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(54) Title: METHODS FOR INDUCING DIFFERENTIATION OF UNDIFFERENTIATED MAMMALIAN CELLS INTO
OSTEOBLASTS

(57) **Abrégé/Abstract:**

The present invention provides methods for inducing differentiation of undifferentiated mammalian cells into osteoblasts, and methods for identifying a compound that induces differentiation of undifferentiated mammalian cells into osteoblasts. In addition, the present invention provides polynucleotides and vectors, and the use thereof as a medicament for the treatment of a disease involving a systemic or local decrease in mean bone density. Moreover, the present invention provides methods for in vitro production of bone tissue, osteoblast cells and methods for diagnosing a pathological condition involving a systemic or local decrease in mean bone density or a susceptibility to the condition.



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(54) Title: METHODS FOR INDUCING DIFFERENTIATION OF UNDIFFERENTIATED MAMMALIAN CELLS INTO OSTEOBLASTS

(57) Abstract: The present invention provides methods for inducing differentiation of undifferentiated mammalian cells into osteoblasts, and methods for identifying a compound that induces differentiation of undifferentiated mammalian cells into osteoblasts. In addition, the present invention provides polynucleotides and vectors, and the use thereof as a medicament for the treatment of a disease involving a systemic or local decrease in mean bone density. Moreover, the present invention provides methods for *in vitro* production of bone tissue, osteoblast cells and methods for diagnosing a pathological condition involving a systemic or local decrease in mean bone density or a susceptibility to the condition.



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METHODS FOR INDUCING DIFFERENTIATION OF UNDIFFERENTIATED
MAMMALIAN CELLS INTO OSTEOLASTS

The present invention relates to methods for
5 inducing the differentiation of undifferentiated
mammalian cells into osteoblasts. The invention further
relates to methods for identifying one or more compounds
that induce said differentiation, as well as to the
compounds per se for inducing said differentiation.

10 Bone contains two distinct cell lineages, bone-
forming cells (e.g. osteoblasts) and bone-resorbing cells
(e.g. osteoclasts). It is a dynamic tissue that is
continuously being destroyed (resorbed) and rebuilt, by
an intricate interplay between these osteoblasts and
15 osteoclasts.

For osteoclasts, a cascade of transcription factors
and growth factors involved in the progression from
progenitor cell to functional osteoclast is well
established. In contrast, little is known about the
20 osteoblast lineage. Osteoblasts originate from
differentiated mesenchymal progenitor cells (MPCs).
During differentiation into osteoblasts bone alkaline
phosphatase activity (BAP) becomes upregulated. Bone
formation in vivo occurs through two distinct pathways
25 during embryonic development: endochondral or
intramembranous ossification (Figure 1). As shown in this
figure, mesenchymal progenitor or stem cells represent
the starting points for both forms of bone formation.
During intramembranous ossification, flat bones such as
30 those of the skull or clavicles, are formed directly from
condensations of mesenchymal cells. During the formation
of long bones, such as limb bones, mesenchymal
condensations first lead to a cartilage intermediate that
is invaded during further development by endothelial

cells, osteoclasts and mesenchymal cells that will differentiate into osteoblasts and osteocytes (From Nakashima and de Crombrughe, 2003).

A number of diseases are known which are caused by a disturbance of the fine-tuned balance between bone resorption and bone build-up. These skeletal diseases represent a large number of patients: hypercalcemia of malignancy, Paget's disease, inflammatory bone diseases like rheumatoid arthritis and periodontal disease, focal osteogenesis occurring during skeletal metastases, Crouzon's syndrome, rickets, opsismodysplasia, pycnodysostosis/Toulouse-Lautrec disease, osteogenesis imperfecta, but the single most important bone disease is osteoporosis.

Osteoporosis affects 1 in 5 women over 50 and 1 in 20 men over 50. A number of treatments are available for the patient. These mostly tackle the net increase in bone resorption, i.e.:

- hormone replacement therapy (HRT)
- selective estrogen receptor modulators (SERMs)
- bisphosphonates
- calcitonin

While these treatments slow down bone resorption, they do not abolish fracturing because the lost bone is not sufficiently replenished. Fracturing will only be stopped when bone formation is sufficiently increased. Therefore, there is great interest in identifying osteogenic pathways that lend themselves to therapeutic intervention with bone anabolism as effect. Currently, only one bone anabolic therapy has reached the osteoporosis market: parathyroid hormone (PTH)1-34. PTH displays bone anabolic effects when administered intermittently. The treatment is very cumbersome because this biopharmaceutical needs to be injected daily by the

patient. In addition, tumor formation was observed when treating animals at high doses. Also, it is a very expensive treatment.

Another class of bone anabolics, bone morphogenetic proteins (BMPs), have been approved but only for niche markets. There are disadvantages to their use as therapeutic agents to enhance bone healing. Receptors for the bone morphogenetic proteins have been identified in many tissues, and the BMPs themselves are expressed in a large variety of tissues in specific temporal and spatial patterns. This suggests that BMPs may have effects on many tissues other than bone, potentially limiting their usefulness as therapeutic agents when administered systemically. Thus, there is a need for novel anabolics that circumvent one or more of the shortcomings of the above mentioned anabolics.

It is an object of the present invention to provide a method for inducing differentiation of undifferentiated mammalian cells into osteoblasts.

This object is achieved by providing a method for inducing differentiation of undifferentiated mammalian cells into osteoblasts, comprising contacting said undifferentiated cells with an inhibitor of any of the polypeptides listed in table 4, and/or fragments and/or derivatives of said polypeptide.

It is to be understood that according to the present invention the term "inhibitor of a polypeptide" relates to any type of molecule that inhibits, e.g. downmodulates the biological activity of said polypeptide, by inhibiting the expression and/or translation of said polypeptide, or by inhibiting the polypeptide per se.

Undifferentiated cells are pluripotent cells which are in an early stage of specialization, i.e., which do not yet have their final function and can be induced to

form almost any given cell type. In particular, these are cells which have not yet differentiated to e.g. osteoblasts or osteoclasts. Such cells are for example blood cells and cells present in bone marrow, as well as
5 cells derived from adipose tissue. In addition, cells which still can be differentiated into mesenchymal precursor cells are contemplated in the present invention, such as, for example, totipotent stem cells such as embryonic stem cells.

10 Osteoblast differentiation can be measured by measuring the level of enzymes that are induced during the differentiation process, such as alkaline phosphatase (BAP), type-1 collagen, osteocalcin and osteopontin. The alkaline phosphatase activity can be measured by adding
15 methylumbelliferyl heptaphosphate (MUP) solution (Sigma) to the cells. The fluorescence generated upon cleavage of the MUP substrate by the AP activity is measured on a fluorescence plate reader (Fluostar, BMG).

In a preferred embodiment of the present invention,
20 the inhibitor is an expression or translation inhibitor inhibiting the expression or translation of a polyribonucleotide encoding the polypeptide.

In another preferred embodiment the inhibitor is an nucleic acid expressing the expression or translation
25 inhibitor inhibiting the expression or translation of a polyribonucleotide encoding the polypeptide.

Preferably, the nucleic acid is included within a vector. More preferably the vector is an adenoviral, retroviral, adeno-associated viral, lentiviral or a
30 sendaiviral vector.

Nucleic acids expressing the inhibitor, e.g. antisense RNA, ribozyme, antisense oligodeoxynucleotide (ODN), and/or siRNA, such as vectors and viral vectors, have the advantage that they produce said inhibitors

continuously. Vectors include plasmids and viruses. In the vector, the nucleic acid sequence may be placed under the control of a appropriate promoter, such as CMV, HSV, TK, SV40, or, the elongation factor among others. A

5 prolonged production of the inhibitor will give a longer inhibitory effect and less transfections are needed.

In a particular preferred embodiment, the inhibitor is selected from the group consisting of an antisense RNA, a ribozyme that cleaves the polyribonucleotide, an
10 antisense oligodeoxynucleotide (ODN), and a small interfering RNA (siRNA) that is sufficiently homologous to a portion of the polyribonucleotide such that the siRNA is capable of inhibiting the polyribonucleotide that would otherwise cause the production of the
15 polypeptide.

Chemically modified variants of the inhibitors form also part of the present invention, and relate to inhibitors which are modified in order to enhance their stability. The inhibitors thus may contain a variety of
20 modifications that confer resistance to nucleolytic degradation, such as, for example, modified internucleoside linkages, modified nucleic acid bases and/or modified sugars and the like. The antisense oligonucleotides of the invention can also be modified by
25 chemically linking the oligonucleotide to one or more moieties or conjugates to enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide. Such moieties or conjugates include but are not limited to lipids such as cholesterol, cholic
30 acid, thioether, aliphatic chains, phospholipids, polyamines, polyethylene glycol (PEG), or palmityl moieties. Other forms of chemical modification, including but not limited to 2'-O-methyl-, Methyl-phosphonate-,

phosphothioate, inverted end- (3'-3'-linkage) modifications, are also possible.

One type of expression-inhibitory agent is a nucleic acid that is antisense to a nucleic acid comprising any of the genes listed in Table 4. For example, an antisense nucleic acid (e.g. DNA) may be introduced into cells in vitro, or administered to a subject in vivo, as gene therapy to inhibit cellular expression of nucleic acids comprising any of the genes listed in Table 4. Anti-sense nucleic acid is intended to mean a nucleic acid that has a nucleotide sequence that interacts through base pairing with a specific complementary nucleic acid sequence involved in the expression of the target such, that the expression of the gene is reduced. Preferably, the specific complementary nucleic acid sequence involved in the expression of the gene is a genomic DNA molecule or mRNA molecule that encodes the gene. This genomic DNA molecule can comprise regulatory regions of the gene, or the coding sequence for the mature gene.

Antisense nucleic acids preferably comprise a sequence containing from about 17 to about 100 nucleotides and more preferably the antisense nucleic acids comprise from about 18 to about 30 nucleotides. Antisense nucleic acids of the invention are preferably nucleic acid fragments capable of specifically hybridizing with all or part of a nucleic acid encoded by any of the genes listed in Table 4 or the corresponding messenger RNA, or they may be DNA sequences whose expression in the cell produces RNA complementary to all or part of the mRNA comprising nucleic acid sequences encoded by any of the genes listed in Table 4. Antisense nucleic acids may be prepared by expression of all or part of a sequence selected from the group nucleic acid sequences encoded by any of the genes listed in Table 4,

in the opposite orientation. Preferably the antisense nucleic acid is prepared by expression of a sequence selected from the groups consisting of SEQ ID NO: 1-226 and SEQ ID NO 247-333, in the opposite orientation.

5 Preferably, the antisense sequence is at least about 17 nucleotides in length.

The term complementary to a nucleotide sequence in the context of antisense nucleic acids and methods therefore means sufficiently complementary to such a
10 sequence as to allow hybridization to that sequence in a cell, *i.e.*, under physiological conditions.

Another type of expression-inhibitor according to the invention is a nucleic acid that is able to catalyze cleavage of RNA molecules. The expression "ribozymes" as
15 used herein relates to catalytic RNA molecules capable of cleaving other RNA molecules at phosphodiester bonds in a manner specific to the sequence. The hydrolysis of the target sequence to be cleaved is initiated by the formation of a catalytically active complex consisting of
20 ribozyme and substrate RNA. All ribozymes capable of cleaving phosphodiester bonds in trans, that is to say intermolecularly, are suitable for the purposes of the invention.

Yet another type of expression-inhibition is RNA
25 interference (RNAi). RNAi is the post-transcriptional process of gene silencing mediated by double stranded RNA (dsRNA) that is homologous in sequence to the silenced RNA and is observed in animals and plants.

According to a particular preferred embodiment of
30 the invention the inhibitor is a siRNA, comprising a first nucleotide sequence of 17-23 nucleotides homologous to a nucleotide sequence selected from any of the target genes listed in table 4, and a second nucleotide sequence of 17-23 nucleotides complementary to said first

nucleotide sequence. Preferably, the inhibitor is a siRNA, comprising a first nucleotide sequence of 17-23 nucleotides selected from the nucleotide sequences identified by SEQ. ID NO. 1-220 and 247-333 in table 4 and 5, and a second nucleotide sequence of 17-23 nucleotides complementary to said first nucleotide sequence.

In another preferred embodiment the siRNA further comprises a third nucleotide sequence connecting the first and second nucleotide sequence, and capable of forming a stem-loop structure within the siRNA.

Such self-complementing single stranded siRNA polynucleotide according to the present invention thus at least comprises a first guide sequence, and a second guide sequence, which complements the first guide sequence, and a third sequence capable of forming a stem-loop structure within said second sequence, which is covalently linked to the distal end of the first sequence and the proximal end of the second sequence. All nucleotides in the first and second sequences may base pair, or alternatively there may be mismatches between the first and second sequences. The nucleotide sequences are preferably between about 17 and 23 nts in length. Preferably the first or the second sequence is a oligonucleotide sequence between about 17 and 23 nt in length selected from the polynucleotide sequences of any of the genes listed in Table 4. Such siRNA polynucleotides with a loop are selfcomplementary and can form stable hairpins. Hairpins are more stable than ordinary dsRNA. In addition, they are more easily produced from vectors.

More preferably the first or second nucleotide sequence comprises a nucleotide sequence consisting of SEQ ID NO: 1-220 and SEQ ID NO 247-333. The nucleotide

sequences of SEQ ID NO 1-220 and SEQ ID NO 247-333 are selected according to siRNA designing rules that give an improved reduction of the target sequences compared to nucleotide sequences that do not comply with these siRNA
5 designing rules (see WO 2004/094636).

Preferably, the third nucleotide sequence is 4-30 nucleotides long, more preferably 5-15 nucleotides long and most preferably 8 nucleotides long. In a most preferred embodiment the third nucleotide sequence is
10 UUGCUAUA (SEQ ID NO: 334).

The present invention further relates to a method for identifying a compound that induces differentiation of undifferentiated mammalian cells into osteoblasts, comprising:

- 15 (a) contacting one or more compounds with a polypeptide listed in table 4, encoded by any of the genes listed in table 4, and/or fragments and/or derivatives of said polypeptide;
- (b) determining the binding affinity of the
20 compound to the polypeptide;
- (c) contacting a population of undifferentiated mammalian cells with the compound that exhibits a binding affinity of at least 10 micromolar; and
- (d) identifying the compound that induces the
25 differentiation of the undifferentiated mammalian cells.

The polypeptides or the polynucleotides of the present invention employed in the methods described above may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly.

30 To perform the methods it is feasible to immobilize either the polypeptide of the present invention or the compound to facilitate separation of complexes from uncomplexed forms of the polypeptide, as well as to accommodate automation of the assay. Interaction (e.g.,

binding of) of the polypeptide of the present invention with a compound can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and
5 microcentrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the polypeptide to be bound to a matrix. For example, the polypeptide of the present invention can be "His" tagged, and subsequently adsorbed onto Ni-NTA microtitre plates,
10 or ProtA fusions with the polypeptides of the present invention can be adsorbed to IgG, which are then combined with the cell lysates (e.g., (35)^S-labelled) and the candidate compound, and the mixture incubated under conditions favorable for complex formation (e.g., at
15 physiological conditions for salt and pH). Following incubation, the plates are washed to remove any unbound label, and the matrix is immobilized. The amount of radioactivity can be determined directly, or in the supernatant after dissociation of the complexes.
20 Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of the protein binding to the protein of the present invention quantitated from the gel using standard electrophoretic techniques.
25 Other techniques for immobilizing protein on matrices can also be used in the method of identifying compounds. For example, either the polypeptide of the present invention or the compound can be immobilized utilizing conjugation of biotin and streptavidin.
30 Biotinylated protein molecules of the present invention can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well

plates (Pierce Chemical). Alternatively, antibodies reactive with the polypeptides of the present invention but which do not interfere with binding of the polypeptide to the compound can be derivatized to the wells of the plate, and the polypeptide of the present invention can be trapped in the wells by antibody conjugation. As described above, preparations of a labeled candidate compound are incubated in the wells of the plate presenting the polypeptide of the present invention, and the amount of complex trapped in the well can be quantitated.

The binding affinity of the compound with the polypeptide or polynucleotide can be measured by methods known in the art, such as using surface plasma resonance biosensors (Biacore), by saturation binding analysis with a labeled compound (e.g. Scatchard and Lindmo analysis), via displacement reactions, by differential UV spectrophotometer, fluorescence polarisation assay, Fluorometric Imaging Plate Reader (FLIPR[®]) system, Fluorescence resonance energy transfer, and Bioluminescence resonance energy transfer. The binding affinity of compounds can also be expressed in a dissociation constant (K_d) or as IC_{50} or EC_{50} . The IC_{50} represents the concentration of a compound that is required for 50% inhibition of binding of another ligand to the polypeptide. The EC_{50} represents the concentration required for obtaining 50% of the maximum effect in vitro. The dissociation constant, K_d , is a measure of how well a ligand binds to the polypeptide, it is equivalent to the ligand concentration required to saturate exactly half of the binding-sites on the polypeptide. Compounds with a high affinity binding have low K_d , IC_{50} and EC_{50} values, i.e. in the range of 100 nM to 1 pM; a moderate

to low affinity binding relates to a high K_d, IC₅₀ and EC₅₀ values, i.e. in the micromolar range.

For high-throughput purposes, libraries of compounds can be used such as peptide libraries (e.g. LOPAPTM, Sigma Aldrich), lipid libraries (BioMol), synthetic compound
5 libraries (e.g. LOPACTM, Sigma Aldrich) or natural compound libraries (Specs, TimTec).

The invention also relates to a method for identifying a compound or mixture of compounds that
10 induces differentiation of undifferentiated mammalian cells into osteoblasts, comprising

- (a) culturing a population of undifferentiated mammalian cells that express a polypeptide listed in Table 4, encoded by a gene listed in table 4, and/or
15 fragments and/or derivatives of said polypeptide;
- (b) exposing said population of cells to a compound or mixture of compounds; and
- (c) selecting the compound or mixture of compounds that induces the differentiation of the undifferentiated
20 cells into osteoblasts.

The compound or mixture of compounds that are identified according to this method can thus be applied to induce differentiation of undifferentiated mammalian cells into osteoblasts.

25 Preferably the compounds identified in these methods of the invention are low molecular weight compounds. Low molecular weight compounds, i.e. with a molecular weight of 500 Dalton or less, are likely to have good absorption and permeation in biological systems and are consequently
30 more likely to be successful drug candidates than compounds with a molecular weight above 500 Dalton (Lipinsky et al, 2001).

According to another preferred embodiment the compounds are peptides. Peptides can be excellent drug

candidates and there are multiple examples of commercially valuable peptides such as fertility hormones and platelet aggregation inhibitors.

According to another preferred embodiment the
5 compounds are natural compounds. Natural compounds are compounds that have been extracted from natural sources, e.g. plants. Using natural compounds in screens has the advantage that more diverse molecules are screened. Natural compounds have an enormous variety of different
10 molecules. Synthetic compounds do not exhibit such variety of different molecules. Other compounds may be selected from carbohydrates, and glycosylated polypeptides.

Moreover, the present invention relates to
15 polynucleotide comprising a sequence of 17-23 nucleotides homologous to a nucleotide sequence selected from any of the target genes listed in table 4, and variants and/or the reverse complements thereof. Preferably, said polynucleotides have a nucleotide sequence selected from
20 the nucleotide sequences identified by SEQ. ID NO. 1-220 and 247-333 in table 4 and 5, or said polynucleotides have a reverse complement of a nucleotide sequence selected from the nucleotide sequences identified by SEQ. ID NO. 1-220 and 247-333 in table 4 and 5. The
25 polynucleotides of the invention have been shown to increase osteoblast differentiation.

The invention also relates to these polynucleotides for use as a medicament.

Furthermore, the invention relates to the use of
30 said polynucleotides in the manufacture of a medicament for the treatment of a disease involving a systemic or local decrease in mean bone density.

As it is shown for the first time in this patent application that the polypeptides comprising an amino

acid sequence selected from the group listed in Table 4 are involved in the differentiation of undifferentiated mammalian cells into osteoblasts, in still another preferred embodiment of the present invention, compounds
5 that are known or will be known to inhibit the activity of any of the polypeptides comprising an amino acid sequence selected from the group listed in Table 4 can now also be used to induce differentiation of undifferentiated mammalian cells into osteoblasts.

10 Therefore, the present invention also relates to the use of compounds that are known in the art to inhibit the activity of any of polypeptides comprising an amino acid sequence selected from the group listed in Table 4, as a medicament. Furthermore, the present invention also
15 relates to the use of compounds that are known in the art to inhibit the activity of any of polypeptides comprising an amino acid sequence selected from the group listed in Table 4, as a medicament for the treatment of a disease involving a systemic or local decrease in mean bone
20 density.

The invention furthermore relates to a vector comprising any of the polynucleotides described above. In addition, the invention relates to said vectors for use as a medicament.

25 In a preferred embodiment, the vector is an adenoviral, retroviral, adeno-associated viral, lentiviral or a sendaiviral vector. Preferably, the vector encodes a siRNA comprising a nucleotide sequence selected from the groups consisting of SEQ ID NO: 1-220
30 and SEQ ID NO 247-333.

The invention further relates to the use of said vectors for the manufacture of a medicament for the treatment of a disease involving a systemic or local decrease in mean bone density. In a preferred embodiment

of the present invention the disease is selected from the group consisting of osteoporosis, hypercalcemia of malignancy, multiple myelomatosis, hyperparathyroidism, and hyperthyroidism.

5 Recombinant viruses are commonly used for gene transfer. To date, the three most commonly used viruses for gene transfer are adenovirus, retrovirus and adeno-associated virus. More recently lentiviruses, a subgroup of the retroviruses, and sendaivirus are being used.

10 Adenoviruses are able to transduce both dividing and non-dividing cells and can be produced at high viral titres. Retroviruses can infect dividing cells only and integrate their genome into the host chromosome. Integration achieves long-term gene expression. Lentiviruses, a sub-

15 family of retroviruses, share all the standard properties of retroviruses but in addition they have the capacity to transduce non-dividing cells. Adeno-associated virus (AAV) is a small, non-pathogenic, single-stranded DNA virus. It requires co-infection with a helper virus

20 (adenovirus or herpes virus) in order to undergo productive infection. In the absence of helper virus, wild-type AAV integrates site-specifically, into the host genome. Similarly to retrovirus, integration facilitates longer gene expression. AAV can infect both non-dividing

25 and dividing cells. Sendai virus is a member of the paramyxoviridae, and is a single-stranded RNA virus that is able to transfect both dividing and non-dividing cells. Its method of entering cells involves sialic acid and cholesterol that are common to many cell types.

30 Sendai viral gene expression and replication are localized in the cytoplasm, in contrast to most viruses that need to enter the nucleus.

Furthermore, the present invention relates to a method for in vitro production of bone tissue, comprising

(a) applying undifferentiated mammalian cells on a substrate to form a cellular substrate;

(b) introducing any of the polynucleotides described above, or a vector comprising said polynucleotide, for a time sufficient to differentiate the undifferentiated cells into osteoblasts, thereby producing a continuous bone matrix. Preferably the continuous bone matrix comprises a thickness of at least 0.5 μm on the surface of the substrate.

The invention thus provides a method for producing a substrate with a matrix grown thereon, which can be used for the provision of load-bearing implants, including joint prostheses, such as artificial hip joints, knee joints and finger joints, and maxillofacial implants, such as dental implants. It can also be used for special surgery devices, such as spacers, or bone fillers, and for use in augmentation, obliteration or reconstitution of bone defects and damaged or lost bone. Bone formation can be optimized by variation in mineralization, both by inductive and by conductive processes.

A combination of the provision of a load-bearing implant (preferably coated with a matrix as described above) with a bone filler comprising a matrix as described, constitutes an advantageous method according to the present invention.

The method of the invention is also very suitable in relation to revision surgery, i.e., when previous surgical devices have to be replaced.

Suitable undifferentiated cells are bone marrow cells, including haematopoietic cells and in particular stromal cells. The marrow cells, and especially the stromal cells are found to be very effective in the bone producing process when taken from their original environment. The undifferentiated cells can be directly

applied on the substrate or they can advantageously be multiplied in the absence of the substrate before being applied on the substrate. In the latter mode, the cells are still largely undifferentiated after multiplication
5 and, for the purpose of the invention, they are still referred to as undifferentiated. Subsequently, the cells are allowed to differentiate. Differentiation can be induced or enhanced by the presence of suitable inductors, such as glucocorticoids, and dexamethasone.
10 Especially suitable inductors of differentiation are the expression inhibitory agents of the present invention.

The use of undifferentiated cells provides several advantages. Firstly, their lower differentiation implies a higher proliferation rate and allows the eventual
15 functionality to be better directed and controlled. Moreover, culturing these cells not only produces the required bone matrix containing organic and inorganic components, but also results in the presence, in the culture medium and in the matrix, of several factors
20 which are essential for growth of the tissue and for adaptation to existing living tissue. Also, the culture medium can be a source of active factors such as growth factors, to be used in connection with the implanting process. Furthermore, such undifferentiated cells are
25 often available in large quantities and more conveniently than e.g., mature bone cells, and exhibit a lower morbidity during recovery. Moreover, the undifferentiated cells can be obtained from the patient for whom the implant is intended. The bone resulting from these cells
30 is autologous to the patient and thus no immune response will be induced. Matrices as thick as 100 μm can be produced as a result of the use of undifferentiated cells.

The substrate on which the undifferentiated cells can be applied and cultured can be a metal, such as titanium, cobalt/chromium alloy or stainless steel, a bioactive surface such as a calcium phosphate, polymer surfaces such as polyethylene, and the like. Although less preferred, siliceous material such as glass ceramics, can also be used as a substrate. Most preferred are metals, such as titanium, and calcium phosphates, even though calcium phosphate is not an indispensable component of the substrate. The substrate may be porous or non-porous. The cells can be applied at a rate of e.g., 10^3 - 10^6 per cm^2 , in particular 10^4 - 2×10^5 cells per cm^2 .

The culture medium to be used in the method according to the invention can be a commonly known culture medium such as MEM (minimum essential medium). Advantageously, the medium can be a conditioned medium. In this context, a conditioned medium is understood to be a medium wherein similar cells have previously been incubated, causing the medium to contain factors such as polypeptides, secreted by the cells which are important for cell growth and cell differentiation.

The cells are cultured for a time sufficient to produce a matrix layer, e.g., a matrix layer having a thickness of at least $0.5 \mu\text{m}$, in particular from 1 up to $100 \mu\text{m}$, more in particular of 10 - $50 \mu\text{m}$. The cells may be contacted with the culture medium for e.g. 2-15 weeks, in particular 4-10 weeks.

The production of the matrix, when applied on a substrate, results in a continuous or quasi-continuous coating covering the substrate for at least 50%, in particular at least 80% of its surface area.

In another embodiment the present invention provides osteoblast cells obtainable by methods of the present invention.

Another embodiment of the present invention involves
5 the use of the osteoblast cells obtainable by methods of the present invention for in vitro production of bone tissue.

The present invention relates further to a method for diagnosing a pathological condition involving a
10 systemic or local decrease in mean bone density or a susceptibility to the condition in a subject, comprising

(a) determining the level of expression of a polynucleotide encoded by any of the target genes listed in table 4, in a biological sample derived
15 from said subject; and

(b) comparing the level of expression with the level of expression of the polynucleotides in a sample derived from a healthy subject;

wherein an increase of the amount of
20 polynucleotide in the sample of the subject compared to the sample of the healthy subject is indicative of the presence of the pathological condition.

In addition, the present invention relates to a method for diagnosing a pathological condition involving
25 a systemic or local decrease in mean bone density or a susceptibility to the condition in a subject, comprising

(a) determining the amount of a polypeptide encoded by the genes listed in table 4 in a biological sample derived from said subject; and

30 (b) comparing the amount with the amount of said polypeptide in a biological sample derived from a healthy subject;

wherein an increase of the amount of polypeptide in the subject compared to the healthy subject is indicative

of the presence of the pathological condition. Preferably the pathological condition is selected from the group consisting of osteoporosis, hypercalcemia of malignancy, multiple myelomatosis, hyperparathyroidism, and
5 hyperthyroidism.

According to the present invention, the term "derivatives of a polypeptide" relates to those proteins, proteinaceous molecules, fractions of proteins, peptides, and oligopeptides that comprise a stretch of contiguous
10 amino acid residues of the polypeptide and that retain the biological activity of the protein, e.g. polypeptides that have amino acid mutations compared to the amino acid sequence of a naturally-occurring form of the polypeptide. A derivative may further comprise additional
15 naturally occurring, altered, glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally occurring form of the polypeptide. It may also contain one or more non-amino acid substituents compared to the amino acid
20 sequence of a naturally occurring form of the polypeptide, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence.

The term "fragment of a polypeptide" relates to
25 peptides, oligopeptides, polypeptides, proteins and enzymes that comprise a stretch of contiguous amino acid residues, and exhibit substantially a similar, but not necessarily identical, functional activity as the complete sequence.

30 The term "polynucleotide" refers to nucleic acids, such as double stranded, or single stranded DNA and (messenger) RNA, and all types of oligonucleotides. It also includes nucleic acids with modified backbones such

as peptide nucleic acid (PNA), polysiloxane, and 2'-O-(2-methoxy)ethylphosphorothioate.

The term "derivatives of a polynucleotide" relates to DNA-molecules, RNA- molecules, and oligonucleotides that comprise a stretch or nucleic acid residues of the polynucleotide, e.g. polynucleotides that may have nucleic acid mutations as compared to the nucleic acid sequence of a naturally occurring form of the polynucleotide. A derivative may further comprise nucleic acids with modified backbones such as PNA, polysiloxane, and 2'-O-(2-methoxy)ethyl-phosphorothioate, non-naturally occurring nucleic acid residues, or one or more nuclei acid substituents, such as methyl-, thio-, sulphate, benzoyl-, phenyl-, amino-, propyl-, chloro-, and methanocarbanucleosides, or a reporter molecule to facilitate its detection.

The term "fragment of a polynucleotide" relates to oligonucleotides that comprise a stretch of contiguous nucleic acid residues that exhibit substantially a similar, but not necessarily identical, activity as the complete sequence.

The term "homologous sequence", whether a nucleic acid sequence or an amino acid sequence, is limited to a sequence that is identical or substantially identical to the parent sequence and has the same biological property as the parent sequence. The term is meant to include naturally occurring mutants and variants of the parent sequence. The parent sequence is either provided in table 4-6, or is indicated by the GenBank accession numbers in these tables. In general, a homologous sequence is at least 95% identical to the parent sequence. However, up to two mismatches are allowed for the 17-21 bp siRNA sequences, provided that the homologous sequence leads to

a similar down-regulation of the corresponding gene as the parent sequence.

Short Description Of The Figures

5

Figure 1. Intramembraneous and endochondral ossification.

Figure 2. Principle of the osteoblast differentiation assay.

10 Figure 3. Lay-out of the 96 well knock-down control plate.

Figure 4. Lay-out of the 384 well control plate.

Figure 5. Performance of the knock-down control plate in the AP assay

15 Figure 6. Dot plot representation of raw data for one SilenceSelect screening plate

Figure 7. Profiling of target expression in MPCs (A) and knock-down of gene expression by Ad-siRNA (B)

20 Figure 8. Profiling of target expression in primary human OBs

Figure 9. Analyzing the upregulation of BAP-mRNA versus PLAP- or IAP-mRNA

Figure 10. Results mineralization assay

25 Figure 11. Pipetting scheme used for screening the Ad-shRNAs at 3 MOIs.

EXAMPLES

30 Example 1. Development of a high-throughput screening method for the detection of endogenous alkaline phosphatase

Principle of the assay

Mesenchymal progenitor cells (MPCs) are determined to differentiate into osteoblasts in the presence of

appropriate factors (e.g. BMP2). An assay to screen for such factors was developed by monitoring the activity of alkaline phosphatase (AP) enzyme, an early marker in the osteoblast differentiation program. MPCs were seeded in 384 well plates and simultaneously co-infected one day later with adenoviruses encoding the human coxsackie and adenovirus receptor (hCAR; Ad-hCAR) and individual siRNA adenoviruses (Ad-siRNA) from the SilenceSelect™ collection. AdC15-hCAR/ AdC20-hCAR co-infection increases the AdC01-siRNA infection efficiency. Cellular AP activity was determined 13 days after the start of the infection (13 dpi). Figure 2 illustrates the principle of the assay.

15 Development of the assay

MPCs were isolated from bone marrow of healthy volunteers, obtained after informed consent (Cambrex/Biowhittaker, Verviers, Belgium).

20 In a series of experiments, carried out in 384 well plates, several parameters were optimized: cell seeding density, multiplicities of infection (MOI) of control viruses (Ad-BMP2 or Ad-eGFP), MOI of Ad-hCAR, duration of infection, toxicity, infection efficiency (using Ad-eGFP) and the day of readout.

25 Using Ad-BMP2 (BMP2 overexpression) as a positive control for assay development, the following protocol resulted in the highest dynamic range for the assay with the lowest standard deviation on the background signal: MPCs were seeded on day0 at 500 cells per well of a 384 well plate and co-infected the next day using a mix of Ad-hCAR (5 μ l of an Ad-hCAR solution: mix total MOI=155.7) and 1 μ l of Ad-control-virus (Ad-BMP2 or Ad-eGFP; corresponds to a theoretical MOI of 5000). On day5, the medium containing virus was removed and replaced by

fresh medium containing no virus. Upregulation of alkaline phosphatase was read at 13 dpi: 15 μ l 4-Methylumbelliferylphosphate (MUP, Sigma) was added to each well, the plates were incubated for 15 min at 37°C and monitored for AP activity using a fluorescence plate reader (Fluostar, BMG).

After optimisation of the assay, a small pilot screen was run (103 different Ad-siRNA viruses) with the use of robotics (96/384 channel dispenser Tecan Freedom 200 equipped with TeMO96, TeMO384 and RoMa, Tecan AG, Switzerland). The hits from this screen were collected and retested in the same assay. The two Ad-siRNAs that scored strongest (H9=H24-010; H10=H24-011) were used to generate a control plate (knock-down (KD) control plate) containing Ad-siRNAs. The control plate, a 96 well plate containing 3 negative (N1,N2,N3) and 3 positive (P1,P2,P3) control viruses is depicted in Figure 3. This "knock-down" control plate contains Ad-H9 (H24-010) and Ad-H10 (H24-011) as positive controls; Ad-eGFP (knock-in virus) as infection control; and Ad-eGFP-siRNA, Ad-M6PR-siRNA and Ad-Luc-siRNA (all 3 are knock-down viruses) as negative controls.

The control viruses were pipetted from 96 well KD control plates into 384 well plates using robotics. The final lay-out of the 384 well plate is depicted in Figure 4.

Figure 5 shows results from the automated screening procedure using the KD control plate. The mean and standard deviations of the KD negative controls (N1-N3) were used to calculate a cut-off for hit analysis, which was set at the mean for N1, N2, N3 ('All negatives') plus 3 times the standard deviation for 'All negatives'. The positive controls (P1 and P2), scored in more than 95% of

the infected wells. The negative control viruses scored in less than 5% of the wells.

Example 2. Screening of 7980 Ad-siRNA adenoviruses

5 in the osteogenesis assay

The optimized protocol for screening the SilenceSelect library is the following: on day 0, MPC cells are seeded in black 384 well plates with clear bottom (Costar or Nunc) in 60 μ l medium at a density of 10 500 cells per well. One day later, 1 μ l Ad-siRNA virus from the SilenceSelect™ collection, stored in 384 well plates (estimated titer of 2.5×10^9 viral particles per ml) and 5 μ l of Ad-hCAR solution (total MOI=155), 15 dispensed in 96 well V-bottom plates, is transferred with the aid of a 96/384 channel dispenser (Tecan Freedom 200 equipped with TeMO96, TeMO384 and RoMa, Tecan AG, Switzerland) from the wells of a 96 well plate containing the Ad-hCAR solution to each of the wells of the 384 well plates containing MPCs. The KD control plate was run 20 under the same conditions as the aliquot plates from the SilenceSelect collection. All Ad-siRNA viruses were screened in duplicate, with each singular on a different MPC plate. Plates were then incubated at 37°C. Four days post infection the medium containing the 25 adenoviruses was replaced by fresh medium free of virus. Thirteen days post infection, the AP activity readout was performed. A typical result of a 384 well screening plate is depicted in Figure 6, in which the relative fluorescence units (RFU) are plotted for each of the data 30 points of the 384 well plate on the Y-axis; while the numbers on the X-axis correspond to positions in the 384 well plate.

This duplicate screen was done twice, and all four data points were used for hit calling (see Example 3).

Example 3. Target identification using the AP assay

After performing these 2 screens, the data obtained from measuring the AP activity were analyzed as follows:
5 the background was calculated by taking the mean of the data points from all the plates except the control plate. A cut-off value for hit calling was calculated by adding 3 times the standard deviation of all data points, excluding the control plate. Each data point was analyzed
10 for scoring above or under the cut-off. Only Ad-siRNAs inducing endogenous AP activity levels above the cut-off were of further interest. Hits were prioritized according to their scoring in single or duplo, in one or both of the screens. Data were collected for 7980 Ad-siRNA virus
15 constructs representing 4091 independent genes. An overview of the constructs is given in Table 4.

One of the identified hits has been shown to be a bone anabolic factor before and therefore validates the assay:

20 H24-034: SRC

Marzia et al. (2000) showed that bone formation was increased in Src null mice compared to wild-type mice. Most relevant to this work, osteoblasts isolated from Src null mice or osteoblasts isolated from wild-type mice but
25 transfected with Src-antisense oligonucleotides showed increased AP activity in vitro.

8 genes identified in the screen were targeted by 2 Ad-siRNAs. These genes are AVPR1B, FLJ22955, IL1F8, PPIA, USP38, C9, LOC254378 and BRS3 (see Table 4).

30

Example 4. Quality control of the target Ad-siRNAs

The Ad-siRNA hits, hereafter referred to as 'targets' were subjected to further analysis to establish their therapeutic application as bone anabolic factor. A

first step entailed a quality control on the Ad-siRNA selected for further analysis (this example). Other validation steps are the validation of targets at the mRNA level (Example 5), screening of the targets in osteogenesis assays such as the mineralization assay (Example 6), and development of additional Ad-siRNAs targeting the identified genes (Example 9). Targets that remain of interest after these validation assays are considered for drug discovery: assays are developed allowing the discovery and optimization of compounds that mimic the bone anabolic actions of the target Ad-siRNAs (Example 7). In addition, the anti-resorptive activities of the identified Ad-siRNAs are validated in osteoclast assays (Example 8).

15

Quality control of target Ad-siRNAs:

Target Ad-siRNAs were propagated using PerC6 cells (Crucell, Leiden, The Netherlands) at a 96 well plate level, followed by rescreening these viruses at several MOIs in the primary assay (see Example 1) and by sequencing the siRNAs encoded by the target Ad-siRNA viruses.

PerC6/E2A cells were seeded in 96 well plates at a density of 40 000 cells per well in 180 μ l PerC6/E2A medium. Cells were then incubated overnight at 39°C in a 10% CO₂ humidified incubator. One day later, cells were infected with 1 μ l of crude cell lysate from SilenceSelect stocks containing target Ad-siRNAs. Cells were incubated further at 34°C, 10% CO₂ until appearance of cytopathic effect (as revealed by the swelling and rounding up of the cells, typically 7 days post infection). The supernatant was collected and the virus crude lysate was treated with proteinase K: 12 μ l crude lysate was added to 4 μ l Lysis buffer (1x Expand High

Fidelity buffer with MgCl_2 (Roche Molecular Biochemicals, Cat. No 1332465) supplemented with 1 mg/ml proteinase K (Roche Molecular Biochemicals, Cat No 745 723) and 0.45% Tween-20 (Roche Molecular Biochemicals, Cat No 1335465) in sterile PCR tubes. These were incubated at 55°C for 2 h followed by a 15 min inactivation step at 95°C. For the PCR reaction, 1 μl lysate was added to a PCR master mix composed of 5 μl 10x Expand High Fidelity buffer with MgCl_2 , 0.5 μl of dNTP mix (10 mM for each dNTP), 1 μl of 'Forward primer' (10 mM stock, sequence: 5' CCG TTT ACG TGG AGA CTC GCC, SEQ ID NO: 245), 1 μl of 'Reverse Primer' (10 mM stock, sequence: 5' CCC CCA CCT TAT ATA TAT TCT TTC C, SEQ ID NO: 246), 0.2 μl of Expand High Fidelity DNA polymerase (3.5 U/ μl , Roche Molecular Biochemicals) and 41.3 μl of H_2O . PCR was performed in a PE Biosystems GeneAmp PCR system 9700 as follows: the PCR mixture (50 μl in total) was incubated at 95°C for 5 min; each of 35 subsequent cycles ran at 95°C for 15 sec, 55°C for 30 sec, 68°C for 4 min.. A final incubation at 68°C was performed for 7 min. 5 μl of the PCR mixture was mixed with 2 μl of 6 x gel loading buffer, loaded on a 0.8% agarose gel containing 0.5 $\mu\text{g}/\mu\text{l}$ ethidium bromide to resolve the amplification products. The size of the amplified fragments was estimated from a standard DNA ladder loaded on the same gel. The expected size was ~500 bp.

For sequencing analysis, the siRNA constructs expressed by the target adenoviruses were amplified by PCR using primers complementary to vector sequences flanking the SapI site of the pIPspAdapt6-U6 plasmid. The sequence of the PCR fragments was determined and compared with the expected sequence.

Multiple MOI rescreen

The propagated target Ad-siRNAs were rescreened at several MOIs in the AP assay (see Example 1). The Ad-siRNAs had to score in duplo at at least one MOI to pass this quality control step.

All hits listed in Table 4 fulfilled this quality control step and thus:

- a) showed the correct length of the PCR fragment
- b) showed the correct sequence of the PCR fragment
- c) induced AP activity in duplo at at least 1 MOI.

Example 5. mRNA validation experiments for identified targets

An initial validation of the target Ad-siRNAs was carried out on RNA isolated from infected MPCs. First, the expression of the targets was analyzed in several isolates of primary human MPCs and osteoblasts (hOBs). Second, the knock-down of the target gene expression by the Ad-siRNA was verified at the mRNA level. Third, the upregulation of endogenous bone AP mRNA versus that of placental or intestinal AP mRNA was analyzed.

MPC and osteoblast expression analysis for the identified targets profiling

Expression levels of target genes were determined in 4 different isolates of MPCs and 2 different isolates of hOBs. The MPCs and hOBs (obtained from Cambrex/Biowhittaker, Verviers, Belgium) were seeded at 3000 resp. 5000 cells/cm² in T180 flasks and cultured until they reached 80% confluency. The cells were washed with ice-cold PBS and harvested by adding 1050 µl SV RNA Lysis Buffer to a T180 flask. Total RNA was prepared using the SV Total RNA isolation System (Promega, Cat # Z3100). The concentration of the total RNA was measured

with the Ribogreen RNA Quantification kit (Molecular Probes, Leiden, The Netherlands, Cat No. R-11490). cDNA synthesis was performed using 40 ng total RNA per reaction using the TaqMan Universal PCR Master Mix, No
5 AmpErase UNG, kit (Applied Biosystems, Warrington, UK, Part number 4324018). For each RT reaction a minus-RT reaction (negative control: no enzyme included in the reaction) was performed.

The real-time reverse transcriptase (rtRT)-PCR
10 reaction was performed with gene specific primers on both cDNA and minus-RT samples, using the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK, Part number 4309155). For the normalisation of the expression levels, a RT-PCR reaction was performed on human β -actin
15 using the Human β -actin kit (Applied Biosystems, Warrington, UK, Part number 4310881E). The following program was run on a real-time PCR apparatus (ABI PRISM 7000 Sequence Detection System): 10 min at 25°C, 30 min at 48°C, 5 min at 95°C. In Figures 7A and 8, relative
20 expression levels for 10 genes are depicted for respectively MPC and hOB isolates. For the data in Figure 7A, total RNA was extracted from 4 different MPC isolates and used to analyze expression levels of target genes identified through the target Ad-siRNAs. RtRT-PCR
25 compatible primer sets were developed for 10 genes and compared to expression levels of β -actin. Data are expressed as $-\log(\text{difference to } \beta\text{-actin})$ (Y-axis). For the data presented in Figure 8, total RNA was extracted from 2 different hOB isolates.

Analysis of the knock-down of the target gene expression by the Ad-siRNA was verified at the mRNA level.

To determine whether the target Ad-siRNAs result in knock-down of expression from the corresponding gene, total RNA was harvested from Ad-siRNA infected MPCs and gene expression was analyzed using gene-specific primers.

MPCs were seeded at 25 000 cells per well of a 24 well plate. After 24 hours the cells were infected with knock-down hit viruses or negative control viruses Ad-gPPAR γ and Ad-GL2.2 that knock down the expression of respectively PPAR γ (all four known splice variants) and luciferase, both of which are not related to osteogenesis. For Ad-siRNAs, cells were co-infected with Ad-hCAR and Ad-siRNA crude lysates (MOI Ad-hCAR: 750; 40, 10, 3.3 μ L virus with average titer of 2.5×10^9 virus particles/ml). At 5 dpi the medium was refreshed and at 14 dpi the cell lysates were prepared. Cells were processed for mRNA analysis as described in the previous section. mRNA levels for a specific gene were normalized for β -actin levels and compared to levels measured for the negative control Ad-siRNAs. An example of these kind of analyses is provided in Figure 7B. Data were normalized for β -actin expression levels and compared to endogenous gene expression for MPCs infected with negative control viruses (Y-axis: percent gene expression of endogenous gene; 100% = endogenous mRNA levels present in negative control sample).

Analysis of the upregulation of endogenous bone AP mRNA versus that of placental or intestinal AP mRNA.

BAP is the physiologically relevant AP involved in bone formation. In order to determine whether the measured AP activities were due to upregulation of BAP

expression or of another AP gene product, mRNA levels for all AP genes were analysed for infected MPCs.

mRNA levels were determined as described in the previous sections. The difference is in the primer set used (see

5 Table 2): one set detects BAP ALPL (human alkaline phosphatase liver/bone/kidney) mRNA expression. Another set detects the expression of the 3 other AP genes (ALPI (human alkaline phosphatase intestinal), ALPP (human alkaline phosphatase placental (PLAP)), and ALPPL2 (human
10 alkaline phosphatase placental-like)). ALPI, ALPP and ALPPL2 are highly similar at the nucleotide level and can therefore be amplified using one primer pair.

The primer pairs were first validated on RNA isolated from MPCs infected with Ad-eGFP and Ad-BMP2.

15 Figure 9 illustrates the strong upregulation of BAP mRNA by Ad-BMP2 and the absence of upregulation of expression of any of the other AP genes. MPCs were infected in 24 well plate format using Ad-eGFP (negative control) or the osteogenic Ad-BMP2. Cells were harvested and RNA was
20 prepared and subjected to rtRT-PCR using primer sets amplifying BAP mRNA or mRNA from the other 3 AP genes (PLAP/IAP). Ad-BMP2 strongly upregulates BAP mRNA levels but not the mRNA levels of the other 3 AP genes.

Both primer sets were then used to measure mRNA
25 levels for all AP genes in RNA isolated from Ad-siRNA infected MPCs.

Example 6. Mineralization

The process of osteogenesis consists of several
30 successive events. During the initial phases of osteogenesis, bone alkaline phosphatase (BAP) becomes upregulated. It is however equally important to look at specific events occurring in later stages of osteogenesis such as mineralization.

Assay setup

The process of osteogenesis consists of several successive events. During the initial phases of osteogenesis, bone alkaline phosphatase (BAP) becomes upregulated. Later, during differentiation, cells deposit (hydroxy)apatite (Ca^{2+} -phosphate precipitate) on an extracellular matrix consisting mostly of collagen type I to form mineralized bone. In the bone cell mineralizing assay (BM assay), primary human MSCs are differentiated *in vitro* into mineralizing osteoblasts using BMP2 (recombinant or delivered by adenoviral transduction) as an osteogenic agent. Mineralization is then visualized by staining the MSCs with Alizarin Red, a dye with a high affinity for calcium (see Figure 10).

Screening and hit calling

The following optimized protocol is used for screening Ad-siRNA and Ad-cDNA targets identified in the primary assay:

100,000 MPCs are seeded in each well of a 6 well plate in 2 ml MSC medium, containing 10% FCS. The next day, after incubation at 37°C, 10% CO₂ in a humidified incubator, cells are co-infected with AdC15-hCAR (final MOI of 750) and Ad-siRNA, Ad-cDNA or control viruses at a final MOI of 1250, 2500 and 5000. Cells are incubated at 37°C, 10% CO₂ in a humidified incubator for a further six days. Virus is removed and replaced by 2 ml fresh MSC medium, 10% FCS. Over the next 22 days, medium is refreshed 3 times in 2 weeks. Every other time, medium is refreshed half or completely. At 28 days after the start of the experiment, the conditioned medium is removed, cells are fixed using 10% paraformaldehyde and the monolayers stained with 1 mL of ~1% Alizarin Red (Sigma, # A5533) in MilliQ water (pH adjusted to 4.2).

Ad-eGFP, to assess infection efficiency, Ad-BMP2 as strong osteogenic inducer and Ad-H4-2 as a weak osteogenic factor are included in each experiment as negative and positive controls, respectively. Every
5 experiment where Ad-H4-2 does not induce mineralization is entirely repeated.

The Ad-shRNAs that induced mineralization are presented in Table 6.

10 Example 7. Drug discovery against the identified targets

Compounds are screened for binding to the polypeptides of the present invention. The affinity of the compounds to the polypeptides is determined in a
15 displacement experiment. Such displacement experiments are well known in the art, and can be considered as a common technique among others to identify compounds that bind to polypeptides.

In brief, the polypeptides of the present invention
20 are incubated with a labeled (radio-labeled, fluorescent- or antibody-labeled, or any other detectable label) ligand that is known to bind to the polypeptide and is further incubated with an unlabeled compound.

The displacement of the labeled ligand from the
25 polypeptide is determined by measuring the amount of labeled ligand that is still associated with the polypeptide. The amount of the labeled ligand associated with the peptide is an indication of the affinity for the unlabeled compound.

30 The amount of labeled ligand associated with the polypeptide is plotted against the concentration of the unlabeled compound to calculate IC50 values. This value reflects the binding affinity of the unlabeled compound

to its target, i.e. the polypeptides of the present invention.

Compounds are considered strong binders, when having an IC₅₀ in the nanomolar and even picomolar range.

5 Compounds that have an IC₅₀ of at least 10 micromol or even better in the nmol to pmol range are applied in either the bone alkaline phosphatase assay (BAP) and/or in assays to determine their effect on the induction of osteoblast markers and osteoblast function. Compounds
10 with a lower IC₅₀ are generally considered as of less interest. The polypeptides of the present invention can be prepared in a number of ways depending on whether the assay will be run on cells, cell fractions or biochemically, on purified proteins. Such preparations
15 are well known in the art, as are the different assays.

Example 8. Osteoclast assays: validate anti-resorptive activity of identified targets

Throughout life, the skeleton is in a constant state
20 of remodeling. Focal areas of bone are resorbed by osteoclasts and then replaced by bone matrix newly formed by osteoblasts. The development of osteoporosis is characterized by severe bone loss due to the deregulation of the balance between osteoclast and osteoblast
25 activity, leading to an increased osteoclast-mediated bone resorption.

Osteoclasts emanate from cells of the monocyte/macrophage lineage. In vivo, the differentiation of osteoclast precursor cells towards osteoclasts is
30 controlled by two central factors expressed by stromal cells (MPCs): receptor activator of NFκB ligand (RANKL) and osteoprotegerin (OPG). RANKL is a membrane bound ligand expressed on the surface of MPCs which drives osteoclast differentiation. OPG is a soluble decoy

receptor for RANKL which inhibits osteoclast differentiation by scavenging active RANKL. The balance between RANKL and OPG expression by MPCs determines the level of osteoclast differentiation.

5 As MPCs control the differentiation of osteoclasts, it is important to know the effect of the identified target Ad-siRNAs on osteoclast differentiation or activity. Target Ad-siRNAs that decrease osteoclast differentiation/activity, are very valuable, as these are
10 expected to increase bone apposition by two mechanisms: increase of differentiation / activity of osteoblasts and decrease in osteoclast activity. As illustrated by various precedents (Thirunavukkarasu et al., (2000) *J Biol Chem* 275 : 25163-72 ; Yamada et al., (2003) *Blood*
15 101 : 2227-34) such a pleiotropic effect of osteogenic factors can be expected.

Osteoclast differentiation assay

The effect of osteogenic factors on
20 osteoclastogenesis is evaluated through two types of assays.

In a first assay setup, a co-culture of MPCs with primary human mononuclear cells is performed. The effect of the infection of the MPC monolayer with a knock-down
25 virus on its capacity to support osteoclastogenesis is evaluated. The desired effect is the following: knock-down of the Ad-siRNA target gene expression in the MPCs should inhibit osteoclast differentiation driven by a physiological trigger as e.g. a mixture of 10nM
30 1,25(OH)₂vitD₃ and 50nM M-CSF. The monocytes used can be derived from bone marrow or peripheral blood. In the present example, a differentiation experiment based on peripheral blood derived mononuclear cells (PBMCs) is described. MPCs (obtained from Cambrex/Biowhittaker,

Verviers, Belgium) are seeded in 96 well plates (1000 cells per well) in α -MEM medium (GIBCO-Life Technologies) supplemented with 10% FBS and a day later, these are infected with a target Ad-siRNA. At least three days later, 100 000 PBMCs per well are added as well as M-CSF (R&D systems, 50ng/ml final concentration). Half the volume of medium is refreshed twice a week by medium + 50ng/ml M-CSF and 10nM 1,25(OH)₂vitD₃. Readout is performed 14 days after addition of the PBMCs to the coculture. Spontaneous osteoclast differentiation driven by the physiologically relevant mixture of triggers can be assessed by multiple readouts. Microscopic assessment of the number of 'TRAP positive', multinucleated cells per well is a generally accepted measure for the level of osteoclast differentiation. 'TRAP positive' means that the cells possess a tartrate resistant acidic phosphatase (TRAP) activity. To assess this, the coculture is subjected to an *in situ* TRAP staining performed according to the Acid Phosphatase detection kit (SIGMA, 386-A). Positive cells acquire a purple color upon treatment. As an alternative readout, a marker specific for mature osteoclasts is measured e.g. TRACP5b (tartrate resistant acidic phosphatase type 5b), calcitonin receptor (CTR) or Cathepsin K (CTSK). Measurement of the amounts of osteoclast-derived tartrate resistant acidic phosphatase protein (TRACP5b) in the coculture supernatant is performed by a commercially available ELISA (BoneTRAP assay, Sba sciences, Turku, Finland). CTR or CTSK are detected by immunocytochemistry, upon application of following general protocol. Medium is removed and the coculture is fixed (4% paraformaldehyde, 0.1% TritonX-100, 4°C, 30 min), washed and blocking buffer (PBS + 1%BSA + 0.1% Tween20) is added for an incubation of at least 4hrs. The blocking buffer is removed and the

primary antibody directed against CathepsinK (e.g. Oncogene, IM55L) or Calcitonin receptor (e.g. Serotec, AHP635), dissolved at the desired concentration in a suited buffer (e.g. 0.05M Tris.HCl pH 7.4, 1% BSA), is
5 added to the wells. Incubation is performed overnight, 4°C. The mixture is removed, the cells washed (PBS + 0.1% Tween20) and the suited, HRP conjugated secondary antibody, diluted in the same buffer as the primary antibody, is added. After an incubation of at least 4hrs,
10 a washing step is performed (PBS + 0.1% Tween20) and luminol (a substrate for HRP yielding a luminescent signal: BM Chemiluminescence ELISA Substrate [POD] (luminol), Roche Diagnostics, Cat No 1582950) is added. After 5 min incubation, readout is performed with a
15 luminometer (Luminoskan Ascent, Labsystem). The 2 assays described (assessment of the amount of multinuclear cells and immunochemistry for the detection of osteoclast-specific markers) allow to assess the differentiation of the mononuclear cells towards osteoclasts, but do not
20 yield information about the bone resorptive activity of the osteoclasts formed.

Activity of the osteoclasts is measured in the pit formation assay. For this purpose, the co-culture and infection of cells is performed as described for assays
25 described above with the difference that a bone-like substrate is present at the bottom of the well in which the co-culture is performed. This bone-like substrate can be a dentin slice (e.g. Kamiya Biomedical Company, Seattle (Cat No KT018)) or equivalent (Calcium carbonate
30 coating, OAASTM, Gentaur; BiocoatTM OsteologicTM, BD Biosciences) that is commercially available. The co-culture is performed for at least 14 days on the bone like substrate. Cells are then removed by treatment with sodium hypochlorite and the area resorbed by the

osteoclasts (the resorption pit) can be assessed microscopically. This can be facilitated by the treatment of the surface of the dentin slice with toluidine blue.

In a second assay setup, the effect of the infection of the osteoclast precursor cells (PBMCs or BMMCs) with a hit virus on its ability to differentiate towards an osteoclast is measured in a monoculture assay. For this purpose, the monocytes (PBMCs or BMMCs) are seeded in a 384 well plate in α MEM medium supplemented with 10% serum and 25ng/ml recombinant M-CSF (R&D systems). One day after seeding, the cells are infected with target Ad-siRNAs. Four days after infection, recombinant RANKL is added to the wells (25ng/ml, R&D systems). Medium is refreshed twice a week. Fourteen days after addition of RANKL, the differentiation of the monocytes towards osteoclasts is measured using one of the readouts described for the former assay setup. This assay allows the identification of factors that are indispensable for the response of osteoclast precursor cells to M-CSF or RANKL.

PBMC isolation

PBMCs are obtained from peripheral blood (obtained from patients after informed consent) subjected to the following protocol. Blood is aseptically poured into 50 ml Falcon tubes and spun at 3000 g for 10 min at 25°C. The buffy coat is then collected and diluted 1:1 with PBS. The diluted buffy coat is poured on top of 20 ml Lymphoprep (Sigma) contained in a 50ml Falcon tube. Upon centrifugation (35 min at 400 g at 25°C), a white layer of mononuclear cells on top of the Lymphoprep is collected and washed twice with PBS (centrifugation at 200 g, 10 min, 25°C) and rediluted in 7 ml PBS. This solution is pipetted onto a layer of 7ml of hyperosmolar Percoll gradient contained in a 15ml Falcon tube and

centrifuged 35 min at 400 g at 25°C. The hyperosmolar Percoll gradient is prepared as follows : 1 volume of 1.5 M NaCl and 9 volumes of Percoll (Pharmacia, d=1,130 g/ml) are mixed. This mixture is added 1:1 to a
5 PBS/Citrate buffer (NaH₂PO₄ 1.49 mM, Na₂HPO₄ 9.15 mM, NaCl 139.97 mM, Na-citrate (dihydrate) 13 mM, pH 7.2). After centrifugation, monocytes form a discrete ring on top of the gradient. Monocytes are collected and washed in culture medium. Cells are then ready to use in assays.

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Example 9. Analysis of 'off-target' knock down effect.

SiRNAs exert knock-down of gene expression through a
15 recently discovered and partially understood mechanism. It is generally accepted that the specific annealing of the siRNA sequence to mRNA is responsible for a gene-specific 'on-target' knock-down. However, it cannot be excluded yet that limited mismatching between the siRNA
20 and another mRNA can induce 'off-target' down-regulation of gene expression.

In order to exclude that the knock-down of (an) 'off-target' mRNA(s) was responsible for the observed osteogenic effect, additional siRNAs/shRNAs were designed
25 for 38 targets that induced mineralization (Example 6) using stringent design criteria. The additional Ad-shRNAs were then tested in the BAP assay.

To address the question of possible 'off-target' effects, additional siRNA sequences were designed that
30 align perfectly with the mRNA targeted by the original siRNA. Preferred siRNA sequences do not align to other mRNAs. However, in some cases only siRNAs could be designed that showed some overlap to other mRNAs. For siRNAs that aligned to a minimal number of 'off-target'

mRNAs (maximum of 2 basepairs non-identity checked for every position of the 19mer) the following rules were applied: the putative 'off-target' mRNAs must be different from the putative 'off-target' mRNAs identified for the original siRNA; and putative 'off-target' mRNAs must be different from the putative 'off-target' mRNAs identified for all original target siRNAs. The only exception to these rules made during the course of these experiments were siRNAs designed for PPIA.

For each of the 38 selected target genes, 7 additional siRNAs were designed and processed to derive recombinant adenoviruses. All siRNAs were sequenced upon cloning, to verify their identity and exclude errors due to the oligonucleotide synthesis.

261 Ad-shRNAs were successfully generated and tested in the BAP assay at 3 MOIs in 2 independent experiments, in parallel with the original 38 Ad-shRNAs.

Recombinant adenoviruses encoding the designed shRNAs (Ad-shRNAs) were produced, titered, aliquoted in 96 well plates and stored at -80°C. These plates were processed in the primary BAP assay as follows:

MPC cells were seeded with a Multidrop 384 (Labsystems) in black 384 well plates with clear bottom (Costar or Nunc) in 60 μ l MSC medium containing 10% fetal calf serum (Progentix, The Netherlands), at a density of 500 cells per well. One day later, a 96 well plate containing aliquoted Ad-shRNAs and another containing negative and positive control viruses (knock-down control plate) were thawed and virus aliquots transferred to the MPC plate using a 96-channel dispenser (Tecan Freedom 200 equipped with a TeMO96 and a RoMa plate handler, Tecan AG, Switzerland). For the control plate, 1 μ L virus stock (average titer of of 2×10^9 viral particles per ml) was transferred to the 384 well screening plates. On the

control plate (see Figure 3), negative (N1, N2, N3) and positive (P1, P2) control viruses are diagonally distributed over the plate. N1, N2, N3: Ad-siRNAs targeting the eGFP, mannose-6-phosphate-receptor and luciferase mRNAs, respectively. P1 and P2: Ad-siRNAs targeting the PRKCN (H24-010) and MPP6 (H24-011) mRNAs. P3: Ad-eGFP: overexpression of eGFP allows monitoring of infection efficiency.

The Ad-shRNAs were screened at 3 multiplicities of infection (MOIs): 12,000, 4,000 and 1,333. Ad-shRNAs were aliquotted into the inner wells of a 96 well plate at a titer of $1.5 \times 10^9/4 \mu\text{l}$ and 1/3 and 1/9 dilutions were derived of this plate (Figure 11). The resulting 3 plates were used to infect 3 x 384 well plates seeded with MSCs, in the A1 and B2 positions of each quadrant using robotics. The viruses from the control plate were pipetted in the A2 and B1 positions.

Next, 5 μl of adenovirus expressing the human coxsackie and adenovirus receptor (hCAR) (AdC15-hCAR/AdC20-hCAR) was transferred into these wells (final MOI of 155) from a 96 well V-bottom plate with the aid of the 96-channel dispenser.

Plates were then incubated at 37°C, 10% CO₂ in a humidified incubator for four days. Four days post infection, the medium containing the adenoviruses was replaced by 60 μl fresh MSC medium containing 10% FCS free of virus. After an additional nine days of incubation, medium was removed, 15 μL of a 4-methylumbelliferylphosphate solution (Sigma, # M3168) was added to each well and the fluorescence of 4-methylumbelliferone released by the alkaline phosphatase activity was measured after 15 min incubation at 37°C using a fluorimeter (excitation: 360 nm; emission: 440 nm; FluoStar, BMG).

All Ad-shRNAs viruses were screened in duplicate at 3 MOIs in two independent screens. Thresholds were calculated for hit calling using either all negative controls present in one screening round ('Global' analysis) or using the negative controls present on one screening plate ('Local' analysis). Threshold was set at a BAP signal higher than the mean plus 3 times the standard deviation of negative controls. The two individual datapoints for each virus in the batch were analyzed independently. Hits were selected if one Ad-shRNAs scored at least at one MOI in duplicate in at least one of the 2 screens above the threshold (see Table 3).

A 'Global' analysis of the data identified 61 siRNAs targeting 32 loci and a 'Local' analysis identified 84 siRNAs targeting 35 loci. The identity of the 38 selected genes is presented in Table 1 together with the final number of siRNAs that scored in the BAP assay. In this Table, the numbers indicate all siRNAs that scored in the BAP assay, including the original 38 siRNAs. Based on the 'Global' respectively 'Local' analysis, an average of 2.61 and 3.21 constructs was finally identified for each of the 38 validated targets.

All original 38 Ad-shRNAs scored in the BAP assay based on both the 'Global' and 'Local' analysis.

In conclusion, additional Ad-shRNAs targeting 38 selected targets were designed and constructed. Negative controls present on the control plates were used per plate ('Local' analysis) or per batch of plates ('Global' analysis) to determine the cutoff for hit calling.

The 'Global' analysis resulted in 61 viruses that scored positive in the BAP assay, confirming 32 of the 38 validated targets. The 'Local' analysis resulted in 84 viruses that scored positive in the BAP assay, confirming

35 of the 38 validated targets. All original 38 Ad-shRNA viruses scored in the BAP assay when using either the 'Global' or the 'Local' analysis. The targets LOC160662, PPIA and SLC39A4 were identified only in the 'Local' analysis.

Table 1: Primer sets used:

	Gene	primer name	sequence	SEQ ID
10	CALCRL	CALCRL_For	catggcgacctgaaggaaag	221
	CALCRL	CALCRL_Rev	agagaccaaaagaccctggaagt	222
	SRC	SRC_For	acagcggcggcttctaca	223
	SRC	SRC_Rev	catcggcgtgtttggagtagt	224
	PSMB3	PSMB3_For	ATCCGGATCACCTGTTTGAAAC	225
	PSMB3	PSMB3_Rev	GTGGTGATTTTGTCTTCTCGAT	226
15	HP43.8KD	HP43.8KD_For	CCATACACAGAGGGAAGCATACG	227
	HP43.8KD	HP43.8KD_Rev	CAGTCTTGCTGTGATCTGGGAGTA	228
	APEX	APEX_For	GCATAGGCGATGAGGAGCAT	229
	APEX	APEX_Rev	GACCTCGGCCTGCATTAGG	230
	GPR38	GPR38_For	CATCGTCGCTCTGCAACTTTT	231
	GPR38	GPR38_Rev	CCGCTCTGTACTTCTTTGAAATGA	232
20	ROCK2	ROCK2_For	CCTGGTGGAGACCTTGTAACCT	233
	ROCK2	ROCK2_Rev	AGCAAGAACAACCTTCAGCAGTGTA	234
	CXCR6	CXCR6_For	GCCATGACCAGCTTTCACTACA	235
	CXCR6	CXCR6_Rev	GTTAAGGCAGGCCCTCAGGTA	236
	OPN3	OPN3_For	CTAACCGTGCTGGCCTATGAAC	237
	OPN3	OPN3_Rev	CAGGCCCAGGAAAAATTGATC	238
	FUK	FUK_For	TTCGCGATCAGCCCCTTAC	239
25	FUK	FUK_Rev	ACTCACTGGCTGAGGAGGTCAT	240

Gene-specific primers were developed for the genes denoted in the left column. Their orientation (For = sense, Rev = antisense) is indicated in the second column. The sequence of the primers is given in the third column.

Table 2. Primers used for identification of PLAP and other phosphatases

Name	Sequence	SEQ ID NO:
JDO-05F (PLAP)	TTCCAGACCATTTGGCTTGAGT	241
JDO-05bis R (PLAP/ALPI/ALPPL2)	ACTCCCCTGACTTTCCTGCT	242
JDO-21F (BAP)	CATGCTGAGTGACACAGACAAGAAG	243
JDO-21R (BAP)	TGGTAGTTGTTGTGAGCATAGTCCA	244

Table 3: Identification of multiple siRNAs for selected validated targets in the BAP assay.

Gene ID	Global analysis - Redundancy	Local analysis - Redundancy
APG4B	2	3
C13orf6	2	2
C20orf121	3	4
CCR1	3	4
CCR3	2	2
CEL	2	4
CLIC6	2	2
CSNK1G1	2	3
DUSP5	2	2
FLJ22955	3	4
FRK	3	3
GPR124	2	3
GPR23	2	3
GPR64	4	5
Grin2a - GRIN2A	7	6
GZMK	2	2
HRMT1L3	2	2
IRAK2	4	4
LOC160662 - LST-3	1	2
LOC167417 - GPR150	4	5
LOC254378	3	4
MAP3K9	4	5
MLNR	4	3
MMP23B -MMP23A	3	2
MNK2	2	3
OR1A2	4	6
PPIA	1	3
RASD1	4	5
RDH11	3	4
SENP3	4	4
SLC16A3	2	3
SLC26A8	1	1
SLC39A4	1	2
SLC4A10	1	1
TAO1	3	4
TAS1R3	2	3
TRPM6	1	1
ULK1	2	3

Table 4. Overview of isolated knock-down constructs and corresponding target gene sequences

								SEQ ID NO:
Hit ID	KD Sequence	Target	Target Symbol	Gene	GenBank Nucleotide Accession	GenBank Polypeptide Accession	Name	KD construct
H24-049	GTACCTGCAGGTGCTCAGC		AVPR1B		NM_000707	NP_000698	arginine vasopressin receptor 1B	1
H24-225	ATGGGGCTTCAACAGCCACC		AVPR1B		NM_000707	NP_000698	arginine vasopressin receptor 1B	2
H24-001	CAACTTGTAACCTGGGCAGC		GPR38		NM_001507	NP_001498	G protein-coupled receptor 38	3
H24-002	GGTGAAGGAGGTGCTGGAC		STK11 - LKB1		NM_000455 - SK208	NP_000446	serine/threonine kinase 11 (Peutz-Jeghers syndrome)	4
H24-003	GAATCTCTTCGCAAGATC		HSM801665 - MGC8407		AL136697 - NM_024046	NP_076951	hypothetical protein MGC8407	5
H24-004	CATGCTGTTTGAGAGCATC		MKNK2 - MNK2		NM_017572 - SK236	NP_060042 - NP_951009	MAP kinase-interacting serine/threonine kinase 2	6
H24-005	TTTGTGCTGATTCCATGGC		CALCL		NM_005795	NP_005786	calcitonin receptor-like	7

H24-006	GACGGTGTTAATGATAGCC	CSNK1G1 - CK1g1	NM_022048 SK647	NP_071331	casein kinase 1, gamma 1	8
H24-007	CTTCGGCACTCCTGAGTTC	HSA247087 CaMLCK - MLCK	AJ247087 - SK536 NM_182493	NP_872299	myosin light chain kinase	9
H24-008	GCACAGTTTGAGAAAGCAGC	ROCK2	NM_004850	NP_004841	Rho-associated, coiled-coil containing protein kinase 2	10
H24-009	ACGCAAAGTGGCCAGGAGC	IPT	NM_017646	NP_060116	tRNA isopentenyltransferase 1	11
H24-010	CGATGTGCCTTCAAGATTC	PRKCN - PKD3	NM_005813 SK489	NP_005804	protein kinase C, nu	12
H24-011	GTGCACGGATTCAGAGAGC	MPP6	NM_016447	NP_057531	membrane protein, palmitoylated 6	13
H24-012; H24-161	CTTCTACACGTCCATGCTC	TYMSTR	NM_006564	NP_006555	G protein-coupled receptor	14
H24-013	CAACCTGCTGGTGCTCGTC	OPN3	NM_014322	NP_055137	opsin 3 (encephalopsin, panopsin)	15
H24-014	CTCTCTTAGATCTGGAACC	GZMK	NM_002104	NP_002095	granzyme K (serine protease, granzyme 3; tryptase II)	16
H24-015	AGCAGGAAGCGGACATAC	AF073344 - USP3	AF073344 NM_006537	NP_006528	ubiquitin-specific protease 3 - ubiquitin specific protease 3	17
H24-016	CTCCAGCTGAGTGAGCAGC	FUK	NM_145059	NP_659496	L-fucose kinase	18
H24-	TAAACCAGCAGAGGAGCTC	MGC26954	NM_145025	NP_659462	hypothetical protein MGC26954	19

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H24-028	TCATGTTGACCAAGCAAGC	ADAM22	NM_004194 NM_016351 NM_021721 NM_021722 NM_021723	- NP_004185 - NP_057435 - NP_068367 - NP_068368 NP_068369	- - - - -	a disintegrin and metalloproteinase domain 22	30
H24-029	CTATGCCATCACCTTCTGC	LOC254378	XM_174812	XP_174812		LOC254378	31
H24-030	TGTGCCGAAGGATGTAAGC	LOC137491	XM_070459	XP_070459		similar to a disintegrin and metalloproteinase domain 25 (testase 2)	32
H24-031	CCGGGACATAACTAAATCC	LOC138529	XM_070951	XP_070951		similar to bile salt-dependent lipase oncofetal isoform	33
H24-032	AGCAGGCTATGGGATCAAC	C9	NM_001737	NP_001728		complement component 9	34
H24-033	CCACAAGTTGCAGCATTC	XYLB	NM_005108	NP_005099		xylokinase homolog (H. influenzae)	35
H24-035	GGGCTCAGCCAGGAGATTC	CABC1 - ADCK3	NM_020247 SK609	- NP_064632		chaperone, ABC1 activity of bcl complex like (S. pombe)	36
H24-036	CAGGTAGACATGGCGGCAC	FRK	NM_002031	NP_002022		fyn-related kinase	37
H24-037	CTCTCCAGGACACTGACTC	GPR113	NM_153835	NP_722577		G protein-coupled receptor 113 (GPR113)	38
H24-038	GCACGATTTGGAGGTCGCC	ULK1	NM_003565	NP_003556		unc-51-like kinase 1 (C. elegans)	39
H24-039	CGCTCTGGAGTCTCTCTCC	NTRK1	NM_002529	NP_002520		neurotrophic tyrosine kinase, receptor, type 1	40

H24-040	CCCCAACTACACGGAGTTC	GSK3A		NM_019884	NP_063937	glycogen synthase kinase 3 alpha	41
H24-041	GGACTCTCAGTTCAGCATC	PIK3C2B		NM_002646	NP_002637	phosphoinositide-3-kinase, class 2, beta polypeptide	42
H24-042	CTTTGCGATACATGAGCCC	PRKR		NM_002759	NP_002750	protein kinase, interferon-inducible double stranded RNA dependent	43
H24-043	CTTCCTGAAGACCAGGTTC	STK36 AF200815 AX040393 Fused	- AF200815 - AX040393 - SK199 NM_015690		NP_056505	FUSED serine/threonine kinase	44
H24-044	GAATGCTGGCAACAAGACC	HSA311797 HSA311798 MLK4 - KIAA1804	- AJ311797 - AJ311798 - SK691 NM_032435		NP_115811	mixed lineage kinase 4alpha - mixed lineage kinase 4beta	45
H24-045	GAAGATGTCAACGCCCATC		NM_001894 SK084 NM_152221		NP_001885 NP_689407	casein kinase 1, epsilon	46
H24-046	TTCTACGCACCAGAACTCC	CASR		NM_000388	NP_000379	calcium-sensing receptor (hypocalciuric hypercalcemia 1, severe neonatal hyperparathyroidism)	47
H24-047	TGACGGTTGCATTTGCCAC	GNRHR		NM_000406	NP_000397	gonadotropin-releasing hormone receptor	48
H24-048	CCAGACCTCGAGCAACTCC	ADRA1B		NM_000679	NP_000670	adrenergic, alpha-1B-, receptor	49
H24-049	GGAGTGCAATCTGGTTTAC	DRD1		NM_000794	NP_000785	dopamine receptor D1	50

H24-050				NM_000796 NM_033658 NM_033659 NM_033663	- - - NP_000787 NP_387507 NP_387508 NP_387512	dopamine receptor D3			
H24-051	CAGTCAGTGCAACAGTGTC	DRD3							
H24-052	CGTGCTTGTGCATGTTCCGGC	OPRD1		NM_000911	NP_000902	opioid receptor, delta 1			
H24-053	TGACAGGTTCCGCTCTGGGC	TACR1		NM_001058	NP_001049	tachykinin receptor 1			
H24-054	GTACCTGCGGCAGTTGTTC	CCR1		NM_001295	NP_001286	chemokine (C-C motif) receptor 1			
H24-055	GACCATCACCATCCTGGCC	FZD2		NM_001466	NP_001457	frizzled homolog 2 (Drosophila)			
H24-056	GCAGGCAGAGGACAGATTCC	BRS3		NM_001727	NP_001718	bombesin-like receptor 3			
H24-057	CAAAGCCATGCCCGTAAGC	BRS3		NM_001727	NP_001718	bombesin-like receptor 3			
H24-058	AAGTGCCCTGGCCTTCTTC	FPR1		NM_002029	NP_002020	formyl peptide receptor 1			
H24-059	CTACCACAAGCAGGTGTCC	FZD5		NM_003468	NP_003459	frizzled homolog 5 (Drosophila)			
H24-060; H24-168	TGCGTGTCTTCTTTGTGGGC	SMOH		NM_005631	NP_005622	smoothened homolog (Drosophila)			
H24-	CTACGTGCATCCTGTGCCTC	MC5R		NM_005913	NP_005904	melanocortin 5 receptor			

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H24-070	TTTGTGCTGACAGCTGCTC	CTLA1	NM_033423	NP_219491	similar to granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) (H. sapiens)	69
H24-071	CACCTGCTTTCTCAATGCC	KIAA1453	NM_025090	NP_079366	KIAA1453 protein	70
H24-072	GGTTCTCTGACTCGAACAC	FETUB	NM_014375	NP_055190	fetuin B	71
H24-073	AGCACCTCGCTGACATTCC	SENP3	NM_015670	NP_056485	sentrin/SUMO-specific protease 3	72
H24-074	CCTGGGCAACACCTGCTAC	DKFZP586D2223	NM_018561	NP_061031	DKFZP586D2223 protein	73
H24-075	CTTAAGGTGGACCTGTGGC	CPA6	NM_020361	NP_065094	carboxypeptidase A6	74
H24-076	TTGCACAGAAAGGAGGTGC	SPPL2A	NM_032802	NP_116191	putative intramembrane cleaving protease	75
H24-077	GCCCCGAGTCTTACACAAC	LOC121302	XM_062575	XP_062575	similar to protease	76
H24-078	GCTTCTGGTGAGAGGAC	TGM6	NM_198994	NP_945345	transglutaminase 6	77
H24-079	GTGTATGAAGTGGTCCACC	LST-3	XM_292093	XP_292093	liver-specific organic anion transporter 3	78
H24-080	GAAATCTCACTGCTTCGAC	LOC160131	XM_090078	XP_090078	similar to caspase 1, isoform alpha precursor; interleukin 1-beta convertase; interleukin 1-B converting enzyme; IL1B-convertase	79

H24-081	CAC TT TATAAGCCTGCGGC	LOC284964	XM_209423	XP_209423	similar to phosphoglucomutase like protein)	(novel bA395L14.5	80
H24-082	TGGGCATCCTGGCTGTAAAC	LOC254378	XM_174812	XP_174812	LOC254378		81
H24-083	ATAGACTGCAGAAATGAGCC	C9	NM_001737	NP_001728	complement component 9		82
H24-084	CAGTGCCAAAGAGGAGCCC	NAV2	NM_018162 NM_145117	- NP_060632 NP_660093	neuron navigator 2		83
H24-085	TGTGCTCGAAGGAGGAATC	D13S106E	NM_005800	NP_005791	highly charged protein D13S106E		84
H24-086	ATGTATGGATTCCAGCACCC	BCHE	NM_000055	NP_000046	butyrylcholinesterase		85
H24-087	GCTCTGCTATGTGTCAGTC	HSHIN1	NM_017493 NM_199324	- NP_059963 NP_955356	Hin-1		86
H24-088	TGTGACTAAGCTGGAAGAC	PKDREJ	NM_006071	NP_006062	polycystic kidney disease (polycystin) and REJ (sperm receptor for egg jelly homolog, sea urchin)-like		87
H24-089	TGCGCACAATACTGGCCAC	PTPN1	NM_002827	NP_002818	protein tyrosine phosphatase, non-receptor type 1		88
H24-090	TTCCCGCTACCAGACCTAC	GRIK4	NM_014619	NP_055434	glutamate receptor, ionotropic, kainate 4		89
H24-091	AGCATGGTCATTCACATCC	KCNK9	NM_016601	NP_057685	potassium channel, subfamily K, member 9		90

H24-092	ATGCAGGTCCATATGTGAC	TRPM6	NM_017662	NP_060132	transient receptor potential channel, subfamily M, member 6	91
H24-093	CCTTTCTCTGAACACGGAC	ATR	NM_001184	NP_001175	ataxia telangiectasia and Rad3 related	92
H24-094	GTCAGGCTGAATGAGGAGC	PAK6	NM_020168 - SK429	NP_064553	p21(CDKN1A)-activated kinase 6	93
H24-095	CAGGTTCTCCTCAAACGGC	TAS1R3	XM_371210	XP_371210	taste receptor, type 1, member 3	94
H24-096	GCATAGGAGTGGTCATATC	LOC223021	XM_167349	XP_167349	similar to tensin	95
H24-097	ACATCCTGCTGTCAGAGCC	TAO1 - PSK	NM_004783 - NM_016151	NP_004774 - NP_057235	thousand and one amino acid protein kinase - prostate derived STE20-like kinase PSK	96
H24-098	CATGGAGTTCAGCAAGATC	SLC10A1	NM_003049	NP_003040	solute carrier family 10 (sodium/bile acid cotransporter family), member 1	97
H24-099	GTTCTCCAGTGCCATTGGC	SLC16A3	NM_004207	NP_004198	solute carrier family 16 (monocarboxylic acid transporters), member 3	98
H24-100	TTCGGCCTGGACGTCTATC	ORCTL3	NM_004256	NP_004247	organic cationic transporter-like 3	99
H24-101	GTGCATTGGTGAAAGAGAC	IL5	NM_000879	NP_000870	interleukin 5 (colony-stimulating factor, eosinophil)	100
H24-102	TGTGCAGGATAATTGCTGC	TGFB2	NM_003238	NP_003229	transforming growth factor, beta 2	101

H24-103	AGGTGTCACGTACAGTCC	TNFRSF10A	NM_003844	NP_003835	tumor necrosis factor receptor superfamily, member 10a	102
H24-104	AGTGCGCATCTTCGGCCTC	FGF14	NM_004115	NP_004106	fibroblast growth factor 14	103
H24-105	TTTGTGGACTCCTACGATC	RHEB2	NM_005614	NP_005605	Ras homolog enriched in brain 2	104
H24-106	GCCCTGATGTCCATCTTCC	NR1	NM_014434	NP_055249	NADPH-dependent FMN and FAD containing oxidoreductase	105
H24-107	CATAGGGAAGGACACTTGC	IL1F8	NM_014438	NP_055253	interleukin 1 family, member 8 (eta)	106
H24-108	CCTGGATGTGAGAGAGAGC	IL1F8	NM_014438	NP_055253	interleukin 1 family, member 8 (eta)	107
H24-109	AAC TTGTACTATGAAGGCC	RASSF2	NM_014737 NM_170774	- NP_055552 NP_739580	- Ras association (RalGDS/AF-6) domain family 2	108
H24-110	GTATTCTGTACACCCTGGC	ARSDR1	NM_016026	NP_057110	androgen-regulated short-chain dehydrogenase/reductase 1	109
H24-111	TTCTCGCAATGGCCAATGC	PP1L1	NM_016059	NP_057143	peptidylprolyl isomerase (cyclophilin)-like 1	110
H24-112	GAAGAACAGCAGCCTGGAC	RASD1	NM_016084	NP_057168	RAS, dexamethasone-induced 1	111
H24-113	TCAGGCGGATCTTGACAGC	DCXR	NM_016286	NP_057370	dicarbonyl/L-xylulose reductase	112
H24-114	TTCGGCACTGGTTCCCTC	CSNK2B	NM_001320	NP_001311	casein kinase 2, beta polypeptide	113
H24-115	GACGAATATCAGCTCTGCC	PRKAG2	NM_016203	NP_057287	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	114

H24-116	CCTCTCTACGCCATCTACC	ABCG8	NM_022437	NP_071882	ATP-binding cassette, sub-family G (WHITE), member 8 (sterolin 2)	115
H24-117	TCTCTCCACACAAACCTTC	C20orf121	NM_024331	NP_077307	chromosome 20 open reading frame 121	116
H24-118	GGTCGGGAGGAGAACAGTC	OKB1	NM_033125	NP_149116	organic cation transporter OKB1	117
H24-119	GCGAATTCACCAAGCATTTC	SLC26A8	NM_052961	NP_443193	solute carrier family 26, member 8	118
H24-120	TGTCCAGGACCTATTGAGC	UGT1A1	NM_000463	NP_000454	UDP glycosyltransferase 1 family, polypeptide A1	119
H24-121	TGACCATCTGCATGATGTC	HMGCR	NM_000859	NP_000850	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	120
H24-122	GCATGGCATAGTIGGATTTC	HPGD	NM_000860	NP_000851	hydroxyprostaglandin dehydrogenase 15-(NAD)	121
H24-123	ATGCAGGACATGAACAACC	PLCG2	NM_002661	NP_002652	phospholipase C, gamma 2 (phosphatidylinositol-specific)	122
H24-124	ATGTGTGAATGTGGTTGC	TXNRD1	NM_182729 NM_003330 NM_182742 NM_182743	- NP_877393 - NP_003321 - NP_877419 NP_877420	thioredoxin reductase 1	123
H24-125	TGCAGGCTATTCTGTGAGC	B4GALT5	NM_004776	NP_004767	UDP-Gal:betaGlcNAc beta galactosyltransferase, polypeptide 5	124
H24-126	TCGGCACAACAATCTAGAC	IDH3B	NM_174855 NM_006899	- NP_777280 - NP_008830	isocitrate dehydrogenase 3 (NAD+) beta	125

				NM_174856	NP_777281			
H24-127	GACAGGTGGACAGAGCATC	SIAT4B	NM_006927	NP_008858	NP_062828	NP_066191	126	sialyltransferase 4B (beta-galactosidase alpha-2,3-sialyltransferase)
H24-128	TGTGCGAGACCTCGATTTC	HRMT1L3	NM_019854	NP_062828	NP_066191	NP_932349	127	HMT1 hnRNP methyltransferase-like 3 (S. cerevisiae)
H24-129	AGTTTGTGTAGGTATCGCC	B3GALT1	NM_020981	NP_066191	NP_932349	NP_932349	128	UDP-Gal:betaGlcNAc galactosyltransferase, polypeptide 1
			ENSG0000000169066	ENSG0000000169066	ENSG0000000169066	ENSG0000000169066	129	
H24-130	AGCATGAAAGAAACCCTGC	DHRS4	NM_021004	NP_066284	NP_932349	NP_932349	129	0 - peroxisomal short-chain alcohol dehydrogenase

H24- 131	GAAGATCACCATTGCTGAC			XM_292085	XP_292085	-	similar to peptidylprolyl isomerase A (cyclophilin A) - similar to Peptidyl-prolyl cis-trans isomerase A (PPIase) (Rotamase) (Cyclophilin A) (Cyclosporin A-binding protein) (SP18)	130
				XM_292085	-	XP_497621		
				XM_497621	-	XP_371409		
				XM_371409	-	NP_982255		
		PPIA		NM_203431	-	XP_497870		
		LOC341457		XM_497870	-	XP_496579		
		LOC126170		XM_496579	-	XP_496579		
		LOC388817		XM_495800	-	XP_495800		
		LOC402102		NM_203430	-	NP_982254		
		LOC440892		XM_291544	-	XP_291544		
		LOC439953		NM_178230	-	NP_839944		
		LOC343384		XM_372452	-	XP_372452		
		OAS2		XM_372452	-	XP_372452		
H24- 132	GCATGAATATTGTGGAGGC			XM_371304	-	XP_371304	similar to peptidylprolyl isomerase A (cyclophilin A) - similar to Peptidyl-prolyl cis-trans isomerase A (PPIase) (Rotamase) (Cyclophilin A) (Cyclosporin A-binding protein) (SP18)	131
				XM_371302	-	XP_371302		
				XM_060887	-	XP_060887		
				XM_373301	-	XP_373301		
				NM_021130	-	NP_066953		
		PPIA		XM_497621	-	XP_497621		
		LOC126170		XM_371409	-	XP_371409		
		LOC388817		NM_203431	-	NP_982255		
		LOC402102		XM_497870	-	XP_497870		
		LOC391062		XM_372785	-	XP_372785		
		PPIA		NM_203430	-	NP_982254		
		LOC390299		XM_372452	-	XP_372452		
		LOC391352		XM_372916	-	XP_372916		
		LOC128192		XM_060887	-	XP_060887		

				NM_021130	NP_066953			
H24-133	TGCAGGCAAGCAGACGGTC	OXCT2		NM_022120	NP_071403	3-oxoacid CoA transferase 2		132
H24-134	TGACGCAGATGATGAATCC	LOC83468		NM_031302	NP_112592	glycosyltransferase		133
H24-135	TGGCGCCATGTCAGAGTGC	HDAC10		NM_032019	NP_114408	histone deacetylase 10		134
H24-136	CTTATTGTTTCACATTGGCC	LOC170327		XM_093255	XP_093255	similar to cytochrome P-450		135
H24-137	TGGCACCTATGAGAGGATC	LOC166624		XM_093980	XP_093980	similar to UDP-glucuronosyltransferase		136
H24-138	TCAGGTGTCCCATTCACGC	IRAK2		NM_001570 SK180	NP_001561	interleukin-1 receptor-associated kinase 2		137
H24-139	CTCAGAGGTGGTGAAGAC	ITK		NM_005546 SK184	NP_005537	IL2-inducible T-cell kinase		138

H24-140	TGCAGGAGGAAATTGATGC	AF182273 - NM_000776 CYP3A3 - CYP3A4	AF182273 - NM_000776 NM_017460	- NP_000767 NP_059488	cytochrome P450, subfamily IIA (naphthalene oxidase), polypeptide 3 - cytochrome P450, subfamily IIA (naphthalene oxidase), polypeptide 4	139
H24-141	GAGTCCAGCCTTCATGCC	HUMCYPIIF CYP2F1	- J02906 NM_000774	NP_000765	cytochrome P450, subfamily IIF, polypeptide 1	140
H24-142	GTCCAGCTGAAGAAGATCC	GRIN2A	NM_000833	NP_000824	glutamate receptor, ionotropic, N-methyl D-aspartate 2A	141
H24-143	TTCGGCACTGAGGTCTTGC	FLJ22955	NM_024819	NP_079095	hypothetical protein FLJ22955	142
H24-144	TTGCACACTGAGCTGGAGC	FLJ22955	NM_024819	NP_079095	hypothetical protein FLJ22955	143
H24-145	CCTGCTCTTGAGCAATAAC	TEM5	NM_032777	NP_116166	tumor endothelial marker 5 precursor	144
H24-146	TGTCCAGACCACATGGAGC	LOC126537	XM_497611	XP_497611	similar to cytochrome P450, subfamily IVF, polypeptide 2; leukotriene B4 omega-hydroxylase; leukotriene-B4 20-monooxygenase	145
H24-147	TTGCAGGAGGACAAGATGC	GPR153	NM_207370	NP_997253	G protein-coupled receptor 153	146
H24-148	GATTGTGGCCAAGAGTAC	CLIC6	NM_053277	NP_444507	chloride intracellular channel 6	147
H24-149	CCTCATTTATCACCATGCTC	GPR150	NM_199243	NP_954713	G protein-coupled receptor 150	148

H24-150	CTGGTTATTGGCGGTATC	FLJ16008	NM_00100166 5	NP_00100166 5	FLJ16008 protein	149
H24-151	TTTGTGCTTCACCAAGTGC	GPR97	NM_170776	NP_740746	G protein-coupled receptor 97	150
H24-152	CTGCACAGTCAACACGGTC	LOC256135	XM_172523	XP_172523	similar to N-formyl peptide receptor	151
H24-153	GCAGAGCCAAATATCAGCC	LOC254502	XM_174355	XP_174355	LOC254502	152
H24-154	GTTCAAGAAGCTGCGCCAC	OPN1MW - OPN1LW	NM_000513 NM_020061	-NP_000504 NP_064445	opsin 1 (cone pigments), medium-wave-sensitive (color blindness, deutan) - opsin 1 (cone pigments), long-wave-sensitive (color blindness, protan)	153
H24-155	CGTCTTCATCAGCGTGAC	GPR30	NM_001505	NP_001496	G protein-coupled receptor 30	154
H24-156	GCAGTTCCAAGCTTGATC	OPN1SW	NM_001708	NP_001699	opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan)	155
H24-157	GTACCTGCGCCACTTCTTC	CCR3	NM_001837 NM_178329	-NP_001828 NP_847899	chemokine (C-C motif) receptor 3	156
H24-158	GACCATCACTATCCTGGCC	FZD7	NM_003507	NP_003498	frizzled homolog 7 (Drosophila)	157
H24-159	GTCCTTCTACATCAATGCC	GPR23	NM_005296	NP_005287	G protein-coupled receptor 23	158
H24-160	GAAGAAGCAACTGGGAGCC	GPR64	NM_005756	NP_005747	G protein-coupled receptor 64	159

H24-162	GTTCCAGACCTTCATGTGC	CCR9	NM_006641 NM_031200	-NP_006632 NP_112477	chemokine (C-C motif) receptor 9	160
H24-163; H24-226	CTGGACCGAGCTGCTTGAC	ADMR	NM_007264	NP_009195	adrenomedullin receptor	161
H24-165	GCAGAGCATGGGCTTAAGC	G2A	NM_013345	NP_037477	G protein-coupled receptor	162
H24-167; H24-228	AGCAGGCGGAACATGTTCC	CACNB3	NM_000725	NP_000716	calcium channel, voltage-dependent, beta 3 subunit	163
H24-169	CAACCTGTTTCATCCTTAAC	GALR2	NM_003857	NP_003848	galanin receptor 2	164
H24-170	ATTGGTGACACGGCTGAC	C10orf112	XM_295865	XP_295865	chromosome 10 open reading frame 112	165
H24-171	CATATGGCTCTTCAAGTAC	LOC121456	XM_062645	XP_062645	similar to solute carrier family 9 (sodium/hydrogen exchanger), isoform 7; nonselective sodium potassium/proton exchanger	166
H24-172	TGGCACAGTAACAGCTGCC	ACAT1	NM_000019	NP_000010	acetyl-Coenzyme A acetyltransferase 1 (acetoacetyl coenzyme A thiolase)	167
H24-173	GTTCTCTCAGCACGTTTCGC	PGF	NM_002632	NP_002623	placental growth factor, vascular endothelial growth factor-related protein	168

H24-174	TGCAGACTTCAGTGCAGCC	RHAG	NM_000324	NP_000315	Rhesus blood group-associated glycoprotein	169
H24-175	CTTTCTGGAAATCCTGCCC	SLC39A1	NM_014437	NP_055252	solute carrier family 39 (zinc transporter), member 3	170
H24-176	CAACACAGCAGACACAGTC	GALGT	NM_001478	NP_001469	UDP-N-acetyl-alpha-D-galactosamine:(N-acetylneuraminy)-galactosylglucosylceramide N-acetylgalactosaminyltransferase (GalNAc-T)	171
H24-177	TGGGACCAGTGACTGCAGC	GPLD1	NM_001503	NP_001494	glycosylphosphatidylinositol specific phospholipase D1	172
H24-178	AGCCAGAGGAGTTTGTGGC	HRMT1L1	NM_001535 - NM_206962	NP_001526 - NP_996845	HMT1 hnRNP methyltransferase-like 1 (S. cerevisiae)	173
H24-179	CTCGCAGGAAATTCTGGAC	GS3955	NM_021643	NP_067675	GS3955 protein	174
H24-180	ATGCAGGTCAGGTTGTTTC	SLC4A10	NM_022058	NP_071341	solute carrier family 4, sodium bicarbonate transporter-like, member 10	175
H24-181	ATCAGGCCTTACATCCAGC	C8FW	NM_025195	NP_079471	phosphoprotein regulated by mitogenic pathways	176
H24-182	CTGTGGGAGAAACAGAGAC	LOC165927	XM_093541	XP_093541	similar to hypothetical protein, MNCb-4779	177
H24-183	CTCTGCGACTACTACCTGC	B3GALT6	NM_080605	NP_542172	UDP-Gal:betaGal beta galactosyltransferase polypeptide 6	178

H24-184	CTGATGAAGGCCTTCGACC	LOC123326	XM_063593	XM_063593	similar to NADH-ubiquinone oxidoreductase PDSW subunit (Complex I-PDSW) (CI-PDSW)	179
H24-185	ACCGTGGAAGGCCCTATCGC	CYP24	NM_000782	NP_000773	cytochrome P450, subfamily XXIV (vitamin D 24-hydroxylase)	180
H24-186	TTCCAGCTGATATATCCAC	FLJ111149	NM_018339	NP_060809	hypothetical protein FLJ111149	181
H24-187	GCCAGACACTGACAAGTTC	TTBK2	SK453 NM_173500	NP_775771		182
H24-188	TCGGCAGGGCCAGCATTTTC	MST1	NM_020998	NP_066278	macrophage stimulating 1 (hepatocyte growth factor-like)	183
H24-189	GTCTTCGACCCTCAGGAAC	HRG	NM_000412	NP_000403	histidine-rich glycoprotein	184
H24-190	TCAGAAAGTTGTGCAGGAC	APG4B	NM_013325 NM_178326	-NP_037457 NP_847896	KIAA0943 protein	185
H24-191	CAACTTGCTGACTACGGC	APP	NM_201414 NM_000484 NM_201413	-NP_958817 -NP_000475 NP_958816	amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)	186
H24-192	TGTGCAAGAGGTTTCGTTGC	PPP2CB	NM_004156	NP_004147	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	187
H24-193	ACCAGTGGTAAATGTCAGC	DUSP5	NM_004419	NP_004410	dual specificity phosphatase 5	188
H24-194	CTCTGTATCCCATTCCTC	MAP3K9	NM_033141	NP_149132	mitogen-activated protein kinase kinase 9	189

H24-195	CTTCTTGAAGCATCTATGC	MAP3K1	XM_042066	XP_042066	mitogen-activated protein kinase 1	190
H24-196	CATGCTGTTGTCCCTTTACC	LNPEP	NM_005575	NP_005566	leucyl/cystinyl aminopeptidase	191
H24-197	CGAAGATGTTGACCTGGTC	RNASEL	NM_021133	NP_066956	ribonuclease L (2',5'-oligoadenylate synthetase-dependent)	192
H24-198	TGTCCTGTTGGATGCACAC	PRKAA2	NM_006252	NP_006243	protein kinase, AMP-activated, alpha 2 catalytic subunit	193
H24-199	ATGCAGACCCCTGGACCAAC	TNFRSF11A	NM_003839	NP_003830	tumor necrosis factor receptor superfamily, member 11a, activator of NFKB	194
H24-200	GTAGCACTCTGCGACATGC	SLC39A4	NM_017767 NM_130849	-NP_060237 NP_570901	-solute carrier family 39 (zinc transporter), member 4	195
H24-201	CTGGAATGGGATCCGACAC	SDHC	NM_003001	NP_002992	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kd	196
H24-202	GTTATTCTTCCACCATGGC	NNMT	NM_006169	NP_006160	nicotinamide N-methyltransferase	197
H24-203	TGCCAGCAGTCTCTTGATC	CNTFR	NM_001842	NP_001833	ciliary neurotrophic factor receptor	198
H24-204	GATCTTCCGGCCCAACAC	IGFBP5	NM_000599	NP_000590	insulin-like growth factor binding protein 5	199
H24-205	AGCATGACAGGAAACCTGC	UGCGL2	NM_020121	NP_064506	UDP-glucose ceramide glucosyltransferase-like 2	200
H24-206	TCCTTCTCTGTGAACATACC	LOC136234	XM_069782	XP_069782	similar to NADH dehydrogenase subunit 1	201

H24-207	CCTTGTTGGCCAATGATTC	AADAC	NM_207365	NP_997248	similar to Arylacetamide deacetylase	202
H24-208	CCAGGTTTGTCAACGTGAC	AZU1	NM_001700	NP_001691	azurocidin 1 (cationic antimicrobial protein 37)	203
H24-209	TTCCAGCTCAGTGCTAATC	LOC161785	XM_091124	XP_091124	similar to microtubule-associated proteins 1A/1B light chain 3	204
H24-210	AGCAGGAACATCTCTTCGC	LOC219756	XM_166676	XP_166676	similar to MNK1	205
H24-211	CAAGTTCTCCTGCAAGTTC	ATP4B	NM_000705	NP_000696	ATPase, H+/K+ exchanging, beta polypeptide	206
H24-212	GAGCATGACCTTCGATGGC	GABRR2	NM_002043	NP_002034	gamma-aminobutyric acid (GABA) receptor, rho 2	207
H24-213	CTGGCAGAAAGCAGCACAAC	ADAMTS1	NM_006988	NP_008919	a disintegrin-like and metalloprotease (repolysin type) with thrombospondin type 1 motif, 1	208
H24-214	TACCTGCTGGGCATCAAGC	FGF4	NM_002007	NP_001998	fibroblast growth factor 4 (heparin secretory transforming protein 1, Kaposi sarcoma oncogene)	209
H24-215	CTGGAAGTCCTGTGTGATC	IFNA8	NM_002170	NP_002161	interferon, alpha 8	210
H24-216	GGACACCTTCCCAAATGTC	IL19	NM_013371 NM_153758	- NP_037503 NP_715639	interleukin 19	211

H24-217	GGCAGAAATTCAGAGAGC	SLC10A2	NM_000452	NP_000443	solute carrier family 10 (sodium/bile acid cotransporter family), member 2	212
H24-218	CAACATCCCAACTGTGGTC	GSR	NM_000637	NP_000628	glutathione reductase	213
H24-219	TATCCTGACCTTCCTGCGC	KCNG1	NM_002237 NM_172318	-NP_002228 NP_758529	potassium voltage-gated channel, subfamily G, member 1	214
H24-220	TGTTTACCAGTCCGAAGCC	FGF21	NM_019113	NP_061986	fibroblast growth factor 21	215
H24-221	AACTGTACCGCAGAGTTCC	LOC131961	XM_067688	XP_067688	similar to INOSINE-5-MONOPHOSPHATE DEHYDROGENASE 1 (IMP DEHYDROGENASE 1) (IMPDH-I) (IMPD 1)	216
H24-222	AGCATGATGATAGTTCCTC	TOP2B	NM_001068	NP_001059	topoisomerase (DNA) II beta (180kD)	217
H24-223	CAAGTGCCGTATCATCCAC	SRPK1	NM_003137 SK358	NP_003128	SFRS protein kinase 1	218
H24-224	TATTCGTGCGGAGGAAGAC	SgK071 C9orf96	-SK521 NM_153710	NP_714921	chromosome 9 open reading frame 96	219
H24-227	GTACCTGCTGGTGAGTTC	P4HB	NM_000918	NP_000909	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	220

Table 5. Overview of additional knock-down constructs and corresponding target gene sequences

				SEQ ID NO:
KD Target Sequence	Target Gene Symbol	GenBank accession	Name	KD constructs
CGTTGGCAGAATCATTTAC	GPR38	NM_001507	G protein-coupled receptor 38	247
TTCGCGGATGATGTACTTC	GPR38	NM_001507	G protein-coupled receptor 38	248
CTCTCAGTACTTTAACATC	GPR38	NM_001507	G protein-coupled receptor 38	249
CATAACAAAGGCATCGCCC	MKNK2 - MNK2	NM_017572 -SK236	- MAP kinase-interacting serine/threonine kinase 2	250
TTAATGATAGCCATCCAGC	CSNK1G1 - CK1g1	NM_022048 - SK647	casein kinase 1, gamma 1	251
ATTCCAGGCCATTTATGGC	GZMK	NM_002104	granzyme K (serine protease, granzyme 3; tryptase II)	252
TGTTAGACTACGTGATGAC	FLJ14906	NM_032859	hypothetical protein FLJ14906	253
CAACCTCACCTACAGGATC	MMP23A - MMP23B	NM_006983 NM_004659	matrix metalloproteinase - 23A - matrix metalloproteinase 23B	254
TCTACCCGATCAACCACAC	MMP23A - MMP23B	NM_006983 NM_004659	matrix metalloproteinase - 23A - matrix metalloproteinase 23B	255
CCCTTGGAGGAACTATGCC	LOC254378	XM_174812	LOC254378	256
CAACTCAGACAACTGCATC	LOC254378	XM_174812	LOC254378	257
TCATTGTAAGGCTGTGGCC	LOC138529	XM_070951	similar to bile salt-dependent lipase oncofetal isoform	258
GGACCAATGGGAGATAGAC	FRK	NM_002031	fyn-related kinase	259
AGCTTATCCAGCTTTATGC	FRK	NM_002031	fyn-related kinase	260
GTGCATTAACAAGAAGAAC	ULK1	NM_003565	unc-51-like kinase 1 (C. elegans)	261
ACCTGCAGCCTTCACTTTC	CCR1	NM_001295	chemokine (C-C motif) receptor 1	262
TCCCTTCTGGATCGACTAC	CCR1	NM_001295	chemokine (C-C motif) receptor 1	263
CAATTATGAGTCCACGGTC	OR1A2	NM_012352 NM_014565	olfactory receptor, family 1, subfamily A, member 2	264
AACGATGGGCATGTATTTC	OR1A2	NM_012352	olfactory receptor, family 1, subfamily A, member 2	265
TGTCTCCTATGTTTCAAGTC	OR1A2	NM_012352	olfactory receptor, family 1, subfamily A, member 2	266
GTATGGACAGAACTGGCTC	SEN3	NM_015670	sentrin/SUMO-specific protease 3	267
GATGAACATGTATGGAGAC	SEN3	NM_015670	sentrin/SUMO-specific	268

			protease 3	
			sentrin/SUMO-specific	
ATTCTTCAAACGTATGGC	SENP3	NM_015670	protease 3	269
GCTCCTGGAGAACATGTAC	TAS1R3	XM_371210	taste receptor, type 1, member 3	270
			thousand and one amino acid protein kinase -	
ACCAACCTCAGAGGTTCTC	TAO1	NM_016151 NM_004783	-prostate derived STE20- like kinase PSK	271
			thousand and one amino acid protein kinase -	
AAGCGGACCTACAACTTC	TAO1	NM_016151 NM_004783	-prostate derived STE20- like kinase PSK	272
			solute carrier family 16 (monocarboxylic acid transporters), member 3	273
CGTCTACATGTACGTGTTT	SLC16A3	NM_004207	androgen-regulated short- chain	
GTGTGTACATCAACTGTTC	ARSDR1	NM_016026	dehydrogenase/reductase 1 androgen-regulated short- chain	274
GGCACAGTCCAATCTGAAC	ARSDR1	NM_016026	dehydrogenase/reductase 1	275
CGCAAGTTCTACTCCATCC	RASD1	NM_016084	RAS, dexamethasone- induced 1	276
GGTGTTCAGTCTGGACAAC	RASD1	NM_016084	RAS, dexamethasone- induced 1	277
GTGTTTCAGTCTGGACAACC	RASD1	NM_016084	RAS, dexamethasone- induced 1	278
ACTTGAAGTCTCTCCACAC	C20orf121	NM_024331	chromosome 20 open reading frame 121	279
GTCTTCAATAACTTGAAGC	C20orf121	NM_024331	chromosome 20 open reading frame 121	280
			HMT1 hnRNP	
CCACAACAAGCACGTGTTC	HRMT1L3	NM_019854	methyltransferase-like 3 (<i>S. cerevisiae</i>)	281
ATCAATCGAAAGATTCTTC	IRAK2	NM_001570 - SK180	interleukin-1 receptor- associated kinase 2	282
CGACGTTGACAATTCCAGC	IRAK2	NM_001570 - SK180	interleukin-1 receptor- associated kinase 2	283
GCAGAGTTGCAGATTGTTC	IRAK2	NM_001570 - SK180	interleukin-1 receptor- associated kinase 2	284
			glutamate receptor, ionotropic, N-methyl D- aspartate 2A	285
TCAGCATTCCTACGATAAC	GRIN2A	NM_000833	glutamate receptor, ionotropic, N-methyl D- aspartate 2A	286
CCGGCAGAAGGATAACCTC	GRIN2A	NM_000833	glutamate receptor, ionotropic, N-methyl D- aspartate 2A	287
CCAGAACTGTGAAGTTTAC	GRIN2A	NM_000833	glutamate receptor, ionotropic, N-methyl D- aspartate 2A	288
GCATGGCAAGAAAGTTAAC	GRIN2A	NM_000833	glutamate receptor, ionotropic, N-methyl D- aspartate 2A	289
AGCATGTTATGCCTTATGC	GRIN2A	NM_000833	glutamate receptor, ionotropic, N-methyl D- aspartate 2A	289

TCATTGTTTCTGCCATAGC	GRIN2A	NM_000833	glutamate receptor, ionotropic, N-methyl D- aspartate 2A	290
GATCTTTAACCAGCCTGAC	FLJ22955	NM_024819	hypothetical protein FLJ22955	291
GCACACCGTGGTAGTATAC	FLJ22955	NM_024819	hypothetical protein FLJ22955	292
GTCCTTCCACATCAAGAAC	TEM5	NM_032777	tumor endothelial marker 5 precursor	293
TGCAACCTCTTACCCAAGC	CLIC6	NM_053277	chloride intracellular channel 6	294
CTCAATCCCTTCGTCTACC	GPR150	NM_199243	G protein-coupled receptor 150	295
CGTGGTCTACGCGTTCTAC	XM_094471	XM_094471	LOC167417	296
CATTATCACCATGCTCGGC	GPR150	NM_199243	G protein-coupled receptor 150	297
TCTCTCTTCTATCAATCC	CCR3	NM_178329 NM_001837	- chemokine (C-C motif) receptor 3	298
CCAAACGTGTCTGGAAGAC	GPR23	NM_005296	G protein-coupled receptor 23	299
GTACCTGTAGCCATCTAAC	GPR64	NM_005756	G protein-coupled receptor 64	300
GCTAGTGAATAATGATTGC	GPR64	NM_005756	G protein-coupled receptor 64	301
ACACGACTATAAGTCTAAC	GPR64	NM_005756	G protein-coupled receptor 64	302
ACATTGCAATGGACAACAC	APG4B	NM_013325 NM_178326	- KIAA0943 protein	303
GGAATATCCTGAGTGTTC	DUSP5	NM_004419	dual specificity phosphatase 5	304
TGCCAGAGGGATGAACTAC	MAP3K9	NM_033141	mitogen-activated protein kinase kinase kinase 9	305
ATGGAAGACTGCTGGAATC	MAP3K9	NM_033141	mitogen-activated protein kinase kinase kinase 9	306
GAGCGCTTCAAACGAGATC	MAP3K9	NM_033141	mitogen-activated protein kinase kinase kinase 9	307
ACCTGTCCCTAGATTCTTC	APG4B	NM_013325	KIAA0943 protein	308
ACGCATCTTGGCAAAGAGC	CSNK1G1 - CK1g1	NM_022048 - SK647	casein kinase 1, gamma 1	309
		XM_372328	-	
		XM_371409	-	
	PPIA - LOC390006	NM_203431	-	
	- LOC388817	- XM_499491	-	
	LOC442744	- XM_292596	-	
	LOC342541	- XM_292963	-	
	LOC344178	- XM_371304	-	
	LOC388687	- XM_372916	-	
	LOC391352	- XM_170597	-	
	LOC256374	- XM_371302	-	
	LOC388686	- NM_021130	-	
	LOC122335	- XM_063084	-	
	LOC390827	- XM_497571	-	
	LOC390956	- XM_372741	-	
	LOC442362	- NM_203430	-	
	LOC343384	- XM_498254	-	
	COAS2	- XM_291544	-	
	LOC440063	- NM_178230	- peptidylprolyl isomerase	
ATTCCAGGTTTATGTGTC	LOC392352	XM_495896	- A	310

		XM_373301	-	
		XM_060887	-	
		XM_377444		
:				
CAACAGTGCATCTCTTATC LST-3	XM_292093		liver-specific organic anion transporter 3	311
CAGCATCTACCTCCTGAAC CCR1	NM_001295		chemokine (C-C motif) receptor 1	312
CAGCGCTCTCAATCCCTTC GPR150	NM_199243		G protein-coupled receptor 150	313
CATCGACTATATAGCAGGC CEL	NM_001807		carboxyl ester lipase (bile salt-stimulated lipase)	314
CCAAGAGTCTATTCAAAGC OR1A2	NM_012352		olfactory receptor, family 1, subfamily A, member 2	315
CCACTAATGTCAACAATGC GPR23	NM_005296		G protein-coupled receptor 23	316
CCACTCGTCAGATGTTTGC LOC254378	XM_174812		LOC254378	317
CCCTGTCATTGATGGAGAC CEL	NM_001807		carboxyl ester lipase (bile salt-stimulated lipase)	318
CCGAGCCATATACTTGACC C20orf121	NM_024331		chromosome 20 open reading frame 121	319
CCTAGAGCTGATTGAGTTC MNK2 - MNK2	NM_199054		- MAP kinase-interacting	
	NM_017572	-SK236	serine/threonine kinase 2	320
CCTCGACACCAAGTCTTGC RASD1	NM_016084		RAS, dexamethasone-induced 1	321
	NM_016151		thousand and one amino acid protein kinase -	
CGCTCTTTAACATGAATGC TAO1	NM_004783		- prostate derived STE20-like kinase PSK	322
CGTGGACATGGAGTACGAC TAS1R3	XM_371210		taste receptor, type 1, member 3	323
CTCGTAATGAGACTATAGC ARSDR1	NM_016026		androgen-regulated short-chain dehydrogenase/reductase 1	324
CTGCAAATCTTCAGGTTTC GPR64	NM_005756		G protein-coupled receptor 64	325
GAAGCACGATTTGGAGGTC ULK1	NM_003565		unc-51-like kinase 1 (C. elegans)	326
GATGATGAAGGAGACGTTTC FLJ22955	NM_024819		hypothetical protein FLJ22955	327

		XM_497621	-	
		XM_371409	-	
		NM_203431	-	
	LOC126170	- XM_496546	-	
	LOC388817	- PPIA XM_170597	-	
	- KBTBD9	- XM_067176	-	
	LOC256374	- NM_021130	-	
	LOC131055	- XM_372741	-	
	LOC390956	- XM_372785	-	
	LOC391062	- NM_203430	-	
	LOC343384	- XM_291544	-	
			- peptidylprolyl isomerase	
GATTTGGTTATAAGGGTTC	LOC401859	XM_377444	A	328
			solute carrier family 39	
		NM_017767	- (zinc transporter),	
GCTACTGCCCTATATGATC	SLC39A4	NM_130849	member 4	329
			tumor endothelial marker	
TCCGGTCTATTTGATCGC	TEM5	NM_032777	5 precursor	330
			mitogen-activated protein	
TCGCCCTTCCTATTCCTTC	MAP3K9	NM_033141	kinase kinase kinase 9	331
			solute carrier family 16	
			(monocarboxylic acid	
TGTACGTGTCATCCTGGC	SLC16A3	NM_004207	transporters), member 3	332
			olfactory receptor,	
			family 1, subfamily A,	
TGTGACATTATGCCTTTGC	OR1A2	NM_012352	member 2	333

Table 6. Mineralising hits

H24-001 CAACTTGTACCTGGGCAGC	GPR38	NM_001507	G protein-coupled receptor 38
H24-004 CATGCTGTTTGAGAGCATC	MKNK2 - MNK2	NM_017572 - SK236	MAP kinase-interacting serine/threonine kinase 2
H24-006 GACGGTGTAAATGATAGCC	CSNK1G1 CK1g1	-NM_022048 - SK647	casein kinase 1, gamma 1
H24-007 CTTCGGCACTCCTGAGTTC	HSA247087 caMLCK	-AJ247087 - SK536	myosin light chain kinase
H24-009 ACGCAAAGTGGCCAGGAGC	IPT	NM_017646	tRNA isopentenyltransferase 1
H24-013 CAACCTGCTGGTGCCTCGTC	OPN3	NM_014322	opsin 3 (encephalopsin, panopsin)
H24-014 CTCTCTTAGATCTGGAACC	GZMK	NM_002104	granzyme K (serine protease, granzyme 3; tryptase II)
H24-015 AGCAGGAAGGCGGACATAC	AF073344 USP3	-AF073344 NM_006537	-ubiquitin-specific protease 3 - ubiquitin specific protease 3
H24-018 TCAGGTAGTTGGTTCTGAC	F13A1	NM_000129	coagulation factor XIII, A1 polypeptide
H24-019 CTGCGCCGAACAAATGTAC	PSMB3	NM_002795	proteasome (prosome, macropain) subunit, beta type, 3
H24-020 TGTGGCGACTTGTGCACAC	CLPX	NM_006660	ClpX caseinolytic protease X homolog (E. coli)
H24-021 TCTCTCAGTGTAGAATGCC	FLJ14906	NM_032859	hypothetical protein FLJ14906
H24-024 GTGTACTGGTACAAGGACC	MMP23A MMP23B	-NM_004659 NM_006983	-matrix metalloproteinase 23A - matrix metalloproteinase 23B
H24-026 TCTCTCATCAATACTGGTC	APEX	NM_001641 NM_080648 NM_080649	-APEX nuclease (multifunctional DNA -repair enzyme)
H24-029 CTATGCCATCACCTTCTGC	LOC254378	XM_174812	LOC254378
H24-030 TGTGCCGAAGGATGTAAGC	LOC137491	XM_070459	similar to a disintegrin and metalloprotease domain 25 (testase 2)
H24-031 CCGGGACATAACTAAATCC	LOC138529	XM_070951	similar to bile salt-dependent lipase oncofetal isoform
H24-032 AGCAGGCTATGGGATCAAC	C9	NM_001737	complement component 9
H24-033 CCACAAGGTTGCAGCATTC	XYLB	NM_005108	xylulokinase homolog (H. influenzae)
H24-035 GGGCTCAGCCAGGAGATTC	CABC1 - ADCK3	NM_020247 - SK609	chaperone, ABC1 activity of bcl1 complex like (S. pombe)
H24-036 CAGGTAGACATGGCGGCAC	FRK	NM_002031	fyn-related kinase
H24-038 GCACGATTTGGAGGTCGCC	ULK1	NM_003565	unc-51-like kinase 1 (C. elegans)
H24-041 GGA CTCTCAGTTCAGCATC	PIK3C2B	NM_002646	phosphoinositide-3-kinase, class 2, beta polypeptide
H24-049 GTACCTGCAGGTGCTCAGC	AVPR1B	NM_000707	arginine vasopressin receptor 1B
H24-054 GTACCTGCGGCAGTTGTTC	CCR1	NM_001295	chemokine (C-C motif) receptor 1
H24-062 TTCGGACACCCACAAATGC	RRH	NM_006583	retinal pigment epithelium-derived rhodopsin homolog
H24-064 GTTGTCTCTGTTCTGACGTC	OR1A2	NM_012352	olfactory receptor, family 1, subfamily A, member 2
H24-071 CACCTGCTTTCTCAATGCC	KIAA1453	NM_025090	KIAA1453 protein
H24-073 AGCACCTCGCTGACATTCC	SEN3	NM_015670	sen3/SUMO-specific protease 3
H24-078 GCTTCTGGTGGAGAAGGAC	TGM3L	XM_066181	transglutaminase 3-like
H24-079 GTGTATGAAGTGGTCCACC	LOC160662	XM_090422	similar to solute carrier family 21 (organic anion transporter), member 8

H24-084	CAGTGCCAAGAAGGAGCCC	NAV2	NM_018162	neuron navigator 2
H24-092	ATGCAGGTCCATATGTGAC	TRPM6	NM_017662	transient receptor potential cation channel, subfamily M, member 6
H24-093	CCTTTCTCTGAACACGGAC	ATR	NM_001184	ataxia telangiectasia and Rad3 related
H24-095	CAGGTTCTCCTCAAACGGC	LOC126788	XM_060177	similar to TPA: G-protein coupled receptor
H24-097	ACATCCTGCTGTCAGAGCC	TAO1 - PSK	NM_004783 NM_016151	-thousand and one amino acid protein kinase - prostate derived STE20-like kinase PSK
H24-099	GTTCTCCAGTGCCATTGGC	SLC16A3	NM_004207	solute carrier family 16 (monocarboxylic acid transporters), member 3
H24-104	AGTGCGCATCTTCGGCCTC	FGF14	NM_004115	fibroblast growth factor 14
H24-106	CCCCTGATGTCCATCTTCC	NR1	NM_014434	NADPH-dependent FMN and FAD containing oxidoreductase
H24-107	CATAGGGAAGGACACTTGC	IL1F8	NM_014438	interleukin 1 family, member 8 (eta)
H24-108	CCTGGATGTGAGAGAGAGC	IL1F8	NM_014438	interleukin 1 family, member 8 (eta)
H24-109	AACTTGTAATATGAAGGCC	RASSF2	NM_014737	Ras association (RalGDS/AF-6) domain family 2
H24-110	GTATTCTGTACACCCTGGC	ARSDR1	NM_016026	androgen-regulated short-chain dehydrogenase/reductase 1
H24-111	TTCTCGCAATGGCCAATGC	PPIL1	NM_016059	peptidylprolyl isomerase (cyclophilin)-like 1
H24-112	GAAGAACAGCAGCCTGGAC	RASD1	NM_016084	RAS, dexamethasone-induced 1
H24-113	TCAGGCGGATCTTGACAGC	DCXR	NM_016286	dicarbonyl/L-xylulose reductase
H24-117	TCTCTCCACACAAACCTTC	C20orf121	NM_024331	chromosome 20 open reading frame 121
H24-119	GCGAATTCCACCAGCATTC	SLC26A8	NM_052961	solute carrier family 26, member 8
H24-120	TGTCCAGGACCTATTGAGC	UGT1A1	NM_000463	UDP glycosyltransferase 1 family, polypeptide A1
H24-128	TGTGCGAGACCTCGATTTC	HRMT1L3	NM_019854	HMT1 hnRNP methyltransferase-like 3 (S. cerevisiae)
H24-130	AGCATGAAAGAAACCCTGC	ENSG00000169066	ENSG00000169066	-peroxisomal short-chain alcohol dehydrogenase
H24-131	GAAGATCACCATTGCTGAC	PP1A	-NM_021130	-similar to peptidylprolyl isomerase A
		LOC127711	-XM_060625	-(cyclophilin A) - similar to
		LOC128430	-XM_066074	-Peptidyl-prolyl cis-trans isomerase A
		LOC138130	-XM_070771	-(PPIase) (Rotamase) (Cyclophilin A)
		LOC165317	-XM_092514	-(Cyclosporin A-binding protein) (SP18)
		LOC257232	XM_172314	
H24-133	TGCAGGCAAGCAGACGGTC	OXCT2	NM_022120	3-oxoacid CoA transferase 2
H24-136	CTTATTGTTACATTTGGCC	LOC170327	XM_093255	similar to glyceraldehyde 3-phosphate dehydrogenase
H24-138	TCAGGTGTCCCATTCAGC	IRAK2	NM_001570	- SK180 interleukin-1 receptor-associated kinase 2
H24-141	GAGTCCAGCCTTCATGCCC	HUMCYPIIF	-J02906	-cytochrome P450, subfamily IIF, polypeptide 1
		CYP2F1	NM_000774	
H24-142	GTCCAGCTGAAGAAGATCC	GRIN2A	NM_000833	glutamate receptor, ionotropic, N-

			methyl D-aspartate 2A
H24-143 TTCGGCACTGAGGTCTTGC	FLJ22955	NM_024819	hypothetical protein FLJ22955
H24-145 CCTGCTCTTGAGCAATAAC	TEM5	NM_032777	tumor endothelial marker 5 precursor
H24-146 TGTCCAGACCACATGGAGC	LOC126538	XM_065152	similar to cytochrome P450, subfamily IVF, polypeptide 2; leukotriene B4 omega-hydroxylase; leukotriene-B4 20- monooxygenase
H24-148 GATTGTGGCCAAGAAGTAC	CLIC6	XM_092804	chloride intracellular channel 6
H24-149 CCTCATTATCACCATGCTC	LOC167417	XM_094471	LOC167417
H24-150 CTGGTTATTGGCGGGTATC	LOC165245	XM_103864	similar to 25-hydroxyvitamin D-1 alpha hydroxylase, mitochondrial precursor (25-OHD-1 alpha- hydroxylase) (25-hydroxyvitamin D3 1- alpha-hydroxylase) (VD3 1A hydroxylase) (P450C1 alpha) (P450VD1- alpha)
H24-154 GTTCAAGAAGCTGCGCCAC	OPN1MW	-NM_000513	-opsin 1 (cone pigments), medium-wave- sensitive (color blindness, deutan) -
	OPN1LW	NM_020061	opsin 1 (cone pigments), long-wave- sensitive (color blindness, protan)
H24-156 GCAGTTCCAAGCTTGCATC	OPN1SW	NM_001708	opsin 1 (cone pigments), short-wave- sensitive (color blindness, tritan)
H24-157 GTACCTGCGCCACTTCTTC	CCR3	NM_001837	chemokine (C-C motif) receptor 3
H24-159 GTCCTTCTACATCAATGCC	GPR23	NM_005296	G protein-coupled receptor 23
H24-160 GAAGAAGCAACTGGGAGCC	GPR64	NM_005756	G protein-coupled receptor 64
H24-169 CAACCTGTTTCATCCTTAAC	GALR2	NM_003857	galanin receptor 2
H24-173 GTTCTCTCAGCACGTTTCGC	PGF	NM_002632	placental growth factor, vascular endothelial growth factor-related protein
H24-180 ATGCAGGTCAGGTTGTTTC	SLC4A10	NM_022058	solute carrier family 4, sodium bicarbonate transporter-like, member 10
H24-185 ACCGTGGAAGGCCTATCGC	CYP24	NM_000782	cytochrome P450, subfamily XXIV (vitamin D 24-hydroxylase)
H24-188 TCGGCAGGGCCAGCATTTTC	MST1	NM_020998	macrophage stimulating 1 (hepatocyte growth factor-like)
H24-190 TCAGAAGGTTGTGCAGGAC	Apg4B	NM_013325	KIAA0943 protein
H24-191 CAACTTGCATGACTACGGC	APP	NM_000484	amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)
H24-193 ACCAGTGGTAAATGTCAGC	DUSP5	NM_004419	dual specificity phosphatase 5
H24-194 CTCTGTATCCCATTCCTTC	MAP3K9	XM_027237	mitogen-activated protein kinase kinase kinase 9
H24-200 GTAGCACTCTGCGACATGC	SLC39A4	NM_017767	-solute carrier family 39 (zinc transporter), member 4
		NM_130849	
H24-202 GTTATTCTTCCACCATGGC	NNMT	NM_006169	nicotinamide N-methyltransferase
H24-205 AGCATGACAGGAAACCTGC	UGCGL2	NM_020121	UDP-glucose ceramide glucosyltransferase-like 2

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H24-207 CCTTGTTGGCCAATGATTC	LOC166161	XM_093702	similar to arylacetamide deacetylase (esterase)
H24-211 CAAGTTCTCCTGCAAGTTC	ATP4B	NM_000705	ATPase, H ⁺ /K ⁺ exchanging, beta polypeptide
H24-218 CAACATCCCAACTGTGGTC	GSR	NM_000637	glutathione reductase
H24-219 TATCCTGACCTTCCTGCGC	KCNQ1	NM_002237	potassium voltage-gated channel, subfamily G, member 1
H24-224 TATTCGTGCGGAGGAAGAC	SgK071	SK521	
H24-225 ATGGGCTTCAACAGCCACC	AVPR1B	NM_000707	arginine vasopressin receptor 1B

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CLAIMS

1. Method for inducing differentiation of undifferentiated mammalian cells into osteoblasts,
5 comprising contacting said undifferentiated cells with an inhibitor of any of the polypeptides listed in table 4, and/or fragments and/or derivatives of said polypeptide.

2. Method according to claim 1, wherein the molecule
10 is an expression or translation inhibitor inhibiting the expression or translation of a polyribonucleotide encoding the polypeptide.

3. Method according to claim 1, wherein the
15 inhibitor is a nucleic acid expressing an expression or translation inhibitor inhibiting the expression or translation of a polyribonucleotide encoding the polypeptide.

20 4. Method according to claim 3, wherein the nucleic acid is included within a vector.

5. Method according to claim 4, wherein the vector
is an adenoviral, retroviral, adeno-associated viral,
25 lentiviral or a sendaiviral vector.

6. Method according to any of the claims 1-5,
wherein the inhibitor is selected from the group
consisting of an antisense RNA, a ribozyme that cleaves
30 the polyribonucleotide, an antisense oligodeoxynucleotide (ODN), and a small interfering RNA (siRNA) that is sufficiently homologous to a portion of the polyribonucleotide such that the siRNA is capable of

inhibiting the polyribonucleotide that would otherwise cause the production of the polypeptide.

7. Method according to claim 6, wherein the
5 inhibitor is a siRNA, comprising a first nucleotide
sequence of 17-23 nucleotides homologous to a nucleotide
sequence selected from any of the target genes listed in
table 4, and a second nucleotide sequence of 17-23
nucleotides complementary to said first nucleotide
10 sequence.

8. Method according to claim 6, wherein the
inhibitor is a siRNA, comprising a first nucleotide
sequence of 17-23 nucleotides selected from the
15 nucleotide sequences identified by SEQ. ID NO. 1-220 and
247-333 in table 4 and 5, and a second nucleotide
sequence of 17-23 nucleotides complementary to said first
nucleotide sequence.

20 9. Method according to claim 7 or 8, wherein the
siRNA further comprises a third nucleotide sequence
connecting the first and second nucleotide sequence, and
capable of forming a stem-loop structure within the
siRNA.

25

10. Method according to claim 9, wherein the third
nucleotide sequence consist of the nucleotide sequence
defined by SEQ ID NO: 334.

30

11. Method for identifying a compound that induces
differentiation of undifferentiated mammalian cells into
osteoblasts, comprising:

(a) contacting one or more compounds with a
polypeptide listed in table 4, encoded by any of the

genes listed in table 4, and/or fragments and/or derivatives of said polypeptide;

(b) determining the binding affinity of the compound to the polypeptide;

5 (c) contacting a population of undifferentiated mammalian cells with the compound that exhibits a binding affinity of at least 10 micromolar; and

(d) identifying the compound that induces the differentiation of the undifferentiated mammalian cells.

10

12. Method for identifying a compound or mixture of compounds that induces differentiation of undifferentiated mammalian cells into osteoblasts, comprising

15 (a) culturing a population of undifferentiated mammalian cells that express a polypeptide listed in Table 4, encoded by a gene listed in table 4, and/or fragments and/or derivatives of said polypeptide;

(b) exposing said population of cells to a compound
20 or mixture of compounds; and

(c) selecting the compound or mixture of compounds that induces the differentiation of the undifferentiated cells into osteoblasts.

25 13. Method according to claim 11 or 12, wherein the compounds are selected from the group consisting of low molecular weight compounds, peptides, lipids, natural compounds, and antibodies.

30 14. Polynucleotide comprising a sequence of 17-23 nucleotides homologous to a nucleotide sequence selected from any of the target genes listed in table 4, and variants and/or the reverse complements thereof.

15. Polynucleotide according to claim 14 having a nucleotide sequence selected from the nucleotide sequences identified by SEQ. ID NO. 1-220 and 247-333 in table 4 and 5.

5

16. Polynucleotide according to claim 14 having a reverse complement of a nucleotide sequence selected from the nucleotide sequences identified by SEQ. ID NO. 1-220 and 247-333 in table 4 and 5.

10

17. Polynucleotide according to claim 14, 15 or 16 for use as a medicament.

18. Use of a polynucleotide according to claim 14, 15 or 16 in the manufacture of a medicament for the treatment of a disease involving a systemic or local decrease in mean bone density.

19. Vector comprising a polynucleotide according to any of the claims 14-17.

20. Vector according to claim 19 for use as a medicament.

21. Vector according to claim 19 or 20, wherein the vector is an adenoviral, retroviral, adeno-associated viral, lentiviral or a sendaiviral vector.

22. Use of a vector according to claim 19, 20 or 21, for the manufacture of a medicament for the treatment of a disease involving a systemic or local decrease in mean bone density.

23. Method for in vitro production of bone tissue, comprising:

(a) applying undifferentiated mammalian cells on a substrate to form a cellular substrate;

5 (b) introducing a polynucleotide according to claim 14-17, or a vector comprising said polynucleotide, for a time sufficient to differentiate the undifferentiated cells into osteoblasts, thereby producing a continuous bone matrix.

10

24. Method for diagnosing a pathological condition involving a systemic or local decrease in mean bone density or a susceptibility to the condition in a subject, comprising:

15 (a) determining the level of expression of a polynucleotide encoded by any of the target genes listed in table 4, in a biological sample derived from said subject; and

(b) comparing the level of expression with the
20 level of expression of the polynucleotides in a sample derived from a healthy subject;

wherein an increase of the amount of polynucleotide in the sample of the subject compared to the sample of the healthy subject is indicative of the
25 presence of the pathological condition.

25. Method for diagnosing a pathological condition involving a systemic or local decrease in mean bone density or a susceptibility to the condition in a
30 subject, comprising:

(a) determining the amount of a polypeptide encoded by the genes listed in table 4 in a biological sample derived from said subject; and

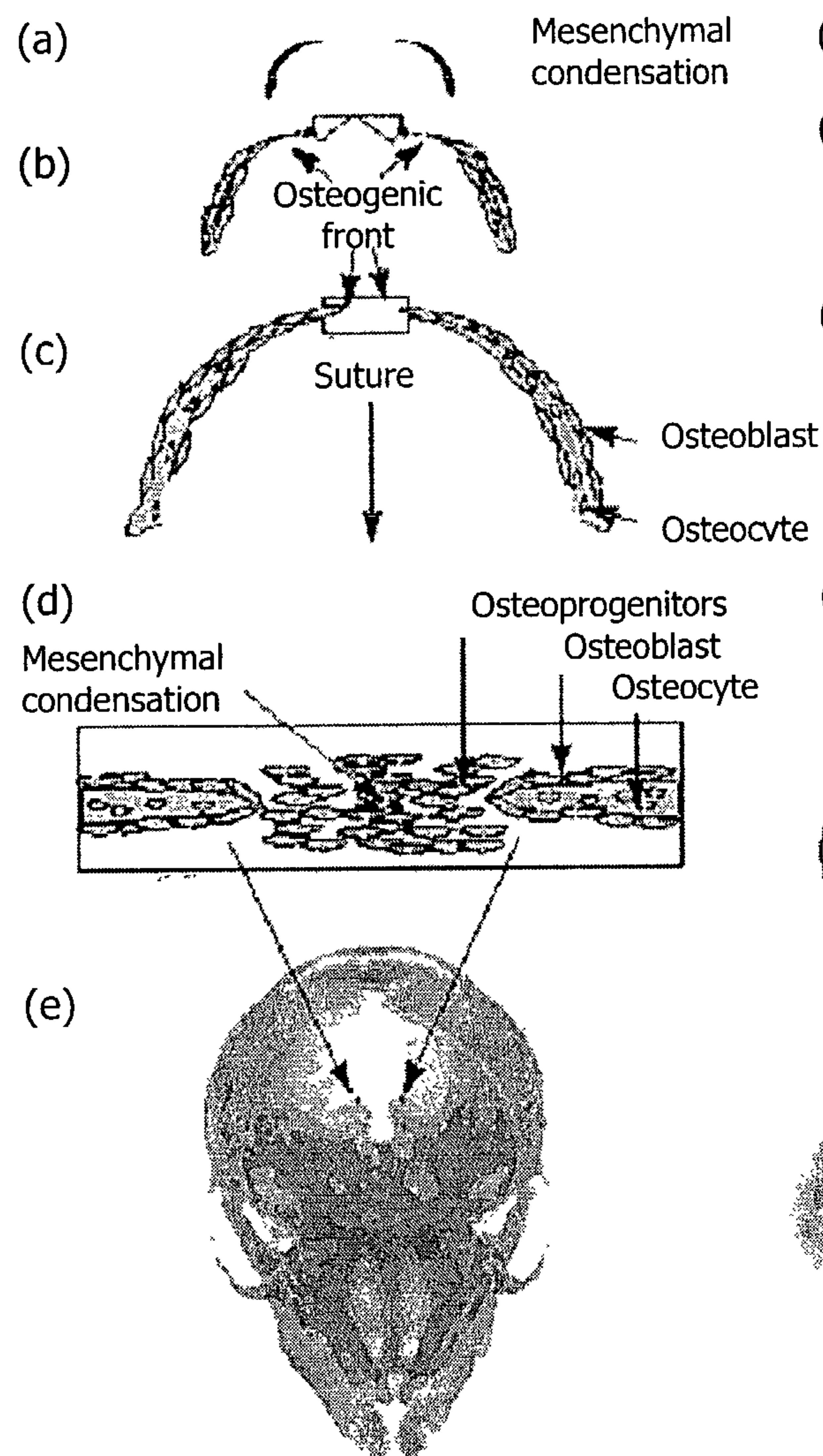
(b) comparing the amount with the amount of said polypeptide in a biological sample derived from a healthy subject;

wherein an increase of the amount of polypeptide in
5 the subject compared to the healthy subject is indicative
of the presence of the pathological condition.

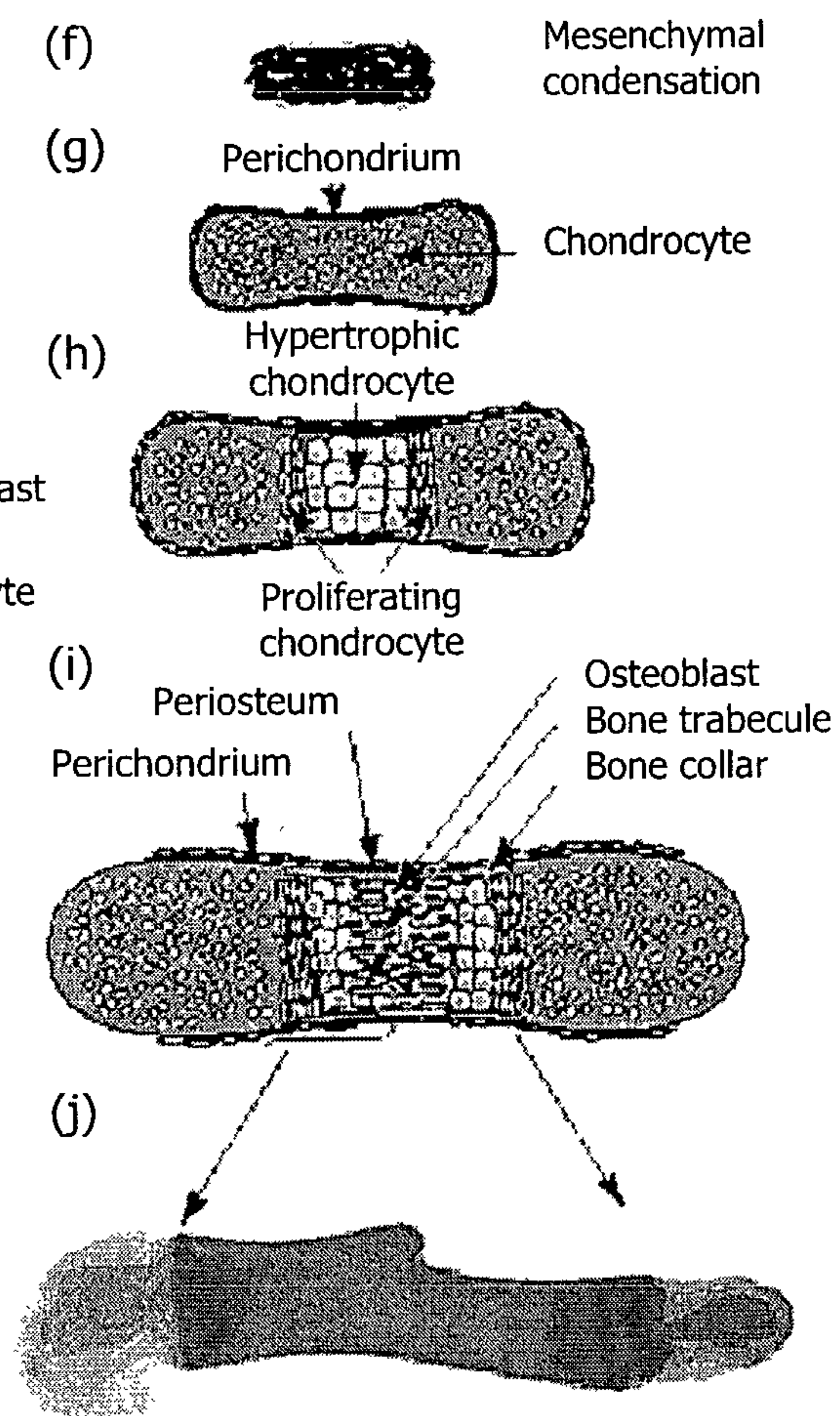
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Intramembranous ossification



Endochondral ossification

FIG. 1

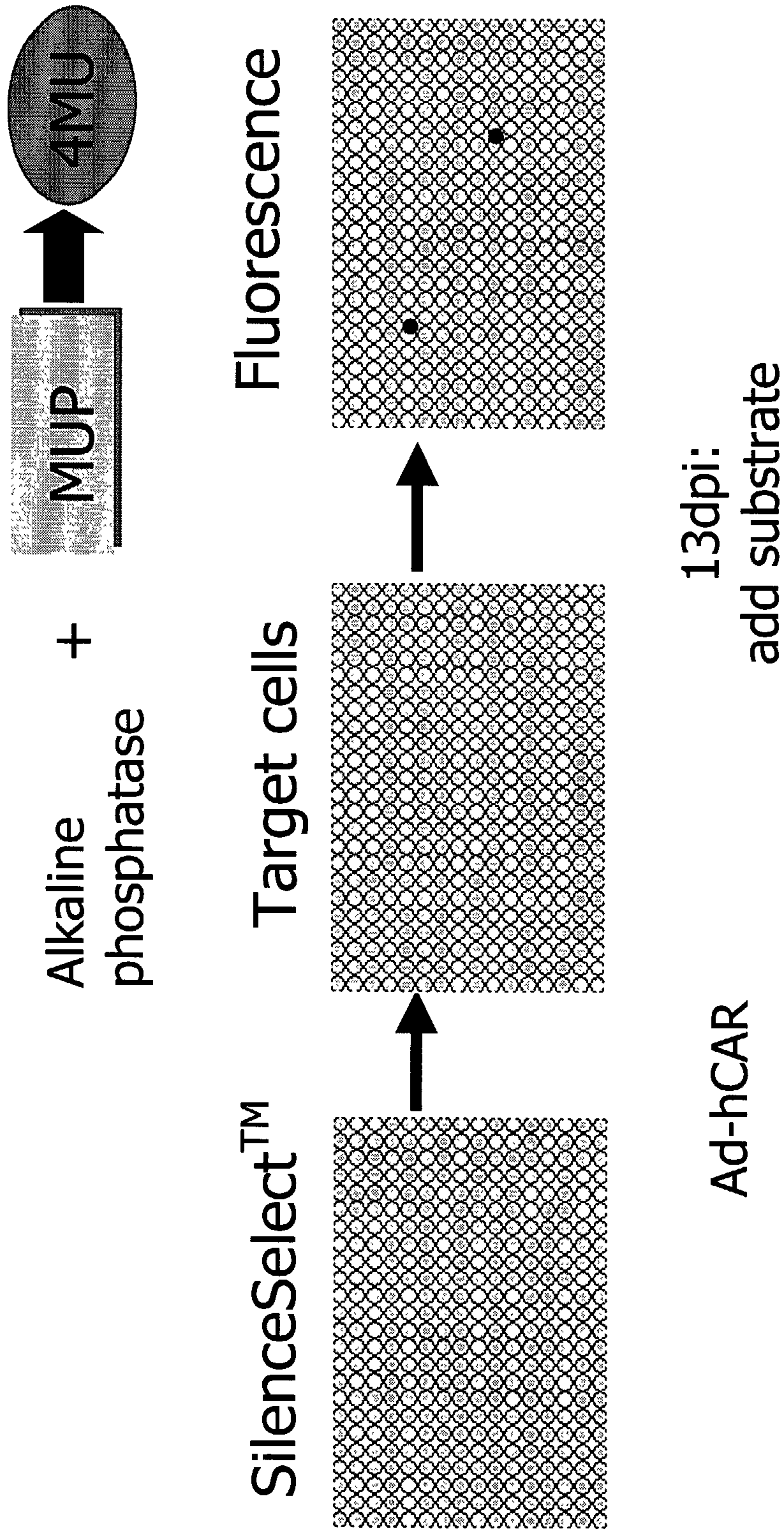


FIG. 2

Lay out disease specific control plate															
96 well plate															
P1	N1	P2	N2	P3	N3	P1	N1	P2	N2	P3	N3	P1	N1	P2	N3
N1	P2	N2	P3	N3	P1	N1	P2	N2	P3	N3	P1	N1	P2	N2	P3
P2	N2	P3	N3	P1	N1	P2	N2	P3	N3	P1	N1	P2	N2	P3	N3
N2	P3	N3	P1	N1	P2	N2	P3	N3	P1	N1	P2	N2	P3	N3	P1
P3	N3	P1	N1	P2	N2	P3	N3	P1	N1	P2	N2	P3	N3	P1	N2
N3	P1	N1	P2	N2	P3	N3	P1	N1	P2	N2	P3	N3	P1	N2	P3
P1	N1	P2	N2	P3	N3	P1	N1	P2	N2	P3	N3	P1	N1	P2	N3
N1	P2	N2	P3	N3	P1	N1	P2	N2	P3	N3	P1	N1	P2	N2	P3
P1: H9	GC-143-193	GC-143-193	GC-143-193	GC-143-193	GC-143-193	N1: eGFP	Ad5001A1101/A1501 00/eGFP.idx 124#32KD GC-143-192	box27							
P2: H10	GC-143-193	GC-143-193	GC-143-193	GC-143-193	GC-143-193	N2: M6PR	Ad5001A1101/A1501 00/M6PR_v1 GC-143-192	box27							
P3: eGFP	GC-143-186	GC-143-186	GC-143-186	GC-143-186	GC-143-186	N3: luc	Ad5001A1101/A1502 00/luc_v13 GC-143-192	box27							

FIG. 3

384 well plate prepared from 1 96 well plate and 1 plate with medium															
This plate is used as source plate for screening															
P1	P1	N1	N1	P2	P2	N2	N2	P3	P3	N3	N3	P1	P1	N1	N1
P1		N1		P2	P2		N2		P3		N3		P1		N3
N1	N1	P2	P2	N2	N2	P3	P3	N3	N3	P1	P1	N1	N1	P2	P2
N1		P2		N2		P3		N3		P1		N1		P2	
P2	P2	N2	N2	P3	P3	N3	N3	P1	P1	N1	N1	P2	P2	N2	N2
P2		N2		P3		N3		P1		N1		P2		N2	
N2	N2	P3	P3	N3	N3	P1	P1	N1	N1	P2	P2	N2	N2	P3	P3
N2		P3		N3		P1		N1		P2		N2		P3	
P3	P3	N3	N3	P1	P1	N1	N1	P2	P2	N2	N2	P3	P3	N1	N1
P3		N3		P1		N1		P2		N2		P3		N2	
N3	N3	P1	P1	N1	N1	P2	P2	N2	N2	P3	P3	N1	N1	P2	P2
N3		P1		N1		P2		N2		P3		N1		P2	
P1	P1	N1	N1	P2	P2	N2	N2	P3	P3	N1	N1	P2	P2	N2	N2
P1		N1		P2		N2		P3		N1		P2		N2	
N1	N1	P2	P2	N2	N2	P3	P3	N1	N1	P2	P2	N2	N2	P3	P3
N1		P2		N2		P3		N1		P2		N2		P3	

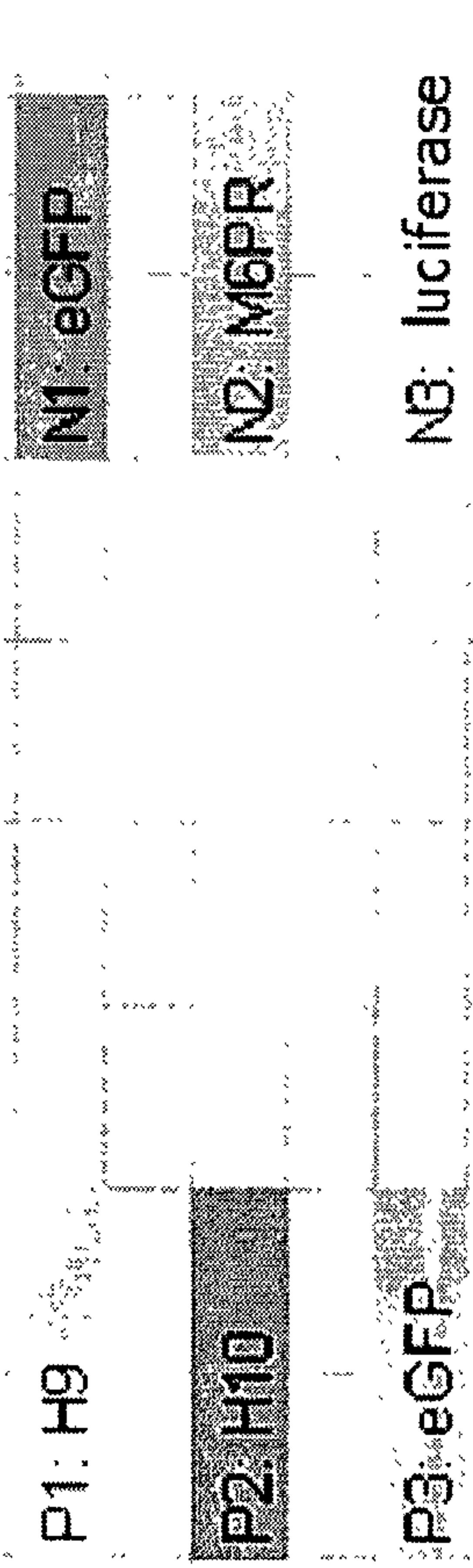


FIG. 4

	Average	Standard deviation	cutoff (=mean (N1-3) + 3 x SD (N1-3))	% positive
P1	20320	5018		98
P2	21063	3138		100
P3	2738	600		0
N1	2940	1584		4
N2	3565	1268		2
N3	3385	962		0
Blanco	3707	1118		1
All negatives	3297	1314	7239	

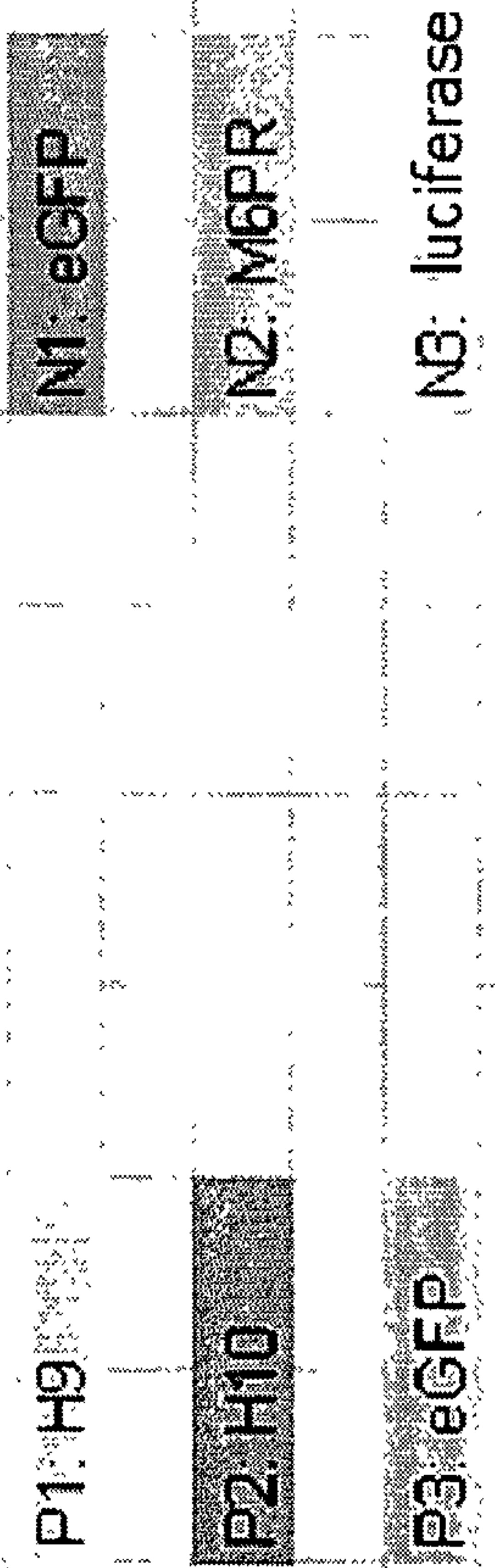


FIG. 5

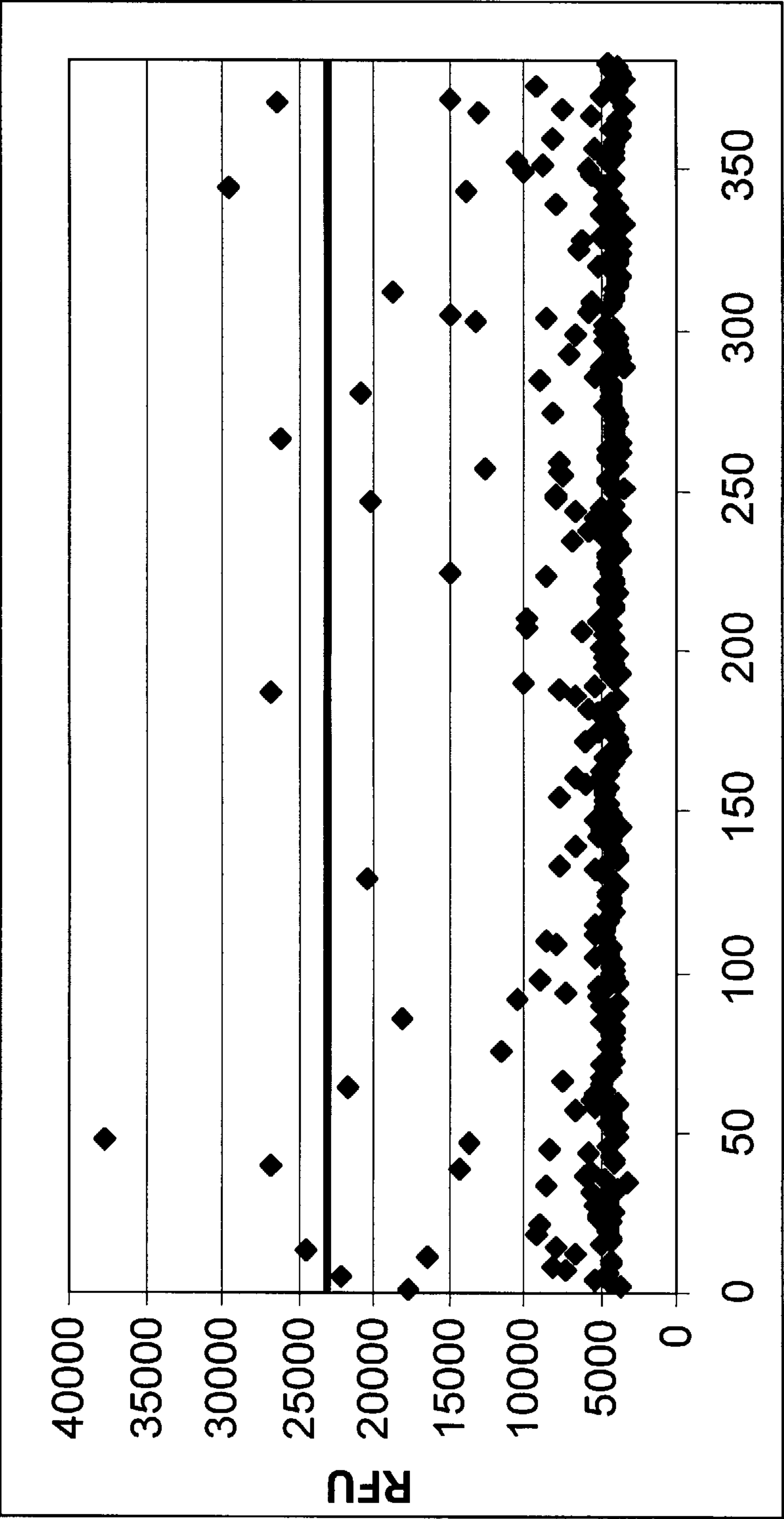
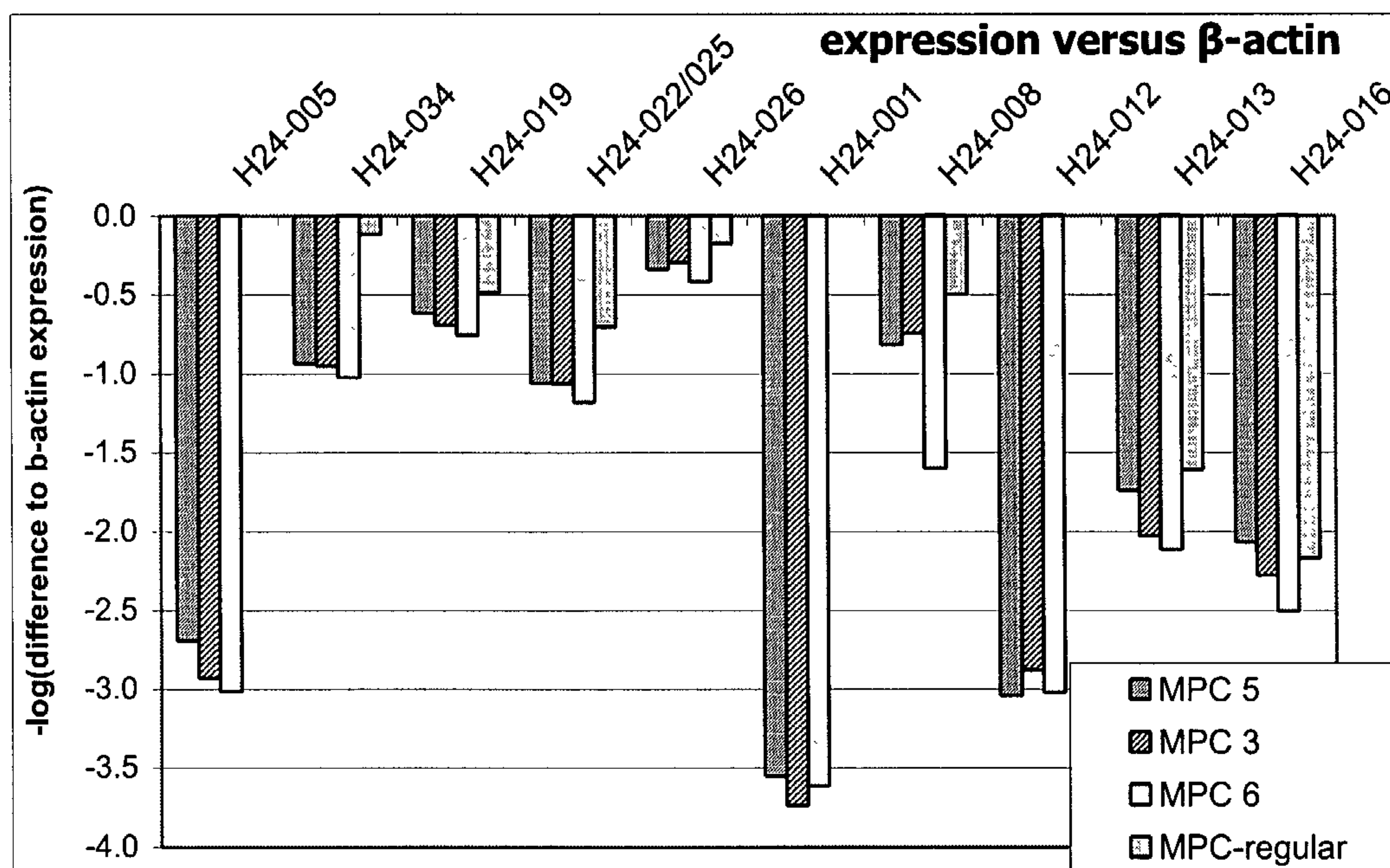
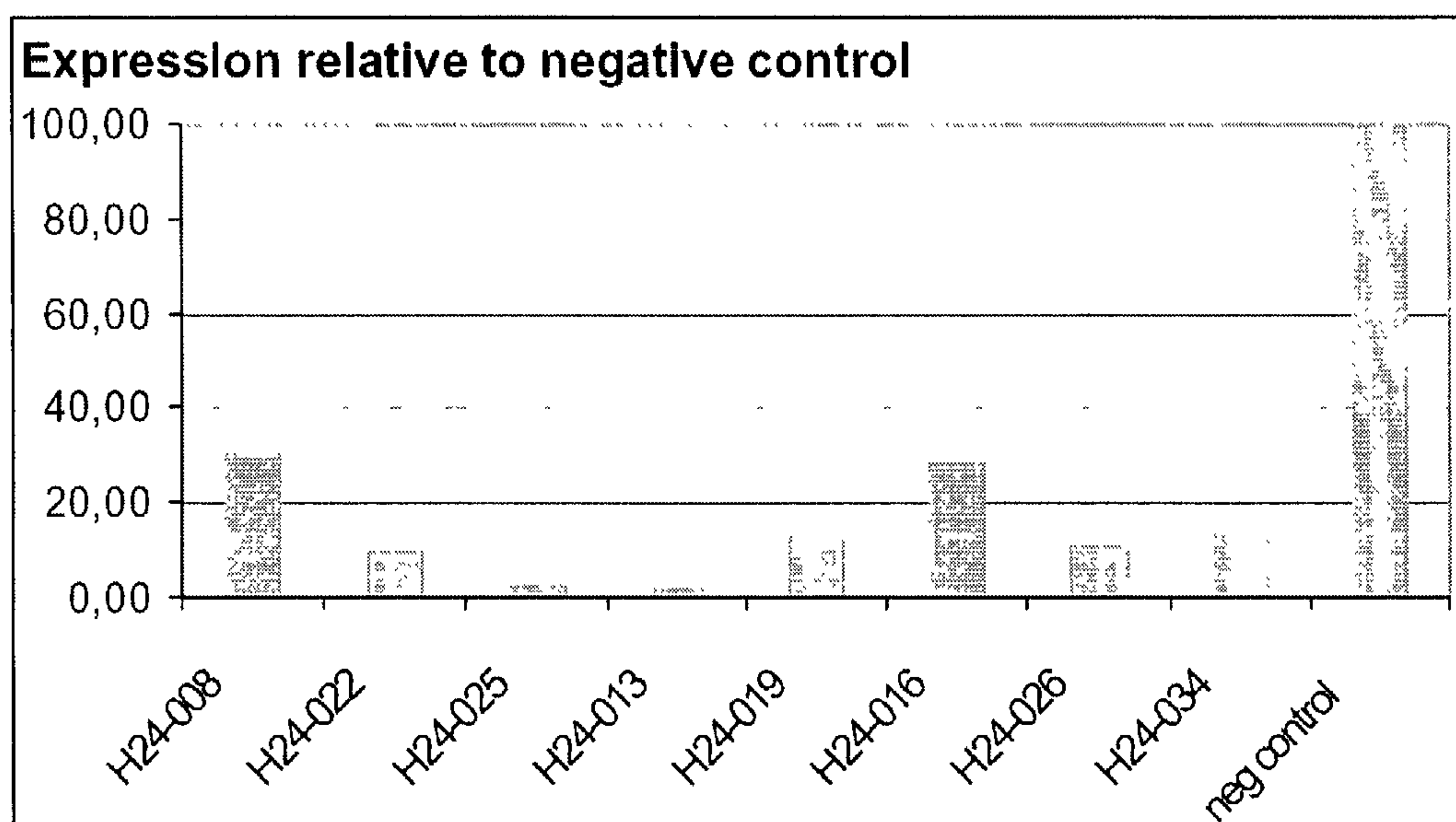


FIG. 6

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FIG. 7AFIG. 7B

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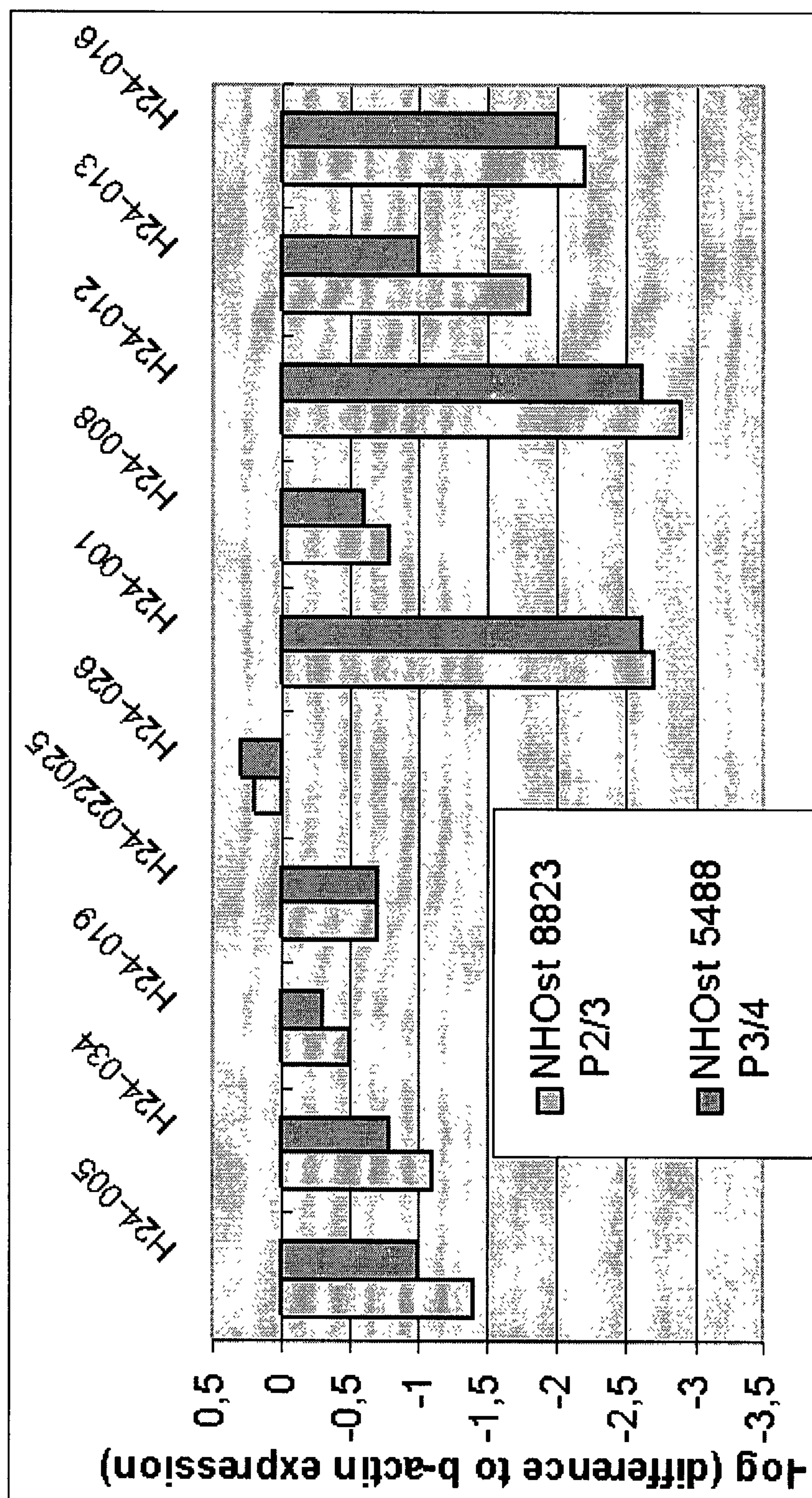


FIG. 8

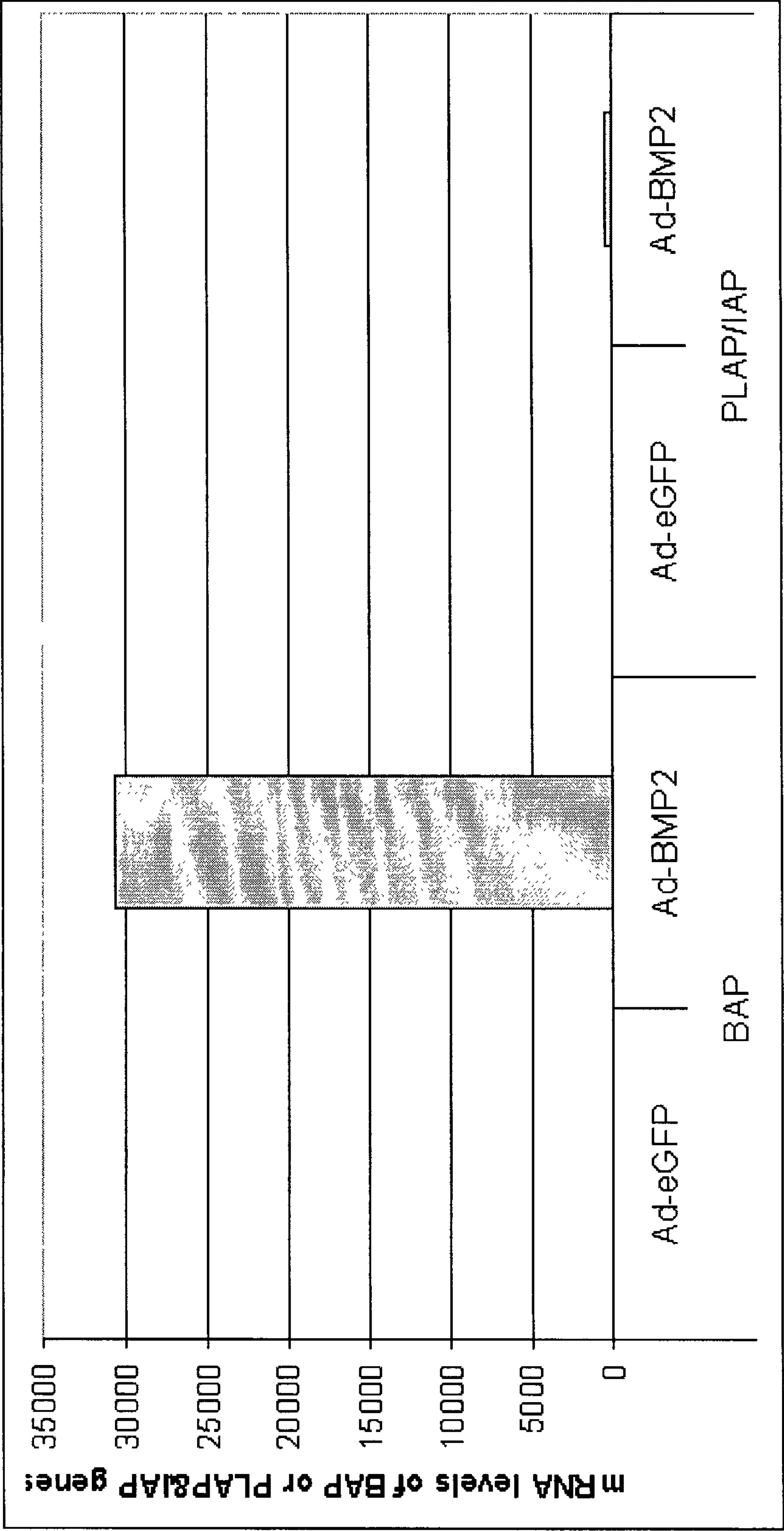
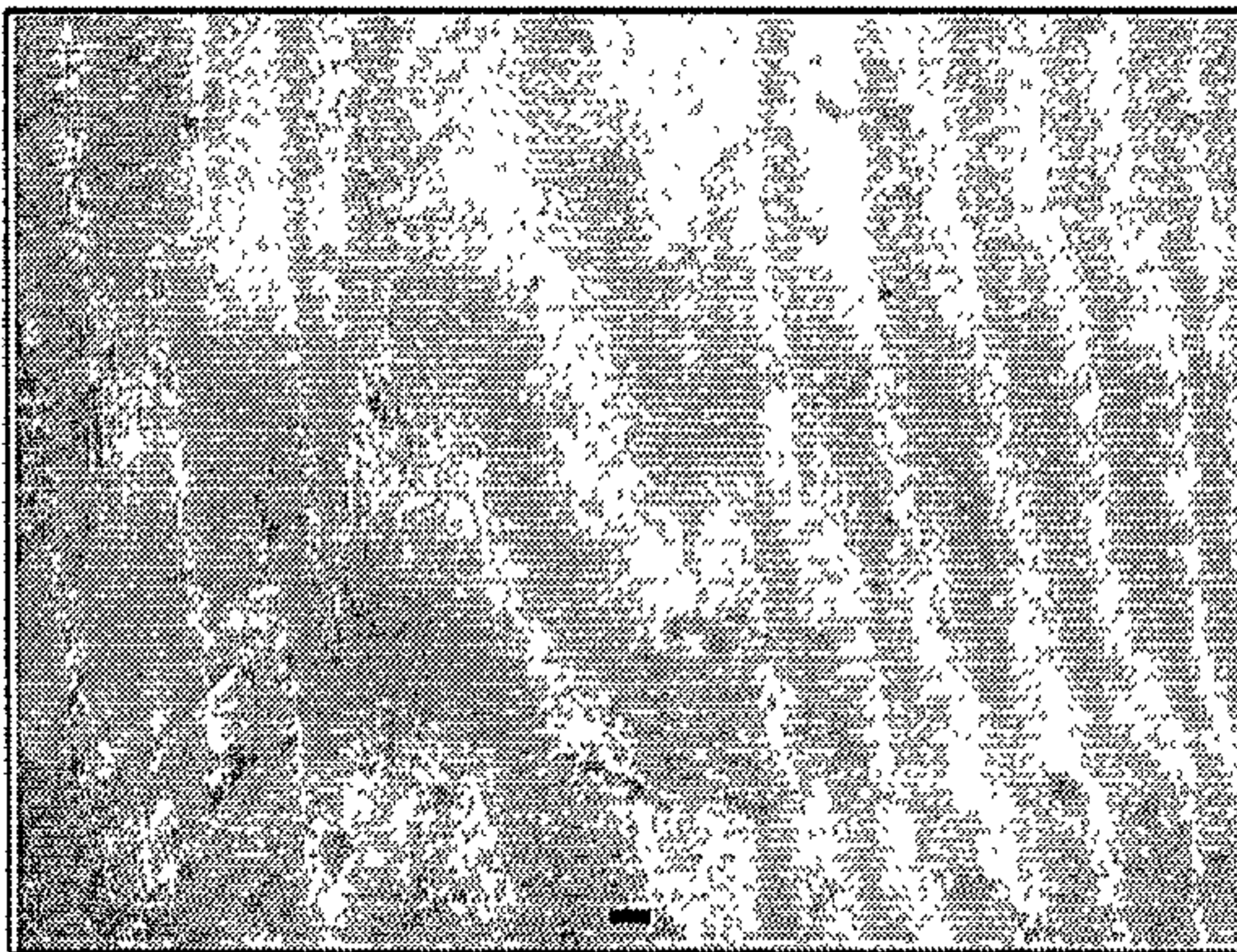


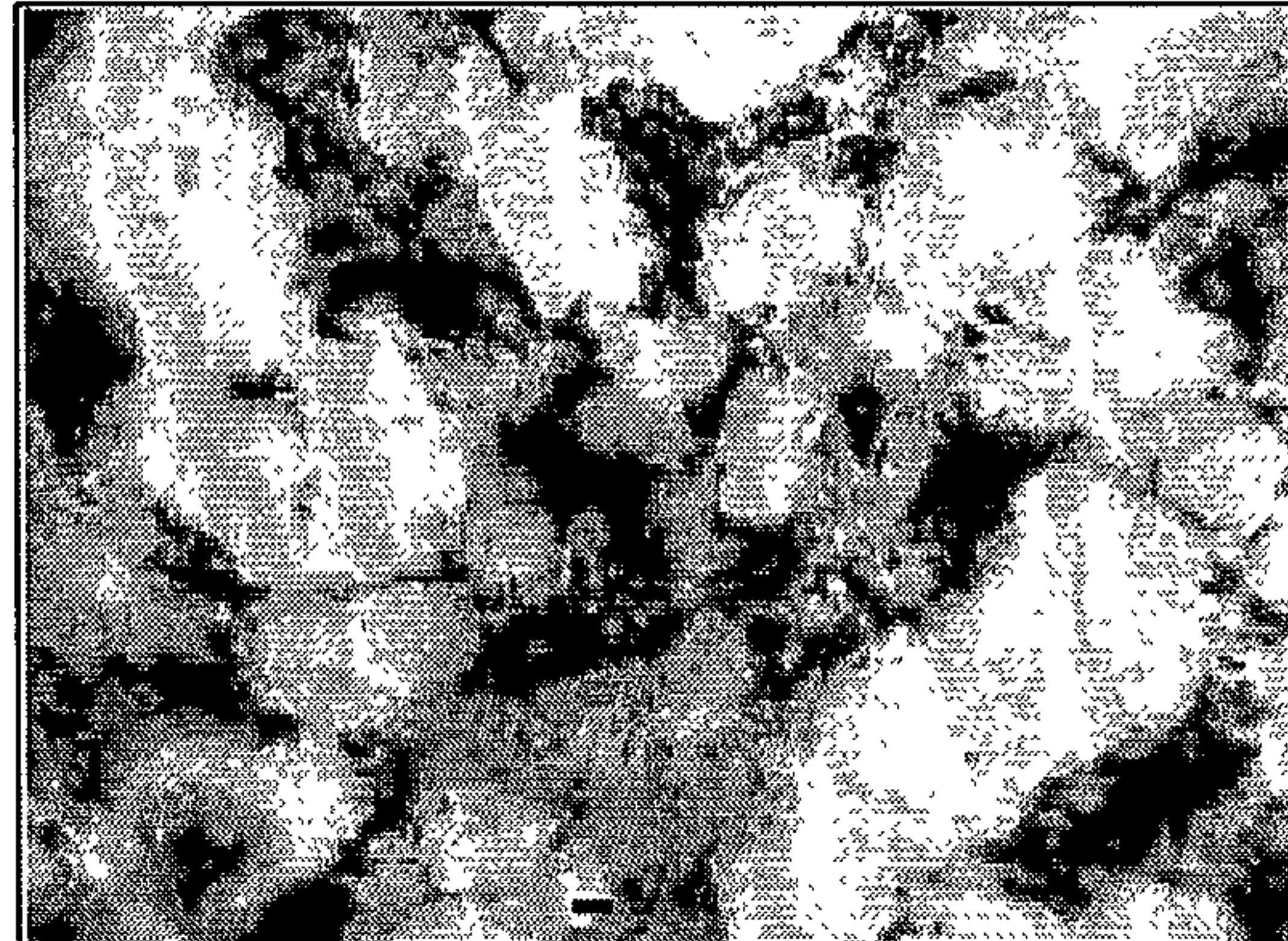
FIG. 9

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Ad5C01-eGFP (KI)

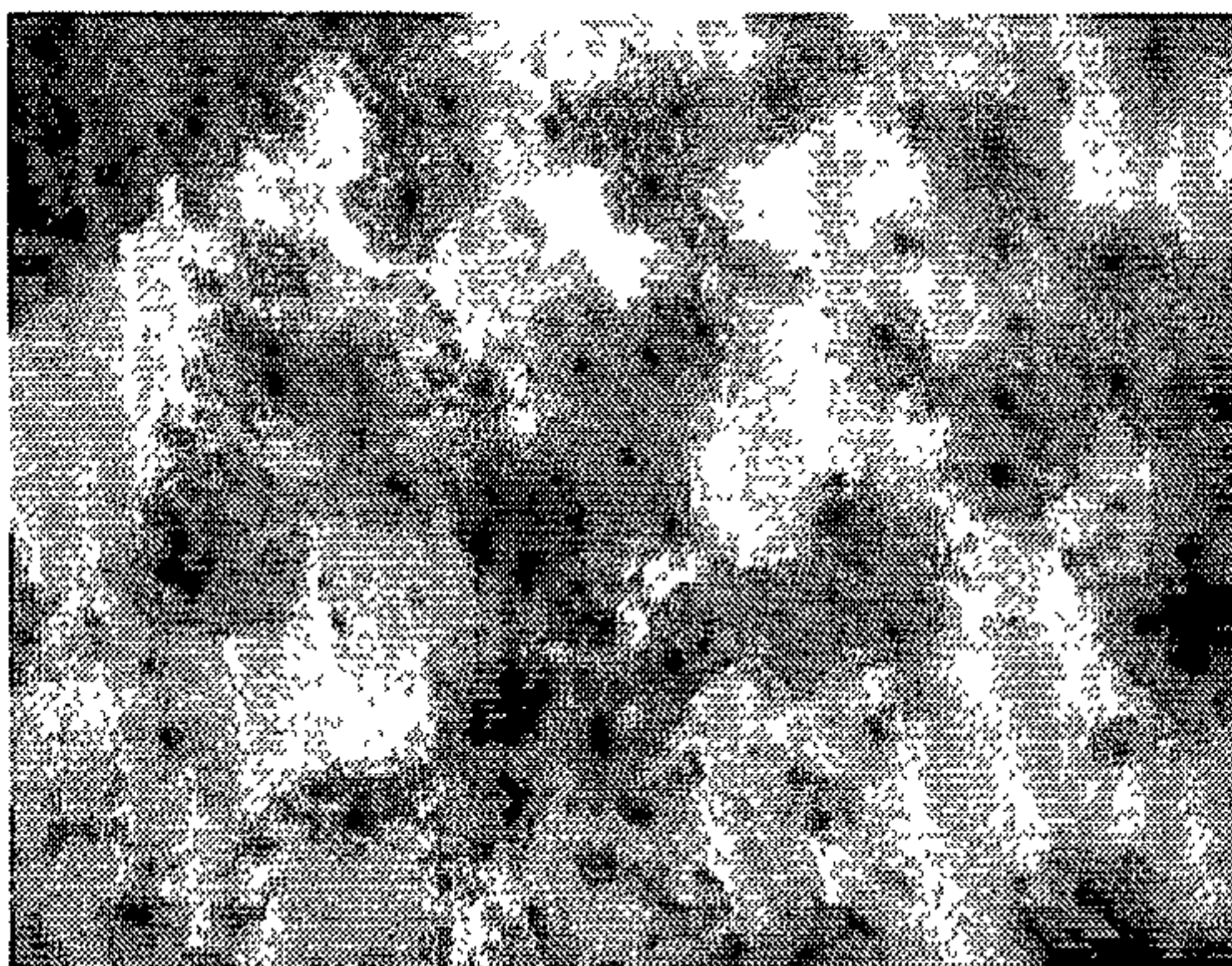


Ad5C01-BMP2

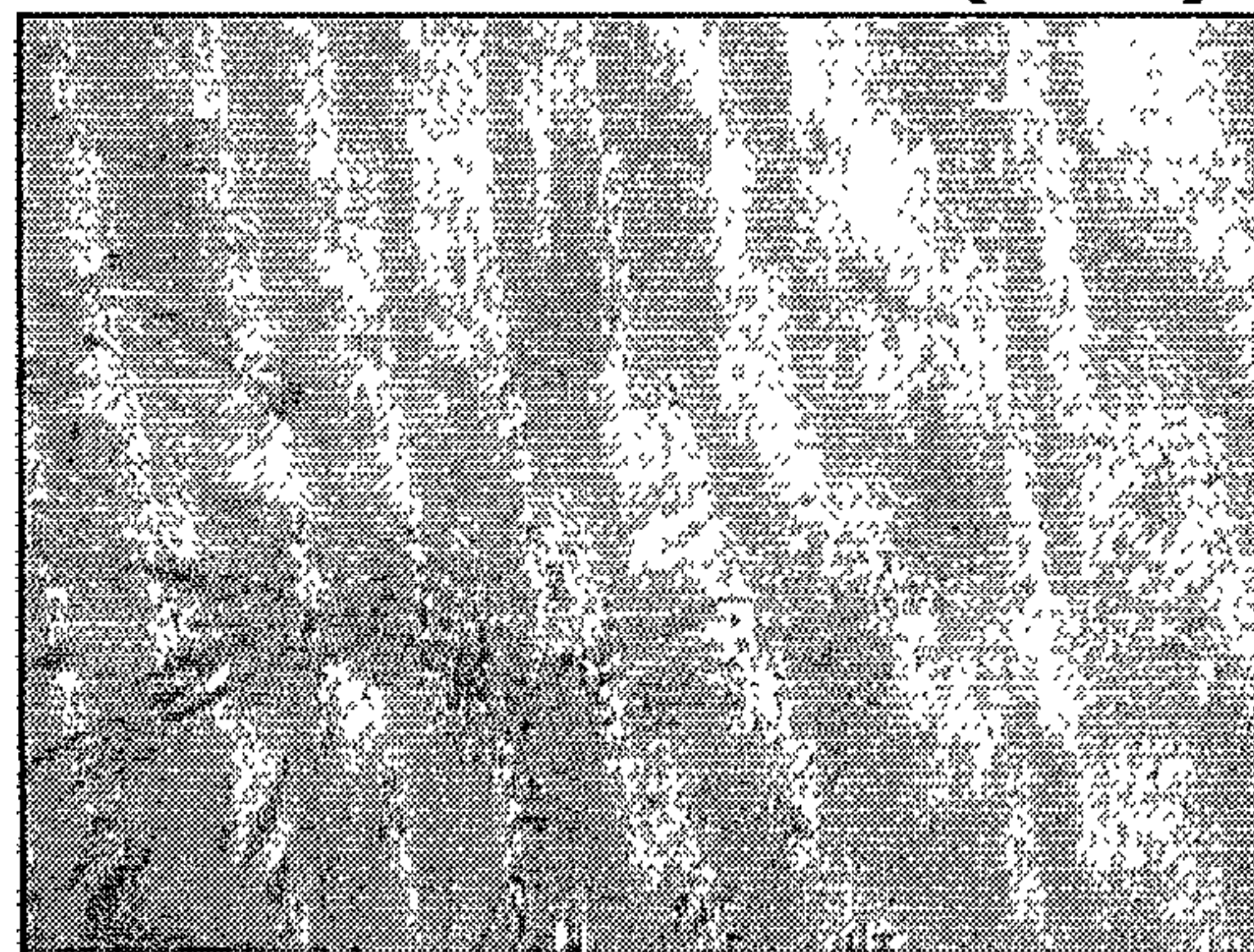


Knock-in

Ad5C01-H24-054



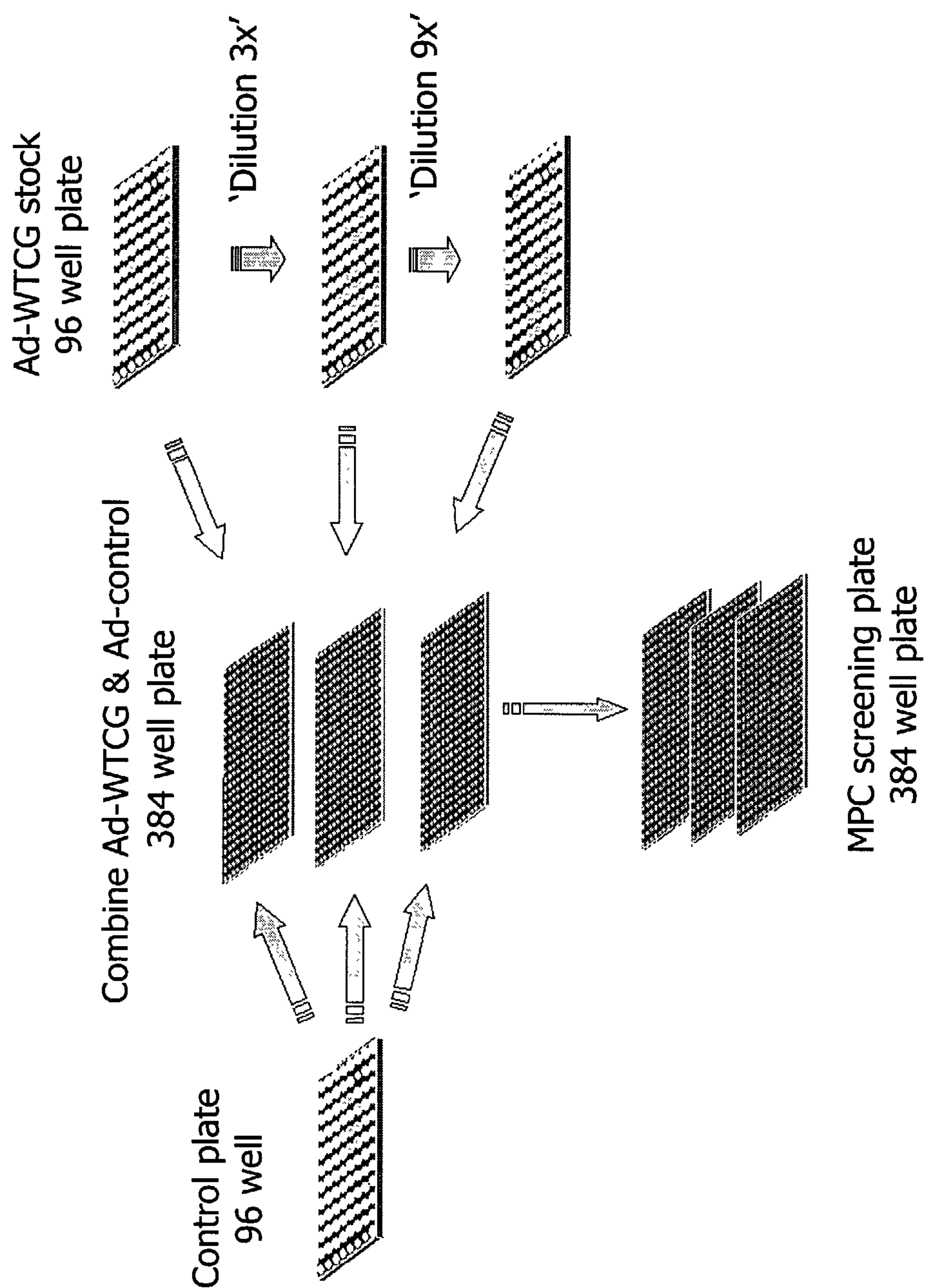
Ad5C01-eGFP (KD)



Knock-down

FIG. 10

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FIG. 11