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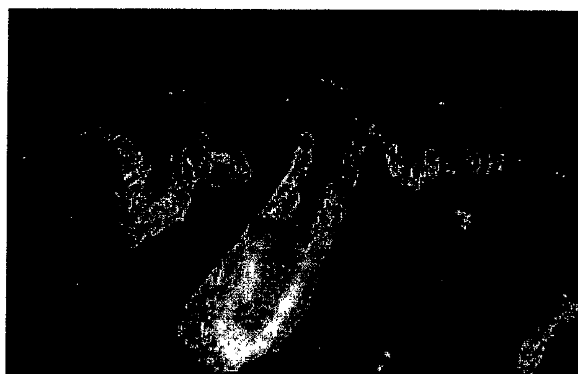
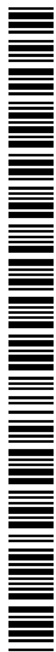


(57) Abstract: The invention provides methods for isolating and identifying epidermal stem cells, and uses thereof. Methods for enriching a population of cells for epidermal stem cells are provided. The methods make use of the MCSP protein. An epidermal stem cell specific promoter and an epidermal stem cell specific delivery vehicle and uses thereof are also described.



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STEM CELLS

The present invention relates to methods of identifying and isolating epidermal stem cells, and uses of isolated epidermal stem cells.

Epidermal stem cells are a sub-population of cells in the germinative/basal layer which are distinguished by their slow-cycling, quiescent nature *in vivo*, greater overall proliferative potential, and unlimited capacity for self-renewal (Turksen and Troy (1998) *Biochem. Cell Biol.* 76: 889-898). These cells are thought to represent between only 1 and 10% of the basal layer of the epidermis (Li *et al* (1998) PNAS **95**:3902-3907). The cell surface antigenic phenotype of epidermal stem cells however, is relatively poorly defined and little is known about these cells largely due to the absence of molecular markers that distinguish them from other proliferative cells within the basal layer. Previous attempts to identify cell surface components unique to epidermal stem cells have focussed on the observation that cells with high levels of integrin β1 ("β1 bright") are known to be capable of generating an epithelial sheet when grafted onto mice, suggesting that this fraction of the basal layer contains epidermal stem cells (Jones, PH, Harper, S, and Watt, FM (1995) *Cell* **80**:83-93). However, as ~ 40% of the basal layer in human foreskin exhibits high levels of β1 integrin *in vivo* (Jones *et al* (1995) *Cell* **80**:83-90), it is highly likely that basal keratinocytes exhibiting high levels of β1 integrin *in vivo* contain both the stem cell population and a significant number of transit amplifying (TA) cells, the progeny of the stem cells which have a limited proliferative capacity. Some researchers have shown that a cell preparation can be enriched for stem cells by rapid adhesion to a variety of substances such as collagen types I and IV, fibronectin and vitronectin, suggesting that epidermal stem cells are 'stickier' than other basal cells. Other attempts to

enrich cell populations for epidermal stem cells have focussed on the quiescent nature of these cells, and used 5-FU or hyperthermic treatment to kill rapidly proliferating cells. More recent purification methods have sought to isolate cells expressing high levels of α6 integrin with concomitant low expression levels of the proliferation-associated cell surface component detected by the monoclonal antibody 10G7 (Kaur, P *et al* (1997) *J. Invest. Dermatol.* **109**:194-199). However, the cells isolated on this basis are only enriched for epidermal stem cells, where not all the isolated cells show an extensive proliferation. Furthermore, it is known that many factors can modify the effectiveness by which a given antibody binds to cell surface integrin molecules such as α6 integrin.

Proteoglycans (CSPG), are glycoprotein molecules that are highly regulated during development. Biochemical studies have established that keratinocytes synthesise a number of proteoglycans with heparan sulfate, chondroitin sulfate and dermatan sulfate glucosaminoglycan side chains. Comparison of the profile of these proteoglycans has revealed differences between proliferating and differentiating keratinocytes and the development of antibodies to specific proteoglycans has demonstrated differences in the distribution of these molecules within normal skin. For example epican, a heparan/chondroitin sulfate proteoglycan is expressed on the surface of basal and suprabasal keratinocytes and syndecan-1 is expressed in all epidermal layers except for the most superficial terminally differentiated cells.

Although the precise function of these molecules is still not clear there is increasing evidence that they play an important role in the regulation of epithelial-mesenchymal interactions and in the control of differentiation. Proteoglycan expression is developmentally regulated and a number of

studies have shown tightly regulated expression of proteoglycans such as versican in the mesenchyme of developing skin appendages. Transfection of the epican core protein cDNA, which is expressed in differentiating keratinocytes, into fibroblasts induces a hyaluron dependent self-aggregating phenotype and the transfected cells acquire the capacity to adhere to keratinocytes. This observation and the altered expression of other proteoglycans during wound healing has led to speculation that these molecules are also important in regulating adhesion during epidermal differentiation.

In a previous study using melanoma cell lines as immunogens a monoclonal antibody (LHM2) was generated which recognises a high molecular weight (>200kD) proteoglycan that is differentially expressed in melanoma cells (Kupsch *et al* (1995) *Melanoma Res.* 5:403-411). Immunoprecipitation studies suggest that the proteoglycan recognised by this antibody is the melanoma associated high molecular weight chondroitin sulfate (MCSP). The core protein of MCSP has been cloned (WO 97/13855) and northern blot analysis indicates that this is highly expressed in more than 90% of human melanoma tissues but is not found in other human cancer cell lines and in a range of human adult and fetal tissues. MCSP is a highly immunogenic N-linked glycoprotein of 250kDa with a proteoglycan component of more than 450kDa. MCSP is reported to have a functional role in growth control, adhesion, cell-substratum interactions and cell-cell contacts. To date, the proteoglycan has been used as a target for radioimmunoimaging and anti-idiotype based melanoma therapies.

Surprisingly, whilst characterising LHM2, we have now observed that this antibody stained human skin selectively which suggested that either the antibody was cross-reacting with a related epitope or MCSP is expressed in

keratinocytes. We now show that MCSP is expressed in human skin and that our antibody recognises keratinocytes in the outer root sheath of the hair follicle and a subset of interfollicular basal keratinocytes. To better define these keratinocytes we have delineated the relationship between MCSP expression and keratin 15 expression. The distribution of the MCSP positive keratinocytes at the tips of the dermal papilla corresponds with that described previously for putative epidermal stem cells within the β1-integrin bright population and indicates that MCSP expression may either be a stem cell marker or a marker of early exit from the stem cell population. Either way, we believe that MCSP is useful as a marker in the identification, isolation and enrichment of epidermal stem cells. The loss of expression of this marker in culture *in vitro* and its re-expression in skin grafted onto immune deficient mice suggests that maintenance of expression of MCSP is dependent on continuing mesenchymal signalling.

Stem cells are thought to be the only cells in the epidermis that have a lifetime that matches the lifetime of the organism. Thus, they would be the cell of choice for long term expression of an engineered gene. Lack of suitable stem cell markers has previously made isolation of stem cells an elusive goal, as it has not been possible to demonstrate the homogeneity of a population of isolated stem cells, or even to conclusively demonstrate the stem cell nature of isolated cells. Our demonstration that MCSP is expressed on the surface of pre-differentiated epidermal cells suggests that it is such a marker.

The ability to identify cells as epidermal stem cells allows selective study and research of this cell type. Furthermore, antibodies specific to MCSP allow identification and selective isolation of epidermal stem cells, enabling their manipulation and use in therapy.

Although it is already possible to culture stem cells *in vitro*, there exists a need for optimal culture conditions to be determined which would allow more long term maintenance in culture and growth of the stem cells outside 5 the donor body without any loss of the stem cell characteristics, particularly to assist in the expansion of a stem cell population for use in therapy. Use of MCSP as a marker of the undifferentiated state provides a means for assessing the efficacy of various culture conditions in achieving this.

10 The sequence of a cDNA encoding MCSP is publicly available (for example, in WO 97/13855 and shown in Figure 8). However, known genomic clones containing the human MCSP coding sequence lack the first 87 nucleotides of the coding region, starting with the start ATG (ie, the 87 most 5' nucleotides of the coding sequence), suggesting that the promoter 15 region is also missing (GenBank Accession Nos AC010725, AC005630, AC010724, AC012064, AC006328 and AC023274). Hence, the MCSP promoter region has not previously been described. We have now obtained and sequenced the promoter region for the MCSP coding region. This is believed to be located in the around 1.8kb of sequence directly 5' to the first 20 nucleotide of the MCSP coding sequence (shown in Figure 7). Database analysis reveals that this sequence is present in non-contiguous fragments in two GenBank database entries; AC019294 and AC068368. Specifically, the corresponding sequence co-ordinates between the sequence in Figure 7 and AC019294 respectively are (1, 64990), (974, 65957), (1198, 147915), 25 (1730, 148450), (1728, 149750) and (1767, 147615). There is no indication given in either of these database entries that the sequence contained therein is associated with the MCSP gene.

One implication of our work is that the MCSP promoter can be used to selectively express a protein other than MCSP which is useful in the isolation, observation or sorting of epidermal stem cells. The promoter is also believed to be useful in directing expression of therapeutic genes for gene therapy.

A first aspect of the invention provides a method of isolating an epidermal stem cell from a sample containing epidermal stem cells, the method comprising selecting cells from the sample which express a polypeptide whose expression is driven by the MCSP promoter.

By "MCSP" we include the polypeptide whose amino acid sequence is given in Pluschke *et al* (1996) *Proc. Natl. Acad. Sci. USA* 93:9710-9715 and which is expressed by cells in the germinative/basal layer of skin, or a homologue thereof in other species such as rat, pig, sheep, cow, mouse etc. Preferably the MSCP from species other than human has at least 60% identity to the amino acid sequence of the human polypeptide, more preferably it has 80% identity, 95% identity and it is particularly preferred if the MCSP from a species other than human has more than 99% identity with human MCSP and is expressed by cells in the germinative/basal layer of skin. Per cent homology can be determined by, for example, the GAP program of the University of Wisconsin Genetic Computer Group. For example, the rat homologue of human MCSP, NG2, displays 82.9% amino acid sequence identity and 86% similarity to human MCSP. An alignment of the human and rat amino acid sequences is given in Figure 8 and an alignment of the human and rat nucleotide sequences is given in Figure 9.

By "MCSP promoter" we mean the promoter which drives expression of the MCSP polypeptide in an epidermal stem cell, or a variant thereof. Thus, the

MCSP promoter includes homologues of the human MCSP promoter present in species other than human, such as rat, pig, sheep, cow etc which are capable of driving transcription, and which are transcriptionally active in cells which naturally express MCSP in those species. The rat homologue of human MCSP is NG2 (GenBank Accession No X56541). A variant of a natural MCSP promoter is also included in the definition of MCSP promoter provided that the variant is one which retains its ability to direct expression in a cell-specific or differentiation state-specific manner essentially the same as natural MCSP promoter. Thus, an MCSP promoter may differ from a natural MCSP promoter by substitutions, insertions, deletions and the like.

It is preferred if the polypeptide whose expression is driven by the MCSP promoter is a detectable polypeptide. By "detectable polypeptide" we include any polypeptide which can be detected. It is particularly preferred that the detectable polypeptide is one which can bind to a partner such as an antibody or has some other physical or chemical attribute, such as being coloured or fluorescent, which allows it to be detected. The detectable polypeptide may conveniently have enzymatic activity which is detectable including an enzymatic activity conferring resistance to cytotoxic drugs or antibiotics. An example of a polypeptide conveying resistance to a cytotoxic drug is the Multidrug Resistance-1 protein (MDR1) as described in Abonour *et al* (2000) *Nature Medicine* 6: 652-658.

In a preferred embodiment of the first aspect of the invention, the polypeptide whose expression is driven by the MCSP promoter is MCSP. This includes the natural situation.

Alternatively, in certain embodiments of the invention which make use of the expression of readily detectable polypeptides which are expressed from the MCSP promoter by recombinant means, the polypeptide whose expression is driven by the MCSP promoter may be, for example, a green 5 fluorescent protein (GFP) or variants thereof which fluoresce yellow or blue as are well known to those skilled in the art. The moiety may be an immunogenic tag, for example a Myc tag. A particularly preferred example of a detectable polypeptide is GFP. In this embodiment, cells in the sample containing epidermal stem cells are typically transfected with a genetic 10 construct in which the expression of detectable polypeptide is expressed from the MCSP promoter and expression of the detectable polypeptide occurs in the epidermal stem cells since they are able to allow transcription from the MCSP promoter.

15 A second aspect of the invention provides a method of enriching for epidermal stem cells in a sample containing epidermal stem cells, the method comprising enriching for cells which express a polypeptide whose expression is driven by the MCSP promoter.

20 By "enrichment" we mean that the proportion of cells in the sample which express a polypeptide whose expression is driven by the MCSP promoter is increased. In a preferred embodiment the proportion of cells which express MCSP is increased. Preferably, a population of cells which is enriched for epidermal stem cells is one where the proportion of cells which express 25 MCSP (MCSP+ve) but do not express keratin 15 (K15-ve) is increased. This is believed to reflect an increase in the proportion of epidermal stem cells in the population.

Preferably the sample containing epidermal stem cells is enriched for epidermal stem cells by at least a factor of 1.2 or 1.5 or 2, more preferably a factor of 3 or 4, and still more preferably by a factor of 6 or 8. It is further preferred if the stem cells are enriched in the sample by a factor of 10.

5

Typically, the sample containing epidermal stem cells is enriched for cells which express a polypeptide (such as MCSP) whose expression is driven by the MCSP promoter by at least a factor of 1.2 or 1.5 or 2 or 3 or 4 or 8 or 10 or 20.

10

According to further embodiments of the first and second aspects of the invention, the method of isolating epidermal stem cells or enriching a sample containing epidermal stem cells for said cells comprises the steps of i) obtaining a sample containing epidermal stem cells; ii) contacting the

15 sample with an agent which selectively binds the polypeptide whose expression is driven by the MCSP promoter; iii) allowing the cells to bind to the agent; iv) removing unbound cells; v) releasing bound cells; and vi) collecting the released cells.

20 In a preferred embodiment, step i) is followed (ie, before step ii) by transfection of the cells with a construct comprising a sequence encoding a polypeptide whose expression is controlled by the MCSP promoter. It will be appreciated that this additional step may not be required where the polypeptide is MCSP which is expressed from chromosomal DNA of the

25 cell.

The agent which selectively binds the polypeptide may be any suitable binding moiety and, typically, is an antibody as described in more detail below. In a preferred embodiment, the polypeptide whose expression is

driven by the MCSP promoter is MCSP and the binding agent is an anti-MCSP antibody.

Although, it is preferred that an agent which selectively binds the polypeptide is used, other methods of selecting cells which express a polypeptide whose expression is driven by the MCSP promoter may be employed. Thus, if the polypeptide itself is coloured or fluorescent spectrophotometric or spectrofluorimetric (eg FACS) methods may be used in the isolation or enrichment process.

Thus, in additional embodiments of the first and second aspects of the invention, the method of isolating epidermal stem cells or enriching a sample containing epidermal stem cells for said cells comprises the steps of i) obtaining a sample containing epidermal stem cells; ii) transfecting or transducing cells in said sample with a genetic construct encoding a detectable polypeptide whose expression is driven by the MCSP promoter; iii) selecting for the detectable polypeptide; and iv) collecting those cells selected for expressing the detectable polypeptide.

The detectable polypeptide may be any detectable polypeptide. Preferably it is a fluorescent polypeptide or one which provides resistance to an exogenously added chemical such as a drug (a "resistance marker"). The resistance provided by such markers include resistance to cytotoxic or cytostatic effects of the chemical or drug.

Typically, where the detectable polypeptide whose expression is driven by the MCSP promoter is a fluorescent polypeptide, the selection is achieved by fluorescence-activated cell sorting (FACS). An advantage of using a fluorescent marker, such as green fluorescent protein or any variants thereof

with emissions at different wavelengths, is that it overcomes any potential difficulty of working with a cell surface protein that might be unstable under certain conditions. Use of this method in neuronal progenitor cells is described in Singh Roy *et al Nature Medicine* (2000) 6, 271-277, and the
5 appropriate methodology is incorporated herein by reference.

Where the detectable polypeptide is a resistance marker, methods of selection are well known in the art. Conveniently, the chemical or drug is a cytotoxic one, and selection may be achieved simply by culturing the cells
10 over a period of time, such as days or weeks. Those cells expressing the resistance marker will be able to survive, or even grow in culture conditions where the appropriate chemical or drug is present. Preferably the chemical or drug used is one which kills non-resistant cells in less than 2 days and more preferably within 24 hours.
15

Suitable methods of transfection of a construct into cells are known in the art. Examples of suitable methods are described in Singh Roy *et al* (2000) *Nature Medicine* 6: 271-277 and Dick (2000) *Nature Medicine* 6: 624-626, incorporated herein by reference.
20

As described above, the cell sample may be any sample of cells which contains epidermal stem cells. Where the sample is a sample of skin cells, the cells may require disaggregation beforehand.

25 A promoter which is active selectively in epidermal stem cells is the MCSP promoter, as described in more detail below.

The sample containing epidermal stem cells may be enriched for epidermal stem cells prior to selection for cells expressing a polypeptide whose

expression is driven by the MCSP promoter. Enrichment can be performed by an equivalent series of steps wherein the sample is contacted with an agent which selectively binds a polypeptide such as β1 integrin or α6 integrin instead of MCSP. Cells which bind the said polypeptide are retained, and those cells which do not are rejected. Enrichment of epidermal stem cells on the basis of β_1 integrin or α_6 integrin expression is described in Jones and Watt (1993) *Cell* 73: 713-724 and WO 99/47644.

An additional or alternative means of enriching the sample containing epidermal stem cells for epidermal stem cells comprises a similar series of steps wherein the sample containing epidermal stem cells is contacted with an agent which selectively binds a polypeptide associated with cell proliferation in keratinocytes, the unbound cells are collected and the bound cells are discarded. Preferably the polypeptide associated with cell proliferation is the transferrin receptor (WO 99/47644).

An alternative or additional means of enriching the sample containing epidermal stem cells includes killing the rapidly proliferating cells present. Such cells can be selectively killed by treatment with an antimetabolite, such as 5-fluorouracil (5-FU), which kills metabolically active cells but spares epidermal stem cells which have a longer cell cycle. Such treatment is described in US 5,827,742.

More preferably, the sample containing epidermal stem cells are enriched for epidermal stem cells by selecting for those cells which attach to dishes coated with collagen type IV, collagen type VII, fibronectin, vitronectin, laminin or a combination of these, in as little as 5 minutes, preferably within 10 minutes and more preferably within 20 minutes. Cells which take longer to attach, for example, longer than 60 minutes, have a lower colony forming

efficiency. A suitable method of enriching for epidermal stem cells is described in Jensen *et al* (1999) *Development* 126: 2409-2418 and in Bickenbach and Chism (1998) *Experimental Cell Research* 244: 184-195.

5 Conveniently, the sample containing epidermal stem cells are enriched for epidermal stem cells on the basis of relative size. Separation of cells on the basis of size can be achieved using either density gradient centrifugation, unit gravity sedimentation or by using a cell sorter. It is less preferred that the sample is enriched for epidermal stem cells on this basis due to potential 10 difficulties caused by inefficiencies in the required cell disaggregation to a single cell suspension and the small size differential between epidermal stem cells and transit-amplifying cells (which may be as small as 1-2 micrometres).

15 Although it is preferred that these putative epidermal stem cell enrichment methods known in the art are used to enrich the sample containing epidermal stem cells prior to selecting for cells which express a polypeptide whose expression is driven by the MCSP promoter, it will be appreciated that it may be useful to use them in conjunction with, or after, the steps 20 described for the first time herein which make use of the expression of a polypeptide driven by the MCSP promoter.

Isolation or enrichment of epidermal stem cells by the methods of the invention may be useful in providing a population of cells of standard 25 origin. Such populations may be useful in studying drug metabolism or studying cellular responses. Furthermore, stem cells are telomerase-expressing cells, and hence isolation of epidermal stem cells provides a cell population which is suitable for the study and examination of telomerase. Such uses are included in the scope of the invention.

A third aspect of the invention provides a method of identifying an epidermal stem cell in a sample containing epidermal stem cells, the method comprising determining which cells in the sample express a polypeptide whose expression is driven by the MCSP promoter.

As for the first and second aspects of the invention, polypeptides whose expression is driven by the MCSP promoter include MCSP, and readily detectable polypeptides such as the green fluorescent protein or variants thereof.

It will be appreciated that expression of a polypeptide whose expression is driven by the MCSP promoter can be determined by any suitable method depending on the properties of the polypeptide. Thus, if the polypeptide is coloured or fluorescent spectrophotometric or spectrofluorimetric methods may be used. For most polypeptides, including MSCP, determination may involve the use of a binding moiety which selectively binds the polypeptide. Suitable binding moieties include antibodies, as described in more detail below.

Preferably, the determination of expression, of the polypeptide, or isolation of or enrichment for cells expressing the polypeptide is by FACS analysis using an antibody capable of specifically binding the expressed polypeptide.

Alternative methods for determining expression of the polypeptide include *in situ* immunofluorescence using fluorescently-labelled antibodies specific for the polypeptide whose expression is driven by the MCSP promoter and slide-based methods, for example where cells are mounted on a slide and the slide is incubated with primary antibody specific for the polypeptide

whose expression is driven by the MCSP promoter, reacted with a biotinylated secondary antibody, bound with an enzyme conjugated to streptavidin and binding is detected using a chromogen substrate.

5 Typically, there will be more than one epidermal stem cell present in a sample, and in this case expression of the polypeptide whose expression is driven by the MCSP promoter may alternatively be analysed by biochemical techniques well known in the art. These include for example, taking a proportion of the epidermal stem cell-containing sample, preparing
10 a protein extract from that proportion, separating the extract by SDS-PAGE and western blotting using an antibody capable of selectively binding to the polypeptide whose expression is driven by the MCSP promoter. In such an approach however, the cells used in the identification are destroyed but the remaining cells may be used further.

15

In preferred embodiments, the isolation, enrichment or identification methods employ a binding agent capable of selectively binding the polypeptide whose expression is driven by the MCSP promoter. Preferably the binding agent is an antibody.

20

Where the polypeptide expressed by the MCSP promoter is the MCSP polypeptide, a suitable antibody is LHM2 as described in Kupsch *et al* (1995) *Melanoma Res.* 5:403-411, although other antibodies which specifically bind MCSP are known in the art. Alternatively, the agent
25 which selectively binds the MCSP polypeptide may be a ligand for MCSP, or a lectin. An example of a ligand which binds MCSP is fibronectin. Hence, fibronectin, or a suitable fragment thereof which selectively binds MCSP, may be used in a rapid adhesion assay to select for MCSP-expressing cells. The interaction of chondroitin sulphate proteoglyans with

the Hep III domain of fibronectin is described in Moyano *et al* (1999) *J. Biol. Chem.* 247: 135-142.

As noted above, human MCSP is a highly immunogenic N-linked glycoprotein with a large proteoglycan component. The antibodies which bind to MCSP may suitably bind the polypeptide component or the carbohydrate component or both.

Antibodies or binding agents which specifically bind other detectable polypeptides as outlined above are also known in the art. For example, antibodies to Myc tags are known in the art and are commercially available.

By "antibody" we include any molecule which binds to the polypeptide in an antibody-like fashion. In particular, we include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299. Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "*Monoclonal Hybridoma Antibodies: Techniques and Applications*", J G R Hurrell (CRC Press, 1982). Antibody-like molecules can also be made or selected using the well known phage display technology.

Epidermal stem cells may be isolated from, enriched in or identified in, any suitable source of cells. Typically, a suitable source of cells is normal skin. It is preferred if cells are from the skin epidermis and it is particularly preferred if the cells are from the germinative/basal layer of the skin epidermis. In an alternative preference, stem cells of the invention may be from the bulge area of the outer root sheath of the hair follicle (Taylor *et al* (2000) *Cell* 102: 451-461). It is less preferred if the cells are from ovary or ileum and even less preferred if the cells are from skin which may include melanoma cells.

The source of stem cells may be a source which has undergone de-differentiation. For example, skin cells may de-differentiate and gain expression of MCSP.

It will be appreciated that the cells which express MCSP are preferably from human skin epidermis, but may also be from the epidermis in other species such as rat, pig, mouse etc.

In a preferred embodiment of the first, second and third aspects of the invention, the MSCP promoter comprises the nucleotide sequence given in Figure 7.

It will be appreciated that only part or only a fragment of the sequence given in Figure 7 may be necessary to impart epidermal stem cell selective expression on a coding sequence operatively linked to it. Determination of a minimal motif necessary for epidermal stem cell selective expression can be done by routine experimentation.

In a further preferred embodiment of the first, second and third aspects of the invention, selection, enrichment or identification of the epidermal stem cell is by fluorescence-activated cell sorting (FACS). FACS is dependent on a marker or antibody conjugated to a fluorescent marker, such as fluorescein, to label specific cells. In this case, it is preferred if the binding agent is an antibody to the polypeptide whose expression is driven by the MCSP promoter. It is more preferred if the polypeptide is MCSP and the antibody is capable of specifically binding to MCSP. Conjugation of the antibody to a fluorescent moiety can be achieved by directly by coupling methods known in the art, or conveniently, by binding the antibody via its constant region to a second antibody which is itself conjugated to a fluorescent moiety and which binds the constant region of the first antibody.

In a further embodiment of the first, second and third aspects of the invention, the binding agent capable of selectively binding the polypeptide whose expression is driven by the MCSP promoter is immobilised. Suitable immobile supports for immobilised binding agent include plastic surfaces, Sepharose™ beads and magnetic beads such as Dynabeads™. Methods of attaching or conjugating binding agents, such as antibodies, to immobile supports are well known in the art.

In still further embodiments, cells selected or enriched or identified according to the method of the first, second and third aspects of the invention may be additionally selected or enriched or identified according to a profile of other components expressed or not expressed on their cell surface. Epidermal stem cells have previously been termed "β1 bright" due to a high level of expression of β1 integrin, and therefore a high expression of β1 integrin may be an additional characteristic of these cells. It is preferred if the epidermal stem cells are additionally selected for, enriched

for, or identified as having high levels of cell surface expression of β1 integrin. This selection, enrichment or identification may be before, during or after selection, enrichment or identification using determination of expression of a polypeptide driven by the MCSP promoter.

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In a similar manner, it is preferred if the epidermal stem cells are selected for, enriched for or identified as having high levels of expression of cell surface α6 integrin in addition to expression of a polypeptide whose expression is driven by the MCSP promoter, or for expression of MCSP.

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By "high levels of expression" we include that the level of expression is sufficient to produce a bright image by immunofluorescence, as is understood in the art (for example, as described in Jensen *et al* (1999) *Development* 126: 2409-2418 and in Jones & Watt (1983) *Cell* 73: 713-24,

15 both of which are incorporated herein by reference). It is preferred if cells displaying a "dim" level of β1 integrin expression are rejected from the selection.

The terms "bright" and "dim" immunofluorescence are known in the art.

20 The judgement of whether fluorescence is either "bright" or "dim" is made objectively, preferably by isolation using FACS of only those cells with a given level of immunofluorescence from the binding of fluorescein conjugated antibodies.

25 It is also preferred if the cells are selected for, enriched for or identified as, not substantially expressing a proliferation marker in keratinocytes such as the transferrin receptor. By "not substantially expressing" we mean that compared to the level of expression detected in differentiated basal keratinocytes, the expression in epidermal stem cells may be detected in

principle but is greatly reduced in comparison to differentiated basal keratinocytes.

Such enrichments as described above may be performed either before or after selection or enrichment for the polypeptide whose expression is driven by the MCSP promoter. It is particularly preferred if any additional selection, for example, for cells with, high expression of β1 integrin is made before the selection or enrichment for a polypeptide whose expression is driven by the MCSP promoter or for cells expressing MCSP. Thus, typically, the sample used for further selection, enrichment or identification has been enriched for epidermal stem cells using any one or more of the putative prior art enrichment methods.

Antibody which selectively binds β_1 integrin may be obtained from Abcam Ltd, Cambridge, UK under product code ab4508-5355-5058.

Typically, where high expression of β_1 integrin is used to select for cells prior to selection for MCSP expression, the 5% of cells with the highest level of β_1 integrin expression are selected.

A fourth aspect of the invention provides a method of isolating an epidermal stem cell from a sample containing epidermal stem cells, the method comprising selecting cells from the sample which express MCSP.

Further embodiments of the fourth aspect are as described for the first aspect of the invention wherein the polypeptide is MCSP.

A fifth aspect of the invention provides a method of enriching for epidermal stem cells in a sample containing epidermal stem cells, the method comprising enriching for cells which express MCSP.

5 "Enrichment" is as defined above. Further embodiments of the fifth aspect are as described for the second aspect of the invention wherein the polypeptide is MCSP.

A sixth aspect of the invention provides a method of identifying an 10 epidermal stem cell in a sample containing epidermal stem cells, the method comprising identifying cells which express MCSP.

Further embodiments of the sixth aspect are as described for the third aspect of the invention wherein the polypeptide is MCSP.

15

A seventh aspect of the invention provides a population of cells enriched for epidermal stem cells obtained by or obtainable by the method of the second or fifth aspect of the invention.

20 The invention also includes a population of cells from the germinative or basal layer of skin enriched for cells which express MCSP on their surface. Thus, MCSP+ve germinative layer or basal layer skin cells are included.

Preferably the population of epidermal stem cells comprises at least 10% 25 epidermal stem cells, more preferably at least 20%, more preferably 30% or 40%, still more preferably epidermal stem cells comprise 50% or 60% or 70% of the total cells. It is particularly preferred if epidermal stem cells constitute more than 80%, or 90% of the total cells in the enriched population. In a still more preferred embodiment, the population of

enriched epidermal cells contains more than 95% or 99% epidermal stem cells.

In one embodiment, the population of cells is enriched for cells expressing a polypeptide whose expression is driven by the MCSP promoter. Preferably, the polypeptide is MCSP.

In a preferred embodiment, the population of cells is enriched for cells expressing MCSP which do not express keratin 15. Thus, the invention also includes a population of cells from the germinative or basal layer of skin enriched from cells which express MCSP on their surface but do not express keratin 15. Thus, MCSP+ve, K15–ve germinative layer or basal layer skin cells are included.

Levels of enrichment may be measured using techniques that indicate cell pluripotency, proliferative capacity or assays for cell differentiation as are known in the art. The presence of stem cells may be implied directly by assessing the proliferative capacity of the population and demonstrating that the stem cells can produce many more daughter cells than the unfractionated cells or the other fractionated populations.

According to a preferred embodiment the population of cells enriched for epidermal stem cell obtained or obtainable by the method of the second aspect of the invention or the population of cells from the germinative or basal layers of skin enriched for cells which express MCSP on their surface further comprises a recombinant nucleic acid molecule within at least one of the cells within the population.

An eighth aspect of the invention provides an isolated epidermal stem cell obtained by or obtainable by the method of the first or fourth aspect of the invention.

5 The invention also includes an isolated cell from the germinative or basal layer of skin which expresses MCSP on its surface.

Epidermal stem cells may also express keratin 15 (K15+ve) (Waseem *et al* (1999) *J. Invest. Dermatol.* 112: 362-369). Preferably, an isolated epidermal 10 stem cell expresses MCSP (MCSP+ve) but does not express keratin 15 (ie, it is MCSP+ve and K15-ve).

Hence, in one embodiment, the isolated epidermal stem cell is further characterised by having substantially no expression of keratin 15 and/or the 15 transferrin receptor.

Expression of keratin 15 and transferrin receptor may be determined by techniques well known in the art and as described above. For example, determination of expression of keratin 15 and transferrin receptor can be 20 achieved using a binding agent capable of specifically binding keratin 15 or transferrin receptor, such as an antibody. Suitable antibodies are well known in the art, and are publicly available. Keratin 15 antibodies are described in Waseem *et al* (1999) *J. Invest. Dermatol.* 112: 362-369 and are available from Imperial Cancer Research Technology, London UK under 25 catalogue number LHK15 (IL28/5 G7.1) or from NovoCastra Laboratories

(Balliol Business Park West, Benton Lane, Newcastle-upon-Tyne NE12 8EW). Transferrin receptor antibodies may be obtained from Abcam Ltd (Cambridge, UK) under product code ab1280-10-T66.

5 Determination of expression of keratin 15 is less preferred if all the isolated cells are required, since keratin 15 is an intracellular protein and hence requires killing the cell to analyse its presence. It will be appreciated that use of keratin 15 expression is best suited to analysis of a representative aliquot of the isolated cells to check for stem cell nature and purity.

10

A cell may further be characterised as a stem cell by determining if it is pluripotent. Cells which do not demonstrate any pluripotency are not stem cells (Clarke *et al* (2000) *Science* 288: 1660-1663; Alison *et al* (2000) *Nature* 406: 257, both of which are incorporated herein by reference).

15

According to a preferred embodiment, the isolated epidermal stem cell obtained or obtainable by the method of the first or fourth aspect of the invention or the isolated cell from the germinative or basal layer of skin which cell expresses MCSP on its surface further comprises a recombinant
20 nucleic acid molecule.

Suitably, the recombinant molecule which is introduced into the isolated epidermal stem cell or population of cells enriched for epidermal stem cells comprises a vector capable of replicating or capable of maintenance as an
25 episome or capable of integrating into the genome of the epidermal stem cell. Preferably, the recombinant molecule expresses an agent such as a polypeptide, and more preferably, the recombinant molecule expresses a therapeutic agent.

By "therapeutic agent" we include therapeutic nucleic acids including antisense agents, and therapeutic polypeptides including an antigenic peptides or polypeptide able to give rise to antigenic peptides within the cell.

5

Specific examples of therapeutic agents and polypeptides are given below.

Once a population of cells has been enriched for epidermal stem cells, or once epidermal cells have been isolated, they may be maintained in culture
10 as is known in the art. Especially where proliferation of the stem cells is desired, the culture conditions specified in WO 99/40107 may be useful.

The invention also includes the population of cells enriched for epidermal stem cells or described above and the isolated epidermal stem cells as
15 described above for use in medicine. That is to say, the cells are packaged and presented for use in medicine. The cells may or may not contain a recombinant nucleic acid molecule, depending on what they are used for.

A ninth aspect of the invention provides a method of treating a patient in
20 need of treatment with epidermal stem cells, the method comprising administering to the patient a population of cells enriched for epidermal stem cells as described above or an isolated epidermal stem cell as described above.

25 In one embodiment of the ninth aspect of the invention, the population of cells or the isolated epidermal stem cell used in the treatment do not further

comprise a polynucleotide. In this embodiment, the epidermal stem cells may be used to regenerate skin and other epithelial cell layers including oral, urethral and corneal epithelia. Suitable patients for treatment with such cells include patients with skin damage.

5

By "skin damage" we mean any condition where the skin is not normal. This includes genetic skin diseases such as epidermolysis bullosa, ectodermal dysplasia and lamellar ichthyosis, and particularly lesions in the skin resulting from surgical intervention, mechanical and thermal injury,

10 infection and so on.

A population of epidermal stem cells may also be useful in treatment of diseased or damaged epithelial tissues, such as in ulcerous conditions (for example, venous leg ulcers, diabetic ulcers and pressure ulcers), acute burn

15 coverage or dermatological surgery wounds or in treating the cosmetic outcome of wound healing.

It will be appreciated that populations of epidermal stem cells which have been isolated or enriched for and subsequently cultured into a graft suitable

20 for the therapies and treatments described herein (such as a transplantable sheet of living keratinous tissue as is known in the art), and use of such populations are included within the scope of the invention.

The invention includes a method of making skin suitable for a skin graft, the

25 method comprising culturing a population of cells enriched for epidermal stem cells or isolated epidermal stem cells of the invention under conditions where they form skin. Skin made in this way is also included in the invention. The method is preferably carried out *in vitro*.

Genetic skin diseases may be treated by administration of a population of cells or an isolated epidermal stem cell which is derived from a source which does not have the genetic abnormality which is responsible for the disease. The alternative source of said cells may be another human, or may be a pig or sheep etc. Administration of the cells may lead to restoration of the lacking biological component required for the correct skin development.

Preferably, the population of cells or the isolated epidermal stem cell expressing MCSP as described above is used in the treatment of skin damage caused by surgical intervention, mechanical and thermal injury, infection and so on. Typically, treatment of skin damage is by production of a skin graft. Epidermal stem cells expressing MCSP can be applied either to an intact acellular dermal matrix which is then transplanted to a skin defect, or applied directly to an acellular dermal matrix which was transplanted to the skin defect prior to application of the epidermal stem cells. Acellular dermal matrices have an intact basement membrane complex which is believed to reduce scarring and contracture at the graft site. Such a matrix may be xenogeneic or of human origin, and is described in WO 97/08295 (Lifecell Corporation).

Alternatively, such a skin graft can be produced by culturing a population of cells or an isolated cell *in vitro* in the presence of a combination of components such as growth factors, serum and irradiated or mitomycin treated mouse fibroblast to increase the number of cells and to produce an intact sheet of cells which is suitable for production of a skin graft. Such a sheet can be released from the culture vessel by treatment with enzymes such as Dispase™ which disrupt the attachment of cells to the substrate but

do not disturb cell-cell contacts. A suitable method is described in US 4,304,866, incorporated herein by reference.

In another embodiment of the ninth aspect of the invention, the population of cells or an isolated epidermal stem cell used in the method of treatment further comprises a polynucleotide. Typically, the polynucleotide is capable of expressing a therapeutic agent such as a polypeptide.

If a gene is integrated into a differentiated cell, repeated treatment can be needed in order to sustain the treatment in an individual. Epidermal stem cells, by virtue of being undifferentiated, offer a cell type which once modified to contain a therapeutic gene can be retained in the body potentially indefinitely. Thus, epidermal stem cells may be useful as a target of genetic manipulation to introduce proteins of therapeutic value (Hengge *et al* (1999) *Exp. Dermatol.* 8:419-431). Such proteins may be ones which are absent from the individual due to a genetic defect.

In one embodiment of the ninth aspect of the invention, an isolated epidermal stem cell or a population of cells enriched for epidermal stem cells further comprising a polynucleotide capable of expressing a therapeutic agent such as a polypeptide are used to treat a metabolic disease, such as a genetic deficiency in adenosine-deaminase which causes severe combined immunodeficiency (SCID). Epidermal stem cells may be manipulated as is known in the art to express a therapeutic polypeptide, such as adenosine deaminase, which may then be secreted into the

circulation. An advantage of using epidermal stem cells is the good blood supply for subsequent systemic circulation of the encoded therapeutic agent.

It will be appreciated that metabolic diseases may also be adequately treated 5 by administration of a isolated epidermal stem cell or a population of cells enriched for epidermal stem cells which do not further comprise a polynucleotide. In such cases the lack of the metabolic polypeptide which gives rise to the disease may be slight or have slight consequences which can be rectified by administration of said cell or cells which are not 10 genetically deficient. In this case, the cell or cells will be from a donor organism which is different to the recipient.

Epidermal stem cells which have been genetically modified so as to express a therapeutic agent may act as a "metabolic sink" wherein the therapeutic 15 agent is an enzyme that can detoxify the body of toxic substances accumulating in certain disorders. Examples of such therapeutic enzymes include ornithine aminotransferase (to treat hyperornithinemia which causes gyrate atrophy), phenylalanine hydroxylase and ornithine transcarbamylase.

20 Further examples of therapeutically useful polypeptides are insulin (to treat diabetes), Factor VIII (to treat haemophilia), erythropoietin, transferrin, Factor IX, apoE and apoA1.

It will be appreciated that other diseases caused by genetic defect which 25 may be amenable to treatment using isolated epidermal stem cells which further comprise a polynucleotide capable of expressing a therapeutic agent

are rare skin diseases such as epidermolysis bullosa, ectodermal dysplasia and lamellar ichthyosis. In these cases, long term gene expression is of particular benefit as the diseases are caused by lack of a specific protein which is required for correct formation of the skin epidermis structure.

In another embodiment of this aspect of the invention, treatment of a patient with epidermal stem cells expressing a therapeutic agent which is a peptide antigen or a polypeptide able to generate an antigenic peptide can be used to vaccinate the patient to immunise them against an infectious disease. It is known that potent cytotoxic T cell and humoral immune responses can be generated due to the antigen processing and presentation in the skin. Proteins which are produced in keratinocytes can by phagocytosed and presented in the MHC class I pathway and lead to CTL priming (Corr *et al* (1996) *J. Exp. Med.* 184:1555-1560).

It will be appreciated that treatment using an isolated epidermal stem cell or a population of cells enriched for epidermal stem cells comprising a polynucleotide according to the ninth aspect of the invention includes the introduction of gene elements such as ribozymes to remove defective gene function.

It will also be appreciated that a cell or cells according to the invention may be subjected to cryopreservation, for example as described in US 5,145,770. Preservation of such cells is of particular use where they comprise a polynucleotide expressing a therapeutic agent, and treatment of a patient with the cells is repeated over a period of time.

A tenth aspect of the invention provides a method of delivering a therapeutic agent to a patient, the method comprising administering to the patient a population of cells enriched in epidermal stem cells as described above or an isolated epidermal stem cell as described above wherein the cells express the therapeutic agent.

The cells which express the therapeutic agent may or may not contain a recombinant nucleic acid. It is preferred that they do.

Isolated epidermal stem cells may be modified to contain a recombinant nucleic acid *ex vivo* using methods of gene transfer known in the art.

Methods of administering a population of cells according to the invention are known in the art. A suitable method is described in WO 99/03505, incorporated herein by reference. Methods of determining the amount of epithelial cells expressing foreign genetic material so to express sufficient therapeutic material are described in US 4,980,286.

The therapeutic agent is typically encoded by a recombinant polynucleotide.

Suitably, therapeutic agents include therapeutic polypeptides as described above, antigenic polypeptides or polypeptides able to give rise to antigenic peptides and ribozymes.

It will be appreciated that the polynucleotide may comprise a promoter sequence which promotes transcription in the desired cell type. For

example, if selective expression of an encoded therapeutic agent is required in an epidermal stem cell, then a promoter which selectively directs expression therein is suitable. Polynucleotides which comprise the nucleic acid sequence shown in Figure 7 may be suitable for expression in
5 epidermal stem cells. Equally, if expression is desired in a range of cell types then the promoter should be included accordingly. A range of promoters are known in the art, which vary in their cell type specificity. An example of a promoter which would become active in differentiating keratinocytes is a keratin promoter.

10

An eleventh aspect of the invention provides a use of a population of cells enriched for epidermal stem cells as described above or an isolated epidermal stem cell in the manufacture of a medicament for treating a patient in need of treatment with epidermal stem cells.

15

The invention also includes use of a population of cells enriched in epidermal stem cells as described above or an isolated stem cell as described above wherein the cells express a therapeutic agent in the manufacture of a medicament for delivering the therapeutic agent to the
20 patient.

A twelfth aspect of the invention provides a use of a reagent which selectively binds to MCSP in isolating epidermal stem cells.

25 Preferably the reagent which selectively binds to MCSP is an antibody.

In a preferred embodiment of the twelfth aspect of the invention, the reagent which selectively binds to MCSP is an antibody and is immobilised. Suitable surfaces for immobilisation of the antibody include magnetic beads, Sepharose™ beads and plastic surfaces such as microtitre plates.

5

A thirteenth aspect of the invention provides a method of determining suitable conditions for culture of epidermal stem cells *in vitro* the method comprising culturing said cells, determining expression of a polypeptide whose expression is driven by the MCSP polypeptide by said cells and if
10 expression of said polypeptide is retained then the conditions are suitable.

The polypeptide whose expression is driven by the MCSP promoter may be any such polypeptide as described with respect to the first three aspects of the invention. Preferably, expression of MCSP is determined.

15

Currently, although they may be cultured *in vitro*, epidermal stem cells exhibit a finite life span in the conditions used. Optimisation of the culturing conditions may allow the culture and expansion of epidermal stem cells for more useful lengths of time.

20

Conditions suitable for culturing epidermal stem cells can be determined by the steps of incubating epidermal stem cells according to the invention, determining over increasingly longer lengths of time if the level of expression from the MCSP promoter by the cells (and, preferably,
25 expression of MCSP) is maintained or decreased. Conditions which increase the length of time before a decrease in expression from the MCSP

A further feature of stem cells which may be determined in order to assess the suitability of culture conditions is telomerase activity. Although telomerase activity is detectable in cultured normal epidermal keratinocytes, research to date (including Yasumoto *et al* (1996) *Oncogene* 13: 433-439;
5 Meyerson (2000) *J. Clin. Oncol.* 18: 2626-2634) indicates that it is expected that telomerase activity will be higher in epidermal stem cells. Hence, the presence of telomerase activity (specifically, a relatively high level of activity) in the cultured cells is an indicator that the conditions are suitable for stem cells, whereas an absence or low telomerase activity is an
10 indication that the conditions are not, or are less, suitable for epidermal stem cell culture. The term "high level of expression" is known and understood in the art.

It will be appreciated that the presence or absence of fibronectin in *in vitro*
15 cell culture may be of utility. As described in Moyano *et al* (1999) *J. Biol. Chem.* 247: 135-142, chondroitin sulphate proteoglycans bind to the HepIII domain of fibronectin which may promote stem cell adhesion when in culture and thereby provide a beneficial effect in determining conditions suitable for culturing epidermal stem cells.

20

Further agents which may be suitable for inclusion in conditions for culturing epidermal stem cells are described in US 4,456,770.

It may be desirable to serially culture the epidermal stem cells during the
25 determination of suitable culture conditions. A process for serial culture for keratinocytes is described in US 4,016,036; however, such conditions may not be suitable for maintaining stem cell characteristics and may instead be useful for subsequent use of stem cells (for example, in the preparation of a skin grant, as described above).

A fourteenth aspect of the invention provides a method of screening for substances which modulate the differentiation state of epidermal stem cells, the method comprising i) providing a population of cells enriched for epidermal stem cells or an isolated epidermal stem cell; ii) contacting said cells with a test substance; and iii) detecting a change in the expression from the MCSP promoter.

Expression from the promoter may be by using a recombinant molecule wherein the promoter is operatively linked to the expression of a detectable polypeptide and expression of the detectable polypeptide is a measure of expression from the promoter. Preferably, in step (iii) a change in the cell surface expression of MCSP is detected.

Cell surface expression of MCSP can be determined by techniques well known in the art. A suitable technique includes FACS analysis using an antibody capable of specifically binding MCSP which is either itself conjugated to a fluorescent marker, or which is bound by a second antibody which is conjugated to a fluorescent marker.

It will be appreciated that the change in expression of MCSP may be an increase or may be a decrease.

Contact of isolated epidermal stem cells with the test substance may produce a decrease in the cell surface expression of MCSP. Such a test substance is likely to be promoting the differentiation of the epidermal stem cells.

Detection of an increase in cell surface expression of MCSP may indicate that the test substance is inhibiting differentiation of the cells or promoting de-differentiation of the cells into stem cells.

In a further embodiment of the fourteenth aspect of the invention, changes in expression of keratin 15 may also be determined, either instead of determination of MCSP or in addition to it. In this embodiment, an increase in expression of keratin 15 is an indicator of differentiation, as epidermal stem cells do not substantially express keratin 15. Detection of keratin 15 expression following contact of the epidermal stem cells with the test substance indicates that the substance is promoting differentiation of the cells.

An alternative or additional marker of differentiation is involucrin. This polypeptide is expressed at an early stage of terminal differentiation as keratinocytes leave the basal layer (Watt (1983) *J. Invest. Dermatol.* 81: 100s-3s). Other structural proteins and keratins may be useful in determining differentation.

Compounds identified or identifiable according to the fourteenth aspect of the invention are useful in methods of treatment according to the ninth aspect of the invention, for example in promoting differentiation of epidermal stem cells administered to damaged skin in order to produce new skin layers, or alternatively in discouraging differentiation of administered epidermal stem cells or a population of cells enriched for epidermal stem cells, in order to allow establishment of the cells within the recipient without terminal differentiation.

Compounds identified or identifiable according to the fourteenth aspect of the invention are also useful as components of cell culture media required for *in vitro* culture of epidermal stem cells.

5 A fifteenth aspect of the invention provides a method of screening for substances which modulate the rate of division of epidermal stem cells, the method comprising i) providing a population of cells enriched for epidermal stem cells or an isolated epidermal stem cell as described above; ii) contacting said cells with a test substance; and iii) detecting a change in the 10 rate of cell division.

According to the fifteenth aspect of the invention, a test substance may either increase or decrease the rate of division of epidermal stem cells. Rate of cell division may be assessed by any routine technique known in the art 15 of cell culture. Test substances which produce an increase in the rate of cell division compared to cells grown in the absence of the substance are likely to be a stimulator of cell division, and in the same way, test substances which decrease the rate of cell division compared to cells grown in the absence of the substance are likely to be growth inhibitors.

20

Compounds which are stimulators of epidermal stem cell division may be useful in the method of treatment according to the ninth aspect of the invention. For example, by decreasing the time required for establishing a useful skin preparation or skin growth, such compounds may improve the 25 efficacy of skin grafting or rate of wound healing (including ulcerous wounds, for example diabetic ulcers and venous leg ulcers) and may be useful in treating the cosmetic outcome of wound healing. Additionally, compounds which are stimulators of epidermal stem cell division may be

useful in the production of large quantities of epidermal stem cells for the purpose of research and therapy.

Diseases or conditions which may be treated, or which may benefit from
5 treatment with substances or compounds which modulate the rate of cell division and/or differentiation of epidermal stem cells include hyperproliferative diseases and psoriasis.

A sixteenth aspect of the invention provides a substance obtained or
10 obtainable by the method of the fourteenth or fifteenth aspects of the invention.

In one embodiment of this aspect of the invention the substances are useful in medicine. Thus, the invention includes such substances packaged and
15 presented for use in medicine.

In an additional embodiment, the substances are useful as components of tissue culture media or as research tools.

20 A seventeenth aspect of the invention provides a method of treating a hyperproliferative skin condition such as psoriasis the method comprising administering to the patient a substance according to the fifteenth aspect of the invention.

25 An eighteenth aspect of the invention provides a use of a substance according to the fifteenth aspect of the invention in the manufacture of a medicament for treating a hyperproliferative skin condition such as psoriasis.

A nineteenth aspect of the invention provides a polynucleotide comprising the nucleic acid sequence shown in Figure 7 or a fragment or variant thereof which is not the clone corresponding to GenBank Accession No. AC019294 or AC 068368.

5

The sequence shown in Figure 7 includes at least a part of the MCSP promoter.

By "fragment" we include a portion of the sequence shown in Figure 7

10 which is useful. A useful portion is one which has a function such as endowing an exogenous promoter or regulatory sequence with epidermal stem cell specificity of activity or regulation of activity, or a function such as promoting transcription.

15 By "variant" we include alleles and variants which occur in nature, and homologues of the sequence in species such as rat, pig, sheep, cow etc. An example of a variant included within the scope of a polynucleotide according to the nineteenth aspect of the invention is the promoter of the rat NG2 polypeptide.

20

The polynucleotide of the nineteenth aspect of the invention is preferably one which has transcriptional promoter activity. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Preferably the

25 transcriptional promoter activity is present in mammalian cells, and more preferable the polynucleotide has transcriptional promoter activity in epidermal stem cells. It is particularly preferred if the transcriptional promoter activity of the polynucleotide is active in epidermal stem cells (eg, MCSP+ve/K15-ve), and not active in normal differentiated epidermal cells

such as transit-amplifying cells (eg, MCSP-ve/K15+ve). By "normal" we mean non-cancerous epidermal cells.

In a further preferred embodiment of the nineteenth aspect of the invention, the polynucleotide is operatively linked to a polynucleotide encoding a polypeptide. Methods for linking promoter polynucleotides to polypeptide coding sequences are well known in the art.

Preferably the polypeptide is a therapeutic polypeptide. Examples of therapeutic polypeptides are described above.

It is also preferred if the polypeptide is a detectable polypeptide, as described above. Detectable polypeptides whose expression is driven by the MCSP promoter are useful in identifying epidermal stem cells. Detectable polypeptides include polypeptides which fluoresce, such as green fluorescent protein, or which have a specific binding agent available, such as an antibody, which selectively bind to them.

In one embodiment of the nineteenth aspect of the invention, the polynucleotide is one suitable for use in medicine. Thus, the invention includes the polynucleotide packaged and presented for use in medicine.

Therapeutic applications of polynucleotides of the nineteenth aspect of the invention include those described above for polynucleotides in the ninth aspect of the invention.

A variety of methods have been developed to operably link polynucleotides, especially DNA, to other polynucleotides such as vectors, for example, via complementary cohesive termini. For instance, complementary

homopolymer tracts can be added to the DNA segment to be inserted into the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

5

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E.coli* DNA
10 polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerising activities.

The combination of these activities therefore generates blunt-ended DNA
15 segments. The blunt-ended segments are then incubated with a larger molar excess of linker molecules in the presence of an enzyme that is able to catalyse the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments
20 are then cleaved with the appropriate restriction enzyme and ligated to an suitable vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease site are
25 commercially available from a number of sources including International Biotechnologies Inc., New Haven, CN, USA.

A desirable way to modify the DNA encoding the polynucleotide of the invention is to use PCR. This method may be used for introducing the

DNA into a suitable vector, for example by engineering in suitable restriction sites, or it may be used to modify the DNA in other useful ways as is known in the art.

5 In this method the DNA to be enzymatically amplified is flanked by two specific primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into suitable vectors using methods known in the art.

10

The DNA (or in the case of retroviral vectors, RNA) is then expressed in a suitable host cell such as an epidermal stem cell, to produce a therapeutic polypeptide.

15 Thus, the polynucleotide of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of a therapeutic polypeptide. Such techniques include those disclosed in US
20 Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to
25 Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA (or in the case or retroviral vectors, RNA) comprising the polynucleotide of the invention may be joined to a wide variety of other DNA sequences for introduction into epidermal stem cells. The companion DNA will depend upon the manner of the introduction of the DNA into the epidermal stem cells, and whether episomal maintenance or integration is desired. The DNA may be introduced into the epidermal stem cells either *in vivo* or *ex vivo*.

Generally, the polynucleotide of the invention is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression of any encoded polypeptide. The vector is then introduced into the epidermal stem cells through standard techniques. Generally, not all of the epidermal stem cells will be transformed by the vector. In the case of *ex vivo* transformation, it will be necessary to select for transformed epidermal stem cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the epidermal stem cell.

A twentieth aspect of the invention provides a method of treating a patient in need of treatment with a therapeutic polypeptide, the method comprising administering to the patient a polynucleotide according to the nineteenth aspect of the invention.

A twenty-first aspect of the invention provides a use of a polynucleotide according to the nineteenth aspect of the invention in the manufacture of a medicament for treating a patient in need of treatment with the therapeutic polypeptide.

Treatments which may involve use of a polynucleotide according to the nineteenth aspect of the invention include genetic diseases, metabolic diseases and vaccination, which are described in further detail above.

5

Preferably, the polynucleotide is used to transfer a gene into an epidermal stem cell, as defined above. The epidermal stem cell may or may not be isolated. By this we mean that use of the polynucleotide according to the nineteenth aspect of the invention may be either *in vivo*, or *ex vivo*.
10 Techniques of both *in vivo*, or *ex vivo* gene transfer are well known in the art of gene therapy (Seitz *et al* (1999) *Gene Therapy* 6: 42-47; Abonour *et al* (2000) *Nature Medicine* 6: 652-658) Suitably, the use of polynucleotide according to the nineteenth aspect of the invention comprises use as demonstrated in Example 4. Preferably, the cell is an epidermal stem cell,
15 and more preferably the epidermal stem cell is one characterised by expression of MCSP.

A twenty-second aspect of the invention provides a use of a polynucleotide according to the nineteenth aspect of the invention in a method to isolate or
20 identify, or enrich for, epidermal stem cells.

A twenty-third aspect of the invention provides a compound comprising a moiety capable of selectively binding to MCSP and a polynucleotide.

25 Such a compound is believed to be useful in targeting the polynucleotide for a cell which expresses MCSP, such as epidermal stem cells.

The nucleic acid delivered to the target site may be any suitable DNA. Nucleic acid molecules suitable for gene therapy are described above. The

polynucleotide may be a polynucleotide according to the nineteenth aspect of the invention. Preferably, the polynucleotide will contain a promoter which is functional in a range of cell types, so that expression of any encoded polypeptide or therapeutic agent is not limited to epidermal stem cells alone but is able to continue during eventual differentiation of the targeted epidermal stem cell progeny. Promoters which are functional over a range of cell differentiation stages are well known in the art.

Examples of promoters which would be useful in this aspect of the invention include the keratin 14, keratin 1, keratin 10 and involucrin promoters.

The moiety capable of binding MCSP may be any suitable moiety as discussed above in relation to the first, second, third, fourth, fifth or sixth aspects of the invention.

The moiety capable of binding to MCSP is preferably an antibody. Antibodies selective for MCSP are described above.

The compound may be a delivery vehicle suitable for introducing genetic material into epidermal stem cells.

The delivery vehicle may be any suitable delivery vehicle. It may, for example, be a liposome containing nucleic acid, or it may be a virus or virus-like particle which is able to deliver nucleic acid. In these cases, the moiety which selectively binds to MCSP is typically present on the surface of the delivery vehicle. For example, the moiety which selectively bind to MCSP, such as a suitable antibody fragment, may be present in the outer surface of a liposome and the nucleic acid to be delivered may be present in

the interior of the liposome. As another example, a viral vector, such as a retroviral or adenoviral vector, is engineered such that the moiety which selectively binds MCSP is attached to or located in the surface of the viral particle thus enabling the viral particle to be targeted to the epidermal stem
5 cells. Targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like particle. Michael *et al* (1995) *Gene Therapy* 2:660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Targeted retroviruses are
10 also available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into pre-existing viral *env* genes (see Miller & Vile (1995) *Faseb J.* 9:190-199 for a review of this and other targeted vectors for gene therapy). Immunoliposomes (antibody-directed) liposomes may be used in which the moiety which selectively
15 binds MCSP is an antibody. For the preparation of immunoliposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* 257:286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the MCSP antibody, or fragment
20 thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures
25 up to 0.8 Mpa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end

rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the skin.

5

A twenty-fourth aspect of the invention provides a method of introducing genetic material into epidermal stem cells, the method comprising contacting the cells with a compound according to the twenty-third aspect of the invention.

10

Suitably, such compounds are useful in the methods of the invention as a means of directing the polynucleotide specifically to epidermal stem cells, enabling targeted delivery of the polynucleotide.

15 A further aspect of the invention provides a method of treating a patient in need of treatment with a therapeutic agent, the method comprising administering to the patient a compound comprising a moiety capable of selectively binding to MCSP and a polynucleotide encoding a therapeutic agent. The therapeutic agent may be any therapeutic agent as described 20 above (including antisense or ribozyme agents) but is preferably a polypeptide.

Patients in need of treatment with a therapeutic polypeptide include those with the genetic disorders outlined above, such as rare skin diseases, 25 metabolic disorders and patients needing immunisation against an infectious disease.

Administration of the compound to the patient may be direct, for example by direct injection into the bloodstream of the patient or sub-cutaneously or

intramuscularly, or by topical application, or it may be indirect, where the compound is administered to a culture of cells according to the invention which are subsequently administered to the patient.

5 A yet further aspect of the invention provides a use of a compound comprising a moiety capable of selectively binding to MCSP and a polynucleotide encoding a therapeutic agent in the manufacture of a medicament for treating a patient in need of treatment with the therapeutic agent.

10

A still further aspect of the invention provides a pharmaceutical composition comprising a population of cells enriched for epidermal stem cells or an isolated epidermal stem cell according to the invention, or a polynucleotide according to the invention or a compound isolated in the 15 screening methods of the invention and a pharmaceutically acceptable carrier.

By "pharmaceutically acceptable" is included that the formulation is sterile and pyrogen free. Suitable pharmaceutical carriers are well known in the art 20 of pharmacy.

Further aspects of the invention provide kits of parts that are useful in carrying out the isolation, identification or enrichment methods of the first, second, third, fourth, fifth and sixth aspects of the invention.

25

Thus, one kit includes a first binding agent capable of selectively binding to MCSP and a further binding agent capable of selectively binding to a component selected from a group consisting of keratin 15, β1 integrin, α6 integrin and transferrin receptor.

Preferably, the binding agents are antibodies. Preferably the kit contains an antibody directed to one polypeptide from the group consisting of keratin 15, β1 integrin, α6 integrin and transferrin receptor. More preferably, the kit contains more than one antibody, each antibody being directed to a different polypeptide chosen from the group consisting of keratin 15, β1 integrin, α6 integrin and transferrin receptor. Thus, for example, a kit may contain three antibodies which bind MCSP, keratin 15 and β1 integrin.

Typically, the antibodies are provided in the kit pre-conjugated to an immobile support, such as Dynabeads™ or Sepharose™ beads to facilitate isolation or enrichment of the cells from the rest of the sample components.

The kit may, or may not further comprise a means for releasing epidermal stem cells bound to the antibodies. Such reagents are well known in the art of cell selection.

Conveniently, the kit further comprises an appropriate means for identifying epidermal stem cells isolated or enriched by methods of the first or fourth aspects of the invention, by detecting the presence of the bound antibodies or the presence of the MCSP polypeptide. Such means may comprise an enzyme-linked chemiluminescent assay. Some of the kit components may be localised on a surface, such as a blotting membrane, or an assay plate for ELISA etc, although the assay can be carried out in solution.

The invention will now be described in more detail with reference to the following Figures and Examples:

Figure 1.

Immunofluorescence showing 3 subpopulations of basal keratinocytes using double staining with LHM2 (upper panel) and K15 antibody (lower panel): MCSP+ve/K15-ve, MCSP-ve/K15+ve and a small population of
5 MCSP+ve/K15+ve cells

Figure 2.

Immunofluorescence showing LHM2 (upper panel; brighter regions) and K15 antibody (lower panel; brighter regions) staining in basal cell
10 carcinomas.

Figure 3.

RT-PCR: MCSP transcripts were identified in all of the basal cell carcinomas (BCCs), normal human skin and keratinocytes samples
15 analysed.

Figure 4.

Comparison of MCSP mRNA expression in A375P cell line, BCCs, normal human skin and cultured keratinocytes. Polyadenylated RNA isolated from
20 A375P (as positive control in lane 1), BCCs (lane 2-4), normal skin (lane 5-6) and keratinocytes was separated on 1% agarose gels, blotted onto nylon filters and sequentially hybridised with MCSP specific and beta-actin probes. Strong MCSP signal is seen in all 3 BCCs but not in normal skin and cultured keratinocytes. Very strong signal is seen in the positive control
25 with A375P cell line.

Figure 5.

In-situ hybridization study showing abundant hybridization signals within the tumour islands in the BCC with the antisense probe but not the sense control. Hybridization signals were also seen in a subpopulation of basal interfollicular keratinocytes

Figure 6.

PCR scheme for walking from the 5' end of the MCSP coding region into previously unidentified sequence.

Figure 7.

DNA sequence of 1.8kb of sequence 5' to the MCSP coding region. The underlined region at the 3' end represents positions 1-9 of the MCSP coding sequence.

Figure 8.

An alignment of the human MCSP amino acid sequence with the rat NG2 sequence generated using the GCG package application GAP.

Figure 9.

An alignment of the human MCSP nucleotide sequence with the rat NG2 sequence generated using the GCG package application GAP. The ATG to STOP codons only were used.

Example 1: Maintenance Of Expression Of The Melanoma Associated Chondroitin Sulphate Proteoglycan In Basal Keratinocytes Is Dependent On Mesenchymal Signalling

5 *LHM2 monoclonal antibody stains keratinocytes in the outer root sheath and a subpopulation of interfollicular keratinocytes.*

Immunostaining demonstrated positive staining in a subpopulation of basal interfollicular keratinocytes and outer hair root sheath keratinocytes. The 10 LHM2 positive interfollicular keratinocytes were preferentially localised at the tips of the dermal papilla. Confocal microscopy revealed that the antigen detected by LHM2 in keratinocytes was predominately localised on the lateral and apical border of the basal keratinocytes. The pattern of staining identified in normal skin was the same for both frozen and formalin 15 fixed specimens. In order to further characterise the LHM2 positive keratinocytes we investigated the relationship between keratin 15 (K15) expression and LHM2 staining. In previous studies we have shown using a monoclonal antibody against K15 (LHK15) that K15 is expressed in the hair follicle outer root sheath and in some basal keratinocytes. Double 20 labelling for LHM2 and LHK15 allowed us to subdivide basal keratinocytes into 3 populations: LHM2+ve/K15-ve, LHM2-ve/K15+ve and a small population of LHM2+ve/K15+ve cells (Fig.1). The LHM2+ve/K15-ve lie at the tips of the dermal papilla and the LHM2-ve/K15+ve cell were located in the rete ridges. In outer root sheath (ORS) keratinocytes the pattern of 25 immunostaining with LHM2 and LHK15 was similar with overlapping expression of both proteins in most ORS keratinocytes. LHM2 expression

was however noted to be slightly more extensive than K15 with a small number of keratinocytes in the distal ORS LHM2+ve/K15-ve.

In order to further characterise the observed heterogeneity of inter-follicular basal keratinocytes with respect to LHM2 staining we examined the pattern of LHM2 staining in two keratinocyte derived skin cancers which show marked differences in their propensity to differentiate. Basal cell carcinomas (BCCs) are composed of undifferentiated keratinocytes which resemble the epithelial cells that give rise to adnexal structures. In contrast squamous cell carcinomas (SCCs) differentiate to a variable degree as cells migrate from the basal to suprbasal layers. LHM2 staining of BCCs (n= 14) showed uniform staining throughout the tumour islands in all of the samples studies (Figure 2). In contrast to LHM2 positive basal keratinocytes in which the apicolateral borders are stained, keratinocytes in BCCs showed more uniform expression on all cell surfaces. In SCCs (n=9) focal LHM2 positivity was observed on undifferentiated keratinocytes in contact with the basement membrane which was lost as the cell differentiated. Double staining of BCCs with LHM2 and LHK15 revealed heterogeneous expression of K15 with clusters of K15+ve keratinocytes within BCC tumour island which suggests that LHM2+ve/K15-ve keratinocytes may be less differentiated than LHM2+ve/K15+ve keratinocytes.

MCSP is expressed in normal human skin and expression is decreased in cultured keratinocytes

Previous studies of MCSP expression suggest that this gene shows a very restricted expression pattern. Having established that LHM2 detects an

antigen in keratinocytes that is overexpressed in basal cell carcinomas we went on to look for evidence of MCSP expression in normal skin. Gene-specific primers based on the published MCSP sequence were designed and used to investigate gene expression in mRNA isolated from A375P melanoma cell-line, normal human skin and cultured keratinocytes. MCSP transcripts were identified in 6 of 6 normal human skin samples and 2 of 2 keratinocytes cell culture (Fig 3). Northern blot analysis detected a hybridising band of 8 kb in the A375P melanoma cell line. No signal was detected in normal skin or primary keratinocyte cultures (Figure 4). The failure to detect MCSP transcripts in RNA isolated from normal skin may be due to its restricted expression in skin. In situ hybridisation of normal skin confirmed that MCSP transcripts are present and like the LHM2 staining the signal was heterogenous in basal keratinocytes. MCSP transcripts were also present in the hair follicle outer root sheath.

In view of the increased LHM2 staining in BCCs we also investigated MCSP gene expression in BCCs. Northern blot analysis revealed that the MCSP gene is markedly upregulated in BCCs and that the transcript size in keratinocytes is identical to that seen in melanoma cell lines (Figure 4).

Maintenance of MCSP protein expression is dependent on mesenchymal signalling

As discussed above immunostaining with LHM2 of normal skin consistently detects positive immunostaining in a subpopulation of basal keratinocytes in addition to outer root sheath keratinocytes of the hair follicle and our RNA expression analysis suggests that the antigen in keratinocytes is the melanoma associated chondroitin sulphate

proteoglycan, MCSP. Primary keratinocytes cultures were established from normal skin which showed positive immunostaining with LHM2. In contrast to the strong staining seen in normal skin no positive immunostaining was observed in cultured keratinocytes. In order to further investigate the regulation of MCSP expression in keratinocytes we examined human whole skin samples maintained in organ culture to determine if maintenance of MCSP expression was dependent of epidermal differentiation. Skin samples maintained in organ culture for 48 hours showed loss of LHM2 staining. By examining skin samples at different time points we were able to establish that the loss of expression occurred within 48 hours of placing the skin in culture. In order to determine the relationship between LHM2 expression and other markers of differentiation in skin organ cultures serial sections were stained for other differentiation markers including keratin 15, keratin 6 and involucrin. Keratin 15 expression was maintained in skin organ cultures at 24 hours but showed an altered expression pattern with the appearance of suprabasal K15 positive keratinocytes and loss of K15 positive basal cells. As reported previously keratin 6 expression was induced in whole skin cultured *in vitro* within 48 hours.

In organ culture the identification of keratin 6 expression suggests that keratinocytes have undergone activation under the experimental condition used in this culture system. The alterations in MCSP and keratin expression could either reflect direct activation of keratinocytes or loss of signals from the mesenchyme that are present *in vivo* but absent in the *in vitro* skin organ culture. In order to further investigate this phenomenon we carried out two further experiments. Firstly we examined MCSP expression in psoriatic skin as keratinocytes are known to be activated in psoriatic lesions. Secondly we cultured skin samples from the same donor *in vitro* and then grafted samples

onto nude mice. In psoriatic skin MCSP expression was lost in the hyperproliferative epithelium of psoriatic plaques. No alteration was observed in the pattern of K15 expression which suggests that the factors regulating MCSP and K15 expression are distinct. Culturing of skin in organ culture for 4 weeks was associated with loss of MCSP expression. It is well recognised that the immunophenotype of skin in organ culture is not identical to that in vivo and that normalisation of the phenotype can be achieved by grafting onto immune deficient mice. In order to determine if MCSP expression could be restored, skin cultured in vitro for 4 weeks was grafted onto nude mice, exposed at 3 weeks and harvested at 6 weeks. In contrast to cultured skin MCSP expression was seen in grafted skin which suggests that the loss of expression may be due to lack of the necessary signals in the *in vitro* model.

MCSP is expressed in rodent skin and shows a similar expression pattern to NG2

As a prelude to further studies to characterise regulation of MCSP
5 expression we have investigated and compared the distribution of MCSP in mouse, rat and human skin. BLAST searching with the human MCSP gene sequence revealed significant homology to NG2 which is a membrane associated chondroitin sulphate proteoglycan originally identified in rats. NG2 has been shown to have a limited distribution in adult tissues being
10 expressed predominantly in neuronal and glial cells and there are no previous reports of expression of NG2 in skin. LHM2 showed weak staining of some mouse and rat tissues including those previously described as being positive for NG2. Using a polyclonalantibodyagainst NG2 (a kind gift from W Stallcup) we observed strong staining of the outer root sheath
15 and interfollicular keratinocytes of both rat and murine skin, in addition to staining of other tissues such as smooth muscle described previously. Immunostaining of chemically induced skin tumours in rats with the NG2 antibody revealed that the expression of NG2 was increased in BCC like tumours in rats which is in keeping with our observations on human BCCs.
20 Immunostaining of human skin with the NG2 antibody revealed an identical staining pattern to that seen with LHM2 with small clusters of positive cells at the tips of rete ridges. In addition to the keratinocyte staining additional staining was seen in smooth muscle cells within the epidermis which was not detected with LHM2.

25

MATERIALS AND METHODS

Immunostaining

Normal human skin was obtained as excess tissue from unrelated surgical procedures and either fixed in formal saline and paraffin embedded or snap

frozen in OCT (Bright Instrument Company Ltd, Huntingdon). BCC samples used were either formalin-fixed paraffin embedded tissue from the pathology archives for patient with BCCs or frozen tumour samples obtained at the time of surgery. Clinical diagnosis was confirmed in all cases by histological examination. Frozen or paraffin sections were immunostained with mouse monoclonal antibodies (DAKO clones C8/144B (recognises CD8 epitope): LHM2 (recognises high MW chondroitin sulfate proteoglycan); LHK15 (binds keratin 15)). Six-micron cryostat sections were cut into silane treated multiwell slides and air-dried. The fixed tissue sections were stained with optimal dilution of the Mabs for 2 hours. Slides were washed for 10 minutes and then stained with goat anti-mouse IgG_1K Texas Red and goat anti-mouse $IgG_{2a}K$ FITC (Harlan Labs). After washing, the slides were mounted and observed by epifluorescence using a Zeiss photo-microscope III.

RT-PCR

RT-PCR using primers based on the published sequence of human MCSP gene (Accession number: X96753) was used to amplify a 288 bp fragment corresponding to position 861-1130 from the coding region of the MCSP gene. Primer sequence used were: forward primer 5'-GCGGAATTCCAGTGTCCACATCAATGCTCA-3' (*EcoR*1 site underlined) and reverse primer 5'-GCGAAGCTTGCCATGTTGCGCGTCAGC-3' (*Hind* III site underlined). The product was subcloned into pGEM-3Z Vector (Promega) and the insert verified by sequencing. Purified insert was labelled by random priming and hybridized to the Northern blots. Riboprobes for in-situ hybridization were generated from the same plasmid.

RNA isolation and Northern analysis

Total RNA was isolated from A375 P cell-line, normal human skin samples, cultured keratinocytes and basal cell carcinomas by the acid guanidinium thiocyanate/phenol/chloroform method (RN*Ace* Total Pure Kit, Bioline Ltd). Polyadenylated RNA extracted from 20 microgram of total RNA using a Dynabeads mRNA Purification Kit (Dynal AS, Oslo, Norway) was size fractionated on 1% formaldehyde-agarose gels and transferred onto nylon membranes (Hybond™-XL, Amersham Pharmacia Biotech). Membranes were hybridised using the cloned ^{32}P labelled MCSP specific probe. Transcript sizes were estimated by comparison with RNA molecule size standard (GIBCO/BRL, Life Technologies™). Hybridizations were done overnight at 42°C. Filters were washed in 2X SSC/0.1% SDS at room temperature for 5 minutes twice followed by another wash in 1X SSC/0.1% SDS at room temperature for 5 minutes thrice. A final wash in 0.1X SSC/0.1% SDS for 15 minutes twice was done at 50°C. Filters were exposed on phospho-imaging screens and hybridization signal was visualised using a Phosphorimager (Molecular Dynamics).

***In-Situ* hybridization**

5 micron paraffin sections were cut onto Superfrost slides (Merk). Sections were deparaffinized followed by treatment with proteinase K at 37°C for 8 minutes and prehybridization at 42°C. Following this, the sections were hybridized with 50 ng/µl of digoxigenin labelled probe in prehybridization buffer containing dextran sulphate overnight at 42°C using a Hybaid Omnislide machine. Following stringency washes to 0.1X SSC/50% formamide, sections were stained with anti-digoxigenin-AP Fab fragments. After washing, the sections were developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) chromagen solution.

Keratinocyte culture and generation of organotypic cultures

Organotypical cultures were generated by growing keratinocytes on a sheet of dead dermis as described previously. Briefly, pieces of de-epidermised dermis (1.5cm-2) were obtained from human skin stored in 80% glycerol (European Skin Bank, The Netherlands) by incubating in PBS for 10 days. Primary human dermal fibroblasts were allowed to attach overnight on the underside of the de-epidermised dermis. The dermis was turned over the following day and seeded with 3 x 10^5 keratinocytes into a stainless steel ring. After the cells were attached the steel ring was removed and the keratinocytes were grown in Dulbecco's modified Eagles medium/Ham F12 (in a ratio 3:1) + 10% fetal calf serum for 4 days before raising the grid to the air liquid interface. The culture was continued for the next 2 weeks before being embedded in OCT and snap frozen.

Organ Culture and Grafting of skin onto nude mice

Skin was maintained *in vitro* using a previously described method (Bickenbach and Holbrook (1987) *J. Invest. Dermatol.* 88: 42-46. Briefly 1cm^2 pieces of skin were supported on millipore filters on stainless steel grids and grown at the air liquid interface in DMEM supplemented with 50μg/ml ascorbic acid, 10ng/ml epidermal growth factor, 4mM glutamine, 10% FCS, 100μg/ml streptomycin and 100U/ml penicillin. Samples grown *in vitro* for specified times were snap frozen in OCT.

In order to investigate the importance of other signals in maintaining MCSP expression, skin maintained in organ culture for 4 weeks was grafted onto balb/c nu/nu mice. Grafting was done according to previously described techniques. Grafts were exposed 3 weeks after grafting and removed for analysis 3 weeks later. Samples were snap frozen in OCT.

DISCUSSION

Maintenance of epidermal integrity requires the existence of epidermal stem cells which have the ability to both self-renew during adult life and produce daughter cells that diffferentiate to form a normal stratified squamous epithelium. Although much is known about the properties of epidermal stem cells, their study has been hampered by the absence of markers that can distinguish stem cells from other basal keratinocytes. In this study we demonstrate that a monoclonal antibody which recognises a high molecular weight chondroitin sulphate proteoglycan specifically labels outer root sheath keratinocytes and a subpopulation of interfollicular keratinocytes. These interfollicular keratinocytes are preferentially localised at the tips of dermal papilla which is the same location within the skin that one finds putative epidermal stem cells as defined by the intensity of staining for β1 integrin. In order to further characterise the LHM2 positive population of basal keratinocytes we carried out double labelling experiments using a monoclonal antibody that detects keratin 15 as this marker has also been proposed as a marker of epidermal stem cells and we have observed in a previous study that keratin 15 shows discontinous expression in basal keratinocytes. By combining these two markers we have been able to subdivide basal keratinocytes into three distinct populations. LHM2 positive, K15 negative cells which are predominately localised at the tips of dermal papilla, LHM2 negative, K15 positive located predominately in the rete ridges and a small population of LHM2 positive/K15 positive cells which lie in the transition zone between these two basal keratinocyte subpopulations. Although we have not been able to directly determine the order of the transition of these different subpopulations as keratinocytes mature from stem cell to transit amplifying cell several lines of evidence suggest that the LHM2 positive/K15 negative cells are likely to be less

differentiated than the K15 positive cells. The transition of basal keratinocytes phenotype observed using these two markers within the basal layer is in keeping with a recent study which suggests that transit amplifying cells migrate along the basement away from β1 integrin putative stem cell clusters at the tips of the dermal papillae into the rete ridges. Further support for this order of transition comes from our results on the staining pattern for these two markers in BCCs which are characterised by downgrowths of predominately undifferentiated keratinocytes and are regarded as puripotential as they not infrequently show slight degrees of differentiation towards, hair follicles, sebaceous or apocrine glands. In a recent study trichoepitheliomas which are more differentiated than BCCs were found to more frequently express K15 than BCCs. This finding and our observation that BCC tumour islands showed uniform staining with LHM2 and only focal staining with K15 suggests that K15 positive keratinocytes are more differentiated than LHM2 positive cells. The loss of LHM2 staining with increasing differentiation is supported by the pattern of immunostaining in SCCs with expression in the undifferentiated basal cell layer which is lost as the keratinocytes differentiate.

Characterisation of the antigen recognised by LHM2 in previous work indicated that the antigen detected is a high molecular weight chondroitin sulphate proteoglycan. This proteoglycan is a highly expressed immunogenic molecule expressed on more than 90% of human melanoma tissues and cultured melanoma cell lines. The cDNA sequence for the core protein of this melanoma associated chondroitin sulphate proteoglycan has been identified and shows 81% homology with the rat chondroitin sulfate proteoglycan, NG2, which was originally isolated from a chemically induced rat neuronal tumour. Both MCSP and NG2 show a restricted expression pattern in adult tissues and they are not known to be expressed in

epithelial cells. Using RT-PCR we have clearly demonstrated that MCSP transcripts are present in normal skin and Northern blot analysis has shown that expression is increased in BCCs and that the transcript size is identical to that seen in melanoma cell lines. Comparison of the immunostaining pattern of human skin with LHM2 and a polyclonal antibody against NG2 suggests that both antibodies recognise a common epitope expressed on keratinocytes and that NG2 also recognises epitopes on muscle cells which are known to express NG2 in rodents. Although LHM2 did not readily stain murine skin, NG2 did recognise basal and outer root sheath keratinocytes. In contrast to human skin where NG2 expression was restricted to only a subpopulation of interfollicular basal keratinocytes, mouse skin showed widespread expression of NG2 in interfollicular keratinocytes. The basis for these differences in the expression pattern between mouse and human skin are not clear and warrant further investigation

The similar distribution of keratinocytes in human skin detected with the NG2 and LHM2 antibodies and the high sequence homology between these two proteins suggest that NG2 and MCSP are either allelic or very closely related. Functional characterisation of MCSP has been largely confined to studies on melanoma cell lines. These studies have implicated MCSP in growth control and cell adhesion and recent work has shown that MCSP interacts with and stimulates integrin-$\alpha 4$ $\beta 1$-mediated adhesion by activating a signal transduction pathway that links Cdc42 activation to cytoskeletal organisation. The localisation of MCSP protein to the apicolateral surface of basal keratinocytes however suggests that the primary role of this proteoglycan in keratinocyte may not be concerned with cell basement membrane interactions. NG2 is known to be expressed in immature cells which are capable of mitosis and often show a high degree of mobility. A characteristic feature of NG2 expressing cells is a degree of

developmental plasticity and in most cell types NG2 expression is downregulated by terminal differentiation. The identification of MCSP expression in more undifferentiated keratinocytes and the loss of expression with differentiation would be compatible with a role for this molecule in 5 controlling cell growth and differentiation in keratinocytes. Morphological analysis of different subpopulations of EGFP marked keratinocytes in confluent sheets of cultured keratinocytes have revealed differences in the compaction of keratinocytes which may be due to differences in motility or intercellular adhesiveness. Given the importance of MCSP/NG2 in 10 regulating motility and adhesiveness in other cell types it will be interesting to determine if MCSP is involved in regulating transit of keratinocytes from the stem cell compartment in human skin.

In addition to establishing that keratinocytes express MCSP we present 15 evidence that the antigen on MCSP detected by LHM2 is lost in both cultured keratinocytes and whole skin grown in organ culture. This loss of antigen expression could be due to loss of MCSP expressing keratinocytes in culture, alterations in MCSP transcription or RNA stability or changes in post-translational protein processing. The re-establishment of MCSP 20 expression in skin grafted onto immune deficient mice suggests that keratinocytes within the grafted skin when placed in the correct environment can re-express MCSP protein. There are at least two possible explanations for this observation. One possibility is that MCSP is being re-expressed on keratinocytes which have previously expressed MCSP and 25 that the loss seen when these cells are cultured is temporary. The second possibility is that MCSP positive cells are selectively lost during primary keratinocyte culture and that there is sufficient developmental plasticity to allow re-expression in the presence of the correct environmental cues. It is well recognised that skin grafted onto immune deficient mice can be

maintained for longer and shows a better morphology than cultured skin and keratinocytes on tissue culture plastic. The basis for these differences are still not clear but it is likely from related studies investigating appendage formation and regulation of keratins expression that mesenchymal signals play a key role in the normalisation of the phenotype of grafted skin. Although at present we have no direct evidence of the basis for loss of LHM2 staining in cultured keratinocytes, the close relationship between MCSP protein levels and mRNA levels in melanoma cell lines and BCCs indicates that it will be important in future studies to determine if the loss of MCSP expression is due to changes in gene transcription as this may shed light on the signals interesting in the maintenance of MCSP expression.

In conclusion, the results of this study indicate that MCSP protein is expressed in normal human skin and BCCs and the expression in normal human skin is restricted to outer hair root sheath keratinocytes and a subpopulation of interfollicular basal keratinocytes (which are believed to be epidermal stem cells). The increased MCSP protein expression seen in BCCs is accompanied by a corresponding increase in MCSP mRNA, indicating that the MCSP expression in BCC may be transcriptionally regulated. The basis for the upregulation of MCSP transcript in BCCs is still not known.

Example 2: Use of MCSP antibody to isolate epidermal stem cells

A selection system, where the wanted cells are immunomagnetically labelled and bound to a magnetic column, may be used. The undesired cells, ie those which do not express MCSP, are allowed to flow through the column. A suitable system involves the selective binding of colloidal magnetic dextran iron particles to stem cells using bispecific tetrameric

antibody complexes. These complexes recognise both dextran and MCSP. Labelled cells are passed over a column placed in a magnetic field. Cells with antibody complexes, and therefore dextran iron, on their surfaces are retain within the column. The undesired cells, which have not been labelled
5 with antibody pass through the column and may be discarded.

The method is essentially as described in the StemSep™ manufacturer's directions (StemCell Technologies Inc, Vancouver, British Columbia, Canada V5Z 4J7) and in US 5,877,299, with the following modifications.
10 The cell sample is one comprising epidermal stem cells.

The tetrameric antibody complex comprises antibody selective for MCSP as the anti-cell antibody. Additional tetrameric complexes with antibody to β1 integrin (for use in an enrichment step) may be made in the same way.

15

The cells are immunomagnetically labelled by adding the MCSP tetrameric antibody complex to the cells as directed.

Stem cell separation is achieved by loading the labelled cell sample into the
20 top of a primed StemSep™ column and feeding it through the column according to the manufacturer's directions. The flow-through fluid is discarded.

To remove the cells from the StemSep™ column, the column is removed
25 from the magnet and adjusted so fluid flow is permitted between the side syringe and column. Media is added to the top of the column and flushed back and forth between the column and side syringe several times. All media is drawn into the side syringe. The side syringe is disconnected and

all media with positively selected cells is expelled into a collection tube. This removal stage is repeated for maximum recovery of stem cells.

Prior enrichment of the sample for epidermal stem cells by preselection of
5 β1 integrin-bright cells may be achieved in the same way as described above, except using a tetrameric antibody complex selective for β1 integrin instead of MCSP. Cells bound to the β1 integrin antibody complex are then used as the cell sample for stem cell isolation using MCSP antibody complex as described.

10

Prior removal of cells expressing proliferation markers such as transferrin receptor may be achieved by using a tetrameric antibody complex selective for the proliferation marker, but retaining all cells which flow through the column and discarding all cells bound to the column.

15

An alternative isolation method is described in WO 91/09938, with the following modifications: an antibody capable of selectively binding MCSP is bound to magnetic particles before of after binding to cells expressing the MCSP polypeptide. The magnetic particles and attached cells are separated
20 from other cells by magnetic aggregation and the cells are liberated from the magnetic particles to leave a positively selected population of cells expressing MCSP.

Example 3

25

Cloning and analysis of the MSCP promoter sequence

The MCSP promoter was cloned using vectorette PCR. Genomic DNA was digested with restriction enzymes and ligated with bubble linkers (DNA
30 duplexes with a region of non-homology in the centre). PCR was

performed using one primer from a sequence of interest and another primer that binds to the linkers (Figure 6).

Walk 1

SSP2	TCTGGCCAACATAGTCAGGGTCAAAGC
Position	49-75 X96753
SSP3	CAAGGCCAGGCCGGGGGCTGGAAGT
Position	24-48 X96753

Walk 2 primers based sequence isolated walk 1

SSP4	CAGTAGGTGTGCAATAAATGCCTGCT
SSP5	CCAATTGCTGCTGTCTGTGGGGATT

Linkers and arbitrary primers used are as described in Clontech Genome walker Kits cat Number K1803-1.

This allowed amplification of sequences adjacent to known sequences. Using this technique, we walked from the 5' end of the MCSP gene into previously unidentified upstream sequences. Several steps were taken to isolate approximately 1 kb of upstream sequence.

A genomic clone was identified by database searching, which contained some MCSP exons. This clone is regarded as complete, yet was missing the first 87 nucleotides of the reported MCSP gene sequence, implying that there was either a large intron (more than 30kb) between the first and second exon, or the that the clone is rearranged.

Using the sequence identified from our vectorette PCR in a BLAST search, we identified an unfinished sequence with homology to our sequence and

which contained the first 87 nucleotides of the MCSP gene (GenBank Accession No AC019294).

The contents of this clone have not been annotated and there is no indication that any of its sequence is associated with the MCSP gene.

Example 4: Isolation of stem cells using GFP expression driven by the MCSP promoter

The method is essentially as described in Singh Roy *et al* (2000) *Nature Medicine* 6: 271-277 with the following modifications.

A sample of cells comprising epidermal stem cells are transfected with plasmid DNA bearing a fluorescent transgene, such as the green fluorescent protein (GFP) placed under the control of the MCSP promoter (shown in Figure 7; the stem cells alone fluoresce and can be identified and isolated by FACS.

Transfection may be achieved according to US 989,837, using the polycationic lipid GeneFECTOR (VennNova), or other methods well known in the art.

Isolation of transfected cells may be done using FACS analysis. 24 hours post-infection, cells may be removed from culture using 0.5mM EDTA and 0.1% trypsin. After short centrifugation (440xg for 5 minutes), cells may be resuspended in serum-containing medium at a density of 5-7x10^6 cells/ml. This suspension may be then filtered through 42μm sterile mesh (Tetko, Inc.). Immediately prior to sorting, cells can be stained with propidium iodide. Transfected cells may then be sorted ion a FACS apparatus such as

a FACStar Plus (Becton Dickinson) equipped with a coherent argon laser tuned to 488 nm. Transfection efficiency data may be obtained with CellQuest software (Becton Dickinson). FACS analysis of β1 bright cells may be performed as described in Jones and Watt (1993) *Cell* 73: 713-724.

5

Expression of GFP may be checked after sorting by use of an IX-70 inverted fluorescent microscope with a GFP short band pass filter, or by confocal microscopy where GFP expression can be analysed using a confocal laser scanning microscope (Nikon Diaphot 200) with excitation at

10 488nM and detection at 500-530 nm bandpass filter.

CLAIMS

1. A method of isolating an epidermal stem cell from a sample containing epidermal stem cells, the method comprising selecting cells from the sample which express a polypeptide whose expression is driven by the MCSP promoter.

2. A method of enriching for epidermal stem cells in a sample containing epidermal stem cells, the method comprising enriching for cells which express a polypeptide whose expression is driven by the MCSP promoter.

3. A method of identifying an epidermal stem cell in a sample containing epidermal stem cells, the method comprising determining which cells in the sample express a polypeptide whose expression is driven by the MCSP promoter.

4. A method according to any one of Claims 1 to 3 wherein the polypeptide whose expression is driven by the MCSP promoter is MCSP.

5. A method of isolating an epidermal stem cell from a sample containing epidermal stem cells, the method comprising selecting cells from the sample which express MCSP.

6. A method of enriching for epidermal stem cells in a sample containing epidermal stem cells, the method comprising enriching for cells which express MCSP.

7. A method of identifying an epidermal stem cell in a sample containing epidermal stem cells, the method comprising identifying cells which express MCSP.

5 8. A method according to any one of the preceding claims wherein the selection, enrichment or identification is made using a binding agent capable of selectively binding the polypeptide whose expression is driven by the MCSP promoter.

10 9. A method according to Claim 8 wherein the binding agent is an antibody to the polypeptide whose expression is driven by the MCSP promoter.

10. A method according to any one of the preceding claims wherein the
15 sample containing epidermal stem cells is the germinative or basal layer of skin.

11. A method according to any one of the preceding claims wherein the selection, enrichment or identification is made using an antibody to
20 MCSP.

12. A method according to any one of the preceding claims wherein the MCSP promoter comprises the nucleotide sequence given in Figure 7 or a part thereof which has epidermal stem cell selective promoter or
25 transcription regulatory activity.

13. A method according to any one of the preceding claims wherein an immobilised binding agent is used to select, enrich for or identify epidermal stem cells.

14. A method according to any one of the preceding claims wherein a fluorescent or resistance marker is used to select, enrich for or identify epidermal stem cells.

5

15. A method according to any one of the preceding claims wherein the selection, enrichment or identification uses drug resistance.

16. A method according to any one of the preceding claims wherein the cells are further selected or enriched for, or identified as, cells which do not substantially express one or more markers of cell proliferation in keratinocytes.

10

17. A method according to any one of Claims 1 to 15 wherein the cells are further selected or enriched for, or identified as, cells which express one or both of $\beta 1$ integrin and $\alpha 6$ integrin.

15

18. A method according to Claims 1 or 2, the method comprising the steps of
    - i) obtaining a sample containing epidermal stem cells;
    - ii) contacting the sample with an agent which selectively binds the polypeptide whose expression is driven by the MCSP promoter;
    - iii) allowing the cells to bind to the agent;
    - iv) removing unbound cells;
    - v) releasing bound cells; and
    - vi) collecting the released cells.

20

25

19. A method according to Claims 1 or 2, the method comprising the steps of
    i) obtaining a sample containing epidermal stem cells;
    ii) transfecting or transducing cells in said sample with a genetic construct encoding a detectable polypeptide whose expression is driven by the MCSP promoter;
    iii) selecting for the detectable polypeptide; and
    iv) collecting those cells selected for expressing the detectable polypeptide.

20. A method according to Claim 19 wherein the detectable polypeptide is a fluorescent or a drug resistance marker.

21. A method according to any one Claims 1 to 12 or 18 or 19 wherein the cell selection, enrichment, identification or selection uses fluorescence activated cell sorting.

22. A method according to any one of Claims 18 to 21 wherein the sample containing epidermal stem cells is pre-enriched for epidermal stem cells by the steps of
    i) contacting the sample containing epidermal stem cells with an agent which selectively binds β1 integrin or α6 integrin;
    ii) allowing the cells to bind to the agent;
    iii) removing unbound cells;
    iv) releasing bound cells; and
    v) collecting the released cells.

23. A method according to any one of Claims 18 to 21 wherein the sample containing epidermal stem cells is pre-enriched for epidermal stem cells by the steps of
    i) contacting the sample with an agent which selectively binds a polypeptide associated with cell proliferation;
    ii) allowing the cells to bind to the agent;
    iii) collecting unbound cells; and
    iv) rejecting bound cells.

24. A method according to either one of Claims 16 or 23 wherein the polypeptide associated with cell proliferation is transferrin receptor.

25. A method according to any one of Claims 18 to 21 wherein the sample containing epidermal stem cells is pre-enriched for epidermal stem cells by the steps of
    i) coating a tissue culture dish with one or more components selected from collagen type IV, collagen type VII, fibronectin, vitronectin and laminin; and
    ii) incubating the sample containing epidermal stem cells in the dish for no longer than 30 minutes; and
    iii) removing unbound cells; and
    iv) collecting the cells bound to the culture dish.

26. A population of cells enriched for epidermal stem cells obtained by or obtainable by the method of either one of Claim 2 or Claim 5.

27. A population of cells from the germinative or basal layer of skin enriched for cells which express MCSP on their surface.

28. A population of cells according to Claim 26 or 27 having substantially no detectable expression of transferrin receptor.

29. An isolated epidermal stem cell obtained by or obtainable by the method of Claim 1 or Claim 4.

30. An isolated cell from the germinative or basal layer of skin which expresses MCSP on its surface.

31. An isolated cell according to Claim 30, further characterised by having substantially no detectable expression of keratin 15.

32. A population of cells according to any one of Claims 26 to 28 or an isolated cell according to any one of Claims 29 to 31 wherein the cells comprise a recombinant nucleic acid molecule.

33. A population of cells or an isolated cell according to any one of Claims 26 to 32 for use in medicine.

34. A method of treating a patient in need of treatment with epidermal stem cells, the method comprising administering to the patient a population of cells according to any one of Claims 26 to 28 or an isolated cell according to any one of Claims 29 to 32.

35. A method according to Claim 34 for treating a metabolic disease.

36. A method according to Claim 34 for treating skin damage.

37. A method of delivering a therapeutic agent to a patient, the method comprising administering to the patient a population of cells according to any one of Claims 26 to 28 or an isolated cell according to any one of Claims 29 to 32 wherein the cells express or contain
5 the therapeutic agent.

38. A method according to Claim 37 wherein the cells express or contain the therapeutic agent due to the presence of a recombinant nucleic acid molecule therein.

10

39. Use of a population of cells according to any one of Claims 26 to 28 or an isolated cell according to any one of Claims 29 to 32 in the manufacture of a medicament for treating a patient in need of treatment with an epidermal stem cell.

15

40. Use of a population of cells according to any one of Claims 26 to 28 or an isolated cell according to any one of Claims 29 to 31 wherein the cells express a therapeutic agent in the manufacture of a medicament for delivering the therapeutic agent to a patient.

20

41. Use of a reagent which selectively binds to MCSP in isolating epidermal stem cells.

42. Use of a reagent according to Claim 41 wherein the reagent is an
25 antibody.

43. Use according to any one of Claims 41 and 42 wherein the binding agent is immobilised.

44. A method of determining suitable conditions for culture of epidermal stem cells *in vitro* the method comprising culturing said cells, determining expression of a polypeptide whose expression is driven by the MCSP promoter by said cells and if expression of said
5 polypeptide is retained then the conditions are suitable.

45. A method according to Claim 44 wherein expression of keratin 15 by the epidermal stem cells is also determined, and if keratin 15 is also expressed then the conditions are not suitable.
10

46. A method of screening for substances which modulate the differentiation state of epidermal stem cells, the method comprising
    i) providing a population of cells enriched for epidermal stem cells or an isolated epidermal stem cell;
15 ii) contacting said cells with a test substance; and
    iii) detecting a change in the expression from the MCSP promoter.

47. A method of screening for substances which modulate the rate of
20 division of epidermal stem cells, the method comprising
    i) providing a population of cells enriched for epidermal stem cells or an isolated epidermal cell according to any one of Claims 26 to 32;
    ii) contacting said cells with a test substance; and
25 iii) detecting a change in the rate of cell division.

48. A substance obtained or obtainable by the method Claims 46 or 47.

49. A substance according to Claim 48 for use in medicine.

50. A method of treating a hyperproliferative skin condition such as psoriasis the method comprising administering to the patient a substance according to Claim 49.

5

51. Use of a substance according to Claim 49 in the manufacture of a medicament for treating a hyperproliferative skin condition such as psoriasis.

10 52. A polynucleotide comprising the nucleic acid sequence given in Figure 7 or a fragment or variant thereof. which is not the clone corresponding to GenBank Accession No. AC019294 or AC 068368.

53. A polynucleotide according to Claim 52 which has transcriptional
15 promoter activity.

54. A polynucleotide according to either one of Claims 52 or 53 which promotes transcription in epidermal stem cells.

20 55. A polynucleotide as defined in any of Claims 52 to 54 operatively linked to a polynucleotide encoding a polypeptide.

56. A polynucleotide according to Claim 55 wherein the polypeptide is a readily dectectable polypeptide.

25

57. A polynucleotide according to Claim 56 wherein the readily detectable polypeptide is a fluorescent polypeptide such as a green fluorescent protein or is a drug resistance polypeptide.

58. A polynucleotide according to Claim 55 wherein the polypeptide is a therapeutic polypeptide.

59. A polynucleotide according to any one of Claims 52 to 58 for use in medicine.

60. A method of treating a patient in need of treatment with a therapeutic polypeptide, the method comprising administering to the patient a polyncleotide according to Claim 58.

61. Use of a polynucleotide according to Claim 58 in the manufacture of a medicament for treating a patient in need of treatment with the therapeutic polypeptide.

62. Use of a polynucleotide according to any of Claims 52 to 57 in a method to isolate or identify, or enrich for, epidermal stem cells.

63. A compound comprising a moiety capable of selectively binding to MCSP and a polynucleotide.

64. A compound according to Claim 63 wherein the binding agent is an antibody.

65. A compound according to Claim 63 or 64 wherein the polynucleotide encodes a polypeptide.

66. A compound according to Claim 65 wherein the polypeptide is a readily detectable polypeptide.

67. A compound according to Claim 63 wherein the polynucleotide encodes a therapeutic agent.

68. A compound according to any one of Claims 63 to 67 for use in
5 medicine.

69. A method of introducing genetic material into epidermal stem cells, the method comprising contacting the cells with a compound according to any one of Claims 63 and 67.

10

70. A method of treating a patient in need of treatment with a therapeutic agent, the method comprising administering to the patient a compound according to Claim 67.

15 71. Use of a compound according to Claim 67 in the manufacture of a medicament for treating a patient in need of treatment with the therapeutic agent.

72. A pharmaceutical composition comprising a population of cells
20 according to any one of Claims 26 to 28 or an isolated cell according to any one of Claims 29 to 31, or a substance according to Claim 48, or a polynucleotide according to any one of Claims 52 to 58, or a compound according to any one of Claims 53 to 58 and a pharmaceutically acceptable carrier.

25

73. A kit of parts comprising a binding agent capable of selectively binding to MCSP and at least one additional binding agent capable of selectively binding to a component selected from the group

consisting of keratin 15, β1 integrin, α6 integrin and transferrin receptor.

74. A kit of parts according to Claim 73 wherein at least one of the
5 binding agents is an antibody.

75. An epidermal stem cell isolatable by the methods herein described.

76. A population of cells enriched for epidermal stem cells obtainable by
10 the methods as herein described.

77. A method of making skin suitable for a skin graft the method comprising culturing a population of cells enriched for epidermal stem cells or an isolated epidermal stem cell according to any one of
15 Claims 26 to 32 under conditions where they form skin.

78. Skin suitable for a skin graft obtained by the method of Claim 77.

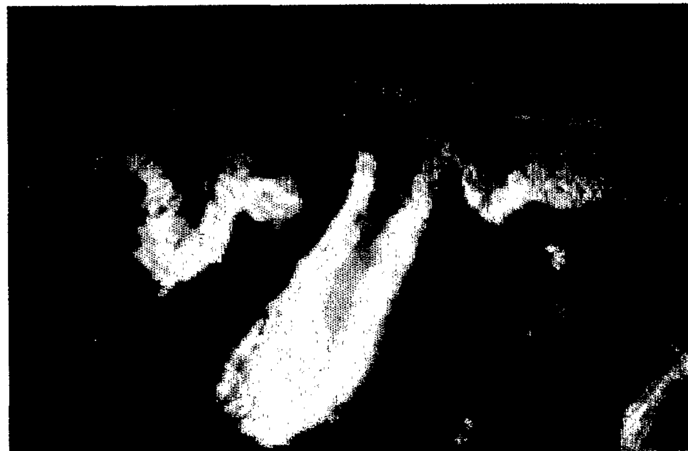

Fig. 1

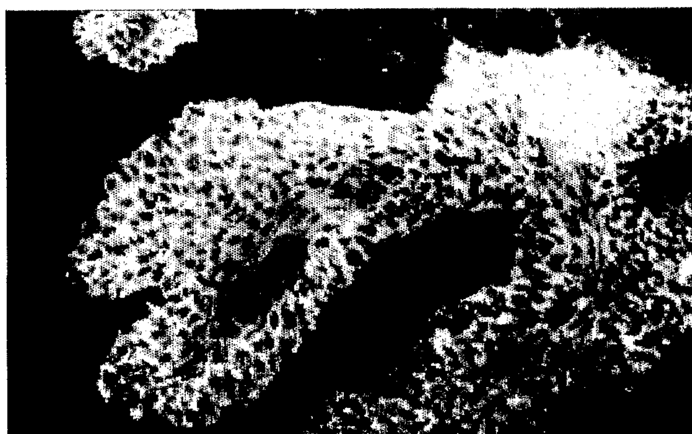

Fig. 2

RTPCR of MCSP Transcripts

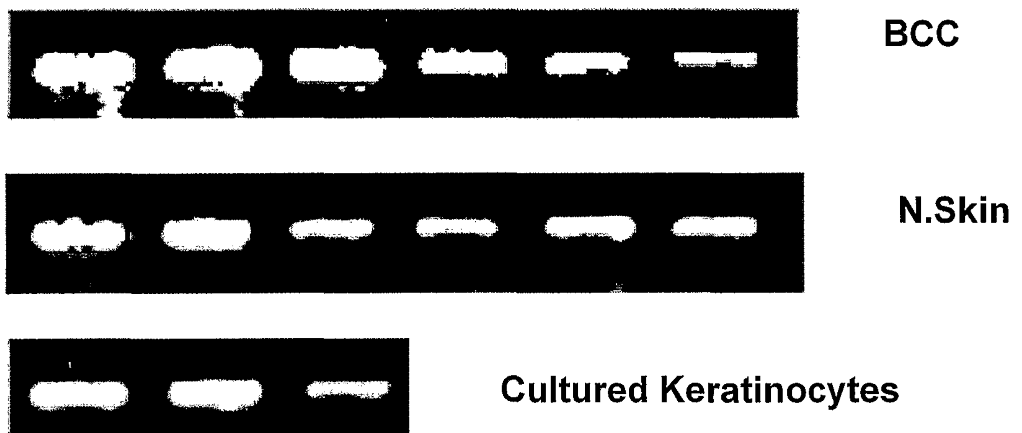

Fig. 3

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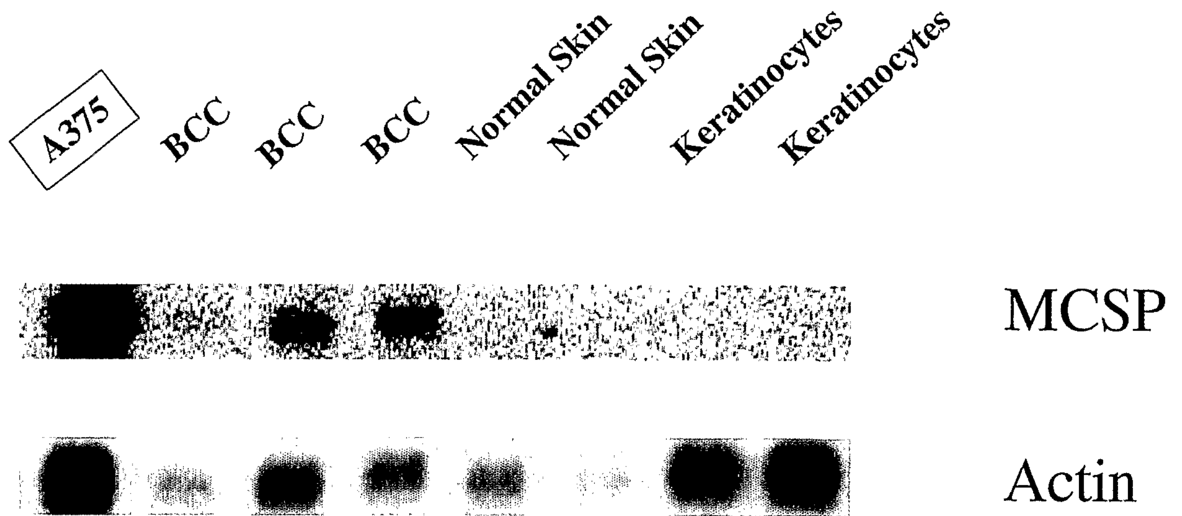

Fig. 4

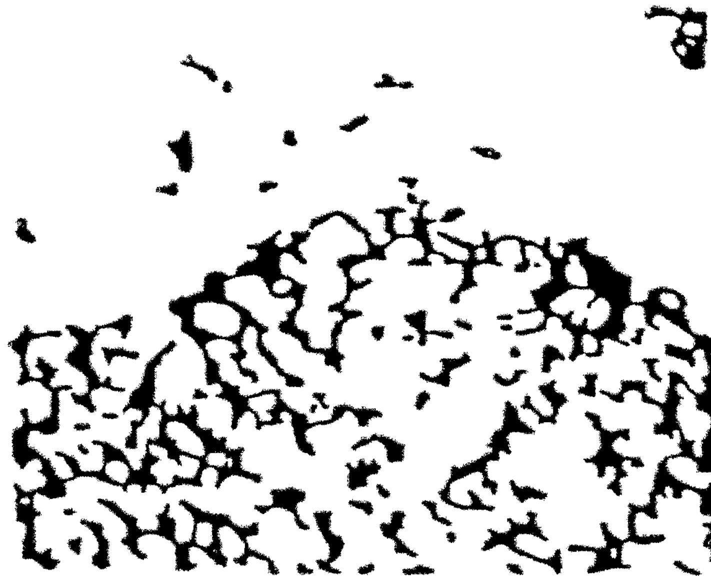

Fig. 5

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Figure 6

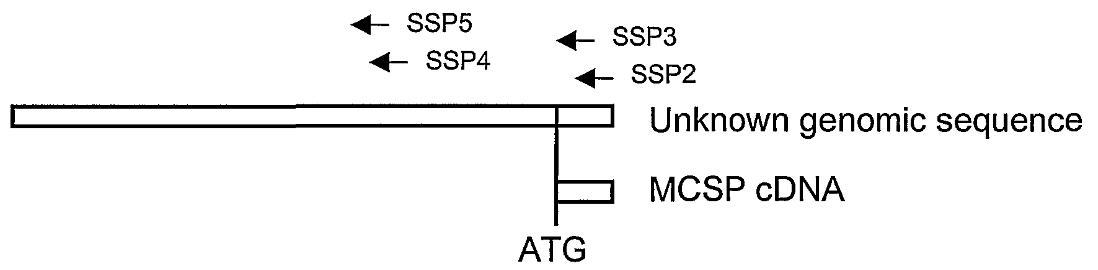

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Figure 7

5′

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GAGCTACGGAGATCAGGACTCCAGGGAATCTTTTGAAAGAGAAGTCGACGTGC
ACGAAGGAGGGCTAGGAGGGCTTGTTGGAGAAAGGTgAGATGTGGCCAGGGAG
GTAGGGGCAGTCAGGGATGgCCACCAAGGGTGCCTGGCCAAGCCATACCGAGG
AAAGTCATGGGAAAGGTTGGAGGGCAGGAGGAAGAAGGTCCGAGCCAGACCTG
AGGATGCCAGAGAGGCCCAGGTCAGCTCTGTTCAGCACTGAGGCTTCAGGGCC
TCGGGGGAgCTTGCTGAGGAGCTGGGGAGGGGACAGTTGAgGAGCAgGCACGG
CTGGTCCTGCAgCCTTTgTTCCAAgACAGTgGGAGGTGGTCAgGGGTCAGTTC
CAAAGGCACACAGGGGCCTcGACTTTCTGCCAGTCTGCCTGGCATGATGCTGC
TCAGCATTGGTCCAGAGTTCCAGCCTAGGTTACGGcTGAGCAAAGGCCAGGCC
TGTGTGCGCTGGAGCCGCAGCATCCCTTTTCCACTGAGAGCCTCAGTTTCCTC
ATCTGAAGCAGGACACAGTTGACCAACACATACGCTTCCCAAAGTTGTCAAGA
GACCAGGTGAATGTTTTTTGTAAACTGGAAAGCCCCAGCAGAGATGCACCAGC
CGGGTTATTACCGCCGTGCTGGTTGCCATTATTTTCCAGGCTTGTGTTTCTCT
GCCCAGCTAGCAACAAGGCAGGGGGAGGCTCCCAGCTAATCCAGACTTGAATC
TCAGCTGTGGGTGGGAGCGCCCTGTCCGGAGGCTCCACAGACATCTGTGTAGG
TGGGGAGCAGGGAAGATGGGGAGGGGGAAGGCGGGGGAGATGGGGCCAGCAG
AGATCTTCACCCAAATTTTTGATTCATGGAACAAAAAGCCTTCTGTCTTATTT
TTTCATTCCTGAAAAGAAAGCTCTTTCCCTCCAAACACAGTCTTCCAAGCAGc
ACCCCCAACCCCAGGCCCACAGTTGGCCAGTAATTAGCCCCTAATGAGGACCA
GGTGTCcTGCTGACTTGAGGAGAAGGAAAAACAAGGGACAAATTGTGGAATCA
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AGGAGAAAAGATAAGCATTTATTGAGCACCTACTGTATGCCAGGTCCTGGGCT
AGGCACTTGAGGTACATTAAAGCATTTAATCCTCATCAACCCAATTTTACAAG
TGAGGAAACTGCCACAGAGAGGTGAGGTGACTTGTTCAAGGTTTGACAGCCAA
GTAGGAGAGCGGGGATTCGAACCCAGGTCTCTCTGGTTTCAAGACCAGTACTC
ATAACCCCCGCATGGATCAGTCACCATCACACCTCGAAAGTCCTGACACTCAG
CCAGCCGGGATGCAGTCC 3′

Figure 8 (Page 1 of 4)

%identity 82.903

%similarity 86.003

Where the gap creation penalty was 8, the gap extension penalty was 2 and the scoring scheme used was the BLOSUM62 table.

Accession numbers: Human MCSP trembl:Q92675, and Rat NG2 tremblnew:CAA39884

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GAP of: mcsp.aa check: 4629 from: 1 to: 2322

REFORMAT of: mcsp.aa check: 4629 from: 1 to: 2322 August 21,
2000 11:40
 (No documentation)

to: ng2.aa check: 1322 from: 1 to: 2326

REFORMAT of: ng2.aa check: 1322 from: 1 to: 2326 August 21,
2000 11:41
 (No documentation)

Symbol comparison table:
/molbio1/software/gcg/gcgcore/data/rundata/blosum62.cmp
CompCheck: 6430

Gap Weight: 8 Average Match: 2.912
Length Weight: 2 Average Mismatch: -2.003

Quality: 10025 Length: 2326
Ratio: 4.317 Gaps: 2
Percent Similarity: 86.003 Percent Identity: 82.903

mcsp.aa x ng2.aa August 21, 2000 11:44 ..

. . . . .
1    MQSGRGPPLPAPGLALALTLTMLARLASAASFFGENHLEVPVATALTDID 50
1    MLLSPGHPLSAPALALILTLALLVRSTAPASFFGENHLEVPVPSALTRVD 50
. . . . .
51   LQLQFSTSQPEALLLLAAGPADHLLLQLYSGRLQVRLVLGQEELRLQTPA 100
51   LLLQFSTSQPEALLLLAAGQTDHLLLQLQSGHLQVRLALGQNELSLQTPA 100
. . . . .
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Figure 8 (Page 2 of 4)

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101  ETLLSDSIPHTVVLTVVEGWATLSVDGFLNASSAVPGAP.LEVPYGLFVG 149
101  DTVLSDSTTHTVVLTVSNSWAVLSVDGVLNTSAPIPKASHLKVPYGLFVG 150

150  GTGTLGLPYLRGTSRPLRGCLHAATLNGRSLLRPLTPDVHEGCAEEFSAS 199
151  SSGSLDLPYLKGISRPLRGCLHSAILNGRNLLRPLTPDVHEGCAEEFSAG 200

200  DDVALGFSGPHSLAAFPAWGTQDEGTLEFTLTTQSRQAPLAFQAGGRRGD 249
201  DEVGLGFSGPHSLAAFPAWSTREEGTLEFTLTTRSQQAPLAFQAGDKRGN 250
. . . . .
250  FIYVDIFEGHLRAVVEKGQGTVLLHNSVPVADGQPHEVSVHINAHRLEIS 299
251  FIYVDIFEGHLRAVVEKGQGTMLLRNSVPVADGQPHEVSVHIDVHRLEIS 300
. . . . .
300  VDQYPTHTSNRGVLSYLEPRGSLLLGGLDAEASRHLQEHRLGLTPEATNA 349
301  VDQYPTRTFNRGVLSYLEPRGSLLLGGLDTEASRHLQEHRLGLTPGAANI 350
. . . . .
350  SLLGCMEDLSVNGQRRGLREALLTRNMAAGCRLEEEEYEDDAYGHYEAFS 399
351  SLVGCIEDFSVNGRRLGLRDAWLTRDMAAGCRPEEDEYEEEVYGPFEAFS 400
. . . . .
400  TLAPEAWPAMELPEPCVPEPGLPPVFANFTQLLTISPLVVAEGGTAWLEW 449
401  TLAPEAWPVMDLPEPCVPEPGLPAVFANFTQLLTISPLVVAEGGTAWLEW 450
. . . . .
450  RHVQPTLDLMEAELRKSQVLFSVTRGAHYGELELDILGAQARKMFTLLDV 499
451  RHVQPTLDLTEAELRKSQVLFSVSQGARHGELELDIPGAQTRKMFTLLDV 500
. . . . .
500  VNRKARFIHDGSEDTSDQLVLEVSVTARVPMPSCLRRGQTYLLPIQVNPV 549
501  VNRKARFVHDGSEDTSDQLMLEVSVTSRAPVPSCLRRGQIYILPIQVNPV 550
. . . . .
550  NDPPHIIFPHGSLMVILEHTQKPLGPEVFQAYDPDSACEGLTFQVLGTSS 599
551  NDPPRIVFPHGSLMVILEHTQKPLGPEIFQAYDPDSACEGLTIQLLGVSA 600
. . . . .
600  GLPVERRDQPGEPATEFSCRELEAGSLVYVHCGGPAQDLTFRVSDGLQAS 649
601  SVPVEHRDQPGEPVTEFSCRDLEAGNIVYVHRGGPAQDLTFRVSDGMQAS 650
. . . . .
650  PPATLKVVAIRPAIQIHRSTGLRLAQGSAMPILPANLSVETNAVGQDVSV 699
651  GPATLKVVAVRPAIQILHNTGLRLAQGSAAAILPANLSVETNAVGQDVSV 700
. . . . .
700  LFRVTGALQFGELQKHSTGGVEGAEWWATQAFHQRDVEQGRVRYLSTDPQ 749
701  LFRVTGTLQFGELQKQGAGGVEGTEWWDTLAFHQRDVEQGRVRYLSTDPQ 750
. . . . .
750  HHAYDTVENLALEVQVGQEILSNLSFPVTIQRATVWMLRLEPLHTQNTQQ 799
751  HHTQDTVEDLTLEVQVGQETLSNLSFPVTIQRATVWMLQLEPLHTQNPHQ 800
. . . . .
800  ETLTTAHLEATLE...EAGPSPPTFHYEVVQAPRKGNLQLQGTRLSDGQG 846
801  ETLTSAHLEASLEEEGEGGPYPHIFHYELVQAPRRGNLLLQGTRLSDGQS 850
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Figure 8 (Page 3 of 4)

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847  FTQDDIQAGRVTYGATARASEAVEDTFRFRVTAPPYFSPLYTFPIHIGGD 896
851  FSQSDLQAGRVTYRATTRTSEAAEDSFRFRVTSPPHFSPLYTFPIHIGGD 900

897  PDAPVLTNVLLVVPEGGEGVLSADHLFVKSLNSASYLYEVMERPRLGRLA 946
901  PNAPVLTNVLLMVPEGGEGVLSADHLFVKSLNSASYLYEVMEQPHHGSLA 950
. . . . .
947  WRGTQDKTTMVTSFTNEDLLRGRLVYQHDDSETTEDDIPFVATRQGESSG 996
951  WRDPKGRATPVTSFTNEDLLHGRLVYQHDDSETIEDDIPFVATRQGEGSG 1000
. . . . .
997  DMAWEEVRGVFRVAIQPVNDHAPVQTISRIFHVARGGRRLLTTDDVAFSD 1046
1001 DMAWEEVRGVFRVAIQPVNDHAPVQTISRVFHVARGGQRLLTTDDVAFSD 1050
. . . . .
1047 ADSGFADAQLVLTRKDLLFGSIVAVDEPTRPIYRFTQEDLRKRRVLFVHS 1096
1051 ADSGFSDAQLVLTRKDLLFGSIVAMEEPTRPIYRFTQEDLRKKQVLFVHS 1100
. . . . .
1097 GADRGWIQLQVSDGQHQATALLEVQASEPYLRVANGSSLVVPQGGQGTID 1146
1101 GADHGWLQLQVSDGQHQATAMLEVQASEPYLHVANSSSLVVPQGGQGTID 1150
. . . . .
1147 TAVLHLDTNLDIRSGDEVHYHVTAGPRWGQLVRAGQPATAFSQQDLLDGA 1196
1151 TAVLHLDTNLDIRSGNEVHYHVTAGPHWGQLLRDGQSVTSFSQRDLLDGA 1200
. . . . .
1197 VLYSHNGSLSPEDTMAFSVEAGPVHTDATLQVTIALEGPLAPLKLVRHKK 1246
1201 ILYSHNGSLSPQDTLALSVAAGPVHTSTVLQVTIALEGPLAPLQLVQHKR 1250
. . . . .
1247 IYVFQGEAAEIRRDQLEAAQEAVPPADIVFSVKSPPSAGYLVMVSRGALA 1296
1251 IYVFQGEAAEIRRDQLEVVQEAVLPADIMFSLRSPPNAGYLVMVSHGASA 1300
. . . . .
1297 DEPPSLDPVQSFSQEAVDTGRVLYLHSRPEAWSDAFSLDVASGLGAPLEG 1346
1301 DGPPSLDPVQRFSQEAINSGRVLYLHSRPGAWSDSFSLDVASGLGDPLEG 1350
. . . . .
1347 VLVELEVLPAAIPLEAQNFSVPEGGSLTLAPPLLRVSGPYFPTLLGLSLQ 1396
1351 ISVELEVLPTVIPLDVQNFSVPEGGTRTLAPPLIQITGPYLGTLPGLVLQ 1400
. . . . .
1397 VLEPPQHGPLQKEDGPQARTLSAFSWRMVEEQLIRYVHDGSETLTDSFVL 1446
1401 VLEPPQHGALQKEDRPQDGTLSTFSWREVEEQLIRYVHDGSETQTDGFIL 1450
. . . . .
1447 MANASEMDRQSHPVAFTVTVLPVNDQPPILTTNTGLQMWEGATAPIPAEA 1496
1451 LANASEMDRQSQPMAFTITILPVNDQPPVITTNTGLQIWEGAIVPIPPEA 1500
. . . . .
1497 LRSTDGDSGSEDLVYTIEQPSNGRVVLRGAPGTEVRSFTQAQLDGGLVLF 1546
1501 LRGIDSDSGPEDLVYTIEQPSNGRIALRVAPDAEAHRFTQAQLDSGLVLF 1550
. . . . .
1547 SHRGTLDGGFPFRLSDGEHTSPGHFFRVTAQKQVLLSLKGSQTLTVCPGS 1596
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Figure 8 (Page 4 of 4)

```
1597 VQPLSSQTLRASSSAGTDPQLLLYRVVRGPQLGRLFHAQQDSTGEALVNF 1646
1601 VQPLSSQSLSASSSTGSDPRHLLYQVVRGPQLGRLLHAQQGSAEEALVNF 1650

1647 TQAEVYAGNILYEHEMPPEPFWEAHDTLELQLSSPPARDVAATLAVAVSF 1696
1651 TQAEVNAGNILYEHEISSEPFWEAHDTIGLLLSSSPARDLAATLAVTVSF 1700
. . . . . .
1697 EAACPQRPSHLWKNKGLWVPEGQRARITVAALDASNLLASVPSPQRSEHD 1746
1701 DAACPQRPSRLWRNKGLWVPEGQRAKITVAALDAANLLASVPASQRGRHD 1750

1747 VLFQVTQFPSRGQLLVSEEPLHAGQPHFLQSQLAAGQLVYAHGGGGTQQD 1796
1751 VLFQITQFPTRGQLLVSEEPLHARRPHFLQSELTAGQLVYAHGGGGTQQD 1800
. . . . . .
1797 GFHFRAHLQGPAGASVAGPQTSEAFAITVRDVNERPPQPQASVPLRLTRG 1846
1801 GFRFRAHLQGPTGASVAGPQTSEAFVITVRDVNERPPQPQASIPLRITRG 1850
. . . . . .
1847 SRAPISRAQLSVVDPDSAPGEIEYEVQRAPHNGFLSLVGGGLGPVTRFTQ 1896
1851 SRAPVSRAQLSVVDPDSAPGEIEYEVQRAPHNGFLSLAGDNTGPVTHFTQ 1900
. . . . . .
1897 ADVDSGRLAFVANGSSVAGIFQLSMSDGASPPLPMSLAVDILPSAIEVQL 1946
1901 ADVDAGRLAFVANGSSVAGVFQLSMSDGASPPIPMSLAVDVLPSTIEVQL 1950
. . . . . .
1947 RAPLEVPQALGRSSLSQQQLRVVSDREEPEAAYRLIQGPQYGHLLVGGRP 1996
1951 RAPLEVPQALGRSSLSRQQLQVISDREEPDVAYRLTQGPLYGQVLVGGQP 2000
. . . . . .
1997 TSAFSQFQIDQGEVVFAFTNFSSSHDHFRVLALARGVNASAVVNVTVRAL 2046
2001 ASAFSQLQVDQGDVVFAFTNFSSSQDHFKVLALARGVNASATVNVTVQAL 2050
. . . . . .
2047 LHVWAGGPWPQGATLRLDPTVLDAGELANRTGSVPRFRLLEGPRHGRVVR 2096
2051 LHVWAGGPWPQGTTLRLDPTVLDASELANRTGSMPRFRLLEGPRYGRVVR 2100
. . . . . .
2097 VPRARTEPGGSQLVEQFTQQDLEDGRLGLEVGRPEGRAPGPAGDSLTLEL 2146
2101 VSQGRAESRTNQLVEDFTQQDLEEGRLGLEVGRPEGRSTGPTGDRLTLEL 2150
. . . . . .
2147 WAQGVPPAVASLDFATEPYNAARPYSVALLSVPEAARTEAGKPESSTPTG 2196
2151 QATGVPPAVALLDFATEPYHAAKFYKVTLLSVPEAARTETEKTGKSTPTG 2200
. . . . . .
2197 EPGPMASSPEPAVAKGGFLSFLEANMFSVIIPMCLVLLLLALILPLLFYL 2246
2201 QPGQAASSPMPTVAKSGFLGFLEANMFSVIIPVCLVLLLLALILPLLFYL 2250
. . . . . .
2247 RKRNKTGKHDVQVLTAKPRNGLAGDTETFRKVEPGQAIPLTAVPGQGPPP 2296
2251 RKRNKTGKHDVQVLTAKPRNGLAGDTETFRKVEPGQAIPLTTVPGQGPPP 2300
. .
2297 GGQPDPELLQFCRTPNPALKNGQYWV 2322
2301 GGQPDPELLQFCRTPNPALRNGQYWV 2326
```

Figure 9 (Page 1 of 11)

Using the GCG package application GAP with default parameters the %id for the cDNAs human mcsp and rat ng2 are:

%identity 82.637

%similarity 82.637

Where the gap creation penalty was 50, the gap extension penalty was 3 and the scoring scheme used was the nwsgapdna table where matches score 10 and mismatches sacore 0, matches to IUPAC ambiguity codes also score 10.

Accession numbers: Human MCSP trembl:X96753, and Rat NG2 embl:X56541

GAP of: mcsp.na check: 8465 from: 1 to: 6969

REFORMAT of: mcsp.na check: 8465 from: 1 to: 6969 August 21, 2000 11:42
(No documentation)

to: ng2.na check: 3137 from: 1 to: 6981

REFORMAT of: ng2.na check: 3137 from: 1 to: 6981 August 21, 2000 11:41
(No documentation)

Symbol comparison table:
/molbio1/software/gcg/gcgcore/data/rundata/nwsgapdna.cmp
CompCheck: 8760

Gap Weight: 50 Average Match: 10.000
Length Weight: 3 Average Mismatch: 0.000

Quality: 57454 Length: 6981
Ratio: 8.244 Gaps: 2
Percent Similarity: 82.637 Percent Identity: 82.637

Match display thresholds for the alignment(s):

mcsp.na x ng2.na August 21, 2000 11:46 ..

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```
1    atgcagtccggccgcggccccccacttccagccccggcctggccttggc 50
1    atgcttctcagcccgggacacccgctgtcagctccagccctggccttgat 50
. . . . . .
51   tttgaccctgactatgttggccagacttgcatccgcggcttccttcttcg 100
51   tcttaccttggccttgttggtcagatctacagctcctgcctccttcttcg 100
. . . . . .
101  gtgagaaccacctggaggtgcctgtggccacggctctgaccgacatagac 150
101  gggagaaccacctggaggtgcctgtgccctcagccctgaccagagtagac 150
. . . . . .
151  ctgcagctgcagttctccacgtcccagcccgaagccctccttctcctggc 200
151  ttactgctccagttctccacatcgcagcccgaagccctgctcctcttggc 200
. . . . . .
201  agcaggcccagctgaccacctcctgctgcagctctactctggacgcctgc 250
201  agcaggccaaacagatcatctcctgctgcagctccagtctggacacctac 250
. . . . . .
251  aggtcagacttgttctgggccaggaggagctgaggctgcagactccagca 300
251  aggtcagacttgccctgggacaaaatgagctgagtctgcagacaccagca 300
. . . . . .
301  gagacgctgctgagtgactccatcccccacactgtggtgctgactgtcgt 350
301  gacacggtgctgagtgactccacaacccacaccgtagtgctcaccgtctc 350
. . . . . .
351  agagggctgggccacgttgtcagtcgatgggtttctgaacgcctcctcag 400
351  caacagctgggctgtgctgtctgttgatggagtgttgaacacctctgccc 400
. . . . . .
401  cagtccc...aggagccccccctagaggtcccctatgggctctttgttggg 447
401  ccatcccaaaagcatcccacctcaaagtcccctatgggctctttgtgggc 450
. . . . . .
448  ggcactgggacccttggcctgccctacctgaggggaaccagccgacccct 497
451  tcctctggaagccttgacctgccttacctgaagggaatcagtcgacccct 500
. . . . . .
498  gaggggttgcctccatgcagccaccctcaatggccgcagcctcctccggc 547
501  gaggggctgcctccactcagccattctcaatggccgcaaccttctccgcc 550
. . . . . .
548  ctctgacccccgatgtgcatgagggctgtgctgaagagttttctgccagt 597
551  cactaacccccgatgttcatgagggttgtgctgaagaattctctgctggt 600
. . . . . .
598  gatgatgtggccctgggcttctctgggccccactctctggctgccttccc 647
601  gatgaagttggcctgggcttctctggaccccactcactggctgccttccc 650
. . . . . .
648  tgcctggggcactcaggacgaaggaaccctagagtttacactcaccacac 697
651  tgcctggagcacgcgggaggaaggcaccctggagtttaccctcaccactc 700
. . . . . .
698  agagccggcaggcacccttggccttccaggcagggggccggcgtggggac 747
701  ggagtcagcaagcacccctggccttccaggccggggacaagcgtggcaac 750
```

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```
748  ttcatctatgtggacatatttgagggccacctgcgggccgtggtggagaa 797
751  tttatctacgtggacatatttgagggccacttgcgggctgtggttgaaaa 800
. . . . .
798  gggccagggtaccgtattgctccacaacagtgtgcctgtggccgatgggc 847
801  gggtcagggtaccatgctgcttcgtaacagcgtgcctgtggctgacgggc 850
. . . . .
848  agccccatgaggtcagtgtccacatcaatgctcaccggctggaaatctcc 897
851 agccccatgaggtcagcgtacacatagatgttcaccggctggaaatctct 900
. . . . .
898  gtggaccagtaccctacgcatacttcgaaccgaggagtcctcagctacct 947
901  gtagatcaataccctacacgtactttcaaccgtggggtcctcagctacct 950
. . . . .
948  ggagccacggggcagtctccttctcgggggctggatgcagaggcctctc 997
951  ggagcctcgtggcagtctcctccttgggggctcgatacagaggcctctc 1000
. . . . .
998  gtcacctccaggaacaccgcctgggcctgacaccagaggccaccaatgcc 1047
1001 gccacctccaagaacaccgtctgggcctgacaccgggggctgccaacatc 1050
. . . . .
1048 tccctgctgggctgcatggaagacctcagtgtcaatggccagaggcgggg 1097
1051 tccctggtaggctgcatagaagatttcagtgttaatggcaggaggctggg 1100
. . . . .
1098 gctgcgggaagctttgctgacgcgcaacatggcagccggctgcaggctgg 1147
1101 cctccgggacgcctggctgacccgtgacatggcagcaggctgcaggcctg 1150
. . . . .
1148 aggaggaggagtatgaggacgatgcctatggccattatgaagctttctcc 1197
1151 aggaggatgagtatgaggaagaggtctatggcccgtttgaagctttctcc 1200
. . . . .
1198 accctggctcccgaggcttggccagccatggagctgcctgagccatgcgt 1247
1201 accctggcgcccgaagcctggccagtcatggatctgccagagccgtgtgt 1250
. . . . .
1248 gcctgagccagggctgcctcctgtctttgccaatttcacccagctgctga 1297
1251 tcctgagccgggactgcctgccgtcttcgcgaacttcacgcagctgctca 1300
. . . . .
1298 ctatcagcccactggtggtggccgagggtggcacagcctggcttgagtgg 1347
1301 ccattagtcccctggtcgtggccgagggtggcacagcctggcttgagtgg 1350
. . . . .
1348 aggcatgtgcagcccacgctggacctgatggaggctgagctgcgcaaatc 1397
1351 cggcacgtgcagcccacgctggacctgacagaggcggagctgcgcaagtc 1400
. . . . .
1398 ccaggtgctgttcagcgtgacccgaggggcacactatggcgagctcgagc 1447
1401 ccaggtactgttcagcgtgagccagggtgcacgccacggcgagctggagc 1450
. . . . .
```

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```
1448 tggacatcctgggtgcccaggcacgaaaaatgttcaccctcctggacgtg 1497
1451 tagacatcccgggagcccaaacccgaaaaatgtttacactgttggacgtg 1500

1498 gtgaaccgcaaggcccgcttcatccacgatggctctgaggacacctccga 1547
1501 gtgaaccgaaaggctcgctttgttcacgatggctctgaagacacctctga 1550
. . . . .
1548 ccagctggtgctggaggtgtcggtgacggctcgggtgcccatgccctcat 1597
1551 ccagctgatgctggaggtatcagtgacttctcgggcacctgtgccctctt 1600
. . . . .
1598 gccttcggaggggccaaacatacctcctgcccatccaggtcaaccctgtc 1647
1601 gcctgcggaggggccaaatttacattctccccatccaggtaaaccccgtc 1650
. . . . .
1648 aatgacccaccccacatcatcttcccacatggcagcctcatggtgatcct 1697
1651 aacgacccacctcgcatcgtcttcccgcacggcagcctcatggtgatcct 1700
. . . . .
1698 ggaacacacgcagaagccgctggggcctgaggttttccaggcctatgacc 1747
1701 ggagcacacacagaagcctctgggacccgagattttccaggcctatgacc 1750
. . . . .
1748 cggactctgcctgtgagggcctcaccttccaggtccttggcacctcctct 1797
1751 cagactctgcttgtgagggtctcaccatccagcttcttggtgtctccgct 1800
. . . . .
1798 ggcctccccgtggagcgccgagaccagcctggggagccggcgaccgagtt 1847
1801 agtgtccctgtggaacaccgagaccagcccggagagccagtgactgagtt 1850
. . . . .
1848 ctcctgccgggagttggaggccggcagcctagtctatgtccactgcggtg 1897
1851 ttcctgtcgagacctagaggcaggcaacatagtctatgtccaccgtggcg 1900
. . . . .
1898 gtcctgcacaggacttgacgttccgggtcagcgatggactgcaggccagc 1947
1901 ggcctgcacaggacctgacattccgggtcagtgatggaatgcaggccagt 1950
. . . . .
1948 cccccggccacgctgaaggtggtggccatccggccggccatacagatcca 1997
1951 gggccagctacactgaaggtcgtggccgtccggccagccatacagatcct 2000
. . . . .
1998 ccgcagcacagggttgcgactggcccaaggctctgccatgcccatcttgc 2047
2001 ccacaacacagggctgcgcctggcccagggctctgctgcagccatcttgc 2050
. . . . .
2048 ccgccaacctgtcggtggagaccaatgccgtggggcaggatgtgagcgtg 2097
2051 ctgccaacctgtcggtagaaacgaatgcagtaggacaggatgtgagcgtg 2100
. . . . .
2098 ctgttccgcgtcactggggccctgcagtttggggagctgcagaagcatag 2147
2101 ctgttccgagtcactggcaccttgcagtttggggagctgcagaagcaggg 2150
. . . . .
```

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```
2148 tacaggtggggtggagggtgctgagtggtgggccacacaggcgttccacc 2197
2151 ggccggaggggtagagggcaccgagtggtgggatacactggccttccacc 2200


2198 agcgggatgtggagcagggccgcgtgaggtacctgagcactgacccacag 2247
2201 agcgcgatgtggagcaaggccgagtgaggtacctgagtactgacccacaa 2250
. . . . .
2248 caccacgcttacgacaccgtggagaacctggccctggaggtgcaggtggg 2297
2251 caccacacccaagacacagtggaggacctgaccttggaggtgcaggtggg 2300
. . . . .
2298 ccaggagatcctgagcaatctgtccttcccagtgaccatccagagagcca 2347
2301 ccaggagacactgagcaatctgtctttcccagtgaccatccagagagcca 2350
. . . . .
2348 ctgtgtggatgctgcggctggagccactgcacactcagaacacccagcag 2397
2351 cagtatggatgctgcagcttgagcctctgcatacacagaaccctcatcag 2400
. . . . .
2398 gagaccctcaccacagcccacctggaggccaccctggaggag........ 2439
2401 gaaaccctcacctcagcccacctagaggcttccctggaggaggagggaga 2450
. . . . .
2440 .gcaggcccaagccccccaaccttccattatgaggtggttcaggctccca 2488
2451 aggaggaccataccctcatatcttccactatgagttggttcaggcccccca 2500
. . . . .
2489 ggaaaggcaaccttcaactacagggcacaaggctgtcagatggccagggc 2538
2501 ggagaggcaacctcctgctccagggtacaaggctgtcagatggccagagc 2550
. . . . .
2539 ttcacccaggatgacatacaggctggccgggtgacctatggggccacagc 2588
2551 ttcagccagagtgacctgcaggccggtcgtgtgacctacagggccacaac 2600
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2601 gcggacctctgaggcagctgaggactccttccgtttccgggtcacatctc 2650
. . . . .
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2651 caccacatttctccccgctctacaccttccctatccacattggcggtgac 2700
. . . . .
2689 ccagatgcgcctgtcctcaccaatgtcctcctcgtggtgcctgagggtgg 2738
2701 ccaaacgctcctgtcctcactaacgtcctgctcatggtacccgagggagg 2750
. . . . .
2739 tgagggtgtcctctctgctgaccacctctttgtcaagagtctcaacagtg 2788
2751 ggagggagtgctgtctgctgaccacctctttgtcaagagtctcaacagtg 2800
. . . . .
2789 ccagctacctctatgaggtcatggagcggccccgccttgggaggttggct 2838
2801 ccagctatctctatgaggttatggagcagccccaccatggcagcttggct 2850
. . . . .
```

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```
2839 tggcgtgggacacaggacaagaccactatggtgacatccttcaccaatga 2888
2851 tggagggacccaaaaggtagagccaccccagtaacatcctttactaatga 2900
. . . . . .
2889 agacctgttgcgtggccggctggtctaccagcatgatgactccgagacca 2938
2901 ggacctgctacacggccgactggtctaccagcacgatgactctgagacca 2950
. . . . . .
2939 cagaagatgatatcccatttgttgctacccgccagggcgagagcagtggt 2988
2951 tagaggatgatatcccatttgtggccacacgccagggcgagggcagcggt 3000
. . . . . .
2989 gacatggcctgggaggaggtacggggtgtcttccgagtggccatccagcc 3038
3001 gacatggcctgggaggaggtgcgtggtgtcttccgagtggccatccagcc 3050
. . . . . .
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3051 tgtgaacgatcacgcccctgtgcagaccatcagccgtgtcttccacgtgg 3100
. . . . . .
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3101 cccggggcggacagcggctgttgactacagatgatgtggccttcagtgat 3150
. . . . . .
3139 gctgactcgggctttgctgacgcccagctggtgcttacccgcaaggacct 3188
3151 gctgattcgggcttcagtgacgcgcaactggtgctgacccgcaaggacct 3200
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3189 cctctttggcagtatcgtggccgtagatgagcccacgcggcccatctacc 3238
3201 cctctttggcagcatcgtggctatggaggagcccacgaggcccatctacc 3250
. . . . . .
3239 gcttcacccaggaggacctcaggaagaggcgagtactgttcgtgcactca 3288
3251 gtttcacccaagaggatctcaggaagaagcaagtcctgtttgtgcactca 3300
. . . . . .
3289 ggggctgaccgtggctggatccagctgcaggtgtccgacgggcaacacca 3338
3301 ggggccgaccatggctggctccagctacaggtgtctgatgggcaacacca 3350
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3351 ggctactgccatgctggaggtgcaggcctcagagccctatctccacgtag 3400
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3401 ccaatagttccagtcttgtggttcctcaaggaggccagggcaccatcgac 3450
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3451 acagccgtgctccacctggacaccaacctagatatacgaagtgggaatga 3500
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3489 ggtccactaccacgtcacagctggccctcgctggggacaactagtccggg 3538
3501 ggtccactaccatgtcacagctggccctcactggggacagctgctccggg 3550
. . . . . .
```

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```
3539 ctggtcagccagccacagccttctcccagcaggacctgctggatggggcc 3588
3551 atggccagtcagtcacctccttctcgcaacgggacttgctggatggggcc 3600
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3589 gttctctatagccacaatggcagcctcagccccgaagacaccatggcctt 3638
3601 attctctacagccacaatggcagcctcagcccgcaagacaccctggccct 3650
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3639 ctccgtggaagcagggccagtgcacacggatgccaccctacaagtgacca 3688
3651 ttctgtggcagcagggccagtacacactagcaccgtcctacaagtgacca 3700
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3689 ttgccctagagggcccactggccccactgaagctggtccggcacaagaag 3738
3701 ttgccctagagggcccccctggctccactacaactggtgcagcacaaaagg 3750
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3739 atctacgtcttccagggagaggcagctgagatcagaagggaccagctgga 3788
3751 atctatgtcttccaaggggaggcagctgagatcagaagggaccagctaga 3800
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3789 ggcagcccaggaggcagtgccacctgcagacatcgtattctcagtgaaga 3838
3801 ggtagtccaggaggcagtgctgcctgccgacatcatgttctcgttgagaa 3850
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3839 gcccaccgagtgccggctacctggtgatggtgtcgcgtggcgccttggca 3888
3851 gcccccccgaacgctggctacttggtgatggtgtcccacggtgcttcagca 3900
. . . . .
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3901 gatgggccacccagcctggaccctgtgcagcgcttctcccaagaggcaat 3950
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3939 ggacacaggcagggtcctgtacctgcactcccgccctgaggcctggagcg 3988
3951 aaattcaggccgggttctctacttgcactctcgccctggagcctggagtg 4000
. . . . .
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4001 attccttctccctggatgtggcctcgggtctgggcgatcctctcgaaggc 4050
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4039 gtccttgtggagctggaggtgctgcccgctgccatcccactagaggcgca 4088
4051 atctctgtggagctggaagtgttgcccaccgtcatcccccctggatgttca 4100
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4101 aaacttcagcgttcctgagggtggcactcgtacgctggcccctccgctga 4150
. . . . .
4139 tccgtgtctccgggccctacttccccactctcctgggcctcagcctgcag 4188
4151 tccaaatcactgggccctacttggggacactgccaggccttgtcctacag 4200
. . . . .
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4201 gtgctagagccaccgcagcacggggccctgcagaaggaggaccgtcctca 4250
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```

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```
4239 agccaggaccctcagcgccttctcctggagaatggtggaagagcagctga 4288
4251 agatgggaccctcagcactttctcctggagagaggtggaagagcagctga 4300

4289 tccgctacgtgcatgacgggagcgagacactgacagacagttttgtcctg 4338
4301 tccgatacgtgcatgatgggagtgagacgcagacggacggcttcatcctg 4350
. . . . .
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4351 ctagctaatgcctcagagatggatcgccagagccagcccatggccttcac 4400
. . . . .
4389 tgtcactgtcctgcctgtcaatgaccaacccccatcctcactacaaaca 4438
4401 tatcaccatcctccctgttaatgaccaacccctgtcatcaccacaaaca 4450
. . . . .
4439 caggcctgcagatgtgggaggggccactgcgcccatccctgcggaggct 4488
4451 caggcttgcagatctgggaggggctattgtgcccatccctcctgaggcc 4500
. . . . .
4489 ctgaggagcacggacggcgactctgggtctgaggatctggtctacaccat 4538
4501 ctcaggggaatagacagtgattcagggccggaagacttggtctacaccat 4550
. . . . .
4539 cgagcagcccagcaacgggcgggtagtgctgcgggggcgccgggcactg 4588
4551 cgagcagcccagcaatggacggatagccttgagggtagcaccagacgccg 4600
. . . . .
4589 aggtgcgcagcttcacgcaggcccagctggacggcgggctcgtgctgttc 4638
4601 aggcccaccgcttcacacaggcccagctggacagcgggcttgtgctgttc 4650
. . . . .
4639 tcacacagaggaaccctggatggaggcttcccgttccgcctctctgacgg 4688
4651 tcacacagaggagccctggaaggaggcttccacttcgacctctctgatgg 4700
. . . . .
4689 cgagcacacttcccccggacacttcttccgagtgacggcccagaagcaag 4738
4701 cgtacacacttctcctggacatttcttccgagtggtagcccagaagcagg 4750
. . . . .
4739 tgctcctctcgctgaagggcagccagacactgactgtctgcccagggtcc 4788
4751 tactcctctccttggagggcagccggaagctgactgtctgtccggagtct 4800
 . . . . .
4789 gtccagccactcagcagtcagaccctcagggccagctccagcgcaggcac 4838
4801 gtgcagccactcagcagccagagcttgagtgccagctccagtacaggctc 4850
. . . . .
4839 tgacccccagctcctgctctaccgtgtggtgcggggcccccagctaggcc 4888
4851 cgaccctcgtcacctgctctaccaggtggtaagaggcccccagctcggcc 4900
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Figure 9 (Page 9 of 11)

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SUBSTITUTE SHEET (RULE 26)

Figure 9 (Page 11 of 11)

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