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## **Integrin signaling modes controlling cell migration and metastasis**

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## **Chapter 3**

Regulation of MacMARCKS by integrin  $\beta 3$  expression

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# The regulation of MacMARCKS expression by integrin $\beta 3$

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## ABSTRACT

Integrin-mediated adhesion regulates multiple signaling pathways. Our group previously showed that ectopic expression of different integrin  $\beta$ -subunits in the neuroepithelial cell line GE11, has distinct effects on cell morphology, actin cytoskeletal organization, and on focal contact distribution. In this report we have investigated changes in gene transcription levels resulting from overexpression of the integrin  $\beta 3$  subunit. We found that  $\beta 3$  overexpression leads to the transcriptional downregulation of MARCKS related protein (MRP) resulting in a decreased expression of the MRP protein. Furthermore, we show that the Ras/MAPK pathway controls the basal level of MRP expression but  $\beta 3$  overexpression bypasses this pathway downstream of ERK to downregulate MRP. Further studies indicate that a region of the cytoplasmic tail of  $\beta 3$  containing part of the NITY motif is responsible for increased cell spreading and MRP downregulation. However, MRP overexpression failed to inhibit the  $\beta 3$ -induced increase in cell spreading while the knock down of MRP expression in GE11 cells did not increase cell spreading. We suggest that the downregulation of MRP by  $\beta 3$  is not required for increased cell spreading but instead that MRP downregulation is a secondary effect of increased cell spreading.

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## Introduction

Integrin-mediated cell adhesion is essential during development and wound healing and influences the characteristics of malignant tumors [1,2]. Members of the integrin family of hetero-dimeric transmembrane proteins connect the extracellular matrix (ECM) to the actin cytoskeleton and modulate adhesion and migration by means of different downstream signaling pathways [3,4]. In this complex mechanism the regulation of gene expression by integrin-dependent adhesion is also thought to play a role. Studies with monocytes revealed that adhesion to diverse substrata regulates the expression of several genes coding for cytokines and transcription-associated factors (see for review: [5]). Fibroblasts upregulate

collagenase, stromelysin, gelatinase and c-fos expression when adhered to a matrix of fibronectin and tenascin but not on fibronectin alone [6]. Furthermore, antibodies binding to the integrin subunits  $\alpha 1$  and  $\alpha 2$  inhibit stromelysin-1 expression [7]. The binding of  $\alpha 5\beta 1$  to fibronectin or of  $\alpha v\beta 3$  to vitronectin increases Bcl-2 levels through the PI3K-Akt pathway [8]. Also, in *Drosophila*, the presence of the integrin PS1 is required for the normal expression of two genes in the midgut [9]. More recently it was reported that the cell cycle regulator cdc2 is upregulated after re-expression of  $\alpha v\beta 3$  in  $\beta 3$  knockout cells resulting in increased migration [10]. Moreover, overexpression of  $\beta 3$  in CHO cells leads to a decrease in uPAR expression by bypassing the Ras/MAPK pathway that regulates basal expression of uPAR. The NITY motif in the  $\beta 3$

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cytoplasmic tail is important for uPAR downregulation and binds to the short isoform of the  $\beta 3$  binding protein,  $\beta 3$ -endonexin, that has been shown to downregulate uPAR transcription [11].

Previously, the profound effects of  $\beta 1$  re-expression in the  $\beta 1$  knockout neuroepithelial cell line, GE11, were investigated [12]. Re-expression of  $\beta 1$  in GE11 cells (GE $\beta 1$  cells) results in the loss of cell-cell contacts while the cells acquire a fibroblast-like appearance. On the other hand, overexpression of  $\beta 3$  in this cell line (GE $\beta 3$  cells) increased cell spreading and focal contact formation while disrupting cell-cell contacts. Expression of  $\beta 1$  or overexpression of  $\beta 3$  enhanced migration albeit through different modes [12–14]. Since  $\beta 3$  overexpression led to drastic morphological changes we hypothesized that the expression of some genes is regulated by  $\beta 3$ . To identify such genes a microarray analysis was performed. Surprisingly,  $\beta 3$  overexpression led to the downregulation of only a single gene that codes for the protein MacMARCKS. MacMARCKS or MARCKS-related protein (MRP) is a small acidic protein with an N-terminal myristoylation site that is inserted into lipid bilayers [15] and a positively charged effector domain (ED) that can bind to negatively charged phospholipids in the plasma membrane [16]. MRP is also thought to bind to actin [17], calmodulin [18] and dynamin [19], and is phosphorylated at serine residues by PKC [20]. MRP is implicated in the activation of integrins and cell spreading by regulating the cortical actin network [21,22]. The expression of MARCKS, a protein closely related to MRP, is downregulated by the oncogenes v-Jun [23], v-Src [24], c-Ras [25] and H-Ras (dataset from [26]). In contrast, stimulation of BV-2 microglial cells with LPS increases MARCKS and MRP expression via Src kinases [27].

We report that the Ras/MAPK pathway regulates MRP expression in GE11 cells because MEK inhibition led to an increase in MRP expression while RasV12 expression in GE11 cells downregulated MRP expression. Interestingly,  $\beta 3$  overexpression bypassed this pathway downstream of ERK to downregulate MRP expression. Furthermore, we excluded other pathways since we found that overexpression of activated Src, or of  $\beta 3$ -endonexin or the inhibition of PI3K did not affect MRP expression. We further show that the presence of the cytoplasmic tail of  $\beta 3$  up to the isoleucine residue of the NITY motif is essential for MRP downregulation but also for increased cell spreading. In another cell line,  $\beta 3$  overexpression also increased cell spreading while MRP expression was downregulated, suggesting that these two effects are linked. However, overexpression of MRP in GE $\beta 3$  cells did not inhibit cell spreading while knocking down MRP expression in GE11 cells did not increase spreading. Finally, we present data showing that  $\beta 3$  overexpression regulates the localization of MRP.

## Materials and methods

### Antibodies and other materials

The following antibodies were used: polyclonal antibodies against MRP (10002-2-Ig, Proteintech Group Inc), MARCKS (m-20, Santa Cruz), monoclonal anti- $\alpha$ -tubulin (clone B-5-1-2, Sigma), monoclonal anti-pan cadherin (clone CH-19, Sigma),

monoclonal anti-paxillin (BD Transduction Labs clone 1665), monoclonal ERK2 (BD Transduction Labs clone 33), phospho-ERK rabbit Ab (Cell Signaling #9101). The monoclonal anti-human  $\beta 3$  Ab 23C6 was kindly provided by Dr. Michael Horton (University College London, London, UK). The monoclonal anti-vinculin (clone V1F9; [34]) and anti- $\beta 3$  (clone C17) antibodies were kindly provided by Dr. Marina Glukhova (Institut Curie, Paris, France) and Dr. Ellen van der Schoot (Sanquin, Amsterdam, the Netherlands), respectively. Texas Red-conjugated phalloidin was obtained from Molecular Probes. PMA, LY294002 and PD98509 were from Sigma.

### Cell lines

GE11 cells have been isolated previously [12] and GE11 cells re-expressing the human  $\beta 1$  or overexpressing the human  $\beta 3$  integrin subunit or expressing the chimera integrin subunits  $\beta 3$ -1 or  $\beta 1$ -3 were established in our laboratory [12,13]. Cells were cultured in DMEM with 10% fetal calf serum, penicillin and streptomycin. mSCC2 is a mouse squamous cell carcinoma cell line isolated from a skin tumor induced by the two-stage chemical carcinogenesis protocol (Karine Raymond, unpublished results). The human pancreatic adenocarcinoma cell line NP18 has been described previously [28].

### cDNA, plasmids and generation of mutants

Full-length MRP was a kind gift from Dr. Deborah Stumpo (National Institute of Environmental Health Science, Research Triangle Park, NC). MRP was cloned into the pEGFP-N1 vector (BD Biosciences, Clontech) by digestion with *Bam*HI and the fragment encoding MRP-GFP was recloned into the retroviral vector LZRS-IRES-zeo [29].  $\beta 3$  deletion mutant constructs were obtained from Dr. Jari Ylännä (University of Oulu, Oulu, Finland) and cloned into the same retroviral vector. The retroviral expression plasmid encoding H-Ras<sup>G12V</sup> (Ras<sup>V12</sup>) was provided by Dr. John Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Both long and short isoforms of  $\beta 3$ -endonexin tagged with GFP were obtained from Dr. Meinrad Gawaz (E.H. University of Tübingen, Tübingen, Germany) and cloned into the retroviral LZRS vector. The activated c-Src (Y529F) cDNA was obtained from Upstate Biotechnology. Restriction sites for *Eco*RI and *Not*I were added on the 5' and 3' ends of the construct along with a *myc* tag and stop codon at the 3' end by PCR using the following primers: 5' GGAAATGAATTCATGGGCAGCAACAAGAGCAA-GAGCAAGCCCAAGGAC and 3' GGACCTTGGCGCCGCTAGTT-CAGATCCTCTCTGAGATGAGTTTGTCTAGGTTCTCCCGGGGCTGGTACTGTGGGCTC. The fragment was subsequently cloned into the LZRS-IRES-EGFP vector.

### Retroviral transductions

Cell lines expressing activated Ras<sup>V12</sup>, c-Src, MRP-GFP,  $\beta 3$  or deletion mutants of this subunit were established using retroviral transduction. Cells were transduced by adding 1 ml virus-containing supernatant to  $10^5$  cells in 8 ml medium and incubated for 16 h in the presence of DOTAP (Boehringer). Transduced cells were maintained in fresh medium and sorted three times for a positive GFP signal by FACS® or were

labeled with anti- $\beta 3$  antibody and FACS sorted after selection with zeocin. A clonal cell line was established from the Src-expressing GE11 cells and used for transduction of  $\beta 3$ . Resulting cells were sorted three times for  $\beta 3$  expression.

### Microarray analysis

Microarray slides were prepared at the central microarray facility (CMF) at the Netherlands Cancer Institute. A list of genes is available at <http://microarrays.nki.nl/download/geneid.html>. Cell lines were grown on plastic in normal growth medium and total RNA was isolated, labeled and hybridized as described at <http://www.nki.nl/nkidep/pa/microarray/protocols.htm>.

### Northern blot analysis

Corresponding clones of the identified genes were obtained from the central microarray facility. DNA was isolated, sequenced and used as templates for PCR reactions to obtain a suitable probe for Northern blot analysis. The following primers were used for MRP (AAGGAGACCCCAAGAAGAA and CTCATTCTGCTCAGCACTGG). Total RNA was isolated using guanidine–isothiocyanate (GIT). Briefly, cells were lysed in a buffer containing 4 M GIT, 25 mM sodium citrate, 0.1 M  $\beta$ -mercaptoethanol, and 0.5% sarkosyl. RNA was isolated after phenol chloroform extraction and was precipitated with isopropanol. Northern blots were performed using standard protocols.

### Cell labeling and immunoprecipitation

Cells were surface-labeled with  $^{125}\text{I}$  using lactoperoxidase as described previously [30]. Cells were lysed in 1% Nonidet P40 in 25 mM Tris-HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu\text{l}/\text{ml}$  leupeptin and 10  $\mu\text{l}/\text{ml}$  soybean trypsin inhibitor. Cell lysates were clarified by centrifugation and immunoprecipitations were performed with antibodies bound to protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) or to protein A-Sepharose conjugated with rabbit anti-rat IgG or anti-mouse IgG. Immunoprecipitates were analyzed by SDS-PAGE under non-reducing conditions and visualized using Kodak Biomax XAR film.

### Western blot analysis

Cell culture plates containing attached cells were washed with PBS and lysis buffer [50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 100 mM NaF, and inhibitor cocktail (Sigma)] was added. Cells were scraped and centrifuged for 2 min at 14,000 rpm. 20  $\mu\text{l}$  of lysate was added to 5  $\mu\text{l}$  3 $\times$  SDS sample buffer (Biolabs) and boiled. Samples were loaded onto SDS-PAGE gels, separated and transferred to polyvinylidene difluoride membranes (Millipore) and analyzed by Western blotting followed by ECL using the Super signal system (Pierce Chemical Co.).

### Immunofluorescence and flow cytometry

For immunofluorescence cells were fixed in 2% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100

for 5 min with PBS washing in-between steps. Coverslips were subsequently blocked with 2% BSA in PBS for 1 h at room temperature (RT). Coverslips were incubated with primary antibodies for 1 h at RT, washed three times in PBS and incubated with FITC or Texas Red secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at RT. Slides were mounted in MOWIOL 4-88 solution supplemented with DABCO (Calbiochem) and examined with a confocal Leica TCS NT microscope. For all immunofluorescence experiments, 4 random fields each of three independent experiments were examined of which a representative image was used for publication. For cell sorting cells were trypsinized, washed twice in PBS supplemented with 2% serum and incubated with primary antibody for 1 h at 4  $^{\circ}\text{C}$ . After three washes, cells were incubated with FITC- or PE-conjugated secondary antibody for 1 h at 4  $^{\circ}\text{C}$ . Finally cells were washed and resuspended in PBS with 2% serum and sorted using a FACStar plus $^{\circ}$  (Becton Dickinson). Cells expressing GFP constructs were trypsinized and washed before being sorted.

### RNAi against MRP

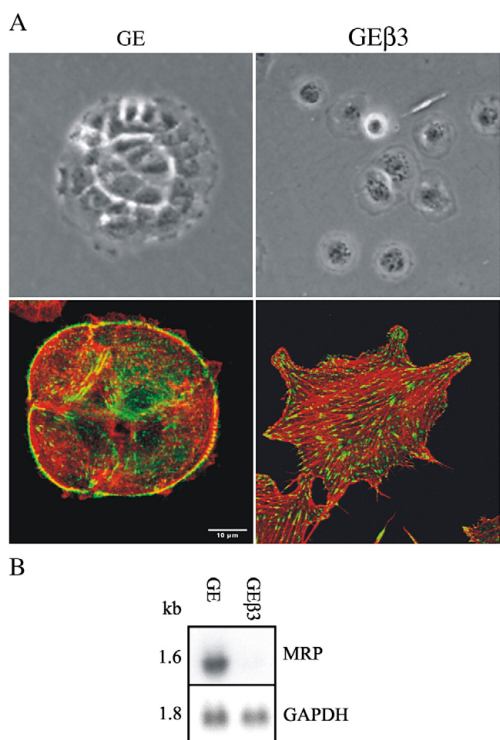
A SMARTpool $^{\circ}$  from Dharmacon consisting of 4 siRNAs (catalog number M-042960-00-0010) against MRP (NM\_010807) was used to transfect GE11 cells along with the standard siCONTROL $^{\circ}$  siRNA from Dharmacon as negative control. Cells were transfected using the standard transfection protocol provided by Dharmacon using the transfection reagent DharmaFECT $^{\circ}$  1. Protein expression was assessed 48 h after transfection while at the same time cells were fixed and stained for confocal analysis.

## Results

### Overexpression of $\beta 3$ in GE11 cells regulates MRP expression

GE11 cells have an epithelial morphology adhering to one another with well-defined cell–cell contacts. They have small, peripheral focal contacts and a thick cortical actin ring present along the circumference of the entire epithelial island. Overexpression of  $\beta 3$  in GE11 cells (GE $\beta 3$  cells), causes a reorganization of the actin cytoskeleton resulting in the loss of the cortical actin ring and the formation of short, thick stress fibers connected to a large number of focal contacts and the loss of cell–cell contacts (Fig. 1A).

Since  $\beta 3$  overexpression induced these dramatic morphological changes in GE11 cells, we hypothesized that the regulation of some genes would be changed. To identify these genes we performed a microarray analysis of the GE $\beta 3$  cell line using GE11 cells as reference. Genes, whose transcription level differed three-fold or more from that in the reference GE11 cells were selected. Surprisingly, despite the dramatic morphological changes induced by  $\beta 3$  overexpression in GE11 cells, the transcription of only a single gene appeared to be affected. The transcription of this gene, *Mlp*, coding for the protein MRP was downregulated by 72% in GE $\beta 3$  cells as compared with GE11 cells. This was confirmed by Northern blot analysis because mRNA levels for MRP were strongly decreased in GE $\beta 3$  cells in comparison to GE11 cells (Fig. 1B). To



**Fig. 1 – Analysis of gene transcription after  $\beta 3$  overexpression.** (A) Phase contrast and confocal analysis of GE11 and GE $\beta 3$  cells. Cells were stained with anti-paxillin antibodies in green and with phalloidin in red. (B) Total RNA was isolated from GE11 and GE $\beta 3$  cells and analyzed using Northern blot. mRNA bands were visualized using radioactively labeled probes.

determine if the decrease in *Mlp* transcription also led to a decrease in MRP expression, lysates of GE11, GE $\beta 3$  and GE $\beta 1$  cells were analyzed by Western blot. Indeed, MRP expression was diminished after  $\beta 3$  overexpression but remained unchanged in GE $\beta 1$  cells (Fig. 2A). To exclude the possibility that the observed downregulation of MRP expression is attributable to clonal variation, we analyzed several independent, bulk-sorted GE $\beta 3$  cell populations. MRP expression was diminished in all of them (data not shown). Western blot analysis showed that  $\beta 3$  overexpression had no effect on the expression of MARCKS (Fig. 2A). Therefore, overexpression of  $\beta 3$  in GE11 cells results in the specific downregulation of MRP expression.

*The cytoplasmic tail of  $\beta 3$  downregulates MRP expression only when it is associated with the  $\alpha v$  subunit*

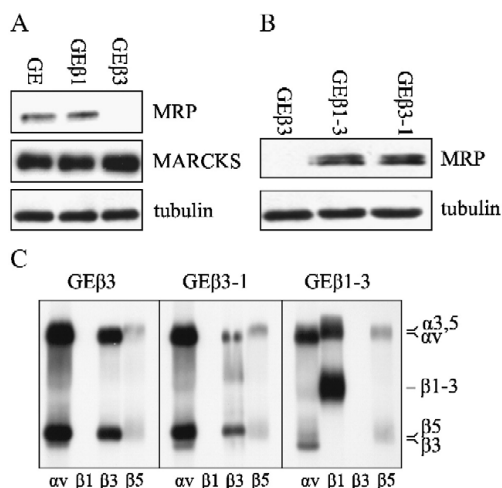
Integrins convert signals from the extracellular environment into intracellular signals via their cytoplasmic tails [3]. We hypothesize that the cytoplasmic tail of  $\beta 3$  is involved in the

downregulation of MRP expression. To test this, a chimera of the  $\beta 3$  extracellular and transmembrane domain, fused to the  $\beta 1$  cytoplasmic tail ( $\beta 3$ -1) and a construct of the  $\beta 1$  extracellular and transmembrane domains fused to the  $\beta 3$  cytoplasmic tail ( $\beta 1$ -3) were expressed in GE11 cells and MRP expression was measured (Fig. 2B). Expression of the chimeras did not affect MRP expression indicating that the presence of the cytoplasmic tail of  $\beta 3$  without the extracellular domain, or vice versa, was insufficient to down-regulate MRP expression.

Possibly, the effect on MRP expression depends on the  $\alpha$  subunit that is associated with  $\beta 3$ . For that reason we identified the integrin complexes present on the surface of the cells expressing full-length  $\beta 3$  or either of the chimeras (Fig. 2C). Both full-length  $\beta 3$  and the  $\beta 3$ -1 chimera were associated with the  $\alpha v$  subunit while the  $\beta 1$ -3 chimera was associated with  $\alpha 5$  and/or  $\alpha 3$ . Therefore, the cytoplasmic tail or the extracellular domain of  $\beta 3$  by themselves cannot affect MRP expression but instead it is the association of the  $\alpha v$  and the  $\beta 3$  subunits that mediates this effect.

*A distinct region of the  $\beta 3$  cytoplasmic tail is responsible for increased cell spreading and MRP downregulation*

To characterize the underlying mechanism responsible for the regulation of MRP by  $\beta 3$ , we sought to identify the region within the  $\beta 3$  cytoplasmic tail that mediates the down-regulation of MRP expression. MRP expression was measured



**Fig. 2 –  $\alpha v\beta 3$  only downregulates MRP expression.** (A) Western blot stained for MRP and MARCKS expression in GE11, GE $\beta 1$  and GE $\beta 3$  cell lines. Tubulin was visualized on the same blot as the loading control. (B) Western blot for MRP expression on lysates of GE $\beta 3$ , GE $\beta 3$ -1 and GE $\beta 1$ -3 cells with tubulin staining used as loading control. (C) Surface-expressed integrins were labeled using  $^{125}I$  and immunoprecipitated using antibodies against the indicated integrin subunit.

in several cell lines expressing different truncated  $\beta 3$  constructs (Fig. 3A). Our data shows that expression of a construct with a deletion of the five C-terminal residues of  $\beta 3$  representing the complete Src-binding motif (RGT) [31] and the tyrosine and threonine residues of the NITY motif still led to downregulation of MRP expression. However, the expression of further truncated constructs of the cytoplasmic tail of  $\beta 3$  no longer had an effect on MRP expression (Fig. 3B). Interestingly,  $\beta 3$ -induced increased cell spreading appeared to depend on the same region of the cytoplasmic tail that influences MRP expression (Fig. 3C). Thus, it is possible that proteins that bind to  $\beta 3$  in the vicinity of the NITY motif are

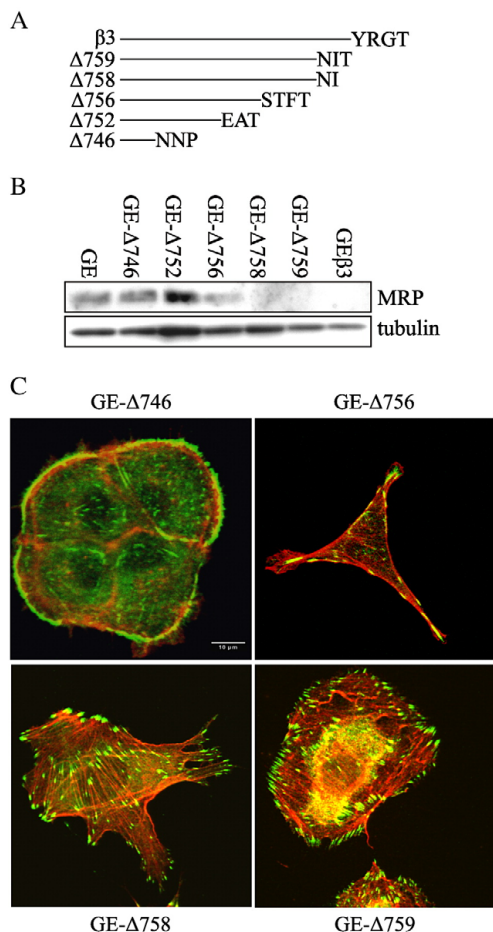
involved in the regulation of MRP expression as well as in increased cell spreading.

### **The Ras/MAPK pathway regulates the basal level of MRP expression in GE11 cells but $\beta 3$ overexpression bypasses this pathway to downregulate MRP**

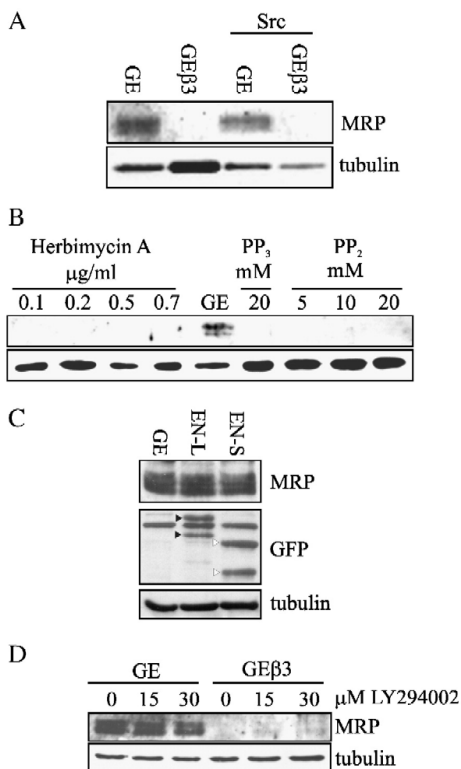
Stimulation of BV-2 microglial cells with LPS upregulates MRP expression through a Src dependent signaling pathway [27]. Moreover, MARCKS expression is downregulated by v-Src and c-Ras expression in 3T3 cells [25]. Thus, the question arose whether  $\beta 3$  overexpression in GE11 cells downregulates MRP expression through a Src-dependent pathway or the Ras/MAPK pathway. GE11 cell lines were established expressing activated c-Src or expressing activated c-Src together with  $\beta 3$ . Western blot analysis of these cell lines indicated that the expression of activated c-Src did not affect MRP expression in the GE11 or  $\beta 3$  overexpressing cell lines (Fig. 4A). Src kinases and protein tyrosine kinases (PTK) were inhibited in GE $\beta 3$  cells using PP2 and Herbimycin A, respectively (Fig. 4B). Neither Src nor PTK inhibition restored MRP expression. Therefore, the regulation of MRP by  $\beta 3$  is independent of Src activity.

To test whether the Ras/MAPK pathway is involved in the regulation of MRP expression, GE11 and GE $\beta 3$  cells were incubated for increasing periods of time with 25  $\mu$ M PD98509. ERK phosphorylation levels decreased after an incubation of 1 h. Interestingly, MRP levels increased sharply after 1 h in GE11 cells but not in GE $\beta 3$  cells (Fig. 5A). Both cell lines were also incubated for 2 h with increasing concentrations of PD98509. ERK phosphorylation levels were decreased in both cell lines after incubation with 10  $\mu$ M, but MRP expression only increased in GE11 cells after incubation with 10  $\mu$ M PD98509 while there was no change in MRP expression in GE $\beta 3$  cells (Fig. 5B). To confirm that the Ras/MAPK pathway regulates MRP expression, a Ras<sup>V12</sup> construct was stably expressed in GE11 cells. Expression of Ras<sup>V12</sup> resulted in increased ERK phosphorylation along with a decrease in MRP expression (Fig. 5C). Therefore, the Ras/MAPK pathway regulates MRP expression in GE11 cells, but  $\beta 3$  overexpression bypasses the effect of ERK phosphorylation to downregulate MRP expression.

Two other pathways that were previously implicated in gene regulation downstream of integrins were investigated for their possible role in the  $\beta 3$ -dependent downregulation of MRP expression. Firstly, it was reported that the short isoform of the  $\beta 3$  integrin-binding protein,  $\beta 3$ -endonexin, is important for the downregulation of uPAR expression after  $\beta 3$  overexpression in CHO cells [11]. To investigate if  $\beta 3$ -dependent MRP downregulation is mediated by  $\beta 3$ -endonexin, constructs with the long or short isoform fused to GFP were expressed in GE11 cells followed by Western blot analysis of MRP (Fig. 4C). MRP expression was not influenced by the expression of either isoform of endonexin. Therefore,  $\beta 3$ -induced downregulation of MRP is not dependent on the presence of  $\beta 3$ -endonexin. Secondly, it was shown that ligand binding by  $\alpha v\beta 3$  and  $\alpha 5\beta 1$  leads to increased Bcl-2 expression through the activity of the PI3K pathway [8]. We investigated if PI3K plays a role in the regulation of MRP expression by incubating GE11 and GE $\beta 3$  cells overnight with the specific inhibitor LY294002 and analyzing MRP expression. The data shows that LY294002 did not alter MRP expression at the concentrations tested (Fig.



**Fig. 3 – The distal NITY motif is essential for downregulation of MRP expression and increased cell spreading. (A)** Diagram depicting the deletion mutants of the  $\beta 3$  and showing the last amino acids of each mutant. **(B)** Cells expressing deletion mutants were analyzed for MRP expression by Western blot. **(C)** Confocal images of these cells stained for paxillin and F-actin indicate the differences in FC formation, F-actin organization and cell shape.



**Fig. 4 – MRP expression is regulated independently of Src activity.** (A) GE11 and GE11-Src cell lines with or without  $\beta 3$  overexpression were analyzed for MRP expression. (B) GE $\beta 3$  cells were incubated with Herbimycin A, PP<sub>3</sub> or PP<sub>2</sub> and analyzed for MRP expression together with GE11 cells as positive control. (C) Long (EN-L) and short (EN-S) forms of endonexin fused to GFP were stably expressed in the GE11 cell line. Western blot analysis was performed for endogenous MRP levels and the same blot was stained for GFP and tubulin. Note that endonexin is present as two distinct bands indicated with filled arrowheads for EN-L and empty arrowheads for EN-S. (D) GE11 and GE $\beta 3$  cells were incubated with 0, 15 or 30  $\mu$ M LY294002 overnight before being lysed and analyzed for MRP expression by Western blot.

4D). Therefore, the PI3K pathway is not involved in  $\beta 3$ -mediated regulation of MRP expression.

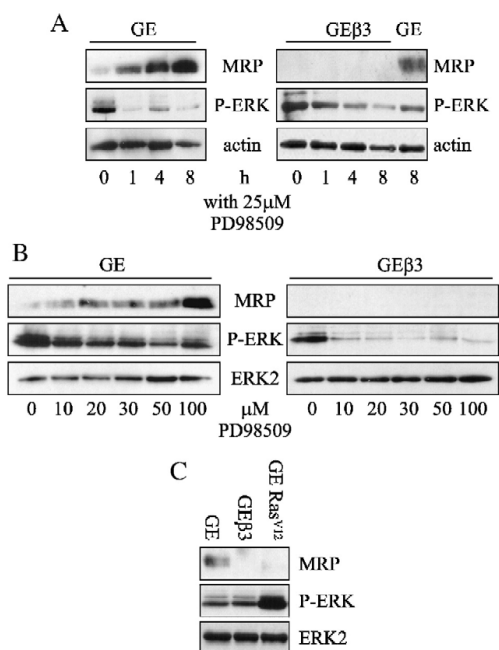
#### MRP downregulation and cell spreading are regulated by $\beta 3$ in the NP18 cell line

To determine if the downregulation of MRP expression by overexpressed  $\beta 3$  only occurs in GE11 cells, we measured MRP expression in two other cell lines overexpressing  $\beta 3$ . In the pancreatic carcinoma cell line, NP18, MRP was down-regulated after  $\beta 3$  overexpression but MRP expression was

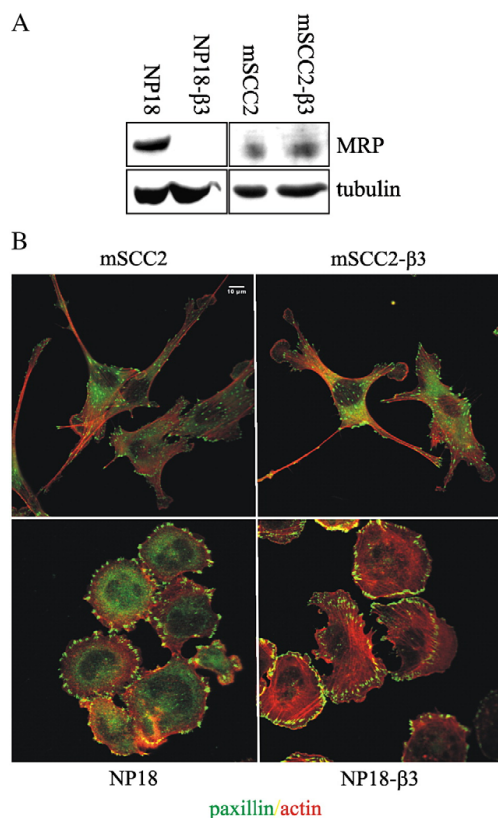
not affected in the mouse squamous cell carcinoma cell line mSCC2 (Fig. 6A). Paxillin and actin staining of the parental NP18 cells indicated that focal contacts were present in clusters evenly spaced at the cell periphery in close proximity to a cortical actin network. In contrast, in the NP18- $\beta 3$  cell line the focal contacts present around the periphery of cells were more often connected to thick actin stress fibers extending across the entire cell. However, overexpression of  $\beta 3$  in the mSCC2 cell line did not noticeably affect the morphology of the cells (Fig. 6B). Thus, the effect of  $\beta 3$  overexpression on MRP expression is not limited to the GE11 cell line. Moreover, the overexpression of  $\beta 3$  induced morphological changes in the NP18 cells similar to those seen in GE11 cells.

#### MRP is not essential for $\beta 3$ -induced effects on morphology but $\alpha v \beta 3$ expression does regulate MRP localization

We showed that the downregulation of MRP expression by  $\beta 3$  overexpression is accompanied by morphological changes. To determine if the loss of MRP is responsible for these morphological changes, we silenced MRP expression in GE11 cells using a siRNA SMARTpool<sup>®</sup> from Dharmacon. The SMARTpool<sup>®</sup> along with a negative control were transfected



**Fig. 5 – MRP expression is regulated by the Ras/MAPK pathway.** GE11 and GE $\beta 3$  cells were incubated with PD98509 for different periods (hours) (A) or at different concentrations (bottom in  $\mu$ M) (B). MRP and phospho-ERK levels were analyzed with actin or ERK2 as loading control. (C) GE11, GE $\beta 3$ , and GE Ras<sup>V12</sup> were analyzed for MRP and phospho-ERK levels with ERK2 as loading control.



**Fig. 6 – Effect of  $\beta 3$  overexpression in NP18 and mSCC2 cells. (A)** Western blot analysis for MRP expression of two cell lines, NP18 and mSCC2, overexpressing  $\beta 3$ . **(B)** mSCC2 cells and NP18 cells with or without overexpressed  $\beta 3$  were stained for paxillin and F-actin.

into GE11 cells and MRP expression was assessed after 48 h. Western blot analysis showed that MRP was absent in the SMARTpool® transfected cells while cells transfected with the control siRNA still expressed MRP (Fig. 7A). Cells were also fixed and stained for vinculin, cadherin and F-actin. Cells transfected with the negative control and with the MRP-directed siRNA formed islands with a normal morphology and normal F-actin and vinculin distribution (Fig. 7B). There was no evidence that cell spreading or cell–cell contact formation was affected by the knockdown of MRP. Additionally, MRP was re-expressed in GE $\beta 3$  cells using a MRP-GFP cDNA construct. Confocal analysis revealed that MRP-GFP expression had no effect on the morphology of GE $\beta 3$  cells (Fig. 8A). The cells remained well spread and the formation of actin stress fibers and focal contacts was not perturbed. To determine if the downregulation of MRP expression in GE $\beta 3$  cells is important for the initial spreading of these cells, MRP-GFP was stably expressed in GE11 cells before  $\beta 3$  was overexpressed through retroviral transduction. 24 h after

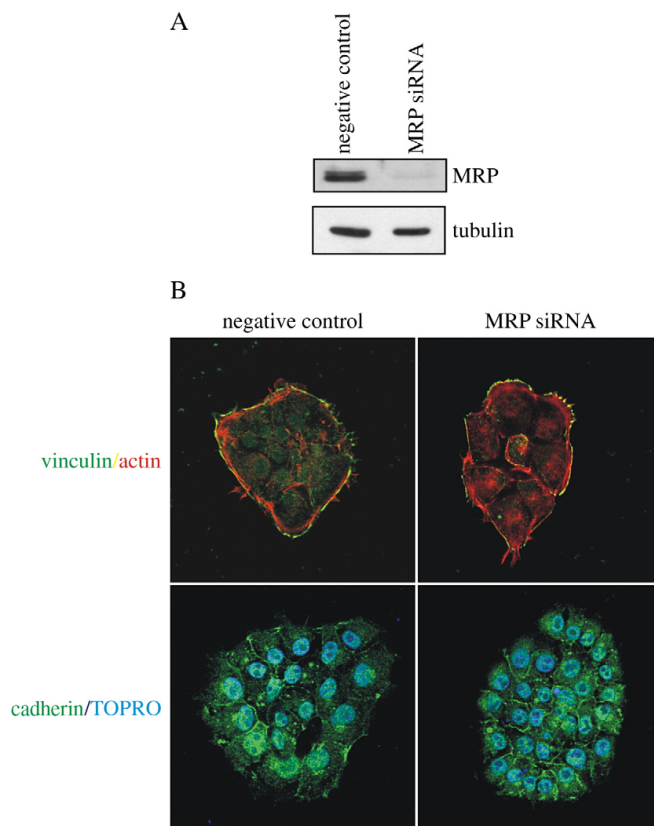
infection, the morphology of cells expressing MRP-GFP and  $\beta 3$  was similar to that of GE $\beta 3$  cells (Fig. 8B). Therefore, we conclude that cell spreading is not influenced by the levels at which MRP is expressed. Interestingly, we noticed that the localization of MRP-GFP was altered after  $\beta 3$  overexpression. In line with a previous report showing that MRP is localized at the basolateral membranes of MDCK cells [32], confocal analysis of GE11 cells indicated that overexpressed MRP-GFP was concentrated at the basolateral membrane and partially co-localized with cadherin but not with paxillin (Fig. 8A). In contrast, after  $\beta 3$  overexpression MRP-GFP was diffusely distributed along the entire cell membrane with occasional staining of internal membranes as well as weak actin filament decoration. In conclusion, MRP is not involved in the  $\beta 3$ -induced morphological effects, but  $\beta 3$  overexpression clearly influences MRP-GFP localization.

## Discussion

It has been suggested that the regulation of gene expression forms part of the mechanism by which integrins control cell migration and invasion [10]. Previously, it was shown that the re-expression of  $\beta 3$  in knockout cells increases cdc2 protein levels [10] and that overexpression of  $\beta 3$  in CHO cells leads to a decrease in uPAR protein levels [11]. In both cell lines migration and spreading were enhanced. Previous work in our laboratory has shown that overexpression of  $\beta 3$  or re-expression of  $\beta 1$  subunit has distinct effects on cell morphology, migration, Rho activation and cofilin phosphorylation [13,14].

We hypothesized that the effect on cell morphology induced by  $\beta 3$  overexpression coincides with changes in the transcription of several genes, possibly including genes such as uPAR and cdc2. To test our hypothesis we performed a microarray analysis of GE $\beta 3$  cells and compared the gene expression profile of these cells to that of the parental GE11 cells. Surprisingly,  $\beta 3$  overexpression resulted in the down-regulation of only a single gene, i.e. the gene coding for MRP. Both uPAR and cdc2 cDNAs were present on the array used for this study but the transcription of these genes was not changed by the overexpression of  $\beta 3$ .

Various signaling pathways have been implicated in gene regulation downstream of integrins. One of these pathways was elucidated in CHO cells in which  $\beta 3$  overexpression inhibits uPAR transcription. It was found that overexpression of the short isoform of the  $\beta 3$  integrin binding protein,  $\beta 3$ -endonexin, also resulted in decreased uPAR transcription [11] suggesting that  $\beta 3$ -endonexin is the downstream effector of  $\beta 3$  in a pathway that leads to uPAR down-regulation [11]. It was also shown that ligand binding by  $\alpha 5 \beta 3$  and  $\alpha 5 \beta 1$  leads to an increase in Bcl-2 expression that was dependent on the PI3K-Akt pathway [8]. We investigated if these signaling pathways were also involved in the  $\beta 3$ -mediated downregulation of MRP expression. We show that overexpression of  $\beta 3$ -endonexin in GE11 cells had no effect on MRP expression and we conclude that this pathway does not regulate the expression of MRP. Our data also indicates that MRP expression was not changed by inhibition of the PI3K pathway.

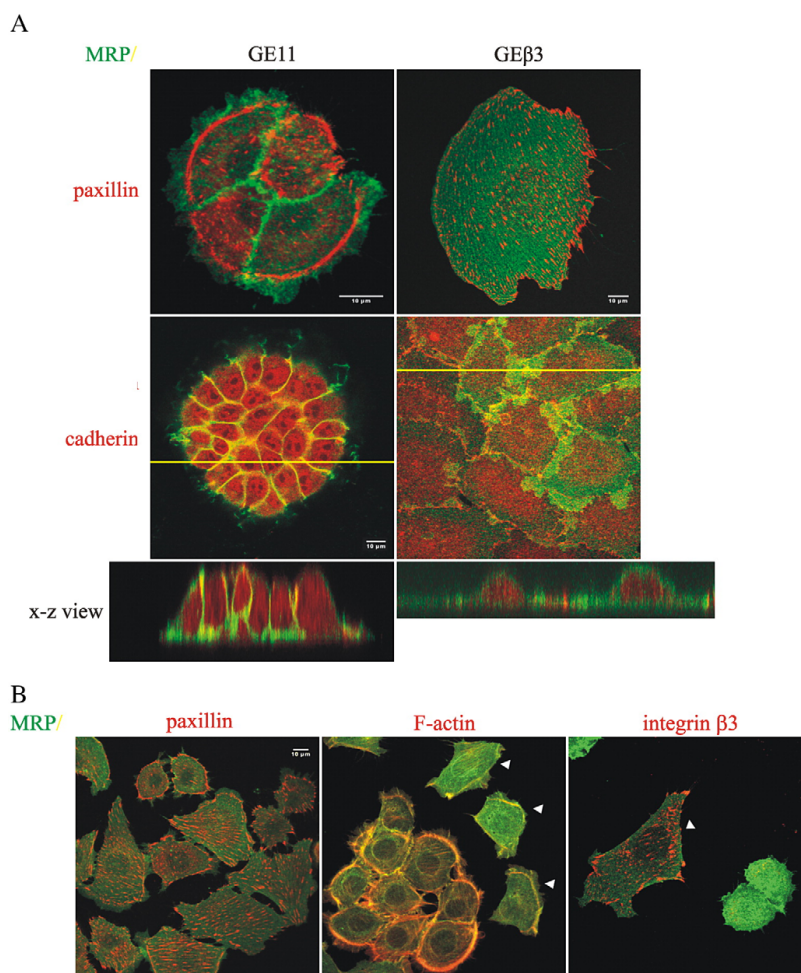


**Fig. 7 – RNAi-mediated downregulation of endogenous MRP in GE11 cells. (A) Western blot analysis of MRP expression in GE11 cells transfected with siRNA against MRP and a negative control. (B) GE11 cells transfected with siRNA directed against MRP or with a negative control were fixed and stained for vinculin and actin or for cadherin together with TOPRO that was used as nuclear staining.**

On the other hand, a number of soluble factors and signaling intermediates have been identified that affect MRP expression. It was shown that LPS stimulation increases MRP expression in BV-2 microglial cells through the activity of Src kinase [27]. Moreover, transformation of 3T3 cells with v-Src or Ras leads to decreased expression of MARCKS [25]. Our results indicate that active Src does not decrease MRP expression. Intriguingly, the inhibition of ERK phosphorylation caused an increase in MRP expression in GE11 cells but had no effect on MRP expression in GE $\beta$ 3 cells while Ras<sup>V12</sup> expression in GE11 cells resulted in increased phospho-ERK levels and decreased MRP protein levels. Therefore, the Ras/MAPK pathway controls the basal level of MRP expression in GE11 cells but  $\beta$ 3 overexpression bypasses this pathway downstream of ERK to downregulate MRP expression.

Our studies with  $\beta$ 3 chimeras show that downregulation of MRP expression by  $\beta$ 3 only occurs when it is associated with the  $\alpha$ v subunit. Furthermore, we show that the  $\beta$ 3 cytoplasmic tail is essential for MRP downregulation. Within the cytoplasmic tail of  $\beta$ 3 several motifs have been identified that are

important for different downstream signaling events. For example, the membrane proximal NPxY motif is important for binding to talin and critical for integrin activation, while the distal NITY motif is important for cell spreading although it appears that the tyrosine residue of this motif is not essential [33]. We tested several deletion mutants of  $\beta$ 3 and show that the loss of the complete NITY motif does not lower the expression of MRP. Inclusion of the asparagine and isoleucine residues of the NITY motif leads to the downregulation of MRP expression while also causing cells to spread like GE $\beta$ 3 cells. Therefore, the same region of  $\beta$ 3 responsible for MRP downregulation also increases cell spreading. In NP18 cells,  $\beta$ 3 overexpression also caused changes in the cytoskeleton while MRP expression was downregulated. On the other hand, overexpression of  $\beta$ 3 in mSCC2 cells did not lead to changes in morphology or to the downregulation of MRP expression, emphasizing that  $\beta$ 3 overexpression is responsible for both these phenomena. MRP has been implicated in cell spreading and cytoskeletal organization [20,21], both of which are altered in GE $\beta$ 3 cells,



**Fig. 8 – Overexpression of MRP has no effect on  $\beta 3$ -induced cell morphology.** (A) GE11 and GE $\beta 3$  cells expressing MRP-GFP were grown on glass coverslips, fixed and stained for paxillin and actin or for cadherin while a z-stack was made of a GE-MRP island and of a GE $\beta 3$ -MRP confluent cell layer stained for cadherin. The vertical slice presented in the right panel is obtained from the region indicated by the white line in the left panel. (B) GE11-MRP cells were grown on slides and infected with  $\beta 3$ -retrovirus. After 24 h of infection, cells were fixed and stained for paxillin, F-actin and integrin  $\beta 3$ . All the cells in the left panel that stained positively for paxillin have the typical  $\beta 3$ -induced morphology while arrowheads in the right panel indicate infected cells.

suggesting that MRP may be important for the  $\beta 3$ -induced effects on cell morphology. However, our data indicates that altering MRP expression by knock down in GE11 cells or by overexpression in GE $\beta 3$  cells does not influence cellular morphology. We, therefore, suggest that either MRP is not involved in cell spreading or morphology or that it is not the only protein involved and that the stronger effects of other proteins mask the effect of MRP.

In conclusion, our studies show that the expression level of  $\beta 3$  can influence cell morphology and the transcription levels

of MRP and that the Ras/MAPK pathway is important for the regulation of MRP expression.

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