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M. Sc. Thesis, Pharmacology

Expression of scinderin in megakaryoblastic leukemia cells induces differentiation, maturation, apoptosis with release of platelet-like particles, and inhibits proliferation and tumorigenesis

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Year 2001

To my parents

Abstract

Proliferation of atypical megakaryoblasts is a characteristic of megakaryoblastic leukemia. Cell lines established from patients with this disorder show presence of gelsolin but absence of scinderin expression, two filamentous actin severing proteins present in normal megakaryocytes and platelets. Vector-mediated expression of scinderin (pcDNA3-Sc) in the megakaryoblastic cell line MEG-01 induced a decrease in F-actin and gelsolin as evaluated by immunocytochemistry, image analysis and immunoblotting. This was accompanied by an increased Rac2 expression followed by activation of PAK/ MEKK.SEK/ JNK/ c-jun, c-fos and the Raf/MEK/ERK transduction pathways. Transduction pathway activation was responsible for cell differentiation, polyploidization, maturation and apoptosis with release of platelet-like particles. Platelet-like particles expressed surface CD41a antigen, had dense core vesicles, a high affinity serotonin transport and a circular array of microtubules. Treatment of platelet-like particles with thrombin induced aggregation and release of serotonin. Cell proliferation and the cells' ability to form tumours in nude mice were also inhibited by expression of scinderin. The lack of scinderin expression in megakaryoblastic leukemia cells seems to be responsible for their inability to enter into differentiation and maturation pathways characteristic of their normal counterparts.

I would like to specially thank Dr. José María Trifaró for accepting, supporting and supervising me in his laboratory; as well as for his interest, persistence and faith in this project.

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Abbreviations

ADP = adenosine diphosphate

AML = acute myelogenous leukemia

AP-1 = activator protein-1

ATP = adenosine triphosphate

Bcl-2 = B-cell lymphoma/leukemia 2

CD41a = platelet antigen (fibrinogen receptor).

CML = chronic myelogenous leukemia

DMS = demarcation membrane system

DTS = dense tubular system

ECL = enhanced chemiluminescence

ECM = extracellular matrix

EDTA = ethylene diamine tetra-acetic acid

ERK = extracellular signal activated kinase

F-actin = filamentous actin

FAK = focal adhesion kinase

FCS = fetal calf serum

GPIIb/IIIa = glycoprotein IIb/glycoprotein IIIa complex (CD41a antigen or fibrinogen receptor)

HEPES = N-[2-ydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

HRP = horse radish peroxidase

[^{3}H]5-HT = tritium-labeled 5-hydroxy tryptamine (serotonin)

[^{3}H]thymidine = tritium-labeled thymidine

IgG = immunoglobulin G

JNK = c-jun amino terminal kinase

MAPK= mitogen activated protein kinase

MLCK = myosin light chain kinase

MK = megakaryocyte

PAK = p21 activated kinase

PBS = phosphate buffer saline

PI3K = phosphatidylinositol 3-kinase

PI5K = phosphatidylinositol 5-kinase

PIP_2 = phosphatidylinositol 4,5-bisphosphate

PIP_3 = phosphatidylinositol 3,4,5-triphosphate

PKB = protein kinase B

PKC = protein kinase C

PPO = platelet peroxidase

PMA = phorbol myristate acetate

PTEN = phosphatase and tensin homologue

RER = rough endoplasmic reticulum

ROK = Rho activated kinase

Sc = scinderin

SCCS = surface connected canalicular system

SDS = sodium dodecyl sulphate

SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TCA = trichloroacetic acid

TEMED = tetramethylethylene diamine

Tpo = thrombopoietin

TUNEL = terminal deoxyuridil end-labeled technique

WASP = Wiskott Aldrich syndrome protein

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1 - Introduction

1 - Introduction

1.1. Normal megakaryocytes biology and platelet formation

In humans, nearly all blood cell production occurs in red bone marrow, which is located in the upper regions of the major long bones, in the axial skeleton and in the skull. Bone marrow derives from a hierarchical developmental system comprised of hematopoietic stem cells, intermediate level progenitors and maturing cells committed to each lineage (fig. 1.1). Although the morphology of all major blood cell types is similar during their initial developmental stages, cells committed to platelet production exhibit a unique structural morphology. Megakaryocytes give rise to blood platelets, growing to 10 times the diameter of most other bone marrow and blood cells, and containing up to 128 times the normal chromosomal complement (blood platelets are responsible for repair, and re-establish vascular integrity following injury (hemostasis)). Like all blood cell precursors, megakaryocytes are derived from pluripotent marrow stem cells, which can either renew themselves or differentiate into all blood elements (Ogawa, 1993). Although stem cell lineage commitment decisions are cell autonomous (Fairbairn et al., 1993), external influences, primarily from the so called cytokines, are required for these programs (Ogawa, 1993). Thrombopoietin, also known as mpl ligand, is the cytokine primarily responsible for the growth and differentiation of megakaryocytes (Vigon et al., 1992).

1.1 (a). Polyploidy formation

Megakaryocytes become polyploid through a process of DNA replication without cytokinesis, referred to as endomitosis (fig. 1.2). Polyploidization in megakaryocytes is

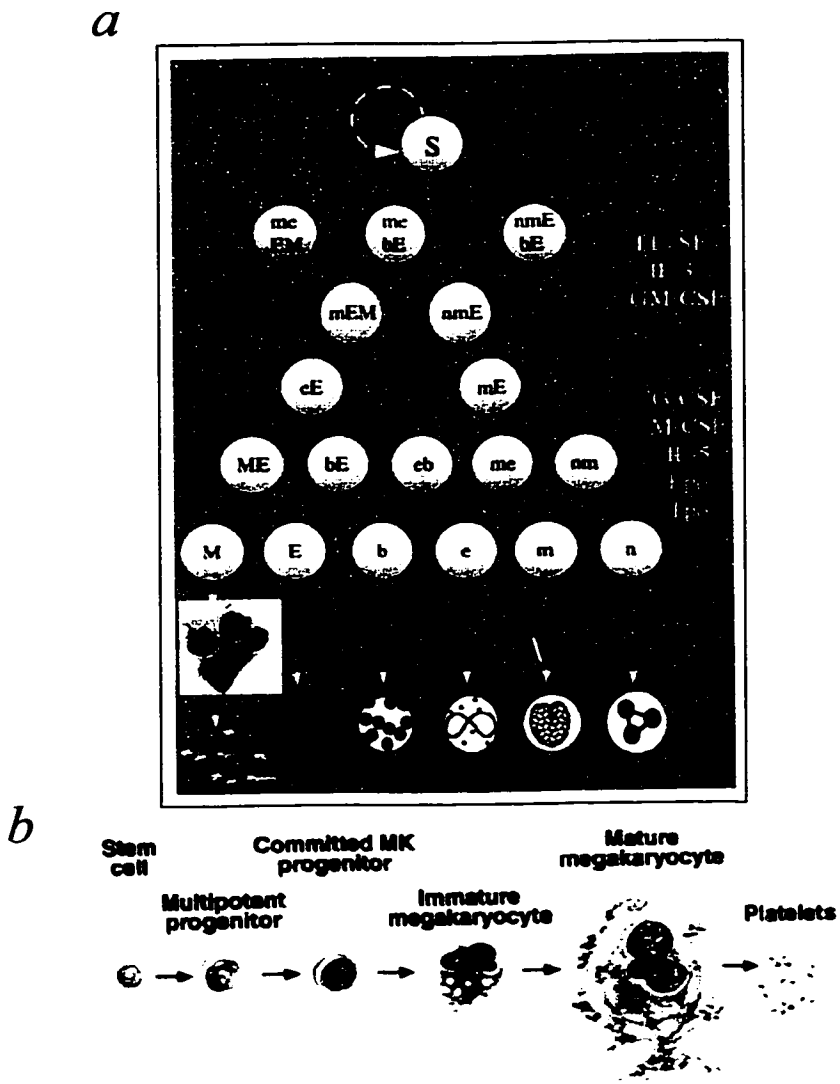

Fig. 1.1: Model of hematopoiesis. *a*, The scheme derives from in vitro hematopoietic colony formation and in vivo properties of bone marrow cells. The hematopoietic stem cell has one of two fates: either to self-renew (circular arrow) or to commit to cell development and undergo cell division and differentiation steps. Stem cells progressively lose developmental potential, giving rise to progressively commited single blood cell lineages. Because each step is dependent on one or more cell divisions, there is a potential for a tremendous amplification of cell numbers during these process. Althouh the cells are autonomous, the decision to commit to specific cell lineages is dependent on hematopoietic growth factors. The Flt-3 ligand (FL), steel factor (SF), interleukin (IL)-3 and granulocyte-macrophage (GM)-colony-stimulating factor (CSF) act principally on multipotent progentors, and G-CFS, IL-5, eryropoietin (Epo) and thrombopoietin (Tpo), work primarily (but not exclusively) on cells commited to specific lineages (granulocytes, monocytes, eosinophiles, erythrocytes, and megakaryocytes, respetively). *b*, Megakaryocytes differentiation lineage. (From: Kaushansky K., review, 1999).

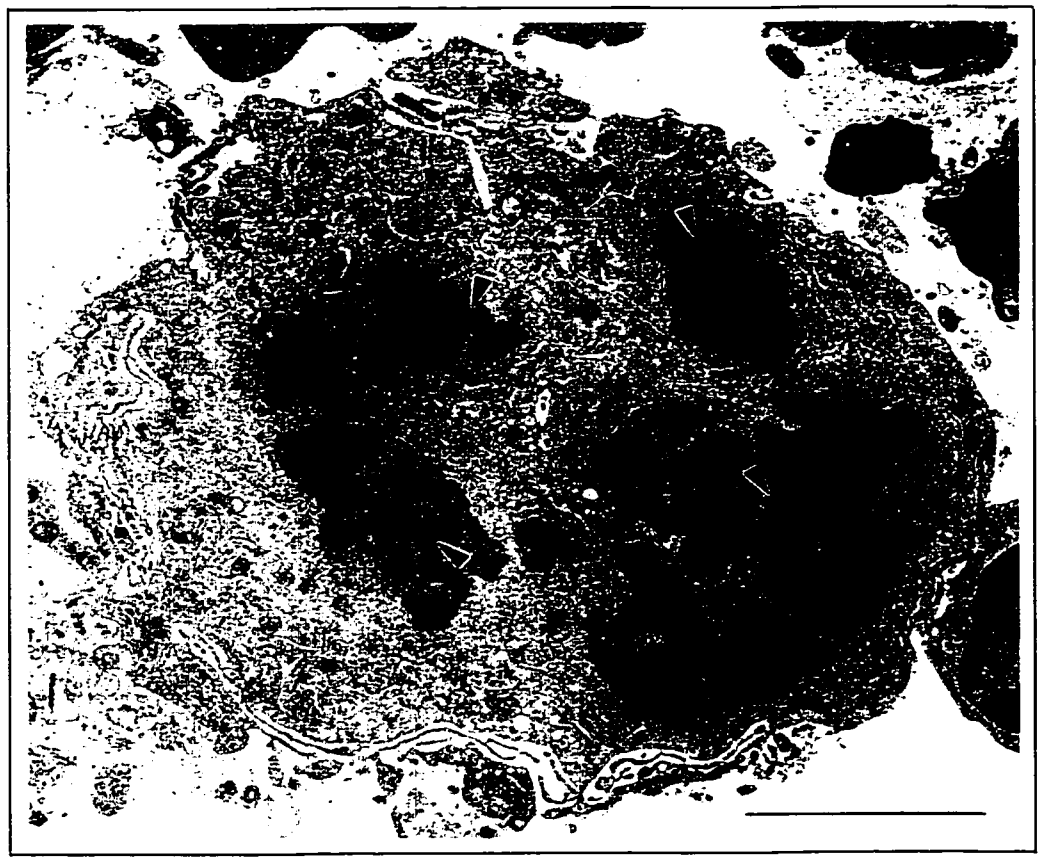

Fig. 1.2: Electron micrograph of a mouse megakaryocyte in endomitosis. There is absence of nuclear membrane. The demarcation system (DMS) is beggining to develop in the cell periphery. The arrowheads indicate the condensed chromosomes. Magnification, x 5700; bar = 5 μm. (From: Jackson et al., 1997).

unique among hemopoietic cells, although hepatocytes also show a small degree of polyploidization. Each endo-mitotic cycle in megakaryocytes results in a doubling of their DNA content, and this process is similar in length to that of a generation cycle in diploid cells (Odell et al., 1968). The total number of endo-mitotic cycles can range from 2 to 6, reaching ploidy stages of up to 128N, although the average ploidy is 16N..

Changes at the molecular level may explain the process of endo-mitosis in megakaryocytes. The expression and function of the primary proteins regulating cell cycle progression are altered in the process of the commitment of a megakaryocyte precursor to an endo-mitotic cycle. (Baatout et al., 1996). Alterations in the levels of cyclin B1 and the consequent inactivation of the cyclin dependent kinase cdc2, a kinase responsible for normal cell cycle progression through mitosis, occur through an increase in the levels of p21 , a known inhibitor of cdc2 activity (Kikuchi et al., 1997). Thus cells skip mitosis and enter another cell cycle, resulting in polyploidization. One of the mechanisms involved in megakaryocyte polyploidization is the control of cell kinetics by cyclin B. This cell cycle regulator is highly expressed in granulocytes and monocytes, yet undetectable in the megakaryocytic lineage (bone marrow cultured megakaryocytic cells), the megakaryocytic cell lines DAMI, HEL, MEG-01 and platelets. Eventually its mRNA is present in similar amounts in all cell types (Gu et al., 1993). This polyploidization process occurs independently of cytoplasmic maturation, which follows polyploidization. Both processes are regulated independently and are not inter-related (fig. 1.3) (Kikuchi et al., 1997).

1.1 (b). Ultra-structure of megakaryocytes

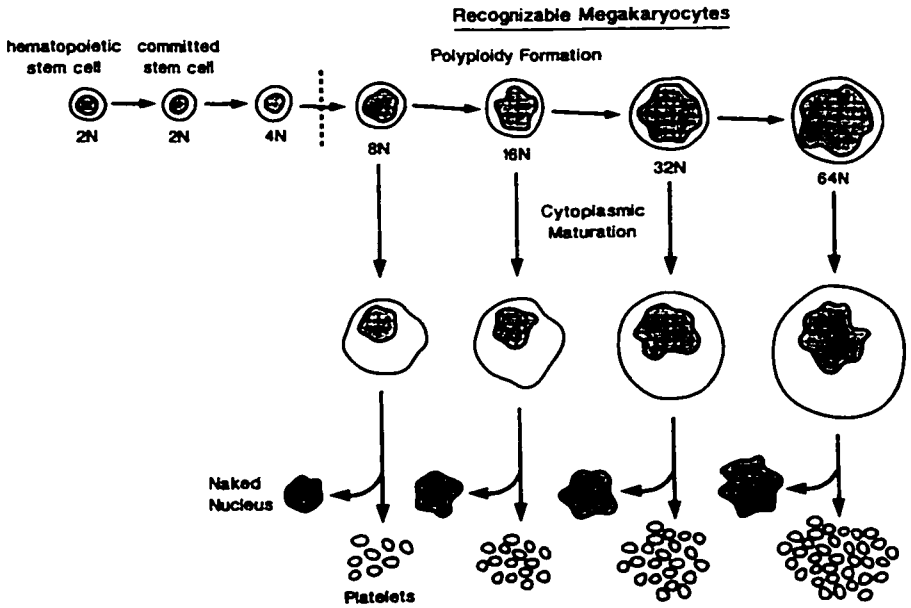

Fig. 1.3: Scheme illustrating the stages of megakaryocytes development. (1) Commitment of hematopoietic precursors to the megakaryocytic lineage; (2) differentiation of megakaryocyte progenitors to recognizable megakaryocytes; (3) polyploid formation; (4) cytoplasmic maturation; and (5) platelet shedding. The scheme shows that polyploidization precedes and is completed before the rapid expansion and maturation of megakaryocyte cytoplasm. Megakaryocytes can stop polyploidization, undergo cytoplasmic maturation and platelet shedding at any level of polyploidy; and megakaryocyte size is related to both the degree of polyploidization and the stage of maturation. (From: Jackson et al., 1997).

The earliest recognizable megakaryocyte detectable by electron microscopy measures 10-12 μm in diameter and contains a large, bilobulated nucleus with prominent nucleoli (fig. 1.4 *a*). Megakaryocytes of intermediate maturity are approximately 15-30 μm in diameter and have an 8-32 N polyploidy (fig. 1.4 *b*). Mature megakaryocytes measure 30-50 μm in diameter (fig. 1.4 *c*). The nucleus is usually positioned at one pole of the cell, and nucleoli are less prominent than in immature cells. The demarcation membrane system (DMS) divides the entire megakaryocyte cytoplasm into "platelet fields". The RER (rough endoplasmic reticuli) and Golgi complex are reduced, whereas the DMS and α-granules are present through the entire cytoplasm. At the stage of platelet production, the megakaryocyte shape becomes irregular and the final stage in the life of the cell is a "naked nucleus" (fig.1.4 *d*) surrounded by a small cytoplasm.

The demarcation membrane system (DMS) is an extensive system of narrow channels homogeneously distributed through the cytoplasm at the late stages of megakaryocytes maturation. The narrow channels are in contact with the external milieu (Behnke, 1968). The DMS was first described by Behnke as a structure which originates from the plasma membrane in the form of tubular invaginations at multiple sites. Moreover, it has been suggested that the DMS compartmentalizes the megakaryocyte cytoplasm into platelet territories, which are released into the circulation as platelets at the end of the megakaryocyte maturation process. An alternative model for platelet formation is the flow model, which describes the role of the DMS as a membrane reservoir for evagination of cytoplasmic

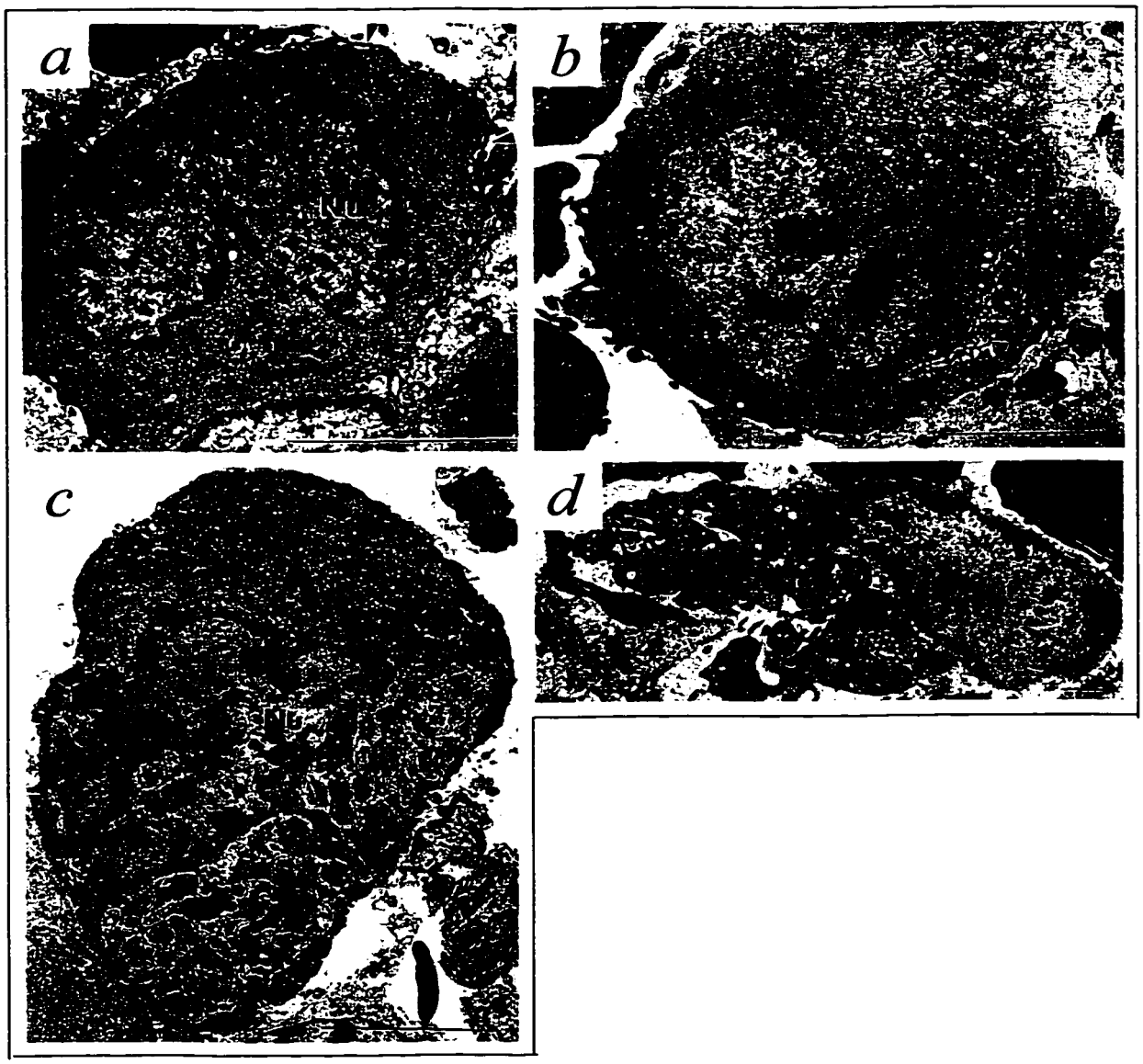

Fig 1.4: a, electron micrograph of an immature mouse megakaryocyte. Arrowheads indicate nascent demarcation membranes. Nu, nucleus. Magnification, x 7900; bar = 5 μm. b, A mouse megakaryocyte of intermediate maturity. A few granules are apparent within the cytoplasm. Arrowheads indicate the development DMS. Nu, nucleus. Magnification, x 7900; bar = 5 μm. c, A mature megakaryocyte. Arrowheads mark the extensive DMS. Nu, nucleus. Magnification, x 7000; bar = 10 μm. d, A naked mouse megakaryocyte nucleus remaining after almost complete shedding of the cytoplasm to form platelets. Nu, nucleus. Magnification, x 11600; bar = 1 μm. (From: Jackson et al., 1997).

processes. These processes then undergo partitioning into platelets (Radley and Scurfield, 1980; Radley and Haller, 1982). Evidence of megakaryocyte processes formation extending through the endothelium of narrow sinusoid vessels, at the ultrastructural level, gives support to this model (Radley and Scurfield, 1980). The DMS is also thought to form the surface-connected canalicular system (SCCS) of platelets, which serves as a conduit for the secretion of platelet granule contents (White, 1970).

The dense tubular system of platelets and the RER and nuclear envelope of megakaryocytes are identical. Although neither the platelets' SCCS nor the megakaryocytes' DMS are connected to the DTS (dense tubular system), both form closely associated membrane complexes (White and Clawson, 1980).

Although platelets' α-granule contents were first thought to be synthesized solely in the MK, it was later demonstrated that both megakaryocytes and platelets are capable of endocytosing soluble proteins and packaging these proteins within their secretory granules until required for hemostasis (Handagama et al., 1987; Handagama et al., 1989; Harrison et al., 1989).

α-Granules are present in the earliest recognizable megakaryocyte, preceding the development of demarcation membranes, and are thought to originate from the Golgi complex at the time of megakaryocyte maturation. α-Granules measure 200-500 nm in diameter, contain a nucleoid within a finely granular matrix, and contain numerous proteins, among which are included albumin, β-thromboglobulin, factor V, fibrinogen, fibronectin, IgG, platelet factor 4, vitronectin, von Willebrand factor, GPIb (glycoprotein Ib), GPIIb/IIIa and P-selectin. α-Granules are homogeneously distributed throughout the cytoplasm between

the demarcation membranes of mature megakaryocytes.

α-Granules' constituents are derived from 3 different mechanisms: (i) endogenous synthesis via the protein synthesis pathway (megakaryocytes only), (ii) receptor-mediated endocytosis (MKs and platelets), and (iii) pinocytosis (megakaryocytes and platelets). Examples of endogenously synthesized constituents are platelet factor 4 (PF 4), β-thromboglobulin, GPIb, GPIIb, GPIIIa, etc. Examples of plasma compounds incorporated via receptor uptake include fibrinogen monomers (taken up via GPIIb/IIIa complex , or fibrinogen receptor, into α-granules). This is done only by platelets and not by megakaryocytes, which are not in direct contact with plasma, but rather with the internal environment of the bone marrow. Although they are not in direct contact with blood vessels, megakaryocytes, are able to take-up blood fibrinogen monomers.

Dense core granules, which are also found in megakaryocytes, are approximately 200-300 nm in diameter and are identified, at the EM level, on the basis of a bull's eye appearance (consisting of a clear halo encircling a dark, central area). The main components of these dense granules are ATP, ADP, pyrophosphate, calcium and various other cations (Holmsem, Weiss, 1979). Electron-dense granules are rarely observed in megakaryocytes and it is believed that this inability to detect dense granules in megakaryocytes is due to the absence of serotonin and calcium storage within the megakaryocyte granules. In normal conditions, the level of serotonin in the bone marrow is too low for adequate uptake by the megakaryocyte, which is capable of taking up plasma compounds such as Ca^{++} and serotonin, into dense core granules.

1.2. The cytoskeleton

1.2 (a). Introduction

It is known that movements of cells and sub-cellular organelles are regulated by dynamic cytoskeletal proteins, such as tubulin, actin, myosin, etc. Proteins responsible for such dynamic changes reside in cells in monomeric states (soluble form) and are capable of polymerization into insoluble or gel form. The sol-gel transformation of linear polymers represents the formation of a giant coherent molecule from dispersed subunits. Such dynamic changes, resulting in polymerization (with consequences in motility), are tightly regulated by a number of proteins (gelsolin, scinderin, villin, alpha-actinin, etc.) and transduction pathways. Actin polymerization and de-polymerization for example, are processes which are regulated by actin-filament binding proteins. Two of the most important proteins regulating actin polymerization/depolymerization include gelsolin and scinderin. These actin-filament-binding proteins have three actin-filament-binding sites, and bind actin filaments with approximately equivalent affinities in the micro-molar range (Matsudaira, 1991). In muscle cells, for example, cell activation induces contraction, presumably by inducing the phosphorylation of myosin moleclules, which cross-link with actin filaments, through transient increases in cytosolic calcium (Conrad et al., 1993). This increase in cytosolic calcium induces cell membrane expansion at the site of the contraction. Simultaneously, at the site of membrane expansion, actin filaments assemble from monomeric subunits to form a tridimensional network. This dynamic network of actin filaments (Theriot et al., 1992) modulates the formation of protrusions and serves as an anchor for adhesion molecules.

Many signal transduction mechanisms (e.g. calcium transients, pH changes, protein phosphorylation reactions, phospholipid turnover, GTP-binding proteins, etc.) are proposed to mediate actin assembly, actin disassembly and cell movement. (Stossel, review, 1993). The first step in the assembly of actin filaments from subunits is nucleation, a step in which two or three monomers aggregate (a highly unfavourable reaction). The next step is based on the "barbed" and "pointed" end polarities of the actin filaments. The barbed ends of actin filaments serve as nuclei onto which actin subunits can rapidly add (thermodynamically a very favourable reaction). This occurs at actin monomers concentrations of a few micro-molars (fig. 1.5). A near 10-fold higher actin monomers concentration is required to add subunits to the pointed ends (Sheterline et al., 1994). Proteins responsible for capping and severing the actin filaments such as gelsolin and scinderin (among others) block subunit exchange on the barbed ends of actin filaments, thus regulating spontaneous actin polymerization. Uncapping of actin-filament barbed ends by removal of these proteins can determine when actin filaments elongation occurs (Stossel, 1989). Gelsolin and scinderin (and other capping proteins) have the ability to bind to the sides of the actin filaments, sever or nibble them and remain firmly attached to the newly formed barbed ends, thus preventing reannealing of the broken filaments and spontaneous polymerization (fig. 1.6). Plasma membrane phosphoinositides, implicated in signal transduction, cause gelsolin and scinderin to dissociate from actin-filament barbed ends, thus promoting actin assembly (fig. 1.7), and providing a link between cell surface stimulation and actin assembly-disassembly (fig. 1.8). During receptor-mediated cell stimulation, reaction cascades lead to the breakdown of

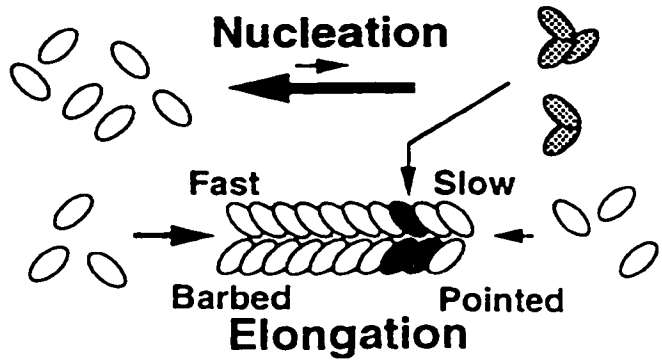

Fig. 1.5: Spontaneous actin polymerization. (From Stossel T., review, 1993).

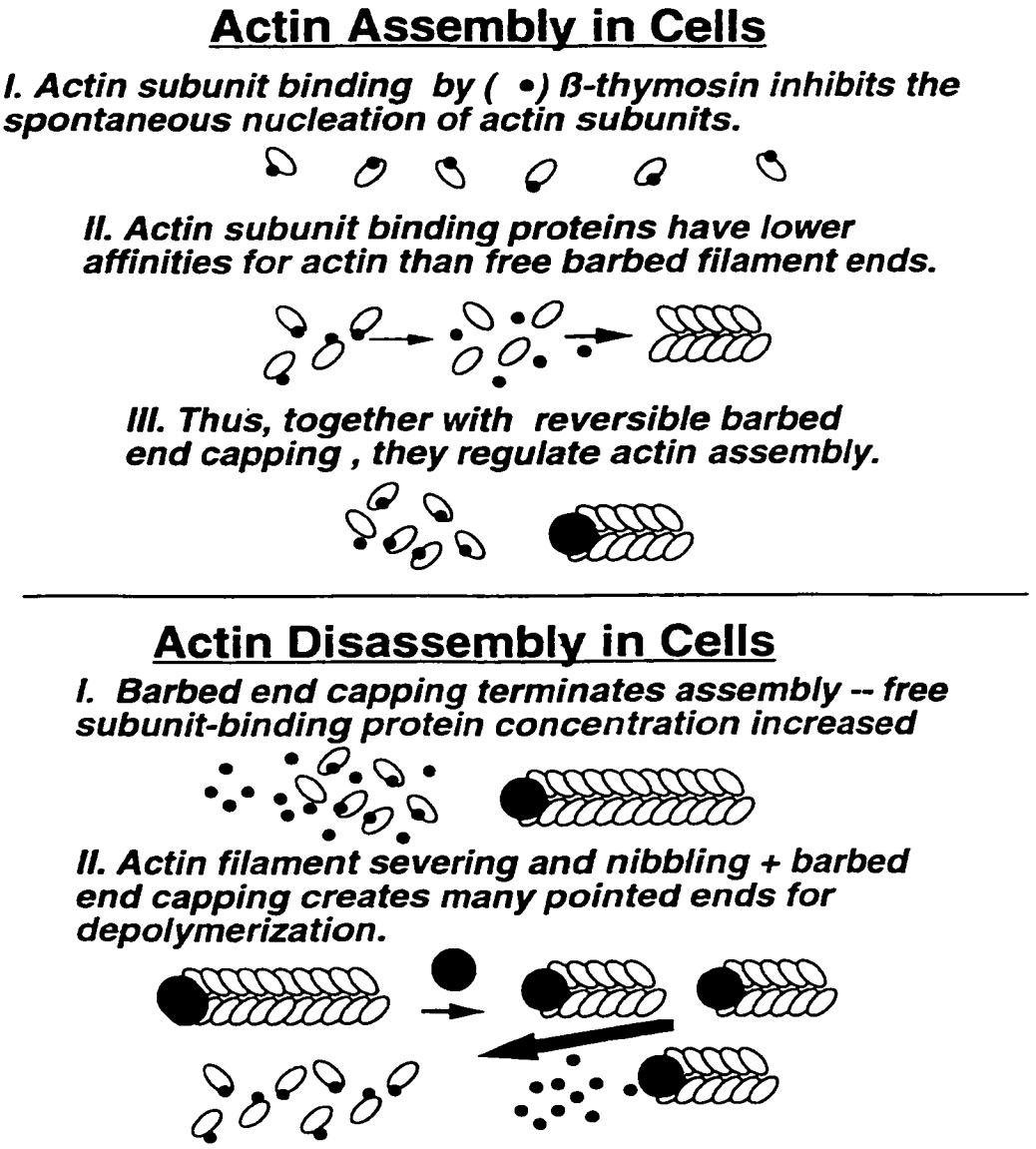

Fig. 1.6: Control of intracellular actin assembly and disassembly. Not shown is that some capping proteins also work by nucleating actin subunits and then remaining bound to the barbed ends of the oligomers formed (From: Stossel T., review, 1993).

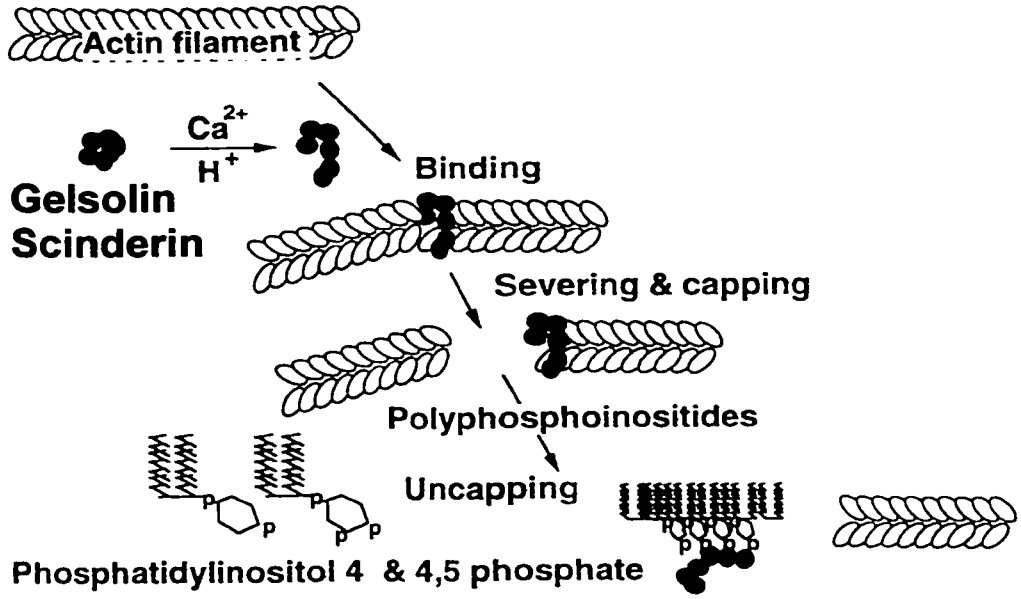

Fig. 1.7: Regulation of gelsolin and/or scinderin activities by ions and polyphosphoinositides. (Modified from: Stossel T., review, 1993).

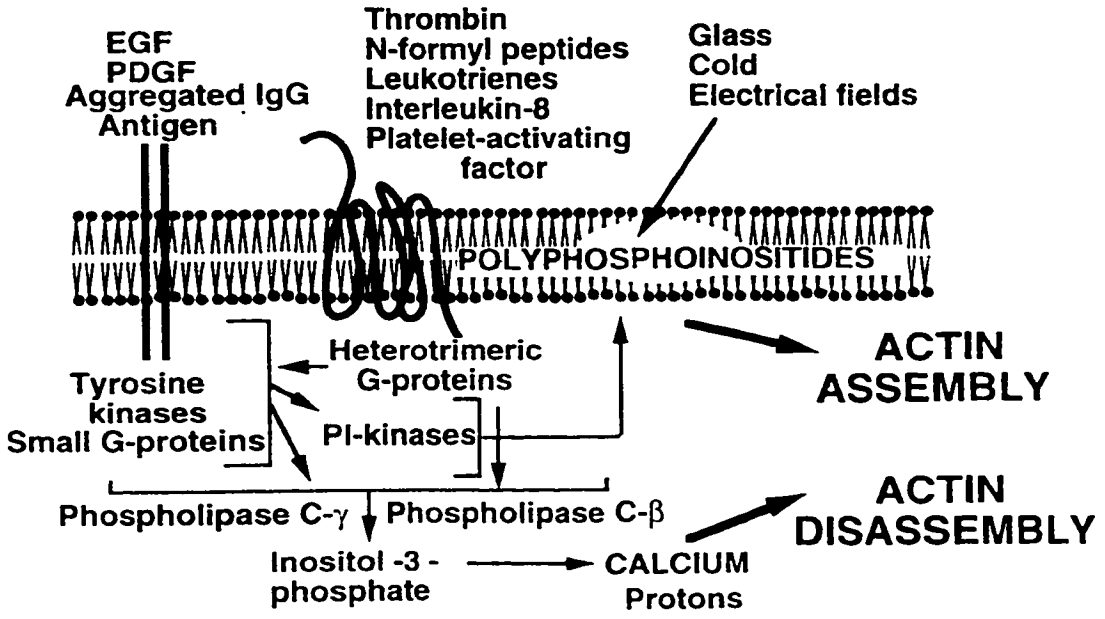

Fig. 1.8: Integrated mechanism for controlling actin assembly and disassembly by cell signalling processes. (From: Stossel T., review, 1993).

phosphoinositides, releasing bound proteins like gelsolin and scinderin. The breakdown products of phosphoinositides cause the release of calcium (and possibly of protons) from intracellular compartments (i.e. endoplasmic reticulum), and the effects of these ions on gelsolin, scinderin and related proteins result in actin filament disassembly. Conversely, reactions (i.e. activation of the GTPases Rac and Rho) leading to the synthesis of phosphoinositides would promote actin assembly by inducing the uncapping of actin filament fragments capped by gelsolin, scinderin and other capping proteins (Stossel, 1994).

The F-actin-severing protein scinderin

Scinderin is a calcium-dependent, filamentous, actin-severing-and-capping protein first discovered in chromaffin cells in our laboratory (Rodríguez del Castillo et al., 1990). This protein is present mainly in tissues with high secretory activity, such as adrenal medulla, hypophysis and testis, including platelets, and it is part of the exocytotic machinery (Zang et al., 1996; Trifaró et al. 1998; Trifaró, 1998). The scinderin gene has also been cloned (Marcu et al., 1994).

Scinderin requires Ca^{++} for activity in its role in the secretory processes (Rodríguez del Castillo et al., 1990). Equilibrium dialysis studies have demonstrated the presence of two scinderin Ca^{++} binding sites (Kd 5.85 x 10^{-7} M, and Kd 2.85 x 10^{-6} M) (Trifaró et al., review, 2000). Complete amino acid sequence analysis of scinderin (715 amino acids) (Marcu et al., 1994) has revealed that it shares homology with gelsolin (63 %) and villin (53 %), two other F-actin-severing proteins (Marcu et al., 1994). Similar to gelsolin and villin, scinderin contains six domains (1-6) (see fig. 1.9). Strong similarities exist between domains 1 and 4,

2 and 5, and 3 and 6 in gelsolin, villin and scinderin. It has also been suggested that this family of actin-filament-severing proteins may have evolved by tandem gene triplication with a predicted 14 kDa monomer unit of 120-139 amino acid residues. This is approximately the size of domain 1 of gelsolin (Trifaró et al., review, 2000).

Scinderin's actin-binding sites 1, 2 and 3 are present in domains 1, 2 and 5 respectively (fig. 1.9). The first two actin-binding sites are involved in the severing of actin filaments. There is high homology between these two actin-binding sites of scinderin and those of gelsolin. Thus, the filament-severing activity of scinderin may reside in the first two domains, since it has been demonstrated for gelsolin that in addition to domain 1, a second binding site in domain 2 is necessary for full severing activity (Trifaró et al., review, 2000). In vitro binding of domain 1 of gelsolin to actin seems to be Ca^{++}-independent (Bryan, 1988) whereas binding of its N-terminus of scinderin to actin requires the presence of Ca^{++} (Trifaró et al., 1992). A third actin-binding site has been demonstrated in domain 5 of scinderin. Scinderin binds actin at this site in a Ca^{++}-independent manner, and thus nucleates actin assembly (Marcu et al., 1998). Therefore, in addition to binding actin on sites present in domains 1 and 2, scinderin must also bind actin on a third site in domain 5 in order to sever or nucleate actin effectively. Under resting conditions, scinderin is bound to plasma membrane phospholipids in a pH and Ca^{++}-dependent manner (Rodríguez del Castillo et al., 1992). Actin and phospholipids compete for binding to scinderin, indicating that a phopholipid binding site and at least one of the two actin-binding sites are localized in the same domain of scinderin (domain 2). Moreover, phospholipids could be more easily displaced from scinderin by actin under acid

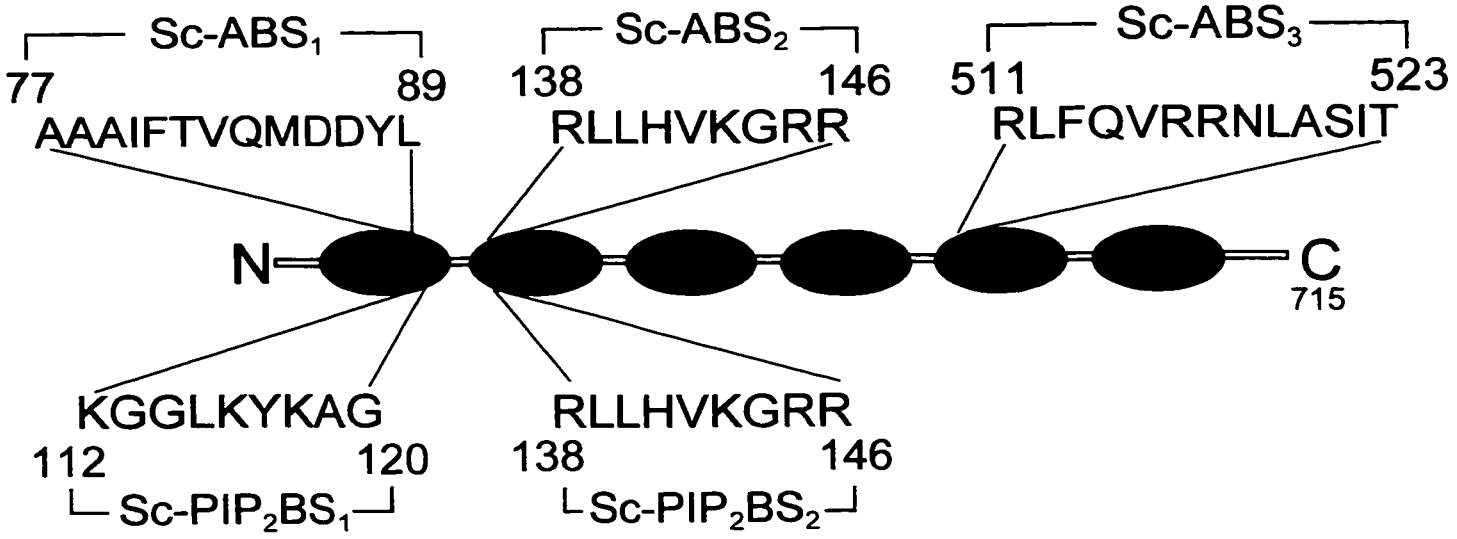

Fig. 1.9: Schematic representation of scinderin domains (Sc 1-6) with the amino acid sequences and position of 3 actin binding sites (Sc-ABS_1, Sc-ABS_2 and Sc-ABS_3) and two PIP_2 binding sites, are shown. (From: Trifaró et al., review, 1998).

rather than alkaline pH, providing 10^{-7} to 10^{-6} M Ca^{++} is present (Rodríguez del Castillo et al., 1992). Therefore, under conditions that produce intracellular acidification and rises in Ca^{++}, scinderin would leave its binding sites (phospholipids) at the plasma membrane to interact and sever actin. Another indication of the competition between actin and phospholipids for scinderin is the fact that PIP_2 (phosphatidylinositol-diphosphate) inhibits the actin-severing activity of scinderin (Rodríguez del Castillo et al., 1992). This suggests that cellular levels of PIP_2 might regulate the activity of scinderin. Indeed, the synthesis of PIP_2 and other phosphoinositides is regulated by small molecular G proteins such as Rac and Rho (Hartwig et al., 1995), suggesting that these proteins might indirectly regulate the activity of scinderin.

Two PIP_2 binding sites on scinderin occur at amino acids 112-119 and 138-146, of domains 1 and 2 respectively (fig. 1.9). The PIP_2 binding site in domain 2 of scinderin overlaps with the second actin-binding site (fig. 1.9), providing an explanation for the competition for scinderin between PIP_2 and actin and for the inhibition by PIP_2 of the actin-severing activity of scinderin (Trifaró et al., review, 2000).

1.2 (b). Role of the cytoskeleton and the importance of cytoskeletal proteins in megakaryocyte spreading and platelet formation

Platelets contain many cytoskeletal proteins synthesized by megakaryocytes, including actin, α-actinin, myosin, spectrin, talin, tensin, tropomyosin, tubulin and vinculin (Fox, review, 1993). Disruption of either microtubules or actin filaments can alter the developmental dynamics of platelet formation. Depolymerization of microtubules in megakaryocytes by

nocodazole results in inability of the cells to form cytoplasmic processes or to undergo fragmentation. Moreover, already-formed pseudopodia undergo retraction when nocodazole is added to megakaryocytes cultures, suggesting that depolymerization of microtubules is responsible for pseudopod or process retraction (Tablin et al., 1990). Similar results are obtained from cells treated with vincristine, an alcaloid that causes rapid disassembly of the mitotic spindle (Haller et al., 1983; Radley, Haller, 1982). Treatment of megakaryocytes in culture with taxol, a drug that polymerizes and stabilizes microtubules, results in the production and extension of abnormally thick processes or extensions that do not bead or fragment. These observations support the concept of a microtubule basis for pseudopodial extension (Tablin et al., 1990). Processes formation and fragmentation proceed and are accelerated in megakaryocytes in cultures treated with cytochalasin B, an inhibitor of actin polymerization. This suggests that arrangement of the actin-rich peripheral zone may be an important step in the process of platelet formation (Tablin et al., 1990; Leven, Yee, 1987). In other words, treatment of megakaryocytes in culture with cytochalasins B or D allows microtubules to expand into formerly dense actin areas. When the peripheral actin-rich zone is disrupted, the arrays of microtubules adjacent to that zone either elongate or reorient into extensions or pseudopodia (Tablin et al., 1990). In summary, a model for platelet formation has been proposed, which involves the following four steps (Tablin et al., 1990):

(1) The cells adhere to the extracellular matrix.

(2) Thin pseudopodia (future pro-platelets) that are not tightly adherent to the extracellular matrix form. This process involves rearrangement of the actin-rich peripheral zone,

permitting microtubule elongation and/or re-orientation.

(3) Microtubule-driven pseudopodia form, and the demarcation membrane system and granules move into the processes.

(4) Pro-platelets and individual platelets pinch off by coalescence of the vesicles related to the demarcation membrane system and their fusion with the plasma membrane (Tablin et al., 1990).

There is, however, another proposed model of platelet formation (Radley and Haller, 1982) in which the demarcation membrane system is the source of the additional membrane necessary for pseudopodia formation and therefore envelopes putative platelets. Another mechanism has also been proposed, in which platelets are formed by a mechanism similar to cytokinesis, in which the actin-rich cleavage furrow separates daughter cells (Radley and Haller, 1982). This model would suggest that the extension of demarcation membranes is not involved in platelet release.

1.2 (c). The release of platelets from megakaryocytes is also a consequence of cell senescence

Primary GPIIb/IIIa positive megakaryocytic cells, directly isolated from the bone marrow of normal individuals, undergo apoptotic cell death in a culture system supplemented with thrombopoietin (Tpo), more rapidly than CD34-derived megakaryocytes (Zauli et al., 1997). However, extrapolation of these observations to "in vivo" megakaryocytopoiesis must be done with caution, because cytokines other than Tpo and complex cell-to-cell and cell-to-bone marrow matrix interactions are known to play a primary role in the development of

megakaryocytes and platelet release in vivo (Hoffman, 1989).

Apoptosis occurs mainly in mature megakaryocytes. The kinetics of platelet release in CD34-derived megakaryocytes is delayed with respect to the peak of megakaryocyte maturation and coincides with the onset of apoptosis in these cells. This correlation suggests that maximal platelet production and megakaryocytes apoptosis are closely related events (Zauli et al., 1997). Apoptosis in primary megakaryocytes is only partially modulated by Tpo, and this occurs only for a short period of time at early stages of maturation (Zauli et al., 1997). The behaviour of Tpo is similar to other hematopoietic growth factors, which show pleiotropic activity on cell survival, growth and maturation, depending on the stage of differentiation of the target cell. Thus, at later stages in primary culture, Tpo-treated CD34 positive cells undergo maturation, followed by a peak of platelet production at approximately days 18 to 21 in culture (fig. 1.10). Platelet production follows a peak of apoptosis at day 18 in culture (Zauli et al., 1997).

It has also been demonstrated that platelets produced "in vitro" by CD34 positive peripherical blood cells, cultured under conditions that promote megakaryocyte formation (Tpo treatment), are ultra-structurally and functionally identical to plasma-derived platelets (Choi et al., 1995). Pro-platelets and platelets produced under these conditions express platelet-specific glycoproteins Ib and IIb, and also contain microtubule coils equal in size to those found in plasma-derived platelets. In the presence of fibrinogen, culture-derived platelets aggregate in response to both thrombin and ADP. This aggregation is specifically inhibited by the addition of anti-GPIIb/IIIa (fibrinogen receptor) antibody (Choi et al., 1995).

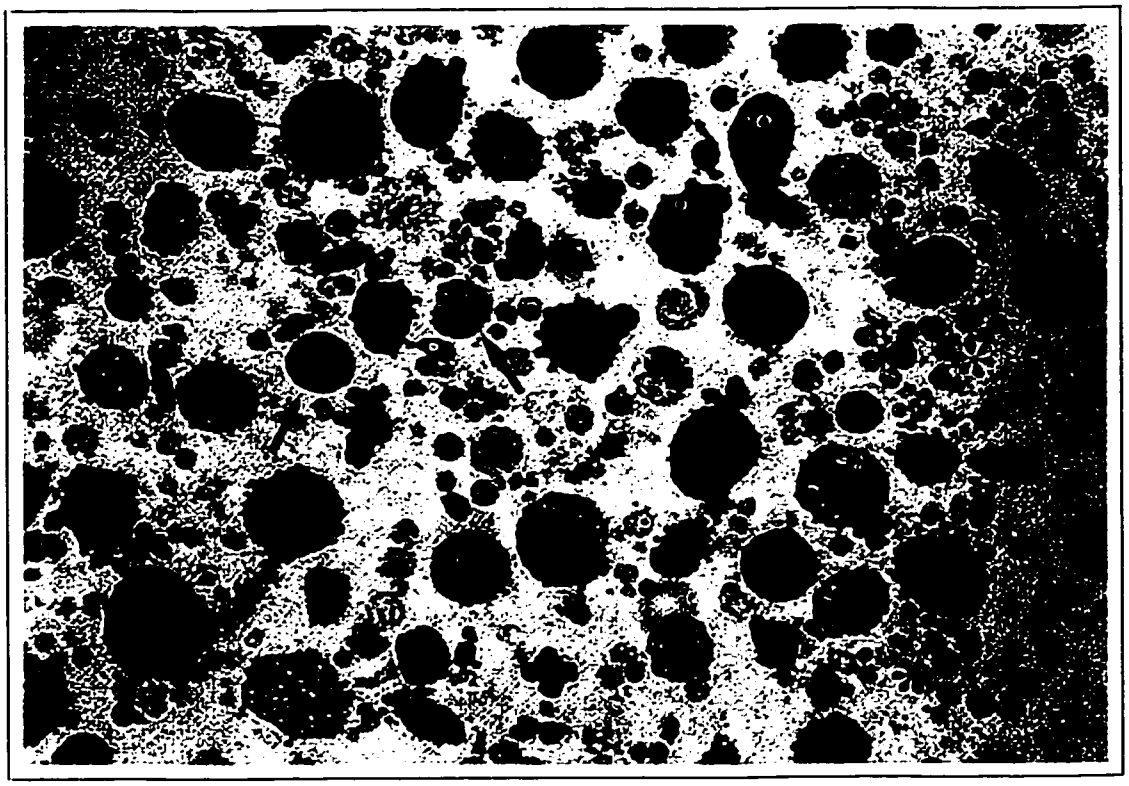

Fig. 1.10: Platelets released in culture supernatants by CD-34 derived megakaryocytes. Arrows show apoptotic megakaryocytes surrounded by clusters of platelet-size fragments (*). OM x 40. (From: Zauli et al., 1997)

1.2 (d). Clinical abnormalities of megakaryocytopoiesis

A. Chronic myelogenous leukemia:

An increase in megakaryocyte numbers and thrombocytosis are frequently observed in patients with chronic myelogenous leukemia (CML). However, the megakaryocytes in CML are usually smaller and of lower than normal ploidy, which seems discordant with the observed thrombocytosis. In thrombocytotic states, megakaryocyte numbers, size and ploidy are usually increased, but this is not the case in CML. An explanation of this is that the smaller size and ploidy of megakaryocytes in CML is a direct consequence of the myeloproliferative effect by itself. Another possibility is that regulation of progenitor proliferation is abnormal, whereas regulation of megakaryocyte polyploidyzation remains normal. In this case, Tpo levels would be reduced as a consequence of negative feedback due to elevated platelet number, and lower Tpo levels would result in decreased megakaryocyte size and ploidy.

Another remarkable thrombopoietic abnormality develops in CML. Platelet-storage pool levels of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) show a significant decrease as CML progresses to the accelerated phase (Jackson et al., 1997). This precipitous decrease in ATP and ADP represents development of a severe abnormality in dense granule formation in megakaryocytes, and its onset is predictive of imminent blast crisis (Jackson et al., 1997).

B. Megakaryoblastic leukemia:

Acute megakaryocytic leukemia (AML) or megakaryoblastic leukemia is classified as M7

by the FAB (French-American-British) classification. This phenotype is prevalent in about 5 % of all cases of AML. In bone marrow aspirates, the leukemic megakaryoblasts and promegakaryocytes are engendered by blasts with budding cytoplasm or blasts that have a lymphoid appearance, and these are accompanied by intense myelofibrosis.

A small proportion of megakaryoblasts are present in other cases of AML, but in megakaryocytic leukemia megakaryoblasts are prominent (>10 %) leukemic cells, with platelet count being normal or elevated at the time of onset.

Marrow aspiration (biopsy sample) is often unsuccessful ("dry trap") because of the extensive marrow fibrosis in most cases. The marrow biopsy contains either small or large blast cells or a combination of both. The former have a high nuclear/cytoplasmic ratio, dense chromatin with distinct nucleoli, and resemble lymphoblasts (when circulating in blood, these cells are often mistaken for lymphocytes). The larger blasts may have some features of maturing megakaryocytes, with agranular cytoplasm with cytoplasmic protrusions, clusters of platelet-like structures, or shedding of cytoplasmic blebs. Confirmation of their megakaryoblastic maturation phenotype requires immunocytologic studies of the presence of von Willebrand factor and the lineage-specific glycoproteins Ib, IIb/IIIa, or IIIa (Lichtman et al., 1990).

1.2 (e). Megakaryocytic cell lines

Human megakaryocytic immortal cell lines derived from the peripheral blood or the bone marrow of patients with leukemia are invaluable tools for studying the biochemistry and differentiation of hematopoietic cell lines. The development of specific monoclonal

antibodies against cell surface antigens has made it possible to identify the lineages of cell lines faster and more precisely (Saito, review, 1997).

Many cell lines have only erythroid, myeloid or lymphoid phenotypes, but some cell lines appear to express primitive multi-lineage characteristics and have the ability to differentiate into granulocytes, erythrocytes and megakaryocytes. A small number of cell lines with pure megakaryocytic features have been isolated. These so called "pure" megakaryocytic cell lines have been useful for studies of megakaryocyte differentiation and maturation, and this is of great advantage and importance, since megakaryocytes constitute only 0.03-0.06 % of all nucleated cells in the bone marrow, and consequently it is difficult to isolate sufficient numbers of megakaryocytes.

Nineteen cell lines displaying megakaryocytic features have been described (Table 1.1). Remarkably, almost all megakaryocytic cell lines have been established during the past 10 years. This is mainly due to the fact that specific megakaryocyte markers have been identified during this period of time. The availability of hematopoietic growth factors (cytokines) for cell culture use has also made it easier to establish cell lines. All the megakaryocytic cell lines described were derived from patients with megakaryoblastic crisis of CML, M6 or M7 (French-American-British classification) acute myelogenous leukemias.

Specific ultrastructural markers for megakaryocytes are platelet membrane glycoproteins GPIIb/IIIa, GPIb, vWF and PPO (platelet peroxidase). Table 1.1 shows that the most consistent marker for the megakaryocytic lineage is the presence of GPIIb/IIIa (CD41a, or fibrinogen receptor).

Table 1.1. Some properties of human cell lines that express megakaryocytic features

Cell line	Source	GP IIb/IIIa	GPIb	vWF	PPO	Glycophorin A	Megakaryocytic differentiation with PMA
K-562	CML blast crisis	+	-	-		+	+
HEL	M6	+	+	-		+	+
MEG-01	CML blast crisis	+	+/-	+	+	-	+
EST-IU	ANLL	+		+	+	-	+
LAMA-84	CML blast crisis	+	-	-	+	+	+
Dami	M7	+	+	+		+	+
KOPM-28	CML blast crisis	+			+/-		+
T-33	CML blast crisis	+	+	+	+/-	-	+
M-07	M7	+	+		+/-	-	
OC1M1, OC1M2	M6	+	-	-		+	+
KU812	CML blast crisis	+	-		+	+	+
CMK	M7	+	+	-	+	+	+
CHRF-288-11	M7	+		+	+	-	+
UT7	M7	+	+		+	+	+
MOLM-1	CML blast crisis	+					
MKPL-1	M7	+	-	-	-	-	
ELF-153	Acute myelofibrosis	+	+	+	-	-	+
MEG-A2	CML blast crisis	+	-	-	-	-	+
NS-MEG	CML blast crisis	+	+	-	+	+	+

GPIIb/IIIa, platelet membrane glycoprotein IIb/IIIa; GPIb, platelet membrane glycoprotein Ib; PPO, platelet peroxidase; vWF, von Willevbrand factor; PMA, phorbol myristate acetate (phorbol ester); CML, chronic myelogenous leukemia; +, positive; +/-, very weakly positive; -, negative (Modified from: Saito H., review, 1997)

Treatment of any of these cell lines with PMA results in an enhanced expression of these markers, including GPIIb/GPIIIa, PPO and some degree of polyploidy. Other antigens such as CD33, CD34 and HLA-DR (Hoffman, 1989), which are found in early hematopoietic progenitors, are not included in table 1.1. These antigens are variably expressed on the surface of many immature megakaryocytic cell lines.

The cell lines in table 1.1 seem to represent a spectrum of leukemic cells at various stages of differentiation. Each cell line has a combination of various erythroid, myeloid and megakaryocytic markers. However, it is possible to classify the 19 cell lines into 2 categories by the presence of glycophorin A. Thus, MEG-01, EST-IU, T-33, M-07, CHRF-288-11, MKPL-1, ELF-153, MEG-A2 and NS-MEG seem to belong to a megakaryocytic-specific cell line, whereas K-562, HEL, LAMA-84, Dami, OCIM1, OCIM2, KU812, CMK and UT7 are erithrocytic-megakaryocytic cell lines. This last group may originate from a common hematopoietic progenitor of both lineages (see fig 1.11). Furthermore, a close association of erythroid and megakaryocytic lineages has been suggested, as both express common transcription factors, including GATA-1 and NF-E_2 (Romeo et al., 1990). To some extent, each of these immortal cell lines are biologically different from normal megakaryocytes (Hoffman, 1989). A typical example is that they all have complex and bizarre karyotype abnormalities (Philadelphia chromosome for example). Therefore these cell lines may express a unique phenotype that is not exactly observed in the normal process of megakaryocytic differentiation and maturation.

The K-562 cell line was first considered to be earlier myeloid cells which fail to

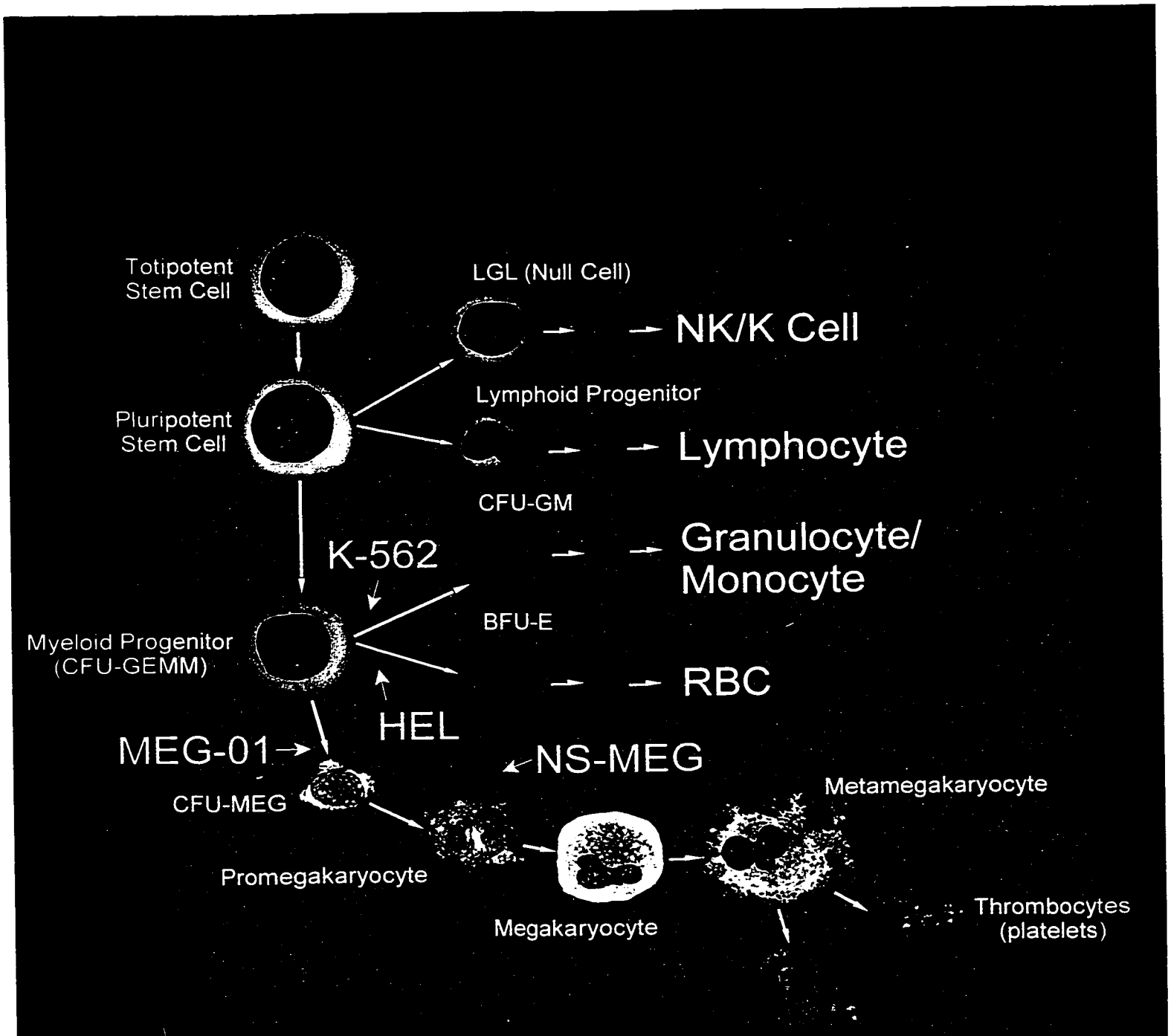

Fig. I.11: Scheme illustrating the different hematopoietic lineages and stages of development in bone marrow. In yellow are indicated the possible stages of maturation and ubication of the megakaryocytic cell lines K-562, HEL, MEG-01 and NS-MEG. (Modified from: Diggs et al., 1985)

differentiate. However, further studies have revealed that this cell line has some phenotypic markers of the erythroid and megakaryocytic lineages (Gewirtz et al., 1982). The HEL cell line also expresses some features of erythroid, macrophage (monocytic) and megakaryocytic lineages (Martin and Papayannopoulou, 1982; Tabilio et al., 1984; Long et al., 1990). Although both K-562 and HEL cell lineages have a rather weak megakaryocyte phenotype, treatment of these cells with low concentrations of PMA (10^{-8} to 10^{-7} M) leads to their commitment to the megakaryocytic lineage. PMA-stimulated HEL cells increase their size and show cytoplasmic maturation, with an increase in surface expression of several platelet-megakaryocyte-associated proteins such as GPIIb/IIIa, GPIb, vWF (von Willebrand factor) and PF4 (Platelet Factor 4). K-562 and HEL cell lines are primitive pluripotent hematopoietic cells useful for examining or studying the process of lineage determination and commitment (differentiation).

The MEG-01 cell line possess many megakaryocytic characteristics (GPIIb/IIIa, vWF and PPO) and has no markers for B or T lymphocytes, myeloid or erythroid cells, except BA-1 antigen and CD14. GPIb was not detected on the surface by immunofluorescence, but in the cytoplasm by immunoperoxidase technique (Ogura et al., 1985). The MEG-01 cell line was established from a patient with blast crisis of Philadelphia (Ph^1) chromosome-positive chronic myelogenous leukemia (Ogura et al., 1985). It has a generation time of about 36-48 hours, and possesses a hyperploid karyotype with a chromosome number of 56 to 58 (Ogura et al., 1985). PMA can stimulate MEG-01 to develop into more mature megakaryocytes, as observed by morphological and biochemical criteria (Ogura et al., 1988). Expression of

GPIIb/IIIa, vWF, fibrinogen, factor XIIIA and β-thromboglobulin (β-TG) is markedly enhanced by PMA treatment (Ogura et al., 1988). PMA treatment also induces the expression of fibronectin and factor V, which are not detected in non-treated cells, and can induce the development and budding of blebs and the multiplication of nuclei (Ogura et al., 1988). Ploidy is increased from 2N to 4N and from 4N to 8N (Ogura et al., 1988). MEG-01 cells spontaneously produce some platelet-like particles which are positively stained with anti-tubulin and GPIIb/IIIa antibodies (Takeuchi et al., 1991). The subcutaneous injection of MEG-01 cells into nude mice resulted in the establishment or formation of transplantable human megakaryoblastic tumours (MEG-01/nu). These tumours have been used to evaluate the in vivo efficacy of anti-tumour agents (Takeo et al., 1993).

1.2 (f) Cytoskeletal proteins and tumoral cells

There is evidence that the cytoskeleton plays a broad and diverse role in a number of cellular functions including cell growth regulation and signal transduction (Zigmond, 1996). Changes in cytoskeletal organization are remarkable in many tumour cells, and in particular, actin microfilaments have been shown to undergo dramatic modifications or rearrangements in tumour cells *in vitro* (Holme et al., 1986). Gelsolin is among the various proteins that bind to filamentous actin and reduce its assembly (Pollard et al., 1986).

Expression of gelsolin, scinderin and cap-G are complementary in high degree (Lueck et al., 1998), suggesting that the distinct functional properties of these proteins lead to distinct patterns of cellular expression. These are determined mainly by the motile capacity of the cell. For example, gelsolin-null cells have increased amounts of F-actin in stress fibres. They

display a five-fold increase in the expression of the Rac GTPase. Rac levels return to normal after gelsolin transfection (Azuma et al., 1998). Signalling from Rac to gelsolin was also originally identified in neutrophils (Arcaro, 1998). The release of gelsolin from actin occurs independently of PI3-kinase and phosphoinositides, and could be produced by Rac-GTP complex or other small GTPases-GTP complexes.

Another property of gelsolin is that it is a substrate of Caspase-3, a protease activated during apoptosis (Kothakota, 1997). Gelsolin is cleaved by Caspase-3 during apoptosis in various cell types, and the amino-terminal cleavage product possesses calcium-independent filamentous actin-severing activity. Expression of this amino-terminal fragment in cells leads to apoptosis. Also, gelsolin-null neutrophils progress to apoptotic cell death more slowly than their wild-type counterparts. Gelsolin, however, when over-expressed in Jurkat cells (a human T-cell line), inhibits apoptosis following stimulation with pro-apoptotic agents (Ohtsu et al., 1997). A possible explanation for this observation is that gelsolin, in complex with components such as phosphoinositides, functions as a competitive inhibitor of Caspase-3. (Azuma et al., 2000). Gelsolin expression is down-regulated in 60-90 % of tumours during carcinogenesis (Kuzumaki et al., 1998). Decreased gelsolin expression has been reported in both mouse and human Ras transformed cells *in vitro* (Mullauer et al., 1993). Taken together, this suggests that gelsolin may have tumour suppressor properties (inhibits proliferation). Also, the expression of gelsolin in normal secretory glands, but not in normal proliferative glands, may be related to the proliferative rate of these cells. Thus, normal colonic epithelial cells, which also have a high proliferation rate, were found to be negative for gelsolin

expression (Porter et al., 1993).

Gelsolin and actin both interact with components of the signal transduction pathways (Zigmond, 1996), and abnormalities or alterations in signal transduction are also a characteristic of malignant cells. An interesting finding is that gelsolin induction has also been found to correlate with differentiation in myeloid cell lines (Kwiatkowski, 1988).

1.3. Statement of the problem

Leukemic megakaryocytic cell lines (HEL, DAMI, K-562, NS-MEG, MEG-01, etc.) do not express scinderin whereas normal megakaryocytes and platelets do (Rodríguez del Castillo et al., 1991). Furthermore, it is known that megakaryocyte maturation and production of platelets are regulated by obvious changes in cytoskeletal dynamics (Tablin et al., 1990). These changes are not observed in megakaryoblastic leukemic cells; and they are unable to produce platelets. It is quite possible that cytoskeleton changes leading to platelet formation are the result of the activation of regulatory proteins such as scinderin, gelsolin, etc.

Could the introduction of scinderin, which is not expressed in megakaryocytic cell lines, decrease F-actin and disrupt the cytoskeleton? If it did, could these effects, together with the activation of signal transduction pathways, and/or other mechanisms, trigger further cellular changes leading to growth suppression, induction of differentiation and apoptosis?

The possibility of such effects prompted us to express scinderin (by transfection with specific vectors) in megakaryocytic leukemic cells in search of clues to understanding the effects of scinderin. A careful consideration of possible lines of inquiry led to the design of experiments to address the following issues:

1. Whether the expressed scinderin is functional in terms of its F-actin severing activity.

2. The effects of scinderin on F-actin content.

3. The effects of changes in F-actin on the rate of cell proliferation.

4. Any changes observed in exploring issues #1, 2 and 3 (above),would be followed up with further experiments to study the effects of scinderin on cell differentiation and maturation.

5. Any observed effects of the expression of scinderin on cell proliferation, differentiation and maturation, would lead to the investigation of the molecular mechanisms (i.e. transduction pathways) involved in scinderin's effects, the degree of cell maturation and the possible production and release of platelet-like particles.

6. Any observed production and release of platelet-like particles would be followed up by the investigation of the properties of these particles including:

   a) expression of platelet antigens; b) uptake and release of serotonin; c) microtubule distribution; d) responses to thrombin; (e.g., aggregation, serotonin release, etc.); e) ultrastructure, etc.

7. Given that megakaryocytic cell lines produce tumours when injected into nude mice, an important goal of this study would be to determine whether these cells expressing scinderin are less malignant than controls transfected with vectors only.

2 - Materials and Methods

2.1. Materials

The polyclonal anti-scinderin rabbit antiserum used was generated in Dr. Trifaro's laboratory. No epitopes were used to immunize the rabbits, but the whole recombinant scinderin protein instead. RPMI 1640 culture media, Geneticin (G-418), Lipofectamine transfection reagent was obtained from Life Technologies. PMA, Wright-Giemsa stain kit, BSA, mouse monoclonal (Mo) anti-α-tubulin antibody, mouse (Mo) anti-gelsolin and rabbit polyclonal (Po) anti-actin antibody were obtained from Sigma Chemical Co. pcDNA3 plasmid was obtained from Invitrogen Corp. Goat anti-rabbit HRP-IgG, Bradford protein reagent and PVDF membranes were obtained from Bio-Rad. Goat anti-mouse HRP-IgG and the ionophore A23187 were obtained from Calbiochem. DAPI and rhodamine- phalloidin were obtained from Molecular Probes. ECL (enhanced chemiluminesence) reagent and [γ^{32}P]ATP were obtained from Amersham. [^{3}H]-5HT was obtained from NEN Life Science. TUNEL kit and Histone H_4 were obtained from Boheringer Mainheim. Mouse (Mo) anti-CD41a was obtained from Biodesign Int. Thrombin was obtained from Chronologue Inc. Purified mouse IgG1, mouse (Mo) anti-fos and mouse (Mo) anti-ERK1 were obtained from Pharmingen. Rabbit (Po) anti -jun, rabbit (Po) anti-JNK, rabbit (Po) anti-pJNK, rabbit (Po) anti p38K and PD98059 were obtained from New England Biolabs. Rabbit (Po) anti-NFE2, rabbit (Po) anti-Rac2, rabbit (Po) anti Cdc42 and mouse (Mo) anti-RhoA were obtained from Santa Cruz Biotechnology. Mouse (Mo) anti-Ras was obtained from Oncogene Research Products. Rabbit (Po) anti-PAK was obtained from Upstate Biotechnology. Triton X-100 was obtained from J. T. Baker Chem. Co., Phillisburg, N. J. Fluoxetine was a generous gift from

Dr. Hrdina's lab., Faculty of Medicine, University of Ottawa.

2.2. Methods

2.2 (a). Suspension cultures

Cell lines (HEL, NS-MEG, K-562 and MEG-01), cultured in RPMI supplemented with 10 % fetal calf serum (FCS), were transfected, with mammalian expression constructs, using Lipofectamine according to manufacturers' specifications . Cells were then cultured in RPMI 1640 medium containing 10 % FCS in the presence of 0.8 mM G-418 (geneticin) at 37 °C in a 5 % CO_2 atmosphere. The media was renewed weekly at a proportion of 1/30 (1 part of cell suspension diluted in 30 parts of fresh medium) to keep cells in exponential growth. For experiments, cells were seeded at a number of 1 x 10^5/ml and grown in antibiotic free conditions in either 12 or 30 ml medium. Samples were taken at 4, 8, 12 or 14 days in culture (with 50 % medium replacement at day 7 when held in culture for 12 to 14 days), both attached and detached cells were included. To obtain particles, cells were harvested at day 22 of culture, with 50 % culture medium renewal at days 7 and 14.

2.2 (b). Preparation of vectors

MEG-01 cells were transfected with control vector without insert, or with vector containing Sc-cDNA. A pGEX-4T2 plasmid containing Sc-cDNA, previously prepared in our laboratory (Marcu et al., 1994) was used as a source of cDNA. After double digestion (see fig. 2.1) with the restriction enzymes BamH I and Not I, a fragment of 2.9 Kb corresponding to the entire Sc sequence (of which 2145 bp encoded for a protein with a 750 bp extension past the termination codon) was sub-cloned into pcDNA3 vector (Invitrogen Corp., San Diego, CA.).

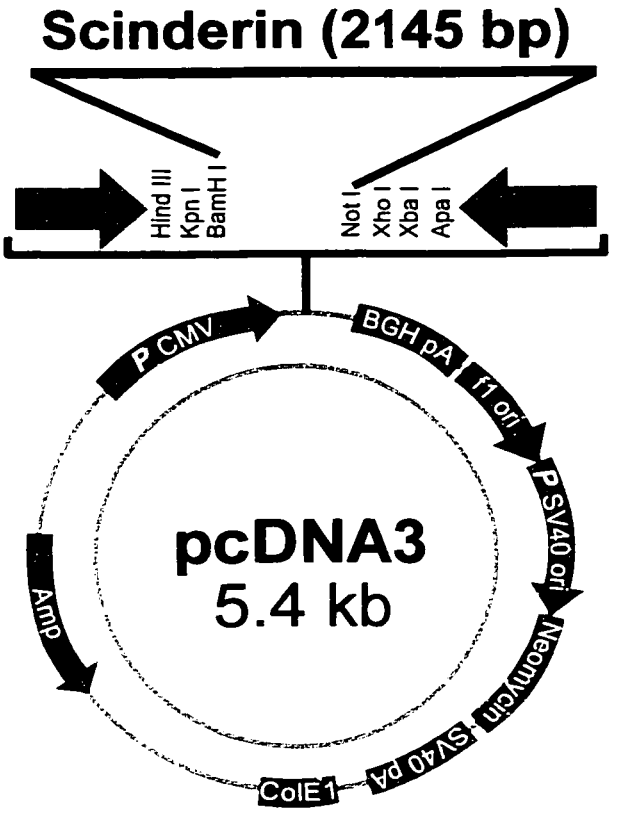

Fig. 2.1: Schematic representation of the pcDNA3 vector containing the full scinderin cDNA insert between restriction sites of BamHI and Not I on its multiple clonning site. The enhancer-promoter for high-level transcription (CMV), the polyadenylation signal and transcription termination sequence (BGH), the SV40 origin of episomal replication, the T7 and Sp6 RNA promoters flanking the multiple cloning site and the Neomycin and Ampicillin resistant-genes, among other features, are also represented in this scheme.

Orientation and insertion sites of Sc-cDNA in pcDNA3 vector were confirmed by sequencing. A preparative batch (see fig. 2.2A) of high quality cDNA (200 μg of pcDNA3-Sc) was obtained from 250 ml bacterial culture (E. Coli JM 105 strain) purified through QIAGEN columns (chromatographic principle). Plasmid DNA concentration was calculated spectrophotometrically and verified by double digestion with either Bam H I and Hind III or Bam H I and Not I that yielded fragments of 700 or 2145 bp (full insert) respectively.

2.2 (c). Transfection and generation of clones

MEG-01cells were transfected (see fig. 2.2B) with either empty vector control (pcDNA3) or Sc-pcDNA expression construct using Lipofectamine (Life Technologies) according to the manufacturers' specifications. Optimum conditions for transfection were obtained using 5 μg of plasmid DNA and 15 μl of Lipofectamine reagent added to 2 x 10^6 cells/ml in serum-free RPMI 1640 media for 5 hours at room temperature. RPMI 1640 (1 ml) supplemented with 20 % serum was added and exposure to DNA-Lipofectamine complexes continued overnight. Cells were then transferred to T75 flasks containing 30 ml RPMI 1640 containing 10 % FCS and geneticin (G-418, Life Technologies) at a concentration of 0.8 mM. Cells were grown in selection medium for 10 days in order to obtain isolated clones. Clones were selected and transferred to 96-well plates and allowed to proliferate. Western-blot analysis and immunocytochemistry were conducted to analyse expression of (exogenous scinderin) for each clone. Clones identified as positive by both methods were frozen and stored in liquid N_2 for later use. Stably transfected cells were maintained in selective medium (0.4 mM G-418 in RPMI 1640 + 10 % FCS). The optimum ratio and concentration of vector

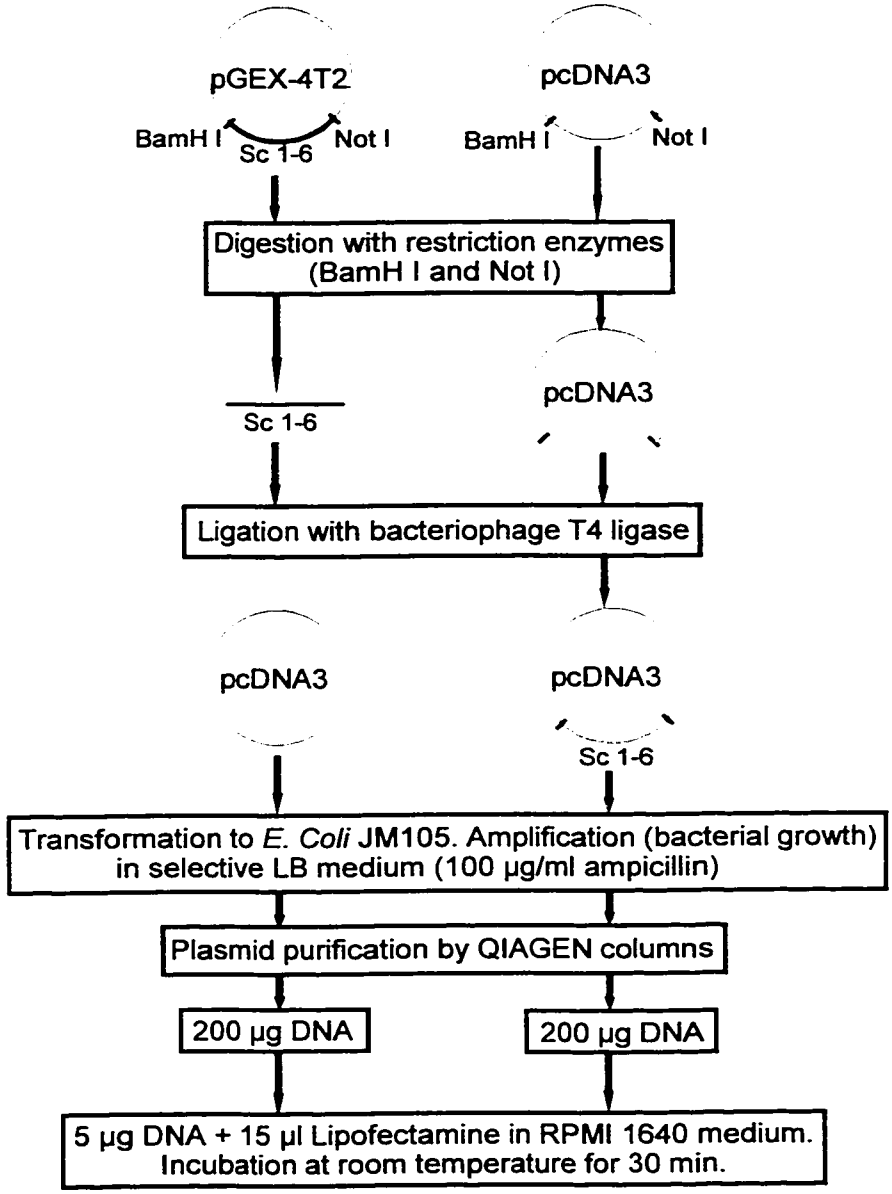

Fig. 2.2A: Scheme of the transfection procedure.

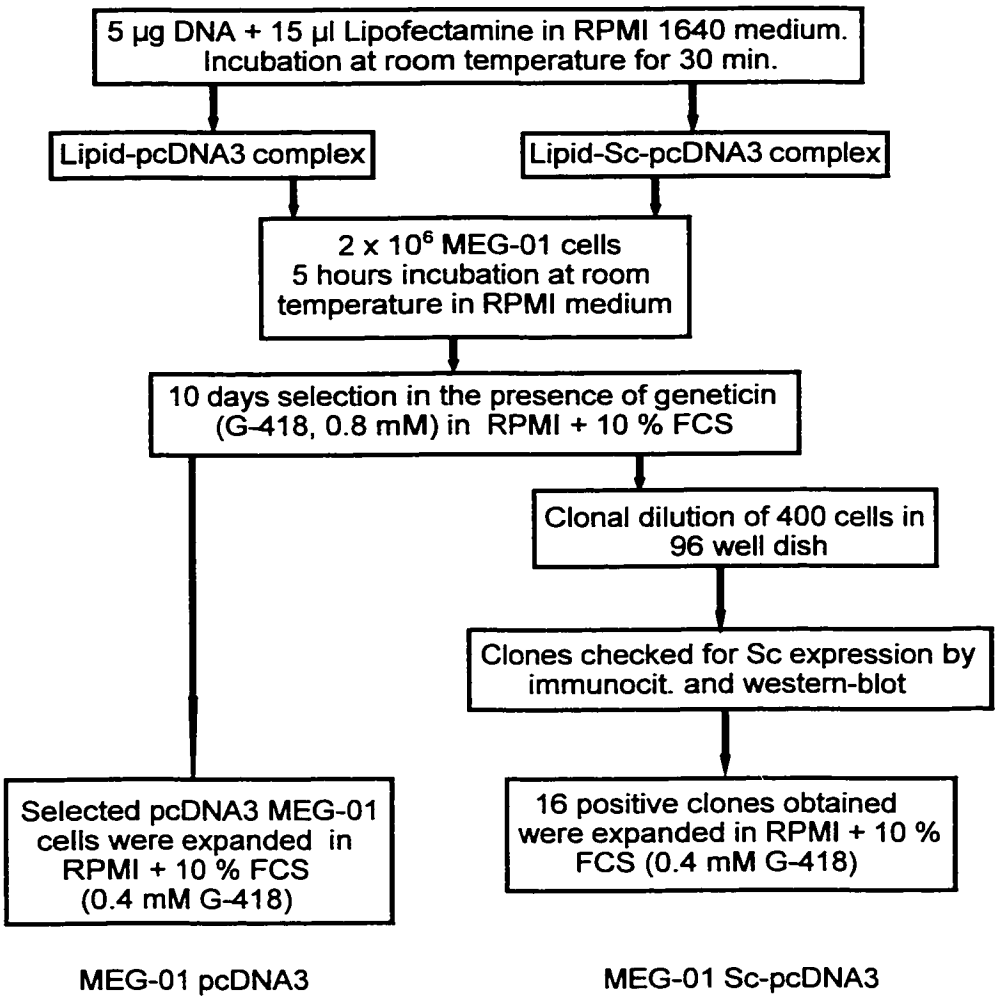

Fig. 2.2B: Scheme of the transfection procedure (continuation).

DNA/Lipofectamine were obtained using a vector containing a βGal fragment (PCMVSport βGal, Life Technologies) following the manufacturer's recommended protocol for development of blue positive cells (the best transfections were less than 3 % for Sc positive cells). Sixteen clones positive for scinderin expression with 100 % of cells expressing scinderin, were obtained, as shown by western-blot and immunocytochemistry. However, the level of scinderin expression among the clones varied and only those with high Sc expression were used in subsequent studies.

2.2 (d). Immunocytochemistry and fluorescence microscopy

Cells were cytospined onto glass slides, fixed with 3.7 % formaldehyde in Locke's solution for 20 min., then permeabilized by 100 % acetone for 1 min. F-actin was detected using rhodamine-phalloidin, a probe for filamentous actin. Scinderin was detected using polyclonal anti-scinderin rabbit antibody (1:500 dilution) previously produced in our laboratory. In some experiments, monoclonal antibodies against α-tubulin (1:200 dilution) and CD41a (1:100 dilution) were used. Secondary antibodies conjugated either to fluorescein isothiocyanate (FITC) or cyanine (Cy3) were used, and cells were washed and mounted as previously described (Lee and Trifaró, 1981). Cells mounted on coverslips were observed under incident light using a Leitz Ortholux fluorescent microscope and cells were photographed using Kodak Tri-X pan film (400 ASA). Colour pictures were taken with a Sony digital camera and digital images were aquired using Northern Eclipse software (Empix Inc.). Digital images were then imported into Adobe Photoshop 4.0 software for further processing and printed with an Epson Stylus Photo Ex colour printer (Epson American Inc.). Quantitative analysis

of rhodamine-phalloidin (F-actin) as well as cyanine (Cy3) (CD41a) fluorescent images was performed using a Hamamatsu Photonic KK Argus 50/CL image processor coupled to a TV3M Zeiss video camera. Video camera parameters (i.e. gain, offset and sensitivity) were kept constant and fluorescence intensity was expressed in arbitrary units. Apoptosis death was assessed by counting the number of fluorescent nuclei following TUNEL (terminal deoxynucleotide transferase + FITC-dUTP) labelling, according to the manufacturer's guidelines (Boehringer Manheim Corp.). Dead cells were identified by staining with 1 % trypan blue.

2.2 (e). Morphometric studies (determination of cell volumes and nuclei count in Wright-Giemsa stained preparations)

Cells were initially placed at a number of 8 x 10^5/ml RPMI 1640 + 10 % FCS and grown for either 4 or 8 days in antibiotic free (G-418) medium. Then cells were harvested, cytospined onto glass slides and stained with Wright-Giemsa. Slides were then mounted and observed under a light Leitz Ortholux microscope. The number of nuclei and nuclear lobules was determined by microscopic examination. Cell volumes were determined using microphotographs of the Wright-Giemsa stained slides. Cell diameters were measured on the printed photographs. The diameters in millimetres were converted to μm by dividing the mm values for a calculated magnification factor.

Example: Mag. Factor = Objective Mag. x Zoom Eyepiece x Tube Factor x 35 mm Camera Factor x Printing Factor

Printing Factor = Length of printed photograph/Length of negative photograph (film)

376 = 63 x 5 x 1 x 0.32 x 3.73 diameter (mm)/0.376 = diameter (μm)

Then the volume values in μm^3 were determined and histograms were constructed.

2.2 (f). Cell proliferation assay

Culture media (15 ml) were inoculated with 1 x 10^5 cells/ml and grown for 24 days. Every 3 days cells were counted using hemocytometer and 1 x 10^6 cells were removed and seeded in new flasks containing 15 ml fresh medium. The mean values of cell counts from each 3-day period were multiplied by the number obtained in the subsequent count. This product was then multiplied by the next count number and, subsequently, until the end of the experiment, in order to obtain the total number of cells grown during the entire period.

2.2 (g). Electrophoresis and immunoblotting

Cells grown in culture medium for either 4, 8, 12 or 14 days, were collected by centrifugation, washed in 1 X PBS (mM: NaCl, 137; KCl, 2.7; Na_2HPO4, 4.3; KH_2PO4, 1.4; pH 7.3) and lysed in RIPA buffer (NaCl, 140 mM; KCl, 2.6 mM; K2HPO4, 1.8 mM; NP-40, 1 %; sodium deoxycolate, 0.5 %; pH 7.2), containing 1mM PMSF, for 15 min. in ice. Samples were then centrifuged at 16.000 g for 10 min. at 4 °C. The clarified supernatants were retained and protein content/concentration was determined using the Bradford (spectrophotometric) method (BioRad). Aliquots (15 μg protein/lane) were taken and mixed with Laemmli's loading buffer (Tris-HCl, 125 mM; glycerol, 20 %; SDS, 4 %; 2β-mercaptoethanol, 10 %; bromophenol blue, 0.05 %; pH 6.8) and heated at 95 °C for 6 min. Protein samples were analyzed by monodimensional 10 % SDS-PAGE according to the method of Doucet and Trifaró (1988). Gels were prepared from stock solutions of 25 %

acrylamide and 0.25 % N,N'methylenebisacrlylamide. The final composition of the separating gel was 10 % acrylamide, 0.1 % N,N'methylenebisacrylamide, 0.4 % SDS, 5 % glycerol, 100 mM glycine and 200 mM Tris, pH 9.0. Polymerization was induced by addition of 0.1 % ammonium persulphate and 0.05 % TEMED prior to casting. Once polymerized, the stacking gel was layered on top of the separating gel. The stacking gel contained 4 % acrylamide, 0.04 % N,N'methylenebisacrylamide, 0.4 % SDS, 5 % glycerol, 4 mM EDTA and 70 mM Tris-HCl. This solution was degassed for 15 min. prior to adding 0.1 % ammonium persulfate and 0.005 % TEMED for polymerization. Once the gel was loaded with samples, tanks were loaded with running buffer (mM: Tris base, 25; glycine, 150; SDS, 0.1). Gels were run under the constant current of 25 mA/gel using a GIBCO Electrophoresis power supply (Life Technologies). Following electrophoresis, some of the gels were incubated for 30 min. with constant agitation, in Coomasie Brilliant Blue (using 0.1 % R-250 coomasie blue stain (BioRad) in 40 % methanol and 10 % acetic acid), to stain separated proteins. Gels were destained overnight in 25 % methanol and 10 % acetic acid. Gels were then dried in a Gel Slab Dryer model 224 (BioRad) for 1.5 hours. After SDS-PAGE, gels were soaked for 15 min. in electro-transferring buffer (mM: Tris-HCl, 25; glycine, 150; and 20 % methanol (v/v)). Proteins were then electro-transferred to nitrocellulose or PVDF membranes (pore size 0.45 μm, BioRad) at 90 V for 1.5 hours in an LKB 2005 transfer electro-blotting unit. Membranes were blocked for 1 hour in 2 % skim milk powder in PBS, then immunoblotted with the appropriate antibodies in 2 % milk-PBS. Membranes were also blotted with mouse monoclonal anti-α-tubulin (1/2000) to ensure equivalent amount of

protein loaded per lane. After 3 washes with 0.05 % Tween 20 in PBS, the membranes were blotted with either 1/2000 or 1/5000 dilutions in 2 % milk in PBS of either anti-mouse or anti-rabbit HRP conjugated secondary antibodies, for another hour at room temperature. Membranes were washed three more times with PBS-0.05 % Tween 20.

Protein bands were then visualized using Enhanced Chemo-Luminescence (ECL) reagent (Amersham). The intensity of fluorogram bands was measured using Scion Beta Image software (Scion Corp.). Areas under peaks were integrated using the same program and results were expressed in arbitrary units as ratios to tubulin bands intensity (gel loading control). For the differentiation experiment (PMA) cells were treated with 10 nM PMA for 5 days, then harvested, subjected to protein extraction, electrophoresed and blotted using the procedures described above. For actin distribution and quantification in Triton detergent extracts, cultured cells were treated with 1 % Triton X-100 in ice for 15 min., and the Triton extracts underwent the procedures described above.

2.2 (h). PAK kinase activity assay

Cells cultured for 14 days were harvested, washed with PBS and lysed in RIPA buffer [mM: Tris-HCl, 50 (pH 7.4); NaCl, 150; EDTA, 1; PMSF, 1, Na_3VO_4, 1; NaF, 1; Aprotinin, leupeptin, pepstatin, 1 µg/ml each; Na-deoxycholate, 0.25%; pH 7.2] for 15 min. at 4 °C for protein extraction. Protein contents were determined, as previously described, on aliquots of the RIPA extracts by the Bradford method. Protein aliquots were mixed with SDS loading buffer, loaded onto polyacrylamide gels prepared with 160 µg/ml Histone H_4 (a substrate for PAK kinase activity) and resolved by electrophoresis. Gels were incubated in 2 changes of

washing buffer for 1 hour each [50 mM Tris (pH 7.4)/5mM β-mercaptoethanol] containing 20 % isopropanol at room temperature. Gels were then denatured by incubating with 2 changes of 6 M guanidine HCl in washing buffer for 1 hour each, followed by re-naturation by incubating gels overnight in 0.04 % Tween 20 in washing buffer at 4 °C. Re-natured gels were equilibrated in kinase reaction buffer [40 mM Hepes (pH 7.4): 5 mM $MgCl_2$: 2 mM dithiothreitol: 0.1 mM EGTA] for 10 min. at room temperature. The kinase reaction was initiated by the addition of 25 μCi of [γ^{32} P]ATP/ml kinase buffer, at room temperature for 60 min (Rudel and Bokoch, 1997). The reaction was stopped by washing gels with at least 10 changes of 1 % pyrophosphate in 5 % trichloroacetic acid. Gels were then dried and subjected to autoradiography (Rudel and Bokoch, 1997).

2.2 (i). Incorporation of thymidine

Cells previously grown for 4 days were resuspended in culture medium at 25,000/tube/200 μl in microtiter tubes, and incubated overnight at 37 °C. Fifty microliters of [^{3}H] (tritiated) thymidine (20 μCi/ml) were added to each tube and cell suspensions were incubated for 1 hour at room temperature. The reaction was then stopped with ice cold PBS containing 1 mM EDTA. The contents were centrifuged and the sediments washed 3 times with PBS, lysed with 10 % TCA and assayed for radioactivity in a liquid scintillation counter (Congote et al., 1989).

2.2 (j). Isolation of particles and techniques used for their characterization

Scinderin-expressing MEG-01 clones were cultured until cells entered apoptosis and released cytoplasmic particles (19-22 days in culture). Samples were collected by centrifugation (150

x g , 15 min.) and retained supernatants were further centrifuged at 750 x g for 15 min. Supernatants clarified were centrifuged at 1,600 x g for 15 min and sediments containing platelet-like particles were resuspended in small volumes of culture medium. For serotonin-uptake studies, particles were labelled (Yang et al., 1996) by incubation with 0.6 nmol [^{3}H]5-HT/ml in Ca^{++}-free Locke's with or without 2 µg/ml Fluoxetine, at 37 °C for 1 hour. The reaction was stopped with PBS, samples were centrifuged, washed again in PBS and re-centrifuged 3 more times. Finally, the particles were lysed with 10 % TCA, heated at 80 °C, and assayed for radioactivity by scintillation counting. For serotonin release studies, particles were labelled (Marcu et al., 1996) with 0.6 nmol [^{3}H]5-HT/ml culture medium for 1 hour. After 6 washes, particles were stimulated with 1 U/ml thrombin for 3 min. The reaction was then stopped by addition of 6 % glutaraldehyde, the contents centrifuged and the supernatants recovered and assayed for radioactivity in a liquid scintillation counter. For aggregation tests, particles were suspended and incubated in filtered human plasma at room temperature for 5 min., then centrifuged and resuspended in Locke's solution (mM: NaCl, 154; KCl, 2.6; K_2HPO4, 2.14; KH_2PO4, 0.85; $MgCl_2$, 1.2; glucose, 10; and $CaCl_2$, 2.2; pH 7.2). The particles' suspensions were placed in a whole blood dual chamber aggregometer (Chronolog Inc.), and stimulated with 1 U/ml thrombin while being stirred. Transmission was recorded in a Chronolog chart recorder model number 707 (Chronolog Inc.). For aggregation tests in the presence of anti-CD41a antibody, the same procedure was followed except that particles were pre-incubated overnight at 4 °C in culture medium containing 1 µg/ml of either mouse (Mo) anti-CD41a or a non-specific IgG. Also the solutions used for the aggregation tests

(human plasma for incubation and regular Locke's for aggregation) contained either the monoclonal antibody or the non-specific IgG. Electron microscopy studies of the particles were performed by incubation (Yang et al., 1996) of particles for 120 min with 1 mM serotonin followed by a series of steps that included: 3 hours of 4 % glutaraldehyde fixation, 2 washes of 5 min with 0.1 M cacodylate buffer, 1 hour post fixation with OsO_4, 2 washes of 5 min with cacodylate buffer, 30 min block with uranyl acetate, 2 washes of 5 min. with distilled water; a series of dehydration steps with 50 % alcohol for 5 min., 70 % for 5 min., 95 % for 8 min., 2 steps of 100 % for 8 min., 1 step of 100 % alcohol/propylene oxide for 5 min. followed by 2 steps of propylene oxide for 5 min. and finally 1 step of propylene oxide/epon mix for 1 hour (technique developed at the Electron Microscopy Laboratory of the Ottawa General Hospital, Ottawa, Ontario, Canada). Later the epon-embebed preparations were cut into micro-slices which were mounted in copper grids and examined with an electron microscope. Microphotographs were taken, developed and printed with a final magnification of 35625 x.

2.2 (k). Experiments on nude mice

Balb/c nude mice were obtained from Charles River Canada (St. Constant, QC), housed at room temperature (26-28 °C) in sterile polycarbonate micro-isolators. Mice were fed with 18 % Charles River autoclavable Agway rodent chow and acidified/autoclaved water *at libitum*. After acclimatisation for 5 days, mice were injected once in their abdominal flank, subcutaneously, with 100 µl saline containing 10^7 cells from 14 day cultures. Tumour growth was determined by measuring the smallest and largest tumour diameters with a caliber, and

volumes were calculated according to standard procedures. Animals with large tumours were sacrificed following institutional animal care policies. Tumours were dissected from mice and fixed in 3.7 % formaldehyde in PBS at room temperature. Preparations were then processed for micro-slicing by dehydration with xylene (3 x 2 min. each), followed by fixation in 95 % alcohol, then rinsed in tap water. Slices were stained with Mayer's hematoxilin (hematoxilin, 1.0 g; $NaIO_3$, 0.2 g; $(NH_4)_2SO_4$, 50.0 g; citric acid, 1.0 g; chloral hydrate, 50.0 g; distilled water, 1000 ml), followed by several dips in tap water. Later tinction was enhanced by several dips in 0.25 % ammonia water until blue colour was visible. After 2 changes in tap water, eosin solution was applied (10 to 20 dips), followed by 10 to 20 changes in 95 % alcohol, 3 changes in absolute alcohol and 3 changes in xylene (Lillie and Fullmer, 1976). Finally micro-slices were mounted with permount and assayed by light microscopy.

2.2 (l). Statistical analysis

Mean and standard error values for the data yielded by experiments were calculated and plotted using Sigma Plot software (version 5). Significant differences among the results were determined with the Student-t test (paired or unpaired), using Slide Write Software (Advanced Graphic Software Inc.). When more than two groups were compared and/or multiple comparisons were performed, the Anova test was used (Corel Quattro Pro, Corel Corp.). The word "significant" refers to a statistical difference with "p" values less than 0.05 ($p < 0.05$), 0.01 ($p < 0.01$) or 0.001 ($p < 0.001$).

3 - Results

3 - Results

3.1. Expression of scinderin in megakaryocytic cell lines

3.1 (a). Expression of scinderin in the MEG-01 cell line

Two megakaryocytic cell lines (K-562 and MEG-01) which do not express scinderin (fig. 3.1), even after PMA treatment, were transfected with pcDNA3 vector alone (control) or the same vector carrying full length scinderin (Sc), cDNA (pcDNA3-Sc), as indicated in Materials and Methods (good transfection results were obtained with both cell lines). However, because cell line K-562 can be induced to show characteristics of the erythroid lineage (Niitsu et al., 1996; Rowley et al., 1981), whereas the MEG-01 cell line can only show properties corresponding to the megakaryocytic lineage, the latter cell line was selected for the experiments.

Following transfection of MEG-01 cells with pcDNA3-Sc and in order to obtain high scinderin-expressing MEG-01 clones, cloning dilution and 3 passes were performed. Sixteen MEG-01 clones expressing scinderin were obtained as demonstrated by immunoblotting and immunocytochemistry with scinderin antibodies (fig. 3.2 *a* and *b* and fig. 3.3 *c* and *d*). In these clones, 100 % of the cells expressed scinderin (fig 3.3 *c* and *d*).

3.1 (b). Gelsolin levels in MEG-01 clones expressing scinderin

Gelsolin is another F-actin-severing protein present in these cells (Kwiatowski, review, 1999). We proceeded to determine its expression in scinderin-positive clones. MEG-01 cells expressing scinderin showed a 40 % decrease in gelsolin levels after 14 days in culture (fig. 3.4).

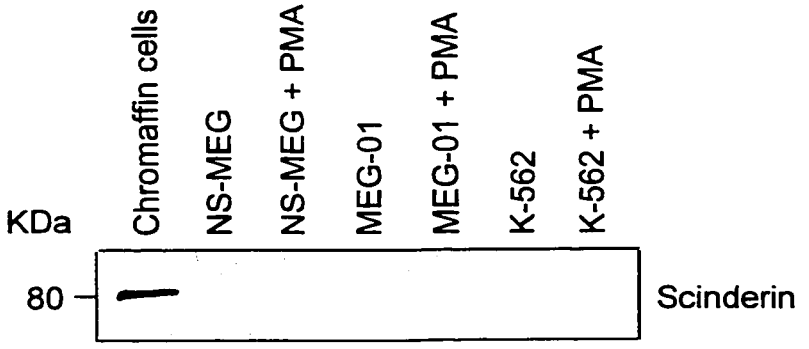

Fig. 3.1: Expression of scinderin in chromaffin cells (positive control) and megakaryoblastic leukemia cell lines NS-MEG, MEG-01 and K-562, in the absence or presence of phorbol ester (PMA). Megakaryocytic cell lines were treated for 5 days with 1 x 10^{-8} M PMA. After the incubation period, untreated (control) and PMA treated cell lines were resuspended in SDS loading buffer and preparations containing proteins corresponding to 50,000 cells were applied to each gel well. Western-Blots with an antibody against recombinant scinderin (1:1,000 dilution) were performed using as a second antibody HRP-conj. anti-rabbit IgG. Blots were developed using an enhanced chemiluminiscence (ECL) method (see Materials and Methods). Under these experimental conditions, no scinderin was detected in any of the cell lines tested.

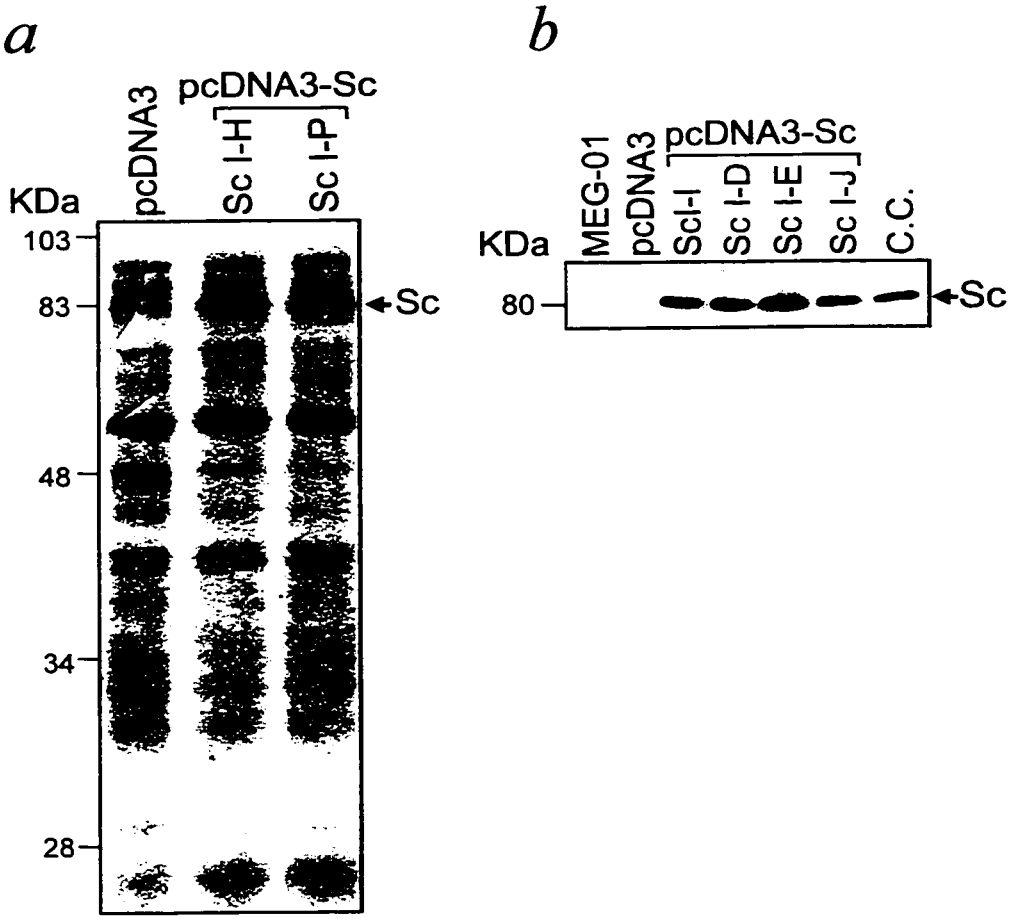

Fig. 3.2: Expression of scinderin in MEG-01 clones. *a*) A Coomasie Blue stained PAGE-SDS gel shows the scinderin band for 2 of the 16 scinderin expressing clones (PcDNA3-Sc I-J and I-P). The scinderin band was not detected in pcDNA3 transfected cells. *b*) Western-blot with polyclonal antiserum raised against recombinant scinderin. Lanes from left to right show: MEG-01wild type cells, pcDNA3 transfected cells, 4 pcDNA3-Sc transfected clones and (C.C.) chromaffin cells (positive control).

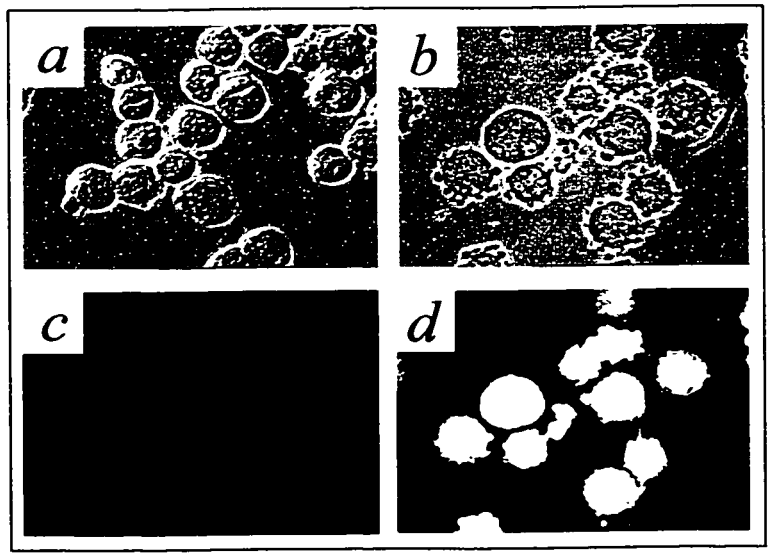

Fig. 3.3: Immunostaining of MEG-01 cells with scinderin antibody: *a*, Phase contrast , and *c*, immunostaining of pcDNA3-transfected cells. *b*, Phase contrast, and *d*, immunostaining of pcDNA3-Sc transfected cells. Following transfection and clonal selection for pcDNA3-Sc cells, cells were grown for 4 days in the absence of G-418. Cells were then harvested, cytospined onto glass slides, stained with scinderin antibody (dil. 1/500), followed by FITC-conj.-antirabbit IgG as second antibody. Preparations were examined by incident fluorescent light (1000 X).

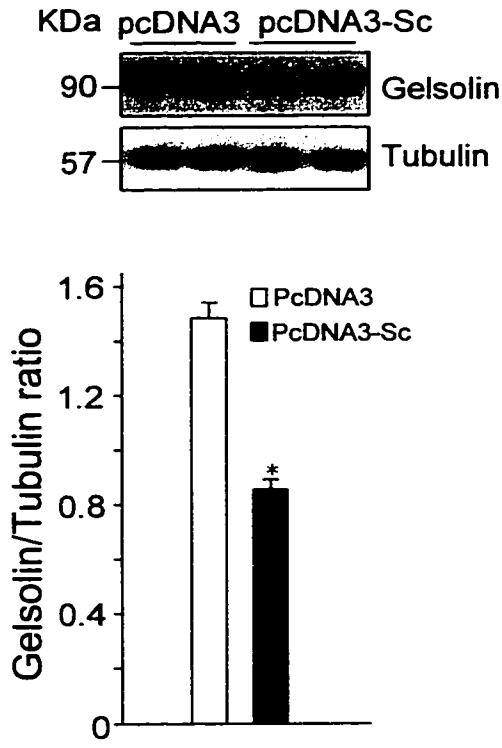

Fig. 3.4: Expression of gelsolin in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc. Cells grown for 14 days in free G-418 culture medium were harvested, washed and lysed to extract proteins. Aliquotes of protein extracts were mixed with loading buffer and resolved by SDS-PAGE. Proteins were transfered to PVDF membranes and blotted with rabbit (Po) gelsolin antibody, followed by HRP-conj.-antirabbit IgG as secondary antibody. Blots were developed using ECL chemiluminiscence method and the results were quantified by scanning densitometry. Tubulin levels detected by a polyclonal antibody were used as control for gel loading.
Bars represent mean ± S.E.M. (n = 4). * $p < 0.001$.

3.2. Characteristics of cells over-expressing scinderin

3.2 (a). Cell volume

Cells expressing scinderin had larger volumes (10.750 ± 341 μm^3; n = 720) when compared to those cells transfected with the pcDNA3 vector alone (4.700 ± 102 μm^3, n = 690) (fig 3.5). The volume distribution of the 2 populations of cells was very different, with cells transfected with vector having more uniform volumes than scinderin-expressing cells, which in addition showed a large volume dispersion (fig. 3.5). Scinderin-positive cells were not only bigger, but also entered endomitosis showing either a hyper-lobulated nucleus or several nuclei (fig. 3.6).

3.2 (b). Cell proliferation

The increased polyploidization observed (fig. 3.6) was also accompanied by a significant decrease in [^{3}H]-thymidine incorporation (fig. 3.7). However, decreased thymidine utilization in cells undergoing endomitosis (DNA replication without mitosis) is not a good indication of a decreased cell proliferation. A much better indication of cell proliferation is obtained by measuring cell number. When this was done a marked reduction was observed in the number of cells in clones expressing scinderin (fig. 3.8). The numbers of scinderin-positive cells were 21 and 9 % of the numbers of cells transfected with the vector alone after 12 and 24 days in culture respectively (fig. 3.9). Cells transfected with the vector alone had the same proliferation rate as the wild-type cells . In all cases, the rates of proliferation observed were exponential.

3.2 (c). Cell morphology

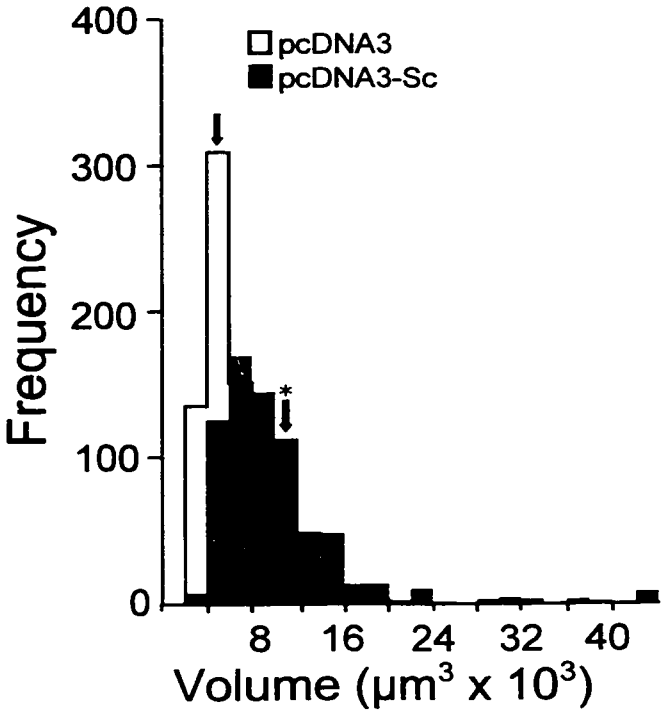

Fig. 3.5: Effect of scinderin expression on MEG-01 cell volume. Cells transfected with either pcDNA3 or pcDNA3-Sc were cultured for 8 days following removal of antibiotic G-418. Cells were then fixed and stained with Wright-Giemsa and volumes of pcDNA3 transfected cells (n = 690 cells) and of pcDNA3-Sc transfected cells (n = 720) were measured. Volumes were calculated by measuring cell diameters. Arrows indicate mean volumes corresponding to the 2 populations of cells. * $p < 0.001$.

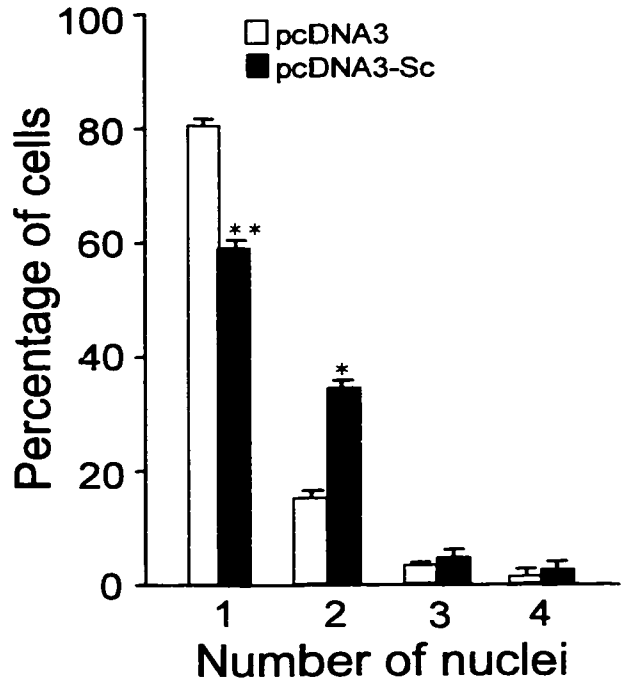

Fig. 3.6: Effect of scinderin expression in nucleus number and nuclear lobulation. Cells transfected with either pcDNA3 or pcDNA3-Sc were cultured for 8 days following removal of antibiotic G-418. Cells were then fixed and stained with Wright-Giemsa and the number of nuclei and/or nuclear lobules for pcDNA3 (n = 1000 cells) and pcDNA3-Sc (n = 1000) were determined. Bars represent mean ± S.E.M. of 4 slides examined for each cell type. Two hundred and fifty cells were examined per slide. ** $p < 0.01$. * $p < 0.001$.

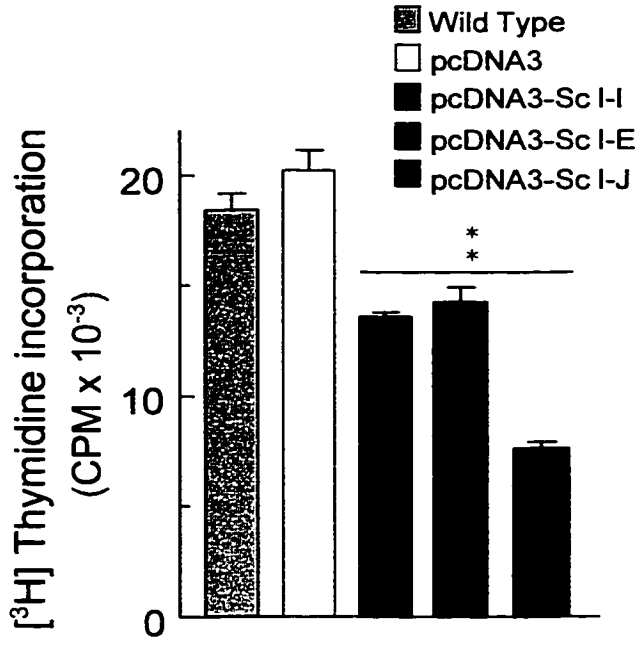

Fig. 3.7: Effect of scinderin expression on [^{3}H]thymidine incorporation. Cells transfected with either pcDNA3 or pcDNA3-Sc (clones I-I, I-E and I-J), and wild type cells, were cultured for 8 days in RPMI + 10 % FCS, in the absence of G-418. Cells (25,000/tube/200 µl medium) were then cultured with 50 µl of [^{3}H]thymidine (20 µCi/ml) for 1 hour at room temperature. The radioactivity incorporated into the cells ([^{3}H]thymidine) was measured in the TCA precipitates. Bars represent mean ± S.E.M. from 4 different experiments. * $p < 0.001$ (Anova test).

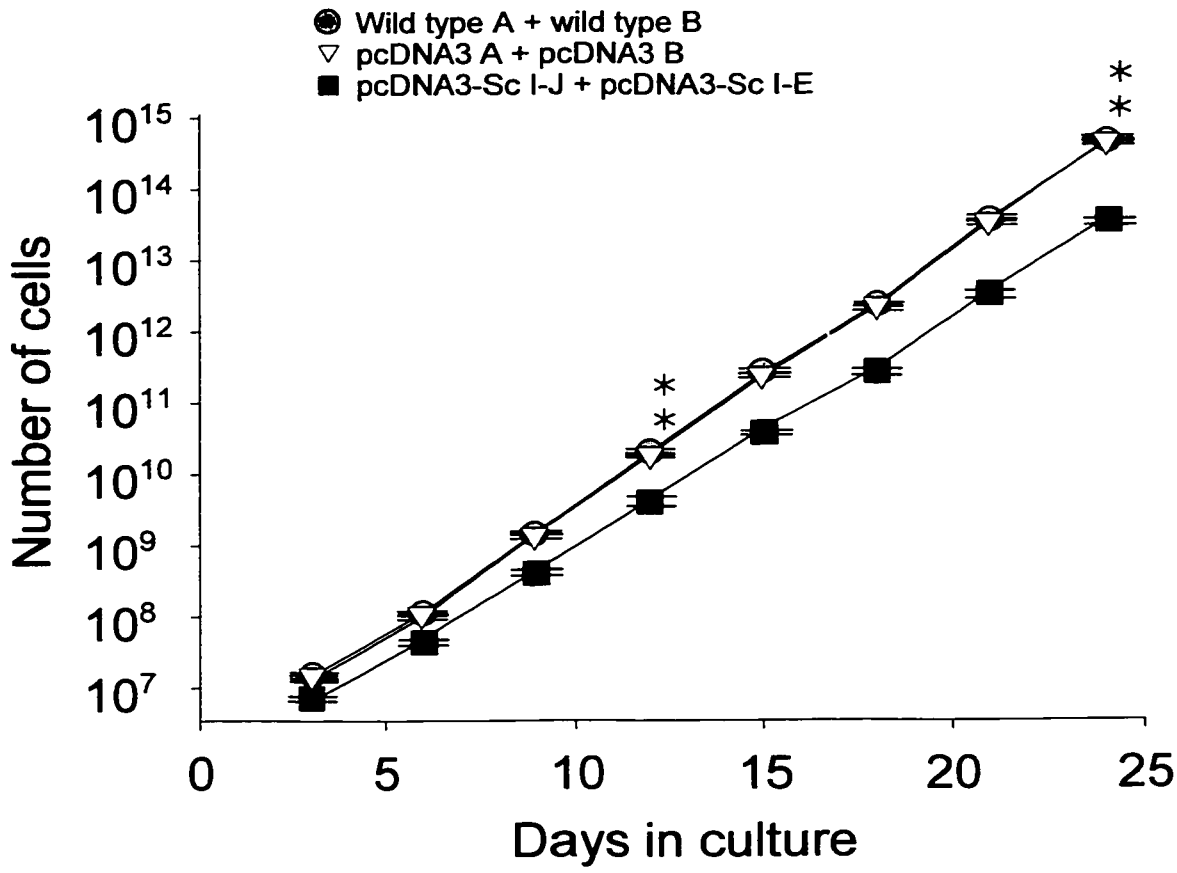

Fig. 3.8: MEG-01 cell proliferation: 1 x 10^6 cells of each cell type were placed in 15 ml RPMI 1640 + 10 % FCS (no G-418) and grown for 24 days. Cell number was determined every 3 days. Values represent mean ± S.E.M. of 6 determinations. * $p < 0.001$ (Anova test).

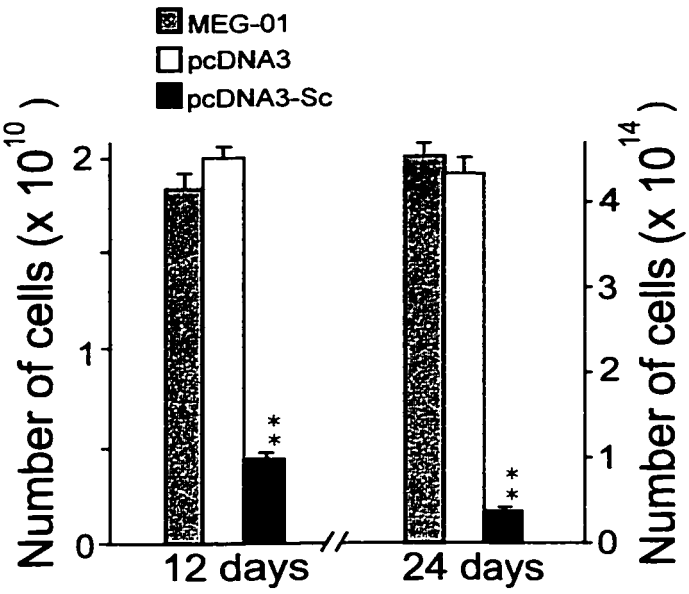

Fig. 3.9: Number of cells after 12 and 24 days in culture. Bars represent mean ± S.E.M. of 6 different experiments. * $p < 0.001$ (Anova test).

In addition to large volumes, cells expressing scinderin display surface morphological changes consisting of protoplasmic extensions with the appearance of beads (fig. 3.10 *b* and *c*). Although protoplasmic extensions were observed in some wild-type cells, as well as in the cells transfected with the vector alone (20.5 ± 3.1 %, n = 500 cells) (fig. 3.10 *a*), most of the scinderin-positive cells displayed extensions (85.6 ± 1.2 %, n = 500). Moreover, the number of extensions per cell was several times greater in cells expressing scinderin (fig. 3.10 *c*). Their protoplasmic extensions also contained scinderin and filamentous actin (fig. 3.10 *d* and *e*).

3.3. F-actin cytoskeleton in cells expressing scinderin

3.3 (a). F-actin content

Scinderin is a filamentous actin-severing-and-capping protein (Rodriguez del Castillo et al., 1990; Marcu et al., 1994), therefore its expression may induce changes in the content and distribution of F-actin. Immunocytochemistry experiments with scinderin antibodies and rodhamine-phalloidin (a probe for filamentous actin) showed some degree of co-localization for the two proteins in scinderin-positive cells (fig. 3.10 *d* and *e* and 3.11). It was also evident from these experiments that the intensity of F-actin fluorescence in vector transfected cells was greater than in scinderin-positive cells, suggesting, in this case, a decrease in filamentous actin, as indicated by image and western-blot analysis (figs. 3.11, 3.12 and 3.13). Differences in fluorescence were apparent when intensity was expressed either per cell or per surface square micron (fig. 3.12). F-actin fluorescence intensity was further and significantly

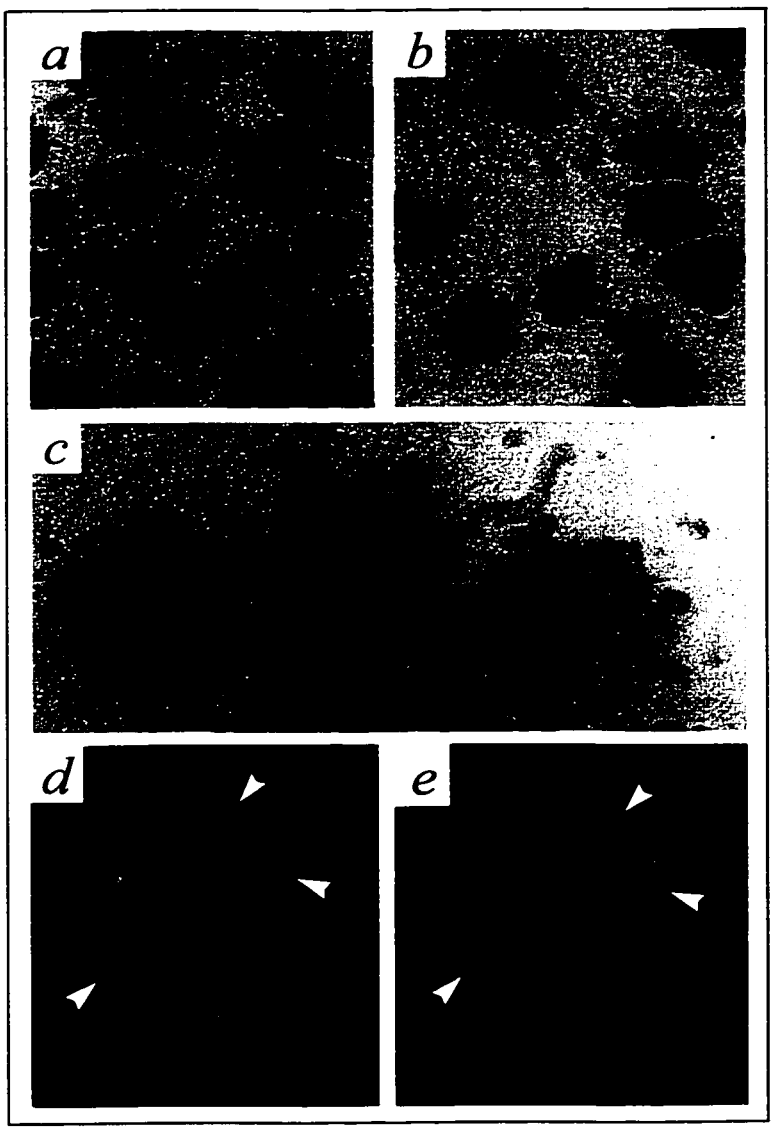

Fig. 3.10: Effect of scinderin expression on MEG-01 cell morphology. Cells transfected with either pcDNA3 or pcDNA3-Sc were cultured for 8 days following removal of antibiotic G-418. *a-c*, Cells were fixed and stained with Wright-Giemsa. Cells were also fixed and stained with either rhodamine-phalloidin, a probe for F-actin (*d*), or a scinderin antibody (*e*). Cells transfected with pcDNA3 (*a*) showed a large single nucleus surrounded by a thin layer of cytoplasm (x 400). *b*, Cells expressing scinderin (pcDNA3-Sc) were much larger multinucleated or with multilobulated nuclei, abundant cytoplasm and with numerous cytoplasmic extensions (x 400). *c*, Same as (*b*) at higher magnification (x 1000). *d*, Distribution of scinderin and (*e*) filamentous actin in a double stained cell (x 1200). There was some degree of correlation between the two markers, especially in the cytoplasmic extensions (arrowheads).

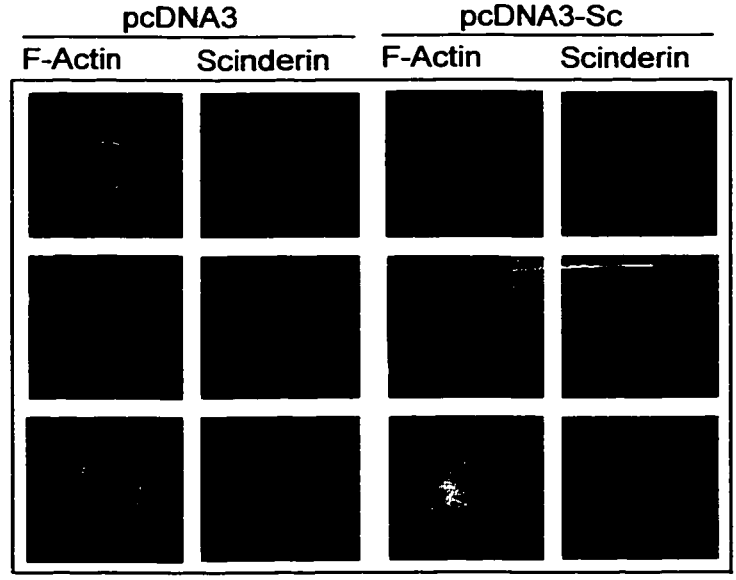

Fig. 3.11: Effect of scinderin expression on filamentous actin content in MEG-01 cells. Fluorescence microscopy images of cells stained with scinderin (Sc) antibodies and rhodamine-phalloidin (a probe for F-actin) following transfection with either vector pcDNA3 (2 left panels) or scinderin carrying vector pcDNA3-Sc (2 right panels). Cells were cultured for 4 days in RPMI + 10 % FCS, in the absence of G-418. Cells were then cytospined onto slides, fixed, permeabilized and stained with rabbit (Po) Sc antibody (1:200), followed by anti-rabbit FITC-IgG as secondary antibody. Cells were also counterstained with rhodamine-phalloidin (1.3 U/ml), and they were finally mounted in glycerol:PBS (1:1; pH 8).

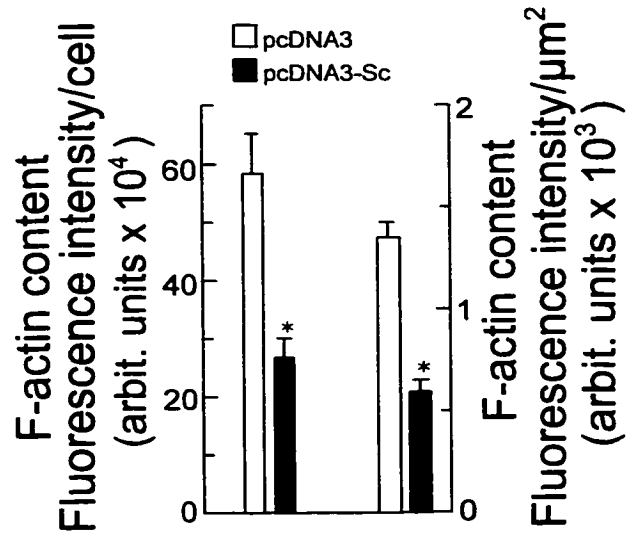

Fig. 3.12: Effect of scinderin expression on MEG-01 cell filamentous actin content. Image analysis quantification of the fluorescence intensity of the cells shown in Fig. 3.11. Quantification of their fluorescence intensity was done with an Argus-50 program. Bars represent the mean ± S.E.M. of 12 preparations analysed for pcDNA3 transfected cells, and 18 preparations (6 from each clone Sc I-I, I-J and I-D) for pcDNA3-Sc transfected cells, each preparation containing 10 to 28 cells. * $p < 0.001$.

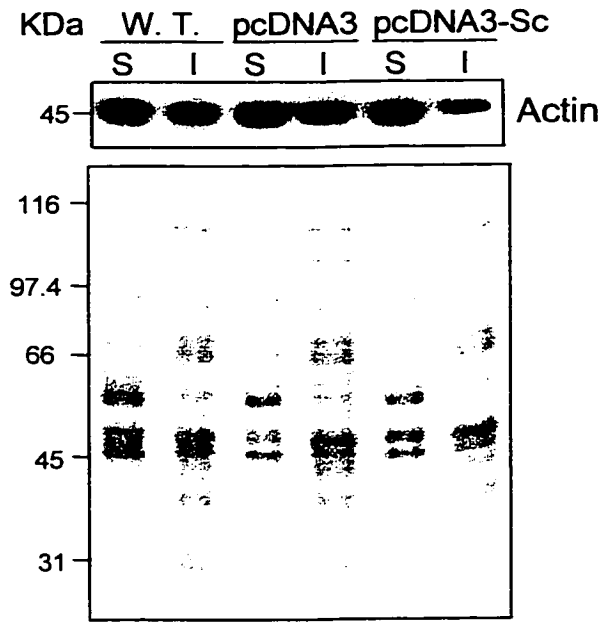

Fig. 3.13: Effect of scinderin expression on MEG-01 cell filamentous actin content. Western-blot carried out with an actin antibody on supernatants (S) and sediments (I) of Triton X-100 extracts prepared from wild type MEG-01 cells (W.T.) and from cells transfected with either pcDNA3 or pcDNA3-Sc. A Coomasie Blue stained gel shows equal amounts of proteins loaded for the different samples.

reduced in scinderin-positive cells upon treatment for 2 minutes with 2 μM of Ca^{++} ionophore A23187 (fig 3.14 and fig. 3.15). A23187 is a carboxylic acid antibiotic that acts as a free mobile carrier to transport Ca^{++} and Mg^{++} from an aqueous medium buffered at pH 7.4 into a bulk organic phase. Cells transfected with vector alone were refractory to this treatment (figs. 3.14 and 3.15), and although all cells expressed gelsolin, the concentration of ionophore was probably high enough to stimulate the disassembly of actin only in cells expressing scinderin (fig. 3.15).

3.4. Expression of platelet markers

3.4 (a). Serotonin uptake system

It has been demonstrated that MEG-01 cells display a serotonin uptake system (Fedorko et al., 1977; Yang et al., 1996). MEG-01 cells have the capacity to take up, store and metabolize serotonin. This uptake of serotonin is accomplished, to a great extent, by specific transporters, as was demonstrated by a 28 % inhibition obtained by pre-treatment with 10 nM fluoxetine (a specific serotonin transporter blocker) (Yang et al., 1996).

MEG-01 cells transfected with vector alone showed serotonin uptake, but the capacity to take up serotonin was larger in scinderin-positive cells (fig. 3.16).

3.4 (b). Expression of glycoprotein IIb/IIIa complex or fibrinogen receptor (CD41a antigen)

Antigen CD41a is another platelet marker expressed in platelets (Phillips et al., 1988), normal megakaryocytes (Nurden et al., 1997) and MEG-01 cells (Ogura et al., 1985). This antigen is also known as glycoprotein IIb/IIIa or fibrinogen receptor. GPIIb/IIIa is a Ca^{++}-

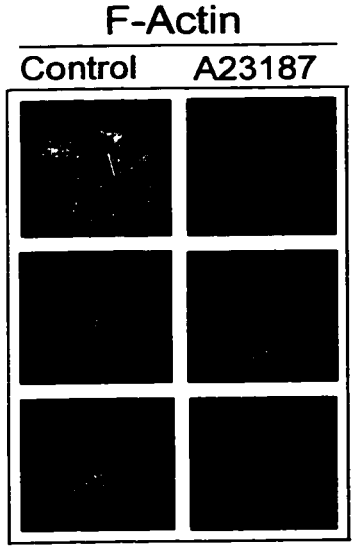

Fig. 3.14: Effect of ionophore A23187 treatment on MEG-01 cell filamentous actin content. F-actin fluorescence of a sample of pcDNA3-Sc transfected cells, incubated for 2 minutes in the absence (control) or presence of 2 μM ionophore A23187. Cells were then fixed, permeabilized and stained with rhodamine-phalloidin, as indicated in Materials and Methods (x 630).

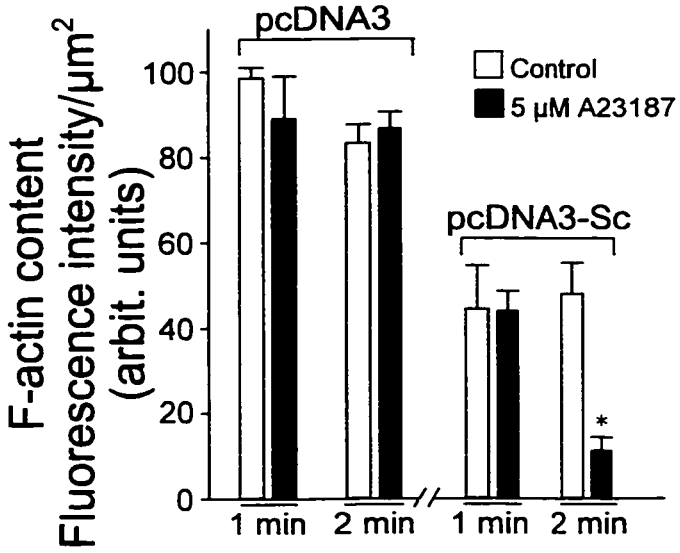

Fig. 3.15: Effect of the ionophore A23187 on MEG-01 cell filamentous actin content. Cells were incubated for either 1 or 2 min. in the absence (control) or prescence of 2 µM ionophore A23187. Cells were then fixed, permeabilized and stained with rhodamine-phalloidin and the F-actin fluorescence intensity was quantified by image analysis (Argus-50 program). Bars represent mean ± S.E.M. of 4 to 5 preparations containing 10 to 34 cells each. * $p < 0.05$ (Anova test).

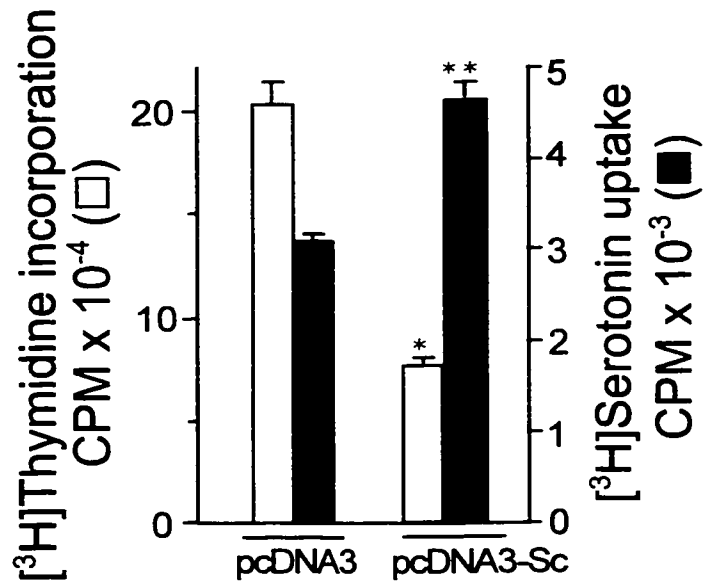

Fig. 3.16: Effect of scinderin expression on [^{3}H]thymidine incorporation and [^{3}H]serotonin uptake. pcDNA3 and pcDNA3-Sc transfected MEG-01 cells were tested for their ability to incorporate [^{3}H] thymidine (open bars) and the ability to take up [^{3}H] serotonin (solid bars). For the [^{3}H] thymidine incorporation experiments, cells were grown for 48 days in RPMI + 10 % FCS in the absence of G-418. Cells (25,000/tube/250 µl medium) were then cultured with 50 µl of [^{3}H] thymidine (20 µCi/ml) for 1 hour at room temperature. Isotope's incorporation was quantified in TCA precipitates by liquid scintillation spectrometry. For [^{3}H] serotonin uptake experiments, cells were grown for 4 days in RPMI + 10 % FCS in the absence of G-418. Cells were then incubated (1 x 10^6 cells/tube)with 1 x 10^{-8} M [^{3}H]5-HT for 1 hour. Cells were then washed and lysed with 10 % TCA. Isotope's incorporation was quantified in the supernatants by liquid scintillation spectrometry. Bars represent mean ± S.E.M. of 4 determinations. ** $p < 0.01$. * $p < 0.001$.

dependent hetero-dimer that, on activated platelets, can bind one of four different adhesive proteins (fibrinogen, fibronectin, von Willebrand factor and vitronectin). The binding of fibrinogen is required for platelet aggregation (Phillips et al., 1988).

Scinderin-positive MEG-01 cells showed an increase in the expression of this receptor, as demonstrated by immunofluorescence with monoclonal antibodies followed by image analysis (figs. 3.17 and 3.18). The increase observed in fluorescence was more than double for scinderin-expressing cells when compared to cells transfected with vector. This was the case when results were expressed either per cell or per surface square micron. However, the difference in fluoresence was less marked when expressed as intensity per surface square micron, because scinderin-expressing cells are bigger than control cells (fig. 3.18).

The expression of CD41a (GPIIb/IIIa) seems to be mediated through activation of the ERK/MAP kinase pathway (Racke et al., 1997). Compound PD98059 prevents activation of the MAP kinase ERK_1 by upstream kinases, which results in the inhibition of the ERK/MAP kinase pathway (Dudley et al., 1995). Therefore, treatment with 75 μM PD98059 for either 4 or 8 days produced a significant inhibition in the expression of CD41a in scinderin-positive MEG-01 cells. The level of CD41a expression in these cells was reduced by the compound to that found in cells transfected with the vector alone (fig. 3.19). PD98059 also reduced cell volumes and the numbers of cytoplasmic extensions (figs. 3.20 and 3.21). All this was accompanied by a reduction in the expression of ERK_1 as demonstrated by immunoblotting (fig. 3.22).

Judging by the changes observed in morphology and in the expression of platelet markers and

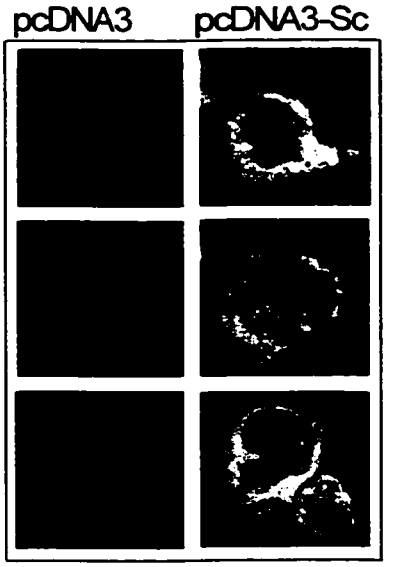

Fig. 3.17: Increased CD41a expression in scinderin positive MEG-01 cells. Cells transfected with either pcDNA3 or pcDNA3-Sc were fixed, permeabilized, treated with mouse (Mo) anti-human CD41 followed by donkey anti-mouse cyanine (Cy3) conj. IgG1. Cells were then examined by epifluorescence (x 630).

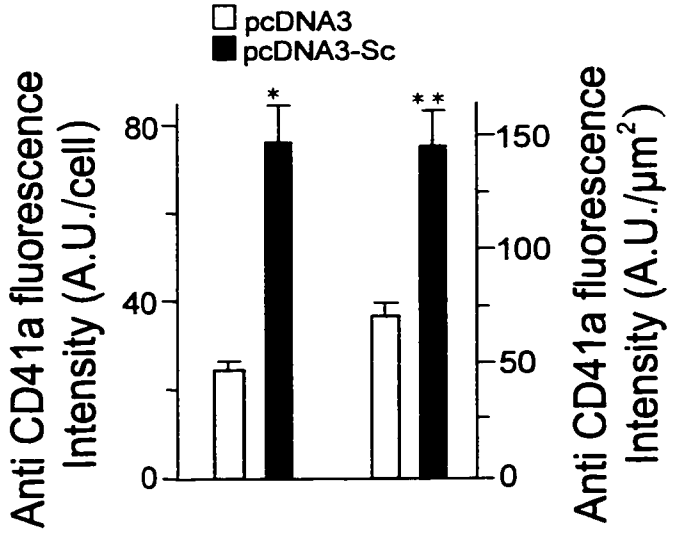

Fig. 3.18: Expression of CD41a (GPIIb/IIIa or fibrinogen receptor) in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc. Fluorescence intensity (A.U. = arbitrary units) of the cells described in Fig. 3.17, measured by image analysis (Argus-50 program). Bars represents mean ± S.E.M. values of 6 preparations containing each 10 to 21 cells in case of pcDNA3 transfected cells, and 19 preparations containing each 5 to 10 cells each in case of pcDNA3-Sc transfected cells. Preparations contained cells from 6 different clones (Sc I-J, I-K, I-N, I-V, I-U and I-W) in case of pcDNA3-Sc transfected cells. $** p < 0.05$. $* p < 0.01$.

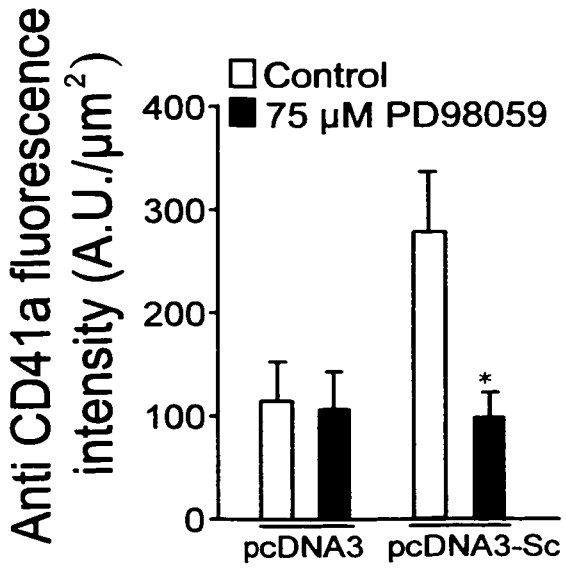

Fig. 3.19: CD41a fluorescence intensity in MEG-01 cells treated with PD98059. Cells transfected with either pcDNA3 or pcDNA3-Sc were cultured for 4 days in RPMI + 10 % FCS (no G-418) in absence (control) or presence of 75 μM PD98059. Cells were then cytospined onto slides, fixed, permeabilized and stained with mouse (Mo) anti-CD41a antibody, and cyanine (Cy3) conj. anti-mouse IgG. Fluorescence intensity of the preparations was quantified by image analysis (Argus-50 program). Bars represent mean ± S.E.M. of 5 preparations for pcDNA3 transfected cells and 10 preparations for pcDNA3-Sc transfected cells (clones Sc I-I and I-J), containing each between 5 and 15 cells. * $p < 0.05$.

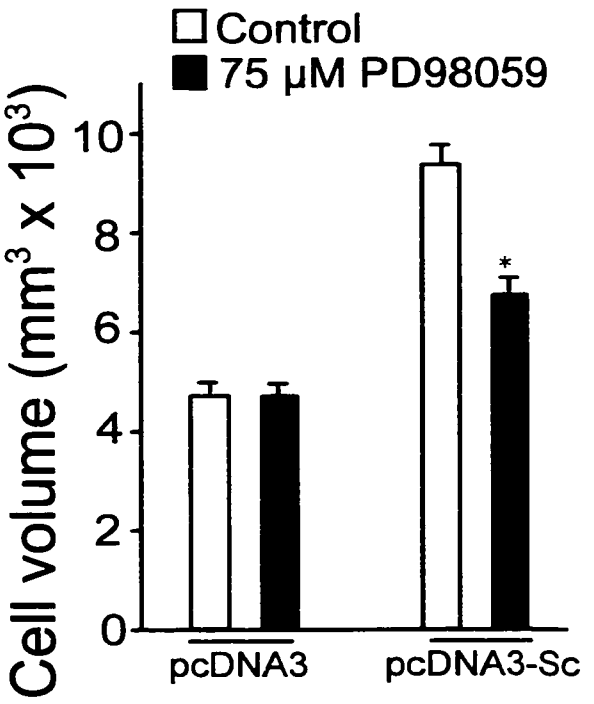

Fig. 3.20: Effect of PD98059 treatment on cell volume. Cells were cultured for 8 days in RPMI + 10 % FCS (no G-418) in absence (control) or presence of 75 µM PD98059. Cells were then cytospined and stained with Wright-Giemsa. Cell volumes were determined as indicated in Materials and Methods. Bars represent mean ± S.E.M. of 240 cells for pcDNA3 transfected cells and 480 for pcDNA3-Sc transfected cells (clones Sc I-I and I-Q). * $p < 0.01$.

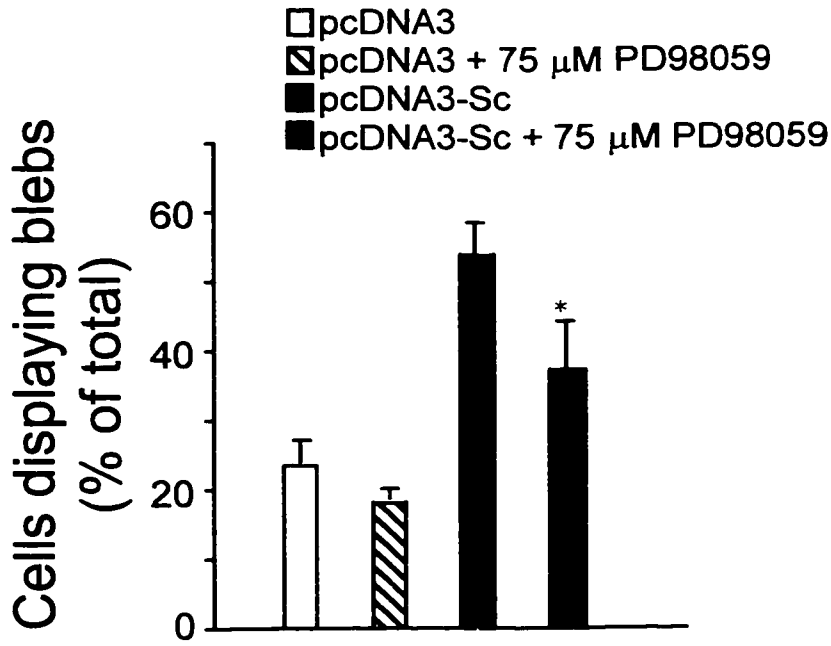

Fig. 3.21: Effect of PD98059 treatment on number of cytoplasmic blebs. Cells transfected with either pcDNA3 or pcDNA3-Sc were cultured in RPMI + 10 % FCS (no G-418) for 4 days in absence (control) or presence of 75 μM PD98059. Cells were then cytospined onto slides and stained with Wright-Giemsa. Numbers of cells displaying blebs were determined by microscopic examination. Bars represent mean ± S.E.M. of 3 preparations of pcDNA3 transfected cells and 6 of pcDNA3-Sc transfected cells (3 from each clone Sc I-I and I-Q), with 130 to 468 cells examined in each case. * $p < 0.05$.

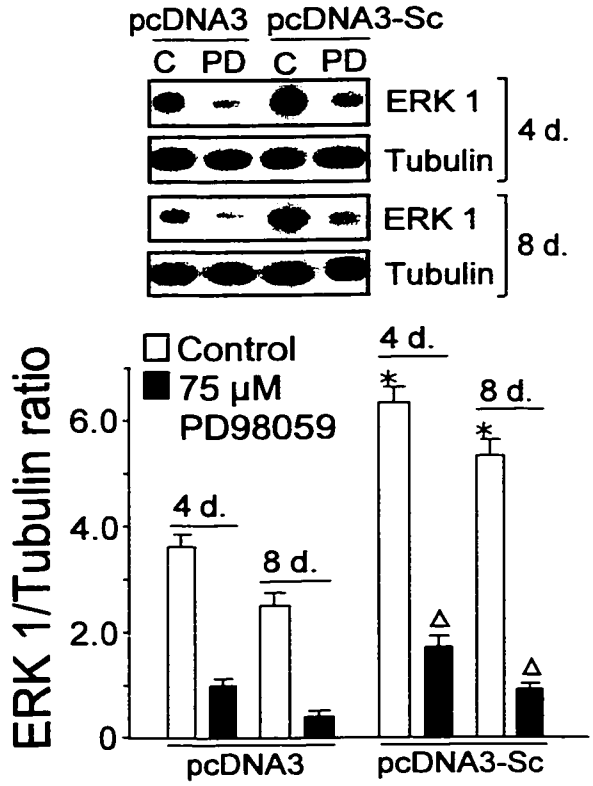

Fig. 3.22: Effect of PD98059 treatment on the expression of ERK1 in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc. Cells were grown for either 4 or 8 days (no G-418), in absence (control) or presence of 75 µM PD98059. Cells were then lysed for protein extraction. Proteins were separated by SDS-PAGE, then transfered to PVDF membranes and blotted with mouse (Mo) anti-human ERK1 followed by HRP-conj. anti-mouse IgG. ECL chemiluminiscence reaction was performed and films were developed by fluorography. Tubulin was used as gel loading control and to express results as ratio between ERK1 and tubulin, following scanning of bands from the fluorograms. * $p < 0.01$, Δ $p < 0.001$ (Anova test).

antigens, it can be concluded that MEG-01 cells expressing scinderin have entered the megakaryocyte differentiation pathway.

3.5. Apoptosis and production of platelet-like particles is the ultimate fate of megakaryoblastic leukemia cells in clones expressing scinderin

3.5 (a). Apoptosis

At the end of the normal process of platelet production, normal megakaryocytes consist of a large nucleus enveloped by a thin layer of cytoplasm (denuded megakaryocyte) (Zauli et al., 1997). At the time of platelet production , megakaryocytes in culture enter apoptosis, a mechanism that can be delayed, only to a certain degree, by thrombopoietin (Williams et al., 1990; Zauli et al., 1997). However, extrapolation from in vitro observations to the in vivo situation must be considered with caution. In fact, cytokines other than Tpo and complex cell-to-cell and cell-to-bone marrow matrix interactions are known to play a primary role in the development of megakaryocytes and platelet production in vivo (Gewirtz, 1986; Mazur, 1987; Zauli and Catani, 1995; Hoffman, 1989; Leven, 1995). Nevertheless, the fact that numerous scinderin-expressing MEG-01 cells die in culture, with numbers increasing over time, prompted us to determine the ultimate fate of these cells. The percentage of dead cells at day 12 in culture was double in scinderin-expressing cells when compared to vector-transfected cells (fig. 3.23). This, of course, was accompanied by an increasing number of cells entering apoptosis as revealed by the TUNEL assay (fig. 3.24 *a* and *b*).

3.5 (b). Production of platelet-like particles by scinderin-expressing MEG-01 cells

Normal human megakaryocytes in culture treated with Tpo undergo a maturation process

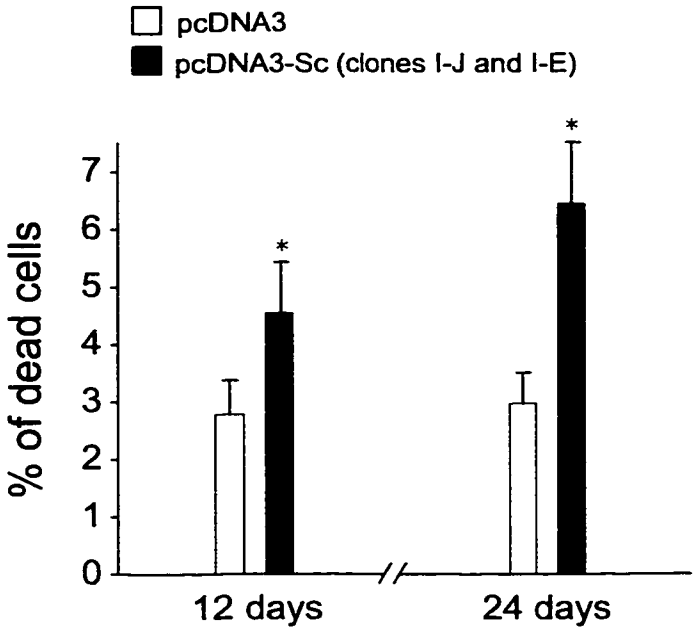

Fig. 3.23: Effect of scinderin expression on the viability of MEG-01 cells. Cells (1 x 10^6) transfected with either pcDNA3 or pcDNA3-Sc (clones I-J and I-E) were placed (per triplicate) in RPMI 1640 + 10 % FCS (no G-418) and grown for 24 days. Every 3 days, the number of cells was determined and the percentages of dead cells (trypan blue positive) were recorded and plotted for each case. The medium was replaced every 3 days. Bars represent mean ± S.E.M. of 6 determinations. * $p < 0.05$.

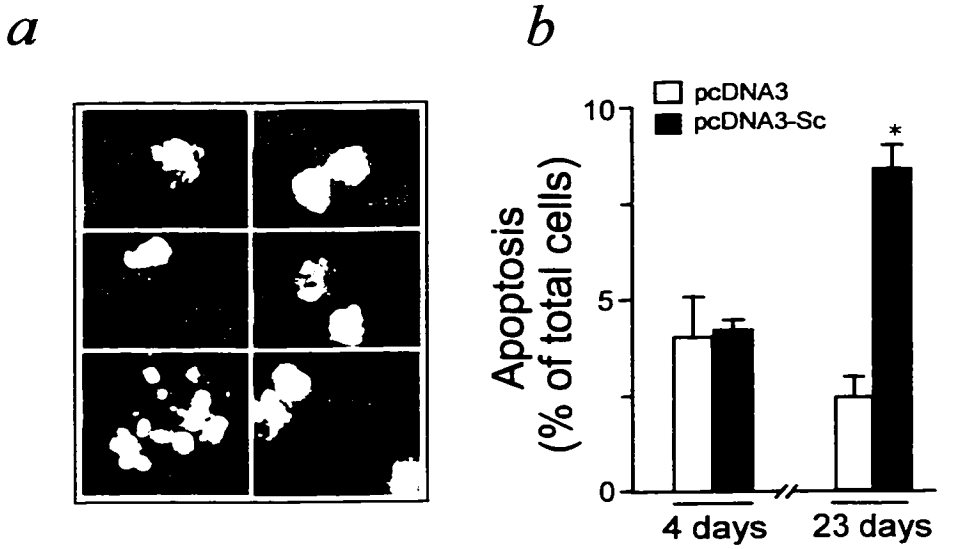

Fig. 3.24: Effect of scinderin expression on MEG-01 cell apoptosis. Cells transfected with either pcDNA3 or pcDNA3-Sc were incubated for either 4 or 23 days in RPMI 1640 + 10 % FCS (no G-418). Cells were then harvested, cytospined onto slides, stained with TUNEL reaction mixture and counter-stained with propidium bromide, as indicated in Materials and Methods. *a*, Microphotographs show apoptotic nuclei stained with FITC-dUTP (yellow), and non-apoptotic nuclei stained with propidium bromide (red). *b*, Number of apoptotic cells was determined and plotted as percentage of total cells. Bars represent mean ± S.E.M. of 6 preparations for pcDNA3 transfected cells and 42 preparations for pcDNA3-Sc transfected cells (clones I-I, I-D, I-E, I-J, I-K, I-E and I-Q). * $p < 0.01$.

ending in apoptosis with release of platelets (Zauli et al., 1997). Platelets generated in vitro from proplatelet-displaying human megakaryocytes are functional, since in the presence of fibrinogen they aggregate in response to both thrombin and ADP (Choi et al., 1995). It has been reported that the human megakaryocytic cell line MEG-01 has the ability to produce platelet-like particles in small amounts (Takeuchi et al., 1991).

Between days 18 and 25 in culture, only scinderin-positive cells release large numbers of cytoplasmic fragments. These particles had an average size of 1.63 ± 0.04 μm (n = 45) (fig. 3.25). They showed CD41a antigen fluorescence (fig. 3.27 *b*), had a high affinity serotonin transport system which was sensitive to fluoxetine (fig. 3.29), dense core vesicles, as demonstrated by electron microscopy (fig. 3.28) and, similar to platelets (White and Sauk, 1984), a circular array of microtubules, as demonstrated by immunocitochemistry with antibodies against α-tubulin (fig. 3.31). Moreover, treatment of these particles with 1U thrombin/ml induced serotonin release (fig. 3.30) and aggregation (fig. 3.26), an effect that was also blocked by 40 % in the presence of an antibody against the fibrinogen receptor (fig. 3.27 *a*).

3.6. Transduction pathways involved in changes observed in scinderin-expressing MEG-01 cells

Stimulation of different kinds of cytokine receptors, including thrombopoietin receptors, activates different transduction pathways leading to cell proliferation, differentiation, maturation and apoptosis (Lieberman, review, 1998; Smithgall, review, 1998). We have examined some of the numerous components of these pathways in an effort to understand

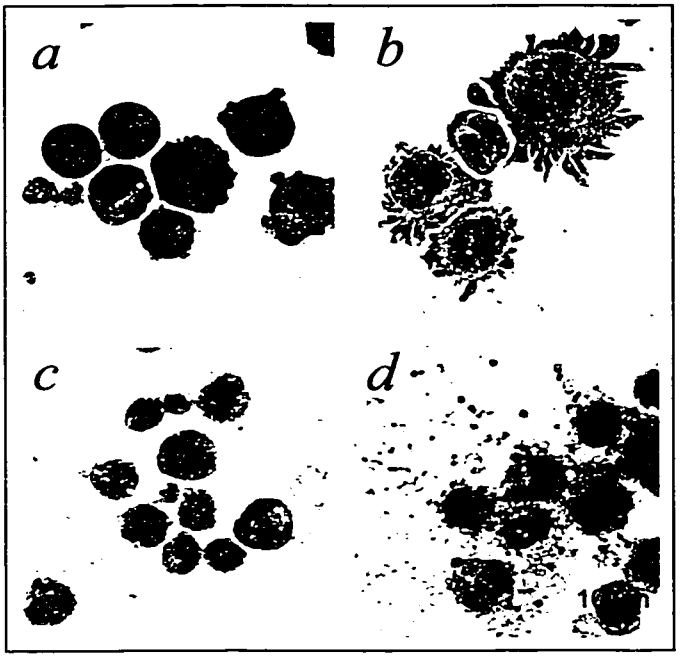

Fig. 3.25: Expression of scinderin in the MEG-01 cell line induced formation and release of platelet-like particles. Cells transfected with either pcDNA3 (*a*, *c*) or pcDNA3-Sc (*b*, *d*) were cultured for 11 (*a*, *b*) and 23 (*c*, *d*)days, and then fixed and stained with Wright-Giemsa. After 23 days in culture, preparations of pcDNA3-Sc transfected cells showed cytoplasmic areas smaller than those observed in *b* and a large number of relatively uniform particles of dimentions similar to platelets.

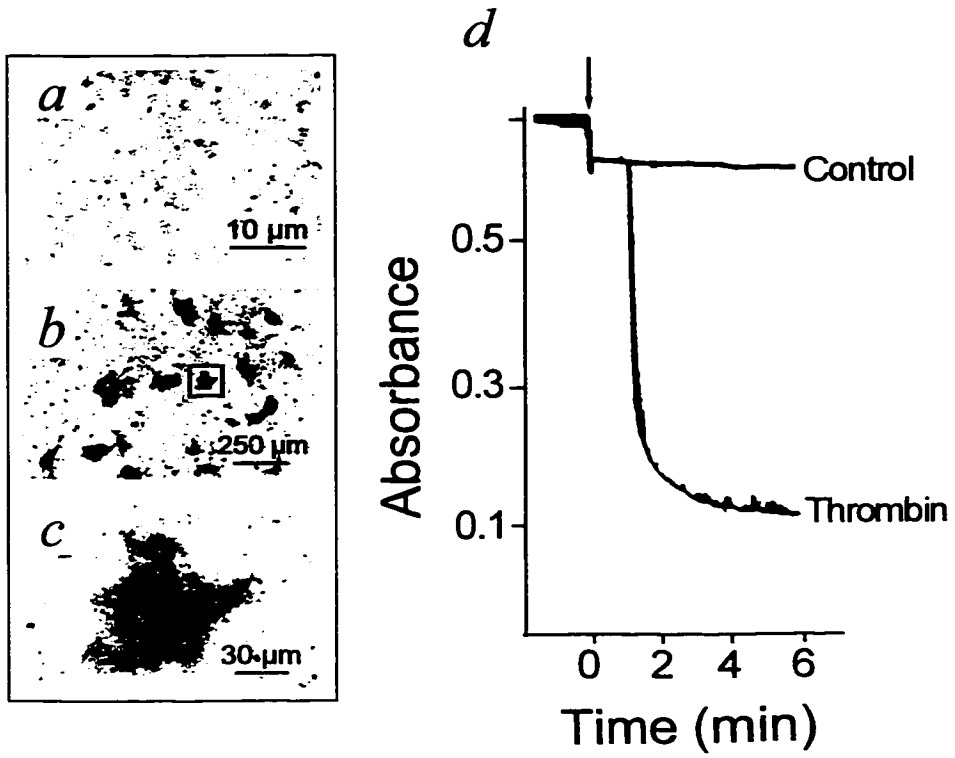

Fig. 3.26: Thrombin-induced aggregation of platelet-like particles released from cells expressing scinderin. Particles were isolated as described in Materials and Methods and were treated with 1U thrombin/ml. This induced aggregation, as shown after fixation and staining with Wright-Giemsa, before (*a*) and 6 min. after addition of thrombin (*b*, *c*). c Shows at larger magnification the particle aggregate contained within the box shown in (*b*). *d* Shows the decrease in light absorbance of the same preparation. The arrow indicates the time of the stimulus. The deflection at 0 min. indicates decrease in absorbance produced by addition of the thrombin aliquot.

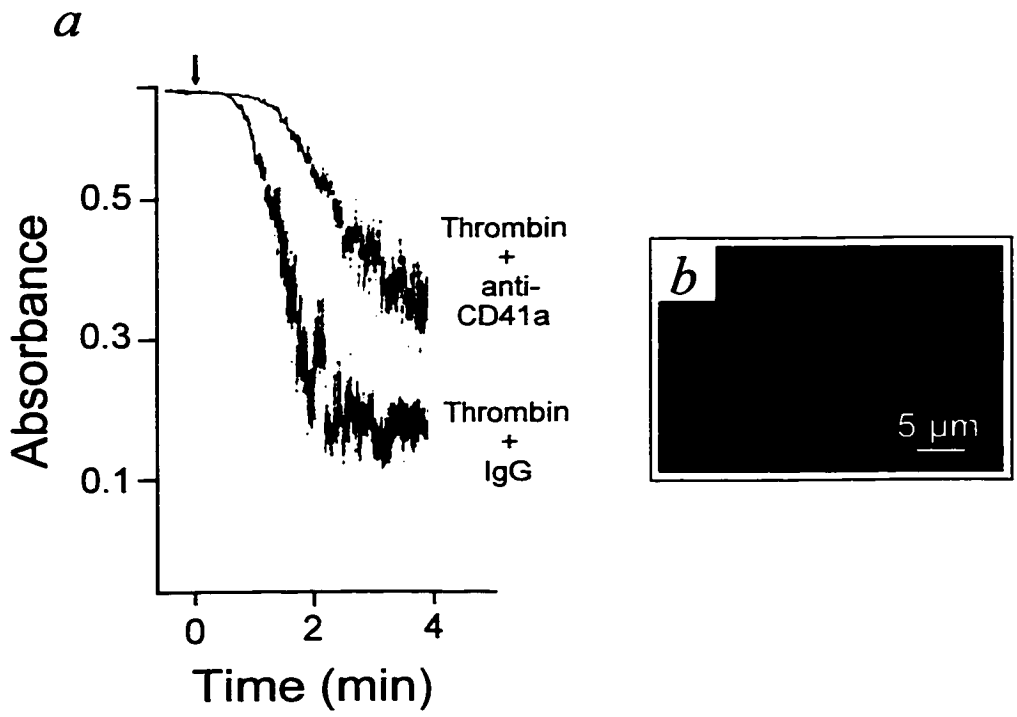

Fig. 3.27: CD41a antibody inhibits thrombin-induced aggregation of platelet-like particles. Particles produced by the scinderin expressing clones were isolated and treated with CD41a antibody as described in Materials and Methods. Particles were then assayed for aggregation in presence of 1 U/ml thrombin. *a*, Traces showing a decrease in absorbance due to aggregation. *b*, Immunostaining with anti-CD41a of particles expressing the fibrinogen receptor (see Materials and Methods).

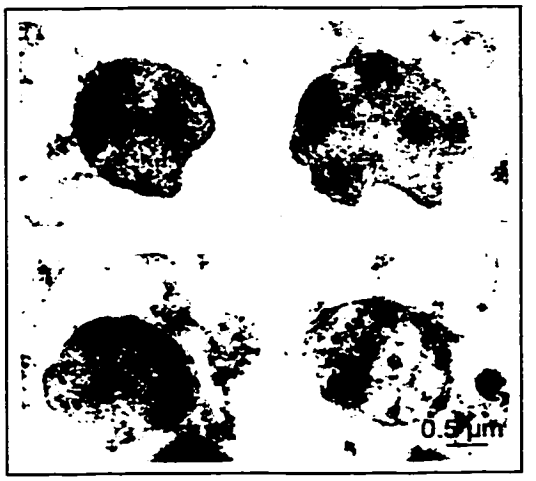

Fig. 3.28: Electron microscopy of platelet-like particles released from MEG-01 cells previously transfected with pcDNA3-Sc (clones I-J and I-E). Platelet-like particles were isolated from 22 days cultures (see Materials and Methods). Isolated particles were incubated in culture medium, in absence or presence of 1 µM serotonin (5-HT) for 60 min. at room temperature. This was followed by fixation in glutaraldehyde and embeding in propylene oxide/epon mixture. Preparations were then processed for electron microscopy. The micrographs show dense core granules within the particles cytoplasm (x 35625).

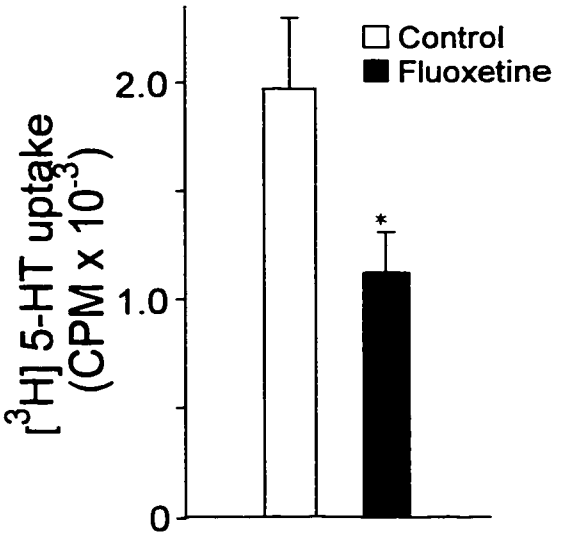

Fig. 3.29: Inhibition of serotonin uptake by fluoxetine. Platelet-like particles were pre-labelled with 10^{-8} M of [^{3}H]5-HT (serotonin) in the absence or presence of 6 nM fluoxetine, in Ca^{++}-free Locke's solution, for 1 hour at 37° C. Particles were then washed with Ca^{++}-free Locke's solution and treated with 10 % TCA. Extracts were assayed for radioactivity in a scintillation spectrometer. Bars represent mean ± S.E.M. of 8 preparations. * $p < 0.05$.

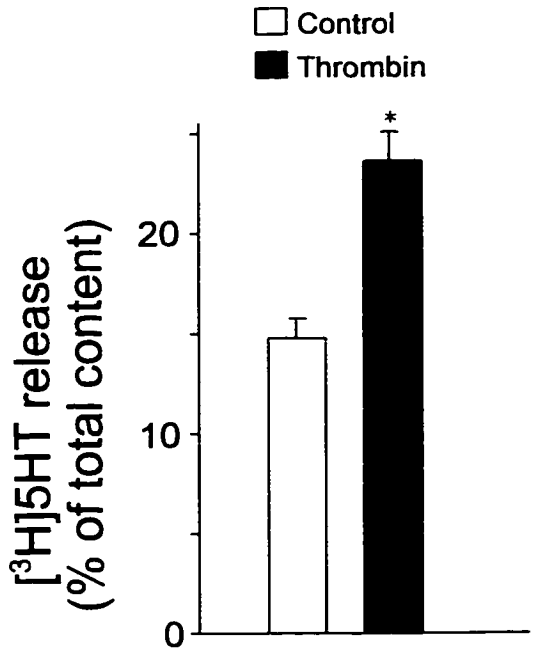

Fig. 3.30: Thrombin-evoked release of serotonin from platelet-like particles. Particles produced by the pcDNA3-Sc transfected MEG-01 cells were isolated by centrifugation after 22 days of culture (see Materials and Methods). Particles were labelled, for 1 hour, in culture medium containing 30 μCi/ml of [^{3}H]5-HT, at room temperature. Particles were then washed and treated with 1 U/ml thrombin for 3 minutes. The reaction was stopped with 6 % glutaraldehyde. Particles were washed again with culture medium, lysed with 10 % TCA and the [^{3}H]5-HT radioactivity in the extracts was determined in a liquid scintillation spectrometer. Bars represent mean ± S.E.M. of 8 preparations. * $p < 0.001$.

how scinderin-expression triggers directly, or most probably, indirectly through F-actin disassembly, the activity of one or more of these pathways. As indicated above, expression of GPIIb/IIIa (CD41a or fibrinogen receptor) was accompanied by an increased expression of ERK_1, and both expressions were blocked by PD98059, a compound known to block the Raf/MEK/ERK pathway (Dudley et al., 1995). This would suggest the involvement of this pathway in the differentiation process triggered by the expression of scinderin.

3.6 (a). Small molecular weight GTP-binding proteins of the Ras family

Mutated Ras genes with oncogenic potential were first detected in human tumours (Satoh et al., 1992). These Ras proto-oncogenes (N-ras, K-ras, H-ras) encode low molecular weight (p21) GTPases, referred to collectively as Ras, that play a central role in growth regulatory signal transduction (Satoh et al., 1992). Activation of Ras proteins is a key step in biochemical pathways triggered by ligand-bound cell surface receptors. This class of receptors responds to a wide variety of cell agonists including growth factors (Satoh et al., 1992).

Association of Ras with GTP induces a major conformational change in the protein, causing it to expose a region responsible for direct interactions with downstream effector molecules, such as Raf, which, in turn, activates the ERK/MAP kinase pathway among others (for review see Smithgall, 1998).

Ras levels were found to be decreased in scinderin-positive cells at 12 and 14 days in culture (fig. 3.32). In this regard it has been suggested that Ras activation is necessary mainly for a proliferative response, and that differentiation requires an additional, or perhaps a separate,

pathway (Smithgall, review, 1998). Therefore, a decrease in Ras levels would agree with the decrease in proliferation found in scinderin-expressing clones.

3.6 (b). Small molecular weight GTP-binding proteins of the Rho family

Members of the Rho family of small GTPases are key regulators of actin cytoskeleton. Indeed, Cdc42, Rac and Rho control the assembly and disassembly of actin cytoskeleton in response to extracellular signals. Furthermore, through their interaction with multiple target proteins, they produce a coordinated control of other cellular activities such as gene transcription and cell adhesion (Hall, review, 1998; Tapon and Hall, review, 1997).

Cdc42 induces actin-rich surface protrusions called filopodia (Nobes and Hall, 1995). Rac activation leads to the assembly of a mesh-work of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles (Ridley et al., 1992). Rho acts as a molecular switch to control a signal transduction pathway that links membrane receptors with cytoskeleton (Ridley et al., 1992).

Rac and Cdc42 have also been reported to regulate the c-jun NH_2-terminal kinase (JNK) and the p38 mitogen-activated protein (MAP) kinase cascades (Coso et al., 1995), and this, in fact, represents a physiological function for these small GTPases. Rho GTPases have also been reported to stimulate transcription of cyclin D through another mechanism (Westwick et al., 1997), thus triggering the progression of the G_1 phase of the cell cycle. (Lamarche et al., 1996).

Our results show a decrease in the expression of Cdc42 in scinderin-positive cells at days 12 and 14 of culture (fig. 3.32). It is possible that low F-actin levels in scinderin-positive cells

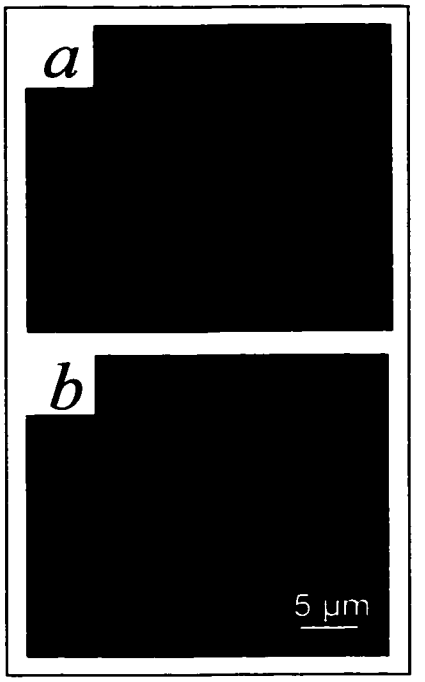

Fig. 3.31: Presence of similar arrays of microtubules in human platelets (*a*) and platelet-like particles produced by pcDNA3-Sc transfected cells (*b*). Particles produced by scinderin expressing cells previously cultured for 22 days were isolated as described in Materials and Methods. Particles were then cytospined onto slides and air dried. Slides were fixed, permeabilized and stained with mouse (Mo) anti-α tubulin, followed by anti-mouse Cy3 (cyanine) conj. IgG as secondary antibody. Preparations were then examined under epifluorescence.

are responsible for the decrease in the expression of Cdc42. Surprisingly, Rac2 (the most common isoform of Rac in hematopoietic cells) was found to increase remarkably in scinderin positive cells (fig. 3.33). Rho levels, on the contrary, were found to decrease (as Cdc42 levels) in scinderin-positive cells (fig. 3.33).

3.6 (c). C-jun amino-terminal pathway and its terminal effectors: -The transcription factors c-jun and c-fos

The induction of actin polymerization and of JNK activity are mediated by two different pathways triggered by Rac and/or Cdc42 (Lamarche et al., 1996). The Ser-Thr kinase called p65 PAK (p21-activated kinase) might mediate activation of JNK by Rac and/or Cdc42 (Lamarche et al., 1996). It has also been reported that activation of the JNK pathway by either Rho GTPases or by expression of the constitutively activated PAK C-terminus, is sufficient to induce apoptosis (Faure et al., 1997). Furthermore, it is known that activation of the (Rac/Cdc42/PAK/MEKK.SEK/JNK) pathway, is responsible for triggering a decrease in proliferation and apoptosis through an increased expression of transcription factors c-jun and c-fos (Deinhardt, review, 1996; Liebermann et al., 1998). The present results show that in scinderin-positive clones, there was a very significant increase in both c-jun and c-fos protein levels (fig. 3.35). Further proof for the activation of this pathway was the observation of increases in expressions of JNK2 and PAK (fig. 3.34). Normally PAK is activated by Rac2 (Minden and Karin, review, 1997), and in cells expressing scinderin, there was a significant increase in PAK levels and activity (figs. 3.34 and 3.36). It is, therefore, possible that the high levels of Rac2 observed in scinderin-positive clones (fig. 3.33) were responsible for the

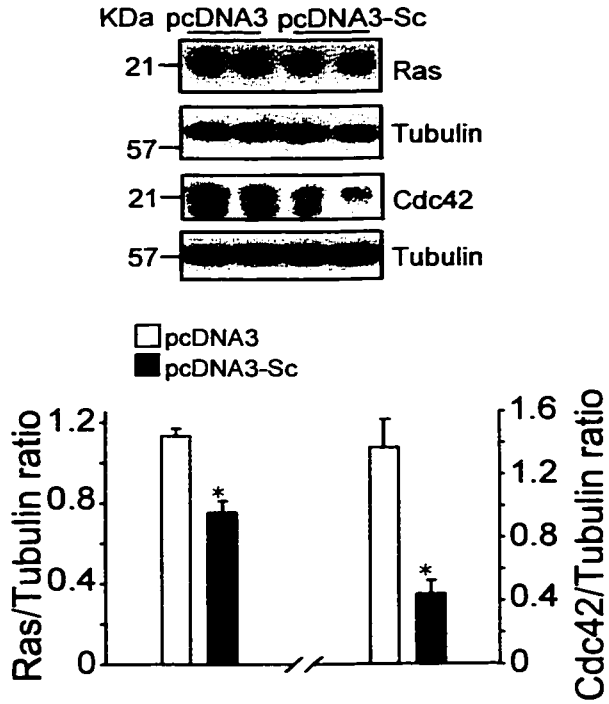

Fig. 3.32: Expression of Ras and Cdc42 in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc (clone I-J). Cells were cultured for 14 days in RPMI + 10 % FCS after removal of antibiotic G-418. Cells were then harvested, washed and treated with RIPA buffer to extract proteins. Aliquotes of protein extracts were mixed with SDS loading buffer and proteins were separated by electrophoresis. Following SDS-PAGE, proteins were transfered to membranes and blotted with either rabbit (Po) anti-Ras or rabbit (Po) anti-Cdc42, followed by anti-rabbit HRP-conj. IgG as secondary antibody. ECL chemiluminiscence reaction was performed and films were developed by fluorography. Tubulin was used as gel loading control and to express results as ratios between each protein and tubulin following scanning of bands from the fluorograms. Bars represent mean ± S.E.M. of 4 separate experiments. $* p < 0.01$.

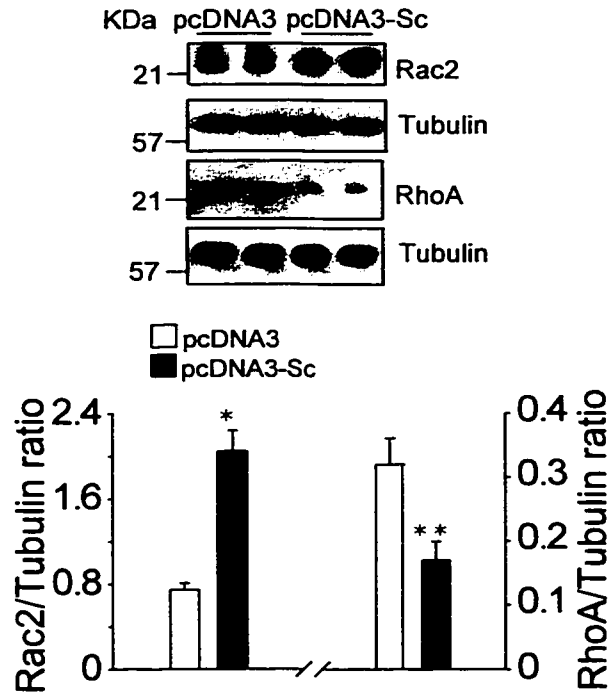

Fig. 3.33: Expression of Rac2 and RhoA in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc (clone I-J). Cells were cultured for 14 days in RPMI + 10 % FCS after removal of antibiotic G-418. Cells were then harvested, washed and treated with RIPA buffer to extract proteins. Aliquotes of protein extracts were mixed with SDS loading buffer and proteins were separated by electrophoresis. Following SDS-PAGE, proteins were transfered to membranes and blotted with either rabbit (Po) anti-Rac2 or mouse (Mo) anti-RhoA, followed by either anti-rabbit or anti-mouse HRP-conj. IgG as secondary antibodies respectively. ECL chemiluminiscence reaction was performed and films were developed by fluorography. Tubulin was used as gel loading control and to express results as ratios between each protein and tubulin, following scanning of bands from the fluorograms. Bars represent mean ± S.E.M. of 4 separate experiments. $** p < 0.05$. $* p < 0.001$.

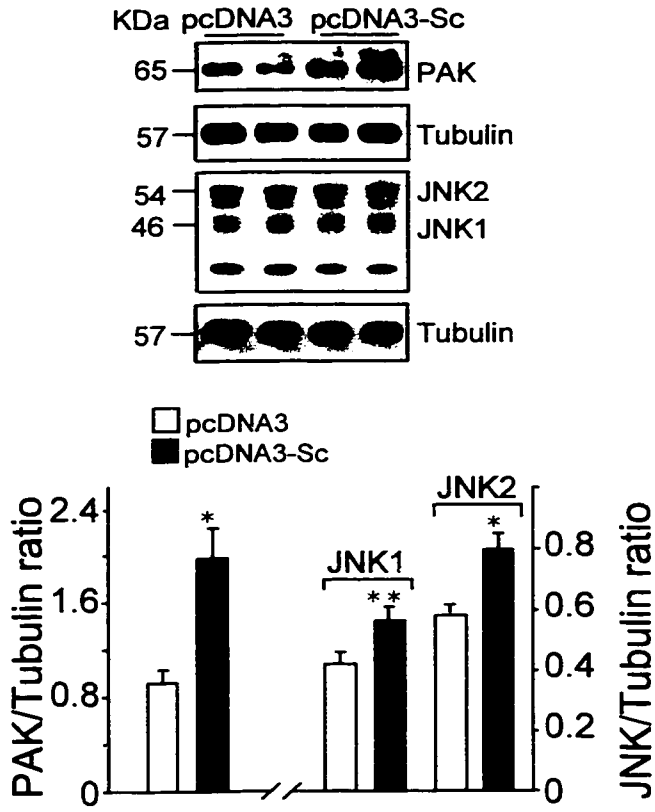

Fig. 3.34: Expression of PAK and JNK in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc (clone I-J). Cells were cultured for 14 days in RPMI + 10 % FCS after removal of antibiotic G-418. Cells were then harvested, washed and treated with RIPA buffer to extract proteins. Aliquotes of protein extracts were mixed with SDS loading buffer and proteins were separated by electrophoresis. Following SDS-PAGE, proteins were transfered to membranes and blotted with either rabbit (Po) anti-PAK or rabbit (Po) anti-JNK, followed by anti-rabbit HRP-conj. IgG as secondary antibody. ECL chemiluminiscence reaction was performed and films were developed by fluorography. Tubulin was used as gel loading control and to express results as ratios between each protein and tubulin, following scanning of bands from the fluorograms. Bars represent mean ± S.E.M. of 4 separate experiments. ** $p < 0.05$. * $p < 0.01$.

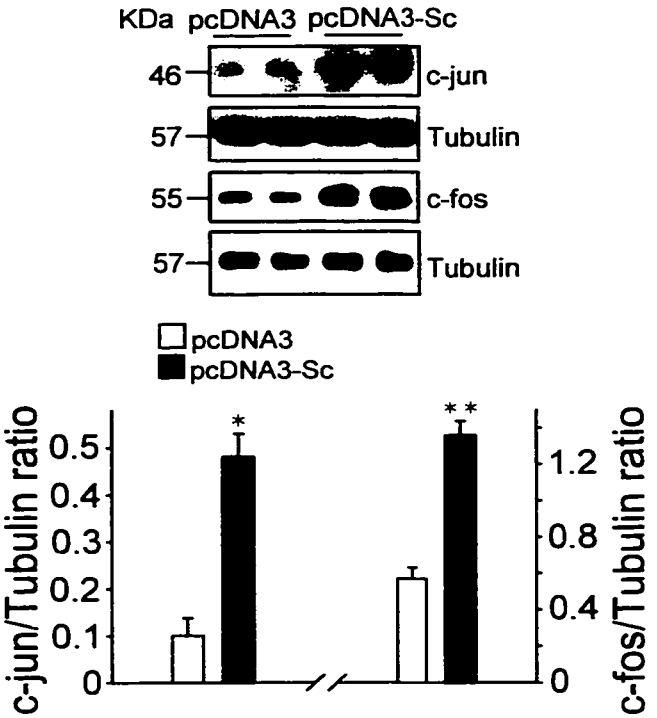

Fig. 3.35: Expression of c-jun and c-fos in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc (clone I-J). Cells were cultured for 14 days in RPMI + 10 % FCS after removal of antibiotic G-418. Cells were then harvested, washed and treated with RIPA buffer to extract proteins. Aliquotes of protein extracts were mixed with SDS loading buffer and proteins were separated by electrophoresis. Following SDS-PAGE, proteins were transfered to membranes and blotted with either rabbit (Po) anti-c-jun or mouse (Mo) anti-c-fos followed by either anti-rabbit or anti-mouse HRP-conj. IgG as secondary antibodies respectively. ECL chemiluminiscence reaction was performed and films were developed by fluorography. Tubulin was used as gel loading control and to express results as ratios between each protein and tubulin, following scanning of bands from the fluorograms. Bars represent mean ± S.E.M. of 4 separate experiments. $**$ $p < 0.01$. $*$ $p < 0.001$.

stimulation of the JNK pathway. It has also been reported that decreases in gelsolin expression are accompanied by increases in the levels of Rac (Azuma et al., 1998). Therefore, additional experiments were performed to examine this possibility. In cells expressing scinderin, Rac2 levels increased with time in culture whereas gelsolin levels decreased (fig. 3.37).

3.6 (d). The Jak/Stat pathway (STAT5)

Tpo and other cytokines, which bind to tyrosine kinase receptors, perform part of their mainly proliferative effects through activation of the Jak/Stat pathway (Mikayawa et al., 1996). Binding of cytokines to tyrosine kinase receptors leads to recruitment of Jak kinases (mainly Jak2), which in turn become activated and phosphorylate Stat transcription factors (mainly Stat3 and Stat5) (Pellegrini and Dusanter-Fourt, 1997). Activated Stats regulate the transcription of genes responsible for cell proliferation (Pellegrini and Dusanter-Fourt, 1997). Scinderin-positive MEG-01 cells showed a significant decrease in the levels of transcription factor STAT5 when compared to cells transfected with vector alone (fig. 3.38). As demonstrated above, scinderin-positive cells grow considerably less than their control (pcDNA3 transfected cells). Therefore it is possible that a lesser amount of this transcription factor would be needed by the cells, as a result of the decrease in proliferation caused by scinderin expression.

3.7. Transcription factor NF-E2

Transcription factor NF-E2 is a tissue-restricted hetero-dimeric protein which recognizes an extended AP-1 like motif (Andrews et al., 1993b; Talbot et al., 1990). This hetero-dimer

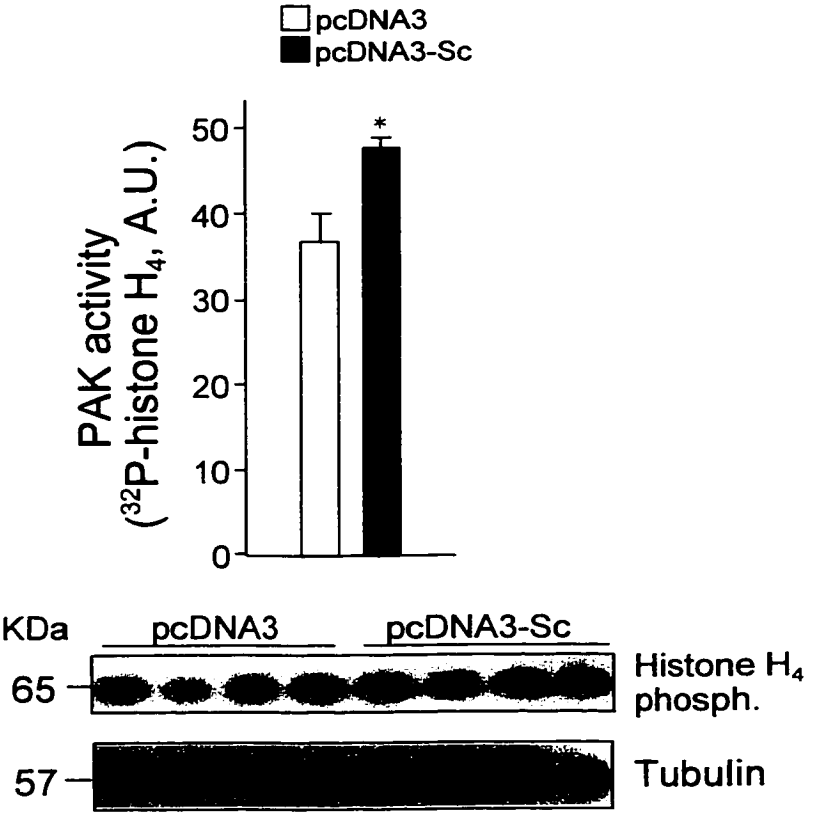

Fig. 3.36: Effect of scinderin expression on PAK activity. Cells were cultured for 14 days in RPMI + 10 % FCS without G-418. Cells were then harvested and treated with RIPA buffer for protein extraction. Aliquotes of protein extracts were mixed with SDS loading buffer and resolved by electrophoresis in SDS polyacrylamide gels containing 160 µg/ml histone H_4 as substrate for PAK. After washing and denaturation with 6 M guanidine-HCl, the praparation was treated with 0.04 % Tween 20 in washing buffer (see Materials and Methods) at 4°C (renaturation), and the kinase reaction was started by the addition of 25 µCi/ml of [γ^{32} P]ATP. The gel was then dried and subjected to autoradiography. Results obtained were then quantified by scanning densitometry. Bars represent mean ± S.E.M. of 4 preparations. * $p < 0.05$.

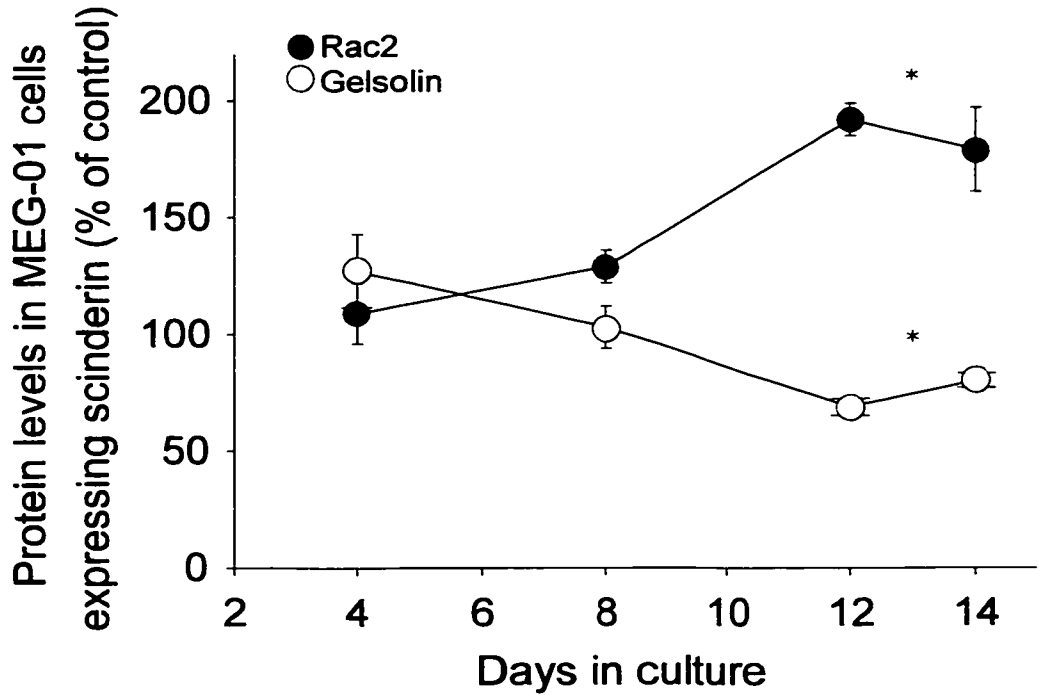

Fig. 3.37: Effect of scinderin expression on Rac2 and gelsolin levels in MEG-01 cells. Wild type cells and cells transfected with either pcDNA3 or pcDNA3-Sc, were grown for 4, 8, 12 and 14 days in RPMI + 10 % FCS after removal of antibiotic G-418. Cells were then harvested, washed and treated with RIPA buffer to extract proteins. Aliquotes of protein extracts were mixed with SDS loading buffer and proteins were separated by electrophoresis. Following SDS-PAGE, proteins were transfered to PVDF membranes and blotted with either rabbit (Po) anti-Rac2 or rabbit (Po) anti-gelsolin, followed by anti-rabbit HRP-conj. IgG as secondary antibody. ECL chemiluminiscence reaction was performed and films were developed by fluorography. Tubulin was used as gel loading control and to calculate ratios between each protein and tubulin, following scanning of bands from the fluorograms. Open and closed circles represent means ± S.E.M. of 4 separate determinations. $**$ $p < 0.05$ and $*$ $p < 0.01$ are the significance between protein levels of cells cultured for 4 days and those cultured for 12 and 14 days (Anova test).

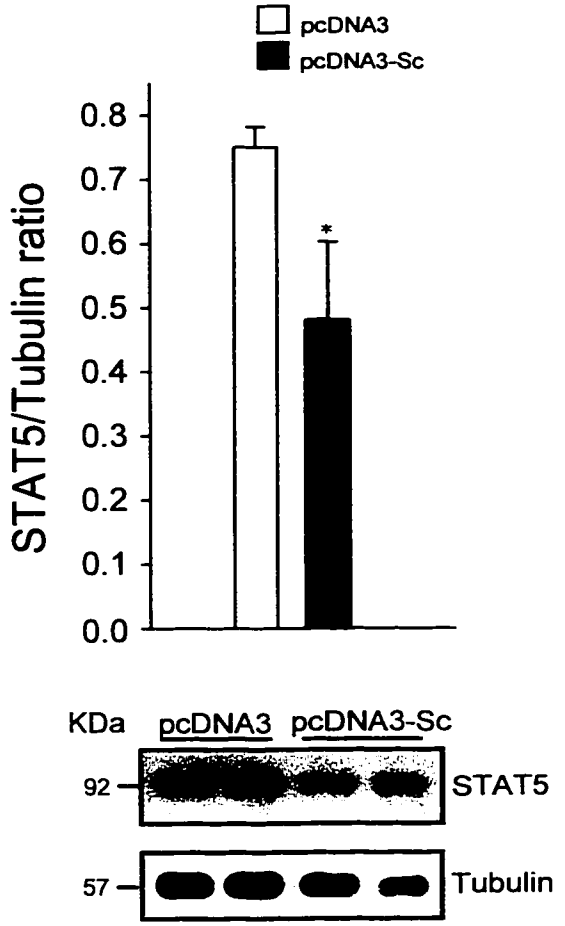

Fig. 3.38: Expression of STAT5 in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc (clone I-J). Cells were cultured for 14 days in RPMI + 10 % FCS after removal of antibiotic G-418. Cells were then harvested, washed and treated with RIPA buffer to extract proteins. Aliquotes of protein extracts were mixed with SDS loading buffer and proteins were separated by electrophoresis. Following SDS-PAGE, proteins were transfered to membranes and blotted with mouse (Mo) anti-STAT5 followed by anti-mouse HRP-conj. IgG as secondary antibody. ECL chemiluminiscence reaction was performed and films were developed by fluorography. Tubulin was used as gel loading control and to express results as ratios between STAT5 and tubulin, following scanning of bands from the fluorograms. Bars represent mean ± S.E.M. of 4 separate experiments. * $p < 0.05$.

consists of two leucine zipper (bZip) proteins of 45 and 18 kDa (Andrews et al., 1993a, b). While NF-E2 p18 is widely expressed in many different cell types, NF-E2 p45 expression is restricted to cells of the erythroid, megakaryocytic and mast cells lineages (Shivdasani et al., 1995). This transcriptionally active hetero-dimer seems to play an important role in later stages of megakaryocytic maturation, such as secretory granule formation and platelet release (Lecine et al., 1998b; Shivdasani et al., 1995).

We have examined the levels of NF-E2 p45 in scinderin-positive cells, and compared them with those in cells transfected with vector alone. As shown in fig. 3.39, scinderin-positive clones expressed less NF-E2 but, quite unexpectedly, these cells showed the appearance in the SDS gels of a protein band with an electrophoretic mobility corresponding to 90 Kda. The nature of this protein band which was recognized by the p45 NF-E2 antibody has yet to be identified.

3.8. Effect of scinderin expression on tumorigenesis

Nine Balb/c mice were injected, subcutaneously, in their abdominal flanks with 1 x 10^7 MEG-01 cells previously transfected with vector pcDNA3 (controls). A similar group of mice received injections of the same number of cells also previously transfected with vectors carrying full length scinderin cDNA insert (pcDNA3-Sc). All cells were cultured for 14 days prior to injection. Seven mice of those which received pcDNA3 transfected cells, developed large tumours and had to be sacrificed 3 weeks following injection according to institutional animal care policies (fig. 3.40). On the other hand, only 2 small tumours were observed in the group of 9 mice injected with clones expressing scinderin (pcDNA3-Sc). Remaining

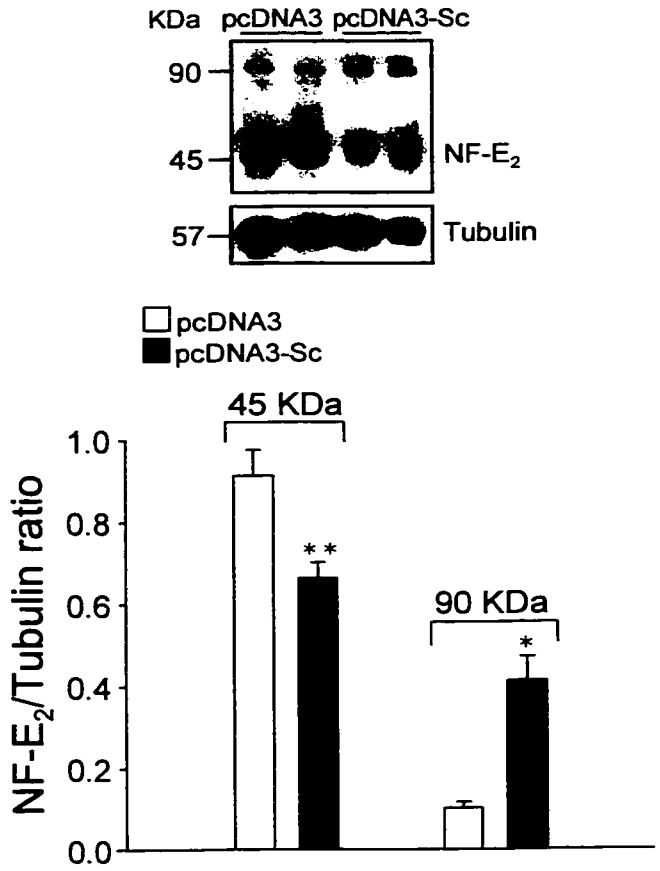

Fig. 3.39: Expression of transcription factor $NF\text{-}E_2$ in MEG-01 cells transfected with either pcDNA3 or pcDNA3-Sc (clone I-J). Cells were cultured for 14 days in RPMI + 10 % FCS after removal of antibiotic G-418. Cells were then harvested, washed and treated with RIPA buffer to extract proteins. Aliquotes of protein extracts were mixed with SDS loading buffer and proteins were separated by electrophoresis. Following SDS-PAGE, proteins were transfered to membranes and blotted with rabbit (Po) anti-$NF\text{-}E_2$ followed by anti-rabbit HRP-conj. IgG as secondary antibody. ECL chemiluminiscence reaction was performed and films were developed by fluorography. Tubulin was used as gel loading control and to express results as ratios between $NF\text{-}E_2$ and tubulin, following scanning of bands from the fluorograms. Bars represent mean ± S.E.M. of 4 separate experiments. $**\ p < 0.05$. $*\ p < 0.01$.

animals in this group were free of tumours (fig. 3.40). The histology of these small tumours was then compared to that of the large tumours found in animals of the control (pcDNA3) group. The latter set were solid tumours of well packed cells showing single nuclei surrounded by small cytoplasmic areas (fig. 41 *a*). Conversely, the two small tumours formed by scinderin-expressing cells showed large areas of cells with apoptotic nuclei surrounded by large numbers of platelet-like particles (fig. 3.41 *b*), a situation similar to that observed with these cells in culture (see above fig. 3.25 *d*). Therefore, it seems that *in vitro* as well as *in vivo*, apoptosis with platelet-like particles release is the fate of cells transfected with pcDNA3-Sc.

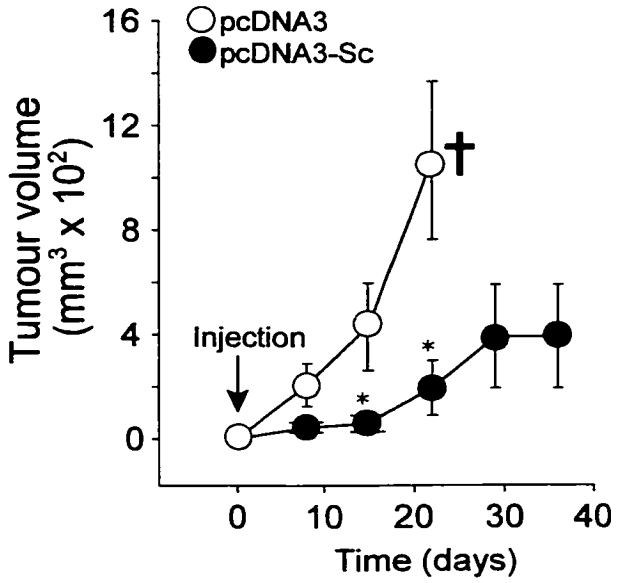

Fig. 3.40: Effect of scinderin expression on tumour growth in nude mice. Balb/c nude mice were injected subcutaneously with cells (1 x 10^7 cells/100 µl saline)cultured for 14 days after transfection with either pcDNA3 or pcDNA3-Sc. Open and closed circles represent mean ± S.E.M. from 9 mice (* $p < 0.05$, Anova test). †, pcDNA3 transfected mice were sacrificed at 3 weeks following injection acoording to institutional animal care policies.

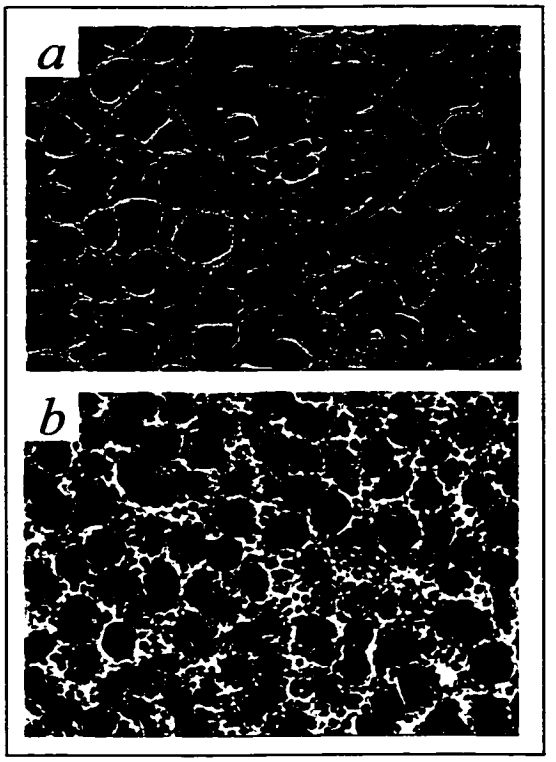

Fig. 3.41: Histology of tumors grown in nude mice: *a*, Hematoxilin-eosin staining (see Materials and Methods) of a section from a large tumor produced by pcDNA3 transfected MEG-01 cells (x 1000) . Histology of 8 remaining tumors in this group was similar, and so it was that of tumors formed by wild type MEG-01 cells (data not shown). *b*, Similar staining of a section from 1 of the 2 small tumors produced by pcDNA3-Sc transfected MEG-01 cells, showing apoptotic nuclei surrounded by a large number of platelet-like particles. The second small tumor in this group had similar histology.

4 - Discussion

4 – Discussion

Scinderin is a Ca^{++}-dependent filamentous actin-severing protein (Rodríguez del Castillo et al., 1990; Rodríguez del Castillo et al., 1992) present in platelets and megakaryocytes. However, as has already been demonstrated here, it is absent from megakaryoblastic leukemia cell lines. The scinderin gene has been cloned in our laboratory (Marcu et al., 1994), and one of scinderin's functions has been demonstrated to be the control of cortical F-actin networks during secretion from cells such as chromaffin cells (Vitale et al., 1991) and platelets (Rodríguez del Castillo et al., 1992). However, it is clear that scinderin might also be involved, to some degree, in the control of other dynamic changes occurring in the reorganization of actin cytoskeleton networks such as pro-platelet formation and platelet release (Leven and Yee, 1987; Tablin et al., 1990; Leven, J. Cell Biology, 1995). It is known that the disruption of either microtubules or actin filaments in normal megakaryocytes can alter the dynamics of platelet formation (Tablin et al., 1990). For instance, the treatment of megakaryocytes in culture with taxol, a drug that stabilizes microtubules, induces pseudopodial (pro-platelet) extension (Tablin et al., 1990), supporting a role for microtubules in this process. However, pseudopod fragmentation and platelet formation is also induced or accelerated by treatment with cytochalasin B, an inhibitor of actin polymerization, suggesting that an actin-rich peripheral network in megakaryocytes is an important component in the process of platelet formation (Tablin et al., 1990). When this actin-rich peripheral network is disrupted in megakaryocytes, microtubules, whose circular array is adjacent to the F-actin peripheral network, either elongate or reorient during the

extension of fillipodia (Tablin et al., 1990). Radley and Haller (1982) have also proposed that platelets are formed by a mechanism similar to cytokinesis in which an actin-rich cleavage furrow separates daughter cells in the pseudopodia. It is tempting to speculate that scinderin, an F-actin-severing protein, would play an important role in the process of pseudopodia formation and platelet release.

Although scinderin was successfully expressed in the cell lines K-562 and MEG-01, we selected the latter cell line, because MEG-01 cells show features of the megakaryocyte lineage, whereas K-562 expresses both erythroid and megakaryocytic lineage characteristics (Rowley et al., 1981; Niitsu et al., 1996).

The experiments described here show that the expression of scinderin cDNA in the MEG-01 cell line decreases cell proliferation and induces polyploidization, differentiation and maturation, followed by apoptosis with release of platelet-like particles.

4.(1). Decrease in F-actin content induced by scinderin expression in MEG-01 cells and its implications

The initial observation in MEG-01 cells expressing scinderin was a decrease in the levels of filamentous actin due to the severing activity of scinderin, which was totally active, as demonstrated by a further decrease in F-actin levels upon treatment of cells with the ionophore A23187. This compound in other cell systems, such as human neutrophils, induces an increase in F-actin content, which is dependent on extracellular free calcium (5 mM) (Howard and Wang, 1987). However, in electro-permeabilized neutrophils, increasing extracellular Ca^{++} beyond the resting level by direct addition of $CaCl_2$ to the medium,

resulted in F-actin disassembly (Downey et al., 1990). Conversely, lowering intracellular Ca^{++} resulted in spontaneous actin assembly, showing that actin assembly is not mediated by an increase in intracellular Ca^{++}, but rather elevated Ca^{++} facilitates F-actin disassembly, an effect possibly mediated by Ca^{++}-dependent actin- filament- severing proteins such as gelsolin (Downey et al., 1990). In other systems such as human umbilical vein endothelial cells (Thurston and Turner, 1993) and human U937 monocyte-macrophage cells (Joseph and Mac Dermot, 1992), agonists such as thrombin, which by a different mechanism, increases intracellular Ca^{++}, (see fig. 4.1), also induce F-actin assembly. There are other mechanisms that induce F-actin network disruption and are independent of the rise of intracellular Ca^{++}, such as PKC activation by PMA (Apgar, 1991), or tyrosine kinase receptor stimulation, followed by an increased phosphatidylinositol turnover (Deanin et al., 1991, see fig. 4.1). In MEG-01 cells, thrombin, ADP and epinephrine treatment induce a rapid increase in intracellular Ca^{++} followed by activation of PKC (Schootemeijer et al., 1994). Thrombin induces actin polymerization with shape changes in MEG-01 cells through activation of PKC, and this effect is also mimicked by A23187 at low concentrations (10^{-5} M) (Goto et al., 1992). On the other hand, in scinderin-expressing MEG-01 cells, concentrations of the ionophore A23187 in the order of 10^{-6} M, induce F-actin disassembly (with no decrease in F-actin in the control MEG-01 cells transfected with vector only), suggesting a direct activation of scinderin by a rapid increase in intracellular Ca^{++}. Moreover, a decrease in F-actin content was not observed in vector transfected cells in spite of the presence of gelsolin in these cells. The difference in the response of these two groups of cells to the ionophore

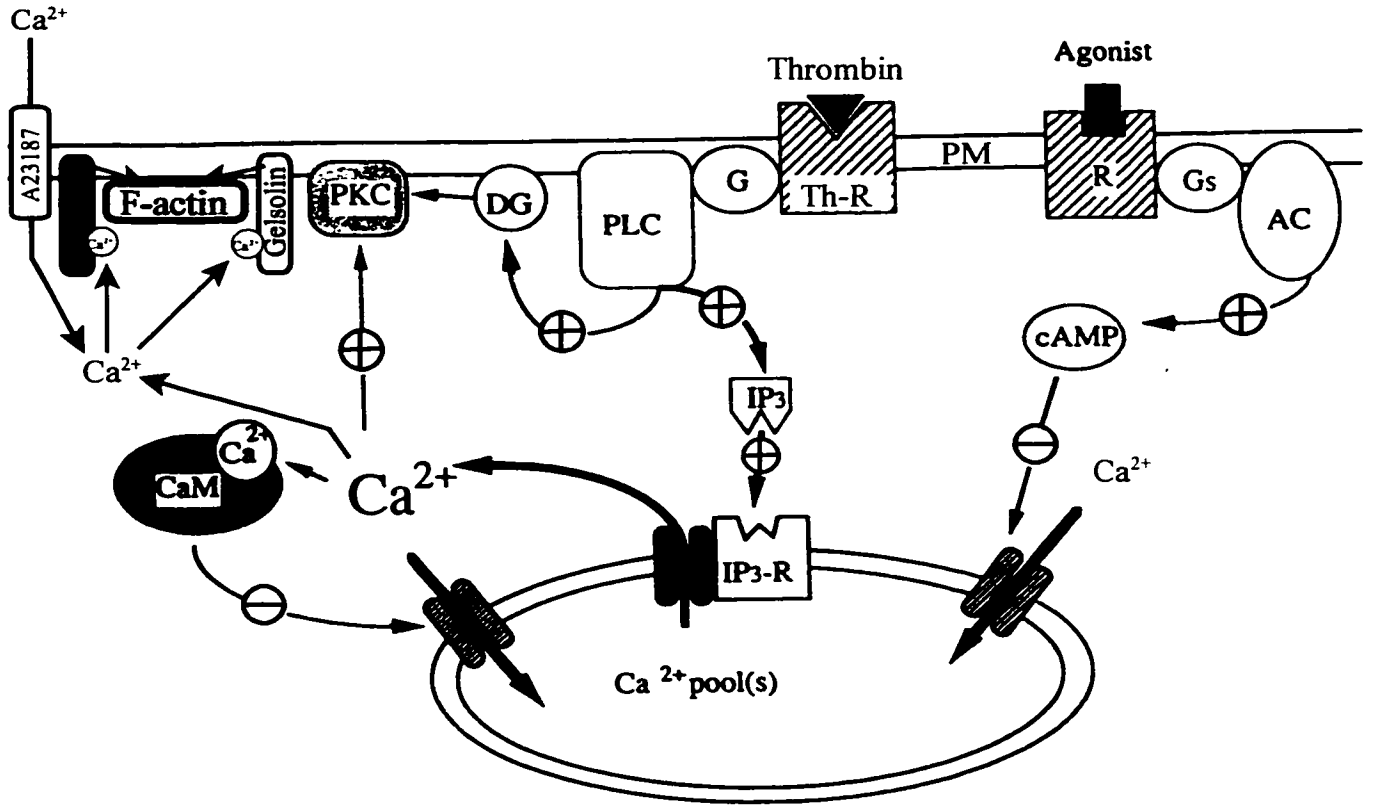

Fig. 4.1: Schematic representation for ionophore A23187 and thrombin-induced Ca^{2+}oscillations in normal megakaryocytes. The (+) marks on the arrows indicate the signal which acts to raise $[Ca^{2+}]i$, and the (-) marks indicate the signal which acts to reduce $[Ca^{++}]i$. AC, adenylate cyclase; G, GTP-binding protein; DG, dyacylglycerol; PLC, phospholipase C; PM, plasma membrane; R, receptor. (Modified from: Uneyama et al., 1993).

might be due to the fact that the intracellular Ca^{++} concentration reached upon ionophore treatment was only high enough to stimulate the over-expressed scinderin.

It has also been shown that cancer cells express low levels of gelsolin (Kwiatkowski, review, 1999). For example, gelsolin expression is down-regulated in 60-90 % of tumours during carcinogenesis of the colon, stomach, bladder (Kuzumaki et al., 1998), breast (Asch et al., 1996), and lung (Dosaka-Akita et al., 1997). However, in other cases such as small-cell lung carcinoma, gelsolin was found to be moderately-to-highly expressed, and present in focal-adhesion complexes, which seem to be involved in cancer cell migration and invasion (metastasis). Thus, higher gelsolin expression might be a prognostic factor for cancer recurrence (Shieh et al., 1998). Moreover, in trans-endothelial invasive cells, lower expression of gelsolin could not be observed when compared to non-invasive cells (Brandt et al., 1999). Therefore, it has been postulated that separate mechanisms should exist for oncogenesis and metastasis (Brandt et al., 1999). In other cell types such as endometrial and ovarian cancer cells, the presence of gelsolin in well-differentiated cells, and its absence from poorly differentiated cells, suggests a late event in tumorigenesis (Afify and Werness, 1998). In addition, the absence of gelsolin expression in normal proliferative glands suggests a more complex relationship than that observed in tumours and that gelsolin level regulation may be associated with either high proliferation rate or state of differentiation (Afify and Werness, 1998).

Another of the known functional properties of gelsolin is its recently discovered role in apoptosis. Kotakhota et al. (1997) have demonstrated that in cultured neutrophils, caspase-3,

a cysteinyl protease, was implicated as a key mediator of apoptosis. This is because of the proteolitic effect of caspase-3 on gelsolin. In vivo gelsolin is cleaved by caspase-3 in cells stimulated by the Fas ligand (Kotakhota et al., 1997). Thus activated, gelsolin severs actin filaments in a Ca^{++}-independent manner (Kotakhota et al., 1997). Moreover, expression of the gelsolin cleavage product in multiple cell types causes the cells to round up, detach from the matrix and undergo nuclear fragmentation, common processes in apoptosis (Geng et al., 1998). Neutrophils from mice lacking gelsolin (knock out) also have delayed onset of blebbing and DNA fragmentation, following apoptosis induction (Kotakhota et al., 1997). Thus, caspase-3-mediated cleaved gelsolin is a physiological effector of morphologic change during apoptosis (Kothakota et al., 1997). Moreover, gelsolin-null mice had about twice the number of circulated neutrophils as wild-type mice (Kothakota et al., 1997). When other cell systems such as smooth muscle cells were exposed to pro-inflammatory cytokines, gelsolin fragmentation mediated by activated caspase-3 was produced (Geng et al., 1998). This was accompanied with a reduction in F-actin content and a marked disruption of cell structure (Geng et al., 1998). Moreover, adenovirus-mediated transfection of the N-terminal generated gelsolin fragment into smooth muscle cells altered cell morphology, reduced cell viability, increased the number of TUNEL-positive cells, and promoted inter-nucleosomal DNA fragmentation (Geng et al., 1998). During DNA fragmentation, the Ca^{++}-insensitive gelsolin fragment would contribute indirectly, by triggering mechanisms ending in nuclease activation (Davoodian et al., 1997). On the other hand, in other cell systems such as Jurkat cells, over-expressed gelsolin strongly inhibits apoptosis induced by anti-Fas antibody, C_2-ceramide or

dexamethasone (Ohtsu et al., 1997). This effect is not accompanied by changes in F-actin content (Ohtsu et al., 1997). This suggests that alterations in the actin network do not contribute to the apoptotic inhibitory effect of gelsolin. Due to the fact that over-expressed scinderin in MEG-01 cells induces disruption of the cytoskeleton (lower F-actin content), it is tempting to speculate that the apoptosis observed in these cells (increased percentage of TUNEL positive cells) would occur through both cytoskeleton disruption (mimicking the effects of cytochalasins) (Takada et al., 1996) and down-regulation of gelsolin levels (Azuma et al., 2000). Moreover, gelsolin was found, through its binding to PIP_2 (phosphatidylinositol 4,5-bi-phosphate), to strongly inhibit caspases-3 and 9 activities (Azuma et al., 2000). Scinderin also has the property of binding PIP_2 (Rodríguez del Castillo et al., 1992). Therefore, it is tempting to speculate that the over-expressed scinderin in MEG-01 cells would bind most of the available PIP_2 molecules. This, in turn, would impede gelsolin from inhibiting the activation of the caspases.

Finally, decreased gelsolin levels in MEG-01 cells could be either the result of scinderin over-expression or the result of low levels of F-actin. More likely, it would be the result of low levels of F-actin, a condition that might regulate the expression of gelsolin since it has been shown that resting platelets from knock-out mice lacking gelsolin have 33 % more F-actin than wild-type platelets (Barkalow et al., 1996).

4.(2). Decrease in proliferation induced by scinderin expression in MEG-01 cells

In order to understand the effects that occurred in scinderin-expressing MEG-01 cells in comparison to their counterparts transfected with vectors only, we first have to consider the fact that conditions in which the effects triggered by scinderin were observed in our experiments were those of cells growing in a serum-containing culture medium. The serum used contained cytokines and growth factors that through their receptors and activation of signal transduction pathways produced effects, mainly of growth and proliferation (Lieberman, review, 1998; Smithgall, review, 1998). These signals would encounter the obstacle of a disrupted cytoskeleton or other modifications in the case of scinderin-expressing cells. Therefore, in many cases, these modifications would translate into disrupted or modified signals, which would cause the phenotypical and biochemical changes observed in these cells.

Among the growth factors present in serum which are important in the proliferation and differentiation of normal megakaryocytes are IL3 (interleukin-3), IL6, IL11 and Tpo (thrombopoietin) (Jackson et al., 1997). Of these, the most important and complete for megakaryocyte growth and development is Tpo. This cytokine has a pleiotropic effect, dependent on the stage of megakaryocyte differentiation. At early stages it mainly promotes proliferation, and at later stages it mainly promotes maturation, pro-platelet formation and platelet release (Gurney et al., 1994). In megakaryoblastic leukemic cell lines such as MEG-01 cells, because of their poorly differentiated state, Tpo mainly promotes proliferation (Gurney et al., 1994). Tpo is also (like other cytokines, especially IL3) associated with

survival (Lotem et al., 1991), promoting viability by inhibiting apoptosis (Borge et al., 1996; Ratajczak et al., 1997).

Tpo transduces its effects through its receptor, Mpl, which is a tyrosine kinase receptor, and this is done by tyrosine phosphorylation through the Jak/Stat pathway (Miyakawa et al., 1996, see fig. 4.2). This pathway, which is activated by a great number of cytokines, growth factors and hormones, involves ligand-dependent activation of a particular class of receptor-associated tyrosine kinases, the Jak proteins (Finbloom and Larner, review, 1995). When activated, these proteins phosphorylate themselves as well as receptor components, creating recruitment sites for Stat transcription factors. Once the Stat is phosphorylated, it dissociates from the receptor-Jak complexes and translocates to the nucleus, where it participates in transcriptional gene activation (Pellegrini and Dusanter-Fourt, 1997). The Jak/Stat pathway is mainly a proliferative one (Finbloom and Larner, review, 1995). This is supported by the finding that in MEG-01 and DAMI cell lines (both megakaryocytic), constitutive activation of the Jak2/Stat5 signal transduction pathway correlates with growth factor-independent proliferation (Liu et al., 1999). Apart from the Jak/Stat pathway, Tpo was also found to activate (through phosphorylation of Shc and Vav) the Ras/Raf-1/MEK/ERK pathway, a transduction system known to be involved in proliferation and differentiation (Nagata and Todokoro, 1995).

We have studied the ERK/MAP kinase (described below in section 4.6) and the Jak/Stat pathways in our experiments on MEG-01 cells. As described in the results chapter, Stat 5 levels are significantly lower in scinderin-positive cells than in cells transfected with vector

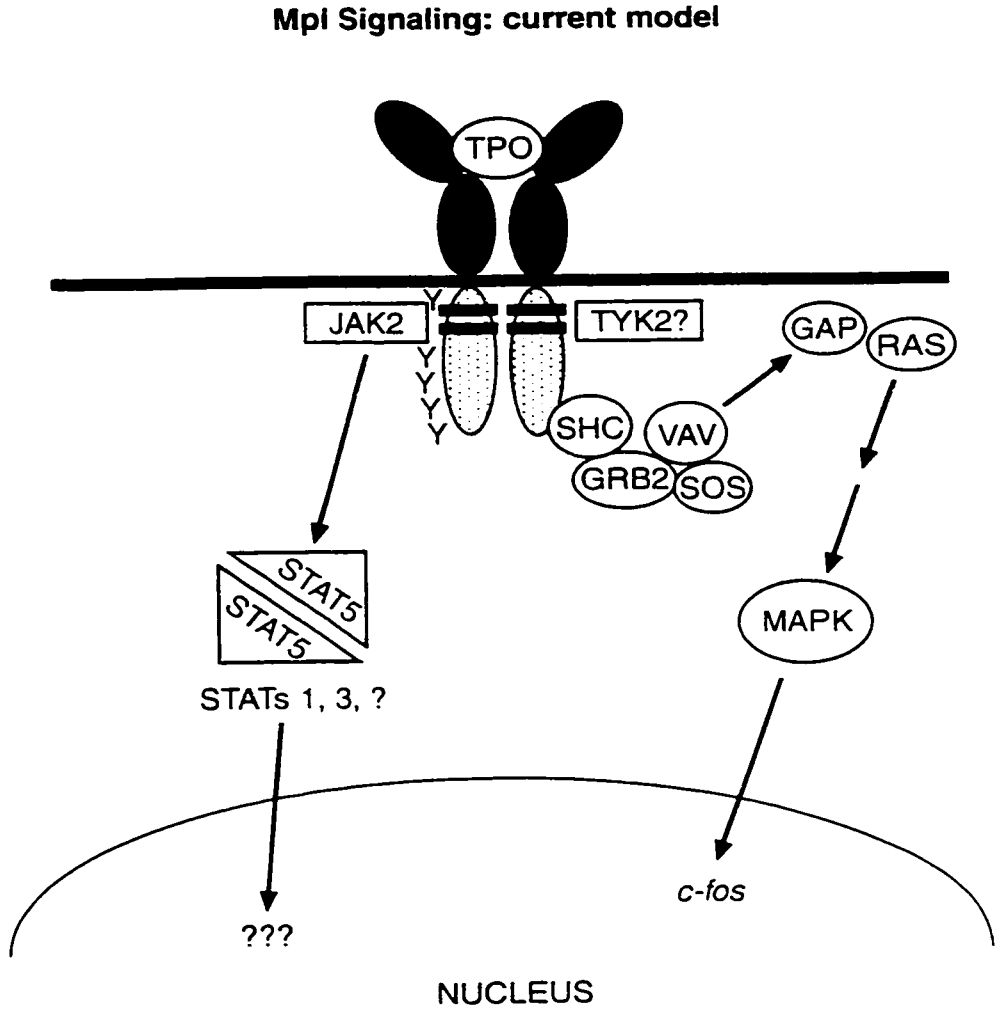

Fig. 4.2: A model of current understanding of how Mpl transduces its proliferative signal. Clear evidence for phosphorylation of JAK2 and TYK2 has been provided, although the physiologic relevance of the latter is not yet certain. Also, evidence has been provided for phosphorylation of STAT5, possibly STAT 1 and STAT3, SHC, and members of the MAPK pathway likely involved in the response. (From: Kaushansky et al., 1997).

alone, with the former expressing less Stat 5. A possible interpretation for this finding is that because scinderin-expressing cells proliferate less than control cells, a lesser amount of this transcription factor would be needed. The decreased proliferation observed (and by extension for lower Stat 5 levels) would be due, at least in part, the low levels of F-actin in these cells, because it is known that filamentous actin plays an important role in cell division (Cao and Wang, 1990). On the other hand, the decrease in proliferation might also be due to the induction of polyploidization and endomitosis that these cells have entered.

4.(3). Scinderin expression in MEG-01 cells induces endomitosis and polyploidization

The development of cultured megakaryocytes is influenced by factors present in the serum (Tpo). Normal serum stimulates both mitosis and endomitosis. Aplastic serum (higher Tpo concentration) favours mitosis over endomitosis, with smaller megakaryocytes having lower levels of ploidity. Thus, both mitotic and endomitotic events in megakaryocytopoiesis are influenced by factors present in the serum (mainly Tpo), with a strong correlation between ploidity and cell diameter (Arriaga et al., 1987).

During the process of polyploidization, megakaryocytes enter mitosis and progress through normal prophase, prometaphase, metaphase and up to anaphase A, but they do not enter anaphase B, telophase and cytokinesis (Nagata et al., 1997). There is no outward movement of the mitotic spindle poles to separate the sister chromatides during anaphase B. Thus the re-assembly of the nuclear envelope encloses all the genetic material at anaphase and then skips telophase and cytokinesis (Nagata et al., 1997). This indicates that megakaryocytes

must have a unique regulatory mechanism in anaphase, e.g., factors regulating anaphase such as microtubule and microfilament motor proteins, that might be involved in the polyploidization process (Nagata et al., 1997; Vitrat et al., 1998). Actin networks might play a role in polyploidization, because it has been demonstrated that treatment with cytochalasin B or D, compounds that depolimerize F-actin, induces polyploidization (Chatelain et al., 1992) and, indeed, scinderin-positive cells had a decrease in F-actin. The described effect of cytochalasins is not accompanied by increased expression of platelet antigens (Mouthon et al., 1994) as is the case in the scinderin-expressing cells described here. In the UT-7 megakaryocytic leukemic cell line, treatment with PMA increases polyploidization, a response accompanied by an increase in p21, and consequent inhibition of the activity of the CDC2/cyclin B complex (M-phase promoting factor) (Kikuchi et al., 1997). Also in the DAMI megakaryocytic cell line, PMA-induced polyploidization is accompanied by a several-fold increase in cyclin D (promoter of entry into the S-phase). This indicates that cyclin D is up-regulated in differentiating megakaryocytic cells and it may contribute to differentiation causing growth arrest (Wilhide et al., 1995). Whether similar increases in p21 and cyclin D expressions are taking place in scinderin-positive cells, which, as we have observed, also underwent a process of polyploidization, remains to be established.

4.(4). Scinderin expression in MEG-01 cells induces apoptosis after several days in culture

The decrease in proliferation observed in scinderin-positive cells was also accompanied by an increase in apoptosis as revealed by the TUNEL assay. Apoptosis is the physiological fate

of normal megakaryocytes (Zauli et al., 1997) and these cells enter programmed cell death at the end of their maturation and differentiation cycle, just before they release platelets (Zauli et al., 1997). Similarly, cells expressing scinderin, after polyploidization and expression of platelet-specific antigens (e.g., glycoprotein IIb/IIIa or fibrinogen receptor), undergo apoptosis (18-23 days in culture) in considerable proportion. This effect is accompanied by the release, at 18-24 days in culture, of cytopasmic particles (platelet-like particles). Apoptotic processes were not seen (or seen in very small proportion) in cells transfected with vector alone.

What might be the possible mechanisms through which scinderin, induces apoptosis? Cytoskeletal network disruption results in fundamental changes in cellular structure and function, including morphological alteration, disruption of mitosis, and consequently, cell death (Rao et al., 1999). Another consideration is the fact that apoptosis is also triggered by modified cell cycle progression (polyploidy) (Rudin and Thompson, review, 1997). Outlined below are several hypothetical mechanisms by which scinderin could trigger apoptosis:

1. Several protein kinases become activated upon exposure to 3-phosphorylated inositol lipids. Among them is PKB/AKT (protein kinase B/product of the retroviral oncogene *v-akt*), which interacts with 3-phosphorylated phosphoinositides via their pleckstrin homology domains (Coffer, Jin and Woodgett, review, 1998). Once targeted to the membrane, it becomes phosphorylated at two residues, which relieve intra-molecular inhibition, allowing the activated complex (kinase-phospholipid) to dissociate and modify its target, Bcl-2

(Coffer, Jin and Woodgett, review, 1998). Bcl-2 inactivates ICE-like proteases (caspases), positive regulators and effectors of apoptosis (Ahmed et al., 1997). In view of the fact that in our cells scinderin is over-expressed, (i.e. present in excess), it is possible that the available phosphoinositides in the cells are bound to scinderin molecules. This might render them unavailable for activating PKB, and in consequence render the cells more prone to apoptosis.

2. The fact that signalling through phosphoinositide 3-kinases (PI3K) is important in regulating the balance between mitogenesis and apoptosis (Yao et al., 1995, 1996; Minshan et al., 1996; Scheid et al., 1996) is confirmed by the role of the tumour suppressor PTEN (phosphatase and tensin homologue). This molecule, present in all mammalian cells, is indeed a major tumour suppressor in human cancer. It has been shown to act through PI3K signalling, by removing the 3-phosphate from 3-phosphoinositides (Leevers, Vanhaesebroeck, Waterfield, review, 1999). Could scinderin mimic the effect of PTEN by binding to 3-phosphoinositides among others? It is tempting to speculate that PTEN expression could thus be lower in MEG-01 cells expressing scinderin.

3. The focal-adhesion kinase or FAK, is a 125 KDa protein tyrosine kinase (PTK), whose name is derived from its intracellular localisation (Schaller et al., 1992). It is an element of a signalling pathway regulated by cell surface receptors called integrins (Shaller and Parsons, 1993, 1994). Integrins are hetero-dimeric, trans-membrane proteins that can simultaneously bind to proteins of the extracellular matrix (ECM), (e.g., fibronectin), and to components of the actin cytoskeleton, e.g. vinculin, talin and alpha-actinin (Burridge et al., 1988). In

addition to their role in cell adhesion to ECM and in anchoring the cytoskeleton, integrins can transduce extracellular cues into cytoplasmic signals, including tyrosine phosphorylation and enzymatic activation of FAK (Burridge et al., 1992; Hanks et al., 1992; Kornberg et al., 1992). Structurally, FAK contains multiple binding sites for cytoskeletal and signalling proteins. Its role is to facilitate the assembly of multi-protein complexes at focal adhesions, and to mediate signal transduction from integrins and growth factor receptors (Hildebrand et al., 1993). It is known that the activation of FAK through its phosphorylation is part of the processes of signal transmission from the extracellular environment to the nucleus (via PKC, PIK and others). This depends, in part, on the integrity of the actin cytoskeleton (Schaller, review, 1996). It is known, for example, that vinculin, a cytoskeletal protein which acts as a bridge between integrins and F-actin through talin (which also binds FAK), depends on phosphatidyl-inositol-4,5-bisphosphate, to unmask its actin and talin binding sites (Gilmore and Burridge, 1996). In this regard it is tempting to speculate that over-expressed scinderin in MEG-01 cells would bind, to a great extent, to phosphoinositides, thus rendering them unavailable for talin binding. Consequently, focal-adhesion complexes would not be assembled; FAK would not be activated; and survival signals from cytokines or other signals from integrin agonists (ECM) would not be transduced to the nucleus, thus triggering apoptotic processes (Gilmore and Burridge, 1996). In megakaryocytes, cytochalasin D, which disrupts the cytoskeleton, abolishes the phosphorylation of FAK upon stem cell factor stimulation (Hiregowdara et al., 1997), producing cell detachment (an apoptotic process).

FAK activation seems a cell-survival factor and FAK de-phosphorylation may lead to decreased activity of signalling cascades that otherwise would block the apoptotic machinery. We can conclude that cytoskeleton integrity is a check-point for transmitting some signals from outside the cell to the nucleus. In this project we have observed that in their days in culture, MEG-01 cells expressing scinderin remained, for the most part, attached to the flasks, and on days 12 to 14 in culture, cells began to detach from the culture surface. Cell detachment was accompanied by an increase in TUNEL positive cells in scinderin-expressing cells. We can speculate that disruption of cytoskeleton and consequently of FAK activity could, for the most part, be involved in triggering apoptosis in scinderin-expressing MEG-01 cells. Finally we can assume that FAK levels would probably be low in scinderin positive cells after 12-14 days in culture.

4. The PAK/MEKK-SEK/JNK-SAPK pathway is activated mainly by cytokines, hormones and various forms of stress (for example, cytoskeletal disruption), utilizing p21 proteins of the Rho family (Rho, Rac, Cdc42, etc) (Smithgall, review, 1998; Janmey, review, 1998; Denhardt, review, 1996). The disruption of the F-actin cytoskeleton, as observed in MEG-01 cells expressing scinderin, could, indeed, induce cellular stress with activation of this pathway. The increase in Rac2 expression observed in scinderin-positive cells may be a cellular response to increase actin polymerization as the result of decreased F-actin cellular levels in these cells. Alternatively, an increase in Rac might be the result of the decreased expression of gelsolin observed in these cells, since it has been demonstrated that there is a reciprocal correlation between gelsolin and Rac expression (Azuma et al., 1998). PAK (p21-

activated kinase) has been found to be an effector of Rac (Minden and Karin, review, 1997) and scinderin-positive cells showed increases in the levels and activity of PAK as well as of JNK2, a factor downstream of PAK in the cascade PAK/MEK-SEK/JNK-SAPK. Activation of JNK is also involved in the activation of c-jun in cells entering apoptosis (Lieberman, Gregory and Hoffman, review, 1998; Smithgall, review, 1998) and in hematopoietic precursor cells during their development into mature cells (Lieberman, Gregory and Hoffman, review, 1998). In this regard, it has been shown that c-jun/c-fos (also known as AP-1 factor) are highly expressed in terminally differentiated megakaryocytic lineages (Kreipe et al., 1986; Panterne et al., 1992). The increases in JNK2, c-jun, c-fos and apoptosis in cells expressing scinderin are clear indications of the activation of this pathway This mechanism of apoptosis, which would, very probably, be triggered by scinderin expression, in MEG-01 cells, will be described in more detail in section 4.(8).

4.(5). The apoptotic process in scinderin expressing MEG-01 cells is followed by the production and release of cytoplasmic fragments (platelet-like particles), which are functional

Leven and Yee (1987) found that treatment of normal megakaryocytes in culture with cytochalasins D or B (inhibitors of actin polymerization), in presence of trombocytopenic plasma (Tpo above normal levels) caused these cells to form processes of 3 to 6 μm diameter. Consequently they proposed a model of platelet formation in which a factor stimulating fragmentation present in thrombocytopenic plasma (Tpo) would bind to a receptor on the surface of the mature megakaryocyte. This binding triggers changes in the

organization of actin filament networks (disassembly of microfilaments performed by actin regulatory proteins). Processes that subsequently form may have a core of microtubules which may elongate by polymerization of tubulin, forming along the processes microtubule rings which may control the intervals at which pro-platelets (detached processes) break into platelets.

It is logical to assume that scinderin would have a role in facilitating process formation and pro-platelet release. Scinderin would even be involved in the breaking of pro-platelets into platelets, as we observed the co-localisation of scinderin and F-actin in the cortical actin ring as well as in the blebs or protrusions formed, in scinderin-positive cells.

It is also known, as mentioned above, that the process of platelet formation in normal megakaryocytes is a consequence of cell senescence (Zauli et al., 1997). In fact Zauli et al. (1997) observed, in CD34-derived megakaryocyte progenitors cultured in the presence of Tpo (100 ng/ml), an increase in apoptotic cells at day 18 in culture and another increase in platelet release at day 21. According to our results, scinderin-expressing cells, after polyploidization and expression of platelet markers (e.g. glycoprotein IIb/IIIa or fibrinogen receptor), released cytoplasmic particles, by day 18-24 in culture. It is known that MEG-01 cells can, spontaneously, release a small number of cytoplasmic particles (Takeuchi et al., 1991). However, cells expressing scinderin produced and released cytoplasmic particles in numbers two or three orders of magnitude greater. Choi et al. (1995) found that platelets produced by culturing megakaryocytes in vitro are functional. They express glycoproteins Ib and IIb/IIIa, contain microtubule coils equal in size to those found in plasma-derived

platelets, and aggregate in response to both thrombin and ADP plus fibrinogen. Finally they found that this aggregation is specifically inhibited by the addition of a function-blocking anti-GPIIb/IIIa antibody.

We found that the cytoplasmic particles produced by scinderin-expressing cells behave very much like platelets:

1. They expressed the fibrinogen receptor (GPIIb/IIIa).

2. They contained a high affinity serotonin uptake system which could be blocked by fluoxetine, a known inhibitor of serotonin and dopamine transporters (Omenn and Smith, 1978). This is evidence of their functionality as a consequence of megakaryocyte maturation. Megakaryocytes do not contain serotonin, and platelets uptake and store serotonin from enterochromaffin cells after their release from megakaryocytes (Toh , 1954). However, megakaryocytes have the capacity to accumulate exogenously supplied serotonin (Fedorko, 1977). Immature megakaryocytes in culture, when incubated in the presence of serotonin, show very few dense granules (Wojenski and Schick, 1993). There is a posibility that they are unable to retain serotonin because of a lack of adenine nucleotides, which are present in mature megakaryocytes (Rudrich et al., 1980), and are thought to form a complex with serotonin, thereby, preventing its diffusion out of the granule. Whether the particles produced by the scinderin-expressing cells contain, apart from serotonin, adenine nucleotides in their dense core granules, remains to be established.

3. In presence of plasma (fibrinogen) the particles responded to thrombin with aggregation, an effect which was also inhibited, to a great extent, as in platelets (Woods et al., 1986), by

antibodies against GPIIb/IIIa (anti-CD41a). Previous investigators have shown that exposure of platelets to thrombin causes an increase in the number of GPIIb/IIIa receptors available on the platelet surface through externalization of receptors from other compartments in the platelet (Wencel-Drake et al., 1984). Woods et al. (1986) found that platelets contain at the SCCS (surface-connected canalicular system), a large internal sequestered pool of GPIIb/IIIa which is not accessible to molecules of big size (antibodies for example). If thrombin stimulation occurred when all external receptors were bound to GPIIb/IIIa antibody, then the free receptors externalized by thrombin would be able to produce some aggregation. In this regard, the partial inhibition of aggregation observed in particles produced by scinderin-positive cells, would reflect a similar situation. The fact that the particles produced by scinderin-positive cells seem to express GPIIb/IIIa in more than one compartment (as in platelets) would be another indication of functionality, and consequently, maturation.

4. Similar to platelets (White and Sauk, 1984), these particles showed a circular array of microtubules. In this regard, it is known that the processes (proplatelets) formed in megakaryocytes in culture posses both longitudinal (intervals) and circumferential or coil (future platelets) microtubules. (Choi et al., 1995). Therefore, the distinctive morphology (coils) of microtubules in particles released by scinderin-positive MEG-01 cells is a clear indication that they are very similar to platelets formed and released from megakaryocyte processes.

We can conclude that conditions which decrease filamentous actin, as in scinderin-positive MEG-01 cells, would favour formation and release of platelets. However, the expression of

scinderin in MEG-01 cells induced cellular changes other than the one produced by the simple treatment of cells with cytochalasins. Moreover, additional mechanisms operating in scinderin-expressing cells, such as activation of specific transduction pathways, might be responsible for the maturation and differentiation changes observed. Transduction pathways involved in cell proliferation, differentiation and apoptosis have been described (Smithgall, review, 1998; Minden and Karin, review, 1997; Sugden and Clerk, review, 1997; Shivdasani and Orkin, review, 1996; Janmey, review, 1998; Finbloom and Larner, review, 1995). These pathways are not completely understood due to the fact that several of their components can stimulate effectors in more than one pathway (cross-talk). Nevertheless, we have made attempts to understand the transduction mechanisms involved in scinderin-expressing MEG-01 cells by measuring the levels and activities of several of these transduction factors.

4.(6). Expression of scinderin in MEG-01 cells induces activation of the ERK/MAP kinase pathway in early days in culture

The ERK/MAP kinase signal transduction pathway plays a role in relaying mitogenic signals from cell surface receptors to the nucleus to stimulate G0/G1 transition (Seger and Krebs, 1995). Thus, signalling via receptor tyrosine kinases (growth factors and mitogens) leads to a chain of biochemical events among which are included sequential activation of p21 Ras, Raf, MEK, ERK/MAPK, and the transcription factor Elk1 (Hill and Treisman, 1995; Cobb and Goldsmith, 1995). Paradoxically, the ERK/MAP kinase pathway also plays a role in cellular differentiation in some systems. For example, nerve growth factor (NGF) induction of neuronal differentiation in PC12 pheochromocytoma cells requires sustained activation

of ERK/MAP kinase (Pang et al., 1995). In hematopoietic cell differentiation, the role of ERK/MAPK pathway has not yet been fully characterized, but it is known to be activated by PKC and to be involved in some models of cellular differentiation. For example, Racke et al. (1997) found that a sustained activation of the ERK/MAPK pathway by PMA is required for megakaryocytic differentiation of K-562 cells. In scinderin- expressing MEG-01 cells, there were signs of early activation of the Raf, MEK, ERK/MAPK pathway in the form of the increased expression of platelet GPIIb/IIIa (CD41a) between days 4 and 8 in culture. The fact that in the present experiments, compound PD98059, a known inhibitor of MEK (Racke et al., 1997), inhibited GPIIb/IIIa expression in both vector-transfected and scinderin-positive cells, is an indication of the involvement of this cascade in the expression of platelet antigens.

4.(7). Expression of scinderin in MEG-01 cells induces modified expression of the ras and rho family of small p21 GTPases

As has been mentioned before, small p21 GTPases are responsible for morphological changes in the F-actin cytoskeleton (F-actin polymerization) that drive cell movement, formation of fillipodia, lamellipodia and membrane ruffles (Boivin et al., review, 1996; Narumiya, review, 1996; Zigmond, review, 1996). They are also involved in the formation of stress fibres and focal-adhesion-complexes (sites of cell attachment to extracellular matrix) (Narumiya, review, 1996, see fig. 4.3). In addition, the p21GTPases play a role in other functions of the cell such as the activation of intracellular pathways (ERK/MAP kinase

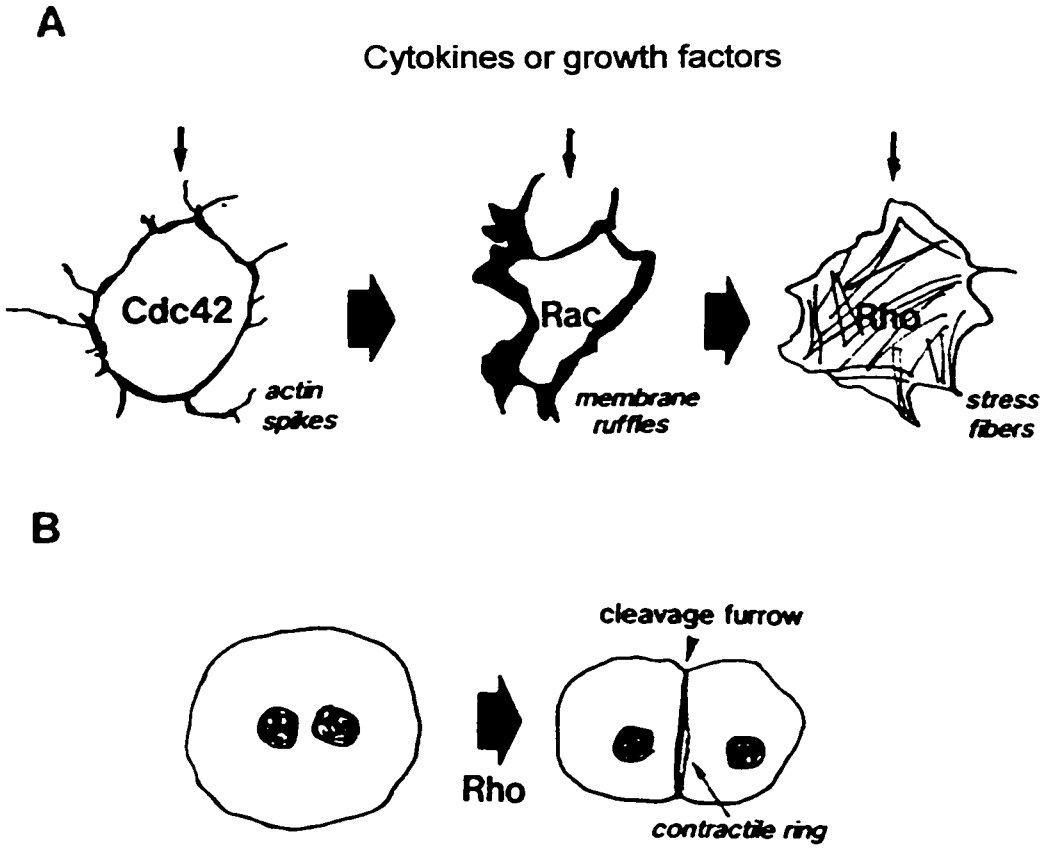

Fig. 4.3: A: changes in cell morphology and the actin cytoskeleton induced by the Rho GTPase cascade. Each of the Rho family of GTPases, Cdc42, Rac and Rho, when activated, directs the organization of a specific type of actin cytoskeleton to induce the characteristic morphological changes. Furthermore, these Gtpases are activated in the sequence Cdc42--Rac--Rho in the cells. This sequence is associated with the time-dependent change in cell morphology from filopodia to lamellipodia to stress fibers. B: involvement of Rho in cytokinesis. Rho is activated during M-phase following nuclear division and induces the formation of a contractile ring in the cleavage furrow. Rho action is required throughout cytokinesis, because inactivation would cause the loss of the preexisting contractile ring. (Modified from: Narumiya, review, 1996).

and JNK kinase), through which they regulate growth, differentiation and cell death (Boivin et al., review, 1996; Zigmond, review, 1996).

1. Ras expression

Ras protein is bound to cell membranes, and in close proximity to G protein-coupled and tyrosine kinase receptors (Peeper and Bernards, review, 1997). Growth factors stimulation induces activation of Ras, which, in turn, stimulates proliferative pathways (ERK/MAP kinase), inducing exit from phase G0 (quiescence) of the cell cycle (Peeper and Bernards, review, 1997). Mutated Ras genes with oncogenic potential were first detected in human tumours (Symons, review, 1996a). Cell transformation by Ras and other oncogenes is accompanied by a dramatic alteration in the organisation of the actin cytoskeleton (Symons, review, 1996a). These changes are reflected in the loss of proteins associated with the actin cytoskeleton, including alpha-actinin, vinculin and certain tropomyosin isoforms (Symons, review, 1996a). If the normal levels of any of these proteins are restored, morphological transformation is inhibited and tumorigenesis decreases, indicating that the reorganisation of the actin cytoskeleton is an essential component of cell transformation (Symons, review, 1996a).

Could scinderin be a missing cytoskeleton-associated protein in transformed cells?

Efficient transformation by Ras requires activation of other direct effectors, in addition to Raf (MAPK pathway), and is inhibited by inactivation of the PI3-kinase pathway (Azuma et al., 1998). Ras activates PI3-kinase directly, and an end product of PI3-kinase activates Rac, which, in turn, activates PI5-kinase (Azuma et al., 1998). This, in turn, produces PI(4,5)P2,

which inactivates F-actin capping proteins such as gelsolin, scinderin, and tensin, thus inducing rapid F-actin polymerization (Azuma et al., 1998). Thus, the ability of activated Ras to stimulate PI3-kinase in addition to Raf is important in Ras transformation of mammalian cells and is essential in Ras-induced cytoskeletal organization (Rodríguez-Viciana et al., 1997; Aspenstrom, review, 1999). On the other hand, Rac, Cdc42 and Rho, quite distinct from Ras, by themselves exhibit limited tumorigenic properties (Rodríguez-Viciana et al., 1997; Aspenstrom, review, 1999).

Several distinct F-actin/PIP2 binding proteins, such as gelsolin, have been shown to suppress Ras-induced malignant transformation when they are over-expressed (Tikoo et al., 1999). Thus, capping of actin filaments at the barbed ends alone would be sufficient to block one of the Ras signalling pathways essential for its oncogenicity. Thus, either F-actin capping or PIP2 binding would be enough for the anti-Ras action of these actin-cytoskeletal proteins (Tikoo et al., 1999). For example, in the fibroblastic NIH3T3 cell line, over-expression of tensin, an F-actin/PIP2 binding protein, induces inhibition of malignant-induced growth (Tikoo et al., 1999).

Another consequence of Ras activation is the stimulation of a kinase called PKB/AKT, through production of PI(3,4)-P2, as a result of PI3-kinase activation. PKB/AKT inhibits apoptosis through activation of Bcl-2 (Fukui, Ihara and Nagata, review, 1998). Thus, Ras signalling through growth factors, apart from inducing proliferation through cytoskeletal changes, among other effects, also inhibits pro-apoptotic mechanisms. The retinoblastoma tumour-suppresser protein (Rb) is an essential G1-specific mediator that links Ras-dependent

mitogenic signalling to cell cycle regulation (Peeper et al., 1997). In growing cells, inactivation of Ras causes a decline in cyclin D1 protein levels, accumulation of the growth-suppressive (non-phosphorylated) form of Rb, and G1 arrest (Peeper at al., 1997). Phosphorylation of Rb by cyclin D1/CDK complexes abolishes Rb-mediated inhibition of transcription factor E2F-1, a molecule which activates genes involved in cell division. An example of this is *c-myc*. Moreover over-expression of E2F-1 in megakaryocytes blocks differentiation during maturation, increasing proliferation and decreasing polyploidization (Guy et al., 1996; Beijersbergen and Bernards, review, 1996; Thompson et al., 1996).

MEG-01 cells expressing scinderin showed a significant decrease in Ras protein levels when compared to vector-transfected cells. This decrease was observed at early stages of culture (4 days). Because over-expressed scinderin would occupy most of the available barbed ends of F-actin, it would somehow impede the action of Ras through Rac to uncap F-actin. Consequently, scinderin capping of actin filaments would impede F-actin polymerization, a condition necessary for proliferation. This would produce a decrease in Ras levels as observed in scinderin-positive MEG-01 cells. A consequence of lower Ras levels would be the down-regulation of PI3-kinase, and this, would be transduced into a decreased production of PI(3,4)P2. On the other hand, it is known that Ras stimulates the production of cyclin D, and thus progression through the G1 phase (Peeper et al., 1997). Through formation of Cyclin D/Cdk complexes, cyclin D stimulates phosphorylation of Rb, thus rendering it inactive to impede the action of the pro-proliferative E2F-1 transcription factor (Peeper et

al., 1997). Consequently, lower Ras levels in scinderin-expressing cells would transduce into a less repressed Rb protein. This would be reflected in decreased cell proliferation.

2. Cdc42 expression

This small p21 GTPase induces F-actin assembly that results in protrusions of hair like filopodia (Symons, review, 1996a). Cdc42 is also an upstream regulator of Rac (Symons, review, 1996a) through stimulation of the PI3-kinase (Symons, review, 1996a) (see fig. 4.4). Cdc42, apart from inducing F-actin assembly indirectly through phospholipid production (PI(3,4)P2) and uncapping of F-actin proteins, also induces this process directly through WASP (Wiskott Aldrich syndrome protein) and profilin (Symons et al., 1996b). WASP, which is only expressed in hematopoietic cells (Symons et al., 1996b), is a specific effector of Cdc42 (Symons, review, 1996a). Cdc42 is also reported to have a role in tubulin polymerization. For example, Stowers et al. (1995) found that in T cells, Cdc42 regulates the polymerization of both actin and microtubules (tubulin), and thus promotes the polarisation of the cells towards the antigen. In this regard it is logical to assume that the low levels of Cdc42 found in MEG-01 scinderin-expressing cells would be transduced into lesser tubulin polymerization (nocodazole or vincristine-like effect). This would also explain, at least in part, the polyploidization observed in scinderin-positive cells.

Cdc42 is an upstream regulator of Rac through PI3-kinase (Clark et al., 1998), therefore, an explanation for its lower levels in scinderin-expressing MEG-01 cells could be the result of the higher Rac levels observed in these cells. Under these conditions, Cdc42 would be down-regulated. Another explanation would be that because F-actin is lower in these cells, this

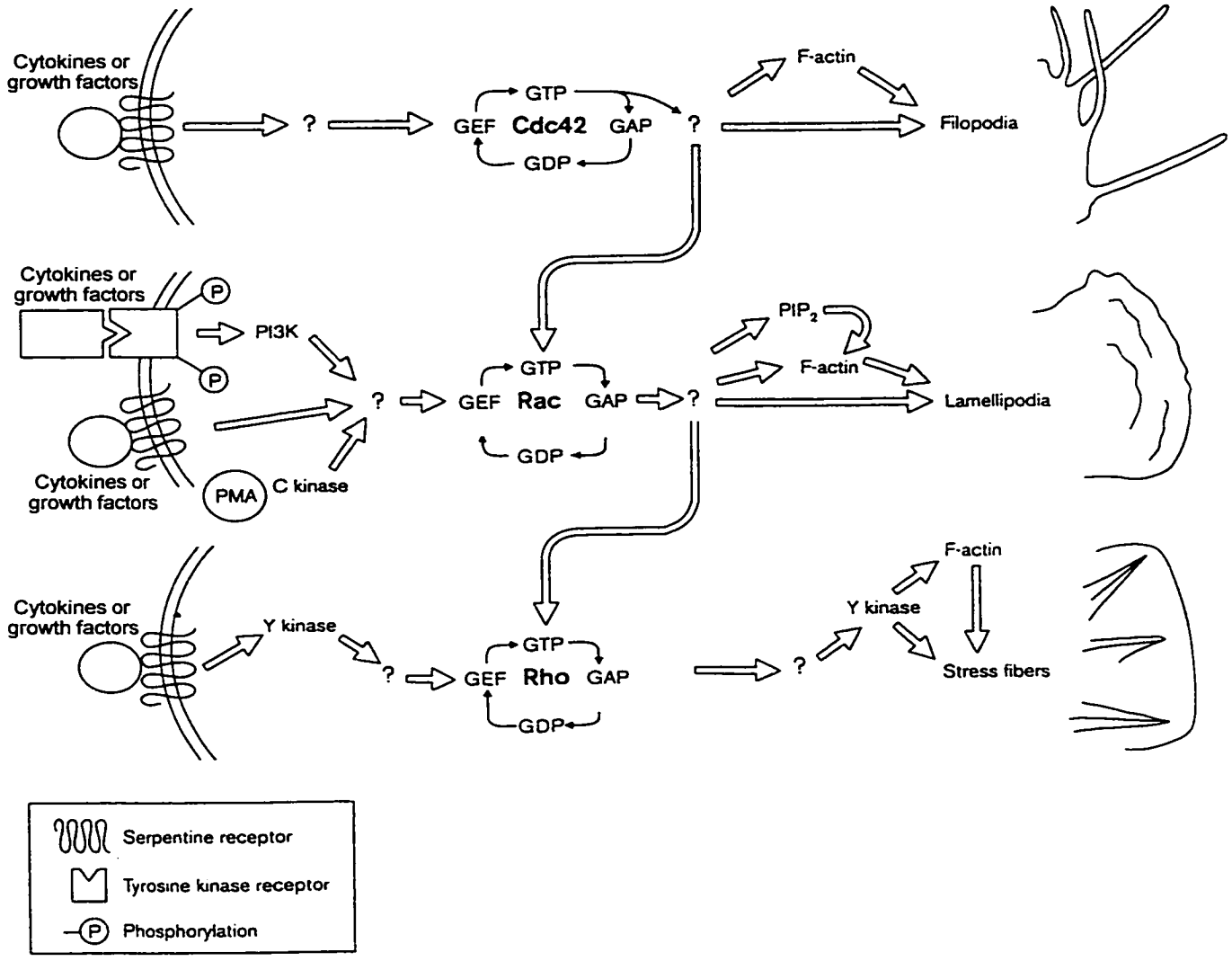

Fig. 4.4: Shematic representation of proposed relationships between receptors, Rho family members, and the cytoskeleton. The receptor for extracellular ligands are serpentine receptors linked to heterotrimeric G proteins, tyrosine kinase receptors, or protein kinase C (C kinase). The kinases are PI3K, C kinase (protein kinase C target of PMA), and Y kinase (the tyrosine kinases). GTP and GDP refer to the nucleotides bound by the Rho family of G proteins. (Modified from: Zigmond, review, 1996).

would be transduced into lower levels of Cdc42 and this would affect its direct interaction with actin through WASP and profilin. Consequently, we can speculate that the levels of WASP and profilin should be higher to account for the low F-actin levels observed in scinderin-positive MEG-01 cells.

3. Rac expression

Growth factors direct actin remodelling through Rac, inducing lamellipodia and pleat-like protrusions, or ruffles, in most cells (Hartwig et al., 1995). In wild-type fibroblasts, EGF (epidermal growth factor) stimulation leads to the activation and recruitment of Rac molecules to membrane sites where ruffling and motility would take place (Hartwig et al., 1995). Rac is then activated at the membrane and this is followed by formation of a multi-protein complex, which includes PI5-kinase (Hartwig et al., 1995). This stimulates PI(4,5)P2 synthesis and the uncapping of gelsolin. This process produces free barbed ends and nucleation sites for actin assembly. The fact that Rac and gelsolin are recruited to the sites of ruffling suggests that there is physical interaction by both with membrane lipids (Arcaro, 1998). In platelets for example, agonists like thrombin lead to activation of Rac, and this, in turn, activates PI5-kinase. The PIP2 generated causes the loss of actin-capping proteins from the barbed ends of filaments, resulting in rapid elongation by the addition of actin monomers (Hartwig et al., 1995). Interestingly, the addition of PIP2-binding peptide derived from gelsolin to platelets, inhibits the actin polymerization induced by Rac (Miki et al., 1998). Up-regulation of Rac in gelsolin-null animals and cells indicates that Rac expression might be the result of a feed-back mechanism that senses cell actin organization (Arcaro, 1998). Re-

expression of gelsolin in gelsolin-null fibroblasts reverts the ruffling response, translational motility and Rac expression to normal (Azuma et al., 1998). We can interprete the increased levels of Rac2, observed in MEG-01 cells expressing scinderin as a result of the down-regulation of gelsolin levels observed in these cells. This would be caused by either scinderin over-expression or the low F-actin content observed.

In summary, it seems that over-expressed scinderin decreases gelsolin expression, by decreasing F-actin or possibly through other mechanisms such as negative feed back at the level of transcription. Therefore, decreased gelsolin levels might be responsible for the increased Rac2 levels observed, due to the known reciprocal regulation between gelsolin and Rac (Azuma et al., 1998). However, increased Rac2 could not induce F-actin polymerization, because the over-expressing scinderin has occupied most of the actin filament barbed ends as well as bound most of the available PIP2 and PIP3. It is tempting to speculate that the over-expressed Rac2 should then be driven to interact with other known Rac effectors such as PAK (p21-activated kinase), triggering signalling to the nucleus.

We can conclude, then, that effects induced by over-expressed scinderin should, in some cases, mimic gelsolin over-expression (low F-actin content transduced in less cell proliferation and higher phospholipid sequestering). In other cases, over-expressed scinderin would mimic gelsolin down-regulation, such as increased Rac levels or induction of apoptosis.

4. Rho expression

Rho is a GTPase that regulates certain actin rearrangements, such as stress fibres, focal-adhesion complexes, cytokinetic contractile-ring formation, (see figs. 4.3, and 4.4) and transcriptional events (Leng et al., 1998; Narumiya, review, 1996; Machesky and Hall, 1997). The view that Rho is involved in oncogenesis is based on observation of its own transforming potential and its close connection to Ras (Boivin et al., 1996). In fact, Rho is essential for Ras-induced transformation (Boivin et al., 1996). Moreover, fibroblasts over-expressing Rho injected in nude mice have been found to produce tumours (Avraham et al., 1990; Perona et al., 1993; Boivin et al., 1996). Unlike Ras, which is, constitutively, a plasma membrane component, Rho GTPases are thought to be at least partially cytosolic (associated with a guanine nucleotide dissociation inhibitor, or GDI), and, therefore, must translocate to the plasma membrane where they would associate with GEF (guanine nucleotide exchange factor) (Hall, review, 1998). Rho is also downstream of Rac, with Rac being able to activate Rho (Arcaro, 1998). This is also evident in gelsolin-null cells, where there is an abundance of stress fibres (Arcaro, 1998).

Rho action on actin polymerization is mediated by PIP5-kinase, which is recruited by Rho to specific sites in the cell (Narumiya, review, 1996). At these sites, PIP5-kinase generates PIP2, which binds to actin-binding proteins, uncapping the barbed ends of F-actin to promote actin polymerization (Narumiya, review, 1996). In fact, Rho recruits PIP5-kinase into a complex at the plasma membrane where localized production of PIP2 initiates actin stress-fiber assembly and focal-adhesion formation (Tapon and Hall, review, 1997). On the other

hand. Rho-binding kinases. such as ROK (Rho-activated kinase). are able to promote formation of focal adhesions and stress fibers when introduced into cells (Clark et al., 1998). These Rho-binding kinases can phosporylate the myosin-binding subunit of myosin-light-chain phosphatase. thereby inactivating it. This results in high levels of MLC phosphorylation (Clark et al., 1998). Phosphorylated MLC induces a conformational change in myosin. increasing its binding to actin filaments and triggering the assembly of stress fibres (Clark et al., 1998). Actin stress fibres are long bundles that end in focal adhesions. points of attachment of the plasma membrane to the extracellular matrix (Lim et al., review. 1996). Focal adhesions contain a complex of proteins including the tyrosine kinases Src. focal-adhesion kinase (FAK). and the actin-binding proteins vinculin. paxillin. alpha-actinin and talin (Lim et al., review. 1996). These proteins are linked to integrins at the cytoplasmic face of the membrane (Lim et al., review. 1996). Sequestration of PIP2 with a micro-injected anti-PIP2 antibody prevented LPA (lysophosphatidic acid)/Rho-induced focal-adhesion assembly and actin stress-fiber formation in Balb/c3T3 cells (a T lymphocite cell line). suggesting that Rho-dependent PIP2 formation contributes to focal-adhesion and actin stress fibre assembly (Gilmore et al., 1996). The fact that Rho intervenes in the formation of focal complexes makes this protein important in cell adhesions and the transmission of signals from integrins to the nucleus (Boivin et al., review, 1996). In detached cells. there is less Rho. or it is inactivated (Boivin et al., review. 1996). Additional evidence for the role of Rho in focal-adhesion assembly was found by Gilmore et al. (1996). who demonstrated that there is a phosphoinositide-regulatable actin-binding site on vinculin (an actin-binding protein that

connects F-actin to integrins), thus indicating the possibility that Rho may promotes vinculin and actin association by stimulation of PI5-kinase (Machesky and Hall, 1997).

Rho also has a role in cytokinesis. The inactivation of Rho completely uncouples nuclear division from cell division (Narumiya, review, 1996). Cytokinesis is carried out by the contraction of the actin contractile ring formed in the cleavage furrow in the middle of the cell body (Narumiya, review, 1996) (see fig. 4.3). Evidence for this was found when working with sperm-induced cytoplasmic division of Xenopus embryos. Cell division in them was completely inhibited by micro-injection of Rho GDI (inhibitory GDP/GTP exchange protein) or C3 (a botulinic toxin that ADP-ribosylates Rho to impair its functioning) (Kishi et al., 1993). Under these conditions, nuclear division occurred normally, but the furrow formation, induced by the contractile ring of acto-myosin just beneath the plasma membrane, was impaired (Kishi et al., 1993), leading to the conclusion that Rho, together with its regulatory proteins, regulates cytoplasmic division through the acto-myosin system.

A role for Rho in megakaryocyte endomitosis was also found, using the botulinic C3 Rho -inactivating enzyme (Takada et al., 1996). The CMK megakaryoblastic leukemia cell line expresses high levels of RhoA. Addition of C3 to the culture medium induced a high frequency of polyploid cells with increased GPIIb/IIIa antigens on the cells (Takada et al., 1996). Cytochalasin B, an actin-polymerization inhibitor, also induced polyploid cells; however, it did not stimulate the expression of GPIIb/IIIa in CMK cells (Takada et al., 1996). This finding suggests that C3-induced increase in the expression of GPIIb/IIIa in CMK cells is not mediated through the actin microfilament disassembly, giving Rho a partially

regulatory role in polyploidization and GPIIb/IIIa expression in CMK human megakaryoblastic leukemia cells (Takada et al., 1996).

Moreover, Yammamoto et al. (1993) found that fibroblasts arrest in the G1/S transition phase following injection of botulinic C3 toxin, which supports the involvement of Rho in cell cycling.

We have found that after 12-14 days in culture, MEG-01 cells expressing scinderin showed low levels of Rho-A when compared to cells transfected with vector alone. The reason for the decrease in RhoA expression should be attributed to low F-actin levels or to the decrease in Ras levels (given that Rho is a closely associated downstream effector of Ras) observed in scinderin-positive cells. However, F-actin and Ras levels were decreased at early days (4) in culture in scinderin-expressing cells. An interesting observation made in our experiments was that from day 14 in culture, scinderin-positive cells began to detach from the flask surface, and to produce and release cytoplasmic particles. This occurred with an increase in apoptosis (see above in section 4.4). Cell detachment would be a consequence of RhoA inactivation, or an indication, that RhoA is down-regulated because it is no longer required to maintain focal complexes. Decreased RhoA levels would also contribute to increased polyploidy and GPIIb/IIIa expression, although increases in both were already detected at early stages of culture. Finally, the marked decrease in proliferation in scinderin-positive cells after 14 days in culture, would fit perfectly with the concomitant decrease in RhoA levels observed at the same time. This, would result in arrest in the G1 phase of the cell cycle.

4.(8). At later stages of culture, MEG-01 cells expressing scinderin undergo increased activation of the c-jun amino-terminal (JNK) pathway, resulting in increased c-jun/c-fos levels

Rac and/or Cdc42 have not been shown to activate MEKK1 directly. The Rac-responsive serine/threonine kinase PAK (p21-activated kinase) acts between Rac and MEKK1 (Minden and Karin, review, 1997). This protein kinase binds GTP-loaded Rac and/or Cdc42 through a specific Cdc42/Rac interacting binding (CRIB) motif, and becomes strongly activated by them in a GTP-dependent manner (Minden and Karin, review, 1997). Activated PAK induces transcriptional up-regulation through activation of JNK and other MAP kinase cascades in appropriate cellular settings (Obermeier et al., 1998). PAK has also been reported to have the ability to phosphorylate Raf. Studies in spleen homogenates indicate that this is done, both *in vitro* and *in vivo*, in residues 338 or 339, the same residues that are phosphorylated in response to Ras, Src and growth factor receptor stimulation(King et al., 1998). This leads to the conclusion that signal transduction through Raf depends on both Ras and activation of PAK (King et al., 1998) (see fig. 4.5).

PAK also has the ability to phosphorylate MEK1 (Schwartz and Baron, review, 1999). This phosphorylation is at a site distinct from the residues phosphorylated by Raf, an usual upstream kinase (Schwartz and Baron, review, 1999). Phosphorylation of MEK1 by PAK enhances the association of Raf with MEK, and over-expressed active PAK greatly enhances the activation of MEK by Raf (Schwartz and Baron, review, 1999).

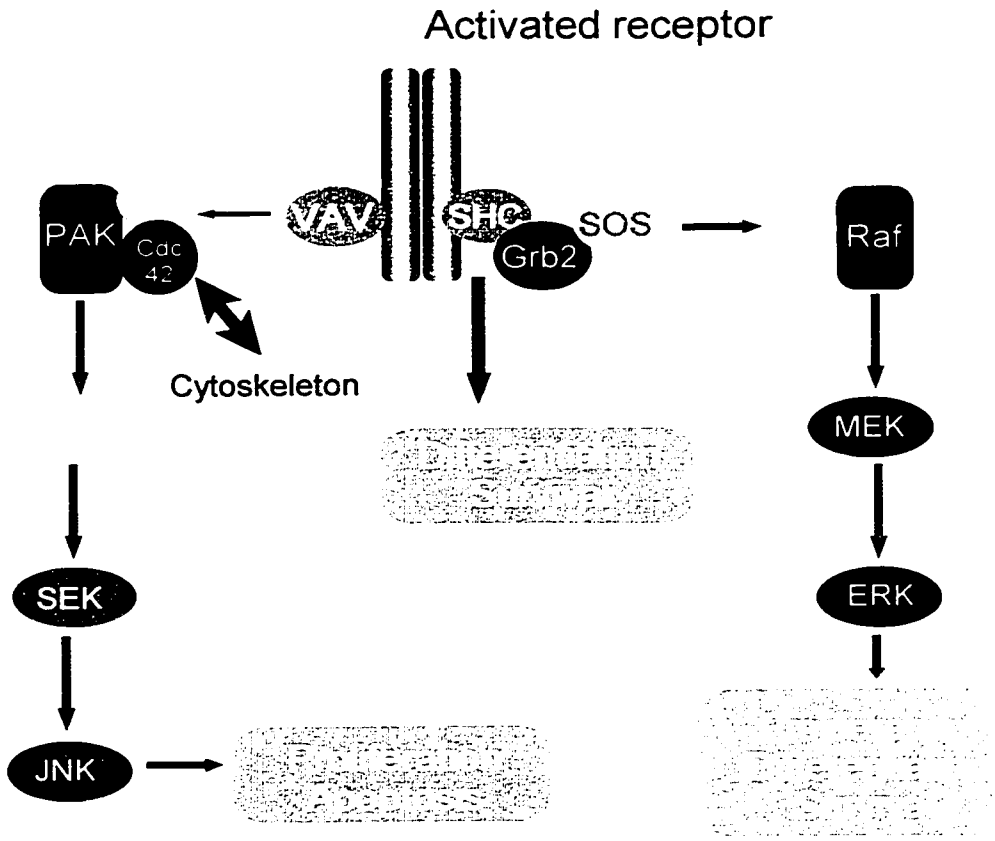

Fig. 4.5: Activation of small G-protein/MAPK pathways downstream of cytokine receptors. Binding of a cytokine to its receptor induces receptor oligomerization and activation of associated tyrosine kinases (kinases not shown for clarity). Tyrosine phosphorylation of the receptor creates high afinity binding sites for effector molecules with SH2 domains, including several involved in the regulation of small G-proteins downstream. Recruitment and tyrosine phosphorylation of Shc promotes association with the Grb2/SOS guanine nucleotide exchange complex for Ras. Once activated, Ras can interact with multiple downstream effectors, including the serine/threonine kinase Raf. Activated Raf leads to the activaton of ERK via the intermediate dual specificity kinase MEK. Activated ERKs can translocate to the nucleus and phosphorylate several transcription factors. This pathway has been shown to influence proliferation, differentiation and survival in hematopoietic cells. SHC might represent a branch point, promoting effects of differentiation and survival that are independent of Ras. Many cytokines also induce tyrosine phosphorylation of VAV, which stimulates its guanine nucleotide exchange activity for Rac and Cdc42. Activation of these small GTPases directly influences cytoskeletal architecture and stimulates a parallel MAPK pathway to the nucleus. This pathway terminates in the activation of c-jun N-terminal kinase (JNK) and involves the kinase intermediates PAK (for p21-activated kinase, which is activated directly by Rac and Cdc42) as well as MCKK and SEK (analogous to Raf and MEK, respectively). Note that the pathway from VAV to Cdc42/Rac/JNK is not an exclusive one, connections between Ras and Rac/Cdc42 as well as crosstalk between several of the downstream kinases have also been observed. (From: Smithgall, review, 1998).

Signalling molecules or enzymes essential for nuclear or cytoplasmic breakdown are sequestered or inhibited by the cytoskeleton (Janmey, review, 1998). Disruption of the cytoskeleton would liberate or activate these sequestered elements and allow them to reach their targets (Janmey, review, 1998). Disruption of microtubule turnover by drugs such as taxol, vincristine and vinblastine leads to phosphorylation of Raf and Bcl-2 (B-cell lymphoma/leukemia 2), and is associated with cell death (Janmey, review, 1998). Moreover, PAK is absolutely required for JNK activation when cells are exposed to nocodazole and taxol (Yuyuri et al., 1999). Microtubule restructuring might activate PAK via the GTP-binding protein Rac (Rac has been shown to bind β-tubulin in a GTP-dependent manner) (Yujiri et al., 1999). It has been proposed that tubulin-Rac association could control Rac signalling (Yuyuri et al., 1999). It has also been shown that inhibitory PAK mutants block Rac activation of JNK (Yuyuri et al., 1999). If Rac functions as a sensor for cytoskeletal changes, it could stimulate PAK resulting in JNK activation (Yujiri et al., 1999).

Disruption of the actin system by cytochalasin D can induce apoptosis in cultured cells, suggesting that the de-polymerization of actin may activate this process (Janmey, review, 1998). The possibility that destabilising F-actin is important for apoptosis is strengthened by the finding that over-expression of the actin-monomer-binding protein tymosin betha-10 can accelerate apoptosis (Janmey, review, 1998). It has been proposed that JNK serves as a "major switch" for programmed cell death (Janmey, review, 1998) because JNK is activated by a variety of cellular stresses, among them cytoskeletal changes (see fig. 4.5).

C-fos/c-jun have also been implicated as positive modulators of apoptosis induced in hematopoietic progenitor cells of the myeloid lineage (Lieberman, Gregory and Hoffman, review, 1998), a function that may relate to the control of blood cell homeostasis, as well as to programmed cell death associated with terminal differentiation (Lieberman, Gregory and Hoffman, review, 1998). It has been observed that c-fos/c-jun are stably induced during terminal differentiation of myeloid cells, with highest expression in the terminally differentiated mature cells. This suggests that c-fos/c-jun are likely to play a role in the initiation, maintenance and progression of the terminal-differentiation program of hematopoietic cell processes, associated with growth arrest and apoptotic cell death (Lieberman, Gregory and Hoffman, review, 1998). High expression of c-fos/c-jun has also been observed in terminally differentiated cells of the megakaryocyte and mastocyte lineages (Lieberman, Gregory and Hoffman, review, 1998).

There are at least two kinases, 46 and 54 Kda in size, which bind to c-jun and phosphorylate it on serines 63 and 73 (Minden and Karin, review, 1997). The 54 Kda JNK2 protein has a much higher affinity towards c-jun than the 46 Kda JNK1 protein (Minden and Karin, review, 1997). As a result of this, JNK2 is a more efficient c-jun kinase than JNK1. Once JNK is activated, it is translocated to the nucleus and forms a transient complex with the N-terminal activation domain of c-jun (Minden and Karin, review, 1997). JNK can also lead to increased expression of c-fos (Minden and Karin, review, 1997).

Transcription factors whose activities are regulated by ERK-mediated phosphorylation include various members of the TCF (translational control factor) family, such as Elk1

(Minden and Karin, review, 1997). JNK phosphorylates Elk1 on the same major sites as do the ERKs, and these sites are necessary for stimulating Elk1 transcriptional activity (Minden and Karin, review, 1997). Like c-jun, Elk1 activity is regulated by phosphorylation, and this stimulates its ability to activate the transcription of genes such as c-fos (Minden and Karin, review, 1997) (see fig. 4.6). Thus, while induction of c-fos genes by growth factors may be primarily mediated by the ERKs, the JNKs are most likely responsible for c-jun gene induction in response to cellular stresses and cytokines. In both cases, induction of the c-fos genes leads to increased production of the c-fos protein, which, in turn, can translocate to the nucleus and form hetero-dimers with c-jun (AP-1 complexes) (Minden and Karin, review, 1997). Therefore, increased production of c-fos is important for AP-1 activity, because c-jun/c-fos hetero-dimers are more stable than c-jun/c-jun homo-dimers, and lead to a more stable AP-1 complex (Minden and Karin, review, 1997) (see fig. 4.6).

We can speculate that in MEG-01 cells expressing scinderin, the elevated Rac levels observed are consequential to cytoskeletal disruption (see above). Elevated Rac levels should induce the observed elevated PAK levels and PAK activation through auto-phosphorylation. PAK activation should induce activation of MEKK1 and through it, activation of JNK2 (54 KDa) (see fig. 4.5). JNK activation should then induce increased levels of c-jun and c-fos proteins. However, the observed increased levels of c-fos protein would probably reflect, to some degree, activation of the ERK/MAP kinase pathway. This would probably happen through activated PAK stimulating both Raf and MEK, which would, in turn, activate ERK. ERK would then activate the transcription factor Elk1, which would induce c-fos production.

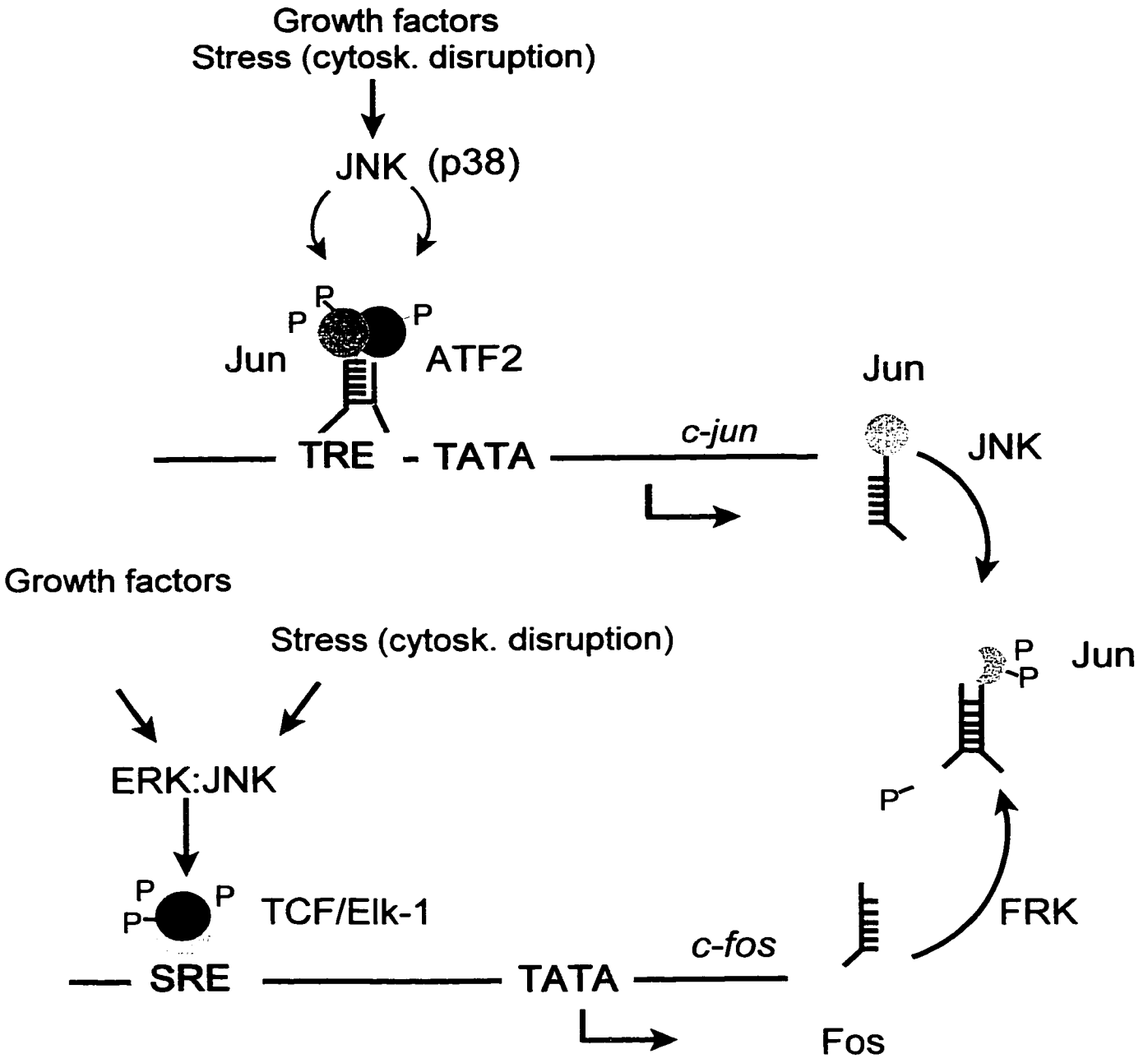

Fig. 4.6: Regulation of AP-1 (Fos/Jun) expression and activity by distinct groups of MAPKs. JNK-mediated phosphorylation of ATF2 and Jun, bound to the c-jun promoter, stimulates their transcription activities, leading to the induction of c-jun. On the other hand, phosphorylation of TCF/Elk1, bound to the c-fos promoter by ERKs or JNKs, stimulates its transcriptional activity, thereby leading to c-fos induction. Newly synthetized Fos and jun proteins dimerize to form stable AP-1 heterodimers; a further increase in AP-1 activity is achieved by JNKs and FRKs phosphorylating Jun and Fos, respectively, on sites that promote their transcriptional activity.
(From: Lieberman et al., review, 1998).

Therefore, increased c-fos levels would be the result of both JNK and ERK/MAP pathway activation. Moreover, the fact that in scinderin-positive MEG-01 cells, c-fos protein levels were found increased, suggests that this protein would contribute, to some degree, to the increased differentiation observed. On the other hand, elevated c-fos protein levels would contribute to the observed induction of apoptosis through the formation of stable AP-1 complexes with c-jun (fig. 4.6). In consequence, there would be a complex process of balance between differentiation and apoptosis in scinderin-positive MEG-01 cells.

4. (9). The transcription factor NF-E2

Critical regulators of hematopoietic differentiation have been identified through the study of nuclear factors binding cis-regulatory elements involved in lineage-specific gene expression (Orkin, review, 1995). No single cis-element is sufficient to establish lineage-specific gene expression. For example, GATA-1, which seems to interact with cis-elements of nearly all erythroid-expressed genes, is also expressed in mast cells, eosinophils, megakaryocytes and progenitors not yet committed to a single pathway (Orkin, review, 1995).

It has been mentioned above that the erythroid and megakaryocytic NF-E2 transcription factor is an obligatory hetero-dimer between a 45 KDa hematopoietic-restricted polypeptide (p45 NF-E2) and widely expressed 18 KDa proteins related to the avian oncogene *v-maf* (Lecine et al., 1998a). Both subunits belong to the bZip (basic leucine zipper) family of transcriptional regulators (Lecine et al., 1998a). Although evidence points to NF-E2 as the major enhancer protein acting at the betha-globin gene locus in developing erythroid cells (Lecine et al., 1998a), mice lacking p45 NF-E2 display only minor red blood cell

abnormalities (Lecine et al., 1998b). The most notable feature in these mice is the virtual absence of circulating blood platelets associated with arrested megakaryocyte maturation (Lecine et al., 1998b). Thus, two distinct "erythroid" transcription factors play critical roles in proper megakaryocyte development and are required to generate normal platelets in vivo (Lecine et al., 1998b).

In primary megakaryocytes, p45 NF-E2 is the only large subunit that dimerises with distinct small maf proteins to form a heterogeneous NF-E2 complex (Lecine et al., 1998a). Whereas p18 mafK is the predominant small maf protein in erythroid cells, the related proteins mafF and/or mafG predominate in megakaryocytes (Lecine et al., 1998a). Therefore, there is differential small maf protein expression among these two closely related blood lineages (Lecine et al., 1998a). Small maf proteins can also dimerise with themselves and with c-jun, but not with c-fos. Although the small maf proteins mainly recognise the consensus NF-E2 sequence as hetero-dimers with either NF-E2 p45, or c-jun, the hetero-dimers are different in their trans-activation potentials (Kataoka et al., 1995). Co-expression of c-jun and small maf could not activate a promoter with tandem repeats of the NF-E2 site (Kataoka et al., 1995). This means that tissue-specific gene expression and differentiation of megakaryocytes and erythroid cells are regulated by competition among c-jun and NF-E2 for small maf protein binding sites (Kataoka et al., 1995). It has been shown that in the Friend erythro-leukemia cell line, c-jun, as a homo-dimer or as a hetero-dimer together with small maf protein NF-E2 p18 could bind to NF-E2/AP-1 binding sites (Francastel et al., 1997). Thus,

it can be speculated that c-jun expression has an inhibitory effect on NF-E2 transcriptional activity (Francastel et al., 1997).

The decrease in p45 NF-E2 levels in MEG-01 cells expressing scinderin came as a surprise in view of the indication that scinderin-positive cells have entered the differentiation and maturation pathway. Equally interesting was the appearance, only in scinderin-positive cells, of a 90 KDa band which was recognised by the p45 NF-E2 antibody. This high molecular weight band has never before been described. It could represent an NF-E2 dimer or a precursor of NF-E2. Moreover, in view of the fact that scinderin-positive cells showed high levels of c-jun protein, it is possible that the latter could act as a repressor for NF-E2 transcriptional activity, through formation of inactive c-jun/p18 NF-E2 complexes. This suggestion is speculative at this point, and further work would be needed to understand the reason for the low expression of p45 NF-E2 in MEG-01 cells expressing scinderin.

4. (10). Tumour formation in nude mice and scinderin expression

Autocrine regulation of megakaryocytopoiesis can occur, at least in vitro (Hoffman, review, 1989). Using the human megakaryocytic cell line CHRF-288, Witte et al. (1988) showed that the cells produce large quantities of TGF-beta and fibroblast-like growth factors.

Several observations have suggested that the megakaryocytic lineage may be pathogenically important in the development of myelofibrotic stroma (Hassan et al., 1995). Certain humoral factors released from the proliferating megakaryoblasts that are unable to store them in their defective alpha-granules could result in increased collagen synthesis by bone marrow fibroblasts (Hassan et al., 1995). Myelofibrosis is a well recognized feature of both acute

megakaryoblastic leukemia and myelodysplasia (Reilly, 1992). Indeed, in bone marrow biopsies of patients with megakaryoblastic leukemia, there is an intense myelofibrosis (myelofibrotic stroma) accompanying the leukemic megakaryoblasts (Lichtman et al., 1990). Myelofibrotic stroma is characterized by an increase in total collagen, fibronectin, and proliferation of fibroblasts (Reilly, 1992). Three observations led to the belief that the megakaryocytic lineage may be pathogenetically important in the development of myelofibrotic stroma, including. These observations revealed that:

(i) megakaryocytic hyperplasia is a constant and prominent characteristic of idiopathic myelofibrosis;

(ii) fibroblast proliferation and collagen deposition are often maximal in areas of megakaryocyte necrosis; and

(iii) morphological abnormalities of megakaryocytes are more prominent in myelofibrosis than in other myeloproliferative diseases (Reilly, 1992).

There is evidence that the release of both PDGF and TGF-beta from MEG-01 cells, enhances the growth of bone marrow fibroblasts (Hassan et al., 1995). It has also been shown that subcutaneous injection of ELF-153 megakaryoblastic leukemic cells into nude mice provoked the formation of tumours, in three out of five mice after 6 weeks. These tumours had a very rigid texture whose histological examination revealed dense infiltration by blast cells and pronounced reticular fibrosis (Hassan et al., 1995).

MEG-01 cells expressing scinderin failed to form tumours or formed only small tumours in nude mice. On the other hand, injection of cells previously transfected with vectors alone

produced large tumours, whose histology revealed actively growing leukemic cells and organized connective tissue. This was in marked contrast with the high proportion of apoptotic nuclei surrounded by cytoplasmic fragments (platelet-like particles), found in the two small tumours produced by the injection of scinderin-positive cells. This observation suggests that scinderin-positive cells may be less malignant than the corresponding MEG-01 wild-type cells.

All of the above findings lead to the conclusion that the restitution of scinderin expression in human megakaryoblastic leukemia cells activates specific transduction pathways leading to cell differentiation and maturation, together with inhibition of proliferation and tumour formation. Whether these cells also acquired all the characteristics of normal cells, including the absence of tumorigenesis, remains to be determined.

5 - Conclusions

5 - Conclusions

Scinderin is a Ca^{++}-dependent filamentous actin-severing protein (Rodríguez del Castillo et al., 1990; Rodríguez del Castillo et al., 1992) present in platelets (Rodríguez del Castillo et al., 1991) and megakaryocytes but absent from megakaryoblastic leukemic cell lines. The findings of this study suggest that scinderin is responsible, to some extent, for the control of dynamic changes occurring in the reorganization of actin cytoskeletal networks during proplatelet formation and platelet release.

MEG-01 cells expressing scinderin were found to have a particular morphology with higher size and presence of protrusions or blebs. Scinderin expression also induced a decrease in the levels of filamentous actin (see fig. 5.1). This particular gene expression, brought about either through decreased F-actin levels or through negative feedback mechanisms at the gene transcription level, induced a decrease in the levels of gelsolin in MEG-01 cells. Decreased F-actin content also resulted in decreased cell proliferation, which in turn was reflected in decreased levels of transcription factor STAT-5 (Jak/Stat proliferative pathway), lower proliferation rate (less cell division) and decreased [^{3}H]thymidine incorporation. This decrease in proliferation was accompanied by an increase in polyploidy, perhaps as the result of lower levels of F-actin (cytochalasin D-like effect).

At early stages in culture, there was evidence of activation of the MAP kinase pathway in scinderin-positive cells. This was accompanied by increased expression of platelet antigens such as integrin GPIIb/IIIa (fibrinogen receptor), providing evidence of cell differentiation. This increase was accompanied by increased numbers of serotonin transporters, providing

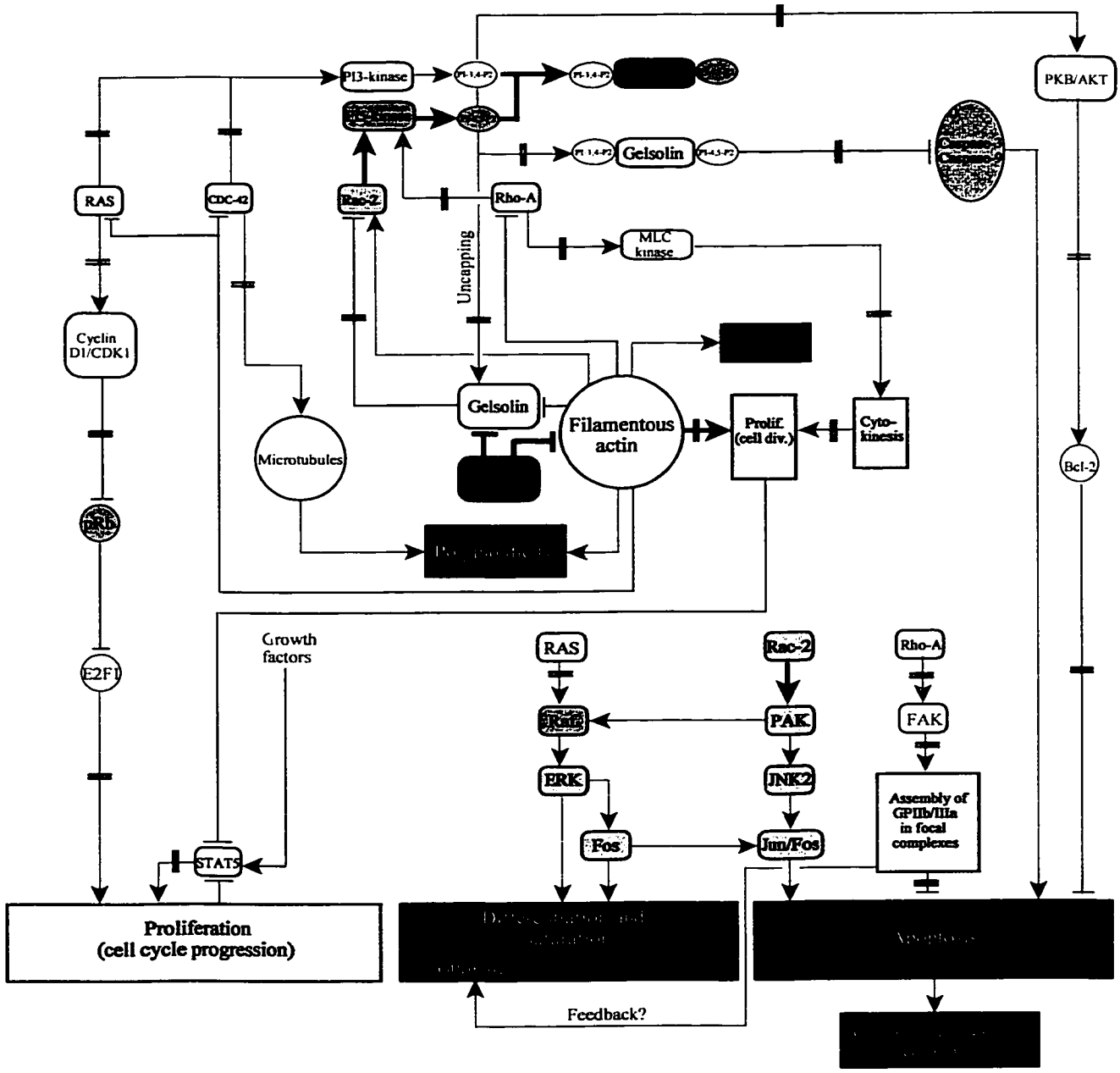

Fig. 5.1: Schematic representation of the effects induced by scinderin expression in MEG-01 cells (some of the mechanisms are hypothetical).

- Stimulatory effect
- Decreased stimulatory effect
- Inhibitory effect
- Decreased inhibitory effect

- ☐ Intermediary whose level and/or activity was found to be decreased
- ☑ Intermediary whose level and/or activity was found to be increased
- ☐ Intermediary whose level and/or activity is presumed to be decreased
- ☒ Intermediary whose level and/or activity is presumed to be increased
- ☐ Decreased cellular effect
- ■ Enhanced cellular effect
- ■ Overexpression

evidence of functional maturation. This enhanced differentiation in cells expressing scinderin was followed by cell detachment and induction of apoptosis at later days of culture (18-22). Another consequence of apoptotic processes was the release of cytoplasmic particles in huge amounts. These cytoplasmic fragments behaved very much like platelets. These evidences included the development of a microtubule ring, expression of GPIIb/IIIa or fibrinogen receptor, aggregation in response to thrombin, thrombin-induced serotonin release, and high affinity serotonin uptake, which was inhibited by specific transporter blockers).

There was also a decrease in the expression of the small p21 GTP-binding protein, Ras, which is responsible for the cell proliferation induced by growth factors (serum), and, to a great extent, for malignancy (by constitutive activation) in many types of cells (Symons, review, 1996a). Ras-mediated activation of PI3 kinase would be ineffective due to the unavailability of phosphoinositides, which would be sequestered, to a great extent, by the excessive amounts ofd scinderin expressed in these cells. Less PI-3,4-P2 would also be available to uncap F-actin-capping proteins, a condition required to induce the F-actin elongation required for cell proliferation. Less PI-3,4-P2 or less F-actin would result, in turn, in the lower levels of Ras observed. Lower PI-3,4-P2 levels would also be responsible for lower activation of PKB/AKT and of Bcl-2 (a broad anti-apoptotic agent), thus lowering apoptotic thresholds in scinderin-positive MEG-01 cells.

The lower Cdc42 levels observed in scinderin-positive cells would be a consequence of lower F-actin levels (due to Cdc42's direct interaction with F-actin through WASP), and, because of this, tubulin polymerization would decrease. Cdc42, apart from regulating F-actin

architecture, has been reported to participate in the regulation of tubulin polymerization. Therefore, lower levels of Cdc42 would also explain the polyploidy observed in scinderin-positive cells.

The decreased Rho levels in scinderin-positive cells could also be due to decreased F-actin levels, on the assumption that Rho interacts with F-actin through activation of PI5 kinase (Narumiya, review, 1996). Decreased Rho levels would provoke decreased cytokinesis (cytoplasmic division), due to reduced activation of MLC kinase, with the consequent uncoupling of cell division from nuclear division. This would explain the larger cell size and the polyploidy observed in scinderin-positive MEG-01 cells. In view of the observation that inhibition of Rho by C3 botulinic toxin in the CMK megakaryocytic cell line causes an increase in GPIIb/IIIa expression (Takada et al., 1996), the lower Rho levels observed in scinderin-positive MEG-01 cells would be also responsible, in part, for the elevated levels of GPIIb/IIIa observed. A hypothetical mechanism for this effect would be an increase in the production of the integrin complex proteins (through increased transcription of the gene responsible for GPIIb, for example). This would compensate the defective assembly of the integrin at focal complexes sites, for which Rho is mainly responsible.

Increased Rac levels in scinderin-positive cells are very probably the consequence of decreased gelsolin levels, due to their well known reciprocal regulation (Azuma et al., 1998). Lower gelsolin levels would induce increased Rac levels, thus inducing actin polymerization. However, actin polymerization would be impeded by over-expressed scinderin, which would tend to occupy most of the available barbed ends and bind free phospholipids. Thus,

scinderin would work as a brake for Rac-induced F-actin polymerization. Over-expressed Rac, would, therefore, be driven to stimulate and activate other effectors, such as PAK kinase. PAK, in turn, would activate the JNK pathway (and probably to some extent also the ERK/MAP kinase pathway through Raf activation), inducing increases in the levels of Jun and Fos proteins. Thes increases would lead to the establishment of a balance between apoptosis (JNK pathway) and differentiation (ERK/MAPK pathway). Lower gelsolin levels in scinderin-positive cells would also be a cause of apoptosis due to the reduction of the gelsolin-phospholipid complexes available to inactivate some caspases (Azuma et al., 2000). Apoptotic processes would cause the senescence of the cells (which might, by then, have attained a certain degree of maturation), and consequently the release of functional platelet-like particles. The process of platelet-like particle formation in scinderin-positive cells was also observed *in vivo*, as was demonstrated by histological examination of the two small tumours formed in nude mice by scinderin-expressing cells. However, in the larger tumours formed by the vector-transfected cells, apoptotic processes do not occur. This suggested that scinderin-positive MEG-01 cells are more mature, and possibly less malignant, than wild-type MEG-01 cells.

In conclusion, the present work shows that expression of scinderin cDNA in the MEG-01 cell line decreases cell proliferation, and induces polyploidization, differentiation and apoptosis, with the release of platelet-like particles. It seems, therefore, that restitution of scinderin expression in human megakaryoblastic leukemia cells activates specific transduction pathways leading to cell differentiation and maturation, together with inhibition of

proliferation and tumour formation. Whether such cells have also acquired all the characteristics of normal cells, including the absence of tumorigenesis, remains to be determined.

6 - Summary of contributions to original knowledge

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The role of cytoskeleton in megakaryocyte development and platelet formation is not well understood. Data presented here contribute to the improved understanding of the mechanisms involved in the differentiation and maturation of this cell lineage.

The main original contributions of this study are listed below:

1. The expression of scinderin in tumoral cells (the megakaryoblastic MEG-01 cell line), with its intact F-actin severing activity, was found to initiate the following effects:

    a. decrease in F-actin content (which was further decreased by ionophore treatment, indicating that scinderin's function was unimpaired);

    b. decrease in cell proliferation;

    c. down-regulation of gelsolin levels with up-regulation of Rac2 levels;

    d. morphological changes such as increase in cell volume, polyploidy and surface bleb formation, thus suggesting cell differentiation;

    e. increase in the expression of platelet markers such as CD41a antigen and serotonin transporters;

    f. induction of apoptosis; and

    g. production of platelet-like particles showing aggregation and serotonin release in response to thrombin.

2. In the course of this study, evidence was also found of:

    a. Rac2 activation of the PAK-JNK pathway and its effectors, the transcription factors c-jun and c-fos;

b. decrease in levels of the transcription factor STAT5 (Jak/Stat pathway), probably as a result of decreased cell proliferation due to low F-actin levels;

c. decrease in the expression of the trancription factor NF-E2 (p45), as well as the appearance of a new 90 kda protein, which cross reacted with the p45 NF-E2 antibody;

d. *in vivo* (by xeno-transplantation in nude mice) inhibition of tumoral cell proliferation, in contrast to their wild-type and vector-transfected counterparts; and

e. *in vivo* production of platelet-like particles by scinderin expressing MEG-01 cells injected into nude mice.

7 - References

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