacgttcacc
     121 gtgccccggc ggaacttgga gagagggcga gtcctaggca gagtgagttt
tgaaggatgt
     181 gctggcctac caaggacagt ctatgtttct gatgacaccc gattcaaagt
gcacacagat
     241 ggcgtgctta cagtcagacg acctgtacac cttcatcgtc cagagctaag
ttttcttgtc
     301 catgcctggg actccaccca caggaagctc tccaccaaag tgacactgga
ggtatcagcg
     361 caccaccacc accaccacag tcatcatgac tctccctctg gaacccagac
agaagtgctc
     421 acatttcctg gcccccacca tggtctcagg agacagaaga gagactgggt
tattcctcct
     481 atcagctgcc cagaaaatga gaaaggccca tttcctaagt cgctggtcca
gatcaaatct
     541 aacaaggaga aagaaaccca agttttctac agcatcactg gccaacgagc
tgatacaccc
     601 cctgtcggtg tttttattat tgaaagagaa acaggatggt taaaagtgac
acagcctctg
     661 gatagagaac agattgccaa gtacattctc ttctctcatg ccgtgtcttc
aaatggacaa
     721 gccattgaag agcctatgga gattgtgatc accgtgaccg accagaatga
caacaagccc
     781 cagttcaccc aggaggtctt caaggcgtct gccctggaag gcgctcttcc
aggaacctct
     841 gtgatgcagg tcacggccac agatatagat gacgaggtga acacctacac
cgctgccatc
     901 ggttacacaa tcccagccca agatcccatg ctgccgcaca acaaaatgtt
caccatcaac
     961 aaggaaacag gcgtcatcag tgtgctcacc accgggctgg accgtgagag
ttttcccaca
    1021 tacaccctga tggtccaagc agcagacctt aacggcgaag gcttgagcac
aactgcaacg
    1081 gccgtgatca cagtcttgga caccaatgat aatgctccca gattcaaccc
aaccacgtac
    1141 gtggggtcgg tgcctgagaa cgaggctaat gtggccatca ccacactcac
agtgactgat
    1201 gccgacgacc ccaacacccc ggcatgggag gctgtttaca cagtattaaa
tgataacgag
    1261 aagcaattta tcgtcgtcac agacccagtc accaatgaag gcactctgaa
aacagctaag
    1321 ggcttggatt ttgaggccaa gcagcagtac atcctgtacg tggcagtgac
aaatgtggcc
    1381 ccctttgaag tcactctccc cacttccaca gccaccgtca ctgtggatgt
gatagatgtg
    1441 aatgaagccc ccatctttgt gcctcctcaa aagagagtgg aagtgcccga
ggactttggc
    1501 gtgggcctgg agatcacatc ctatactgcc cgggagccag acacatttat
ggaacagaag
    1561 atcacgtatc ggatttggag ggacactgcc aactggctgg agattaatcc
agaaacgggt
    1621 gccatttcca ctcgggctga gttggacaga gaggatgtcg atcatgtgaa
gaacagcacg
    1681 tacacggccc tcattatagc cactgacaat ggttctccac ctgccactgg
gacaggcacc
```

Figure 11 – part 2

1741 ctgctcttgt tcctcgatga tgtgaatgac aatggccccg taccagaacc ccggaccatg
1801 gacttctgcc agaggaatcc tgagcctcat atcatcaaca tcaatgatcc tgatctccct
1861 ccgaacacct ccccctttac agcagaactg acacatgggg cgagtgtcaa ttggaccatt
1921 gagtacaatg accaagaacg tgagtctctg attttgaagc caaagaaaac cttagagctg
1981 ggtgaccaca aaatcaatct caagctcata gacaaccaga acaaagacca ggtgaccaca
2041 cttgatgtgc acgtgtgtga ctgtgatggg atcgtcagca actgcaggaa ggcacggcct
2101 gctgaagcag gattgcaagt tcccgccatc ctggggatcc ttggaggcat ccttgctttt
2161 ctgatcctta ttttgctgct tctgctactt gttcggagga gaagggtggt caaagagccc
2221 ttactgcccc cagaagatga cacccgggac aatgtgtatt actatgatga agaaggaggt
2281 ggagaagaag atcaggactt tgacttgagc cagttacata ggggcctgga tgctcggcct
2341 gaagtgactc gcaatgacgt ggcaccaacc ctcatgagtg tgccccagta ccgaccccgc
2401 cctgccaatc ctgatgaaat tggaaacttt attgatgaaa acctgaaggc agctgatagt
2461 gaccccactg ccccacccta tgactctctg ctggtgtttg attatgaagg aagtggttcc
2521 gaagctgcta ctctgagctc cctgaactcc tcagagtcag accaagacca ggactatgac
2581 tacctgaatg aatggggcaa tcgcttcaag aagctggcgg acatgtatgg aggcggcgag
2641 gacgactag

Figure 11 – part 3

Sequence ID No. 10 Protein sequence – NCBI NP_001002763

mgpwsrslsa lccccrcnpw lcrepepcip gfgaesytft vprrnlergr vlgrvsfegc
61 aglprtvyvs ddtrfkvhtd gvltvrrpvh lhrpelsflv hawdsthrkl stkvtlevsa
121 hhhhhhshhd spsgtqtevl tfpgphhglr rqkrdwvipp iscpenekgp fpkslvqiks
181 nkeketqvfy sitgqradtp pvgvfiiere tgwlkvtqpl dreqiakyil fshavssngq
241 aieepmeivi tvtdqndnkp qftqevfkas alegalpgts vmqvtatdid devntytaai
301 gytipaqdpm lphnkmftin ketgvisvlt tgldresfpt ytlmvqaadl ngeglsttat
361 avitvldtnd naprfnptty vgsvpenean vaittltvtd addpntpawe avytvlndne
421 kqfivvtdpv tnegtlktak gldfeakqqy ilyvavtnva pfevtlptst atvtvdvidv
481 neapifvppq krvevpedfg vgleitsyta repdtfmeqk ityriwrdta nwleinpetg
541 aistraeldr edvdhvknst ytaliiatdn gsppatgtgt lllflddvnd ngpvpeprtm
601 dfcqrnpeph iinindpdlp pntspftael thgasvnwti eyndqeresl ilkpkktlel
661 gdhkinlkli dnqnkdqvtt ldvhvcdcdg ivsncrkarp aeaglqvpai lgilggilaf
721 lilillllll vrrrrvvkep llppeddtrd nvyyydeegg geedqdfdls qlhrgldarp
781 evtrndvapt lmsvpqyrpr panpdeignf idenlkaads dptappydsl lvfdyegsgs
841 eaatlsslns sesdqdqdyd ylnewgnrfk kladmyggge dd

Figure 12

Sequence ID No. 11 – amino acid sequence of CAD-HAV domain of E-cadherin

HAV

Figure 13 – part 1

Sequence ID No. 12 Slug - Human

1 mprsflvkkh fnaskkpnys eldthtviis pylyesysmp vipqpeilss gayspitvwt
61 taapfhaqlp nglsplsgys sslgrvsppp psdtsskdhs gsespisdee erlqsklsdp
121 haieaekfqc nlcnktystf sglakhkqlh cdaqsrksfs ckycdkeyvs lgalkmhirt
181 htlpcvckic gkafsrpwll qghirthtge kpfscphcnr afadrsnlra hlqthsdvkk
241 yqckncsktf srmsllhkhe esgccvah

Sequence ID No. 13 **Slug – Mouse**

1 mprsflvkkh fnaskkpnys eldthtviis pylyesypip vipkpeilts gayspitvwt
61 ssaaplhspl psglspltgy ssslgrvspp pssdtsskdh sgsespisde eerlqpklsd
121 phaieaekfq cnlcnktyst fsglakhkql hcdaqsrksf sckycdkeyv slgalkmhir
181 thtlpcvcki cgkafsrpwl lqghirthtg ekpfscphcn rafadrsnlr ahlqthsdvk
241 kyqckncskt fsrmsllhkh eesgccvah

Sequence ID No. 14 **Snail – Human**

1 mprsflvrkp sdpnrkpnys elqdsnpeft fqqpydqahl laaipppeil nptaslpmli
61 wdsvlapqaq piawaslrlq esprvaelts lsdedsgkgs qppsppspap ssfsstsvss
121 leaeayaafp glgqvpkqla qlseakdlqa rkafnckycn keylslgalk mhirshtlpc
181 vcgtcgkafs rpwllqghvr thtgekpfsc phcsrafadr snlrahlqth sdvkkyqcqa
241 cartfsrmsl lhkhqesgcs gcpr

Sequence ID No. 15 **Snail – Mouse**

1 mprsflvrkp sdprrkpnys elqdacveft fqqpydqahl laaipppevl npaaslptli
61 wdsllvpqvr pvawatlplr espkavelts lsdedsgkss qppsppspap ssfsstsass
121 leaeafiafp glgqlpkqla rlsvakdpqs rkifnckycn keylslgalk mhirshtlpc
181 vcttcgkafs rpwllqghvr thtgekpfsc shcnrafadr snlrahlqth sdvkryqcqa
241 cartfsrmsl lhkhqesgcs ggpr

Sequence ID No. 16 **SIP1 – Human**

1 mkqpimadgp rckrrkqanp rrknvvnydn vvdtgsetde edklhiaedd gianpldqet
61 spasvpnhes sphvsqallp reeeedeire ggvehpwhnn eilqasvdgp eemkedydtm
121 gpeatiqtai nngtvknanc tsdfeeyfak rkleerdgha vsieeylqrs dtaiiypeap
181 eelsrlgtpe angqeendlp pgtpdafaql ltcpycdrgy krltslkehi kyrhekneen
241 fscplcsytf ayrtqlerhm vthkpgtdqh qmltqgagnr kfkctecgka fkykhhlkeh

Figure 13 – part 2

301 lrihsgekpy ecpnckkrfs hsgsysshis skkciglisv ngrmrnnikt gsspnsvsss
361 ptnsaitqlr nklengkpls mseqtgllki ktepldfndy kvlmathgfs gtspfmnggl
421 gatsplgvhp saqspmqhlg vgmeapllgf ptmnsnlsev qkvlqivdnt vsrqkmdcka
481 eeisklkgyh mkdpcsqpee qgvtspnipp vglpvvshng atksiidytl ekvneakacl
541 qslttdsrrq isnikkeklr tlidlvtddk mienhnistp fscqfckesf pgpiplhqhe
601 rylckmneei kavlqpheni vpnkagvfvd nkalllssvl sekgmtspin pykdhmsvlk
661 ayyamnmepn sdellkisia vglpqefvke wfeqrkvyqy snsrspsler sskplapnsn
721 pptkdsllpr spvkpmdsit spsiaelhns vtncdpplrl tkpshftnik pvekldhsrs
781 ntpsplnlss tssknshsss ytpnsfssee lqaepldlsl pkqmkepksi iatknktkas
841 sisldhnsvs sssensdepl nltfikkefs nsnnldnkst npvfsmnpfs akplytalpp
901 qsafppatfm ppvqtsipgl rpypgldqms flphmaytyp tgaatfadmq qrrkyqrkqg
961 fqgelldgaq dymsglddmt dsdsclsrkk ikktesgmya cdlcdktfqk ssllrhkye
1021 htgkrphqcq ickkafkhkh hliehsrlhs gekpyqcdkc gkrfshsgsy sqhmnhrysy
1081 ckreaeerea aerearekgh leptellmnr aylqsitpqg ysdseeresm prdgesekeh
1141 ekegedgygk lgrqdgdeef eeeeeesenk smdtdpetir deeetgdhsm ddssedgkme
1201 tksdheednm edgm

Sequence ID No. 17 **SIP1 – Mouse**

1 mkqpimadgp rckrrkqanp rrknvvnydn vvdagsetde edklhiaedd slanpldqdt
61 spasmpnhes sphmsqgllp reeeeeelre svvehswhsg eilqasvagp eemkedydam
121 gpeatiqtti nngtvknanc tsdfeeyfak rkleerdgha vsieeylqrs dtaiiypeap
181 eelsrlgtpe angqeendlp pgtpdafaql ltcpycdrgy krltslkehi kyrhekneen
241 fscplcsytf ayrtqlerhm vthkpgtdqh qmltqgagnr kfkctecgka fkykhhlkeh
301 lrihsgekpy ecpnckkrfs hsgsysshis skkciglisv ngrmrnnikt gsspnsvsss
361 ptnsaitqlr nklengkpls mseqtgllki ktepldfndy kvlmathgfs gsspfmnggl
421 gatsplgvhp saqspmqhlg vgmeapllgf ptmnsnlsev qkvlqivdnt vsrqkmdckt
481 edisklkgyh mkdpcsqpee qgvtspnipp vglpvvshng atksiidytl ekvneakacl
541 qslttdsrrq isnikkeklr tlidlvtddk mienhsistp fscqfckesf pgpiplhqhe
601 rylckmneei kavlqpheni vpnkagvfvd nkalllssvl sekgltspin pykdhmsvlk
661 ayyamnmepn sdellkisia vglpqefvke wfeqrkvyqy snsrspsler tskplapnsn
721 pttkdsllpr spvkpmdsit spsiaelhns vtscdpplrl tksshftnik avdkldhsrs
781 ntpsplnlss tssknshsss ytpnsfssee lqaepldlsl pkqmrepkgi iatknktkat
841 sinldhnsvs sssensdepl nltfikkefs nsnnldnksn npvfgmnpfs akplytplpp
901 qsafppatfm ppvqtsipgl rpypgldqms flphmaytyp tgaatfadmq qrrkyqrkqg
961 fqgdlldgaq dymsglddmt dsdsclsrkk ikktesgmya cdlcdktfqk ssllrhkye
1021 htgkrphqcq ickkafkhkh hliehsrlhs gekpyqcdkc gkrfshsgsy sqhmnhrysy
1081 ckreaeerea aerearekgh lgptellmnr aylqsitpqg ysdseeresm prdgesekeh

Figure 13 – part 3

1141 ekegeegygk lrrrdgdeee eeeeeesenk smdtdpetir deeetgdhsm ddssedgkme
1201 tksdheednm edgmg

Sequence ID No. 18 **E2A – Human – Amino acid sequence**
MNQPQRMAPVGTDKELSDLLDFSMMFPLPVTNGKGRPASLAGAQ

FGGSGLEDRPSSGSWGSGDQSSSSFDPSRTFSEGTHFTESHSSLSSSTFLGPGLGGKS

GERGAYASFGRDAGVGGLTQAGFLSGELALNSPGPLSPSGMKGTSQYYPSYSGSSRRR

AADGSLDTQPKKVRKVPPGLPSSVYPPSSGEDYGRDATAYPSAKTPSSTYPAPFYVAD

GSLHPSAELWSPPGQAGFGPMLGGGSSPLPLPPGSGPVGSSGSSSTFGGLHQHERMGY

QLHGAEVNGGLPSASSFSSAPGATYGGVSSHTPPVSGADSLLGSRGTTAGSSGDALGK

ALASIYSPDHSSNNFSSSPSTPVGSPQGLAGTSQWPRAGAPGALSPSYDGGLHGLQSK

IEDHLDEAIHVLRSHAVGTAGDMHTLLPGHGALASGFTGPMSLGGRHAGLVGGSHPED

GLAGSTSLMHNHAALPSQPGTLPDLSRPPDSYSGLGRAGATAAASEIKREEKEDEENT

SAADHSEEEKKELKAPRARTSPDEDEDDLLPPEQKAEREKERRVANNARERLRVRDIN

EAFKELGRMCQLHLNSEKPQTKLLILHQAVSVILNLEQQVRERNLNPKAACLKRREEE
KVSGVVGDPQMVLSAPHPGLSEAHNPAGHM

Sequence ID No. 19 **E2A – human – DNA encoding sequence**
1 gcctgaggtg cccgccctgg ccccaggaga atgaaccagc cgcagaggat ggcgcctgtg
61 ggcacagaca aggagctcag tgacctcctg gacttcagca tgatgttccc gctgcctgtc
121 accaacggga agggccggcc cgcctccctg gccggggcgc agttcggagg ttcaggtctt
181 gaggaccggc ccagctcagg ctcctggggc agcggcgacc agagcagctc ctcctttgac
241 cccagccgga ccttcagcga gggcacccac ttcactgagt cgcacagcag cctctcttca
301 tccacattcc tgggaccggg actcggaggc aagagcggtg agcggggcgc ctatgcctcc
361 ttcgggagag acgcaggcgt gggcggcctg actcaggctg gcttcctgtc aggcgagctg
421 gccctcaaca gccccgggcc cctgtcccct tcgggcatga aggggacctc ccagtactac

Figure 13 – part 4

481 ccctcctact ccggcagctc ccggcggaga gcggcagacg gcagcctaga cacgcagccc
541 aagaaggtcc ggaaggtccc gccgggtctt ccatcctcgg tgtacccacc cagctcaggt
601 gaggactacg gcagggatgc caccgcctac ccgtccgcca agacccccag cagcacctat
661 cccgcccccct tctacgtggc agatggcagc ctgcacccct cagccgagct ctggagtccc
721 ccgggccagg cgggcttcgg gcccatgctg ggtgggggct catccccgct gcccctcccg
781 cccggtagcg gcccggtggg cagcagtgga agcagcagca cgtttggtgg cctgcaccag
841 cacgagcgta tgggctacca gctgcatgga gcagaggtga acggtgggct cccatctgca
901 tcctccttct cctcagcccc cggagccacg tacggcggcg tctccagcca cacgccgcct
961 gtcagcgggg ccgacagcct cctgggctcc cgagggacca cagctggcag ctccggggat
1021 gccctcggca aagcactggc ctcgatctac tccccggatc actcaagcaa taacttctcg
1081 tccagccctt ctacccccgt gggctccccc cagggcctgg caggaacgtc acagtggcct
1141 cgagcaggag cccccggtgc cttatcgccc agctacgacg ggggtctcca cggcctgcag
1201 agtaagatag aagaccacct ggacgaggcc atccacgtgc tccgcagcca cgccgtgggc
1261 acagccggcg acatgcacac gctgctgcct ggccacgggg cgctggcctc aggtttcacc
1321 ggccccatgt cgctgggtgg gcggcacgca ggcctggttg gaggcagcca ccccgaggac
1381 ggcctcgcag gcagcaccag cctcatgcac aaccacgcgg ccctccccag ccagccaggc
1441 accctccctg acctgtctcg gcctcccgac tcctacagtg ggctagggcg agcaggtgcc
1501 acggcggccg ccagcgagat caagcgggag gagaaggagg acgaggagaa cacgtcagcg
1561 gctgaccact cggaggagga gaagaaggag ctgaaggccc cccgggcccg gaccagccca
1621 gacgaggacg aggacgacct tctccccccca gagcagaagg ccgagcggga gaaggagcgc
1681 cgggtggcca ataacgcccg ggagcggctg cgggtccgtg acatcaacga ggcctttaag
1741 gagctggggc gcatgtgcca actgcacctc aacagcgaga agccccagac caaactgctc
1801 atcctgcacc aggctgtctc ggtcatcctg aacttggagc agcaagtgcg agagcggaac
1861 ctgaatccca aagcagcctg tttgaaacgg cgagaagagg aaaaggtgtc aggtgtggtt
1921 ggagaccccc agatggtgct ttcagctccc cacccaggcc tgagcgaagc ccacaacccc
1981 gccgggcaca tgtgaaaggt atgcctccgt gggacgagcc acccgctttc agccctgtgc
2041 tctggcccca gaagccggac tcgagacccc gggcttcatc cacatccaca cctcacacac
2101 ctgttgtcag catcgagcca acaccaacct gacaaggttc ggagtgatgg gggcggccaa
2161 ggtgacactg ggtccaggag ctccctgggg ccctggccta ccactcactg gcctcgctcc
2221 ccctgtcccc gaatctcagc caccgtgtca ctctgtgacc tgtcccatgg atcctgaaac
2281 tgcatcttgg ccctgttgcc tgggctgaca ggagcatttt tttttttcc agtaaacaaa
2341 acctgaaagc aagcaacaaa acatacactt tgtcagagaa gaaaaaaatg ccttaactat
2401 aaaaagcgga gaaatggaaa catatcactc aagggggatg ctgtggaaac ctggcttatt
2461 cttctaaagc caccagcaaa ttgtgcctaa gcgaaatatt tttttaagg aaaataaaaa
2521 cattagttac aagatttttt ttttcttaag gtagatgaaa attagcaagg atgctgcctt
2581 tggtctctgg tttttttaag ctttttttgc atatgttttg taagcaacaa attttttgt
2641 ataaaagtcc cgtgtctctc gctatttctg ctgctgttcc tagactgagc attgcatttc

Figure 13 – part 5

2701 ttgatcaacc agatgattaa acgttgtatt aaaaagaccc cgtgtaaacc tgagcccccc
2761 ccgtcccccc ccccggaagc cactgcacac agacagacgg ggacaggcgg cgggtctttt
2821 gttttttga tgttgggggt tctcttggtt ttgtcatgtg gaaagtgatg cgtgggcgtt
2881 ccctgatgaa ggcaccttgg ggcttccctg ccgcatcctc tcccctcagg aaggggactg
2941 acctgggctt gggggaaggg acgtcagcaa ggtggctctg accctcccag gtgactctgc
3001 caagcagctg tggccccagc ggtaccctac acaacgccct ccccaggccc ccctaagctg
3061 ctctcccttg gaacctgcac agctctctga aatggggcat tttgttggga ccagtgaccc
3121 ctggcatggg gaccacaccc tggagcccgg tgctggggac ctcctggaca ccctgtcctt
3181 cactccttgc cccagggacc caggctcatg ctctgaactc tggctgagag gagtctgctc
3241 aggagccagc acaggacacc ccccacccca ccccaccatg tccccattac accagagggc
3301 catcgtgacg tagacaggat gccaggggcc tgaccagcct ccccaatgct ggggagcatc
3361 cctggcctgg ggccacacct gctgccctcc ctctgtgtgg tccaagggca agagtggctg
3421 gagccggggg actgtgctgg tctgagcccc acgaaggcct tgggctgtgg ctccgaccct
3481 gctgcagaac cagcagggtg tcccctcggg cccatctgtg tcccatgtcc cagcacccag
3541 gcctctctcc aggtctcctt ttctggtctt ttgccatgag ggtaaccagc tcttcccagc
3601 tggctgggac tgtcttgggt ttaaaactgc aagtctccta ccctgggatc ccatccagtt
3661 ccacacgaac tagggcagtg gtcactgtgg cacccaggtg tgggcctggc tagctggggg
3721 ccttcatgtg cccttcatgc ccctccctgc attgaggcct tgtggacccc tgggctggct
3781 gtgttcatcc ccgctgcagg tcgggcgtct ccccccgtgc cactcctgag actccaccgt
3841 tacccccagg agatcctgga ctgcctgact cccctcccca gactggcttg ggagcctggg
3901 ccccatggta gatgcaaggg aaacctcaag gccagctcaa tgcctggtat ctgccccccag
3961 tccaggccag gcggagggga ggggctgtcc ggctgcctct cccttctcgg tggcttcccc
4021 tgcgccctgg gagtttgatc tcttaaggga acttgcctct ccctcttgtt ttgctcctgc
4081 cctgccccta ggtctgggtg gcagtggccc catagcctct ggaactgtgc gttctgcata
4141 gaattcaaac gagattcacc cagcgcgagg aggaagaaac agcagttcct gggaaccaca
4201 attatggggg gtggggggtg tgatctgagt gcctcaagat ggttttcaaa aaattttttt
4261 taaagaaaat aattgtatac gtgtcaacac agctggctgg atgattggga ctttaaaacg
4321 accctctttc aggtggattc agagacctgt cctgtatata acagcactgt agcaataaac
4381 gtgacatttt ataaag

Sequence ID No. 20 E2A – Mouse – Amino acid sequence

MMNQSQRMAPVGSDKELSDLLDFSMMFPLPVANGKSRPASLGGT

QFAGSGLEDRPSSGSWGSSDQNSSSFDPSRTYSEGAHFSDSHSSLPPSTFLGAGLGGK

GSERNAYATFGRDTSVGTLSQAGFLPGELSLSSPGPLSPSGIKSSSQYYPSFPSNPRR

Figure 13 – part 6

RAADGGLDTQPKKVRKVPPGLPSSVYPPSSGDSYSRDAAAYPSAKTPSSAYPSPFYVA

DGSLHPSAELWSTPSQVGFGPMLGDGSSPLPLAPGSSSVGSGTFGGLQQQDRMGYQLH

GSEVNGSLPAVSSFSAAPGTYSGTSGHTPPVSGAAAESLLGTRGTTASSSGDALGKAL

ASIYSPDHSSNNFSPSPSTPVGSPQGLPGTSQWPRAGAPSALSPNYDAGLHGLSKMED

RLDEAIHVLRSHAVGTASDLHGLLPGHGALTTSFTGPMSLGGRHAGLVGGSHPEEGLT

SGASLLHNHASLPSQPSSLPDLSQRPPDSYSGLGRAGTTAGASEIKREEKEDEEIASV

ADAEEDKKDLKVPRTRTSSTDEVLSLEEKDLRDRERRMANNARERVRVRDINEAFREL

GRMCQLHLKSDKAQTKLLILQQAVQVILGLEQQVRERNLNPKAACLKRREEEKVSGVV
GDPQLPLSAAHPGLGEAHNPAGHL

Sequence ID No. 21 **E2A – Mouse – DNA encoding sequence**

1 gcgccggcgg ctgcgggcgt agcgggccac cgcgggccac cgccgcgcgc cgccgcctct
61 gctacagtcc cttcccgcgg ggcctgctct gagagaagct cgagagagac caggcgacgc
121 gaacgcgagt ggggaggagg aaggacgcgc gacccсgagc cctgcgcgct cccgccgccc
181 acgcgcgacc ctcggggacg cgcccgccac ccttttgtcc ccggggtccc cgagggcggt
241 gggcagcagg gagccccggt gcacccggtg catgcccccg cccagcaggg ctgtctctag
301 acctggggga cgcaccccag ttccaacacc tgctgtcctg ggtggatgat gaaccagtct
361 cagagaatgg cacccgtggg ctctgacaag gaactgagtg acctcctgga cttcagcatg
421 atgttcccgc tacctgtggc caatgggaag agccggcccg cctccctcgg gggaacccag
481 tttgcaggct caggactgga ggaccgaccc agctcaggct cctggggcag cagtgaccag
541 aacagttctt cctttgaccc tagccggaca tacagcgaag gtgcccactt cagtgactcc
601 cacagcagcc tgccgccttc cacgttccta ggagctgggc ttggaggcaa gggcagtgag
661 cggaatgcct atgccacctt tgggagagac accagtgttg gcaccttgag tcaggctggc
721 ttcctgccag gtgagctgag cctcagcagt cccgggccac tgtccccatc gggcatcaag
781 agcagctccc agtattaccc ctcattcccc agcaaccctc gtcggagagc tgcagatggt
841 ggcctggata ctcagccgaa gaaggtccgg aaggttccgc ctggtctccc ttcctcggtg
901 tatccgccca gctcaggtga cagctacagc agggatgctg cagcctaccc ctccgccaag
961 acccccagca gcgcttaccc ctccccсttc tacgtggcag atggcagcct gcacccatca
1021 gctgagctct ggagtacgcc tagccaggtg ggctttgggc ccatgctagg tgacggctct

Figure 13 – part 7

1081 tcccctctgc cccttgcacc gggcagcagc tccgtgggca gtggtacctt tgggggcctc
1141 cagcagcagg atcgcatggg ctaccagctg catggatctg aggttaatgg ctcgctccca
1201 gctgtatcca gcttttcggc tgcccctggc acttacagtg ggacttccgg ccacacgccc
1261 cctgtgagtg gggccgcagc tgaaagcctc ctaggcaccc gagggactac agccagcagc
1321 tcaggggatg cccttgggaa ggcactggcc tcgatctact ccccggatca ctccagcaat
1381 aatttctcac ctagcccctc aacgcctgtg ggttcacccc agggcctgcc agggacatca
1441 cagtggcccc gggcaggagc gcccagtgcc ttatccccca actacgatgc aggtctccat
1501 ggcctgagca agatggagga ccgcttggac gaggccatcc atgtcctgcg aagccacgct
1561 gttggcaccg ctagcgatct ccatgggctt ttgcctggcc atggcgcact gaccacgagc
1621 ttcaccggcc ccatgtcact gggcgggcgg catgccggcc tggtcggggg aagccatcct
1681 gaggagggcc tcacaagtgg ggccagtctt ttgcataacc atgccagcct ccccagccag
1741 cccagttccc tccctgacct ctcacagaga cctcccgact cctatagtgg actcgggagg
1801 gcaggcacaa cagcgggtgc cagcgagatc aagcgggagg agaaagagga tgaggaaatc
1861 gcatcagtag ccgacgccga agaggacaag aaggacctga aggtcccacg cacgcgcacc
1921 agcagtacag atgaggtgct gtccctggag gagaaggacc tgagggaccg ggagaggcgt
1981 atggccaata acgctcggga gcgggtgcgc gtgcgggaca ttaacgaggc cttccgggag
2041 ctgggccgca tgtgccagct gcacctcaag tcggataagg cgcagaccaa gctgctcatc
2101 ctgcagcagg cggtgcaggt catcctgggc ctggagcagc aggtgcgaga acgcaacctg
2161 aaccccaaag cagcctgctt gaagcggagg gaggaggaga aggtgtctgg cgtggtcggg
2221 gacccacagc tgcccctgtc agccgcccac ccgggcctgg gtgaggccca caacccagcc
2281 gggcacctgt gagccgtcac agcttcttcg ttggaccagg gaccaccata tctctgcccg
2341 gggtgcatca ggacggttct ggatgagaca ggtctccatc gaagcatgag cagagagagg
2401 gctctgggga cacttcaggg cctggggagg gtggcactga acagctccct gcttggcccc
2461 agtgaccaag cagaaaagtt ccttcctctc ggttaaccag aactggaaac aaagcagcat
2521 gctccctttt caaaaaggaa agaaagatgc cttaactatg taagacggaa gagtcggacc
2581 gtgccctggc agggcggcct gggactggct tctacttcag agccaccagc acatcgtgcc
2641 taagcatttt tcgttttttt aaaggagaat aaaggaacat tagttttcag attttttttt
2701 taaatgtaga caaaagttag caagaacgag gccttccgtg tctttttttt ttcccttagc
2761 tttttttcc gtatgttttg taagcaacaa atttttgtat aaaagtctca tgtctgtttc
2821 tgtttctaga aaaaaaaaaa aaaaaaaaaa aaaaaatatt taaaaaaaaa aaaaaaaaaa
2881 aaaaaaaaaa aaaaaaaa

Figure 14 – part 1

5 x human E-cadherin in pRNAtin-H1.2neo

DQ090940 Homo sapiens cadherin 1, type 1, E-cadherin (epithelial) (CDH1)gene, complete cds.

Sequence ID No. 22. siRNA insert 1: 76 bp. start at 2258

```
BamH I                                                                  Hind III
GGATCCCGTATTTACGACCTTTCTTGGCATTGATATCCGTGCCAAGAAAGGTCGTAAATATTTTTTCCAAAAGCTT      2258-2278
      ^| Antisense          | Loop      | Sense                  | Termination Signal
```

Sequence ID No. 23. siRNA insert 2: 76 bp. start at 339

```
BamH I                                                                  Hind III
GGATCCCGTTTCTTGAGCCATAAATGCTCTTGATATCCGGAGCATTTATGGCTCAAGAAATTTTTTCCAAAAGCTT      339-359
      ^| Antisense          | Loop      | Sense                  | Termination Signal
```

Sequence ID No. 24. siRNA insert 3: 76 bp. start at 478

```
BamH I                                                                  Hind III
GGATCCCGTTAGTGAGTCAGCAAATTGATTTGATATCCGATCAATTTGCTGACTCACTAATTTTTTCCAAAAGCTT      478-498
      ^| Antisense          | Loop      | Sense                  | Termination Signal
```

Sequence ID No. 25. siRNA insert 4: 76 bp. start at 986

```
BamH I                                                                  Hind III
GGATCCCGTGTGAGCCATGAGCCACTGAGTTGATATCCGCTCAGTGGCTCATGGCTCACATTTTTTCCAAAAGCTT      986-1006
      ^| Antisense          | Loop      | Sense                  | Termination Signal
```

Sequence ID No. 26. siRNA insert 5: 76 bp. start at 2976

```
BamH I                                                                  Hind III
GGATCCCGCATAGTCAACAACCAGGCAGGTTGATATCCGCCTGCCTGGTTGTTGACTATGTTTTTTCCAAAAGCTT      2976-2996
      ^| Antisense          | Loop      | Sense                  | Termination Signal
```

Figure 14 – part 2

3 x mouse E-cadherin in pRNAtin-H1.2neo

```
BC098501 Mus musculus cadherin 1, mRNA (cDNA clone MGC:107495
         IMAGE:30023851), complete cds.
```

Sequence ID No. 27. siRNA insert 1: 76 bp. start at 2126

```
BamH I                                                              Hind III
GGATCCCGTTGTTCTGGTTATCCGCGAGCTTGATATCCGGCTCGCGGATAACCAGAACAATTTTTTCCAAAAGCTT
      ^| Antisense           | Loop     | Sense                | Termination Signal
```

Sequence ID No. 28. siRNA insert 2: 76 bp. start at 1385

```
BamH I                                                              Hind III
GGATCCCGTCTGTGACGACAACGAACTGCTTGATATCCGGCAGTTCGTTGTCGTCACAGATTTTTTCCAAAAGCTT
      ^| Antisense           | Loop     | Sense                | Termination Signal
```

Sequence ID No. 29. siRNA insert 3: 76 bp. start at 369

```
BamH I                                                              Hind III
GGATCCCGTAGATGCCGCTTCACTGTGATTTGATATCCGATCACAGTGAAGCGGCATCTATTTTTTCCAAAAGCTT
      ^| Antisense           | Loop     | Sense                | Termination Signal
```

Figure 15 – part 1

Sequence ID No. 30 Human E-cadherin mRNA NM_004360

```
1 agtggcgtcg gaactgcaaa gcacctgtga gcttgcggaa gtcagttcag actccagccc
       61 gctccagccc ggcccgaccc gaccgcaccc ggcgcctgcc ctcgctcggc
gtccccggcc
      121 agccatgggc ccttggagcc gcagcctctc ggcgctgctg ctgctgctgc
aggtctcctc
      181 ttggctctgc caggagccgg agccctgcca ccctggcttt gacgccgaga
gctacacgtt
      241 cacggtgccc cggcgccacc tggagagagg ccgcgtcctg ggcagagtga
attttgaaga
      301 ttgcaccggt cgacaaagga cagcctattt ttccctcgac acccgattca
aagtgggcac
      361 agatggtgtg attacagtca aaaggcctct acggtttcat aacccacaga
tccatttctt
      421 ggtctacgcc tgggactcca cctacagaaa gttttccacc aaagtcacgc
tgaatacagt
      481 ggggcaccac caccgccccc cgccccatca ggcctccgtt tctggaatcc
aagcagaatt
      541 gctcacattt cccaactcct ctcctggcct cagaagacag aagagagact
gggttattcc
      601 tcccatcagc tgcccagaaa atgaaaaagg cccatttcct aaaaacctgg
ttcagatcaa
      661 atccaacaaa gacaaagaag gcaaggtttt ctacagcatc actggccaag
gagctgacac
      721 accccctgtt ggtgtcttta ttattgaaag agaaacagga tggctgaagg
tgacagagcc
      781 tctggataga gaacgcattg ccacatacac tctcttctct cacgctgtgt
catccaacgg
      841 gaatgcagtt gaggatccaa tggagatttt gatcacggta accgatcaga
atgacaacaa
      901 gcccgaattc acccaggagg tctttaaggg gtctgtcatg gaaggtgctc
ttccaggaac
      961 ctctgtgatg gaggtcacag ccacagacgc ggacgatgat gtgaacacct
acaatgccgc
     1021 catcgcttac accatcctca gccaagatcc tgagctccct gacaaaaata
tgttcaccat
     1081 taacaggaac acaggagtca tcagtgtggt caccactggg ctggaccgag
agagtttccc
     1141 tacgtatacc ctggtggttc aagctgctga ccttcaaggt gaggggttaa
gcacaacagc
     1201 aacagctgtg atcacagtca ctgacaccaa cgataatcct ccgatcttca
atcccaccac
     1261 gtacaagggt caggtgcctg agaacgaggc taacgtcgta atcaccacac
tgaaagtgac
     1321 tgatgctgat gcccccaata ccccagcgtg ggaggctgta tacaccatat
tgaatgatga
     1381 tggtggacaa tttgtcgtca ccacaaatcc agtgaacaac gatggcattt
tgaaaacagc
     1441 aaagggcttg gattttgagg ccaagcagca gtacattcta cacgtagcag
tgacgaatgt
     1501 ggtacctttt gaggtctctc tcaccacctc cacagccacc gtcaccgtgg
atgtgctgga
     1561 tgtgaatgaa gcccccatct ttgtgcctcc tgaaaagaga gtggaagtgt
ccgaggactt
     1621 tggcgtgggc caggaaatca catcctacac tgcccaggag ccagacacat
ttatggaaca
```

Figure 15 – part 2


```
1681 gaaaataaca tatcggattt ggagagacac tgccaactgg ctggagatta atccggacac
1741 tggtgccatt tccactcggg ctgagctgga cagggaggat tttgagcacg tgaagaacag
1801 cacgtacaca gccctaatca tagctacaga caatggttct ccagttgcta ctggaacagg
1861 gacacttctg ctgatcctgt ctgatgtgaa tgacaacgcc cccataccag aacctcgaac
1921 tatattcttc tgtgagagga atccaaagcc tcaggtcata aacatcattg atgcagacct
1981 tcctcccaat acatctccct tcacagcaga actaacacac ggggcgagtg ccaactggac
2041 cattcagtac aacgacccaa cccaagaatc tatcattttg aagccaaaga tggccttaga
2101 ggtgggtgac tacaaaatca atctcaagct catggataac cagaataaag accaagtgac
2161 caccttagag gtcagcgtgt gtgactgtga aggggccgcc ggcgtctgta ggaaggcaca
2221 gcctgtcgaa gcaggattgc aaattcctgc cattctgggg attcttggag gaattcttgc
2281 tttgctaatt ctgattctgc tgctcttgct gtttcttcgg aggagagcgg tggtcaaaga
2341 gcccttactg cccccagagg atgacacccg ggacaacgtt tattactatg atgaagaagg
2401 aggcggagaa gaggaccagg actttgactt gagccagctg cacaggggcc tggacgctcg
2461 gcctgaagtg actcgtaacg acgttgcacc aaccctcatg agtgtccccc ggtatcttcc
2521 ccgccctgcc aatcccgatg aaattggaaa ttttattgat gaaaatctga aagcggctga
2581 tactgacccc acagccccgc cttatgattc tctgctcgtg tttgactatg aaggaagcgg
2641 ttccgaagct gctagtctga gctccctgaa ctcctcagag tcagacaaag accaggacta
2701 tgactacttg aacgaatggg gcaatcgctt caagaagctg gctgacatgt acggaggcgg
2761 cgaggacgac taggggactc gagagaggcg ggccccagac ccatgtgctg ggaaatgcag
2821 aaatcacgtt gctggtggtt tttcagctcc cttcccttga gatgagtttc tggggaaaaa
2881 aaagagactg gttagtgatg cagttagtat agctttatac tctctccact ttatagctct
2941 aataagtttg tgttagaaaa gtttcgactt atttcttaaa gctttttttt ttttcccatc
3001 actctttaca tggtggtgat gtccaaaaga tacccaaatt ttaatattcc agaagaacaa
3061 ctttagcatc agaaggttca cccagcacct tgcagatttt cttaaggaat tttgtctcac
3121 ttttaaaaag aaggggagaa gtcagctact ctagttctgt tgttttgtgt atataatttt
3181 ttaaaaaaaa tttgtgtgct tctgctcatt actacactgg tgtgtccctc tgcctttttt
3241 ttttttttta agacagggtc tcattctatc ggccaggctg gagtgcagtg gtgcaatcac
3301 agctcactgc agccttgtcc tcccaggctc aagctatcct tgcacctcag cctcccaagt
3361 agctgggacc acaggcatgc accactacgc atgactaatt ttttaaatat ttgagacggg
3421 gtctccctgt gttacccagg ctggtctcaa actcctgggc tcaagtgatc ctcccatctt
```

Figure 15 – part 3

3481 ggcctcccag agtattggga ttacagacat gagccactgc acctgcccag ctccccaact
3541 ccctgccatt ttttaagaga cagtttcgct ccatcgccca ggcctgggat gcagtgatgt
3601 gatcatagct cactgtaacc tcaaactctg ggctcaagc agttctccca ccagcctcct
3661 ttttattttt ttgtacagat ggggtcttgc tatgttgccc aagctggtct taaactcctg
3721 gcctcaagca atccttctgc cttggccccc caaagtgctg ggattgtggg catgagctgc
3781 tgtgcccagc ctccatgttt taatatcaac tctcactcct gaattcagtt gctttgccca
3841 agataggagt tctctgatgc agaaattatt gggctctttt agggtaagaa gtttgtgtct
3901 ttgtctggcc acatcttgac taggtattgt ctactctgaa gacctttaat ggcttccctc
3961 tttcatctcc tgagtatgta acttgcaatg ggcagctatc cagtgacttg ttctgagtaa
4021 gtgtgttcat taatgtttat ttagctctga agcaagagtg atatactcca ggacttagaa
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4681 ttgagtgtat acatgtgtgg gtgctgataa ttgtgtattt tctttggggg tggaaaagga
4741 aaacaattca agctgagaaa agtattctca aagatgcatt tttataaatt ttattaaaca
4801 attttgttaa accataaaaa aaaaaaaa

Figure 16 – part 1

Sequence ID No. 31 Mouse E-cadherin mRNA BC098501

1 agccgcggcg cactactgag ttcccaagaa cttctgctag actcctgccc ggcctaaccc
61 ggccctgccc gaccgcaccc gagctcagtg tttgctcggc gtctgccggg tccgccatgg
121 gagcccggtg ccgcagcttt tccgcgctcc tgctcctgct gcaggtctcc tcatggcttt
181 gccaggagct ggagcctgag tcctgcagtc ccggcttcag ttccgaggtc tacaccttcc
241 cggtgccgga gaggcacctg gagagaggcc atgtcctggg cagagtgaga tttgaaggat
301 gcaccggccg gccaaggaca gccttctttt cggaagactc ccgattcaaa gtggcgacag
361 acggcaccat cacagtgaag cggcatctaa agctccacaa gctggagacc agtttcctcg
421 tccgcgcccg ggactccagt catagggagc tgtctaccaa agtgacgctg aagtccatgg
481 ggcaccacca tcaccggcac caccaccgcg accctgcctc tgaatccaac ccagagctgc
541 tcatgtttcc cagcgtgtac ccaggtctca gaagacagaa acgagactgg gtcatccctc
601 ccatcagctg ccccgaaaat gaaaagggtg aattcccaaa gaacctggtt cagatcaaat
661 ccaacaggga caaagaaaca aaggttttct acagcatcac cggccaagga gctgacaaac
721 cccccgttgg cgttttcatc attgagaggg agacaggctg gctgaaagtg acacagcctc
781 tggatagaga agccattgcc aagtacatcc tctattctca tgccgtgtca tcaaatgggg
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901 cagagtttac ccaggaggtg tttgagggat ccgttgcaga aggcgctgtt ccaggaacct
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1081 atagggacac cggggtcatc agtgtgctca cctctgggct ggaccgagag agttacccta
1141 catacactct ggtggttcag gctgctgacc ttcaaggcga aggcttgagc acaacagcca
1201 aggctgtgat cactgtcaag gatattaatg acaacgctcc tgtcttcaac ccgagcacgt
1261 atcagggtca agtgcctgag aatgaggtca atgcccggat cgccacactc aaagtgaccg
1321 atgatgatgc ccccaacact ccggcgtgga aagctgtgta caccgtagtc aacgatcctg
1381 accagcagtt cgttgtcgtc acagacccca cgaccaatga tggcattttg aaaacagcca
1441 agggcttgga ttttgaggcc aagcagcaat acatccttca tgtgagagtg gagaacgagg
1501 aaccctttga ggggtctctt gtcccttcca cagccactgt cactgtggac gtggtagacg
1561 tgaatgaagc ccccatcttt atgcctgcgg agaggagagt cgaagtgccc gaagactttg
1621 gtgtgggtca ggaaatcaca tcttataccg ctcgagagcc ggacacgttc atggatcaga

Figure 16 – part 2


```
1681 agatcacgta tcggatttgg agggacactg ccaactggct ggagattaac ccagagactg
1741 gtgccatttt cacgcgcgct gagatggaca gagaagacgc tgagcatgtg aagaacagca
1801 catatgtagc tctcatcatc gccacagatg atggttcacc cattgccact ggcacgggca
1861 ctcttctcct ggtcctgtta gacgtcaatg ataacgctcc catcccagaa cctcgaaaca
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1981 cccccaacac gtccccctt actgctgagc taacccatgg ggccagcgtc aactggacca
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2281 tgctgattct gatcctgctg ctcctactgt ttctacggag gagaacggtg gtcaaagagc
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3301 aaaaaaaaaa aaaa
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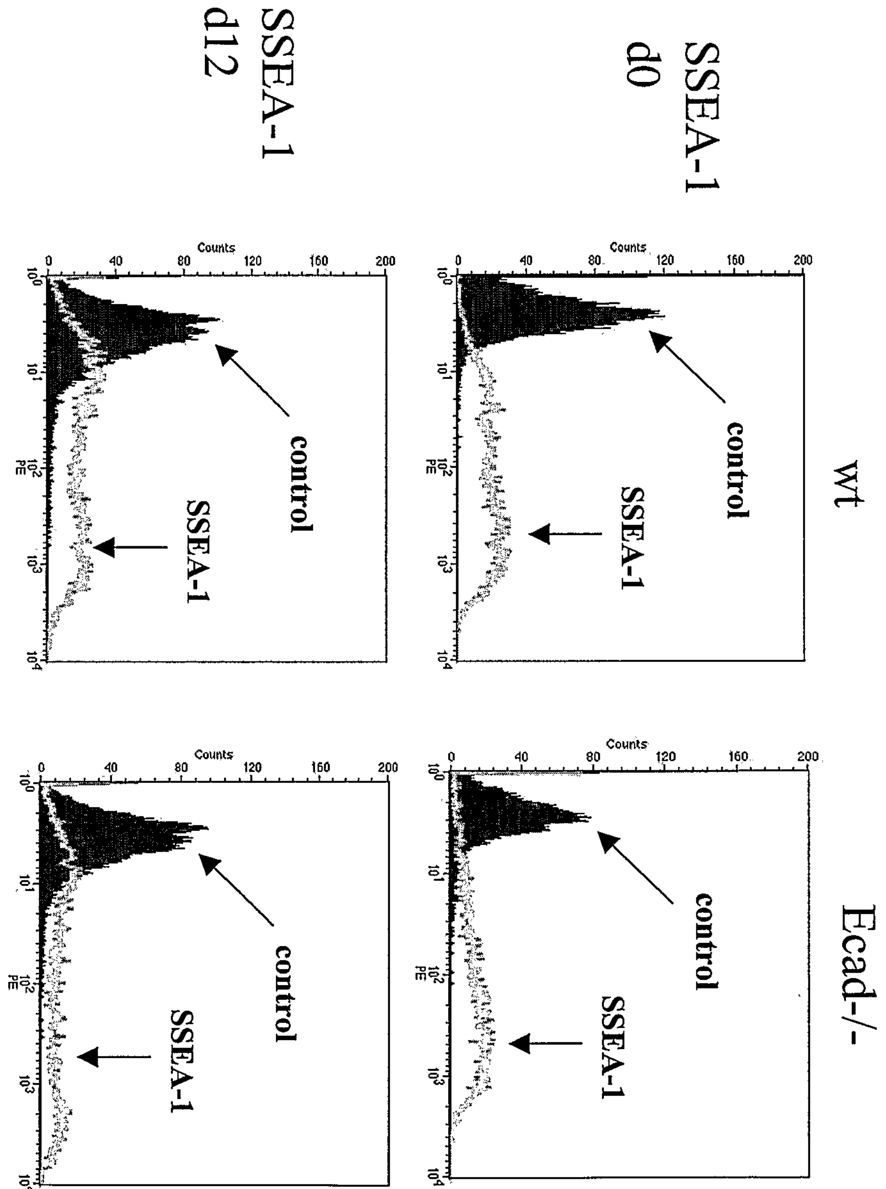
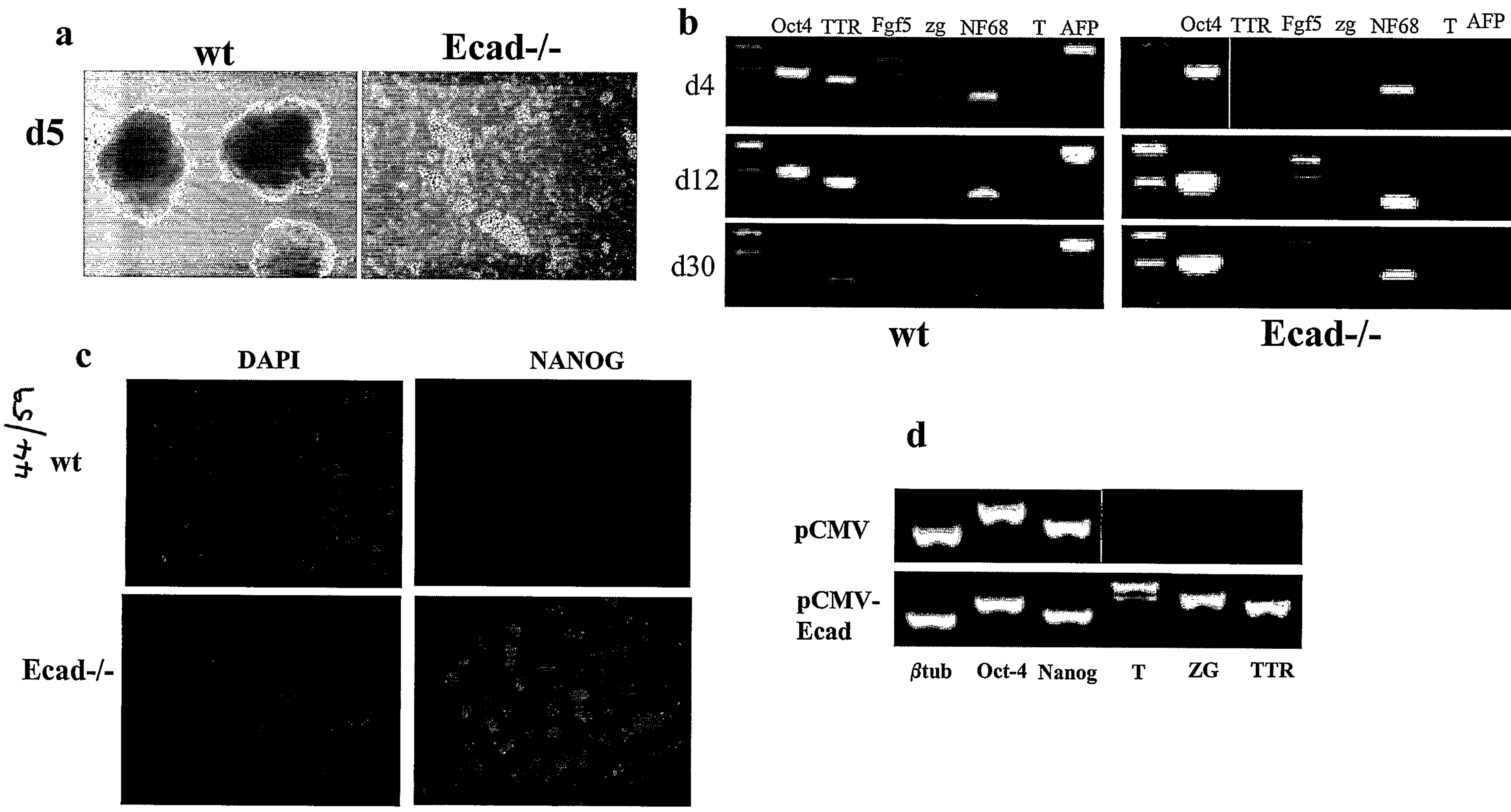


Figure 17.



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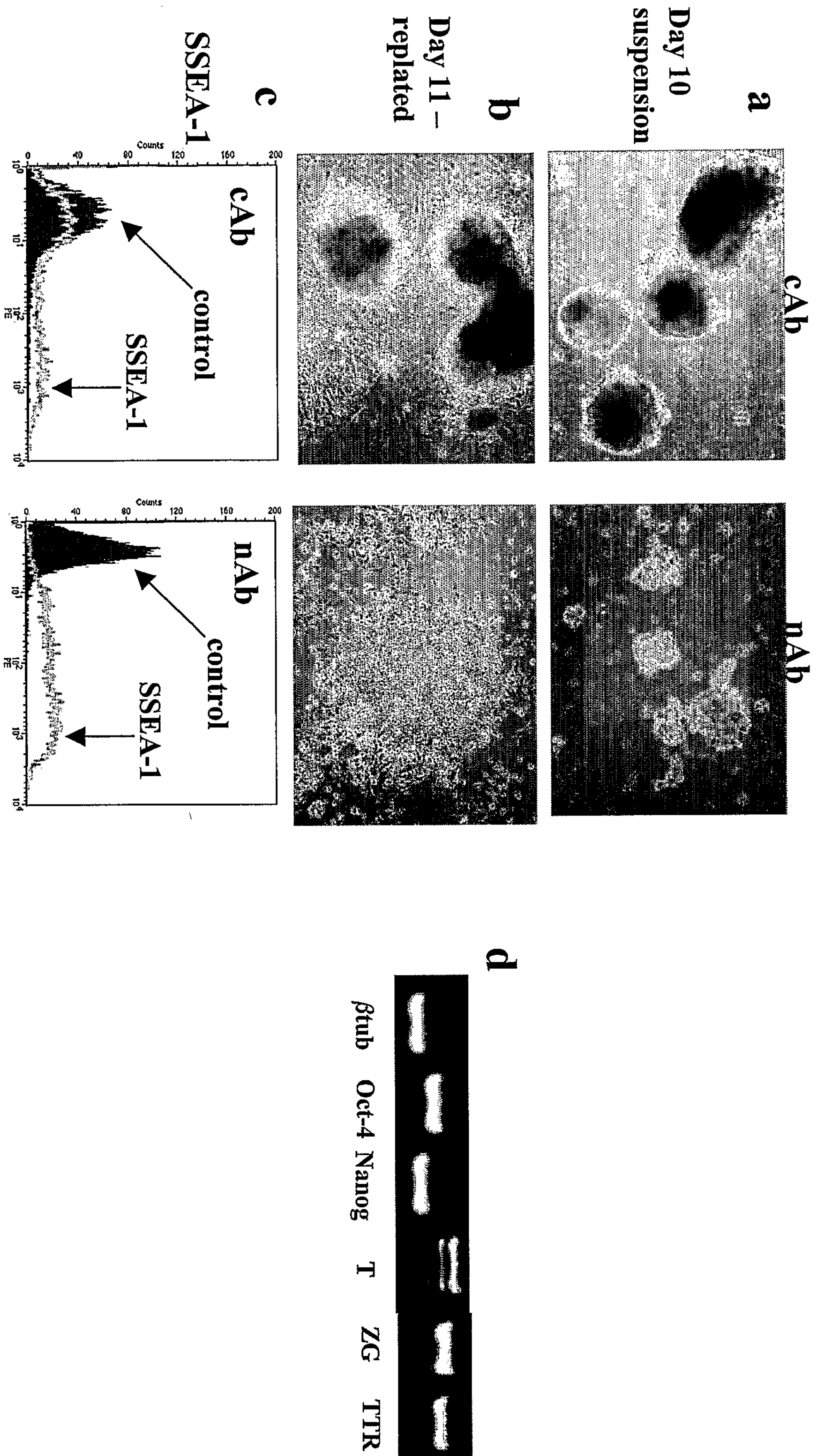

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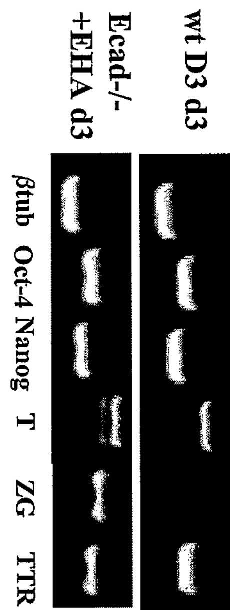

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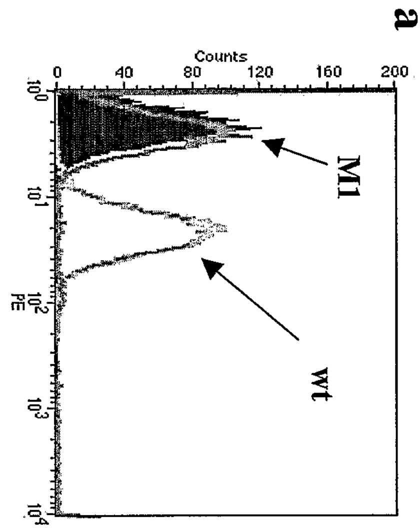

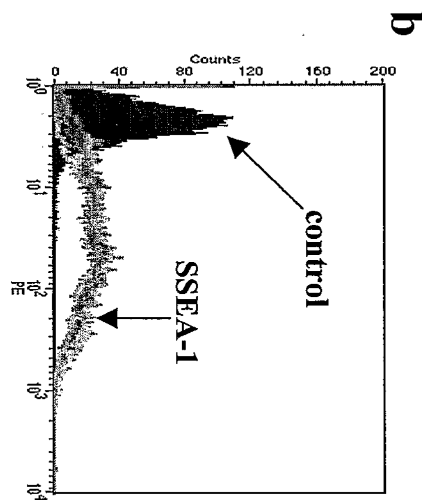

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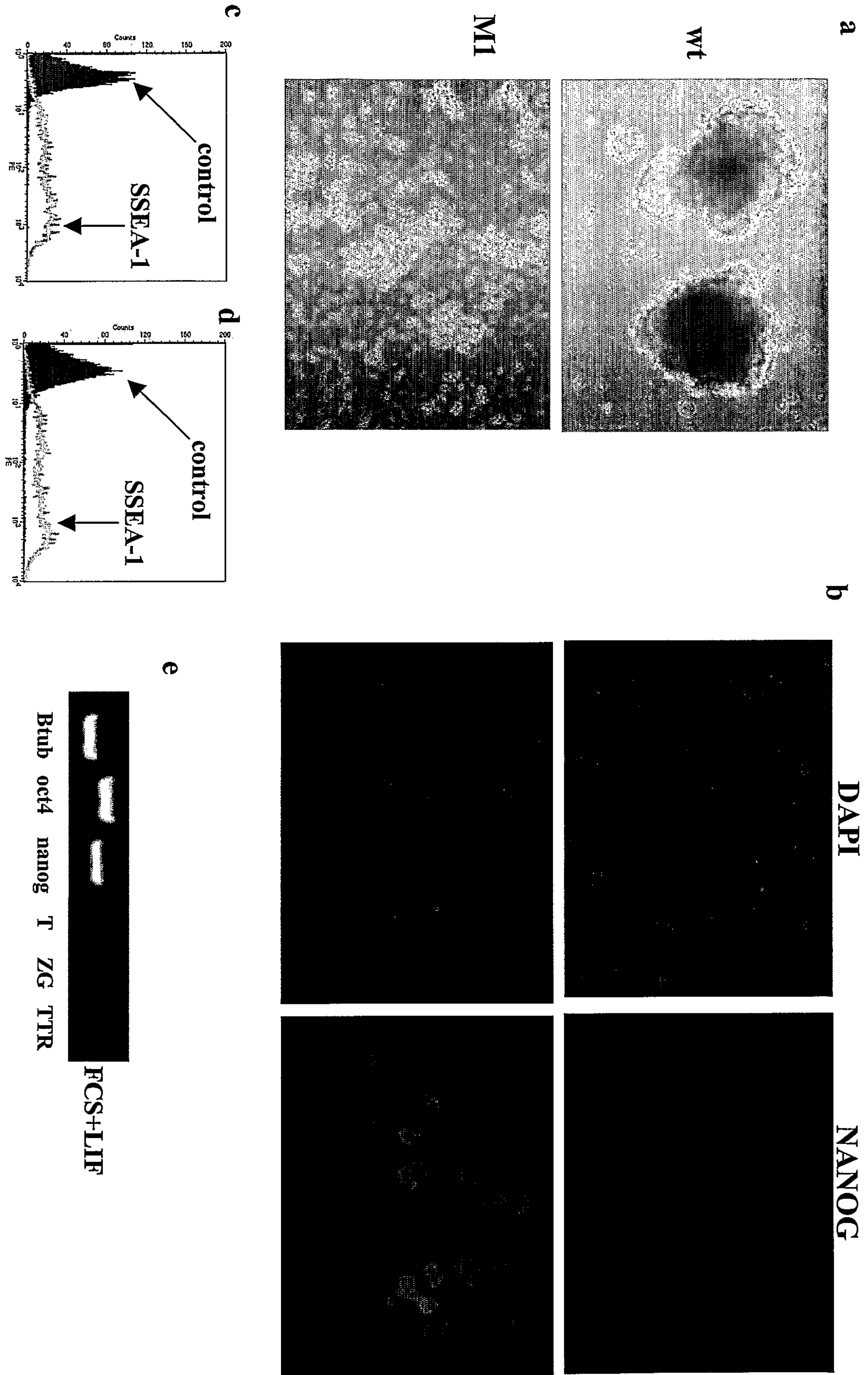

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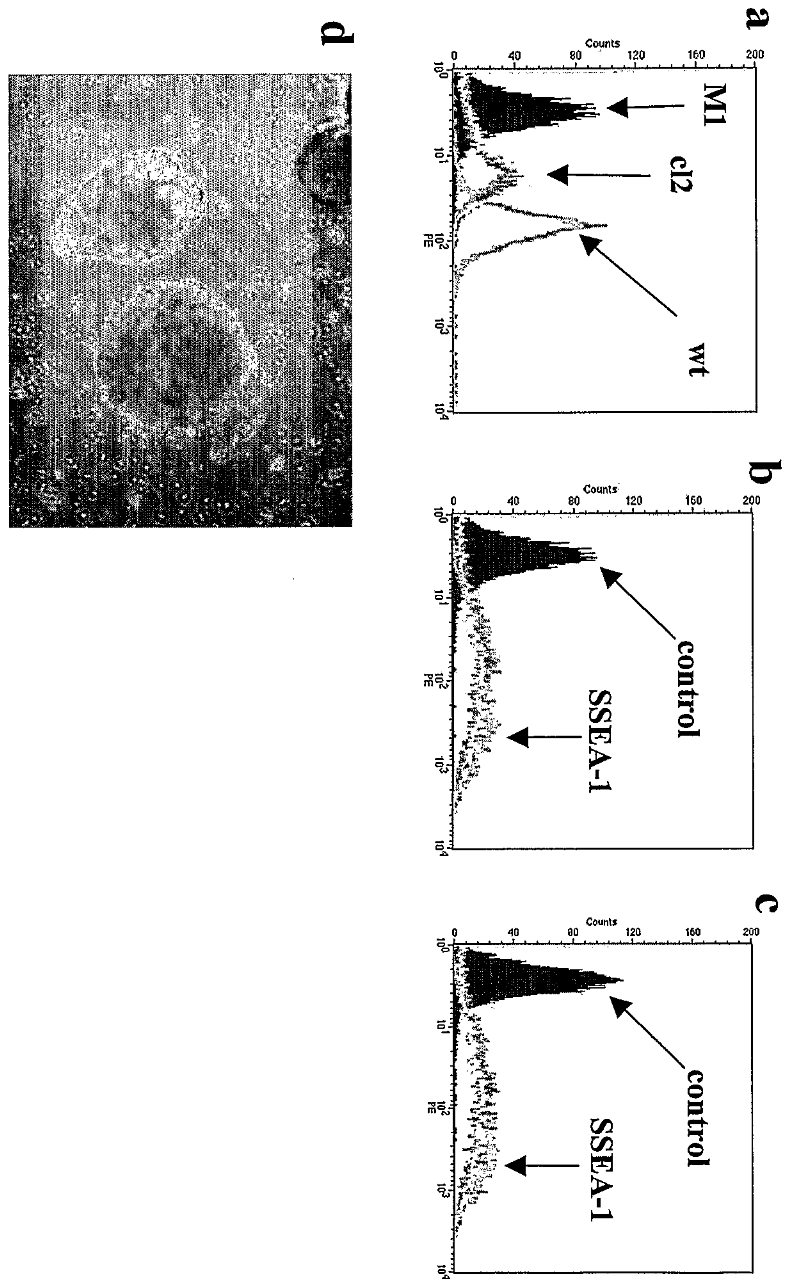

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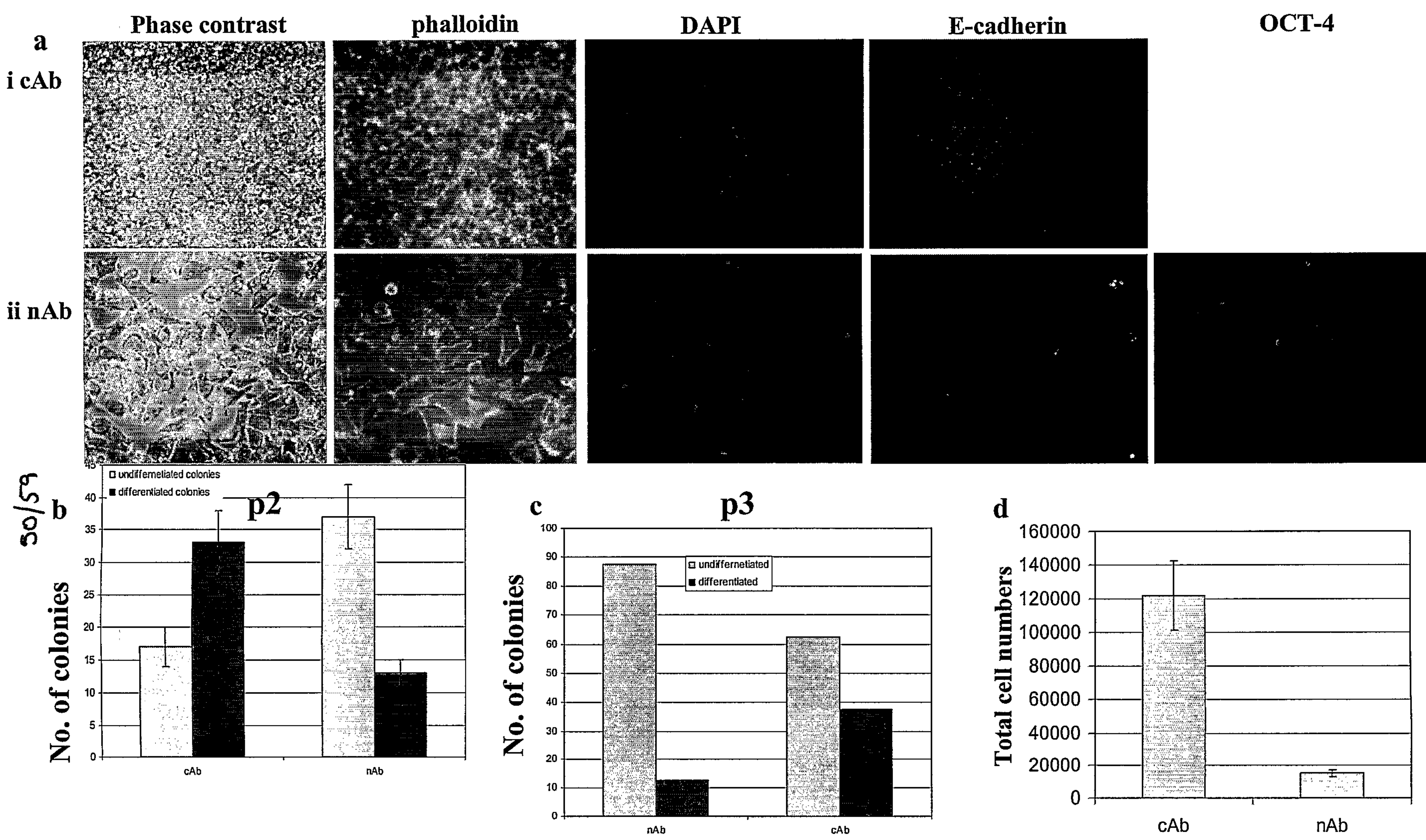

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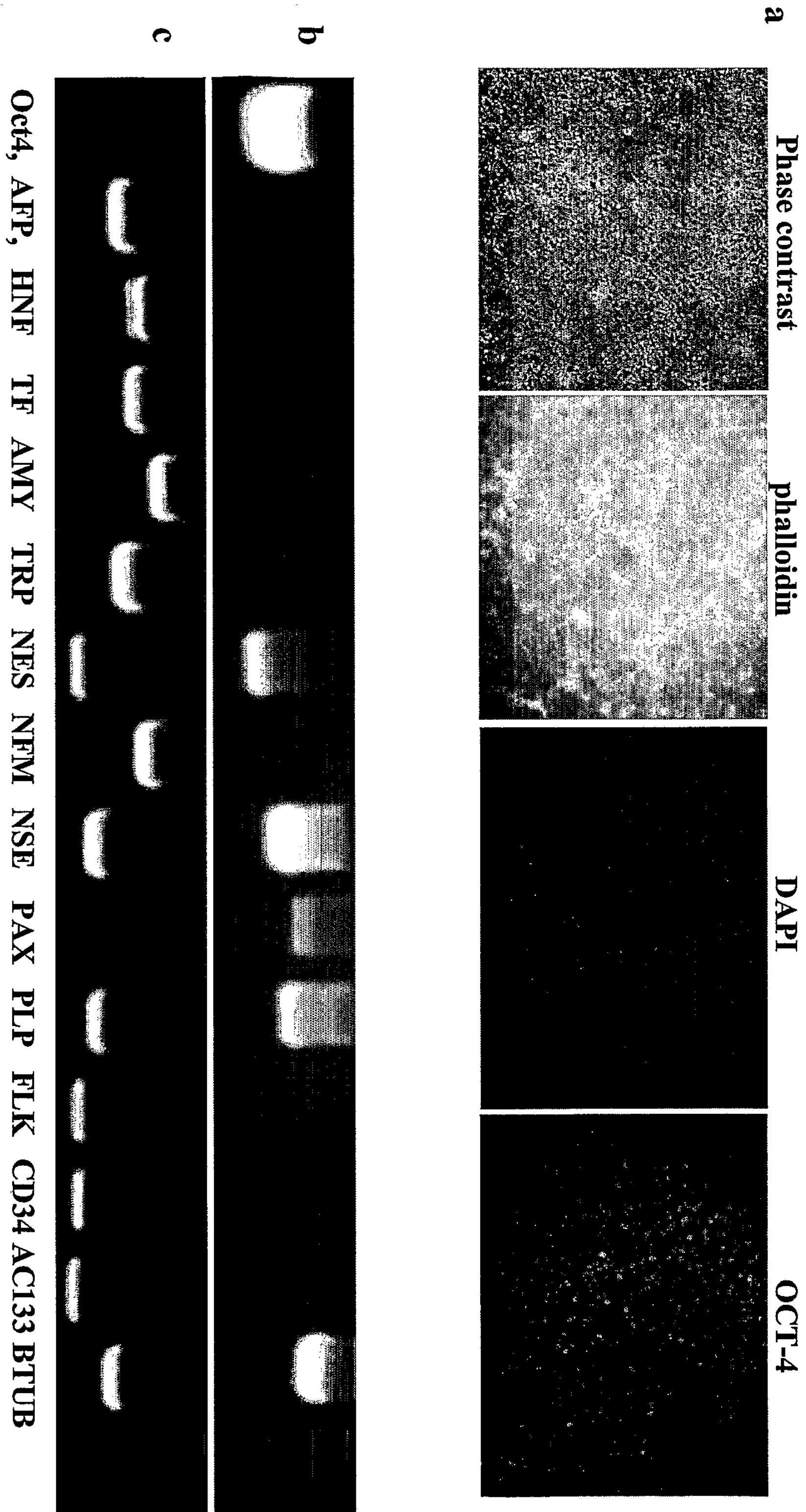

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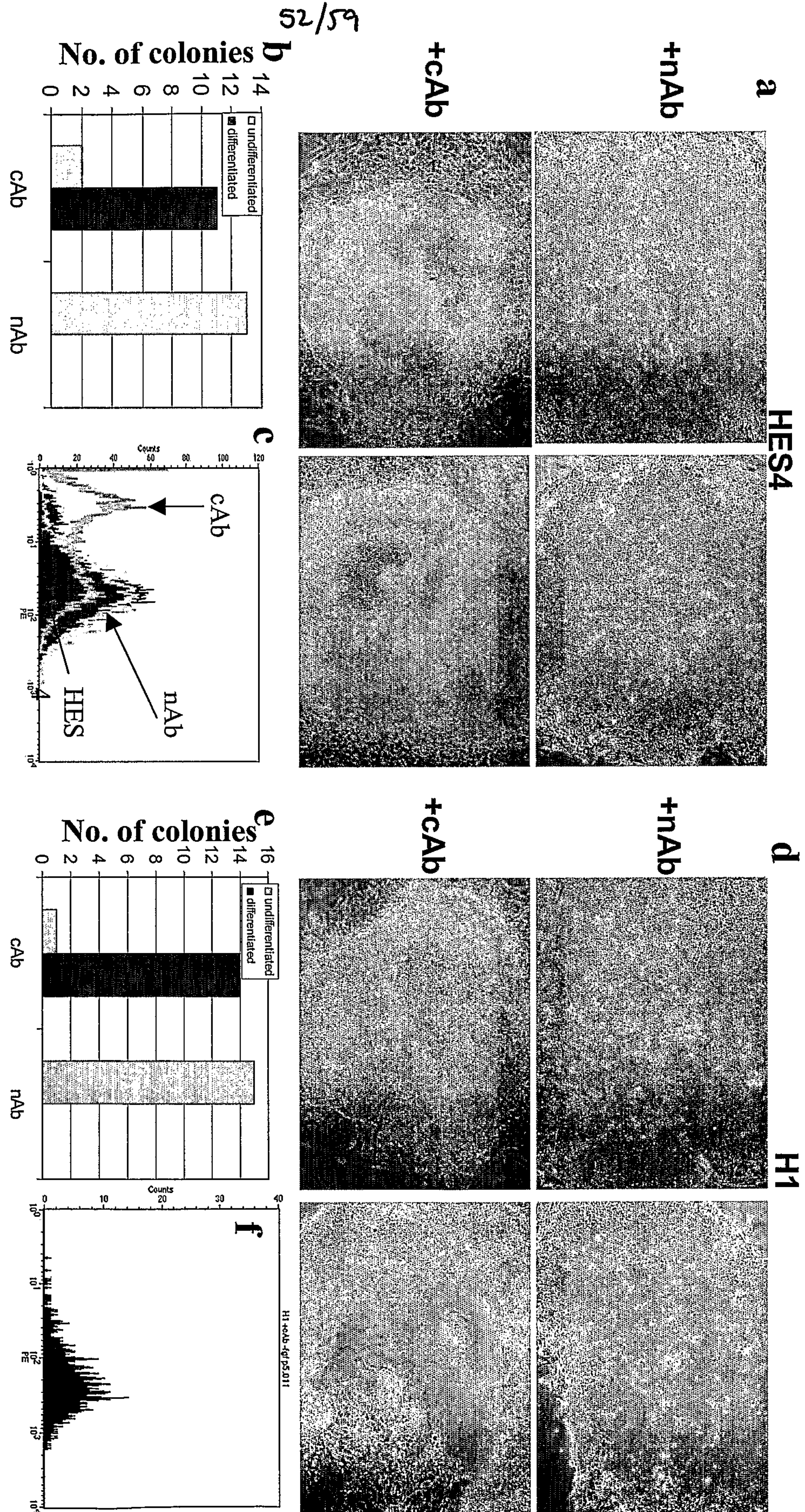

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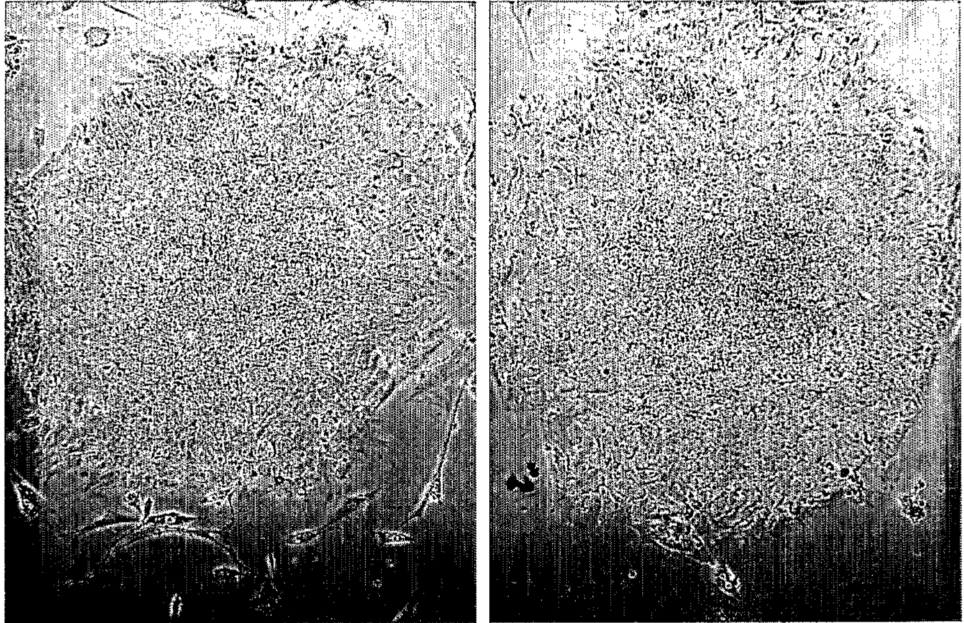


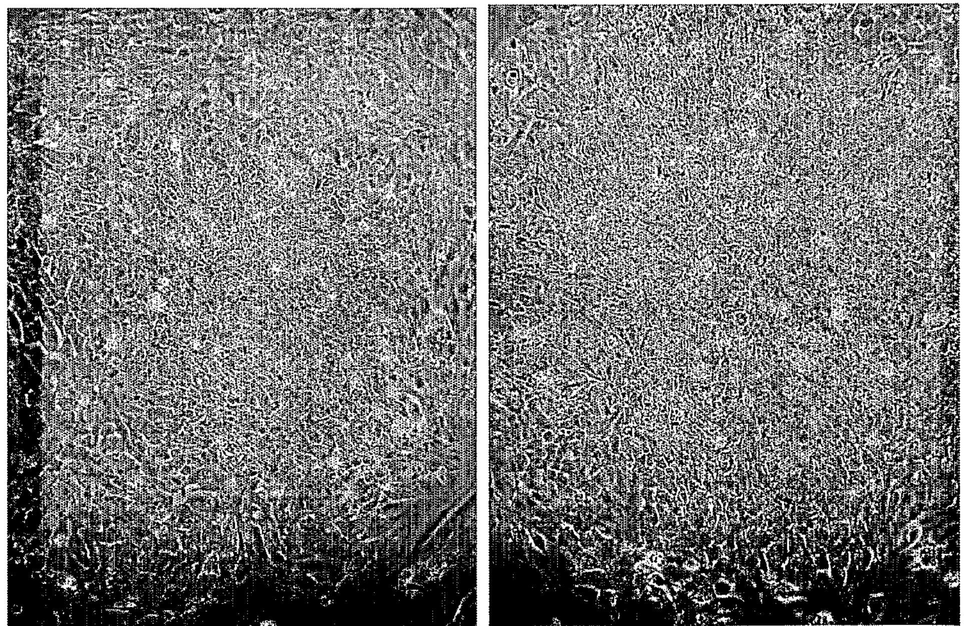
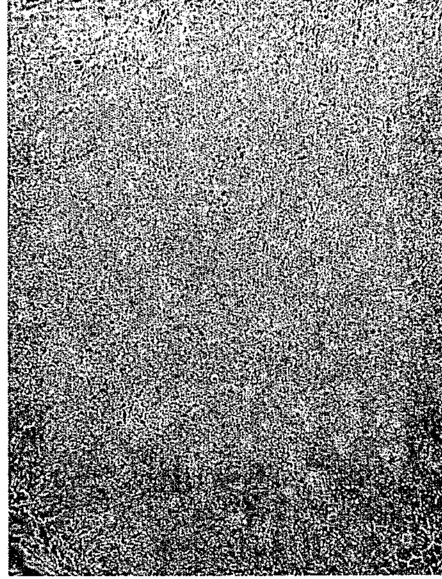
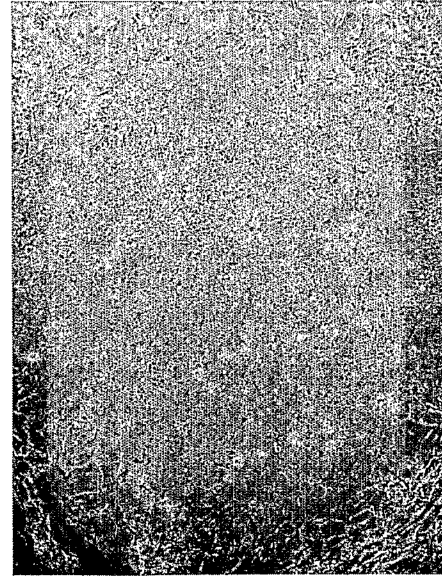


Figure 27.

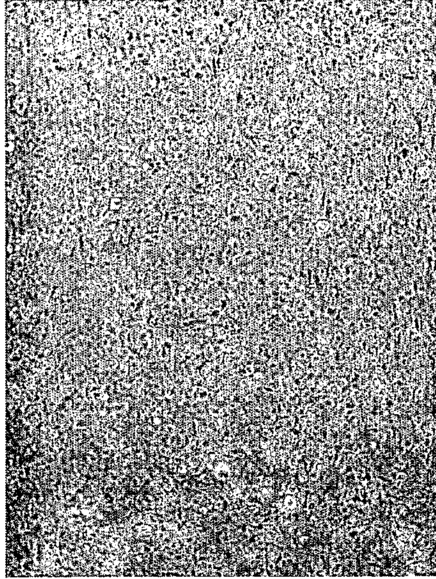
HES4

x100





x200



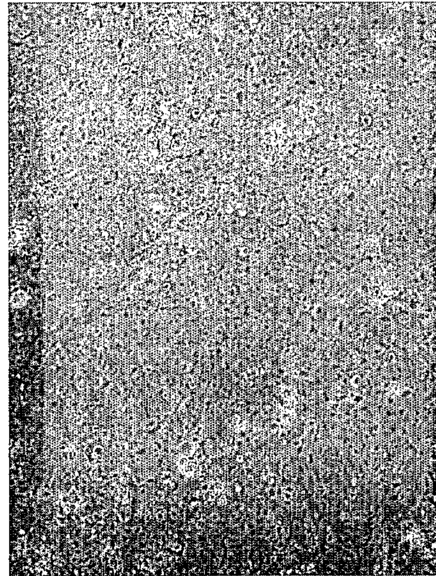
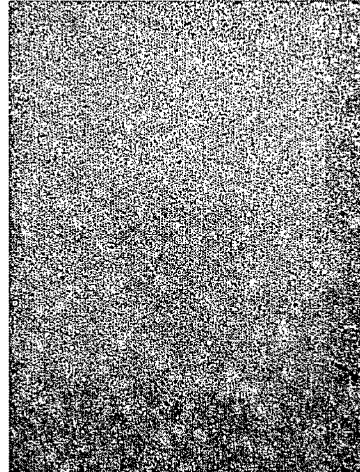
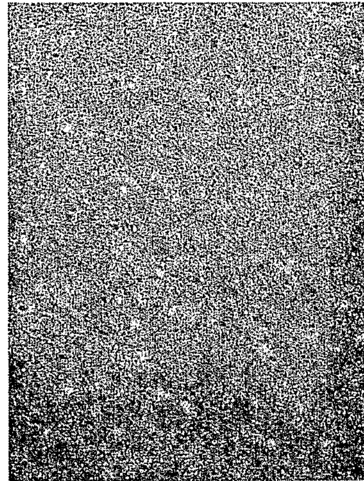


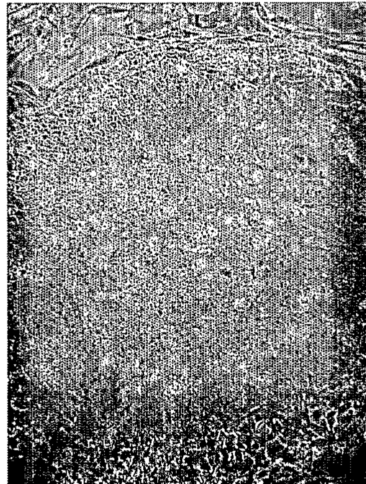
Figure 28.

H1

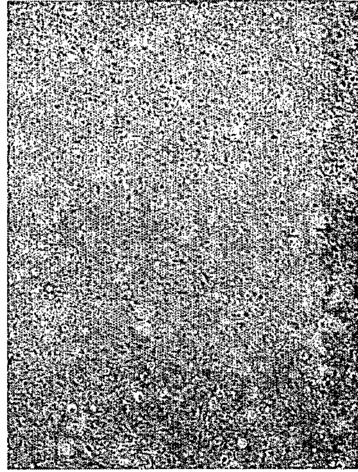
x100

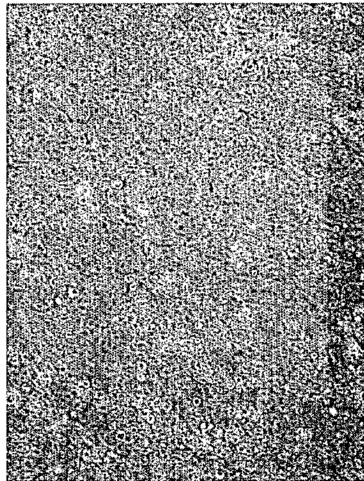


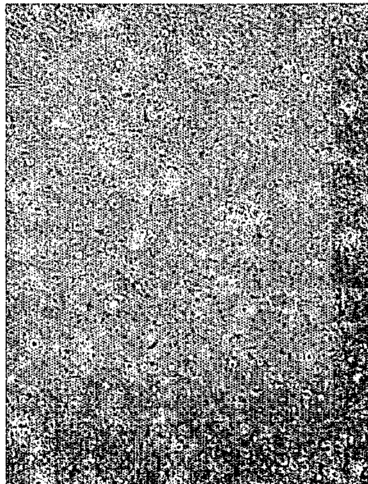




x200







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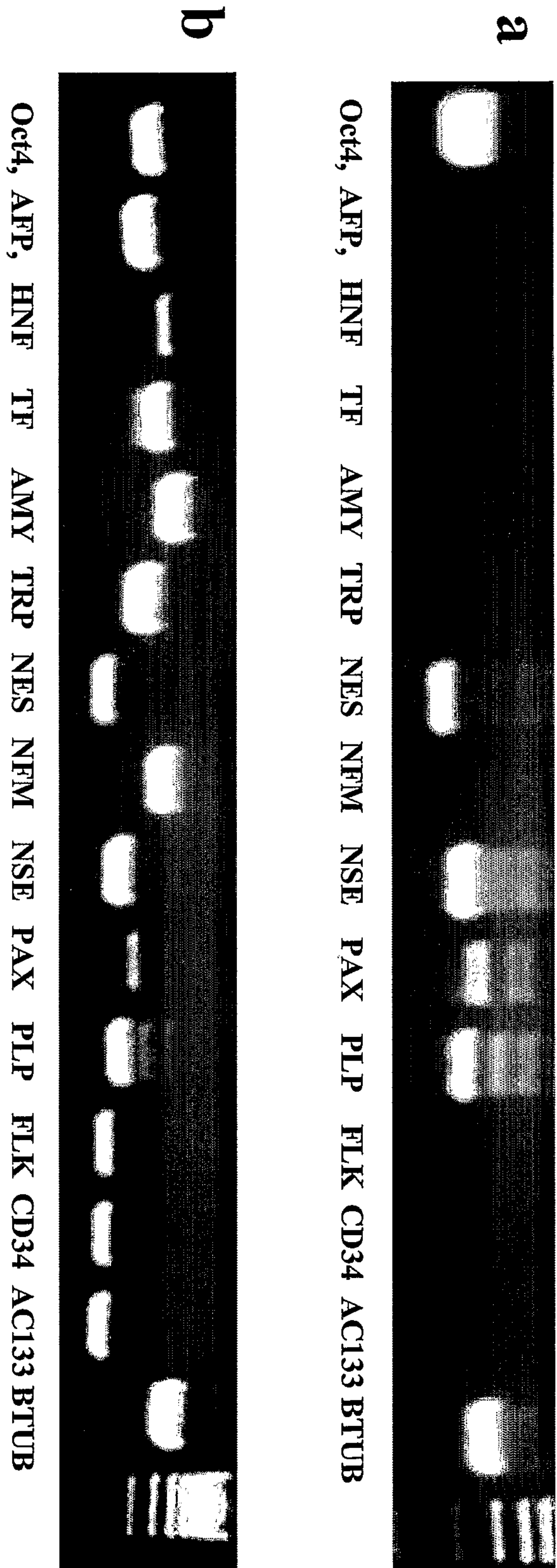

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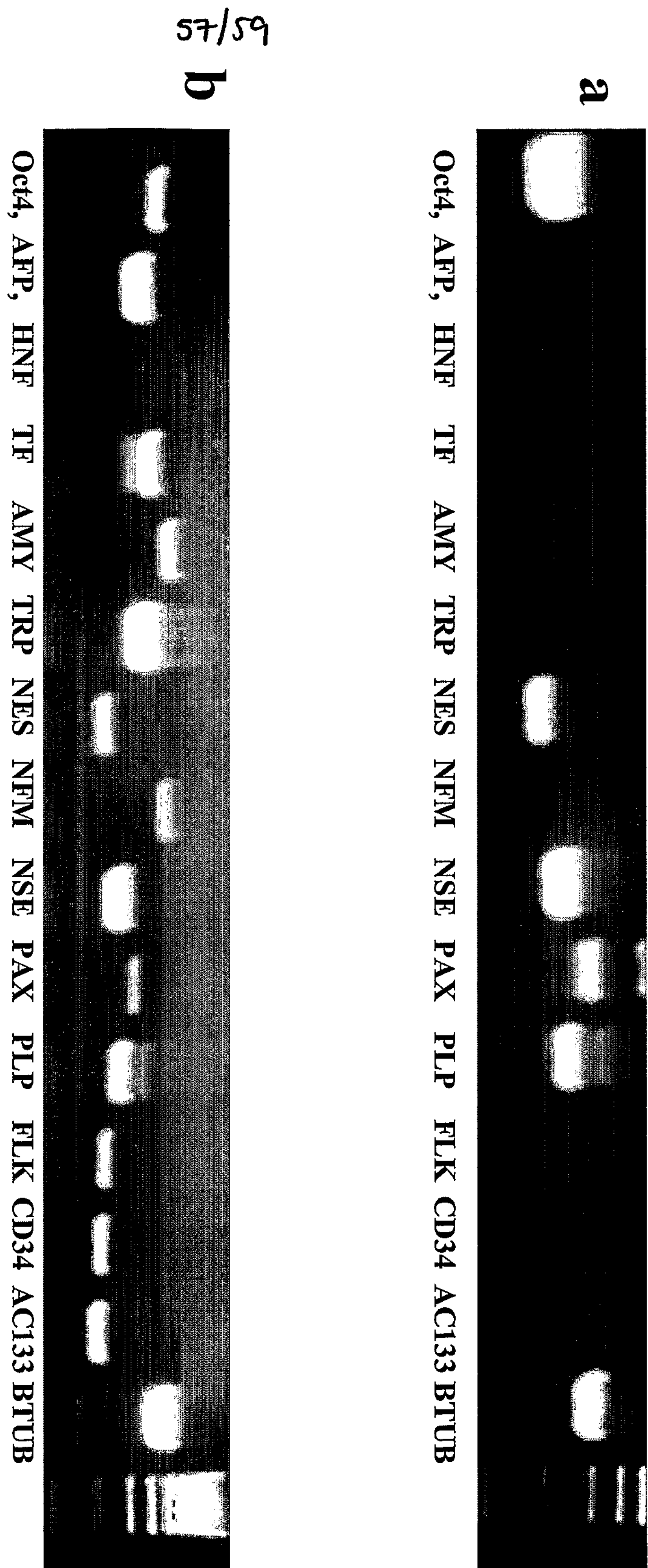

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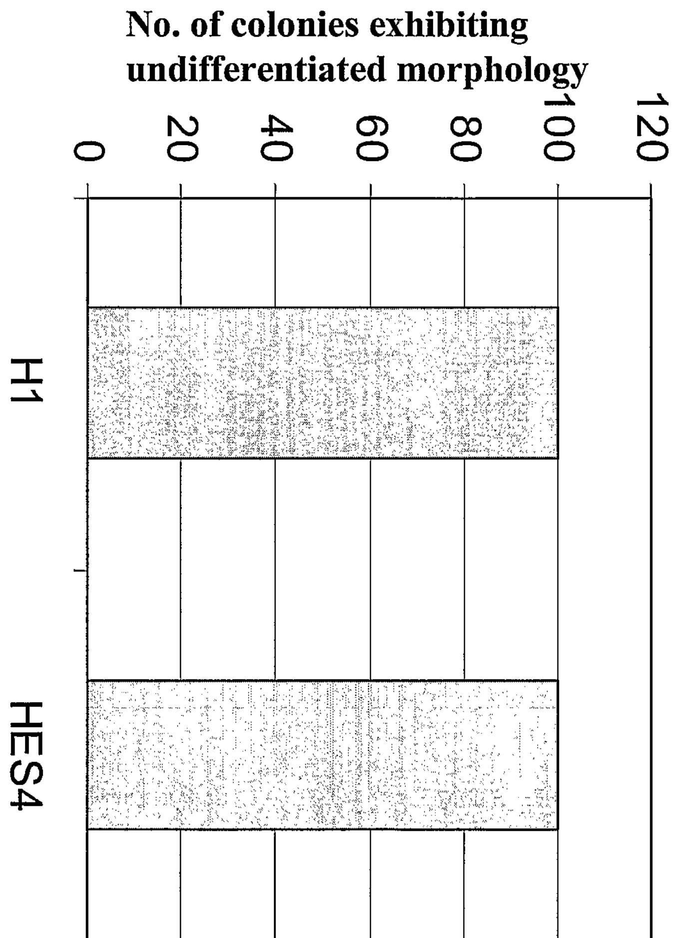

Figure 32.

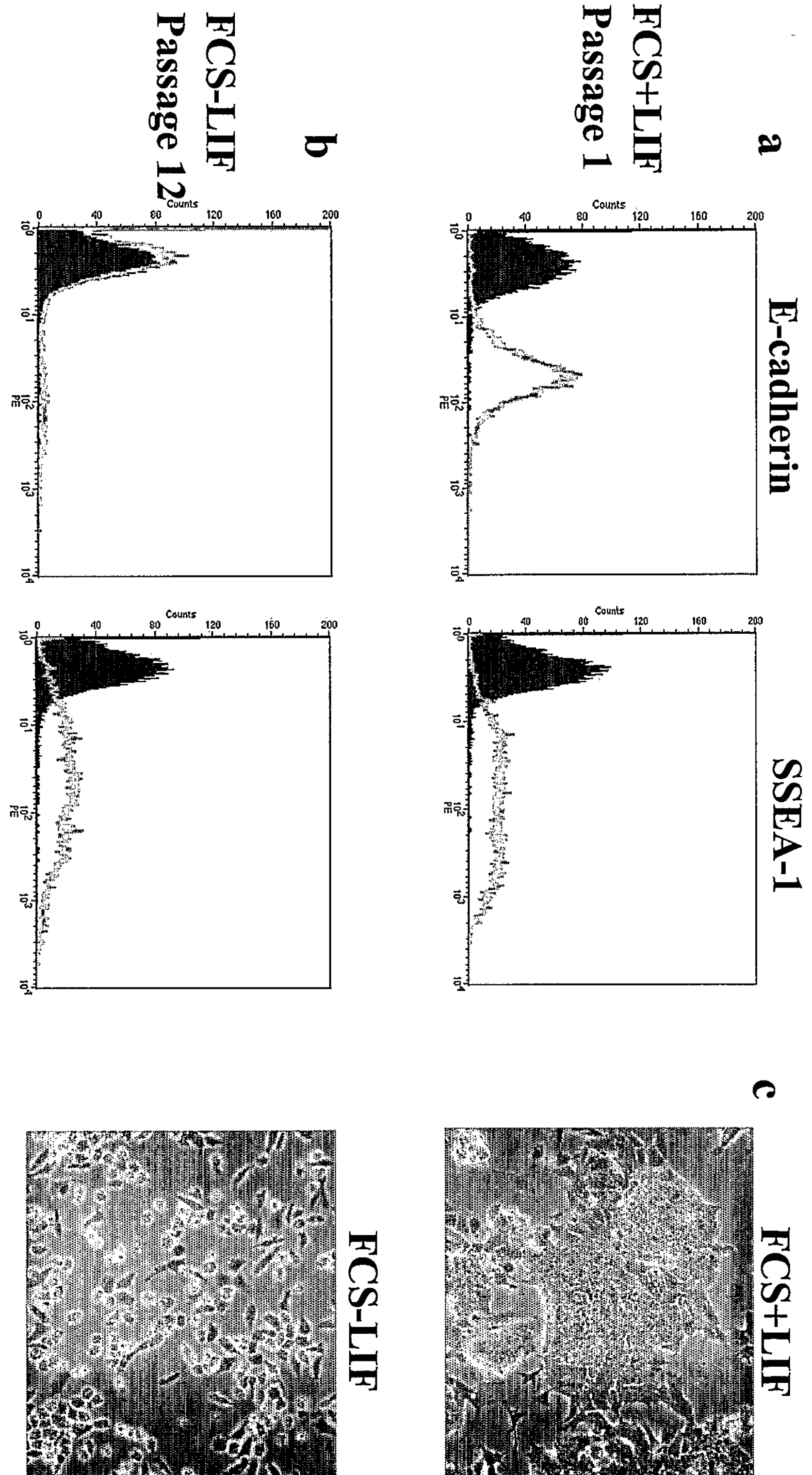

Figure 33.