

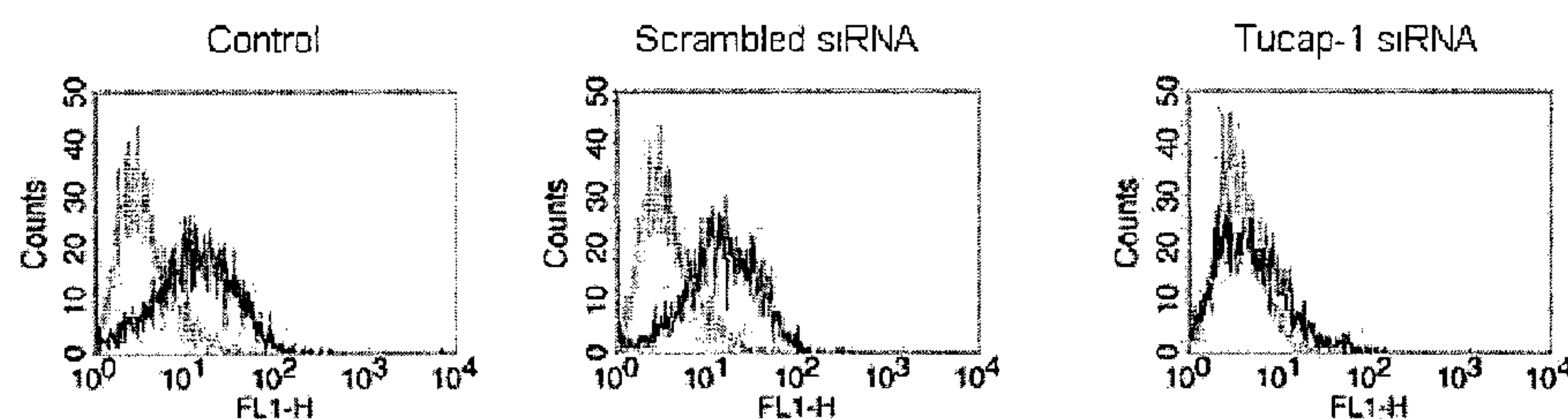


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(54) Titre : NOUVELLE MOLECULE ASSOCIEE A UNE TUMEUR HUMAINE METASTATIQUE, PROCEDES POUR
DETECTER A LA FOIS UNE PROTEINE ET UN GENE ACTIVES ET POUR INTERFERER AVEC L'EXPRESSION
DU GENE

(54) Title: A METASTATIC HUMAN TUMOR ASSOCIATED MOLECULE, METHODS TO DETECT BOTH ACTIVATED
GENE AND PROTEIN AND TO INTERFERE WITH GENE EXPRESSION



(57) **Abrégé/Abstract:**

This disclosure characterizes the function and the expression of the human protein encoded by tm9sf4. The protein is highly expressed in malignant tumor cells and therefore is a novel marker for malignancy. Moreover, the protein is involved in the phagocytotic character of tumor cells. This disclosure provides methods and tools to diagnose and follow up malignancy of tumors. Furthermore, means to inhibit phagocytotic character of tumor cells as well as means to treat cancer are provided.



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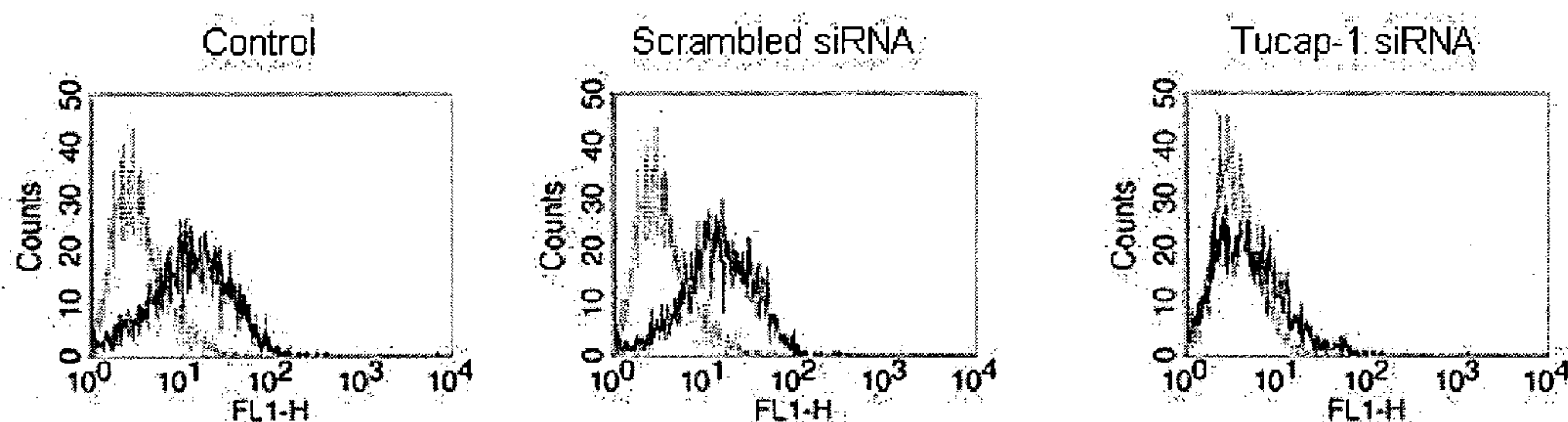
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(54) Title: A NEW METASTATIC HUMAN TUMOR ASSOCIATED MOLECULE, METHODS TO DETECT BOTH ACTIVATED GENE AND PROTEIN AND TO INTERFERE WITH GENE EXPRESSION

**Figure 8**(57) Abstract: This disclosure characterizes the function and the expression of the human protein encoded by *tm9sf4*. The protein is highly expressed in malignant tumor cells and therefore is a novel marker for malignancy. Moreover, the protein is involved in the phagocytotic character of tumor cells. This disclosure provides methods and tools to diagnose and follow up malignancy of tumors. Furthermore, means to inhibit phagocytotic character of tumor cells as well as means to treat cancer are provided.

**A METASTATIC HUMAN TUMOR ASSOCIATED MOLECULE, METHODS
TO DETECT BOTH ACTIVATED GENE AND PROTEIN AND TO INTERFERE
WITH GENE EXPRESSION**

TECHNICAL FIELD

- 5 The present invention relates generally to the field of cancer research and more specifically to identification of new tumorigenesis and metastasis related proteins and genes. The invention relates to a new marker of malignancy. The invention further relates to the field of gene therapy.

BACKGROUND ART

- 10 In 2005, 7.6 million people died of cancer out of 58 million deaths worldwide. Based on projections, cancer deaths will continue to rise with an estimated 9 million people dying from cancer in 2015, and 11.4 million dying in 2030 (WHO data). Cancer treatment may involve surgery, radiation therapy, chemotherapy, hormonal therapy, or some combination of these, but presently survival rates for the most of cancer patients is very low. According to World
15 Health Organization, one third of the cancer burden could be cured if detected early and treated adequately. Pathological research provides means for establishing the diagnosis of the most of solid tumors. Although many cases can be classified reliably with current pathological criteria, there is a significant subset of cases in which no consensus can be reached even among expert pathologists. Diagnostic ambiguity has significant adverse
20 consequences for patients. Misclassifying a tumor as benign may be fatal, and diagnosing a benign lesion as malignant may result in significant morbidity. Currently there is no method to definitively resolve these ambiguities. Therefore, there is a clear need for a diagnostic test that could reduce these uncertainties.

- Phagocytosis is the process by which cells internalize large particles (typically 0.1 mm
25 diameter), such as bacteria or cell debris. The early stage of phagocytosis can be tentatively divided into distinctive steps: cell membrane binding around the particle, phagosome formation, and internalization of the phagosome. In the process of phagosome formation and internalization, actin cytoskeleton has been proposed to drive these steps to allow engulfment.

- Phagocytic cells have been identified in malignant tumors up to a century ago, and, more
30 recently, cells with phagocytic behavior (also defined as cannibalistic behavior) have been

detected in tumors of different histology, such as oat cell carcinoma of the lung, breast cancer, bladder cancer, medulloblastoma, gastric adenocarcinomas, melanoma and squamous cell carcinoma of the skin.

We have recently observed that phagocytosis is a character of metastatic melanoma cells able to phagocytose apoptotic cells, plastic beads stained yeasts, and live lymphocytes displaying efficient phagocytic machinery responsible for a macrophage-like activity, while melanoma cells derived from primary lesions did not display any cannibalistic or phagocytic activity. Moreover, cannibal cells can be detected in 100% metastatic melanoma lesions (Lugini et al., 2004; Lugini et al., 2006).

One of the main features of cannibal cells is an increased acidity of lysosomal-like vesicles and an over expression of cathepsin B, a proteolytic enzyme reported to be involved in tumor invasion and metastasis (Sloane et al., 1981) . Differently from professional phagocyte -like macrophages, cannibal tumor cells do not utilize structures like ruffles or any pseudopodial movement. Instead, live or dead material that touches the tumor cell's external membrane is immediately endocytosed and digested through a sort of *quicksand* mechanism that seems not to involve any specific receptor.

These findings have led us to speculate that cannibal cells feed of other cells, perhaps with no particular need of blood-derived nutrient supply, but also that cannibalism of lymphocytes by tumor cells may represent a rudimentary mechanism of tumor immune escape. Moreover, these findings led us to a novel, revolutionary interpretation that cancer cells, in their habit to use other cells for feeding, may behave as unicellular eukaryotes whose unique purpose is to survive in a continuous fighting against other cells and the unfavourable environment. This theory further led us to speculate that amoebas and metastatic cells might share the same framework with the same regulatory elements allowing their surviving in adverse micro-environmental conditions. However, so far no genes have ever been specifically associated with the cannibal behaviour of cancer cells.

The cellular slime mold *Dictyostelium discoideum* has been previously used as a model organism to study phagocytosis. Mechanisms involved in phagocytosis by *Dictyostelium* cells are very similar to those used by mammalian phagocytes, and involve the actin cytoskeleton and RacF1, a member of the Rho family of GTPbinding proteins. However, no phagocytosis associated specific proteins have ever been identified in mammals.

It has been recently found that the protein encoded by *PHG1A* gene was implicated in cell adhesion and phagocytosis in the amoeba *Dictyostelium discoideum*. This protein belongs to TM9 superfamily and genes encoding TM9 proteins can be unambiguously identified in eukaryotic genomes. The family includes many members in organisms ranging from yeast
5 to plants and human. To mention some example, there are three members of this family in *Saccharomyces cerevisiae*, *Dictyostelium* amoebae, and *Drosophila* flies and four in humans and mice. All of them exhibit a similar overall structure, with a rather variable potential luminal domain followed by a more conserved membrane domain and nine or ten putative transmembrane domains.

10 SUMMARY OF THE INVENTION

Based on our theory that cancer cells use other cells for feeding and behaving as unicellular eukaryotes and possibly share the same framework with the same regulatory elements as amoebas, we compared *PHG1A* gene with human genome. Three homologues of *phg1* have been fully sequenced in human (TM9SF4, U81006 and U94831), and we found the closest
15 homologue of *phg1* of *Dictyostelium dicoideum* in human to be *tm9sf4* (other aliases: KIAA0255, dJ836N17.2) locating in chromosome 20q11.21. Even if this gene is fully sequenced, its function or expression product of this has never been characterized.

Accordingly, we have studied the function and the expression of the protein encoded by *tm9sf4* in more details. In this disclosure, we show the function of the protein and provide
20 a number of applications.

This disclosure shows that the protein is highly expressed in malignant cells. Observations on several melanoma cell lines deriving from patients show that TM9SF4 is a marker of malignancy, since this protein is undetectable on the cell lines deriving from primary lesions. Furthermore, this protein is involved in the phagocytic behavior of metastatic melanoma cells,
25 since silencing the gene encoding this protein strongly inhibits the phagocytic behavior of metastatic cells. Based on these observations we have named this protein as TUCAP-1 (Tumor associated cannibal protein 1). The gene coding for this protein is here accordingly called *tucap-1* gene.

This invention addresses the need for a rapid test to detect malignant cells and diagnose
30 melanoma and other tumors. Accordingly, this disclosure provides antibodies, primers,

oligopeptides and polypeptides useful for TUCAP-1 detection, analysis and potential therapeutic effects.

This invention provides polynucleotides corresponding or complementary to all or part of the *tucap-1* gene, and polynucleotides or oligonucleotides, which hybridize to the *tucap-1* gene, 5 mRNAs, or to TUCAP-1-encoding polynucleotides. Recombinant DNA molecules containing TUCAP-1 polynucleotides, cells transformed or transduced with such molecules, and host-vector systems for the expression of *tucap-1* gene products are provided. The disclosure further provides TUCAP-1 protein and polypeptide fragments thereof.

The invention further provides antibodies that bind to TUCAP-1 proteins and polypeptide 10 fragments thereof, including polyclonal and monoclonal antibodies, murine and other mammalian antibodies, chimeric antibodies, humanized and fully human antibodies, and antibodies labelled with a detectable marker, and antibodies conjugated to radionucleotides, toxins or other therapeutic compositions. The invention further provides methods for detecting the presence of TUCAP-1 polynucleotides and proteins in various biological samples, as well 15 as methods for identifying cells that express TUCAP-1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Molecular structure of TM9SF4/TUCAP-1 protein.

(A) Hydropathy profile of TUCAP-1 protein sequence. Hydrophobic regions are indicated above the line by positive values. Amino acid numbering is indicated on the abscissa.

20 Hydrophilic stretch in the N-terminal region is followed by nine hydrophobic regions.

Analysis was performed according to Claros and von Heijne using TopPred prediction Program.

(B) Graphic representation of TUCAP-1 secondary structure according to TopPred predictor server.

25 Figure 2. Detection of TM9SF4/TUCAP-1 transcripts and characterization of TUCAP-1 antibodies.

(A) RT-PCR analysis of TUCAP-1 (upper panel) and GAPDH (lower panel) on five metastatic (MM1-5) and five primary melanoma (PM1-5) cell lines recently established *in vitro* from metastatic lesion, and peripheral blood cells from two different donors (PBL1-2);

30 M size marker.

5

(B) Western blots of 6-histidine or TUCAP-1 in the six-histidine tagged TUCAP-1 peptide used to immunize mice and in uninduced bacterial lysates. Equal amount of purified protein and bacterial lysate was loaded on reducing gels and blotted with the 6-His-antibody or TUCAP-1 mice antisera. Proteins were visualized using HRP-conjugated secondary antibodies and revealed with ECL system (Pierce).

(C) Western blotting for GFP-TUCAP-1 and GAPDH on Triton soluble (lane 1) and Triton insoluble (lane 2) fractions of GFP-TUCAP-1 (GFP-Tuc) transfected MM1 cells, and Triton soluble and insoluble fractions of untransfected MM1 cells (lanes 3-4). M size marker. Proteins were visualized using HRP conjugated secondary antibodies and revealed with ECL (Pierce). As molecular weight markers Rainbow™ (Amersham UK) prestained standards were used.

Figure 3. Western blotting analysis of TUCAP-1.

Western blotting for TUCAP-1 detection on GFP-Tuc Transfected MM2 cells, Four metastatic melanoma cell lines (MM2-5), and CCD-1064SK human skin fibroblasts (HSC). Loading amount was controlled by immunodetection of actin. Proteins were visualized using HRP conjugated secondary antibodies and DAB system (DAKO, Denmark) as chromogen. Rainbow™ (AmershamUK) prestained standards were used as molecular weight markers.

Figure 4. Immuno-cytochemical and immuno-histochemical analysis of TUCAP-1. Figure 4A is the same in black and white.

Mice pre-immune serum immunocytochemical analysis of (A) MM2 cells; (B) peripheral blood lymphocytes; (C) *in vitro* differentiated macrophages.

TUCAP-1 immunocytochemical analysis of: (D) M2 cells; (E) peripheral blood cells; (F) Macrophages.

Immunohistochemical analysis of malignant melanoma tissues stained with: (G) preimmune mouse serum; (H) TUCAP-1 immune serum; and (I) anti-GP100.

Immunohistochemical analysis of healthy skin stained with: (J) mouse preimmune serum, (K) TUCAP-1 immune serum, and (L) anti-ezrin antibody. Magnification 10X.

Figure 5. Subcellular localization of TUCAP-1/ - I. Figure 5A is the same in black and white.

(A) Western blot analysis of subcellular fractions of TUCAP-1 immunoprecipitates from MM2 total lysates. M size marker, TE total extracts.

(B) Immunofluorescence (IF) double staining analysis of TUCAP-1 (green) and Rab5 (Red).

(C) IF double staining analysis of TUCAP-1 (green) and Lamp-1 (red).

(D) IF double staining analysis of TUCAP-1 (green) and Mitotracker stained mitochondria (red). Yellow/orange areas indicate co-localization. Nuclei were stained with Hoechst 33258. Magnification 100X.

- 5 Figure 6. Subcellular localization of TUCAP-1 – II. Figure 6A is the same in black and white. Immunofluorescence (IF) double staining analysis of TUCAP-1 (green) and EEA1 (Red) on MM2 cells. Yellow/orange areas indicate co-localization. Nuclei were stained with Hoechst 33258. Magnification 100X.

Figure 7. TUCAP-1 detection in cannibal cells. Figure 7A is the same in black and white.

- 10 (A) Detection and localization of TUCAP-1 in metastatic melanoma MM1 cells co-cultured with living lymphocytes. IVM analysis of TUCAP-1 (green). Picture highlights that TUCAP-1 is detectable exclusively on melanoma cells.
- (B) Double fluorescence analysis of TUCAP-1 (green) and EEA-1 (red) in metastatic melanoma MM1 cells co-cultured with living lymphocytes. Yellow/orange areas indicate co-
- 15 localization. Nuclei are stained with Hoechst 33258.

Figure 8. FACS analysis of TUCAP -1 expression.

Facs analysis of TUCAP-1 expression in untransfected MM2 cells, Scrambled siRNA and TUCAP-1 siRNA (TM9SF4 siRNA) transfected MM2 cells 48 hours after transfection.

Figure 9. Functional analysis of TM9SF4/TUCAP-1.

- 20 (A), FACS analysis of phagocytic activity of Scrambled siRNA (SC-siRNA) or TUCAP-1 siRNA (TM9SF4 siRNA) transfected MM2 cells. Gray: unstained control cells; red: negative control Scrambled siRNA transfected cells; green: TM9SF4 siRNA transfected cells.
- (B), FACS analysis of cannibal activity of SC-siRNA or TM9SF4 siRNA transfected MM2 cells incubated 18 hours with DHR123-stained lymphocytes. Gray: unstained control cells;
- 25 red: SC-siRNA transfected cells; Green: TM9SF4 siRNA transfected. Cells. To exclude DHR123-stained lymphocytes not ingested with melanoma cells, exclusively melanoma cell fluorescence emission was evaluated.
- (C) FACS analysis of LysoTracker DND-26 staining of SC-siRNA or TM9SF4 siRNA transfected MM2 cells. Gray: unstained control cells; red: SC-siRNA transfected; Green:
- 30 TM9SF4 siRNA transfected.

Figure. 10. TUCAP-1 overexpression enhance cell invasion through Matrigel. Figure 10A is the same in black and white. Phase micrograph of invading WM743 melanoma cells as compared to GFP-Tagged full length TUCAP-1 WM743 melanoma cells (TWM). Invading cells were Fixed in formaldehyde and stained with crystal violet. Picture clearly show that the number of invading cells was significantly higher for TWM , with a mean of 25 cells for untransfected versus a mean of 132 cells for TUCAP 1 transfected TWM.

DESCRIPTION OF THE INVENTION

TUCAP-1/TM9SF4 (HUGO nomenclature official committee official full name: transmembrane 9 superfamily protein member 4) belongs to the transmembrane 9 superfamily (TM9SF), a highly conserved family of proteins characterized by the presence of a large variable extracellular N-terminal domain and nine to ten putative transmembrane domains. However, function and localization of TM9SF4 has never been described in human cells. In this disclosure, we localize the protein expression and provide useful and novel applications to this protein. This disclosure identifies the protein as a novel oncoprotein and shows that the protein is expressed in malignant tumor cells while undetectable in variety of healthy cells and tissues. This disclosure also shows the structure of the protein and by analogy with other proteins shows that the protein may be involved in pH regulation of intracellular vesicles. Throughout this disclosure we call the protein TUCAP-1 and the gene coding for the protein is accordingly called *tucap-1*.

Table 1 below shows the physicochemical parameters of TUCAP-1 protein

Tupac-1 ProtParam computation of physicochemical parameters:	
Number of amino acids:	625
Molecular weight:	72541.3
Theoretical pI:	6.22
Extinction coefficients:	132880 Abs 0.1% (=1 g/l) 1.832, assuming ALL Cys residues appear as half cystines
	132130 Abs 0.1% (=1 g/l) 1.821, assuming NO Cys residues appear as half cystines
Estimated half-life:	30 hours (mammalian)

Hydropathy analysis of TUCAP-1 through TopPred predictor server revealed a mostly hydrophilic, amino terminal portion that extends up to amino-acid 262, while the remaining portion of the protein is extremely hydrophobic and contains nine potential transmembrane domains (Fig. 1A) On the basis of this predicted structure it may be hypothesized with great confidence that TUCAP-1 is an integral membrane protein as shown in Fig. 1B Our finding that there is a fairly high homology between TUCAP-1 and PHG1(45% that rises to 63% if NCBI Blast protein program positive alignment is considered) led us to a novel hypothesis that TUCAP-1 may have a role in cannibal activity of human metastatic melanoma cells.

According to one preferred embodiment of the present invention are polynucleotides corresponding or complementary to all or part of the *tucap-1* gene, mRNAs, and/or coding sequences, preferably in isolated form, including polynucleotides encoding TUCAP-1 proteins and fragments thereof, DNA, RNA, DNA/RNA hybrid, and related molecules, oligonucleotides complementary to the *tucap-1* gene or mRNA sequences or parts thereof, and polynucleotides or oligonucleotides which hybridize to the TUCAP-1 genes, mRNAs, or to TUCAP-1-encoding polynucleotides.

According to another preferred embodiment of the invention is an “antibody” which is a whole antibody molecule or fragment thereof that recognizes (or can bind to) “specific sequences” of TUCAP-1 protein, which is its antigen. Antibody may be either a polyclonal antibody or a monoclonal antibody. In this embodiment, TUCAP-1 protein is a polypeptide having the amino acid sequence according to SEQ ID NO:1, and the *specific sequences* are polypeptides having an amino acid sequence containing deletion, substitution or addition of one or more amino acids as compared to the amino acid sequence of SEQ ID NO:1 or a fragment of these. The antibody of the present invention encompasses antibody mutants. An “antibody mutant” is a mutant in which one or more amino acid residues in the antibody have been modified from the original.

According to further embodiments are TUCAP-1 inhibitors. Such inhibitor molecules may be polynucleotide sequences that are substantially complementary to the sequence of SEQ ID NO: 2 or part of it, and oligonucleotide sequences substantially complementary to a fragment of SEQ ID NO:2.

According to yet another embodiment of the invention are methods for treating cancer in a human patient comprising the step of administering to the patient a therapeutically

effective amount of a composition comprising a TUCAP-1 binding agent conjugated to a chemotherapeutic drug. Antibodies and fragments that specifically bind to TUCAP-1 protein can be used to treat cancers. The invention includes the use of antibodies and antibody fragments that are fused to other moieties that can have a cytotoxic effect on cancer.

- 5 Another preferred embodiment of this invention is cell lines producing monoclonal antibodies against TUCAP-1 protein or fragments thereof.

According to yet another preferred embodiment are recombinant DNA or RNA molecules containing full-length wild type or mutated TUCAP-1 sequence, or deletion mutants of TUCAP-1, including but not limited to phages, plasmids, phagemids, cosmids, YACs,
10 BACs, as well as various viral and non-viral expression vectors well known in the art, and cells transformed or transfected with such recombinant DNA or RNA molecules. Using these expression vectors, TUCAP-1 may be preferably expressed in several malignant tumor cell lines. Preferred embodiments comprise host-vector systems that are useful for the production of a TUCAP-1 protein or fragment thereof. Such host-vector systems may be employed:
15 i) to study the functional properties of TUCAP-1 and TUCAP-1 mutations; ii) as model to develop a gene therapy protocol based on the utilization of TUCAP-1 silencing vectors and TUCAP-1 mutants expressing vectors.

Accordingly, this invention provides a unique tool to measure, study and detect TUCAP-1, as marker of malignancy of many cancer types. Moreover, this invention introduces a new tool
20 for clinical oncologist in the management and follow up of cancer patients. Moreover, peptides of other molecules able to interfere the expression or acting as TUCAP-1 blocking agents are within the scope of this invention as antineoplastic compounds.

Yet another preferred embodiment of this invention is a kit to detect TUCAP-1 from tissue specimens and body fluids of tumor patients as a diagnostic and or prognostic tool such as
25 a detection kit. Such kit comprises: a) anti TUCAP-1 antibodies; b) a positive control consisting of the purified TUCAP-1 protein, and the necessary buffers.

The invention is now described by means of examples, which are not meant to limit the scope of the invention. The scope of the invention is defined by the appended claims.

EXAMPLE**EXAMPLE 1**

***Tucap-1* transcripts and TUCAP-1 protein are detectable in human malignant melanoma cells, but not in primary melanoma cells, in peripheral blood mononuclear cells or in healthy skin cells.**

Cell culture. Human primary and metastatic melanoma cell lines were respectively derived from primary or metastatic tumor lesions of patients surgically resected at the Istituto Nazionale dei Tumori, Milan, Italy. All cells employed in the current study were designated by PM (primary melanoma) or MM (metastatic melanoma), followed by a progressive number. Human peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque (Pharmacia) density gradient of buffy coats from healthy donors. Monocytes were separated from PBMC by using CD14 labeled Miltenyi microbeads according to manufacturer's indications and were left to differentiate for 2 weeks at 37°C in RPMI 1640 plus 15% FCS. Remaining peripheral blood lymphocytes (PBL), were obtained after CD14 beads mediated monocyte ablation. All the cells were seeded in RPMI 1640 supplemented with 100 IU/mL penicillin, 100 Ag/mL streptomycin, 10% FCS in a 5% CO₂ environment at 37°C. (All reagents were purchased from Cambrex).

PCR analysis. Expression of *Tucap-1* transcripts was assessed by rt-PCR on several primary and metastatic melanoma cell lines obtained from melanomas of patients surgically resected at Istituto Nazionale Tumori, Milan, as compared to peripheral blood lymphocytes (PBL). Total RNA from the cells was obtained by the RNazol (Invitrogen) method and RNA templates were used for RT-PCR amplification. Primers for TUCAP-1 detection were:

tgtgtgaaacaagcgccttc (SEQ ID NO: 3, and
atgaggtggacgtagtagt (SEQ ID NO: 4).

These primers amplify a fragment of 349 base pairs.

Primers used to direct TUCAP-1 His-tagged N-terminal domain synthesis were:

gaattcatgtgtgaaacaagcgcctt (SEQ ID NO:5) and
gtcgacagaaaaccagtggatctg (SEQ ID NO:6).

Primers to detect GAPDH were:

ccatggagaaggctgggg (SEQ ID NO:7) and
caaagttgcatggatgacc (SEQ ID NO:8).

TUCAP 1 cloning and expression of TUCAP 1 fusion protein in human melanoma cells:

PCR products were cloned into pTopo vector (Invitrogen) and then excised with
5 the appropriate pair of restriction enzymes (EcoRI, Sall) to acquire a single fragment that was
subsequently ligated in the pTrcHis2 vector (Invitrogen). The expressed recombinant protein
was purified employing Ni NTA agarose resin (Qiagen) following manufacturer's instructions
and utilized to immunize mice.

Primers that were used to direct GFP-tagged full length TUCAP-1 were:

10 gaattcatgtgtgaaacaagcg (SEQ ID NO:9), and
gtcgatgtctatcttcacagcata (SEQ ID NO:10)

PCR products were cloned into pTopo vector (Invitrogen) and then excised with
the appropriate couple of restriction enzymes (EcoRI-Sall) and ligated to acquire a single
fragment that subsequently was ligated in the pEGFPN1 vector (Clontech) at the EcoRI and
15 Sall sites to produce the GFP-TUCAP-1 fusion protein. Plasmids encoding the GFP-TUCAP-
1 fusion protein were transfected into MM1 and MM2 cells by using the Lipofectamine 2000
transfection kit (Invitrogen) according to the manufacturer's instructions, thus obtaining GFP-
TUCAP-1.

(GFP-Tuc) MM1 or MM2 cells. The percentage of transfected cells was evaluated by
20 Fluorescence-activated cell sorting analysis.

Western Blotting and immunoprecipitation

Bacterial lysates, whole melanoma cell lysates and CCD-1064SK healthy skin fibroblasts
(SantaCruz) were resuspended in SDS sample buffer, denaturated by boiling, separated by
SDS-PAGE, and analyzed by Western blot. 6xHis tagged protein, GFP, TUCAP-1, and
25 GAPDH, were respectively detected with anti6His mAb (Sigma), anti GFP (clone 1E4 MBL),
anti TUCAP-1 mouse serum and antiGAPDH (SantaCruz). TUCAP-1 proteins were
immunoprecipitated overnight at 4°C in the presence of protein A+G-Sepharose beads (Pierce)
from precleared cell lysates, by using rabbit anti TUCAP-1 pAb antibody. Rabbit preimmune
serum was used as negative control. Actin was detected with anti actin mAb (Sigma).

30 In order to characterize *tucap-1* gene product, cDNA derived from MM1 cells was cloned in
bacterial expression vectors to obtain TUCAP-1 first 265 amino acids fused to a 6-Histidine

12

N-terminal tag (6H-Nt- TUCAP 1) (SEQ ID NO:11) Western blot analysis of purified recombinant protein resulted in a translation product of about 30 kDa absent in control bacterial whole lysates (negative control). Therefore, His-tagged TUCAP-1 recombinant peptide was employed as immunogen to produce anti- TUCAP-1 antibodies in mice.

5 The specificity of the TUCAP-1 antiserum was determined by Western blot analysis of the purified 6H-Nt- TUCAP-1 immunoblotted with anti 6His and TUCAP-1 mouse antisera (Fig. 2 B). TUCAP-1 mouse antiserum was further analyzed by Western blot on Triton soluble and Triton insoluble fractions of MM1 cells transfected or not transfected with a GFP-tagged full length TUCAP-1 (GFP- TUCAP-1). The anti-GFP antibody revealed a single
10 specific translation product in the 100 kDa range, while anti-TUCAP-1 antibodies recognized both the GFP-tagged and the endogenous TUCAP-1 corresponding to a 70 kDa protein detectable in both cell lines (Fig. 2C). Interestingly, TUCAP-1 was more represented in the Triton insoluble fractions (GAPDH negative, cytoskeletal proteins enriched fraction), thus supporting the provisional models proposing TUCAP-1 as a transmembrane protein.
15 To further support PCR results, the anti-TUCAP-1 antibodies were blotted in cellular extracts of four metastatic melanoma cells (MM2-MM5), previously analyzed for their cannibal behavior as compared to healthy skin fibroblasts (HSC) and GFP- TUCAP-1 transfected MM2 cells, as a control. TUCAP-1 was exclusively detectable in melanoma cells, while undetectable in skin cells (Fig. 3).

20 EXAMPLE 2

Immunochemistry shows TUCAP-1 exclusively in melanoma cells

Immunocytochemistry and immunohistochemistry. For immunocytochemistry melanoma cells and macrophages, cultured on glass chamber slides (Falcon), and PBL, cytopun on glass slides, were fixed with 80% methanol 10 minutes at 4°C and stained for TUCAP-1, TUCAP-1
25 mouse serum or preimmune control serum. Malignant melanoma and corresponding normal skin tissue from Biomax array slides (Biomax) were immunostained with pre-immune serum, for anti-TUCAP-1 mouse antiserum. Melanoma was also stained for anti-gp100 (Immunotech) while normal skin was also stained for anti-ezrin (Sigma). Proteins were visualized using the peroxidase antiperoxidase method in single staining (Dako) and counterstained with Mayer's
30 hematoxylin.

Fig.4 A-C shows that MM2 cell lines (A), Peripheral blood lymphocytes (B), and *in vitro* differentiated Macrophages (C), were negative for mouse preimmune serum. Consistently with PCR results malignant melanoma cultured cells showed clear positive staining for TUCAP-1 (Fig 4 D) while PBL (Fig. 4E) and macrophages (Fig. 4 F) were negative for TUCAP1 staining. Immunohistochemical analysis of malignant melanoma tissues as compared to healthy skin suggested that TUCAP-1 was detectable only in melanoma tissues (Fig. 4 H) while undetectable in healthy skin (4K). As positive control markers for melanoma and normal skin GP100 (Fig 4I), and ezrin (Fig. 4L) were used respectively. Pre-immune mouse serum staining was always negative in both tissues (Fig. 4G, 4J). These results provide clear evidence that TUCAP-1 was exclusively detectable in melanoma cells and thus support the results of Example 1.

EXAMPLE 3

Subcellular localization of TUCAP-1

Further experiments were performed to analyze the intracellular localization of TUCAP 1.

Cell compartment fractionation. Cells were harvested and processed according to Qproteome plasma membrane kit protocol (Quiagen) in order to obtain non denatured fractions of cellular compartments corresponding to purified plasma membranes and cytosol. The latter fractions were then precipitated with acetone and resuspended in immunoprecipitation buffer B (0.1% SDS, 1% NP40, 0.5% sodium cholate) in order to be subjected to immunoprecipitation with rabbit anti TUCAP-1. Residual pellet from cellular compartment fractionation, containing intact cells and organelles, was deprived of the former through centrifugation and subjected to Triton X-100 extraction in order to obtain soluble and insoluble fractions which were immunoprecipitated with rabbit anti TUCAP-1. Following electrophoresis of samples, the nitrocellulose was blotted with mouse anti-TUCAP-1.

Immunofluorescence analyses. MM2 cells were seeded on cover glass placed in 60-mm Petri dishes. Cells were fixed with 2% paraformaldehyde and permeabilized (Triton X-100 (0.1%) or 24hous. For TUCAP 1 and Rab5 double staining cells were labeled with mouse anti-TUCAP-1 serum and rabbit anti-Rab5 (SantaCruz) and respectively revealed with Alexa Fluor 488-conjugated anti-mouse IgG and anti-rabbit Alexa Fluor 594- conjugated IgG (Molecular

Probes). For TUCAP-1 and Lamp-1 detection cells were labeled with rabbit anti-TUCAP-1 pAb and mouse anti Lamp-1 Mab, (BD Pharmingen) respectively, stained with Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488- conjugated anti-mouse IgG. TUCAP-1 and mitochondria were detected by staining TUCAP-1 with anti-TUCAP-1 mouse pAb and
5 labeled with Alexa Fluor 488-conjugated anti-mouse IgG, while mitochondria were labeled with Mithotracker Red (Invitrogen). After washings, all samples were mounted with glycerol:PBS (2:1) and observed with a Leica DM 2500 fluorescence microscope. Images were recorded with a Spot Insight digital camera (Delta Sistemi) equipped with IAS 8.2 system of image analysis (Delta Sistemi).

- 10 MM2 whole cell lysates were immunoprecipitated with anti-TUCAP-1 antibodies and various subcellular fractions were separated and analyzed by Western blot. The results revealed that TUCAP-1 was mainly recovered in fractions enriched for cellular organelles, while undetectable in cytosolic and plasma membrane fractions (Fig. 5A). In order to identify subcellular localization of TUCAP-1, MM2 cells were double stained for TUCAP-1, and
15 either for the early endosomal markers Rab5, or for the component of late endosomes and lysosomes Lamp-1, or the mitochondrial marker Mitotracker TM. Fluorescence microscopy analysis showed that TUCAP-1 co localized with both Rab5 (Fig. 5B) and EEA-1 (Fig. 6 and Fig. 7B), while it did not co-localize with either Lamp-1 (Fig. 5C), Mitotracker TM (Fig. 5D) or Hoechst stained nuclei.
- 20 Moreover, Figure 7 shows the double staining fluorescence on same cells co-cultured with living lymphocytes. TUCAP-1 is detectable exclusively on the surface of melanoma cells, while lymphocytes are completely unstained (Figure 7A). Again, TUCAP-1 co-localizes with the primary endosome marker EEA-1 (Figure 7B) confirming the expression of this protein on early endosomes.

25 **EXAMPLE 4**

Functional analysis of TUCAP-1

***Tucap-1* silencing inhibits phagocytic behaviour**

The role of TUCAP-1 protein in human metastatic melanoma cells was evaluated by inhibiting its expression through *Tucap-1* silencing.

15

The following StealthR RNAi duplexes (Invitrogen) were used for *tucap-1* silencing:

gagugacguccagauccacugguuu (SEQ ID NO:13), and

aaaccaguggaucuggacgucacuc (SEQ ID NO: 14), and annealed according

to the manufacturer's instructions. As a negative control Stealth RNAi Negative control

5 medium GC duplexes (Invitrogen) was used. Melanoma cells were transfected using

Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions.

Briefly the day before transfection, melanoma cells were seeded in six-well plates (1×10^5 per

well), and after 24 hours, cells were transfected with 30pmol of siRNA per well. 48 hours

after transfection, cells were analyzed for TUCAP-1 expression by FACS analysis.

10 Three different phagocytic metastatic cell lines were transfected with small interfering RNA

to *Tucap-1* (Tucap-1 siRNA), or transfected with an irrelevant siRNA oligo (SC-siRNA).

Fig.8 shows FACS analysis of TUCAP-1 expression on untransfected MM2 cells and

Scrambled siRNA or TUCAP-1 siRNA transfected MM2 cells 48 hours after transfection.

Similar results were obtained in scrambled and TUCAP-1 silenced MM3 cells (not shown).

15 This confirmed an effective knockdown of TUCAP-1.

To assess the phagocytic activity of these cells, we first measured the ability of untransfected,

SC-siRNA transfected, or *Tucap-1* silenced melanoma cell lines to ingest stained yeast cells or

living lymphocytes. 48 hours after transfection, SC-siRNA or TUCAP-1 siRNA transfected

MM2 and MM3 melanoma cells were incubated at 37° C with FITC stained *Saccaromyces*

20 yeasts FITC (1:60), or 10 uMol dihydrorhodamine 123 (DHR123) (Molecular Probes) stained

living lymphocytes (1:10). Phagocytosis/cannibalism was measured after 4 hours by washing

away the excess lymphocytes or yeast cells with PBS and adding a PBS solution containing

trypsin (1.5 g/L) EDTA (0.44g/L). After washings, melanoma cells were harvested and

analyzed on a cytometer equipped with a 488-nm argon laser. At least 10,000 events were

25 acquired and analyzed by a Macintosh computer using CellQuest software (Becton

Dickinson). Melanoma cells that appeared fluorescent in green were considered as

phagocytic/cannibal. The results showed that the TUCAP-1 knocking-down markedly

inhibited both the phagocytic and the cannibal activity of melanoma cells (Fig. 9A, 9B, Table

1), proving that TUCAP-1 plays a key role in the cannibal behavior of metastatic human

30 melanomas and the protein thus can be used as a marker of malignancy.

Table 2. Role of TUCAP-1 in phagocytosis/cannibalism.

16

Phagocytic/Cannibal activity of scrambled siRNA transfected (SC-siRNA) and Tucap-1 silenced (Tucap-1 siRNA) MM2 and MM3 metastatic melanoma cells against FITC stained yeasts and DHR123 stained live lymphocytes. The phagocytic activity was expressed as % of phagocytic cells. Numbers are means.d. of 5 different experiments.

	Yeasts		Live lymphocytes	
	SC-siRNA	Tucap-1 siRNA	SC-siRNA	Tucap-1 siRNA
MM2	37 ± 1	4 ± 2	45 ± 5	17 ± 9
MM3	43 ± 14	14 ± 12	40 ± 9	9 ± 7

5 EXAMPLE 5

TUCAP-1 has a role in regulating acidification of endosomal vesicles

Scrambled siRNA transfected and TUCAP-1 silenced MM2 and MM3 cells were stained with 1 µM LysoTracker probe (Molecular Probes) for 30 minutes at 37 °C and immediately analyzed by a cytometer. Comparisons among different melanoma cell lines were conducted by CellQuest software using the median values of fluorescence intensity histograms. Based on the result that TUCAP-1 localizes on Rab5 bearing endosomes (see EXAMPLE 3), we tested a hypothesis that TUCAP-1 protein may have a role in the pH regulation of phago/endosomal compartments of malignant tumor cells. To verify this hypothesis, control SC-RNAi and TUCAP-1-siRNA transfected cells were stained with the acidotropic probe LysoTracker green and analyzed by flow symmetry. *Tucap-1* gene silencing induced appearance of less acidic vesicle within melanoma cells, as compared to SC-RNA transfected control cells (FIG 9C). These experiments support the hypothesis that TUCAP-1 has a role in regulating acidification of internal vesicles, such as early endosomes.

EXAMPLE 6

20 TUCAP-1 involvement in early phases of metastatic process

Ongoing experiments based on using TUCAP-1 overexpressing cells suggest that this protein is involved in tumor cell invasiveness during early phases of metastatic process. Cell invasion capability of these cells is assayed by using Matrigel invasion chambers (Becton-Dickenson, Bedford, MA, USA). Briefly, untransfected WM743 or GFP-Tagged full length TUCAP-1 WM743 melanoma cells (TWM) were resuspended in serum free medium and loaded into

the top chamber, while in the bottom chamber was placed in medium added with 10 % FCS as a chemoattractant. Cells were incubated at 37°C in a humidified atmosphere and allowed to migrate through the chemotaxis chamber for 48 hours. After incubation, the cells remaining at the upper surface were completely removed using a cotton carrier. The migrated cells
5 on the bottom of chemotaxis chamber were stained with crystal violet. Invading cells were counted microscopically (40×) in four different fields per filter. Figure 10 shows the lower side of transwell membrane, clearly indicating that the number of invading cells was significantly higher for TWM, with a mean of 25 cells for untransfected versus a mean of 132 cells for TUCAP 1 transfected TWM cells.

10 EXAMPLE 7

TUCAP-1 involvement in cisplatin resistance of melanoma cells

Several publications show the role of proteins involved in ion trafficking and the role of endo-lysosomal compartment in drug sequestering, inactivation and extrusion as mechanisms of drug resistance. TUCAP 1 expression in early endosomes and its involvement in pH regulation of
15 endosomal vesicles (as shown in above examples) led us to hypothesize a role for this protein in drug resistance of cancer cell. To prove this, MM2 melanoma cells, highly expressing TUCAP-1, were pretreated with Scrambled (SC-siRNA) or *Tucap-1* si-RNA for 48 hours (as shown in the previous examples), and after transfection cells were treated with 2uM cisplatin. 48 hours after cisplatin induced cytotoxicity was evaluated by FACS analysis of early
20 (annexin-V single positive) and late (PI/Annexin V double positive) apoptosis. *Tucap-1* silencing markedly increased cytotoxic effects of cisplatin as compared to Scrambled-si-RNA treated WM743 cells that behaved as the untransfected control cells. With a mean of 63 % of live cells in control transfected cells versus a mean of 37 % in TUCAP-1 silenced cells.

This set of experiments proves that TUCAP-1 is involved in drug resistance of TUCAP-1 over
25 expressing cells and that *tucap-1* silencing is a promising method to inhibit phagocytotic character of tumor cells.

Based on the results shown in above examples, the present disclosure also provides highly sensitive and specific methods for detection of melanoma and several other tumors characterized by a described phagocytic behaviour i.e. breast cancer, lung carcinoma, bladder
30 cancer, medulloblastoma, and gastric adenocarcinoma. Moreover, this disclosure provides means to distinguish malignant from benign cancer lesions by showing that TUCAP-1 is

exclusively expressed in malignant tumors. Methods for cancer detection comprise evaluation of a biological sample from a putative cancer lesion, typically by *in situ* hybridization, rt-PC, or immunoenzymatic methods.

EXAMPLE 8

5 Polyclonal and Monoclonal Antibodies to TUCAP-1

Based on the examples above, polyclonal (pAb) and monoclonal antibodies (mAb) specific for TUCAP-1 would be useful for various purposes, including for example diagnostics to determine malignancy of a tumor, and treatment of cancer.

10 In order to produce polyclonal antibodies, cDNA from MM1 cells was cloned in bacterial expression vectors to obtain TUCAP-1 aminoacids 18-282 fused to a 6-Histidine N-terminal tag (SEQ ID NO:11). Purified recombinant peptide was used to produce anti-TUCAP-1 antibodies in mice. The anti-TUCAP-1 antibodies recognized immunogen, GFP-tagged full length protein as positive control as well as endogenous TUCAP-1 protein.

15 Polyclonal antibodies were also generated by immunizing rabbit, goat and donkey with a purified peptide fragment having an amino acid sequence according to SEQ ID NO: 12 . The antibodies generated were able to recognize human TUCAP-1 protein by binding to a peptide fragment that consists of amino acids 221-235 of SEQ ID NO: 1. Polyclonal antibodies are also obtained by immunizing goat and donkey.

20 In order to produce monoclonal antibodies, mice were immunized with a peptide fragment having amino acid sequence according to SEQ ID NO:11 or SEQ ID NO:12 or a fragment thereof. Alternatively mice are immunized with peptide fragment having amino acid sequence according to SEQ ID NO: 15 . Selected hybridoma clones were generated by using spleen cells of selected mice. Briefly B-cells deriving from spleen of immunized mice were fused with a myeloma tumor cell line specifically selected for hybridoma production. The deriving
25 fused (hybrid) cells that can grow indefinitely in culture with consequent production large amounts of the desired antibodies. Hybridoma production was performed according to standard protocols. After screening the selected hybridomas, the hybridomas are cloned and grown to large-scale for antibody production. Various positive hybridomas are selected for different uses, for example: a) laboratory experimental uses (Western Blot, immuno-

precipitation, FACS analysis, immunofluorescence and immunohisto-and immunocyto-chemical analysis of human tissues and cultured cells); b) preclinical and clinical studies, c) tumor diagnosis and prognosis tools, such as detection kit. The monoclonal antibodies produced bind to conformational or linear epitopes of TUCAP-1 protein amino acids 18-282
 5 of SEQ ID NO: 1 or peptide fragment consisting of amino acids 221-235 or consisting of amino acids 303-352 of SEQ ID NO: 1. The antibodies also bind to TUCAP-1 protein of mouse, rat, cat, dog, and sheep origin.

The examples above disclose particular embodiments of the invention in detail. However, this has been done by way of example and for the purposes of illustration only. The examples
 10 are not intended to limit the scope of the appended claims, which define the invention.

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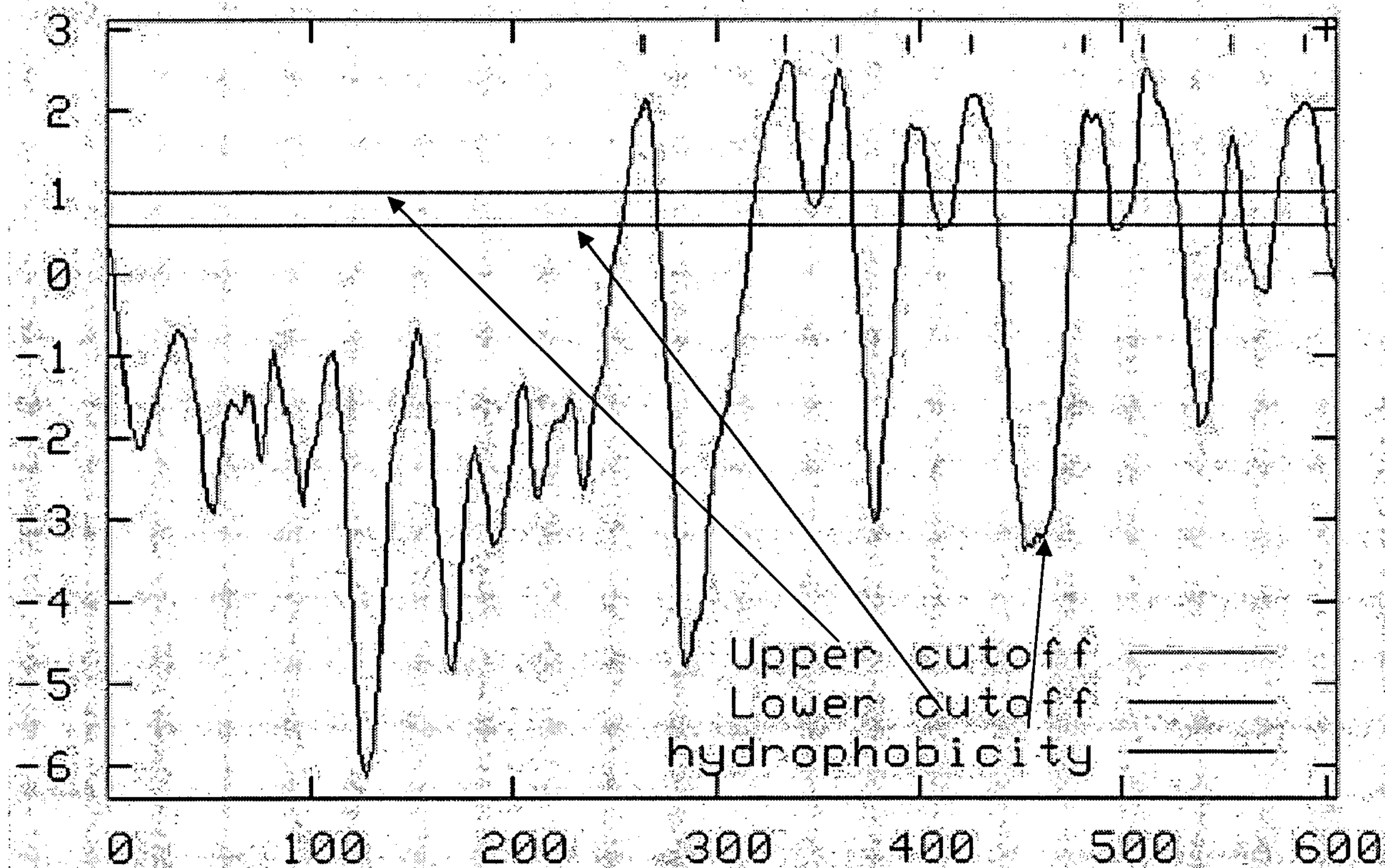
CLAIMS

1. A polypeptide consisting of amino acid sequence according to SEQ ID NO:1 for use as a marker in distinguishing metastatic melanoma tumor cells from healthy skin cells.
2. The polypeptide of claim 1 which specifically binds to antibodies against SEQ ID NO:11; SEQ ID NO:12 or SEQ ID NO:15.
3. An antibody for detection of metastatic melanoma tumors in vitro by recognizing human TM9SF4 protein by specifically binding to a peptide fragment selected from the group consisting of amino acids 18-282, 221-235 and 303-352 of SEQ ID NO:1.
4. The antibody of claim 3 wherein the antibody is a monoclonal antibody and belongs to IgG isotype IgG2A, or IgG isotype IgG1.
5. A kit for detecting metastatic melanoma tumors in vitro, said kit comprising antibodies capable of recognizing human TM9SF4 protein by specifically binding to a peptide fragment selected from the group consisting of amino acids 18-282, 221-235 and 303-352 of SEQ ID NO:1; and a titred positive control comprising a peptide fragment with an amino acid sequence as set forth in SEQ ID NO: 1, SEQ ID NO: 11, SEQ ID NO: 12 or SEQ ID NO: 15, wherein the antibody is used to detect an oncogenic protein according to SEQ ID NO: 1 from a tissue sample or cell culture of a melanoma tumor or suspected melanoma lesion.
6. A method to diagnose metastatic melanoma tumors in vitro by analyzing a tissue sample or cell culture of a melanoma tumor, said method comprising the step of determining presence or absence of expression of a polypeptide with an amino acid sequence set forth in SEQ ID NO: 1 in tumor cells, wherein presence of the expression indicates metastatic activity of the tumor and wherein the presence or absence of the polypeptide having amino acid sequence according to SEQ ID NO: 1 is defined by recognition of TM9SF4 protein by an antibody capable of recognizing human TM9SF4 protein by binding to a

peptide fragment selected from the group consisting of amino acids 18-282, 221-235 and 303-352 of SEQ ID NO: 1.

7. A method to monitor development of metastatic activity of a melanoma tumor by analyzing a tissue sample or cell culture of a melanoma tumor, said method comprising the step of determining presence or absence of expression of a polypeptide having amino acid sequence set forth in SEQ ID NO: 1 in a tumor cell, wherein presence of the expression indicates metastatic activity of the tumor and wherein the presence or absence of the polypeptide having amino acid sequence according to SEQ ID NO: 1 is defined by recognition of TM9SF4 protein by an antibody capable of recognizing human TM9SF4 protein by binding to a peptide fragment selected from the group consisting of amino acids 18-282, 221-235 and 303-352 of SEQ ID NO: 1.
8. A method of inhibiting phagocytic activity of metastatic melanoma tumor cells in vitro, said method comprising a step of inhibiting expression of a polypeptide having an amino acid sequence according to SEQ ID NO: 1 by silencing expression of *tm9sf4*-gene.
9. A method of inhibiting phagocytic activity of metastatic melanoma tumor cells in vitro, said method comprising a step of inhibiting expression of a polypeptide having an amino acid sequence according to SEQ ID NO: 1, wherein tumor cells are transfected with an expression vector comprising an isolated nucleic acid sequence that is complimentary to SEQ ID NO: 2, or complementary to TM9SF4 mRNA.
10. A use of an antibody of claim 3 for preparing a medicament comprising a chemotherapeutic drug and the antibody for targeting the medicament to TM9SF4 expressing metastatic melanoma tumor cells.

1 / 15

A**B**

unknown

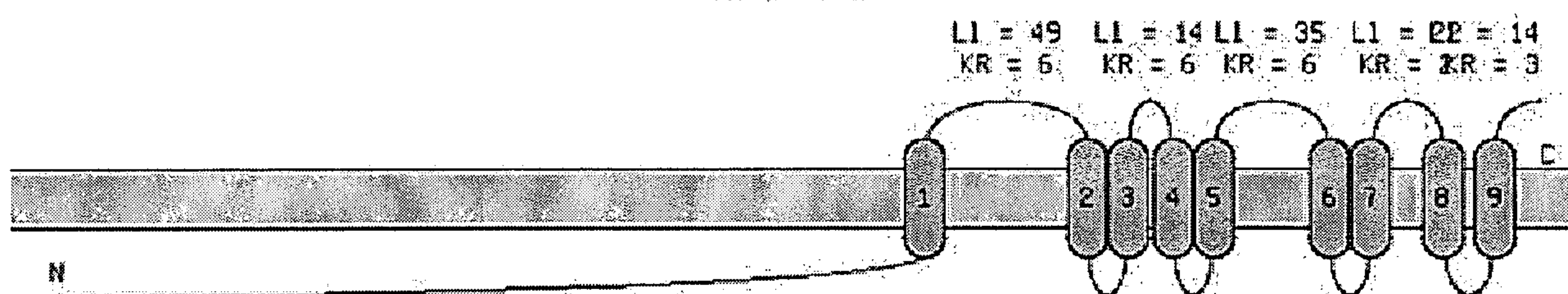
Structure no. 1

Segments included: 1 2 3 4 5 6 7 8 9

□ Segment Putative

■ Segment Certain

CYTOPLASM

LI = 264
KR = 29

LI = 11 KR = 1 LI = 8 KR = 0 LI = 15 KR = 1

LI: Loop length

KR: Number of Lys and Arg

EXTRACELLULAR

Figure 1

2 / 15

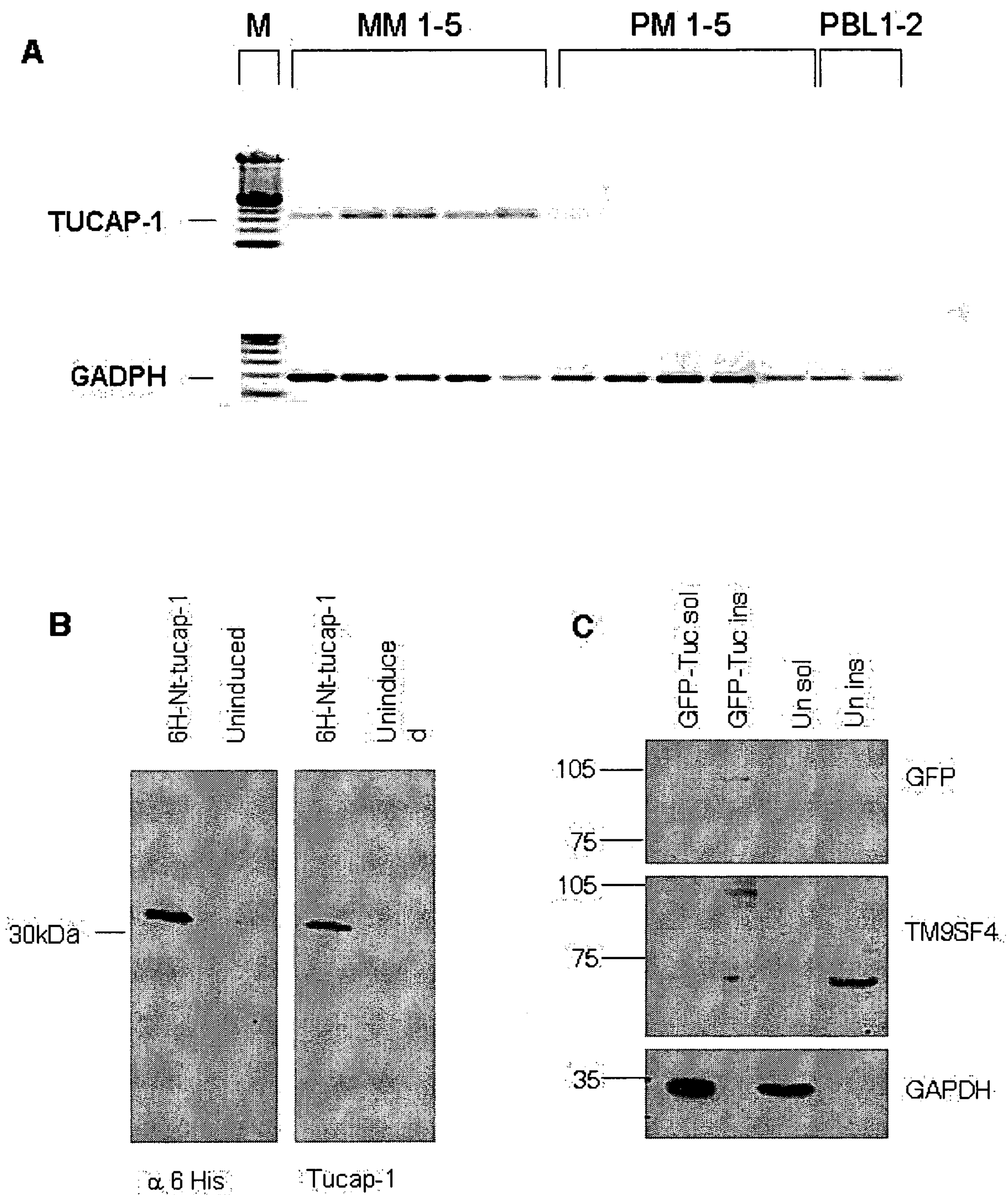
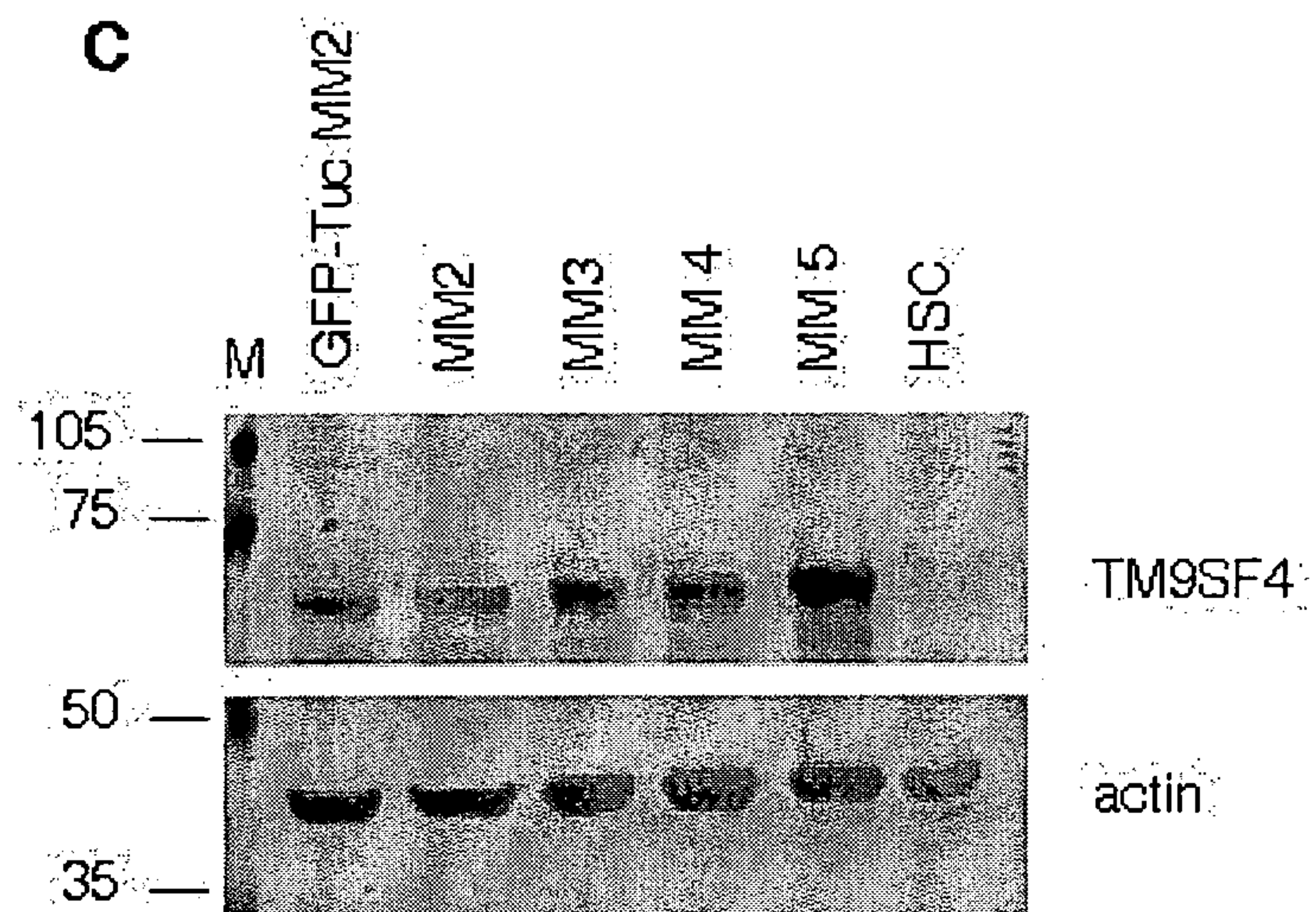


Figure 2

3 / 10**Figure 3**

4 / 15

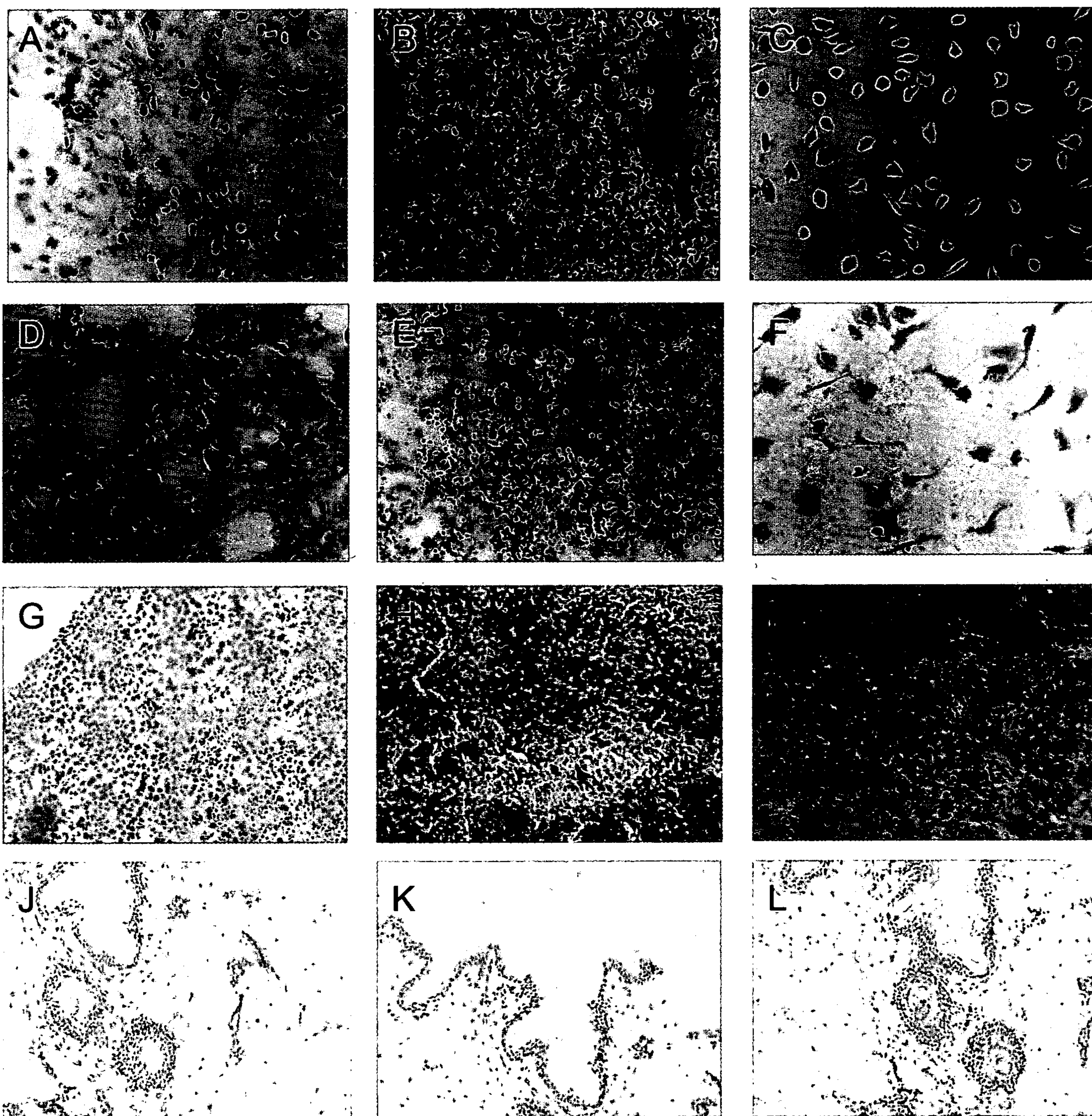


Figure 4

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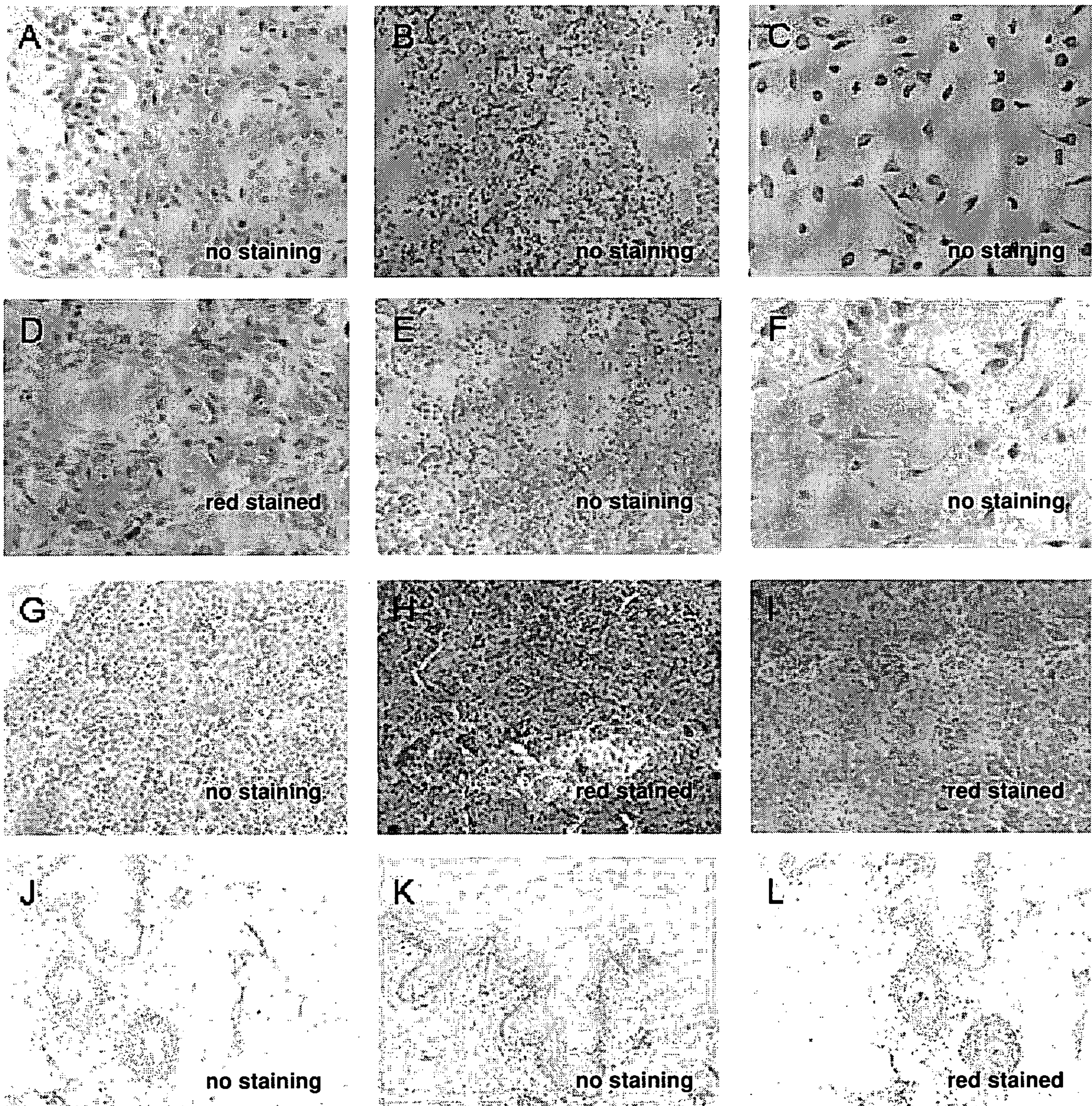


Figure 4A

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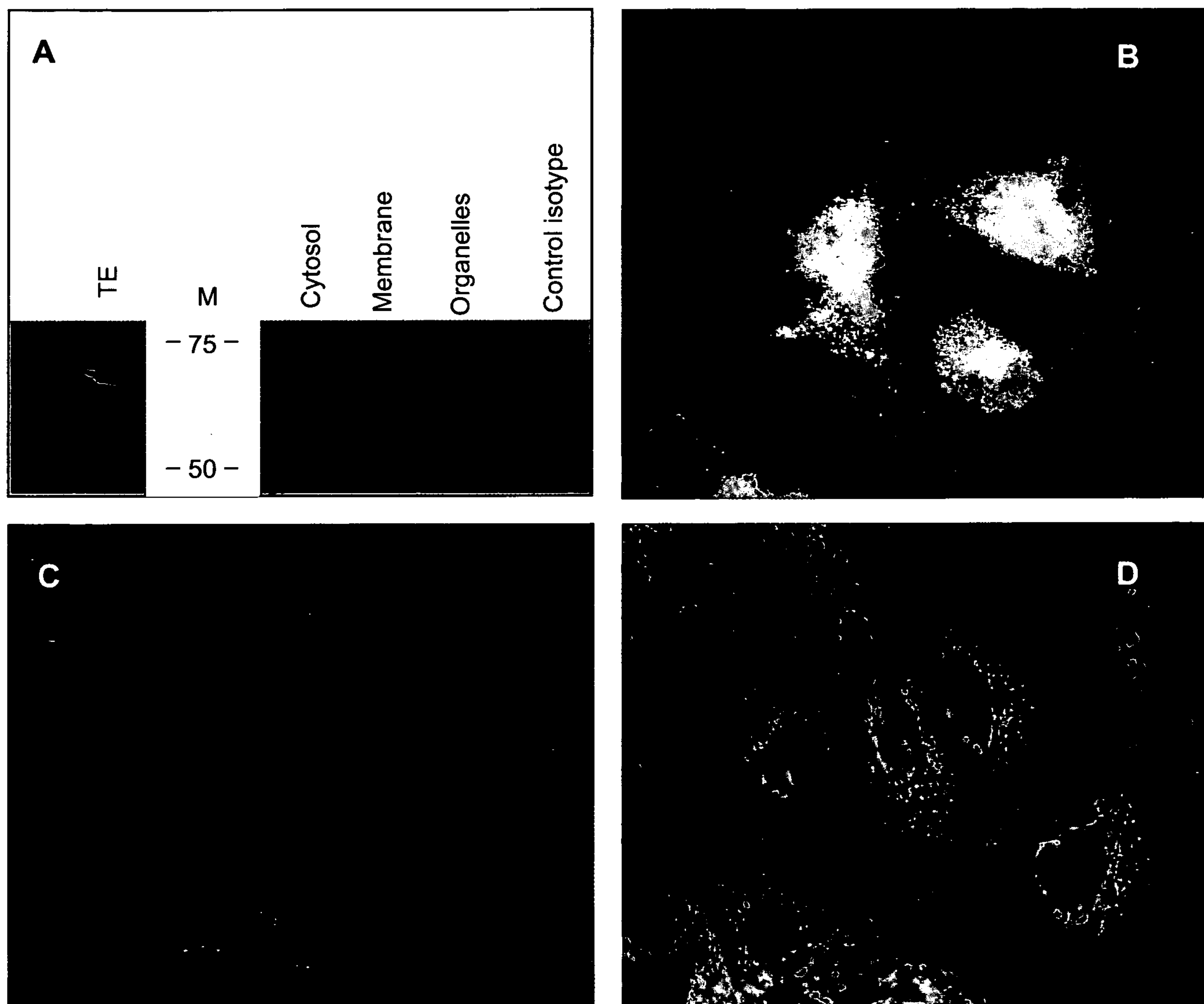


Figure 5

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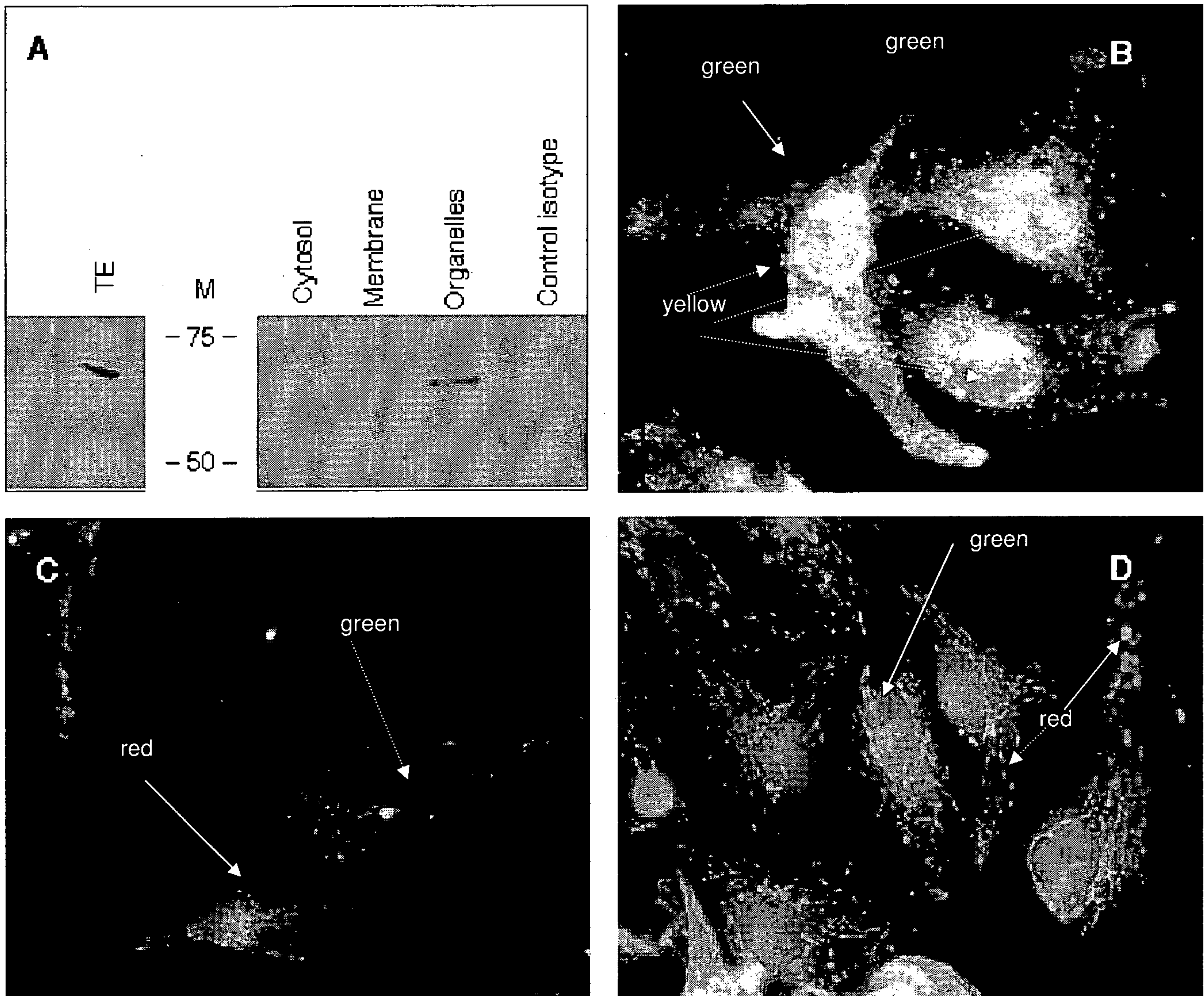


Figure 5A

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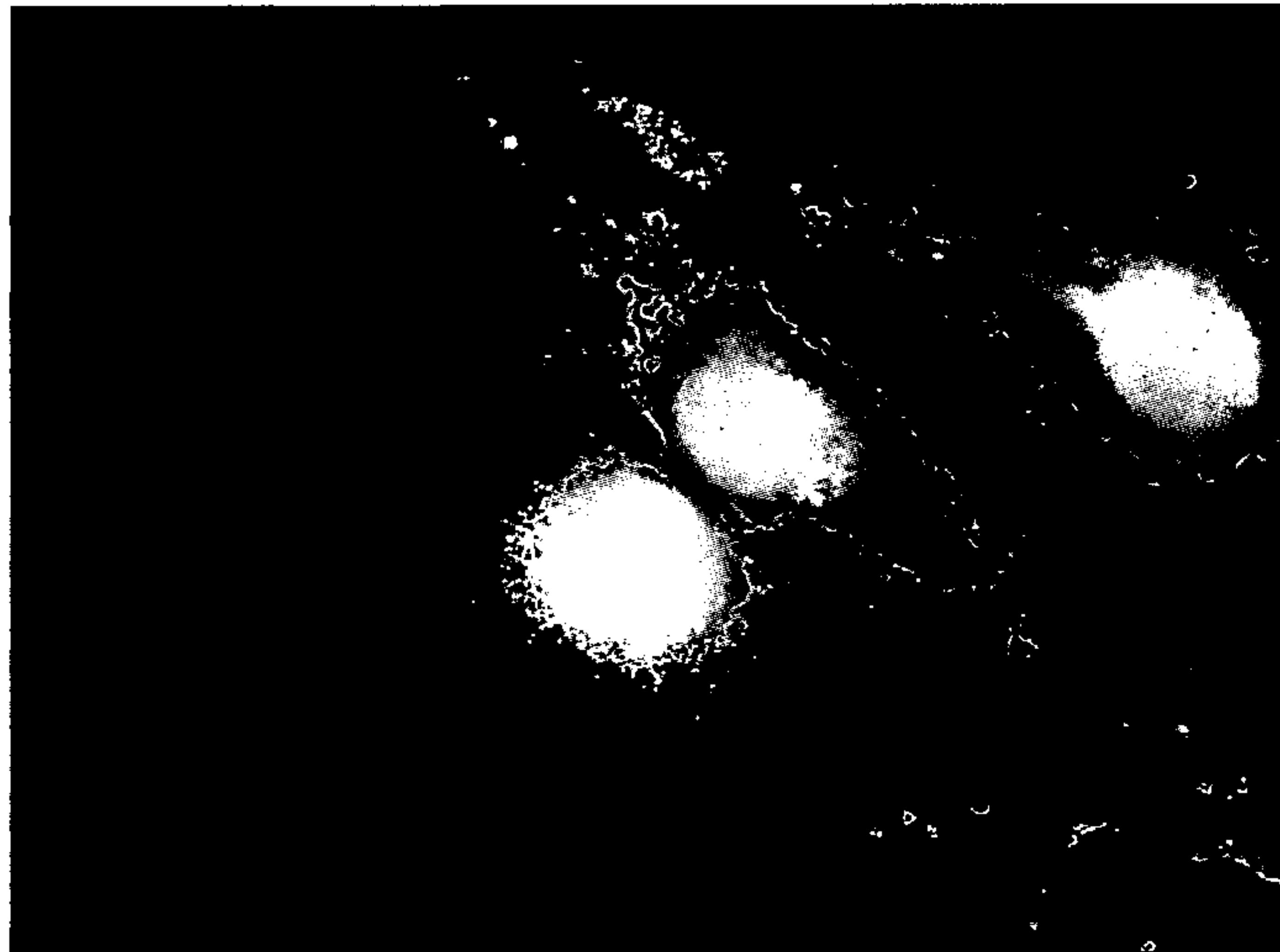


Figure 6

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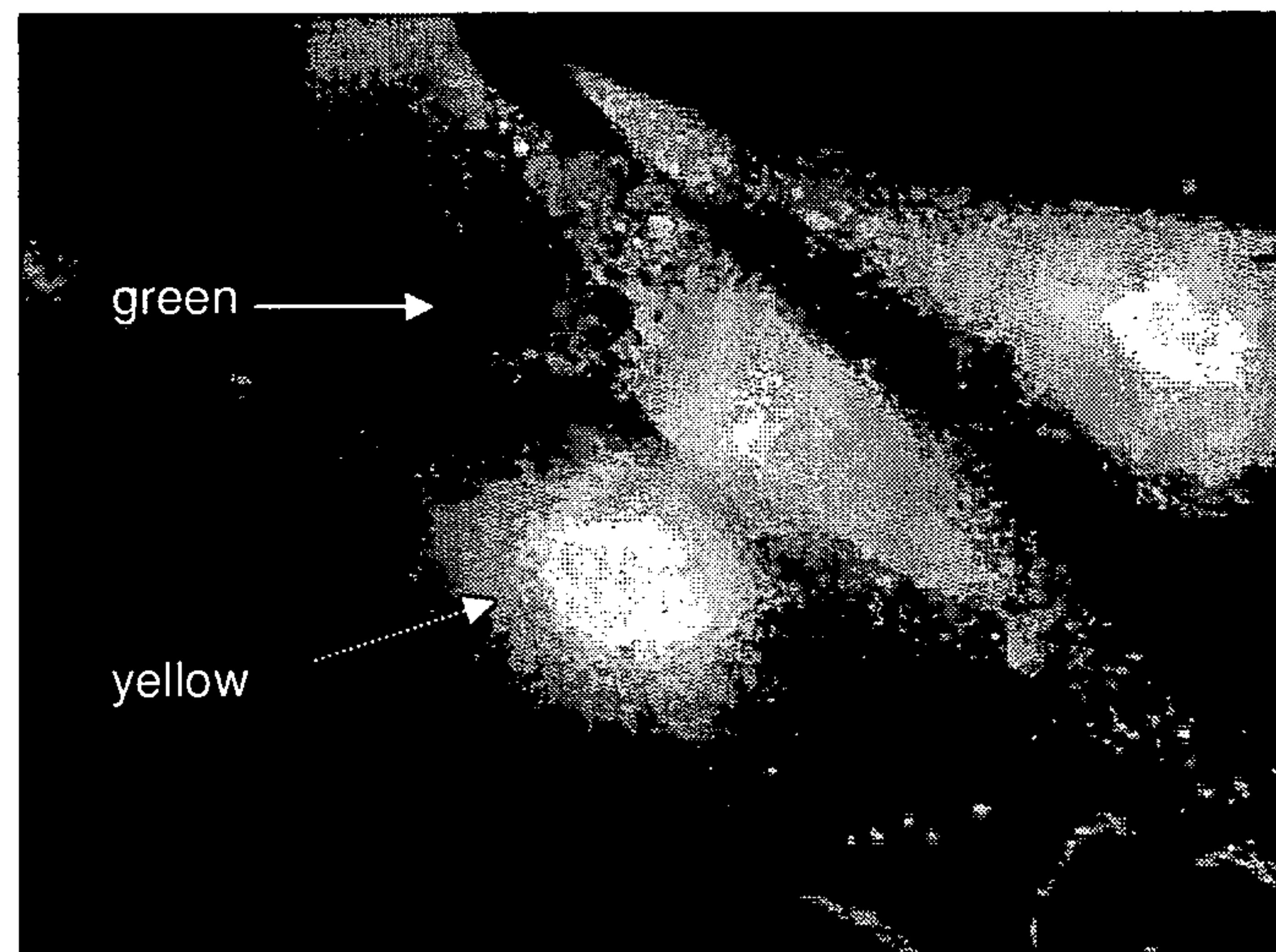


Figure 6A

10 / 15

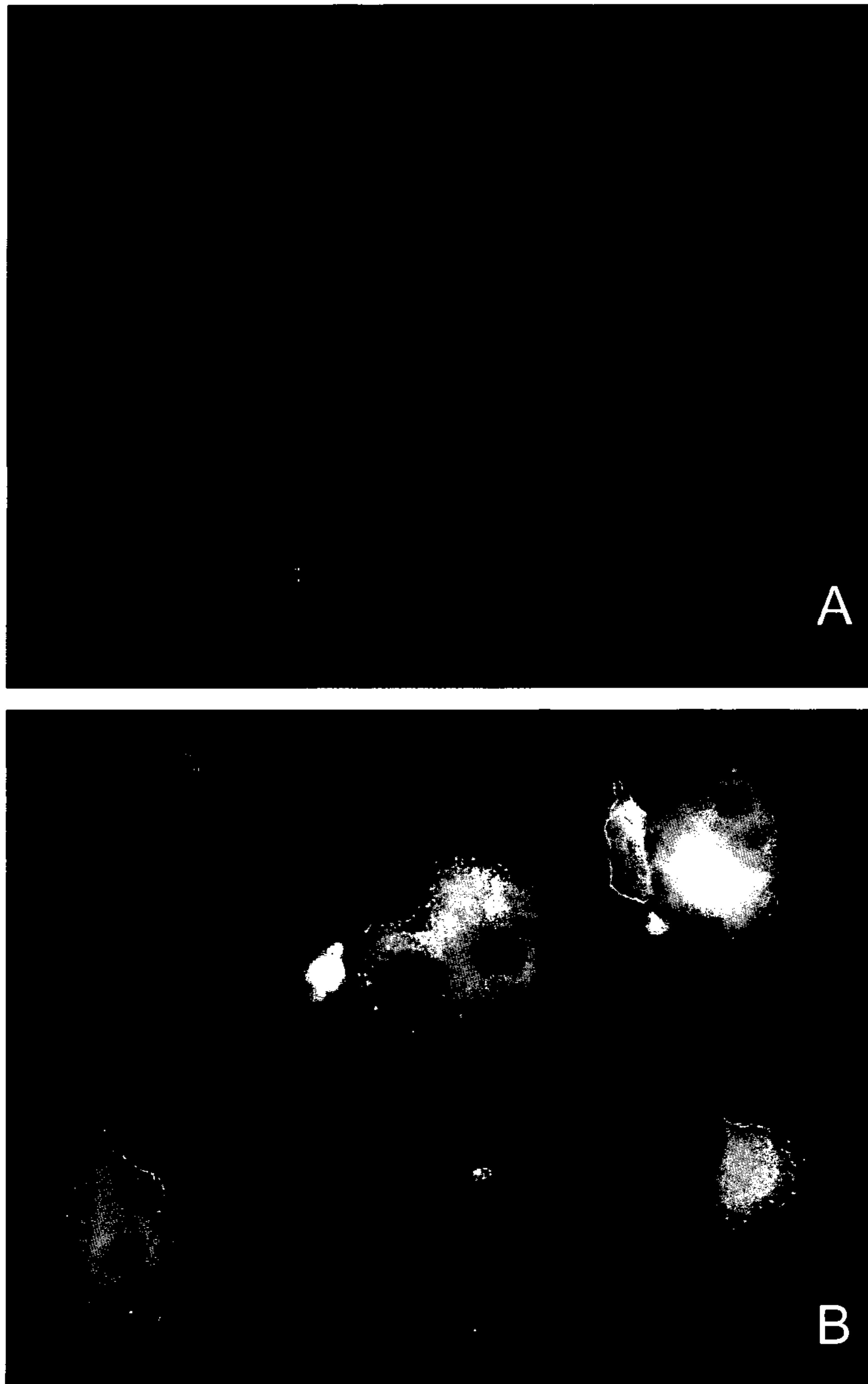


Figure 7

11 / 15

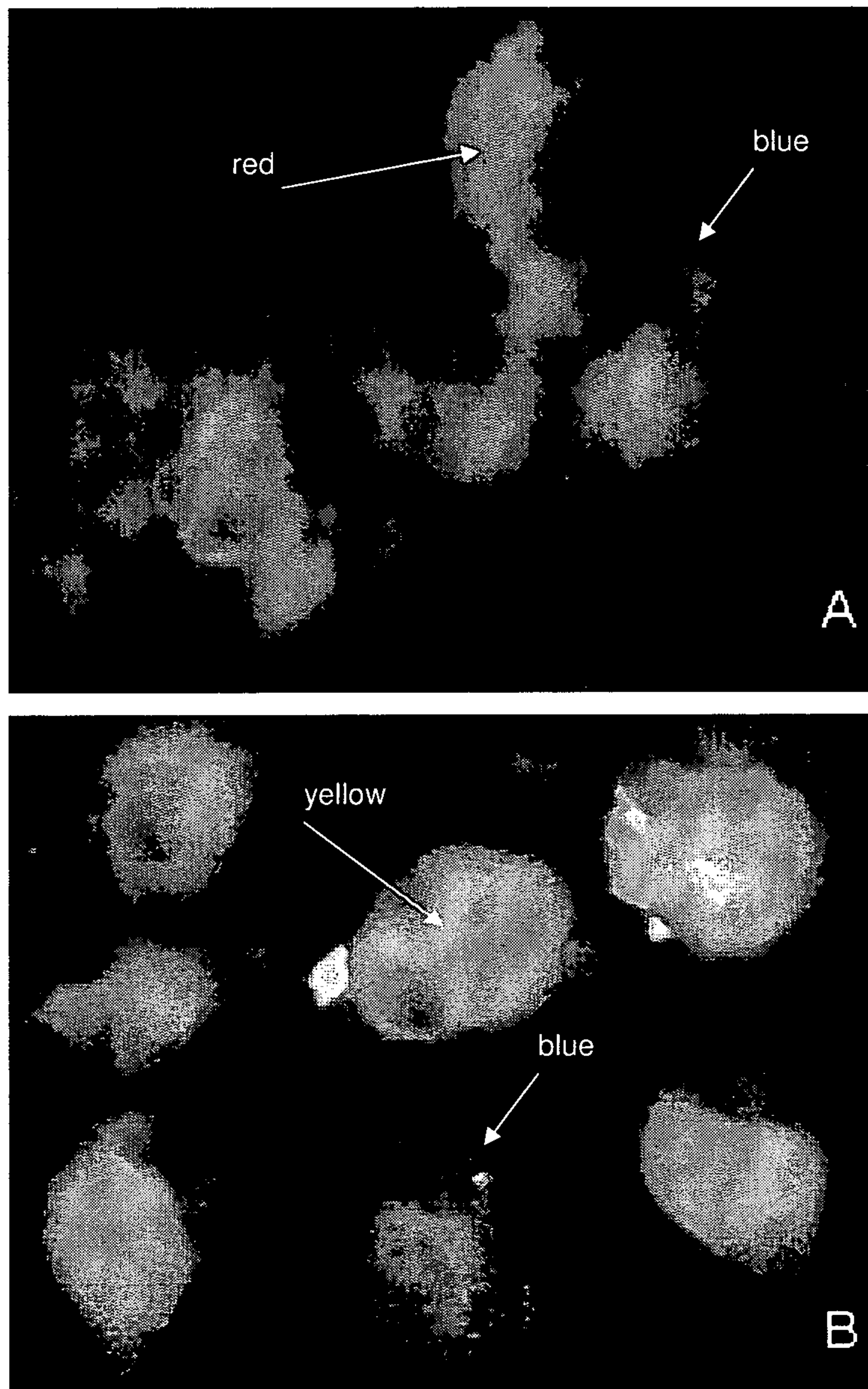
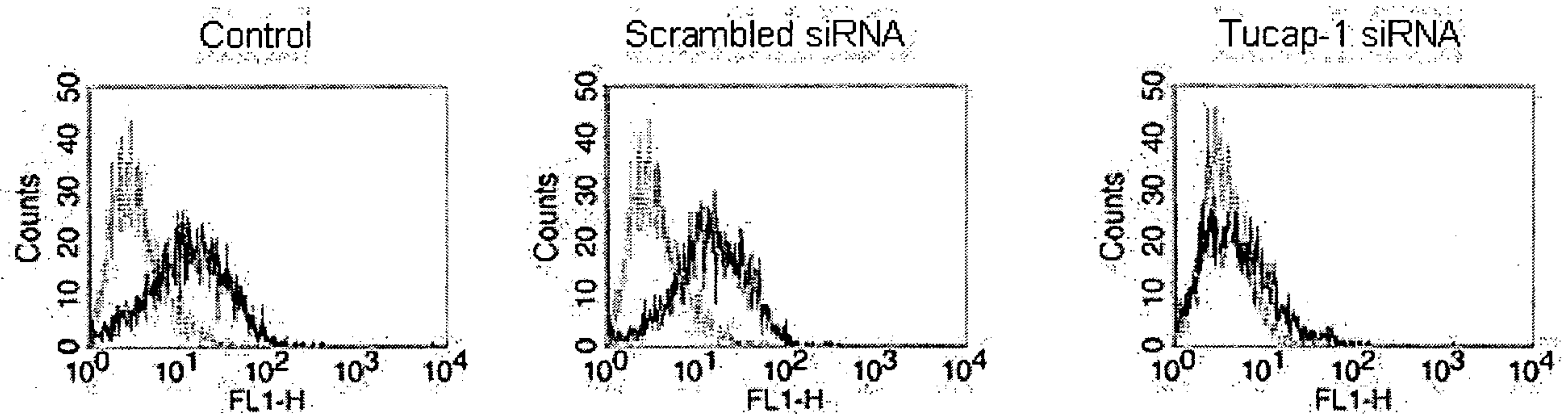


Figure 7A

12 / 15**Figure 8**

13 / 15

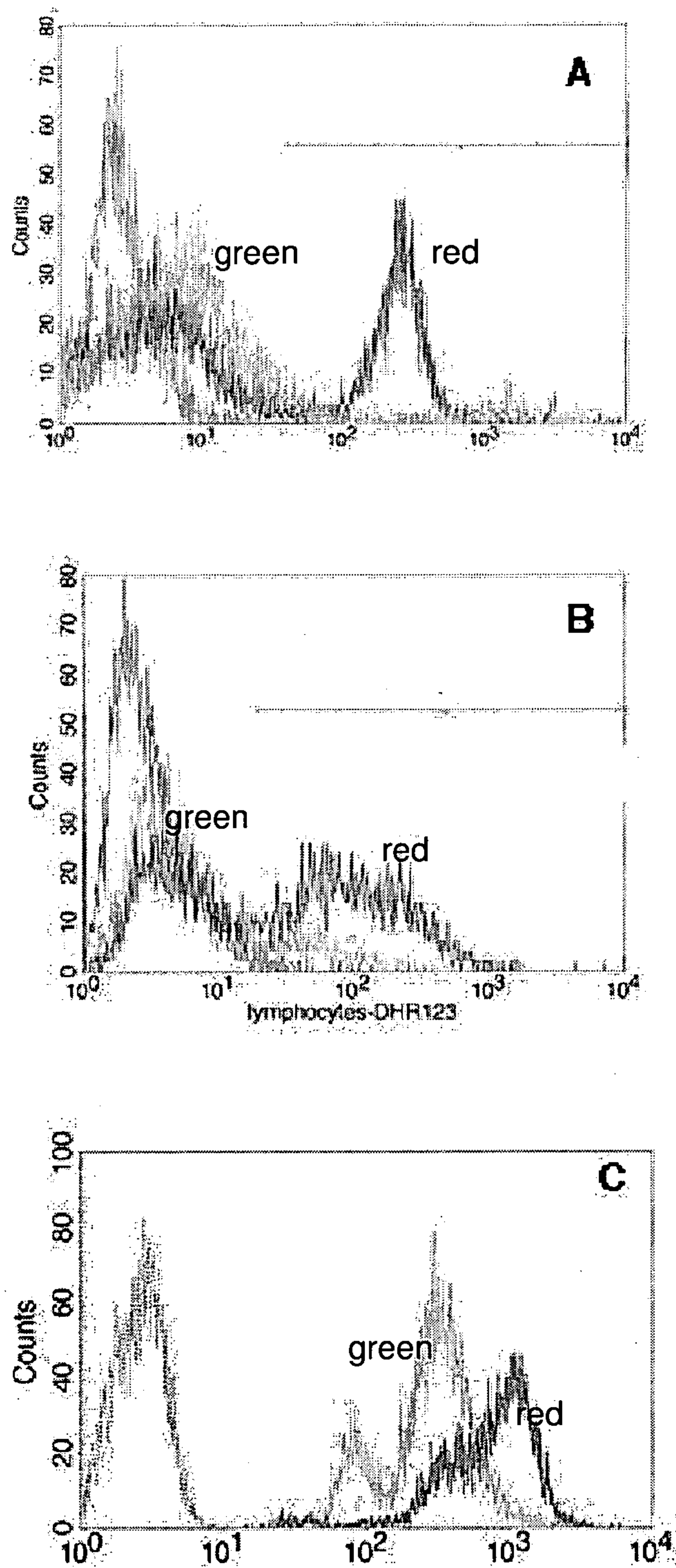
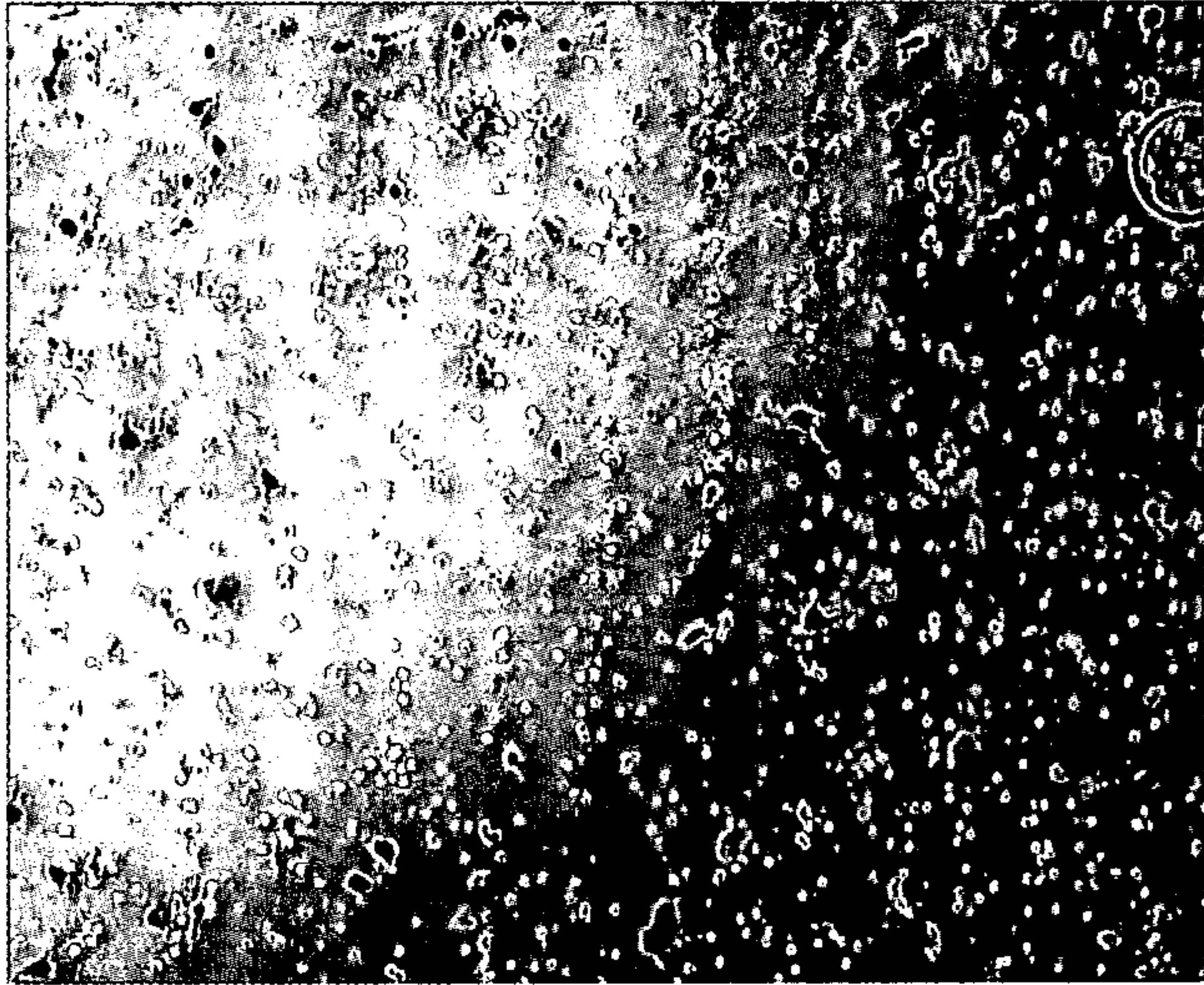
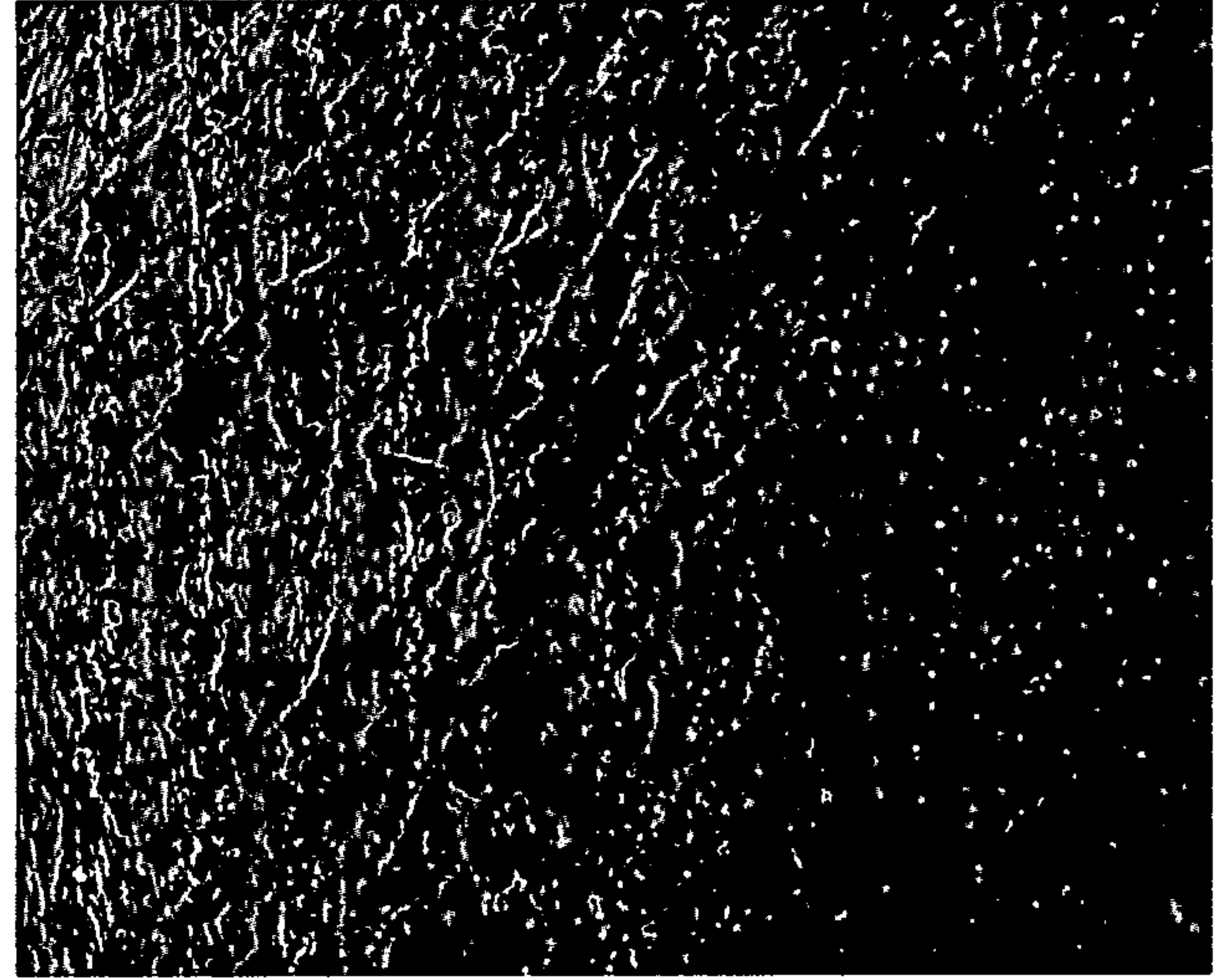


Figure 9

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WM743

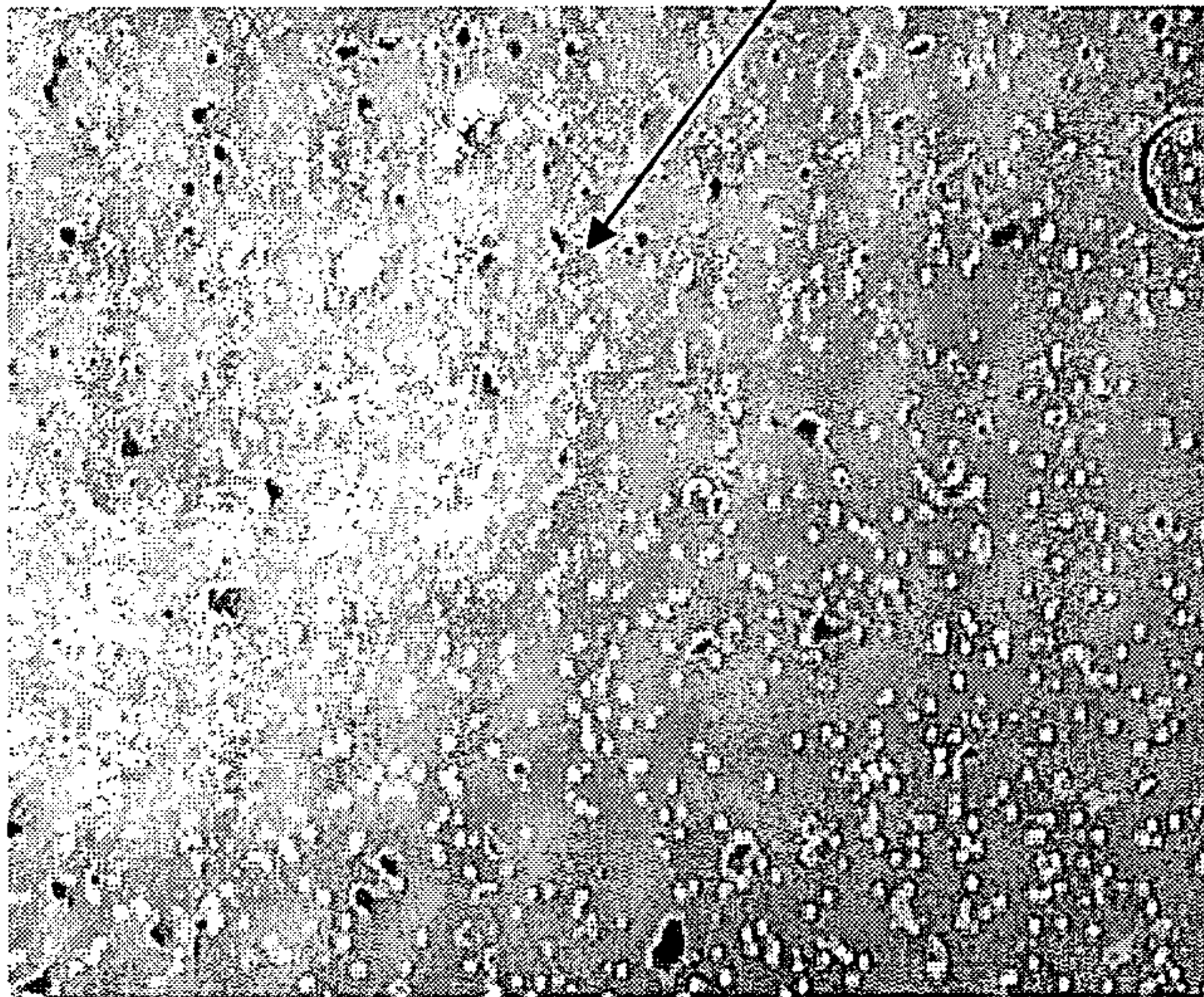


TWM

Figure 10

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Arrows indicate examples of stained cells



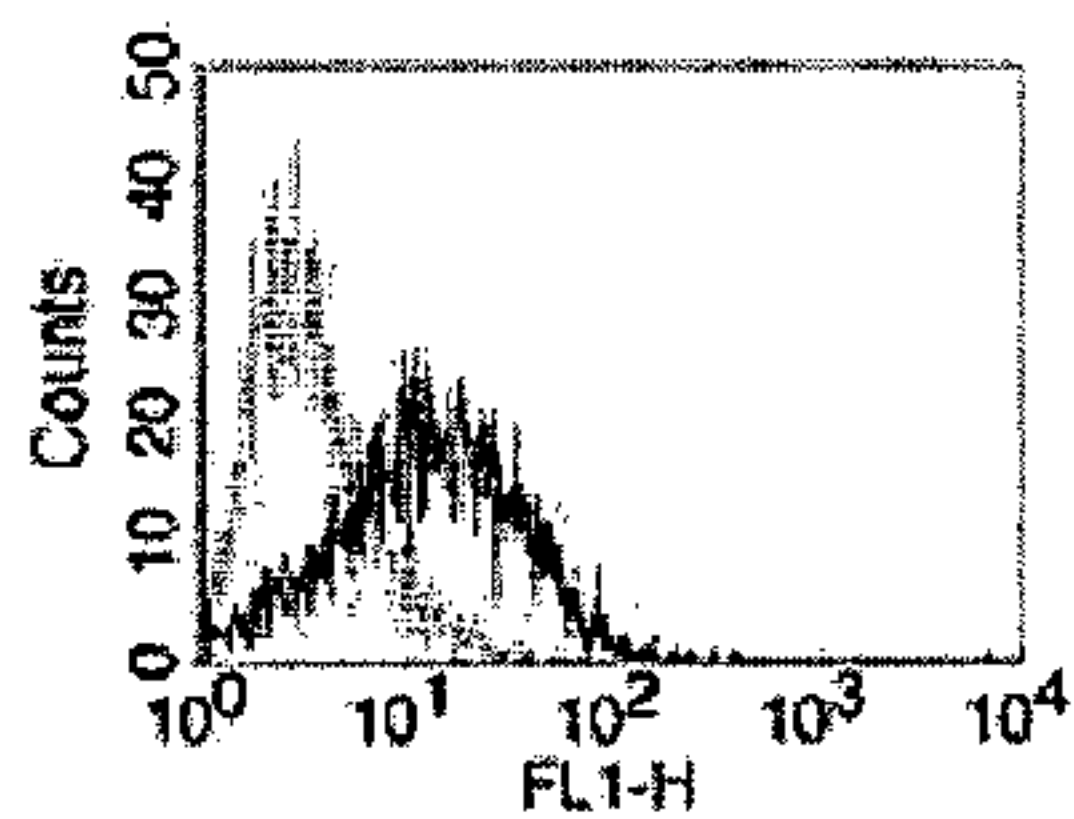
WM743



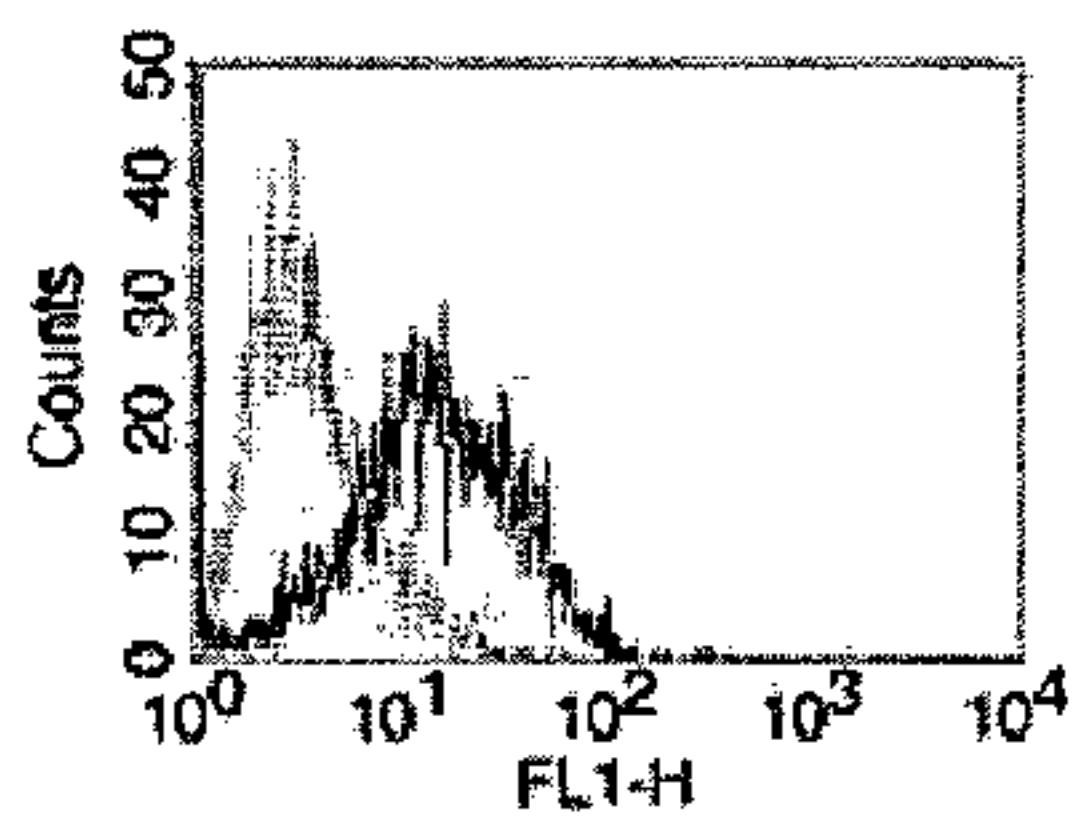
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Figure 10A

Control



Scrambled siRNA



Tucap-1 siRNA

