Medizinische Fakultät Charité – Universitätsmedizin Berlin
Campus Benjamin Franklin
aus dem Institut für Molekularbiologie und Bioinformatik
Direktor: Univ.-Prof. Dr. Burghardt Wittig

20 – 40 kDa OPN isoforms mediate chemotherapy resistance of mouse WAP-SVT/t breast cancer cells and prevent cell death by activation of a phospholipase C regulated mechanism.

Inaugural-Dissertation zur Erlangung des Grades Doctor rerum medicarum
Charité – Universitätsmedizin Berlin
Campus Benjamin Franklin

vorgelegt von
Hugo Ernesto Molina Leddy
aus San Salvador

Referent: Frau Prof. Dr. Monika Graessmann

Korreferent: Herrn Prof. Dr. Burkhardt Dahlmann

Gedruckt mit Genehmigung der Charité - Universitätsmedizin Berlin
Campus Benjamin Franklin

Promoviert am: 21.11.2008

.

ZUSAMMENFASSUNG

Abweichende zelluläre Signalprozesse innerhalb der fein abgestimmten komplexen Regulationsmechanismen für Zelltod und Zellüberleben sind maßgeblich beteiligt an der Therapie-Resistenz und der Tumorprogression von Brustkrebs. Zwei unterschiedliche Zelllinien, isoliert aus einem einzelnen Brusttumor einer WAP-SVT/t transgenen Maus, unterscheiden sich erheblich in ihrem Ansprechen auf apoptotische Stimuli. Während die ME-A Zellen empfindlich sind gegenüber der Apoptose Induktion durch Entzug von Wachstumsfaktoren (Serumentzug) und der Behandlung mit Chemotherapeutika wie Doxorubicin, sind die ME-C Zellen unempfindlich gegenüber diese Apoptose Stimuli. Die ME-C Zellen enthalten eine in Krebszellen häufig auftretenden Mutation im p53 Gen. Co-Kultivierungs Experimente ergaben, dass das Sekret der ME-C Zellen die ME-A Zellen vor der Stress induzierten Apoptose schützt. Microarray und Western Blot Analysen zeigten, dass das Glycoprotein Osteopontin (OPN) in den ME-C Zellen überexprimiert und sezerniert wird. Dieses Protein scheint der Hauptfaktor, für die antiapoptotische Aktivität im Sekret der ME-C Zellen zu sein. Apoptoseresistenz wird erreicht, wenn das Medium Konzentrat (MC) der ME-C Zellen den ME-A Zellen zugefügt wird. Die ME-C und die ME-A Zellen sezernieren unterschiedliche OPN Isoformen. Der Vergleich der Zellsekrete führte zu dem Ergebnis, dass die 20 – 40 kDa OPN Isoformen, die nur in den ME-C Zellen MC vorkommen, für die antiapoptotische Wirkung verantwortlich sind. ME-A Zellen, die nach Transfektion eines OPN-Plasmids OPN überexprimieren, aber die 20-40 kDa OPN Isoformen nicht sezernieren, sind weiterhin sensitiv gegenüber der Stressinduzierten Apoptose. Das deutet darauf hin, dass zusätzliche zellspezifische posttranskriptionale OPN Modifikationen stattfinden müssen, die zur Entstehung der Isoformen mit der antiapoptotischen Aktivität führen. Die durch OPN eingeleitete antiapoptotische Signalkaskade wird über die Phospholipase C (PLC) vermittelt, da die Inkubation der ME-A Zellen mit dem Phospholipase C Inhibitor U73122 die antiapoptotische Wirkung des ME-C Zellsekrets aufhebt. Die Analyse der ME-A und ME-C Zellen auf das Vorkommen von OPN Rezeptoren mittels Durchflußzytometrie und Immunofluoreszenz zeigte die Expression von Integrin β1 und CD44 auf der Zelloberfläche. Beide Proteine könnten an der antiapoptotischen Signaltransduktion von OPN beteiligt sein.

ABSTRACT

Aberrant cellular signalling influencing the complex regulatory network of cell death and survival is a common cause of therapy resistance and tumour progression in breast cancer. Two independent cell lines established from a breast tumour originated in a WAP-SVT/t transgenic animal differ in their resistance to apoptosis. ME-A cells are sensitive to apoptotic stimuli such as growth factor depletion and treatment with anti-tumour agents (e. g. doxorubicin). ME-C cells are very insensitive to apoptotic stimuli and they carry a p53 missense gene mutation frequently observed in breast cancer. In co-cultivation experiments ME-C cells protected the ME-A cells from stress induced apoptosis. Osteopontin (OPN), a secreted glycoprotein, is selectively overexpressed by the ME-C cells as demonstrated by microarray and Western blot analysis and this protein seems to be the main anti-apoptotic factor. ME-C cell medium concentrate (MC) containing OPN prevented ME-A cell death induced by stress stimuli. ME-C cells secrete various OPN isoforms that were not produced by the ME-A cells. There are strong indications that the 20 – 40 kDa OPN isoforms are responsible for the anti-apoptotic activity. ME-A cells engineered to overexpress OPN did not produce the 20 – 40 kDa OPN isoforms, which indicate that additional cell-specific post transcriptional modifications must occur to obtain the specific isoforms which mediate the anti-apoptotic activity. The anti-apoptotic signal involves the activation of phospholipase C (PLC) as the phospholipase inhibitor U73122 restored ME-A cell apoptosis in the presence of ME-C cell MC. When both ME-A and ME-C cells were analysed for OPN receptors by flow cytometry and immunofluoresence experiments, it was demonstrated that both cell lines presented at their surfaces the integrin β1 subunit and CD44 receptors, which might be involved in the anti-apoptotic OPN signal transduction pathway.

ACKNOWLEDGMENTS.

To my supervisor Prof. Dr. Monika Graessmann who gave me the opportunity to work under her directions. I want to thank her for allowing me to work in an interesting topic, for the good counselling in the professional and personal matters, for the patience and interest in discussing every detail of my work, for the inspiration she gave me to continue doing good science, and last but not least, for her friendship.

To Prof. Dr. Adolf Graessmann for the interesting discussions, the technical guidance and scientific ideas.

To Dr. Andreas Klein for his availability to discuss scientific questions with me, for his good counselling and support, for reading and correcting part of this manuscript, and for the time we spend together outside the laboratory as friends.

To Bianca Berg for the exceptional good counselling in technical aspects, for her ability to obtain perfect results, for the endless examples of hard, fine and accurate work. I want to thank her for having taught me the Berliner humor in our talks and for listening as a friend everytime I had some problems.

To Ms. Lenz for the preparation of laboratory material and solutions which made this study possible, for all the talks about personal matters and her friendly attention.

To Eva Guhl for the technical guidance in cell culture matters.

To Dr. Kathrin Danker for some of the material used in this study, and for scientific counselling.

To Mr. Piepno for his patience and efficient work with the “bestellungen”.

To Sandra Massmann for being my intern and the good time that we spent together working in the lab.

To my parents, brother and sister for encouraging me to pursue my goals and supporting me even when they were so far away and when sometimes is hard to understand and accept the dreams of others.

To my girlfriend Katja Freitag for pushing me through hard days with the sweetest love I ever had, for her understanding and patience, for not letting me down even when I thought I had lost my stamina.

To Paul Wafula for reading and correcting this manuscript but most of all for being a good Massala friend. Hakuna Matata!

And last but not least, to the Kommission für wissenschaftlichen Nachwuchs from the Charité for the scholarship I got to work for my doctorate.

CONTENTS

TABLE OF FIGURES

TABLES

Abbreviations.

AP-1	Activating protein 1
cDNA	Complementary DNA
CEFs	Chicken embryo fibroblasts
CMV	Cytomegalovirus
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
EMT	Epithelial-mesenchymal transition.
ERK	Extracellular signal-regulated kinase
ERM	Ezrin, radixin and moesin
ESCC	Esophageal squamous cell carcinomas
FAK	Focal adhesion kinase
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GM-CFS	Granulocyte-macrophage colony stimulating factor
HA	Hyaluronan
HAS2	Hyaluronan synthase 2
HCC	Hepatocellular carcinoma.
HGF	Hepatocyte growth factor
HPLC	High Performance Liquid Chromatography
IκB	Inhibitor of NFκB
IKK	I-kappa-B kinase or IkB kinase complex
IL	Interleukin
kDa	Kilodaltons
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MC	Medium concentrate
ME-A	Mammary epithelial cell line A
ME-C	Mammary epithelial cell line C
MM	Multiple myeloma
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid.
NFκβ	Nuclear factor κβ
OC	Osteoclast
OPN	Osteopontin.
PCR	Polymerase chain reaction
PI3-k	Phosphoinositide-3 kinase
PKB	Protein kinase B/Akt
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog deleted on chromosome Ten
PVDF	Polyvinylidenfluoride
RGD	Arginine-glycine-asparagine
ROCK	Rho kinase

RT-PCR	Reverse transcriptase-Polymerase chain reaction
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
shRNA	Small hairpin RNA
Spp1	Secreted phosphoprotein 1
SV40	Simian virus 40
SV40 T	Simian virus 40 large tumor antigen
SV40 t	Simian virus 40 Small tumor antigen
TGFβ	Tumor growth factor β
WAP	Whey acidic milk protein promoter
TPA	12-O-tetradecanoylphorbol-13-acetate

1. INTRODUCTION

1.1. ME-A and ME-C cell lines isolated from transgenic WAP SVT/t mouse breast tumors differ in their apoptotic program

ME-A and ME-C cell lines isolated from a breast tumor (e.g. tumor 8/61) originated in a WAP-SVT/t transgenic mouse (Tzeng *et al*., 1993) differed in their resistance to apoptotic stimuli (Tzeng *et al*., 1998, Graessmann *et al*., 2006). The ME-A cells maintain SV40 T/t antigen expression in tissue culture. The ME-A cell line has a high apoptotic rate in culture similar to the rate observed by SV40 T/t antigen synthesis in mammary glands. Mammary epithelial cells of WAP-SVT/t transgenic animals undergo elevated apoptosis during late pregnancy which induces premature involution of the gland (Tzeng *et al*., 1993; Tzeng *et al*., 1998). Furthermore, ME-A cells are sensitive to apoptotic stimuli such as cultivation under stress conditions (e.g. serum starvation), chemotherapeutic treatment (e.g. doxorubicin) and oxidative stress (Kohlhoff *et al*., 2000; Graessmann *et al*., 2006). The ME-C cell line was isolated from the same tumor. However, ME-C cells have spontaneously lost transgene expression and present a p53 missense mutation on codon 242 ($p53_{242}$) frequently found in human breast cancers (mouse codon 242 correspond to human p53 codon 245) (Tzeng *et al*., 1998). ME-C cells are resistant to apoptotic stimuli under stress conditions such as serum starvation and chemotherapeutic treatment (Tzeng *et al*., 1998; Graessmann *et al*., 2006).

ME-A and ME-C cell co-cultivation experiments demonstrated that ME-C cells mediate cell death resistance to ME-A cells in a paracrine manner. Microarray and Western blot analyses revealed that osteopontin (OPN) is overexpressed and about 1000-fold more efficiently secreted by the ME-C cells than by the ME-A cells. Induction of ME-A cell apoptosis by growth factor withdrawal (e.g. serum starvation) or doxorubicin treatment could efficiently be inhibited by addition of OPN containing ME-C cell medium concentrate (MC) which activated a survival signal mediated by the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)1/2 (Graessmann *et al*., 2006).

1.2. Osteopontin an ECM protein involved in cell survival and cancer.

Osteopontin was first isolated from bovine bone calcified matrix and characterized as a phospho-sialic-acid-rich phospho protein (Franzén *et al*., 1985). Its primary structure was deduced from cDNA clones isolated from rat osteosarcoma (Oldberg *et al*., 1986). Mouse

OPN is a 294 amino acids protein; it received its name from its ability to "bridge" (Latin *pons*) cell binding to osteoid matrix. OPN binds to hydroxylapatite, the most abundant component of bone calcified matrix, and the protein presents an Arg-Gly-Asp (RGD) sequence similar to that found in fibronectin, that mediates interactions with cell-surface receptors, like integrins. Apart from its function as an important adhesion factor in the extracellular matrix (ECM) OPN has been associated with diverse cellular processes. OPN plays a role as a cytokine in immune and inflammatory responses, as a protective agent in infectious diseases and remodelling agent in wound healing. OPN is also a regulator of haematopoietic stem cell pool size (reviewed in Weber G., 2001; Stier *et al*., 2005).

Since its discovery as a bone matrix protein, OPN expression has been found in other tissues and secreted by different cell types including osteoclasts, macrophages, endothelial cells, smooth muscle cells and epithelial cells and found in all body fluids (Mazzali *et al*., 2002; Khan *et al*.,2002; Wai *et al*., 2006). In several types of cancer and premalignant lesions osteopontin expression is markedly elevated, and has been implicated in promoting migration and survival of tumor cells (reviewed in Weber G.; 2001; Wai *et al*., 2004; Cook *et al*., 2005; Rangaswami *et al*., 2005). The strong secretion of OPN by the ME-C tumor cell line appears to be responsible for the survival mechanisms that act in an autocrine/paracrine manner and to influence positively the resistance to chemoterapeutic treatment and survival in stress conditions of ME-C and ME-A cells (Graessmann *et al*., 2006).

Apart from the role of OPN overexpression in tumor progression, OPN normally participates in different stress response processes and has different functions in the homeostasis of different tissues. OPN´s ability to be involved in these different processes might contribute to different steps in carcinogenesis and malignancy once its expression is subject of aberrant regulation. Osteopontin is involved in:

1. *Macrophage homing and cellular immunity*. OPN induces immune cell migration and invasion to sites of inflammation. OPN acts to mediate chemotaxis through interaction with CD44 or haptotaxis through binding to integrin receptors. The same mechanisms are used by tumor cells in the process of metastasis formation. OPN aids homing of immune cells by preventing apoptosis. Activated macrophages secrete reactive oxygen intermediates after phagocytosis. Oxidative stress kills germs and promotes apoptosis of the phagocyte itself. OPN is induced by macrophages concomitantly with phagocytosis and reduces the level of

ensuing programmed cell death through anti-oxidant effects in an autocrine fashion (reviewed in Weber, G., 2001).

2. *OPN mediates neovascularization and inhibits apoptosis*. OPN is one of three genes differentially expressed during *in vitro* angiogenesis (Prols *et al.*, 1998). Stress-dependent angiogenesis that occurs after injury or hypoxia activates OPN expression. During vascular growth, vascular smooth muscle cells promote the secretion of OPN and a splice variant of CD44 that are involved in endothelial cell proliferation and migration. Engagement of the OPN receptor integrin $\alpha_v\beta_3$ facilitate migration and prevents apoptosis of endothelial cells (reviewed in Weber, G., 2001).

3. *OPN is involved in bone resorption*. Osteoclasts are multinucleated giant cells with bone-resorbing activity. Bone resorption appears to proceed by the intricate coordination of the processes of attachment to bone, polarized secretion of acid and proteases, and active motility of osteoclasts along bone surface (Chellaiah *et al*., 2002). OPN plays a key role in anchoring osteoclasts to bone surface through recognition by $\alpha_v\beta_3$ integrin receptors. Large quantities of OPN secretion by osteoclasts suggest additional roles for OPN´s besides its function as anchorage protein. OPN ability to mediate adhesion to and resorption of bone may play an important role in the observed ability of tumor cells overexpressing OPN to migrate and metastasise to bone (reviewed in Weber, G., 2001).

4. *OPN is involved in ECM composition and remodelling*. Matrix metalloproteinases (MMPs) degrade the ECM and play critical roles in tissue repair, tumor invasion and metastasis. OPN can stimulate activation of pro-MMP-2 through NFκβ-mediated induction of membrane type MMP-1 and this affects positively tumor growth and proliferation (Philip *et al*., 2001). OPN can also induce urokinase-type plasminogen activator (Upa) secretion, which is another remodelling factor of the ECM (Das *et al*., 2004). Mice lacking OPN have an altered matrix assembly which causes disorganization in wound healing processes that may be a consequence of altered expression of metalloproteinases (Liaw *et al*., 1998). OPN itself is part of the ECM and has been found to stimulate migration, proliferation and survival of a variety of cell types by influencing multiple signal transduction pathways upon binding to cell-ECM adhesion receptors (Tuck *et al*., 2003).

1.2.1. Osteopontin gene expression is upregulated in cancer

OPN mRNA expression has been analysed during mammary gland development in mice and its expression is found in non-lactating mammary glands. During pregnancy, levels of OPN mRNA expression increase rapidly in the first day of lactation and decrease at the end of it. During mammary gland involution, levels of OPN mRNA expression are increased again (Rittling *et al*., 1997). Targeted inhibition of OPN expression through anti-sense RNA in the mammary gland caused abnormal morphogenesis and mammary epithelial cell differentiation, lack of mammary alveolar structures and lactation deficiency (Nemir *et al*., 2000).

Several studies point to a link between OPN gene upregulation and cancer. In transformed avian cells OPN was found as one of three strongly activated genes stimulated by v-myc/v-mil(raf)-oncogenes (Hartl *et al*., 2006). In human esophageal squamous cell carcinomas (ESCC), colon tumors and uveal melanomas OPN mRNA upregulation is associated with tumor progression and malignancy (Agrawal *et al*., 2002; Kadkol *et al*., 2005; Ito *et al*., 2006).

Furthermore, different studies corroborate the existence of a strong relation of OPN overexpression to breast cancer. In human breast cancers, upregulation of OPN RNA and protein expression has been associated to the metastatic phenotype (Urquidi *et al*., 2002). In a human mammary carcinoma cell line, OPN overexpression influences expression levels of 99 known genes involved in tumor progression (Cook *et al*., 2005). OPN was highly overexpressed in a mouse cell line containing a p53 mutation, isolated from mammary gland tumors originated by WAP-SV T/t transgene expression (Graessmann *et al*., 2006). Recently, in an study that analysed a group of 303 breast cancer patients followed for up to 20 years, S100P, S100A4, and OPN gene mRNA overexpression were the most significant independent indicators of death in this group of patients (Wang *et al*., 2006).

1.2.2. Transcriptional regulation of osteopontin mRNA expression

Human, murine and porcine OPN promoters have been cloned and diverse consensus regulatory sequences have been found to control the OPN gene expression (reviewed in Wai *et al*., 2004) but the numbers of consensus sequences shared by the three species is relatively small (Hijiya *et al*., 1994). In the murine OPN promoter a characteristic TATA box, and inverted CCAAT box, a positive transcription element and a negative transcription element were identified (Craig *et al*., 1991).

Although expression of OPN is not ubiquitous, OPN is expressed in a variety of cells and shows diverse features of expression. Constitutive expression of OPN is found in bone, kidney, placenta, mammary gland, nerve cells, macrophages and other tissues and cell types, whereas inducible or enhanced expression of OPN is observed in T-lymphocytes (Hijiya *et al*., 1994).

OPN transcription regulation is complicated and involves different complex regulatory pathways. Consensus sequences for binding of several transcription factors have been found in the osteopontin promoter (reviewed in Wai *et al*., 2004). Several molecular pathways involved in tissue homeostasis, which are found deregulated in cancer, activate OPN mRNA transcription. Among the most prominent, activation of MAPK, Myc, p53, Ras, Src, EGF, HGF (Met) and TGFβ signaling induces OPN mRNA overexpression as demonstrated for different cell lines (Hijiya *et al*., 1994; Guo *et al*., 1995; Geissinger *et al*., 2002; Morimoto *et al*., 2002; Tuck *et al*., 2000; Tuck *et al*., 2003; Zhang *et al*., 2003; Wai *et al*., 2004).

Importantly, there exists a connection between p53 and OPN mRNA regulation. OPN was identified as a p53-target gene using mRNA differential display analysis of embryonic fibroblasts from wild type against p53-deficient mice. OPN expression was upregulated by DNA damage-induced p53 activity and adenovirus-mediated transfer of the human p53 gene. Chromatin immunoprecipitation assays confirmed interaction between OPN promoter and p53 protein *in vivo* (Morimoto *et al*., 2002). In the same study it was demonstrated that OPN expression by p53 is conserved across multiple species (rat and mouse) but OPN gene inducibility by p53 varied among cell types. In ME-C cells that contain a p53 mutation frequently found in breast cancer, OPN expression is upregulated (Tzeng *et al*., 1998; Graessmann *et al*., 2006). Recent studies showed that OPN mRNA and protein levels were upregulated in melanoma tissue microarrays in which also phosphatase and tensin homolog deleted on chromosome ten (PTEN) mutations were present. PTEN, a transcriptional target of p53, prevents constitutive activation of PI3-k, and in cells lines where PI3-k is inhibited there is a reduced OPN protein expression (Packer *et al*, 2006).

Another prominent pathway involved in cell transformation, the Ras pathway, also controls OPN expression. Craig *et al*, (1988) discovered OPN protein (named ar2 in this study) as a tumor promoter-inducible protein secreted by mouse JB6 epidermal cells. Transformation of

NIH 3T3 cells with H-Ras resulted in a large increase of OPN protein and mRNA expression mediated through H-Ras activation of AP-1/c-Jun transcription factors. Induction of OPN appears to be mediated by protein kinase C (PKC), which can be activated through different upstream pathways dependent on the cells proliferation state (Smith *et al*., 1989). OPN expression correlated with the extent of Ras activation in mouse fibroblasts (Chambers *et al*., 1992) and in T24 H-Ras-transformed NIH 3T3 cells the OPN promoter contained a protein-binding motif which enhanced OPN mRNA expression upon Ras activation (Guo *et al*., 1995). Mouse primary embryo fibroblasts transfected with an activated-Ras mutant increased the OPN mRNA levels and consequently, secretion of OPN protein, which enhanced the ability of transformed fibroblast for tumor formation *in vivo* (Wu *et al*., 2000). Ras-activation protects cells against hypoxia. Hypoxia is a common feature in solid tumors and is an important microenvironmental factor that decreases the effectiveness of conventional chemotherapy, increases malignant tumor progression, enhances tumor cell invasion and metastasis and is prognostic for tumor control by conventional treatment modalities. OPN is induced by hypoxia by a Ras-activated enhancer. Thus, OPN overexpression and secretion might play a role in the protection of cells against hypoxia (Zhu *et al*., 2005).

Src is another important oncogene that is involved in the regulation of OPN mRNA expression. v-Src was the first defined oncogene and encodes the first recognized tyrosine kinase (Hunter *et al*., 1980). v-Src and its cellular homologue (c-Src) are tyrosine kinases that modulate the actin cytoskeleton and cell adhesions. They are targeted to cell-matrix integrin adhesions or cadherin-dependent junctions between epithelial cells where they phosphorylate substrates that induce adhesion turnover and actin re-modelling (Frame *et al*., 2002). Src can be activated upon ligand binding to growth factor receptors. Epidermal growth factor (EGF) ligation to its receptor can induce OPN gene expression mediated by constitutive Protein Kinase B/Akt activation inducing the constitutive expression of OPN in malignant but not in benign transformed breast cells (Zhang *et al*., 2003). Src activation in epithelial cells reduces cell-cell adhesion by phosphorylation of E-cadherin-catenin complexes. Dissociation of E-cadherin adhesions promotes β-catenin cytoplasmic relocalization and activation of gene transcription. OPN can be regulated by PEA3, β-catenin/Tcf, and AP-1 transcriptions factors, which also synergize in the up-regulation of the matrix metalloproteinase matrilysin (MMP-7) another protein associated with tumor invasion and metastasis (El-Tanani *et al*., 2004). Interestingly, c-Src associates in a macromolecular complex at the cell membrane upon OPN induction and induces the phosphorylation of ERK1/2 and PI3K as well as AP-1 activation

(Tuck *et al*., 2000) pointing to a positive feedback loop mechanism in the regulation of OPN mRNA expression.

Similar to Src positive feedback loop, upon ligand binding and activation of CD44 receptors, the c-Ras pathway is activated (Fitzgerald *et al*., 1999). OPN is a ligand for CD44 receptors. Thus, uncontrolled OPN expression would lead to activation of Src and Ras pathways activating the positive feedback loop of OPN mRNA expression leading to the protein overexpression observed in tumor progression and metastasis (Coppola *et al*., 2004).

1.2.3. Osteopontin protein overexpression in cancer.

OPN protein expression is a key molecular event in tumor progression and metastasis (Coppola *et al*., 2004). Thus, OPN has started to be considered as a potential marker in cancer malignancy and survival prognosis as OPN can be readily measured in blood and tumor tissue. OPN protein expression as prognostic value for metastasis and poor survival of breast cancer patients has been recognized (Bramwell *et al*., 2006). Importantly, a search for prognostic factors in a study of hepatocellular carcinoma (HCC) confirmed that p53 mutation and osteopontin overexpression correlated closely with lower than 5-years survival rates (Yuan *et al*., 2006). A Carboxy-terminal OPN fragment was elevated in urine samples of patients with ovarian cancer (Ye *et al*., 2006). Serum OPN levels were significantly higher after metastasis than before detection of metastasis of uveal melanomas to the liver (Kadkol *et al.*, 2005). OPN protein overexpression in ESCC tumors was implicated in the acquirement of malignant potential. Conditional OPN down-regulation using an inducible short-hairpin RNA (shRNA) vector decreased cell motility, invasion *in vitro*, tumor formation and lymph node metastasis *in vivo* (Ito *et al*., 2006).

In breast cancer patients undergoing sentinel node biopsy OPN levels were significantly higher in lymph node metastases than in primary tumor (Allan *et al*., 2006). In the same study, *in vitro* experiments with the MDA-MB-468 human breast cancer cell line showed the importance of the OPN RGD sequence in integrin-mediated cell adhesion and anchorage-independent growth. Following injection of cells in the mammary fat pad of nude mice, OPN expression increased lymphovascular invasion, lymph node metastases and lung micrometastases. In cells of the hematopoietic cell system, OPN has also been implicated in the induction of hyaluronan synthase 2 (HAS2) expression. High level of HAS2 is associated with increased hyaluronan (HA) production and matrix retention. HA is necessary for tumor

cell adhesion to CD44 receptors allowing anchorage-independent growth, an event associated with tumor progression and malignancy (Cook *et al*., 2006).

In vivo, OPN-null mutant mice subjected to repeated applications of a mutagen/carcinogen to induce cutaneous squamous cell carcinoma exhibited accelerated tumor growth and progression and had a greater number of metastases per animal compared with wild-type animals. However, the size of metastases were smaller in OPN-null animals. Furthermore, when tumor cells coming from OPN-null mice were isolated and injected into nude mice, they were able to form tumors. However, when the cell lines were engineered to reexpress OPN, they showed improved survival at secondary sites (Crawford *et al*., 1998). This study also demonstrated that inactivation of OPN eliminates activities both detrimental and beneficial to tumor progression. Differences between host-derived and tumor-derived OPN were found. It was postulated that host-derived OPN functions as a macrophage chemoattractant and tumor-derived OPN is able to inhibit macrophage function and enhances the growth and survival of metastases. It was proposed that OPN may affect phenotypic characteristics specific to later stages of progression. Selective escape and tumor survival as a consequence of OPN production could explain the observation that a greater number of tumor cells produce OPN as the tumor progresses and that OPN expression is maintained in metastases.

1.2.4. Osteopontin mediated cell survival

Extracellular matrix (ECM) proteins play a fundamental role in growth, survival and differentiation of mammary epithelial cells during mammary gland development which, unlike in other tissues and organs, occurs post-natally. Stromal-epithelial interactions are involved in the mechanism of lumen formation and thus regulate programmed cell death in a mechanism similar to the formation of the proamniotic cavity during mammalian development (Murray *et al*., 2000). The human mammary gland epithelium overexpress OPN specifically during pregnancy and lactation. OPN is also abundant in milk (Senger *et al*., 1989; Rittling *et al*., 1997; Nemir *et al*., 2000). During pregnancy and lactation the gland goes through extreme changes where epithelial cells expand and must be protected from apoptosis in order to establish the milk producing structures. Inhibition of OPN expression during these processes promotes disruption of the milk producing alveolar structures and lactation deficiency (Nemir *et al*. 2000).

Diverse studies *in vitro* have demonstrated the ability of OPN to mediate survival in normal and tumor cells under diverse conditions. Lopez *et al*. (1996) demonstrated that OPN could inhibit heregulin-induced apoptosis of the breast cancer cell line SKBr3. Addition of soluble OPN rescued adherent human umbilical vein endothelial cells from apoptosis induced by deprival of growth factors (Khan *et al*.; 2002). In BA/F3 murine pro-B cell line, binding of OPN to its receptor CD44 contributed to the survival activities of interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CFS) through phosphoinositide 3-kinase (PI3-k)/Akt signalling pathway (Lin *et al*., 2001). OPN can also induce survival by upregulation of Gas6. Gas6 has been found to protect cells from apoptosis by binding the oncogenic receptor Axl and subsequent activation of PI3-k/Akt pathways (Cook *et al*., 2005). In melanocytes, the pigment-producing cells of the skin, which under physiological conditions are normally restricted to *stratum basale* of the epidermis, are able to leave the epidermis and survive in the dermal environment upon transformation, where normal melanocytes cannot survive. Activation of growth factor receptor tyrosine kinases induced the overexpression of OPN protein which mediated survival of melanocytes in three-dimensional dermal collagen gels through activation of integrin $\alpha_v\beta_3$/MAPK pathway in an autocrine way (Geissinger *et al*., 2002).

Trophoblasts like metastatic cancer cells are invasive and must escape host immune surveilliance to survive. One of those immune surveillance pathways is the complement-mediated attack and lysis. OPN has been implicated in the evasion of trophoblasts to this surveillance mechanism by its ability to bind cell receptor integrin $\alpha_v\beta_3$ and sequester factor H to the cell surface of human breast cancer and myeloma cells (Fedarko *et al*., 2000). Multiple myeloma (MM) almost exclusively develops in the bone marrow and generates devastating bone destruction by osteoclasts (OCs) recruited around MM cells. MM cells secrete chemokines that act on MM cells in an autocrine/paracrine fashion and enhance MM cell adhesion to stromal cells through activation of integrins. Stromal cells are stimulated to produce interleukin-6 (IL-6) which promotes proliferation of MM cells and prevents them from apoptosis induced by cancer agents. Osteoclasts, which are among the major components of the bone marrow microenvironment have been shown recently to protect MM cells from apoptosis induced by serum depletion and doxorubicin. OCs produce osteopontin and IL-6 and adhesion of MM cells to OCs further increased the IL-6 production from OCs. Cell-cell adhesion mediated through integrins was proposed as a necessary mechanism for MM cell growth and survival mediated by OCs (Abe *et al*., 2004).

In vivo experiments have also demonstrated the role of OPN in tumor cell survival. In a model of two-stage skin chemical carcinogenesis, OPN-null mice showed a marked decrease both in tumor papilloma incidence and multiplicity compared with wild type mice. OPN expression was induced after treatment with the tumor-promoting chemical 12-O-tetradecanoylphorbol-13-acetate (TPA) and after induction, OPN prevented tumor cell apoptosis (Hsieh *et al*., 2006). In other example of an *in vivo* model of carcinogen-induced cutaneous squamous cell carcinoma in OPN-null mice, tumor–derived OPN have been implicated in the survival of tumor cells at late stages of tumor progression. *In vitro*, cells from tumors originated in OPN-null mice transfected with an OPN construct, were able to survive when plated at low density in comparison to non-OPN expressing tumor cells. *In vivo*, wild-type mice showed larger metastases to lung than OPN-null mice, indicating that one function of OPN is to increase cell survival and growth at a secondary site and the inactivation of host immune responses (Crawford *et al*., 1998).

In other context, Osteopontin has been shown to have a negative effect on the hematopoietic stem cell pool size (Stier *et al*., 2005). When OPN was absent, there was an increase in the stem cells numbers. It was demonstrated that there is a direct effect of OPN in the stem cells apoptotic rate. Exogenous OPN provided a proapoptotic stimulus in primitive cells that was abrogated with a neutralizing antibody to OPN. Matrix proteins create specialized microenvironments for stem cells that regulate stem cell pool size. This is important because deregulation of matrix proteins in neighbour cells to a stem cell niche may disrupt the tight controls in stem cells, promoting uncontrolled expansion. Thus, OPN is able to control the fate of several types of cells. How OPN can regulate its different effects it is not completely understood. However the ability of OPN to be post-translationally modified in several ways (glycosylation/phosphorylation/cleavage) and to be able to interact with different cell membrane receptors made OPN signaling a complicated network that allows for flexible and specific signal regulation.

1.2.5. Osteopontin cell membrane receptors

1.2.5.1. CD44 receptor

Once tumors have grown at the primary sites, the development of metastatic spread depends on the acquisition, alteration, or deletion of a number of properties characteristic of malignant transformation. While moving to new anatomical locations that were previously foreign to

them, new tumor cells use a new set of adhesion, migration and homing proteins. This process depends, in great part, on the ability to attach to and detach from various types of extracellular components and cells. Therefore the modulation of the cell-extracellular matrix (ECM) adhesion and cell-cell adhesion is paramount to the completion of the metastatic cascade. Furthermore, positive and negative effects of these new adhesions act on tumor cell growth. Among the many cell-cell and cell-ECM adhesion molecules used by cancer cells, CD44 plays an important role (reviewed in Jothy, S., 2003).

Osteopontin interacts and activates CD44 receptors. CD44 is the major receptor for hyaluronan, also known as hyaluronic acid (HA). HA is a non-sulfated glycosaminoglycan consisting of 2,000 to 25,000 repeating disaccharide subunits of glucoronic acid and *N*-acetylglucosamine. It is ubiquitously distributed and is the major type of glycosaminoglycan present in the ECM. CD44 is a family of transmembrane proteins encoded by a single gene with at least 19 exons. Structural and functional diversities arise from alternative splicing and variation in *N*- and *O*- glycosylation (reviewed in Götte *et al*., 2006).

The extracellular domain of CD44 is involved in adhesion and migration on extracellular components, it can recruit cytokines and proteases and activate transmembrane proteins like growth factor receptors. CD44 extracellular domain is cleaved during some biological processes and acts in a soluble form as part of the ECM (reviewed in Cichy *et al*., 2003). The cytoplasmic domain is involved in anchorage to the cytoskeleton in an indirect form, binding to cytosolic proteins like ankyrin and members of the ezrin, radixin and moesin (ERM) family of proteins. HA binding to CD44 leads to cytoskeletal reorganization. CD44 associates with Src family of non-receptor tyrosine kinases and through this association controls multiple biological functions in a variety of cells and tissues (reviewed in Marhaba *et al*., 2004; Ponta *et al*., 2003; Bourguignon, L.Y., 2001).

CD44 is expressed at high levels on many cell types, but not all cells that express the receptor are capable of inducing downstream activation events, which points to a high regulation on CD44 functions. Three activation states of CD44 (inactive, inducible and active) have been described based on the ability to bind FITC-labeled HA (FITC-HA) (Lesley *et al*., 1992). Most adhesion proteins are specialized as either cell-cell or cell-ECM adhesion and a remarkable property of CD44 is that it is involved in both. Consequently, dysregulation in the

expression of CD44 has the potential to alter the invasive and metastatic potential of tumor cells (Jothy, S., 2003)

Furthermore, CD44 expression is a marker of metastatic potential in certain tumors. CD44 functions have been linked to lymphocyte migration and activation, inflammation, differentiation and apoptosis (Lakshman *et al*., 2005; Jothy, 2003). These apparently distinct functions may be attributed to multiple mechanisms and structural variations including diversity in ligand binding, signalling pathways, glycosylation patterns, and the expression of variant exons through alternative CD44 gene splicing which confer variability to the extracellular part of the protein (Larkin *et al*.; 2006).

Indeed, overexpression of a myriad of CD44 splice variants (CD44v) and dysregulation of the standard isoform (CD44s) have been observed in the process of malignant transformation and metastasis. CD44 was recognized as a lymphocyte membrane molecule involved in adhesion to endothelial cells leading to the question of whether cancer cells use the same attachment mechanism to migrate (Jalkanen *et al*.; 1989; Stoolman, L. M., 1989). Original studies in animal models discovered CD44v6 isoform associated with metastasis (Gunthert *et al.*, 1991). There is conflicting data about the relation of CD44 splicing variants expression and metastasis progression. In colon carcinomas CD44v6 overexpression correlates with metastatic potential and in prostate carcinomas, metastatic progression correlated with a downregulation of CD44v6 expression. These and other studies demonstrated that tumor progression is characterized by a generalized dysregulation of the splicing process, leading to the expression of a multitude of CD44 isoforms in tumors of increasing malignancies, which is also dependent on the cell type (Jothy, S., 2003).

In contrast, CD44 standard isoform expression prevents the establishment of metastases in experimental animal studies (Pereira *et al*.; 2001). CD44s is the most widely expressed isoform of CD44. Unlike HA, increased expression of CD44s correlates with overall patient survival and in types of cancers that rarely metastasise. In mice that are susceptible to carcinogenesis due to p53 mutation, the absence of CD44 gene leads to abolition of osteosarcoma metastasis but not to changes in tumor incidence or growth (Weber *et al*., 2002) which points to a CD44 role in late stages of tumor progression similar to OPN activity.

In recent years evedence has been accumulated sugesting that CD44 is a receptor for OPN. However, the specific isoforms and posttranslational modifications necessary for this interaction, both in the receptor and the ligand, remain obscure. Smith *et al*. (1999) used four splicing CD44 variants in binding experiments to human recombinant OPN, recombinant OPN treated with thrombin and for OPN coming from other sources (human urine and rat smooth muscle cells). However, Smith *et al*. (1999) did not find support for the interaction between CD44 and OPN, in contrast to previous studies done by Weber *et al*. (1996) which demonstrated that a CD44-transfected cell-line could mediate cation-independent adhesion and migration to OPN. These conflicting results suggested that CD44-OPN interactions may not be a common event *in vivo*, and may be limited to specific CD44 splice variant(s) and/or a particular modified form of osteopontin (Smith *et al*., 1999).

Recently, OPN has been found to induce expression of different CD44 splicing variants thus influencing CD44 activities in tumor progression (Chellaiah *et al*., 2003a; Chellaiah *et al*, 2003b ; Zhu *et al*., 2004; Lee *et al*., 2007). OPN overexpression under CMV promoter in 21NT tumorigenic human breast cancer cell line provoked significant up-regulation of CD44s isoform mRNA expression and at protein levels CD44s and CD44v6 were markedly increased suggesting that OPN can regulate expression of both transcriptional and post-transcriptional (both amount and localization of protein) levels. CD44, CD44v6 and CD44v9 blocking antibodies reduced the OPN-mediated cell migration ability in this model (Khan *et al*., 2006). In another context, previous studies also demonstrated a link between OPN and CD44 expression levels. In osteoclasts and peritoneal macrophages from OPN-null mice CD44 showed a marked decrease in surface expression (Chellaiah *et al*., 2003a; Chellaiah *et al*, 2003b ; Zhu *et al*., 2004). Recently, Lee *et al*., (2007) found that in gastric cancer, where OPN expression is elevated, CD44v6-10 splicing variants were also expressed at elevated levels. OPN mediated anti-apoptotic effect is dependent on the expression of CD44v6 or CD44v7 and the OPN-RGD sequence, which allows interaction with integrins, is dispensable for the OPN-mediated anti-apoptosis effect. The anti-apoptotic signal was dependent on Src activity which activates integrins and subsequently focal adhesion kinase (FAK).

OPN may regulate CD44 activation by other means as direct receptor ligation. In 21NT breast cancer cells, OPN overexpression increases levels of hyaluronan synthase 2 (HAS2), which is associated with increased HA production and matrix retention and is necessary for tumor cell adhesion to bone marrow endothelial cells and anchorage-independent growth (Cook *et al*.,

2006). Increased HAS2 expression has been shown to contribute to enhanced cell survival and anchorage-independent growth of human HT1080 fibrosarcoma (Kosaki *et al*., 1999) and mesothelioma cells (Li *et al*., 2001). The specific accumulation of HA was widely observed in human tumors, including colon cancer (Roponnen *et al*., 1998), lung cancer (Horai *et al*., 1981), breast cancer (Bertrand *et al*., 1992), and glioma (Delpech *et al*., 1993). CD44 function in tumor cells may be to facilitate the penetration of stromal-cell derived HA, which in some tumor cell types may be a critical step toward establishment of metastatic colonies. Metastatic mammary carcinoma cells transfected with cDNAs encoding soluble CD44 receptors reduce the HA binding and internalizing ability of cells and abrogate their ability to form lung metastasis (Yu *et al*., 1997). In inflammatory disease states like rheumathoid arthritis and pulmonary fibrosis, HA has been found at elevated concentrations with preponderance toward lower molecular mass fragments. It has been hypothesized that in a non-inflammatory milieu, inert high molecular weight HA keep CD44 molecules in an inactive inert conformation. Lower molecular weight fragments generated under inflammatory conditions displace the high molecular HA and thereby facilitate CD44 activation. The increase in low molecular weight HA during inflammation and malignant disease might originate from multiple mechanisms like *de novo* biosynthesis, hyaluronidase activity and accumulation of reactive oxygen species (Fitzgerald *et al*., 2000).

Activation of CD44 with HA fragments could be demonstrated in diverse cell lines. HA binding to CD44 leads to activation of Ras and PKCζ, a downstream effector of phospholipase C (PLC) followed by activation of the IkB kinase complex (IKK). IKK activation promotes phosphorylation and degradation of IκBα and Nuclear factor κβ (NFκB) activation leading to induction of proinflammatory genes which might contribute to the pathological development of chronic inflammation and cancer (Fitzgerald *et al*., 2000). Ras activation induces OPN protein expression and CD44 activation is able to induce Ras activation pointing to a positive feedback loop for OPN expression.

Transformation by oncogenic Ras requires the function of Rho family GTPases. However, Rho activation does not seem to be a direct result of signalling pathways activated directly by Ras but it is an event selected in the isolation of transformed cell lines. Ras can activate the MAPK/ERK pathway inducing levels of p21/Waf1 that are inhibitory to cell cycle progression. High levels of Rho activity are selected for because they counteract the high levels of p21/Waf1 induced by oncogenic Ras. Activation of ERK-MAP kinases by oncogenic

Ras opposes RhoA activation of Rho kinase (ROCK) signalling to the cytoskeleton, thereby promoting motility. Thus, during the process of transformation, there is selective cross-talk between Ras and Rho signalling pathways (Sahai et al., 2001).

In the breast cancer cell line MDA-MB-231, the CD44v3 isoform is tightly coupled with RhoGEF in a complex that can up-regulate RhoA signalling and Rho kinase activity. Activated ROCK then phosphorylates certain cellular proteins, including the linker molecule Gab-1. Gab-1 phosphorylation by ROCK promotes the membrane localization of Gab-1 and PI3-k to CD44 and activates certain isoforms of PI3-k to convert PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 leading to Akt activation, cytokine (macrophage-colony stimulating factor (M-CSF)) production and tumor cell behavior (e.g. tumor cell growth, survival, and invasion) required for breast tumor progression (Bourguignon *et al*., 2003). Rho kinase promoted the interaction of CD44v3, CD44v8-10 with ankyrin and increased the migration of metastatic breast cancer cells (Bourguignon, L. Y., 2001). These results are in agreement with data from recent studies where Fujita *et al*., (2002) demonstrated for a human lung cancer cell line that binding of CD44 by HA induced tyrosine phosphorylation of focal adhesion kinase (FAK), which then associated with PI3-k, activated the MAPK pathway and protected cells from apoptosis. A dominant negative Rho mutant abolished FAK phosphorylation and cell survival. The cells used in this study were resistant to apoptosis induced by etoposide (a chemotherapeutical used also in the treatment of breast cancer) and inhibition of Rho, FAK or PI3-k made them sensitive.

Thus, a positive feedback mechanism exists between OPN and CD44, where OPN can induce the expression of different CD44 splicing variants and enzymes that contribute to activate CD44, which concomitantly activate the Ras pathway and the OPN mRNA transcription. For example, in human glioma cells OPN was induced by HA in cells lacking functional PTEN, and expression of OPN depended on Akt/PKB activation downstream of PI3-k (Kim *et al*., 2005).

Ras activation has been implicated in cell survival but also other pathways downstream of CD44 activation may control the anti-apoptotic signal apart from Ras, as has been demonstrated that CD44 can activate hepatocyte growth factor receptor (Met). Met can be associated with the transmembrane death receptor Fas and this interaction prevents Fas self-aggregation and ligand binding, thus inhibiting Fas activation and the induction of cellular apoptosis (Corso *et al*., 2005).

Met activation evokes pleiotropic biological responses, both *in vitro* and *in vivo*, often referred to as the "invasive growth". This is a complex genetic program that is specifically induced by the scatter factor receptors *Met* and *Ron*. It consists of a series of obligatory rate-limiting steps that occur physiologically during embryogenesis and tissue repair. In the first step of this process, cells acquire the ability to dissociate from their neighbours by breaking intercellular adherent junctions (scattering), leave their original environment and reach the circulation ("directional migration" and "invasion"). Cell survival in the bloodstream is facilitated by Met-induced protection from apoptosis and the ability to transiently grow in an anchorage-independent manner. Finally, cells extravasate, face the new environment, proliferate and eventually undergo terminal differentiation (Corso *et al*., 2005).

CD44 has been implicated in inducing proliferation and angiogenesis in tumors. The CD44v3 splicing isoform can be modified by heparan sulfate. Heparan sulfate is a reservoir on which heparin-binding growth and angiogenic factors aggregate, i.e. fibroblast growth factor 2 (FGF2) and hepatocyte growth factor. Heparanase, an enzyme able to break down heparan sulfate, releases these signalling molecules which act to promote tumor growth and invasion and stimulate angiogenesis (Götte *et al*., 2006). p53 binds to the heparanase promoter and inhibits its activity in breast cancer cells (Baraz *et al*., 2006). In contrast p53 mutations activate heparanase expression. Presence of CD44v3 significantly correlates with tumor infiltration by T lymphocytes and cancer metastases to draining lymph nodes, together with a loss of p53 protein expression (Rys *et al*., 2003)

CD44 is also involved in the remodelling of the ECM. CD44 recruits MMP-9 to the surface of cell membrane in keratinocytes and promotes activation of latent TGF-β (Yu *et al*., 2000). In breast cancer cells, hyaluronan-mediated stimulation of CD44v3 phosphorylation by transforming growth factor-β receptor I kinase also resulted in enhanced binding of CD44v3 to ankyrin and promotion of breast cancer migration (Bourguignon, L. Y., 2001; Bourguignon *et al*., 2003). In a model of metastasis of breast cancer to the lung, tumor cells were protected from apoptosis by a mechanism involving CD44/MMP-9 complex activation of latent TGF-*β*2 (Yu *et al*., 2004). Recently OPN expression was correlated with MMP-9 expression in adenocarcinomas (Frey *et al*., 2007) and OPN has been shown to induce pro-MMP-9 activation (Rangaswami *et al*., 2006).

Thus, osteopontin activation of CD44 is complex and involves the activation of other ECM regulated pathways. Osteopontin binding to CD44 activates signal transduction pathways involved in several cellular functions, including survival.

1.2.5.2. Integrin receptors

Integrin receptors are non-covalently associated, heterodimeric, cell-surface glycoproteins with α- and β-subunits. The various combinations of the α- and β-subunits form integrin dimmers with diverse ligand specificity and biological activities. Integrins serve as receptors for a wide variety of ligands including ECM constituents, immunoglobulins, cadherin class cell adhesion molecules and cell surface-associated and soluble members of the disintegrin family. In addition to mediating cell adhesion, integrins make transmembrane connections to the cytoskeleton and activate many intracellular signalling pathways. Integrins play an important role in development, wound healing, immune responses and cancer (reviewed in Hynes, 2002).

Several integrins have been found to be receptors for OPN. Integrin OPN receptors include $\alpha_v\beta_1$ $\alpha_v\beta_3$, $\alpha_v\beta_5$ $\alpha_4\beta_1$, $\alpha_5\beta_1$ $\alpha_8\beta_1$ and $\alpha_9\beta_1$. Receptor-ligand interaction depends on cell type (reviewed in Wai *et al*., 2004). For example, OPN interaction with $\alpha_8\beta_1$ integrin is suggested to be involved in mouse kidney morphogenesis by regulating the epithelio-mesenchymal interactions during kidney development (Denda *et al*., 1998; Rogers *et al*., 1997), however in endothelial cells and osteoclasts, osteopontin interacts with $\alpha_v\beta_3$ integrin receptor (Weber *et al*., 2001; Chellaiah *et al*., 2002; Zhao *et al* ., 2005).

In cancer, during epithelial-mesenchymal transition (EMT) induced by oncogenic Ras signalling, mammary epithelial cells and fibroblasts enhance *de novo* integrin expression and localization (Maschler *et al*., 2005). In breast cancer, tumor progression into malignancy involves changes in the integrin receptor expression. In tumor mammary cells correlative evidence has shown that the $\alpha_v\beta_3$ integrin receptor appears to be preferentially used by more malignant breast epithelial cell lines in binding and migrating toward OPN or vitronectin (Wong *et al*., 1998; Tuck *et al*., 2000). Experiments with the non-metastatic 21NT cell line, which responds to OPN through integrins $\alpha_v\beta_5$ and $\alpha_v\beta_1$, demonstrated that expression of integrin β_3 and OPN proteins converts this cell line into a more aggressive kind. Stable transfected integrin β_3-expressing cells showed increased adhesion, migration and invasion to OPN in vitro, and when injected in nude mice, these cells demonstrated an increased primary

tumor take, decreased tumor doubling time, decreased tumor latency period and increased metastasis to lymph nodes (Furger *et al*., 2003).

OPN contains an RGD sequence responsible for integrin receptor interaction. The integrin-binding sequence of OPN seems to be important for MDA-MB-468 breast cancer cells ability to migrate to lymph nodes (Allan *et al*., 2006). Ligation of OPN to $\alpha_v\beta_3$ integrin present in osteoclasts and bone surfaces has been associated with the ability of breast cancer cells to migrate and metastasise to bone. Chellaiah *et al*. (2002) demonstrated that osteoclasts secrete osteopontin in their basolateral surfaces where it binds to $\alpha_v\beta_3$ integrin in an autocrine manner. Anti-bodies against $\alpha_v\beta_3$ integrin and CD44 blocked the osteopontin-stimulated motility in osteoclasts. Ligation of $\alpha_v\beta_3$ integrin has also been implicated in osteoclast survival (Zhao *et al*., 2005). Furthermore, tumor cells may be able activate signal transduction on cells from a different tissue compartment in a paracrine fashion. The breast cancer cell line MDA-MB-231 conditioned media had potent and direct anti-apoptotic effects in mature osteoclasts, mediated through the MAPK and PI3-k pathways, which greatly contributed to their osteolytic potential. (Gallet *et al*.; 2004).

In MCF-7 and MDA-MB-231 breast cancer cells Das *et al*. (2004) demonstrated that ligation of OPN with $\alpha_v\beta_3$ integrin induces kinase activity and tyrosine phosphorylation of EGFR mediated by Src tyrosine kinase. $\alpha_v\beta_3$ integrin, EGFR and c-Src associate in a macromolecular complex at the cell membrane upon OPN induction. OPN induced integrin-dependent migration of human mammary epithelial cells and activation of hepatocyte growth factor receptor (Met) (Tuck *et al*., 2000) and EGF receptors (Tuck *et al*., 2003). OPN also induces the phosphorylation of ERK1/2 and PI3-k and AP-1 activation, inducing the expression and secretion of urokinase-type plasminogen activator (uPA). Met activation has also been associated with uPA expression. Other growth factors known to promote cell motility like basic fibroblast growth factor (FGF) and transforming growth factor-α, can activate uPA expression. The simultaneous expression of uPA and its receptor has been associated with localized plasminogen activation and pericellular matrix degradation (Yebra *et al*., 1996).

In melanoma cells, treatment with soluble OPN increased $\alpha_v\beta_3$ integrin-mediated Src activity. Increased Src activity is commonly associated with reorganization of epithelial adhesion systems that leads to migratory phenotypes (reviewed in Frame *et al*., 2005). Elevated Src

kinase activity has a negative effect on the ability of epithelial cells to assemble or to maintain cadherin-based cell-cell adhesions and is also essential for the dynamic regulation of cell-matrix adhesions in different cell types. α_v integrin subunit was implicated in the regulation of Src activity.

Recent studies with MCF7 cells derived from breast tumors demonstrated the importance of Src in tumorigenesis (Gonzales *et al*., 2006). An inducible dominant negative c-Src (c-SrcDN: K295M/Y527F) induced altered cell morphology and caused a significant impairment of cell migration, adhesion and spreading. These effects were associated with delocalization and reduced tyrosine phosphorylation of critical proteins involved in integrin signalling and adhesion dynamics such as FAK (tyr^{925}), $P130^{CAS}$ and Paxillin. Expression of SrcDN reduced cell proliferation *in vitro*, and led to decreased AKT phosphorylation and higher expression of cell cycle inhibitor $p27^{Kip1}$. In nude mice, c-SrcDN expression reduced tumor growth and importantly, induced tumor regression. The anti-tumorigenic ability of induced SrcDN was associated with decreased proliferation, induction of apoptosis and reduction of angiogenesis potential. It has been postulated that activation of Src antagonizes p53 function. Thus, integrin activation under a p53 mutation background might activate a pro-survival positive feedback mechanism.

There is abundant evidence that v-Src-transformed fibroblasts are finely balanced between proliferation and death, particularly when serum survival factors are limited. Neoplastic transformation of avian neuroretina cells by $p60^{v\text{-}Src}$ tyrosine kinase resulted in dramatic morphological changes and disregulation of apoptosis (Néel *et al*., 2005). Datta *et al*., 2001 demonstrated that in normal chicken embryo fibroblasts (CEFs), increases in the phosphorylation of FAK at its Src SH2 domain binding site (Tyr^{397}) occur in dependence on levels of fibronectin binding and v-Src binds and phosphorylates FAK without need of this signal. Over time, v-src CEFs showed decreased adhesion due to the reduction of integrin $\alpha5\beta1$-fibronectin bonds. This was explained not by a reduction in the affinity of the integrin to its ligand but by the increase in protease secretion and excess synthesis of hyaluronic acid. Thus, OPN-mediated Src kinase activation and integrin binding would be involved in the HA-mediated activation of CD44.

Indeed, engagement of $\alpha_v\beta_3$ integrin by OPN has been implicated in endothelial cell survival and migration, which may be important for the neovascularization observed in tumors

(Weber, G.; 2001). Recently, Rice *et al*. (2006) demonstrated that the survival pathway is dependent on OPN-induced activation of NFκβ. In mammary tumors, NFκβ activation by autocrine laminin signalling to integrins has been associated with anchorage-independent survival of mammary epithelial cells (Zahir *et al*., 2003).

OPN induces $\alpha_v\beta_3$ integrin-mediated phosphorylation and activation of nuclear factor inducing kinase (NIK) in B16F10 cells. This effect was mediated by the ERK pathway and resulted in the activation of NFκβ, uPA secretion and uPA-dependent pro-MMP-9 activation and cell motility (Rangaswami *et al*., 2006). Previously, OPN was found to induce pro-MMP-2 production and activation in those cells. This was associated with the ability of osteopontin to activate NFκβ via $\alpha_v\beta_3$ integrin activation. Activation of NFκβ induces MT1-MMP induction which activates pro-MMP-2. Inhibition of MMP-2 protein expression in the cells suppressed OPN-induced cell migration, ECM invasion and tumor growth. OPN overexpression promoted CD44 cell surface expression and accumulation of MMP-2 activity in conditioned medium (Samanna *et al*., 2006). Recently OPN ligation to CD44 has been found to activate integrins, suggesting the existence of cross-talk mechanisms between CD44 and integrin pathways.

2. RESEARCH GOALS.

The ME-A and the ME-C cell lines were isolated from a breast tumor (e.g. tumor 8/61) originated in a WAP-SVT/t transgenic mouse. These cell lines differ in their resistance to apoptotic stimuli such as cultivation under serum starvation conditions and doxorubicin treatment. The ME-A cells maintained the SV40 T/t antigen transgene expression and they have a high apoptotic rate in culture. In contrast, the ME-C cells spontaneously lost transgene expression and are very insensitive to apoptotic stimuli. Furthermore, ME-C cells presented a p53 missense mutation frequently found in human breast cancers. Co-cultivation experiments with both cell lines revealed that ME-C cells mediate cell death resistance to the ME-A cells. Microarray and Western blot analysis showed that osteopontin (OPN), a secreted glycoprotein, was selectively overexpressed by the ME-C cells and these cells accumulated large amounts of OPN in culture medium. ME-C cell medium concentrate (MC) prevented apoptosis of ME-A cells cultivated under stress conditions.

Previous experiments performed in our group demonstrated that the MC survival signalling involved activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)1/2 pathway. ME-C cell MC prevented ME-A cell death under serum starvation conditions by blocking the caspase-3 activity. Doxorubicin increased caspase-3 activity and MC prevented apoptosis, although caspase-3 remained active. Thus depending upon the apoptotic stimuli, ME-C cell medium concentrate (MC) prevents ME-A cell apoptosis either upstream or downstream of caspase-3 (Graessmann et al. 2007).

The anti-apoptotic signal mediated by ME-C cell MC might depend on activation of specific cell surface receptors. OPN is a ligand to different cell membrane receptors such as integrins and CD44.

The following questions were addressed in this study:

1. Which of the multiple OPN isoforms, secreted by the ME-C cells, is responsible for the anti-apoptotic activity?

2. Which signaling molecules upstream of ERK/MEK 1/2 are involved in the transduction of ME-C cell MC anti-apoptosis signal upon addition to the ME-A cells in culture?

3. Which OPN receptors are present in the ME-A and the ME-C cells that might mediate the OPN-derived anti-apoptotic signal?

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals subtances

Acetone. (Roth)
Acrylamide solution 30% (Roth)
Ampicillin (Serva)
Agarose (Gibco BRL)
Ammoniumpersulfate (IBI)
Bromophenol Blue (Merck)
Bovine Serum Albumin (Serva)
$CaCl_2$ (Merck)
Chloroform, p.a. (Roth)
CsCl (Roth)
DEPC (Sigma)
Doxorubicin (Sigma)
DTT (Sigma)
EDTA (Roth)
Ethanol (Roth)
Ethidiumbromide (Sigma)
Formaldehyde (Merck)
Fetal Calf Serum (Gibco BRL)
Geneticin/G418 (Gibco BRL)
Glacial acetic acid (Roth)
Glycerine (Roth)
HEPES (Gerbu)
Isoamylalcohol (Roth)
KCl (Merck)
KH_2PO_4 (Merck)
LY294002 (Calbiochem)
Methanol, p.a. (Roth)
$MgCl_2$ (Merck)
Skim Milk Powder (Glücksklee)
Sodium Acetate (Merck)
NaCl (Merck)
Sodiumdodecylsulfate, SDS (Roth)
NaOH (Merck)
$NaH_2PO_4xH_2O$
$Na_2HPO_4x2H_2O$
Nucleotides and Nucleic Acids:
dATP, dCTP, dGTP, dTTP (Pharmacia)
DNA ladder, 1 Kb (Gibco BRL).
Oligo(dT) (Pharmacia)
PD98059 (Biolabs)
Phenol Tris pH 7.4 saturated (rotiphenol, Roth).
PMSF (Serva)
PP2 (Calbiochem)
Ponceau Black (Roth)
Propidium Iodide (Sigma)
Protease Inhibitor (Sigma)
Protein ladder, 10 kDa (Gibco BRL)
RNAse Inhibitor, HPRI (Pharmacia)
Sea Plague Agarose (FMC Bioproducts)
TEMED (Roth)
Tris (Roth)
Triton X-100 (Merck)
Trypsin-EDTA (Gibco BRL)
Tween 20 (Merck)
U73122 (Tocris Biosciences)

3.1.2. Antibodies

Table 1. Antibodies

Primary Antibody	**Type**	**FACS**	**IF**	**WB**	**Company**
Anti-mouse CD44 (Clone IM7)	Rat monoclonal	1:10	1:10, 1:25	-	BD Pharmigen
Anti-intregrin α_V	Mouse monoclonal	1:10	1:10- 1:50	-	Bd Transduction Labs.
Anti-integrin α_V (H9.2b8)	Armenian hamster monoclonal	1:10	1:10- 1:50	-	Santa Cruz
Anti-Integrin β1 (ASK3A6)	Rabbit polyclonal	1:10- 1:50	1:10- 1:50	1:1000	Chemicon
Anti-Integrin β1 (ASK2A4)	Rabbit polyclonal	1:10– 1:50	1:10– 1:50	1:1000	Chemicon
Anti-Integrin β3 (2C9.G2)	Armenian hamster monoclonal	1:10 – 1:100	1:10 – 1:100	-	Santa Cruz
Anti-mouse Integrin β5	Rabbit polyclonal	1:10, 1:25	-	-	BIOZOL
Anti- Integrin β5 (E-19)	Goat polyclonal	1:10-1:50	-	-	Santa Cruz
Anti-Osteopontin	Mouse monoclonal	-	-	1:5000	Santa Cruz
Anti-Osteopontin	Goat polyclonal	-	-	1:5000	R&D systems, AF808
Secondary Antibody	**Type**	**FACS**	**IF**	**WB**	**Company**
Anti-armenian hamster FITC conjugated	Goat polyclonal	1:10, 1:50	1:10, 1:50	-	Santa Cruz
Anti-goat FITC conjugated	Rabbit polyclonal	1:20, 1:50	1:20, 1:50	-	Abcam
Anti-rabbit hamster FITC conjugated	Goat polyclonal	1:50	1:50	-	Santa Cruz
Anti-rat FITC conjugated	Goat polyclonal	1:10	1:10	--	Santa Cruz
Anti-mouse-HRP-conjugated	Rabbit polyclonal	-	-	1:5000	Dako
Anti-goat HRP-conjugated	Rabbit polyclonal	-	-	1:5000	Dako
Anti-rabbit HRP-conjugated	Goat Polyclonal	-	-	1:5000	Dako

3.1.3. Enzymes

DNase RQ I (Promega)
Proteinase K (Boehringer, Mannheim)
Restriction enzymes: EcoRI and EcoRV, Not I, Kpn I (Gibco BRL)
RNase A (Boehringer Mannheim)
Superscript Reverse Transcriptase (Gibco BRL)
Taq-DNA-Polymersae (Gibco BRL).

3.1.4. Molecular biological and biochemical test kits

FuGene transfection reagent (Roche)
Protein assay (BioRad)
Qiagen plasmid maxi kit (Qiagen)
Concert^{TM} gel extraction kit (Gibco BRL)
ECL- detection system (Perkin Elmer Life Science)

3.1.5. Instruments

FACSscan (Becton Dickinson).
High Performance Liquid Chromatography (HPLC) instrument (Amersham Pharmacia)
Laborzentrifuge 1K15 (Sigma).
Megafuge 1.0R (Heraeus Instruments).
RC-5B refrigerated superspeed centrifuge (Sorvall).
Spectrophotometer UV-160 (Shimadzu).
Superdex 200 column (Amersham Pharmacia).
TRIO thermocycler (Biometra).
Western Blot minigel apparat (BioRad).

3.1.6. Special materials

Film Developer and Fixer GBX solutions (Kodak)
PVDF-membrane (Roth)
XRay film X-OMAT AR (Kodak)
Cell Culture materials (Nunc, Falcon)

3.1.7. Solutions

All solutions were used in sterile conditions. Solutions used for RNA analysis were treated with DEPC (100 µl/l) to avoid RNAse contaminations (Sambrook et al., 1989).

Table 2. Solutions

Ammoniunpersulfate solution	10% Ammoniumpersulfate (w/v)
Blocking Buffer (Western Blots)	5% Skim milk powder (w/v) in PBS-T
PBS	137 mM NaCl, 2.68 mM KCl, 6.5 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.68 mM $CaCl_2$, 0.5 mM $MgCl_2$
PBS-T	137 mM NaCl, 2.68 mM KCl, 6.5 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.68 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% Tween 20 (v/v)
Ponceau-S-lösung	0.2% Ponceau Schwarz (w/v), 3% Thricloracetic acid (w/v), 3% Sulfosalicyl acid (w/v)
DNA Loading Buffer	0.25% Bromophenol Blue (w/v), 0.25% Xylene Cyanol FF (w/v), 30% Glycerin (v/v)
Protein Loading Buffer	50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% SDS (w/v), 0.1% Bromophenol Blue (w/v), 10% Glycerin (v/v).
RNA Loading Buffer	50% Glycerin (v/v), 1 mM EDTA, 0.4% Bromophenol Blue (w/v), 0.4% Xylenecyanol FF (w/v)
Reaction Buffer Not I/Kpn I	100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreiol (pH 7.9), suplemented with 1000 µg/ml BSA
Reaction Buffer EcoRI/EcoRV	50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl2, 0.025% Triton X-100 (pH 7.5).
SDS Stock Solution	10% SDS (w/v)
50xTAE (electrophoresis buffer for DNA gels)	2 M Tris (pH 8.0), 50 mM EDTA, 250 mM Sodium-Acetate
TE-Puffer	10 mM Tris (pH 8.0), 8 mM NaOH
Anode Buffer I (Western Blot)	0.3 M Tris (pH 10.4), 10% Methanol in 25 ml dH_2O
Anode Buffer II (western Blot)	0.025 M Tris (pH 10.4), 10% Methanol in 100 ml dH_2O
Cathode Buffer (Western Blot)	0.3 g Glycine, 2.5 ml 1M Tris pH 9.4, 10 ml Methanol add dH_2O to 100 ml
$CaCl_2$ Solution (Bacterial Transformation)	2.4 g HEPES (pH 7.0), 7.4 g $CaCl_2$, add 100 ml dH_2O
Lysis Buffer (Cell lysates)	50 mM Tris/HCl pH 7.8, 150 mM NaCl, 2.5 mM EDTA, 1% NP40, 1 mM PMSF, 1µg/ml Peptastin, Aprotinin, Leupeptin
Solution I (plasmid isolation from 5 ml bacterial culture)	50 mM glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA (pH 8.0)
Solution II (plasmid isolation from 5 ml bacterial culture)	0.2 N NaOH, 1% SDS
Solution III (plasmid Isolation from 5 ml bacterial culture)	5 M Potassium acetate, glacial acetic acid, H_2O
Solution 1 (plasmid isolation from 20 – 200 ml bacterial culture)	50 mM Tris-Cl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A
Lysis buffer (plasmid isolation from 20 – 200 ml bacterial culture)	200 mM NaOH, 1% SDS
Neutralizing solution (plasmid isolation from 20 – 200 ml bacterial culture)	3M potassium acetate pH 5.5
Elution buffer (plasmid isolation from 20 – 200 ml bacterial culture)	1.25M NaCl, 50 mM Tris-Cl pH 8.5, 15% Ethanol
10XLigation buffer	200 mM Tris-Cl pH 7.4, 50 mM $MgCl_2$, 50 mM DTT, 500 µg/ml BSA, .2 mM ATP)

3.1.8. Plasmids

Table 3. Plasmids.

Name	DNA Insert	Vector	References
pcDNA 3.0OPN	Mouse OPN complete coding region.	pcDNA 3.0 The insert was isolated from pTracer-OPN and cloned into the NotI and KpnI sites in the vector. Confers ampicillin and neomycin resistance. See material and methods 3.2.3.10.2.	Vector purchased from Invitrogen.
pTracer OPN	Mouse OPN complete coding region.	pTracer The insert was amplified from cDNA from ME-C cells with OPN specific primers containing EcoRI and EcoRV sites for cloning into multiple restriction sites in the vector. See material and methods 3.2.3.10.1.	Vector purchased from Invitrogen.

3.1.9. Media

3.1.9.1. SOC-Medium

2.0% (w/v) Trypton

0.5% (w/v) Yeast Extract

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM Glucose

3.1.9.2. Luria-Bertani (LB) medium (Gibco BRL)

5g Yeast Extract

10g Bacto-Trypton

10g NaCL add 1l dH_2O.

LB ampicillin media (LB media with 100 µg/ml ampicillin)

LB Agar (LB media with 15g Agar/ lt LB-media)

3.1.9.3. Dulbeccos modified Eagle Medium, DMEM (Gibco BRL)

L-Alanine 35.6 mg/l
L-Arginine 84.0 mg/l
L-Asparagine 60.0 mg/l
L-Asparagin Acid 53.2 mg/l
L-Cystine 48.0 mg/l
L-Glutamic Acid 58.8 mg/l
L-Glutamine 580.0 mg/l
Glycine 30.0 mg/l
L-Histidine x HCL 38.4 mg/l
L-Isoleucine 105.0 mg/l
L-Leucine 105.0 mg/l
L-Lysine 146.0 mg/l
L-Methionine 30.0 mg/l
L-Phenylalanine 66.0 mg/l
L-Proline 46.0 mg/l
L-Serine 42.0 mg/l
L-Threonine 95.0 mg/l
L-Tryptophan 16.0 mg/l
L-Tyrosine 72.0 mg/l
L-Valine 94.0 mg/l
D-Calcium-Pantothenat 4 mg/l
Cholinchlorid 4.0 mg/l
Folic Acid 4.0 mg/l
i-inositol 7.2 mg/l
Nicotinamide 4.0 mg/l
Pyridoxal HCL 4.0 mg/l
Riboflavin 0.4 mg/l
Thiamin x HCL 4.0 mg/l
$CaCl_2$ 264.0 mg/l
$Fe(NO_3)_3$ x $9H_2O$ 0.10 mg/l
KCl 400 mg/l
$MgSo_4$ x $7H_2O$ 200 mg/l
NaCl 6.4 g/l
NaH_2PO_4 x H_2O 140 mg/l
$NaHCO_3$ 3.7 g/l
Phenol red 17 mg/l
D-Glucose 4.5 g/l

3.1.10. Bacteria

E Coli XL-Blue	Genotype: D(mcrA) 182; D(mcrCB-hsdSMR-mrr)172, endA1, sup E 44, Thi-1, recA1, gyrA96, relA1, lac, I'[F´, proAB, $laci^qZDM$ 15, Tn10, (tet^r)]. Stratagene.

3.1.11. Mammalian cell lines

Table 4. Cell lines

Cell Line	Description	References
ME-C (8/61C)	Cell line established from tissue segments of mammary gland or breast tumor 8/61 originated in a WAP-SVT/t transgenic female mouse. ME-C cells remain highly tumorigenic, they grow with high efficiency in soft agar and form tumors after injection in NMRI mice. These cells have spontaneously switched off the expression of the WAP-SVT/t transgene. ME-C cells contain a missense mutation of the p53 gene commonly found in human cancers. These cells are very resistant to apoptotic stimuli such as stress conditions and chemotherapy drugs.	Tzeng *et al*., 1998. Kohlhoff *et al*., 2000. Graessmann *et al*., 2006.
ME-A (8/61A)	Cell line established from tissue segments of mammary gland or breast tumor 8/61 originated in a WAP-SVT/t transgenic female mouse. ME-A cells are immortalized, grow with high efficiency in soft agar and form tumors after injection in NMRI mice. These cells express the WAP-SVT/t transgene with high efficiency and exhibit high levels of spontaneous apoptosis. ME-A cells are very sensitive to apoptotic stimuli such as stress conditions and chemotherapy drugs.	Tzeng *et a*l., 1998. Graessmann *et al*., 2006.

3.2. Methods

3.2.1. Bacteria

3.2.1.1. Bacterial transformation

To obtain bacterial competent cells (E. coli, XL Blue strain) cells, were treated with $CaCl_2$, which promotes perforations in the bacterial cell membrane and allows the uptake of DNA molecules. 1 ml from a bacterial overnight culture was cultivated in 100 ml LB-medium at 37°C, with continuous shaking at 225 rpm until the optical density reached OD_{600} = 0.6. Cell growth was restrained by incubation of the cells in ice for 10 min. Afterwards, cells were centrifuged at 5000 g and the cell pellet resuspended into 20 ml $CaCl_2$ solution (100 mM). Cells were incubated in ice for 30 min and centrifuged again. The pellet was resuspended into 1 ml $CaCl_2$ solution (100 mM) and incubated in ice (1-24 h). Competent cells were stored in aliquots at -80°C.

The heat shock treatment was employed for the transformation of bacterial cells. Plasmid DNA (100 ng) was added to 100 µl of competent cell suspension and incubated for 30 min in ice. Cells were transformed by heat shock (1 min, 42°C) and afterwards incubated in 1 ml SOC-medium for 60 min at 37°C and continuous shaking at 225 rpm. The bacterial suspension was plated onto LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. Ampicillin selection prevented the growth of non-transformed cells. For plasmid isolation, single colonies of transformed bacteria were selected and incubated overnight in 3 ml LB ampicillin medium at 37°C with continuous shaking (225 rpm).

3.2.1.2. Isolation of plasmid DNA

3.2.1.2.1 Plasmid isolation from 5 ml of bacterial culture

One single transformed bacterial colony was incubated overnight in 3 ml of LB medium containing ampicillin (100µg/ml) at 37°C with continuous shaking (225 rpm). Afterwards, 1.5 ml of the overnight culture was transfered into a 1.5 ml tube and centrifuged at 12000 g for 30 sec at 4°C in a microcentrifuge. The supernatant was removed and the pellet resuspended by vigorous vortexing in 100 µl Solution I (50 mM glucose, 25 mM Tris.Cl pH 8.0, 10 mM EDTA pH 8.0). To the suspension, 200 µl Solution II (0.2 N NaOH, 1% SDS) was added and incubated for 5 min in ice. Afterwards, 150 µl of Solution III (5 M Potassium acetate, glacial acetic acid, H_2O) was added and the tube was gently vortexed followed by 3-5 min incubation in ice. The suspension was centrifuged at 12000 g for 5 min at 4°C and the supernatant was transferred into a new tube. DNA was precipitated with two volumes of ethanol at room temperature followed by centrifugation at 12000 g for 5 min at 4°C. The supernatant was then removed and the pellet washed two times with 70% ethanol. The DNA pellet was air dried and redissolved in 50 µl of TE (pH 8.0) containing 20µg/ml RNase. Isolated plasmid DNA was stored at –20°C.

3.2.1.2.2. Plasmid isolation from 20-200 ml of bacterial culture

One single transformed bacterial colony was incubated overnight in 3 ml of LB medium containing ampicillin (100µg/ml) at 37°C with continuous shaking (225 rpm). From this culture, 100 µl were added in 250 ml of LB-medium containing ampicillin and incubated overnight (37°C, 225 rpm). The culture was centrifuged and the bacterial pellet resuspended in 10 ml of solution 1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Bacterial

cells were lysed by adding 10 ml lysis buffer (200 mM NaOH, 1% SDS). The suspension was incubated 5 min at room temperature. In this step, cell membrane proteins and phospholipids were solubilized and both the chromosomal and the plasmid DNA were denatured by NaOH addition. To neutralize the lysis suspension, 10 ml of potassium acetate solution (3M, pH 5.5) was added. In this step plasmid-DNA renatured and it remained in solution while chromosomal-DNA and denaturated proteins precipitated into salt-detergent complexes. The solution was centrifuged and the plasmid-DNA in the supernatant was column-purified (Qiagen-tip-500, Qiagen Plasmid Maxi-Kit) and eluted with 15 ml elution buffer (1.25M NaCl, 50 mM Tris-Cl pH 8.5, 15% ethanol). The plasmid DNA was precipitated from the eluate by adding 10.5 ml isopropanol and by centrifugation. The DNA-pellet was then washed with 70% ethanol, air dried and resuspended in 200 µl TE buffer.

3.2.2. Cell culture

3.2.2.1. Cell culture conditions and treatment with signal transduction inhibitors

ME-A and ME-C cells (Tzeng *et al*., 1998) were plated at 1 x 10^5 cells/ml and cultured under standard conditions (5% CO_2, 37°C) in Dulbecco´s modified Eagles medium (DMEM) supplemented with 5% fetal calf serum (FCS) purchased from Invitrogen. ME cell apoptosis was induced by serum withdrawal (0% FCS, DMEM) or by treating the cells with DMEM medium plus 5% FCS containing 1 µM doxorubicin (Sigma) for 22 h at day two after plating. Apoptotic cells were identified by propidium iodide (Sigma) staining (2 µg/ml) for 10 min and counted under the fluorescence microscope (Zeiss, Axioskop) or subjected to FACS analysis. The following inhibitors were applied for 22 h: Src inhibitor PP2 (1 – 10µM, Calbiochem), Phosphatidylinositol 3-Kinase (PI 3-k) inhibitor LY294002 (1 – 10 µM, Calbiochem), Phospholipase C (PLC) inhibitor U73122 (1- 10 µM, Tocris Biosciences) in the presence or absence of medium concentrate (20 µg/ml total protein).

3.2.2.2. Preparation of ME-A and ME-C cell medium concentrates

ME-A and ME-C cells were plated at 1 x 10^5 cells/ml and grown for two days under standard culture conditions and were further cultivated in DMEM without serum (0% FCS) for 48 h. The medium was collected and centrifuged at 1200 g for 10 min to remove cells. The supernatant was then dialyzed against 0.1% PBS at 4°C and concentrated under vacuum to 1/10 of the original volume. The protein content in the medium concentrate (either from the

ME-A or the ME-C cells) was determined using the Bradford protein assay (Bio Rad) and the final protein concentration was adjusted to 500 µg/ml PBS.

3.2.2.3. Cell transfections

Cells were transfected using the FuGENE® HD transfection reagent (Roche). One day before transfection cells were treated with trypsin solution (0.05% Trypsin, 0,02% EDTA, Gibco BRL) and 3-6 x 10^5 cells were plated in 2 ml 5%FCS, DMEM and incubated overnight at 37°C, 5% CO_2 conditions. An 8:2 ratio of transfection reagent (µl) to pcDNA3-OPN plasmid-DNA (µg) was adequate for positive ME-A cell transfection. pcDNA3-OPN plasmid DNA (2 µg) was resuspended in 100 µl DMEM medium without serum. To the DMEM/DNA mixture 8 µl of FuGENE® HD transfection reagent was added. The suspension was mixed vigorously and incubated for 15 min at room temperature. The suspension was added to the cells and further incubated for 24 h under standard conditions.

3.2.2.4. Antibiotic selection of transfected cells

To select for ME-A cells containing the pcDNA3-OPN plasmid, cells were cultivated in 5%FCS, DMEM containing 500 µg/ml of Geneticin (G418, Gibco BRL) 24 h after transfection. The cells were maintained under antibiotic selection for 3 to 4 weeks, with medium change every three days. The presence of pcDNA3-OPN DNA in the antibiotic selected clones was analysed by isolation of the cell mRNA and DNA by the TRIzol method and subjected to analysis by RT-PCR using adequate primers for the OPN construct (T7 and OPN2 or PCMV and OPN2, see Table 6).

3.2.2.5. Protein isolation.

ME cell lysates were obtained using lysis buffer (20mM Na_3PO_4 pH 7.8, 300 mM NaCl, 5mM EDTA pH 8.0, 1% NP40, 1 mM DTT, 1 mM PMSF, and 1 µg/µl of protease inhibitors leupeptin, aprotinin and pepstatin A). Confluent cells were washed once with PBS, resuspended in 250 µl of ice-cold lysis buffer and scraped from a 6 mm plate with the help of a rubber policeman. The cell suspension was incubated at 4°C while rotating for 30 min and afterwards, it was then centrifuged at maximum speed (14000 rpm) in a table top centrifuge for 10 min at 4°C. The supernatant was recovered and protein concentration was measured by the Bradford method. Cleared cell lysates were stored at -80°C if not used immediately.

3.2.2.6. Western Blot analysis

Protein amount was determined from cell lysates (See Material and methods 3.2.2.5.), medium concentrates (Material and methods 3.2.2.2) or secreted proteins concentrated using Ultrafree Biomax -30K columns (Millipore) by using the Bradford method and measuring absorbance at 595 nm (Bio-Rad, Hercules. CA). The amount of protein indicated in each experiment was solubilized by boiling in loading buffer (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 0.03% bromophenol blue, and 10% glycerin). Proteins were fractionated in 10% or 12% SDS-PAGE gels. Electrophoresis was done in Tris-Glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for 1 h at 200 volts. Proteins were electrophoretically transferred (45 min, 200 mA) onto Immobilion P membrane (PVDF, Millipore) by a semidry transfer apparatus (Biometra). Membranes were incubated in methanol for 10 sec and air-dried for 45 min. Afterwards, membranes were blocked using 5% skim milk in PBS containing 0.1% Tween (PBS-T) for 30 min. OPN detection was achieved by incubating the membranes with mouse-monoclonal anti-OPN (Santa Cruz, sc-21742), or goat polyclonal anti-OPN (R&D systems, AF808). For detection of integrin β, two rabbit polyclonal antibodies (Chemicon, ASK3A6 and ASK2A4) were used. Antibody concentrations are indicated in table 1, WB column. After incubation with primary specific antibody, membranes were washed with PBS-T three times for 5 min. Afterwards membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) at 1:5000 dilution (see material and methods, table 1 column WB). Membranes were washed three times with PBS-T for 5 min and the signal detected with the ECL-detection system (Perkin Elmer Life Science, Germany) following the producer´s instructions. Results were documented by exposing XRay film X-OMAT AR (Kodak) to the produced light signal.

For quantification of immunoreactive bands, the computer software Image J was employed (Abramoff *et al*., 2004). The commands in the gel submenu, which use a simple graphical method that involves the generation of lane profile plots, were used to analyze one-dimensional electrophoretic gels.

3.2.2.7. Flow cytometry (FACS)

To measure apoptosis, cells were plated $1x10^5$ cells/ml, cultivated under standard conditions 24 h at 37°C, 5% CO_2 and then further incubated 24 h under serum starvation conditions in the presence of specific inhibitors 1 μM-2,5 μM LY294002, 1 μM-2,5 μM PP2, 5 μM

U73122, or under standard conditions in the presence of Doxorubicin (1 μM) and specific inhibitors. Cells in suspension in culture medium were recovered and the remaining adherent cells were washed once with PBS, incubated with trypsin for 5 min at 37°C, and resuspended. Samples were centrifuged at 700 g for 10 min at 4°C and washed twice with PBS. Samples were then centrifuged once at 700 g for 5 min at 4°C and resuspended in PBS containing 2 μg/ml propidium Iodide.

To identify cell membrane receptors, $1x10^6$ cells were suspended in 50-100 μl DMEM medium with 5% FCS and incubated with primary antibodies for 1 h at 4°C (concentrations for antibodies used are given in table 3.1. column FACS in Materials and methods). Afterwards cells were washed twice with ten fold volume of ice-cold PBS, centrifuged at 700 g for 5 min at 4°C and incubated with secondary antibodies (see Table 3.1. in Materials and methods) 30-60 min at 4°C in the dark. Cells were washed twice with ice-cold PBS and centrifuged at 700 g for 5 min at 4°C.

Flow cytometry was performed using a FACScan cell sorting machine (Becton Dickinson). The Cell Quest software was used to acquire and analyse statistically flow cytometer data such as immunostaining of cell surface receptors and propidium iodide staining of apoptotic cells. Emission of FITC-conjugated antibodies was detected at around 530 nm and propidium iodide at around 620 nm.

3.2.2.8. Immunofluorescence staining.

ME-A and ME-C cells were plated at $1x10^5$ cells/ml in 5% FCS DMEM medium onto glass slides inside 60 mm cell culture plates. After 24 h incubation cells were washed once with PBS. Cells were air dried and afterwards fixed by adding a 4% paraformaldehyde solution at room temperature for 20 min. Alternatively, ice-cold methanol/acetone (2:1 v/v) solution applied for 10 min was used to fix the cells in the glass slides. Cells were washed twice with PBS for 5 min, air dried and incubated with specific primary antibody for 1-30 min at 37°C (antibody details and concentrations are provided in Table 1. under IF column in Materials and methods). Afterwards cells were washed three times with PBS for 5 min. Cells were air-dried and incubated with the specific secondary antibody conjugated to the fluorochrome FITC (see Table 1 in Material and methods) for 30 min at 37°C in absence of light. Cells were washed twice with PBS for 2 min and incubated in DAPI solution for 10 min for nuclei

staining. PBS was added and fluorescence microscopy was done with a Zeiss Axioskop microscope.

3.2.2.9. Gel permeation chromatography.

1 mg of ME-C cell MC (see Material and methods 3.2.2.2.) was chromatographed on a Superdex 200 (Amersham Pharmacia) column, eluted 0.5 ml/min with PBS by using a FPLC instrument (Amersham Pharmacia). Protein concentration was spectrophotometrically measured for each fraction using the Bradford method (Bio-Rad, Hercules. CA) and either 35 µg or 10 µg of protein were added to ME-A cells growing under serum starvation conditions (see Material and methods 3.2.2.1) to test for its anti-apoptotic activity. 5 µg of protein was used for Western blot analysis of OPN presence in the fractions (see Materials and methods 3.2.2.6). 15 µg of protein was used for SDS-PAGE and silver staining to compare proteins present in the different fractions (see Material and methods 3.2.2.10).

3.2.2.10. Silver staining of proteins.

15 µg of protein for each fraction obtained from the Superdex 200 gel permeation chromatography (see Material and methods 3.2.2.9) was loaded into a 10% polyacrylamide gel. Electrophoresis was done in Tris-Glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for 1 h at 200 volts. After electrophoresis separation of proteins by molecular weight, gels were fixed in 40% ethanol and 10% acetic acid overnight, washed twice in 30% ethanol for 20 min each, and three times in H_2O for 30 min each. The gel was then sensitized by incubation in 0.02% $Na_2S_2O_3$ for 1 min and after three washes with water for 20 sec each, impregnated with 0.15% $AgNO_3$ for 20 min at 4°C. For image development, the gel was incubated in development solution containing 2% Na_2CO_3, 0.04% HCHO and 0.0005% $Na_2S_2O_3$ for 7 min. The reaction was stopped with 0.5% glycine for 30 min.

3.2.3. DNA and RNA analysis

3.2.3.1.Total RNA isolation

To isolate total RNA from ME cells, 5-10x10^6 cells were homogenized by adding 1 ml TRIzol reagent (Invitrogen) and incubated for 5 min at room temperature. Following incubation, 0.2 ml chloroform was added to the tubes and vigorously shaken for 15 sec and incubated for another 2-3 min at room temperature. The aqueous and organic phases were separated by centrifugation at 12000 g for 15 min at 4°C. The aqueous phase was recovered and the organic phase used for total DNA isolation (see Material and methods 3.2.3.2). RNA which remains in the aqueous phase was mixed with 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent used for initial cell homogenization. Samples were incubated for 15-30 min at room temperature and centrifuged at 12000 g for 10 min at 4°C. Afterwards, the supernatant was discarded and the RNA pellet washed with 75% ethanol, adding 1 ml of ethanol per 1 ml TRIzol reagent used for initial homogenization. The sample was mixed by vortexing and centrifuged at 7500 g for 5 min at 4°C. The RNA pellet was vacuum-dried and resuspended in 30-35 µl of DEPC H_2O. RNA concentration was measured photometrically (see Materials and methods 3.2.3.3.) and stored in ethanol at –20°C.

3.2.3.2. Total DNA isolation

Cells were homogenized with TRIzol reagent. After aqueous phase/organic phase separation (see Materials and methods 3.2.3.1.) DNA was precipitated from the organic phase by adding 0.3 ml of 100% ethanol per 1 ml TRIzol used for initial cell homogenization. Samples were mixed and incubated for 2-3 min at room temperature. Afterwards, samples were centrifuged at less than 2000 g for 5 min at 4°C. The supernatant was removed and the pellet washed two times with 1 ml of a 0.1M sodium citrate in 10% ethanol solution and incubated for 30 min at room temperature. Samples were centrifuged at 2000 g for 5 min at 4°C and the DNA pellet was washed in 75% ethanol (1.5 ml 75% ethanol per 1 ml of TRIzol used for initial cell homogenization) for 10 – 20 min at room temperature (with periodic mixing). The samples were centrifuged at 2000 g for 5 min at 4°C. The DNA pellet was air-dried and dissolved in an 8M NaOH solution (300-600 µl). DNA concentration was measured photometrically (see Material and methods 3.2.3.3.) and stored in ethanol at –20°C.

3.2.3.3. RNA and DNA quantification

Nucleic acids absorb ultraviolet light and show a maximum absorbance at a wavelength of 260 nm. At this wavelength, measurement of OD_{260} = 1, with a pathlength of 1 cm, is equivalent to a concentrations of 50 µg/ml for double-stranded DNA and 40 µg/ml for RNA. Concentration of DNA and RNA solutions were estimated by measuring the absorbance at OD_{260} of RNA and DNA.

3.2.3.4. Agarose gel electrophoresis

DNA-fragments of different size can be separated in electrophoretic agarose gels. DNA and RNA was analysed in agarose gels (1.0, 1.5 or 2.0%) in TAE electrophoresis buffer (40 mM Tris pH 8.0, 5 mM Na-acetate, 1 mM EDTA) containing 10 µg/ml of ethidium bromide. A maximum of 20 µl sample were mixed with 1/5 volume of DNA-sample buffer (0.25% bromophenol blue, 0.25% xylencyanol FF, 30% glycerol) and loaded into agarose gels. Electrophoresis ran at 70-100 V for 30-45 min. Visualization of DNA and RNA was done under U.V. light and recorded photographically.

3.2.3.5. Phenol/chloroform extraction

To eliminate impurities in DNA solutions, one time volume of phenol (saturated with 1M Tris-Cl, pH 7.4), phenol/chloroform (1:1 v/v) or chloroform was added and mixed vigorously followed by centrifugation (5 min, 12000 rpm). The aqueous phase was taken and DNA in suspension was ethanol-precipitated (Material and methods 3.2.3.6.) (Sambrook *et al*., 1989).

3.2.3.6. DNA/RNA precipitation

Ethanol precipitation was performed to concentrate and purify DNA/RNA. To DNA/RNA solutions, a three fold volume of ethanol and one tenth of sodium acetate solution (3M) were added and the DNA (at room temperature) and RNA (at -70°C) were precipitated for 30 min. After centrifugation at 12000 rpm for 15 min at 4°C, salts were removed by washing the pellet with 70% ethanol, followed by centrifugation at 12000 rpm for 15 min at 4°C. Pellet was air dried and solubilized in desired buffer.

3.2.3.7. DNA Restriction Analysis

The ability of restriction enzymes to cut double stranded DNA at specific recognition sequences was used to generate and characterize recombinant plasmids. To cut 1-5 µg of plasmid DNA, 1-5 units of enzyme were necessary in a 20 µl reaction.

Restriction cuts with NotI/KpnI enzymes were performed under following conditions: 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol pH 7.9, supplemented with 1000 µg/ml BSA. Samples were incubated at 37°C for 1 h. Restriction cuts made with EcoRI/EcoRV enzymes were performed under the following conditions: 50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl2, 0.025% Triton X-100 (pH 7.5). Samples were incubated at 37°C for 1 h.

3.2.3.8. Isolation and purification of DNA fragments

To isolate and purify DNA fragments obtained from the digestion with restriction enzymes, fragments were separated in agarose gels by electrophoresis (Materials and methods 3.2.3.4.). Fragments were extracted from gels using the Concert^{TM} gel extraction system (Gibco BRL) following the manufacturer´s instructions. DNA fragments were suspended in TE buffer pH 8.0 after purification.

3.2.3.9. T4-DNA-ligase ligation of DNA fragments

The T4-DNA-ligase allows for ligation of 3´hydroxyl ends of one nucleotide with the 5´phosphate end of another. The vector and insert were ligated in a concentration ratio of 3:1 using ligation buffer (20mM Tris-Cl pH 7.4, 5 mM $MgCl_2$, 5 mM DTT, 50 µg/ml BSA, 0.2 mM ATP) in a 10 µl reaction volume. 10 units of T4-DNA-ligase were employed to ligate 100 ng of plasmid DNA to 300 ng of insert DNA. The reaction was incubated for 30 min at room temperature. Ligated DNA was used to transform E. coli (XL-Blue) competent cells (see Material and methods 3.2.1.1).

3.2.3.10. Plasmid constructs

pTracer and pcDNA3 plasmids were purchased from Invitrogen

3.2.3.10.1. pTracer-OPN

To clone the mouse OPN complete coding region into the pTracer vector, the OPN sequence was amplified by PCR (see Materials and methods 3.2.3.12) using as template cDNA obtained by RT-PCR (see Material and methods 3.2.3.11) from the ME-C cells RNA. The OPN specific primers OPN CDS 1 and OPN CDS 2 (see Table 6 in Material and methods) were used for OPN amplification. OPN CDS1 primer contained at its 5´end an overhang sequence with sites recognized by KpnI and EcoRI restriction enzymes before the OPN start codon. OPN CDS 2 primer contained at its 5´end overhang sequences for recognition of EcoRV and PstI after the OPN stop codon. The PCR product obtained with OPN CDS 1 and OPN CDS 2 primers (insert) and the pTracer vector were digested with the EcoRI and EcoRV restriction enzymes (See Material and methods 3.2.3.7 for reaction conditions). The vector and insert were purified by gel electrophoresis and ligated as indicated (see Material and methods 3.2.3.8. and 3.2.3.9). After transformation of E. coli (XL-Blue) with the ligation product and ampicillin selection, plasmid DNA from positive bacterial clones was analysed by digesting it with EcoRI and EcoRV restriction enzymes. Plasmids extracted from positive bacterial clones were sent to a company (AGOWA, Germany) for DNA sequencing analysis. The primers used for sequencing were T7 and SP6 (see Material and methods Table 6.).

3.2.3.10.2. pcDNA3-OPN

To clone the mouse OPN complete coding region into the pcDNA3 vector, the pTracer-OPN plasmid was digested with KpnI and NotI restriction enzymes for the insert (see Material and methods 3.2.3.7 for reaction conditions). The vector and insert were purified by gel electrophoresis and ligated as indicated (see Materials and methods 3.2.3.8. and 3.2.3.9.). After transformation of E. Coli (XL-Blue) with the ligation product and ampicillin selection, plasmid DNA from positive bacterial clones was analysed by digestion with KpnI and NotI restriction enzymes. Plasmids extracted from positive bacterial clones were sent for DNA sequencing analysis to a company (AGOWA, Germany). The primers used for sequencing were T7 and SP6 (see Materials and methods table 3.6.).

3.2.3.11. Reverse Transcription

The enzyme reverse transcriptase is able to create DNA copies of single-stranded RNA. 1-5 µg of isolated RNA (TRIzol method) was treated with 2 units of RQ I DNase (in 40 mM Tris pH 7.9, 10 mM NaCl, 6 mM MgCl2, 10 mM CaCl2) to avoid DNA contamination and the enzyme was removed by phenol/chloroform extraction. The purified RNA was hybridized with Oligo (dT)-primer (70°C, 10 min) and mixed in buffer (50 mM Tris pH 8.3, 75 mM KCl, 3 mM MgCl2. 10 mM DTT) with 200 units of reverse transcriptase (Superscript RT, Gibco BRL) in 20 µl final volume. The mix was incubated 10 min, 37°C, 50 min 42°C and 5 min 95°C. The resulting cDNA was treated with RNAse (20 µg/ml) for 15 min at 37°C to remove RNA. The cDNA was resuspended in 80 µl TE buffer (pH 8.0) and 10 µl used for DNA Polymerase Chain Reaction (PCR) as described in Material and methods 3.2.3.12.

3.2.3.12. cDNA polymerase chain reaction (PCR)

The PCR reaction was employed for *in vitro* amplification of DNA fragments. Two primers with free 3´hydroxyl ends are used complementary to the desired DNA sequences. The amplification was achieved by employing a thermostable DNA-Taq-polymerase. The PCR reaction was performed in a solution containing 100 ng of cDNA in 20mM Tris-Cl pH 8.4, 50 mM KCl, 1.25 units Taq-Polymerase (Gibco BRL), 20 pmol of each primer, 2 mM MgCl2 and 0.2 mM of each dNTP nucleotide to a final reaction volumen of 50 µl, under the temperature schedule described in Table 5:

Table 5. PCR reaction temperature schedule

Temperature	Duration	Number of Cycles
94°C	5 min	
94°C	30 sec	20, 25, or 30 cycles.
T_{AN} (see Table 6)	T_{AN} (see Table 6)	
72°C	T_{EXT} (see Table 6)	
72°C	7 min	

For electrophoretic analysis 10 µl of PCR product was loaded in 2% agarose gels

Table 6. Primers

Name	Gene	Sequence (5´ to 3´)	T_{AN} (°C)	T_{AN} (sec)	T_{EXT} (sec)	Fragment Size
OPN 1	Osteopontin	CGA GGA TT CTG TGG ACT CG	57	45	60	OPN 1/OPN 2 = 400 bp
OPN 2	Osteopontin	CCT GGC TCT CTT TGG AAT G	57	45	60	OPN 1/OPN 2 = 400 bp
OPN CDS 1	Osteopontin	GGT ACC GAA TTC ATG AGA TTG GCA GT ATT	52	45	60	OPN CDS1/Opn CDS 2 = 908 bp
OPN CDS 2	Osteopontin	CTG CAG GAT ATC TTA GTT GAC CTC AGA AGA TGA	52	45	60	OPN CDS1/ OPN CDS 2 = 908 bp
T7	T7 sequence	TAA TAC GAC TCA CTA TAG GG	57	45	60	T7/OPN 2= 770bp
PCMV	Promoter CMV	GGG TCA TTA GTT CAT AGC CC	57	45	60	PCMV/OPN2 = 1357 bp
GADPH 1	GADPH	CCC CTT CAT TGA CCT CAA CTA C	55	45	60	GADPH 1/GADPH 2 =756 bp
GADPH 2	GADPH	TTG AAG TCG CAG GAG ACA ACC	55	45	60	GADPH 1/GADPH 2 =756 bp

4. RESULTS

4.1. ME-A and ME-C cells differed in their OPN expression

4.1.1. WAP SVT/t transgenic mice and ME-A and ME-C breast tumor cell lines

To study tumorigenesis, apoptosis and cell survival, different transgenic mice (e.g. WAP-SVT/t) were previously established. WAP SVT/t transgenic animals synthesized the SV40 T/t-antigen under the transcriptional control of the whey acidic milk protein promoter (WAP) in mammary epithelial (ME) cells. SV40 T/t-antigen synthesis caused premature mammary gland involution during late pregnancy by inducing apoptosis of ME cells. SV40 T/t-antigen activation of the apoptotic program in ME-cells caused a lactation deficiency in females and they were not able to feed their litters (Tzeng *et al*., 1998).

Despite the dramatic ME-cell death rate caused by expression of SV40 T/t antigen, the mammary glands still contained multiple islands of T-antigen positive ME-cells after the lactation period, and as a consequence, all WAP-SVT/t females developed breast cancer after the first pregnancy in both p53 positive (WAP-SVT/t) and p53 negative double transgenic animals (WAP-SV-T/t•p53-/-). Elevated ME cell apoptosis rates were maintained in breast tumors and in corresponding tissue culture cell lines (Tzeng *et al*., 1993; Tzeng *et al*., 1998).

The ME-A and the ME-C cells are immortalized breast cancer cells derived from the WAP-SVT/t tumor 8/61 (Transgenic animal WAP-SVT/t line 8, female 61) (Tzeng *et al*., 1993). ME-A cells grew efficiently in soft agar and formed tumors after injection in NMRI mice. These cells expressed the WAP-SVT/t transgene with high efficiency and exhibited a high apoptosis rate (Tzeng *et al*., 1998).

The ME-C cells were obtained from the same tumor as the ME-A cells. However, in ME-C cells, the SV40 T/t antigen transgene expression is switched off. These cells have the morphology and growth characteristic of tumor cells, they grew with high efficiency in soft agar and formed tumors after injection in NMRI mice. ME-C cells carried a p53 missense mutation on codon 242 ($p53_{242}$) frequently found in human breast cancers (mouse p53 codon 242 correspond to human p53 codon 245) (Tzeng *et al*., 1998). Furthermore, ME-C cells were very resistant to apoptotic stimuli such as cultivation in serum starvation conditions and chemotherapeutic treatment (Tzeng *et al*., 1998; Graessmann *et al*., 2006).

4.1.2. ME-A and ME-C cells apoptosis

ME-A and ME-C cells had different apoptotic rates. ME-A cells expressed the T/t antigen and when cultivated under standard tissue culture conditions (Dulbecco´s modified Eagle´s medium (DMEM) + 5% fetal calf serum (FCS)), they exhibited an elevated spontaneous apoptosis rate of 2-5% (Tzeng *et al*., 1998; Graessmann *et al*., 2006). ME-A cells were very sensitive to growth factor depletion, DNA-damaging agents such as doxorubicin or etoposide, oxidative stress and ultraviolet (UV)-irradiation (Tzeng *et al*., 1998; Kohlhoff *et al*., 2000). Under serum starvation conditions for 22 h, about 30% of the ME-A cells were apoptotic. After 22 h addition of doxorubicin (1 µM) under standard culture conditions, large numbers of the ME-A cells exhibited caspase-dependent morphological changes such as chromatin condensation, nucleus fragmentation, apoptotic bodies (figure 1 and Graessmann *et al.*, 2006) and further apoptotic hallmarks such as oligonucleosomal DNA fragmentation (Tzeng *et al*., 1998). In contrast to ME-A cells, the ME-C cells exhibited a low spontaneous cell death rate and the cells were very insensitive to apoptotic stimuli (Figure 1 and Tzeng *et al*., 1998; Graessmann *et al.*, 2006). In co-cultivation experiments with both breast-tumor cell lines, the ME-C cells mediated cell death resistance to ME-A cells indicating the existence of anti-apoptotic paracrine signals.

4.1.3. Microarray data analysis of gene overexpression in ME-A and ME-C cells

ME-C cells were very resistant to apoptotic stimuli. In contrast to ME-C cells, ME-A cells were very sensitive to apoptotic stimuli. Co-cultivation experiments with both ME-A and ME-C cells demonstrated that the ME-C cells mediated cell death resistance to the ME-A cells. This observation pointed to the existence of an ME-C cell paracrine signal that was able to act on ME-A cell death program. To find out more about the anti-apoptotic factor secreted by ME-C cells, ME-A and ME-C cells were subjected to microarray data analysis of differential gene expression. As the ME-C cell anti-apoptotic factor works in a paracrine manner, the analysis focused on overexpression of secreted proteins.

Microarray data analysis revealed that several genes of secreted proteins had a high expression profile in both ME-A and ME-C cells. However, to search for genes overexpressed in the ME-C cells in comparison to the ME-A cells, the ratio of individual gene expression profile between both cell lines was compared and overexpressed genes were selected as those with the ME-C cell/ME-A cell expression-ratio values of at least of 2 (arbitrary units).

Osteopontin (OPN) gene was found to be one of the highest overexpressed genes in ME-C cells. OPN is involved in protection of cells from apoptosis (reviewed in Weber, G., 2001). Thus, OPN gene expression level in ME-A and ME-C cells was examined.

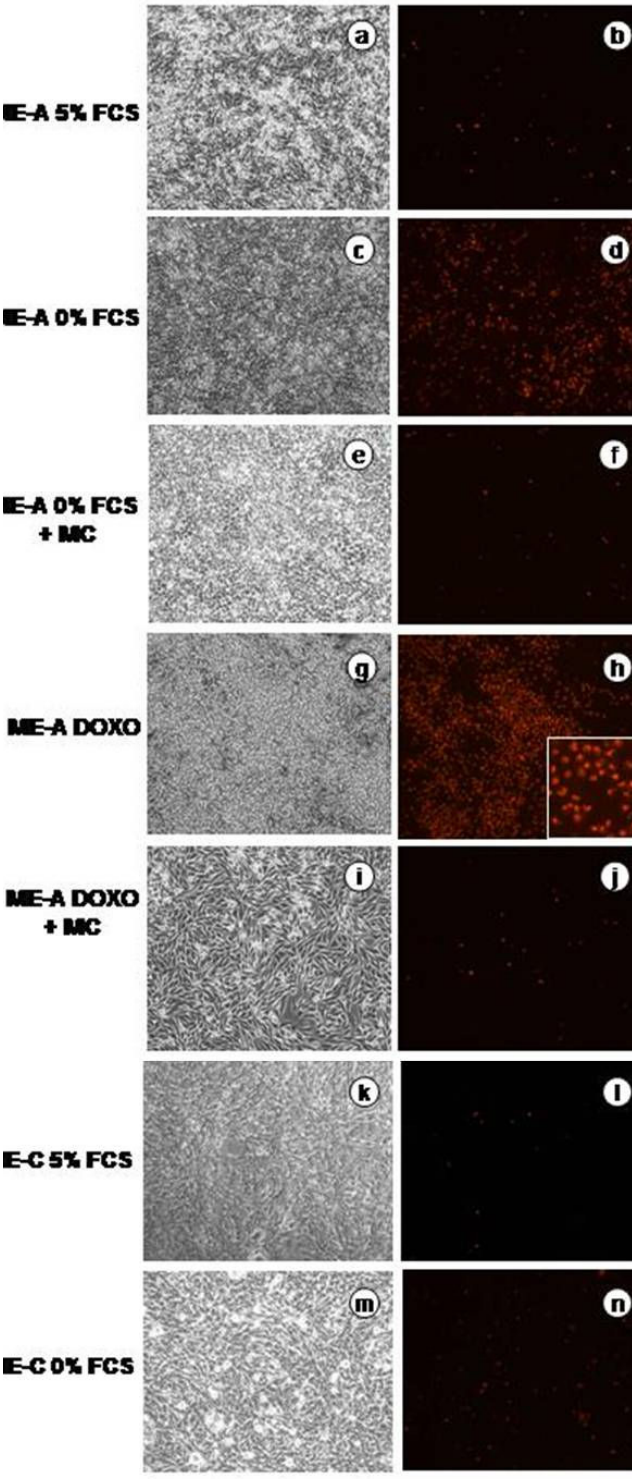

Figure 1. ME-A and ME-C cell apoptosis. (A) Fluorescence microscopy of ME-A and ME-C cells after staining with 2 µg/ml propidium iodide (Nicoletti *et al*., 1991; Riccardi *et al*., 2006) for 10 min (Zeiss, Axioscop; magnification x 100) and (B) phase-contrast microscopy of the cells shown in (A). (a) and (b) ME-A cells cultivated under standard conditions (DMEM with 5% FCS); (c) and (d) ME-A cells cultivated for 22 h in serum free medium (0% FCS in DMEM); (e) and (f) ME-A cells cultivated for 22 h in serum free medium in the presence of 20 µg/ml ME-C cells medium concentrate (MC); (g) and (h) ME-A cells cultivated in the presence of 1µM doxorubicin. Insert: Apoptotic cells at magnification x250; (i) and (j) ME-A cells cultivated for 22 h in the presence of doxorubicin and ME-C cell medium concentrate; (k) and (l) ME-A cells cultivated under standard conditions (DMEM with 5% FCS) (m) and (n) ME-C cells cultivated under standard conditions for 22 h in the presence of 1 µM doxorubicin.

Microarray data analysis demonstrated that OPN RNA expression level was 3.5 times higher in the ME-C cells than in the ME-A cells cultured under standard conditions (as shown in Figure 2). The differences in OPN RNA expression levels remained high under serum starvation conditions with 2.5 times higher in ME-C than in ME-A cells (see Figure 2). The change in OPN mRNA expression levels in ME-A cells growing under standard culture conditions to serum starvation conditions was around 53% (Figure 2). In ME-C cells, the change of OPN mRNA expression levels by the modification of culture conditions was of 12% (see Figure 2). The increase in gene expression levels observed is in agreement with the role of OPN as a stress-response protein.

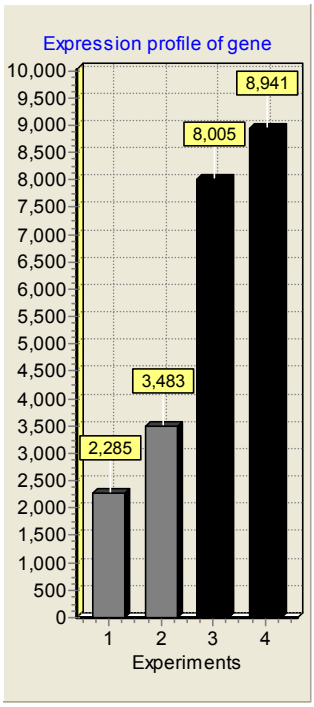

Figure 2. Microarray data analysis of OPN expression in ME-A and ME-C cells. Total RNA extracted from ME-A and ME-C cells cultivated either under standard conditions or in serum free medium was subjected to microarray data analysis using the Affimetrix GeneChip Mouse Expression Set 430A 2.0. Microarray data was analyzed using the CorrXpression software (Wessel *et al.*, 2006). Experiments (1) OPN expression levels in ME-A cells cultivated under standard conditions and (2) in serum free medium. ME-C cells cultivated under (3) standard conditions and (4) in serum free medium.

4.1.4. OPN mRNA expression in ME-A and ME-C cells

To confirm the results obtained in the microarray data analysis, OPN mRNA levels in ME-A and ME-C cells were analysed by RT-PCR and agarose-gel electrophoresis (shown in Figure 3). ME-A and ME-C cells were cultivated under standard conditions for 48 h followed by cultivation of the cells under serum starvation for further 24 h. Total RNA was extracted by TRIzol reagent method. After cDNA synthesis, mRNA transcription levels were analysed by the DNA-polymerase chain reaction (PCR). To analyse gene expression levels PCR reactions differing in the amount of cycles (20, 25, 30) were performed and the samples were analysed by agarose-gel (2% w/v) electrophoresis. The PCR product appearance at a lower number of

PCR reaction cycles and/or differing DNA-band intensities can be used to compare gene transcription levels of specific RNA from the total amount, in this case for the OPN gene.

The ME-A and the ME-C cell OPN mRNA transcription levels after the RT-PCR analysis (20 cycles) and agarose-gel electrophoresis are shown in Figure 3. The OPN-specific primers OPN CDS 1 and CDS 2 (see Material and methods Table 6) were used to verify the complete coding region of the gene. The length of the OPN gene in mouse is 885 bp from the ATG start codon to its stop codon. Figure 3 demonstrates that OPN transcription level was higher in ME-C cells than in ME-A cells cultivated under stress conditions (48 h serum starvation) as can be observed in the DNA-band intensity. However, the ME-A cells also transcribed efficiently OPN under serum starvation conditions. This prompted us to investigate the differences in OPN protein expression levels in both cell lines.

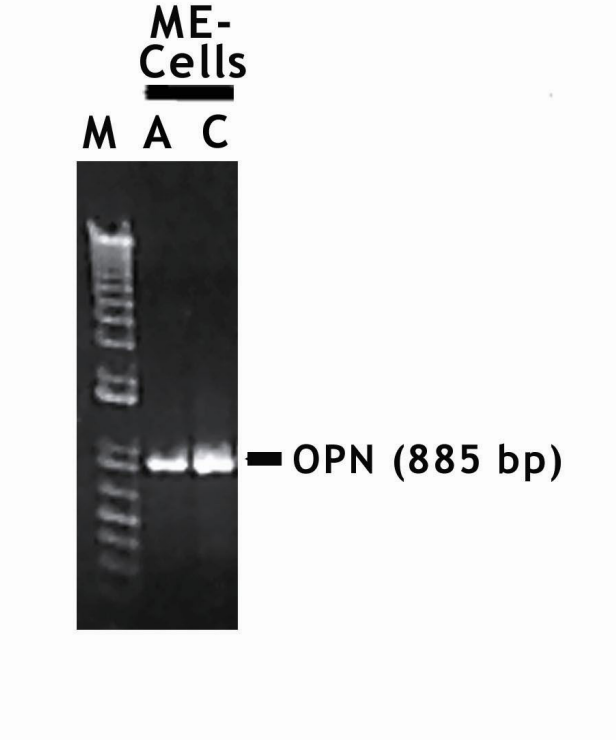

Figure 3. ME-A and ME-C OPN mRNA expression analysis by RT-PCR and agarose-gel electrophoresis. Agarose gel with PCR products obtained from cDNA (amplified from mRNA) of ME-A and ME-C cells using OPN specific primers (OPN CDS 1 and OPN CDS 2, see Material and methods Table 6). (A) ME-A and (C) ME-C cells cultivated 22 h in serum free medium. (M) DNA-ladder marker. OPN DNA size is indicated in nucleotide base pairs.

4.1.5. ME-A and ME-C cell OPN protein expression and secretion

ME-C and ME-A cells were cultivated 48 h under standard conditions and further incubated for 24 h under serum starvation. Cells were collected and suspended in lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/ml of each of the protease inhibitors pepstatin, aprotinin and leupeptin) for protein isolation (see Materials and methods 3.2.2.5). For preparation of medium concentrate (MC) of ME-A and ME-C cells (see material

and methods 3.2.2.2), secreted proteins were harvested by collecting the cell culture media after 48 h incubation under serum starvation conditions, dialysing them against 0.1 x PBS and finally lyophilizing them to obtain medium concentrate (MC).

OPN protein presence in cell lysates and MCs from the ME-A and the ME-C cells was analysed by SDS-PAGE-gel electrophoresis and Western blot analysis (Material and methods 3.2.2.6). Immunoblot analysis of ME cell lysates using a mouse monoclonal anti-OPN antibody (Santa Cruz, sc-21742) demonstrated the presence of OPN in ME-C cells but not in ME-A cells. For the ME-C cells, 10 µg total protein was enough to observe a pair of immunoreactive bands in the 56-58 kDa range (Figure 4C and Graessmann *et al*., 2006). We were not able to observe immunoreactive bands in lysates of ME-A cells, even when 100 µg of protein was loaded into SDS-PAGE-gels.

As OPN is a secreted protein we examined the culture media of both ME cell lines. As shown in Figure 4A (and Graessmann *et al.*, 2006), ME-C cells efficiently secreted OPN into culture media. SDS-PAGE-gel electrophoresis and Western blot analysis of 200 ng protein from ME-C cell medium concentrate (MC) demonstrated the presence of strong and various OPN immunoreactive bands. Mouse OPN encodes a 294 amino-acid protein, with a calculated 32.5 kDa molecular weight, thus the slower migrating isoforms (37 – 115 kDa) observed in Figure 4A, might represent post-translational OPN modifications. OPN has been described as a highly glycosylated (N- and O- glycane) phosphorylated protein (Denhart *et al*., 1993; Shanmugam *et al*., 1997; Christensen *et al*., 2005).

The differences between the amounts of OPN found in cell lysates and culture media of ME-C cells point to OPN secretion as a rapid event. Furthermore, extensive OPN modifications occur at the extracellular level.

The ME-A cells also secreted OPN into the culture media, but at lower levels than the ME-C cells. SDS-PAGE gel electrophoresis and Western blot of 20 µg total protein (that is 100 times more protein than in the analysis of ME-C cell medium concentrate) demonstrated only two major bands at 40-42 kDa (Figure 4B and Graessmann *et al*., 2006) in ME-A cell MC. Thus, it was evident that there were strong differences in the ability of the ME-A and the ME-C cells to produce, secrete and modify OPN.

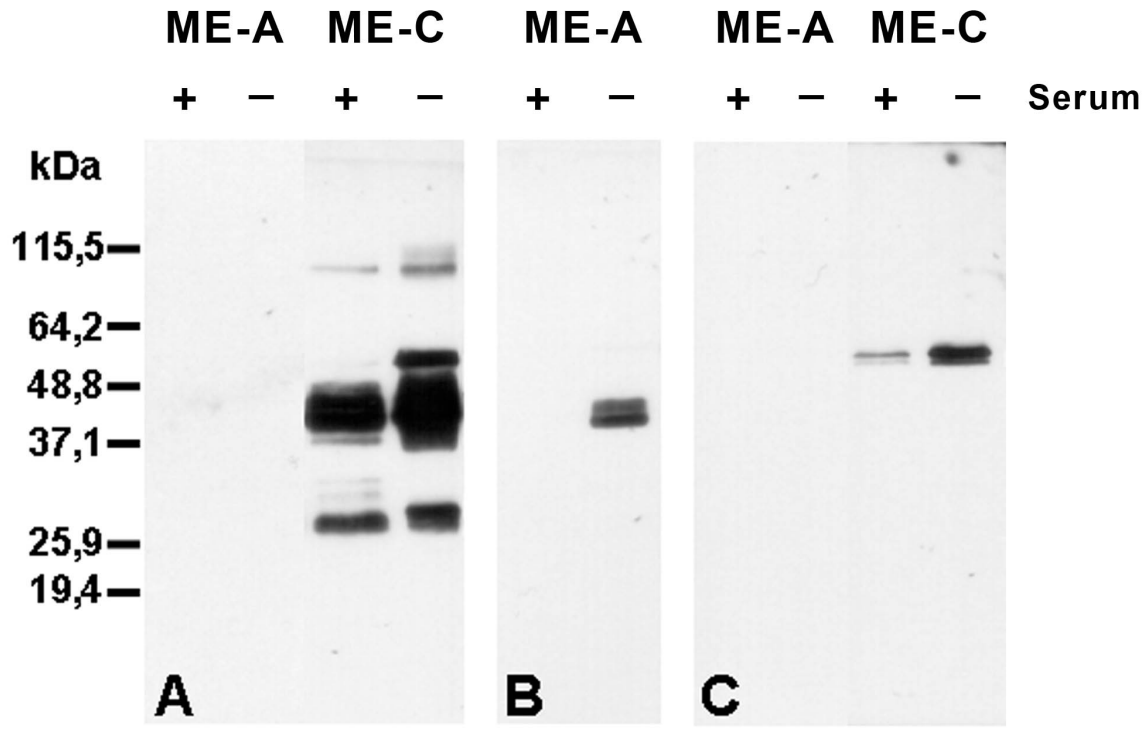

Figure 4. OPN SDS-PAGE gel electrophoresis and immunoblot analysis of cell lysates and medium concentrates of ME-A and ME-C cells. ME-A and ME-C cells are indicated at the top of the image. Cells were incubated either under cultivation in standard conditions (+) or in serum free medium (-). (A) 200 ng of ME-A and ME-C cell MCs, (B) 20 µg of ME-A cell MC and (C) 10 µg of the ME-A and ME-C cells lysates were subjected to 10% SDS-PAGE gel electrophoresis, transferred onto a PVDF membrane (Immobilion P) and hybridized with a mouse monoclonal anti-OPN antibody (Santa Cruz, sc-21742, 1:5000 dilution). It was followed by incubation with an anti-mouse HRP-conjugated secondary antibody (Dako, 1:5000 dilution) and signal detected by chemiluminescence using the ECL-detection system (Perkin Elmer Life Science).

4.1.6. Effect of ME-C cell medium concentrate on ME-A cell apoptosis

The ME-C cells were able to influence the apoptosis rate of the ME-A cells in a paracrine form and the immunoblot analysis demonstrated the presence of OPN in large amounts and in various isoforms in ME-C cell medium concentrates. In contrast, ME-A cell medium concentrates contained OPN in relative small amounts when compared to ME-C cell medium concentrates. To investigate whether the ME-C cell medium concentrate had an anti-apoptotic effect against ME-A cell apoptosis, ME-A cells were cultivated for 48 h under standard serum conditions and apoptosis was induced by cell cultivation for further 22 h under serum starvation (Figure 5) or by treatment with 1 µM doxorubicin (Figure 6). The apoptosis rate was measured by adding 2 µg/ml propidium iodide (Nicoletti *et al*., 1991; Riccardi *et al*., 2006) and fluorescence microscopy or FACS analysis of stained cells.

In Figure 5 is shown the ME-C cell MC effect on ME-A cell apoptosis induced by cultivation under serum starvation conditions. ME-A cells were cultivated under standard serum conditions (Figure 5, control), serum starvation (Figure 5, under 0) and with different concentrations of MC as indicated in Figure 5 (under 20, 10, 5, 2 µg/ml). Apoptosis levels increase to around 20% in ME-A cells cultivated under serum free medium conditions. However, 20 µg/ml of the ME-cell MC is able to rescue ME-A cells from apoptosis to control levels (standard culture conditions). As shown in Figure 5 the ME-C cell MC had a dose-dependent anti-apoptotic effect on the ME-A cells cultivated under serum starvation conditions.

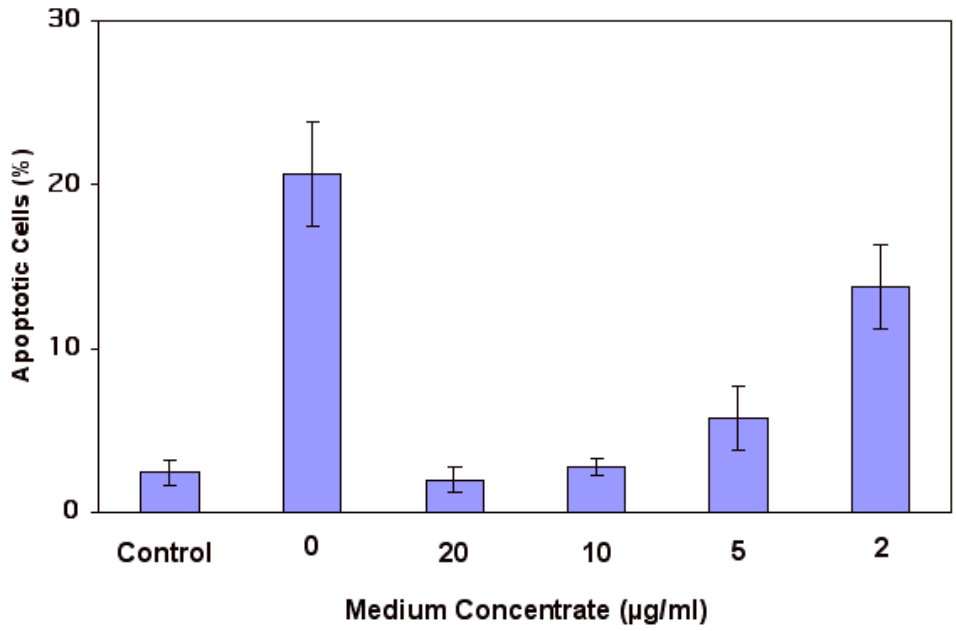

Figure 5. ME-C cell medium concentrate anti-apoptotic activity on ME-A cells cultivated under serum starvation. ME-A cells cultivated 22 h under standard conditions (control), under serum starvation conditions (0) and in serum starvation with the indicated concentrations (20, 10, 5, 2, µg/ml) of ME-C cell MC. Cells were stained with propidium iodide (2 µg/ml) for 10 min and apoptosis rates (%) were measured by fluorescence microscopy or FACS analysis. Numbers given are mean values with their respective standard deviation from three independent experiments.

When ME-A cells were cultivated under standard culture conditions with 1 µM doxorubicin for 22 h the apoptosis rate increased to around 15%. As shown in Figure 6, 20 µg/ml total

protein from ME-C cell medium concentrate was able to rescue ME-A cells from doxorubicin-induced apoptosis to levels comparable to the control. As observed in Figure 6, the anti-apoptotic effect of ME-C cell MC in ME-A cells under treatment with doxorubicin was also dose-dependent.

In both cases (serum starvation and doxorubicin treatment), 20 µg/ml ME-C cell MC was enough to reduce apoptosis rates in ME-A cells to control levels. In the following signal transduction experiments (see Results 4.3), the ME-C cell MC was applied at a final concentration of 20 µg/ml.

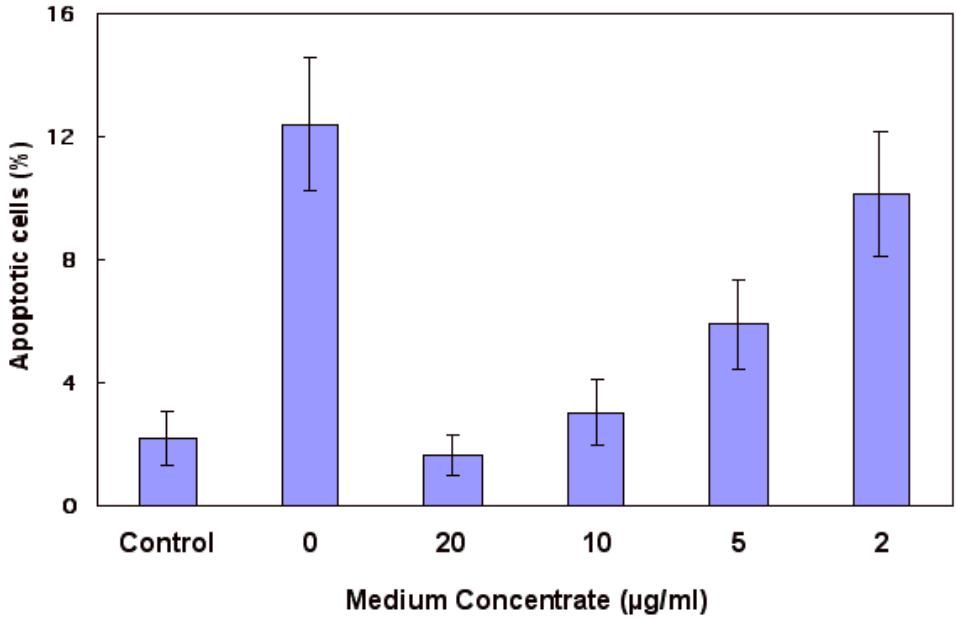

Figure 6. ME-C cell medium concentrate anti-apoptotic activity in ME-A cells cultivated under standard culture conditions and doxorubicin treatment. ME-A cells cultivated 22 h under standard conditions (control) with 1 µM doxorubicin (0) and with 1 µM doxorubicin and the indicated concentrations (20, 10, 5, 2 µg/ml) of ME-C cell MC, were stained with propidium iodide (2 µg/ml) for 10 min and apoptosis rates (%) measured by fluorescence microscopy or FACS analysis. Numbers given are mean values with their respective standard deviation from three independent experiments.

4.2. Gel permeation chromatography of ME-C cell medium concentrate

ME-C cells secretion contained different OPN immunoreactive bands that may represent different post-translational modifications of the protein or different products derived of

alternative gene splicing. Different OPN isoforms have been demonstrated to influence the OPN biological activity in other cell lines and tumor cells (He *et al*., 2005; Agnihotri *et al*., 2001; Crawford *et al*., 1998). To investigate which OPN immunoreactive bands might be involved in the anti-apoptotic signal, the ME-C cell MC was subjected to size-exclusion gel permeation chromatography experiments using a Superdex 200 column (Amersham Pharmacia). Proteins present in the ME-C cell MC were thus fractionated by molecular weight. The different protein fractions were analysed by Western blot analysis for the presence of OPN immunoreactive bands and silver-stained to compare protein patterns in the biological active fractions.

4.2.1. Biological activity of ME-C cell MC protein chromatographic fractions

Upon size-exclusion gel permeation chromatography of proteins present in the ME-C cells MC (1 mg of total protein each time) using a Superdex 200 column (Amersham Pharmacia) several fractions were obtained that contained OPN immunoreactive bands. To further narrow the analysis down to those fractions that contained the OPN isoforms responsible for the anti-apoptotic activity, fractions were tested for their biological activity. From each fraction either 35 µg or 10 µg total protein were tested in ME-A cells. The different fractions were added to the ME-A cells and cells were further cultivated for 24 h under serum starvation conditions. Apoptosis was measured by fluorescence microscopy of propidium iodide stained cells. These experiments demonstrated that fractions enriched with proteins in the 20-40 kDa molecular weight range were the most active ones, and that fractions above 40 kDa did not have any anti-apoptotic activity. 10 µg of total protein from some of the concentrated fractions were sufficient to reduce apoptosis to similar levels as the reduction observed by adding 100 µg (20 µg/ml) of the ME-C cell MC to the ME-A cells growing under serum starvation conditions (see Figure 7). These results suggested that the anti-apoptotic OPN modifications were concentrated in a narrow molecular weight range.

4.2.2. OPN immunoblot detection in ME-C cell MC chromatographic fractions

After examination of the anti-apoptotic activity of the chromatographic fractions, 5 µg of proteins were loaded into 12% SDS-PAGE gels. Proteins were then transferred to PVDF membranes and OPN immunoreactive bands were detected using a mouse monoclonal anti-OPN antibody (Santa Cruz) followed by incubation with an anti-mouse HRP-conjugated secondary antibody (Dako) and the signal was detected by chemiluminscence using the ECL-

detection system (Perkin Elmer Life Science). As shown in Figure 7, 10 µg of total protein from fractions 14-17 had a low anti-apoptotic activity. However, fractions 18-19 which accumulated OPN immunoreactive bands exclusivelly in the 20-32 kDa molecular weight range (as shown in Figure 8) had the highest anti-apoptotic activity. This suggested that the lower molecular weight OPN modifications were responsible for the anti-apoptotic activity observed in ME-A cells. However, higher molecular weight proteins (or OPN immunoreactive bands) in other fractions in the range 32-40 kDa cannot be ruled out from the anti-apoptotic activity as with 35 µg of total protein it was possible to observe anti-apoptotic activity on ME-A cells cultivated under serum starvation conditions. From these experiments it was possible to conclude that fractions above 40 kDa do not have any anti-apoptotic activity. However, it was not possible to conclude from these experiments whether OPN was the only factor in these fractions with an anti-apoptotic activity.

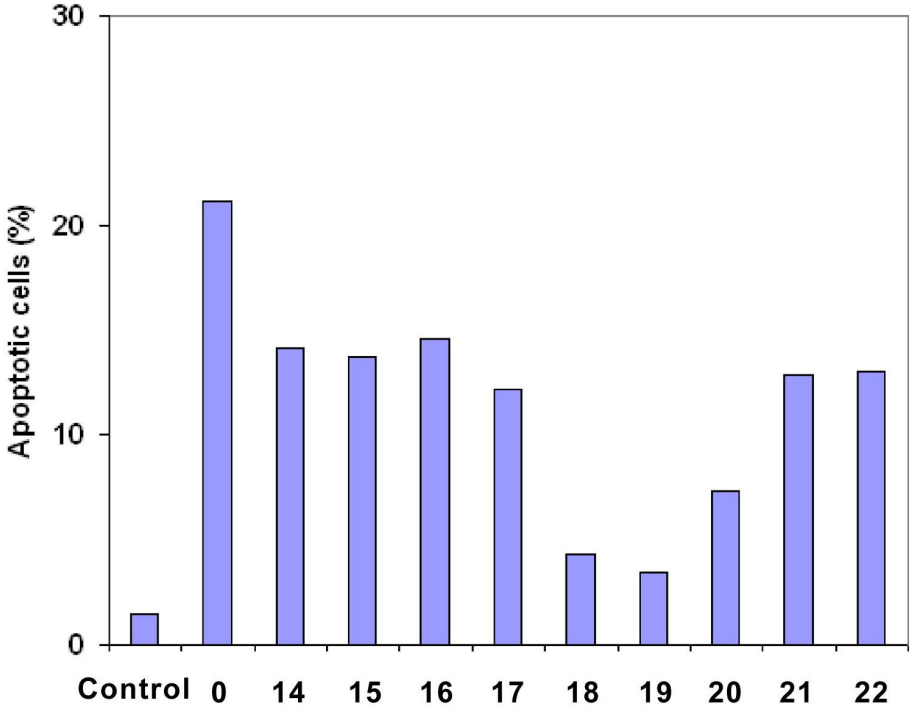

Figure 7. ME-C cell MC chromatographic fractions anti-apoptotic activity on ME-A cells cultivated under serum starvation. ME-A cells were cultivated 22 h under standard conditions (control), in serum free medium (0) and in serum free medium with 10 µg of protein from the different ME-C cell MC size exclusion-chromatographic fractions (14 to 22) obtained by separation in a Superdex 200 column (Amersham Pharmacia). Cells were stained with propidium iodide (2 µg/ml) for 10 min and apoptosis rates (%) were measured by fluorescence microscopy. Results shown represent two independent experiments.

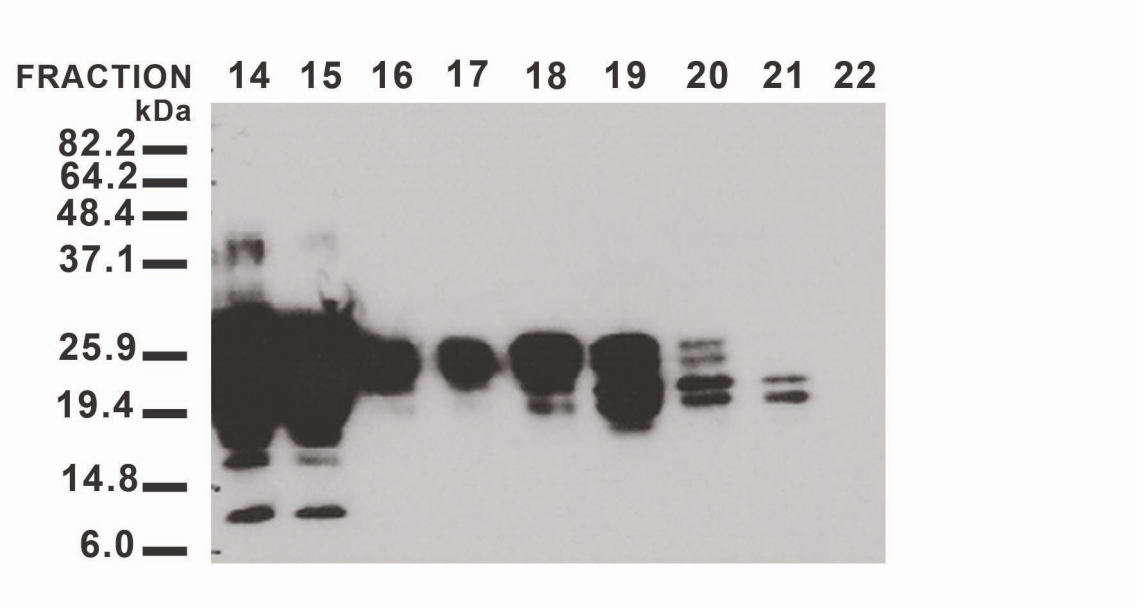

Figure 8. OPN SDS-PAGE gel electrophoresis and immunoblot analysis of ME-C cell MC chromatographic fractions. 5 µg of total protein from ME-C cell MC was separated by size-exclusion chromatography using a Superdex 200 column (Amersham Pharmacia). The resulting fractions were loaded into 12% SDS-PAGE gels and subjected to electrophoresis, transferred onto a PVDF membrane (Immobilion P) and hybridized with a mouse monoclonal anti-OPN antibody (Santa Cruz, sc-21742, 1:5000 dilution) followed by incubation with an anti-mouse HRP-conjugated secondary antibody (Dako, 1:5000 dilution) and the signal was detected by chemiluminescence using the ECL-detection system (Perkin Elmer Life Science).

4.2.3. Silver staining of ME-C cell MC chromatographic fractions

To investigate the amount of protein bands present in each active fraction, chromatographic fractions were subjected to silver staining. Silver staining can be used to detect proteins with high sensitivity in the nanogram range after separation in polyacrylamide gels. 15 µg of total protein in the fractions were subjected to 10% SDS-PAGE gel electrophoresis. The procedure for the protein staining direct in SDS-PAGE gels was described in Material and methods 3.2.2.10. As shown in Figure 9, fractions 18-19, where the highest anti-apoptotic activity was measured (see Figures 7 and 8), accumulated proteins mostly in the range of 20-32 kDa. Furthermore, it is possible to observe in Figure 9 that there were still several bands in the 20-40 kDa range. A strong band of around 28 kDa (marked by an arrow in the figure) was enriched in fractions 18-19. Furthermore, a double-band running around 20 kDa, similar to a double-band immunoreactive to OPN observed in fraction 19-20 (see Figure 8 and 9, lanes 19-20), was enriched in the same fraction as observed by silver staining. As fraction 20 contained relative high anti-apoptotic activity these bands might be involved in the anti-apoptotic activity. However it was not clear whether these bands corresponded to OPN immunoreactive bands observed in Western blot experiments and further experiments would be necessary to clarify this question.

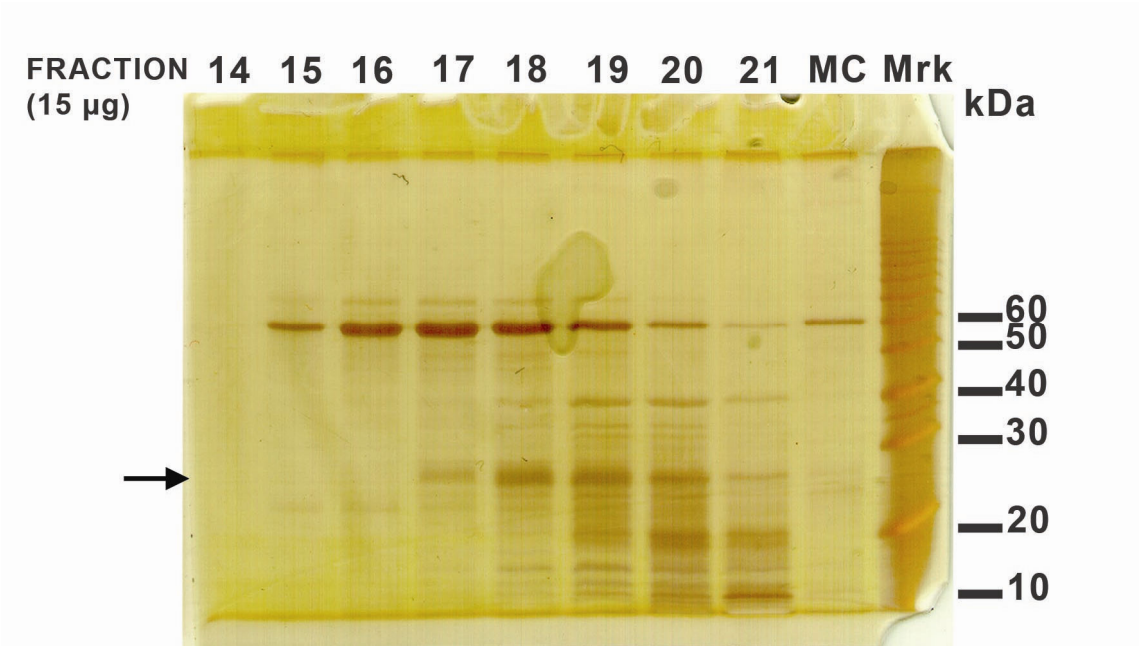

Figure 9. OPN SDS-PAGE gel electrophoresis and silver staining of proteins contained in ME-C cell MC chromatographic fractions. 15 µg of total protein from ME-C cell MC were separated by size-exclusion chromatography using a Superdex 200 column (Amersham Pharmacia) were loaded into 10% SDS-PAGe gels and subjected to electrophoresis. Fractions numbers are given at the top of the figure (14-21). 15 µg total protein from ME-C cell MC (MC) were also loaded into the gels to assess the results of the fractionation. Molecular weight marker (Mrk) sizes are given at the right side of the figure. The arrow indicates a strong stained band of around 28 kDa enriched in fractions 18-19.

4.3. Anti-apoptotic signal transduction pathway activated by ME-C cell medium concentrate in ME-A cells

In previous work it was demonstrated that the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 pathway was involved as an effector in the anti-apoptotic signal transmitted by ME-C cell MC in ME-A cells (Graessmann *et al*., 2006). However, it is not clear which signal transducer transmits the signal from cell receptors to the MAPK kinase cascade. Upon OPN activation of its cell transmembrane receptors integrins and CD44, the non-receptor Src family of tyrosine kinases, phosphatidylinositol 3-kinase (PI3-k) and phospholipase C (PLC) family of proteins have been found to be activated (as described in 1. Introduction). These molecules are upstream regulators of MAPK kinases activation. Thus, Src family, PI3-k and phospholipase C (PLC) family were selected as possible candidates in the activation of the MEK 1/2-mediated ME-C cell MC anti-apoptotic pathway in the ME-A cells. To disrupt each signaling pathway, specific inhibitors against Src family, PI3-k and PLC family were applied to ME-A cells

growing under serum starvation or under doxorubicin treatment in the absence or presence of ME-C cell MC.

4.3.1. Effects of ME-C cell medium concentrate and Src kinase inhibition on ME-A cell apoptosis

To test the involvement of Src tyrosine kinase in the anti-apoptotic signal transduction activated by the ME-C cell MC, ME-A cells were treated with PP2 (Calbiochem), a specific inhibitor of Src-family of tyrosine kinases. PP2 has been widely employed in other studies to interrupt the enzymatic activity of these kinases (Lee et al., 2004; Park *et al*., 2004; Golubovskaya *et al*., 2003; Nam *et al*., 2002). For example, in HT29 human colon cancer cells, 2 μM PP2 reduced Src activity for 2 days and prevented cell growth. Src inhibition by PP2 treatment and focal adhesion kinase (FAK) inhibition in colon and breast cancer cell lines have shown to promote cell detachment and apoptosis.

Preliminary experiments were performed to test the sensitivity of ME-A cells to Src inhibition by PP2. ME-A cells incubated under both serum starvation and standard culture conditions in the presence of different PP2 concentrations demonstrated that the ME-A cells were sensitive to Src inhibition in a dose-dependent manner. Under serum starvation conditions 2.5 μM PP2 promoted around 40% apoptosis after 24 h incubation (Figure 10).

ME-A cells growing under serum starvation conditions were more sensitive to the PP2 inhibitory effect on Src. Incubation with 1 μM and 2.5 μM PP2 for 24 h was able to increase the cell death rate of ME-A cells to around 30% and 40%, respectively, as measured by propidium iodide staining and fluorescence microscopy (shown in Figure 10). Similar results were obtained by measuring cell apoptosis by FACS analysis of propidium iodide staining and annexin V-FITC conjugated detection. Morphological changes relating to the ability of Src to modify the status of cell adhesion were also observed. Increasing the concentration of PP2 at levels above 2.5 μM promoted extensive damage in the cell monolayer (Figure 14, panel B4). A considerable amount of cells detached from the bottom of the plates and of neighbouring cells making it difficult to measure apoptotic cells by fluorescence microscopy.

The ME-C cell MC was able to prevent ME-A cells from undergoing apoptosis under serum starvation conditions in the presence of 2.5 μM PP2 concentrations. As shown in Figure 10,

20 µg/ml of ME-C cell MC was able to reverse the effect of PP2 on ME-A cells apoptosis rate and restore it to the levels of standard serum conditions.

ME-A cells cultivated under 1 µM doxorubicin treatment were also efficiently rescued from apoptosis by the ME-C cell MC even when 7.5 µM PP2 was applied to the cells. The Src inhibition by PP2 demonstrated that Src was not involved in the survival signal promoted by the ME-C cell MC on ME-A cells cultivated under stress conditions thus suggesting that the activation of a different signal transduction is necessary for the survival mechanism other than Src activation.

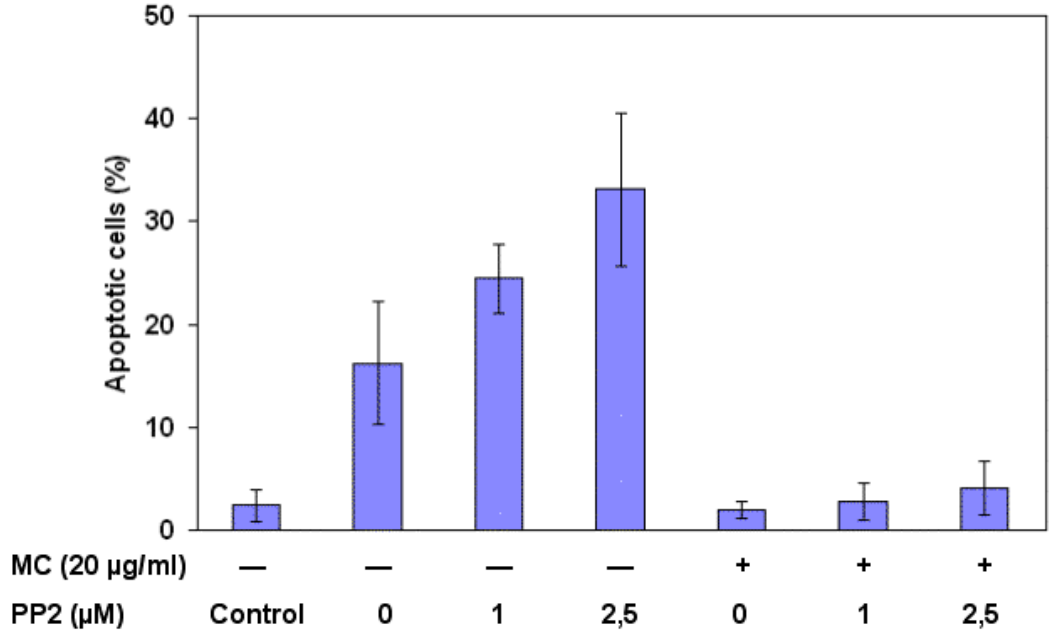

Figure 10. Effect of Src kinase inhibition on ME-A cell apoptosis. ME-A cell apoptosis was induced by serum starvation. ME-A cells were cultivated 22 h under standard conditions (control), in serum free medium (0) and, in serum free medium with two different concentrations of Src inhibitor PP2 (Calbiochem, 1 and 2.5 µM) in the absence (-) or presence (+) of 20 µg/ml ME-C cell MC. Cells were stained with propidium iodide (2 µg/ml) for 10 min. Apoptosis rates (%) were measured by fluorescence microscopy or FACS analysis. Numbers given are mean values with their respective standard deviation from three independent experiments.

4.3.2. Effects of ME-C cell medium concentrate and PI3-kinase inhibition on ME-A cell apoptosis

To investigate the involvement of the PI3-kinase (PI3-k) in the anti-apoptotic signalling of ME-C cell MC, ME-A cells were treated with the PI3-k inhibitor LY294002 (Calbiochem).

The inhibitor has been used in various types of cells to inactivate the enzymatic function of PI3-k (Wu *et al*., 2005; Gallet *et al*., 2004).

To test the sensitivity of ME-A cells to PI3-k inhibition, preliminary experiments were performed in which ME-A cells were cultivated under standard or serum starvation conditions for 22 h in the presence of different concentrations of LY294002. ME-A cells cultivated under serum starvation conditions were sensitive to LY294002 in a dose dependent manner. 2.5 μM PI3-k inhibitor increased apoptosis of ME-A cells to around 60% (Figure 11). Under standard culture conditions apoptotic rates were lower than those observed in serum starvation conditions.

As shown in Figure 11, ME-A cells cultivated under serum starvation conditions in the presence of 1 or 2.5 μM of PI3-k inhibitor for 22 h increased their apoptotic rates to around 30 and 60%, respectively, as measured by fluorescence microscopy and FACS analysis of ME-A cells stained with 2 μg/ml propidium iodide. ME-A cells presented characteristic morphologies of programmed cell death such as apoptotic bodies. (Figure 14. panel C3). As shown in Figure 11, ME-A cells cultivated under serum starvation conditions and PI3-k inhibition in the presence of 20 μg/ml ME-C cell MC had apoptotic rates similar to control levels after 22 h incubation. Results were also confirmed by FACS analysis of propidium iodide staining and annexin V-FITC conjugated detection.

20 μg/ml of ME-C cell medium concentrates were able to rescue ME-A cells from apoptosis when cells were cultivated under standard serum conditions in the presence of 5 μM LY294002 and 1 μM doxorubicin after 22 h incubation.

This result is in agreement with previous results of our group (Graessmann *et al*., 2006) which demonstrated that the protein kinase B (PKB/Akt), a downstream effector of the survival signal mediated by PI3-k, was dispensable for the anti-apoptotic signal of the ME-C cell MC in the ME-A cells.

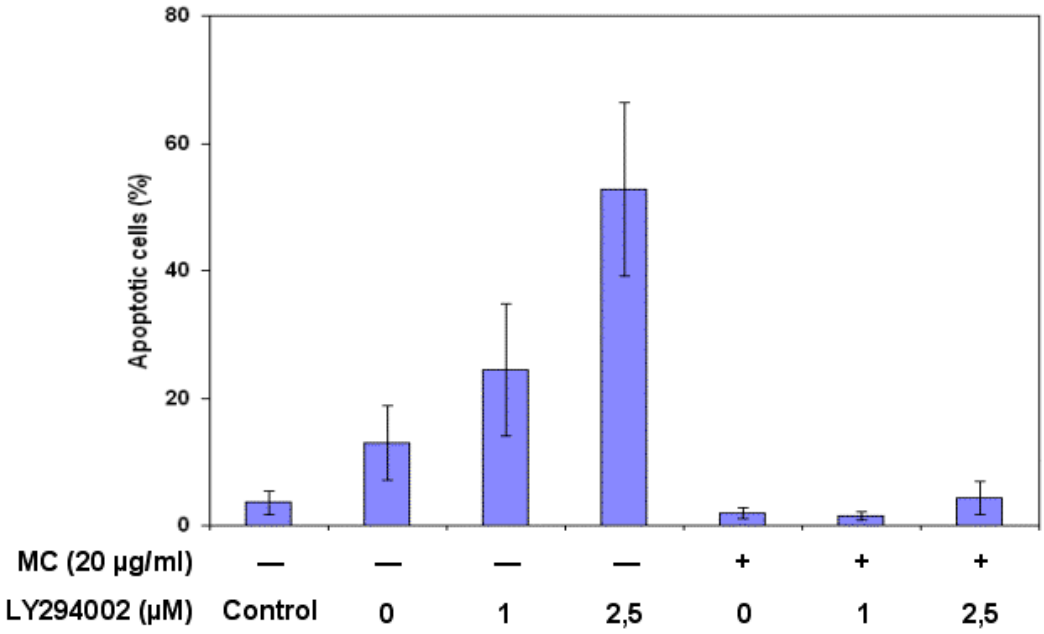

Figure 11. Effect of PI3-kinase inhibition on ME-A cell apoptosis. ME-A cell apoptosis was induced by serum starvation. ME-A Cells were cultivated 22 h under standard conditions (control), in serum free medium (0), serum starvation or serum starvations with two different concentrations of the PI3-k inhibitor LY294002 (Calbiochem, 1 and 2.5 µM) in the absence (-) or presence (+) of 20 µg/ml ME-C cell MC. Cells were stained with propidium iodide (2 µg/ml) for 10 min. Apoptosis rates (%) were measured by fluorescence microscopy or FACS analysis. Numbers given are mean values with their respective standard deviation from three independent experiments.

4.3.3. Effects of ME-C cell medium concentrate and phospholipase C inhibition on ME-A cell apoptosis

Preliminary experiments to investigate the sensitivity of ME-A cells to the phospholipase C (PLC) inhibitor demonstrated that ME-A cells were sensitive to the inhibitor in a dose dependent manner. ME-A cells were cultivated with increasing amounts of phospholipase C (PLC) inhibitor U73122 (Tocris Biosciences) in the presence of serum and under serum starvation conditions for 22 h and ME-A cell apoptosis rate augmented with increasing concentrations of U73122.

PLC inhibition caused marked effects on ME-A cell adhesion. 2 h after the addition of 10 µM U73122 cells changed their morphology, becoming round and detached from the bottom of the culture plates and from neighbouring cells. By further incubation it was possible to observe that cells were still viable even if detached from culture plates. They would sit again

at the bottom of the culture plates and start to build cell-cell adhesions. Most of the cells presented morphological characteristics of cell death, as apoptotic bodies and nucleosomal-DNA condensation under serum starvation conditions and the presence of PLC inhibitor after 24 h incubation.

As shown in Figure 12, ME-A cells growing under serum starvation conditions in the presence of 10 µM U73122 showed apoptosis rates higher than 60% after 22 h incubation. The ME-C cell MC (20 µg/ml) was not able to rescue the cells from programmed cell death (Figure 12). Because cells detached from the culture plates it was difficult to measure the apoptosis rate in the cell monolayer by fluorescence microscopy. Apoptosis rates were measured by FACS analysis after staining of cells with 2 µg/ml propidium iodide.

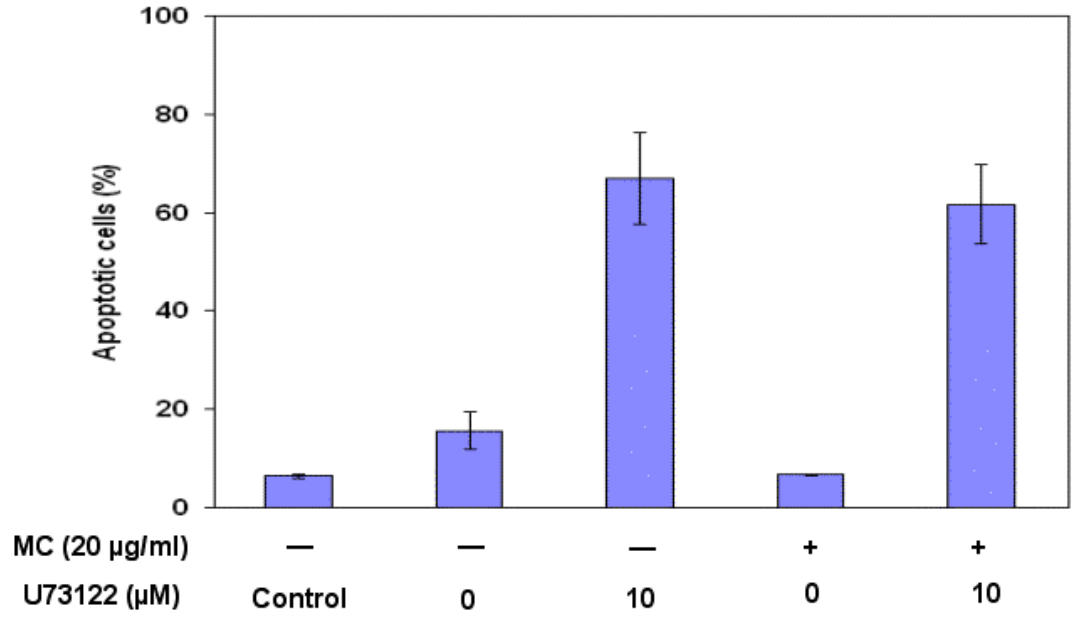

Figure 12. Effect of PLC inhibition on ME-A cell apoptosis under serum starvation conditions. ME-A cell apoptosis was induced by serum starvation. ME-A cells were cultivated for 22 h under standard conditions (control), under serum starvation (0) or under serum starvation and 10 µM U73122 (Tocris Biosciences) in the absence (-) or presence (+) of 20 µg/ml ME-C cell MC. Cells were stained with propidium iodide (2 µg/ml) for 10 min. Apoptosis rates (%) were measured by FACS analysis. Numbers given are mean values from three independent experiments with their respective standard deviation.

As shown in Figure 13, similar effects were obtained when ME-A cells were treated with 10 µM U73122 and 1 µM doxorubicin under standard culture conditions after 22 h incubation. Under doxorubicin conditions, where inhibition of PI3-k and Src family of tyrosine kinases in

the ME-A cells did not have an effect on the anti-apoptotic properties of the ME-C cell MC (see figures 10 and 11) PLC inhibition abolished the protective effect of the ME-C cell MC and promoted apoptosis of the ME-A cells.

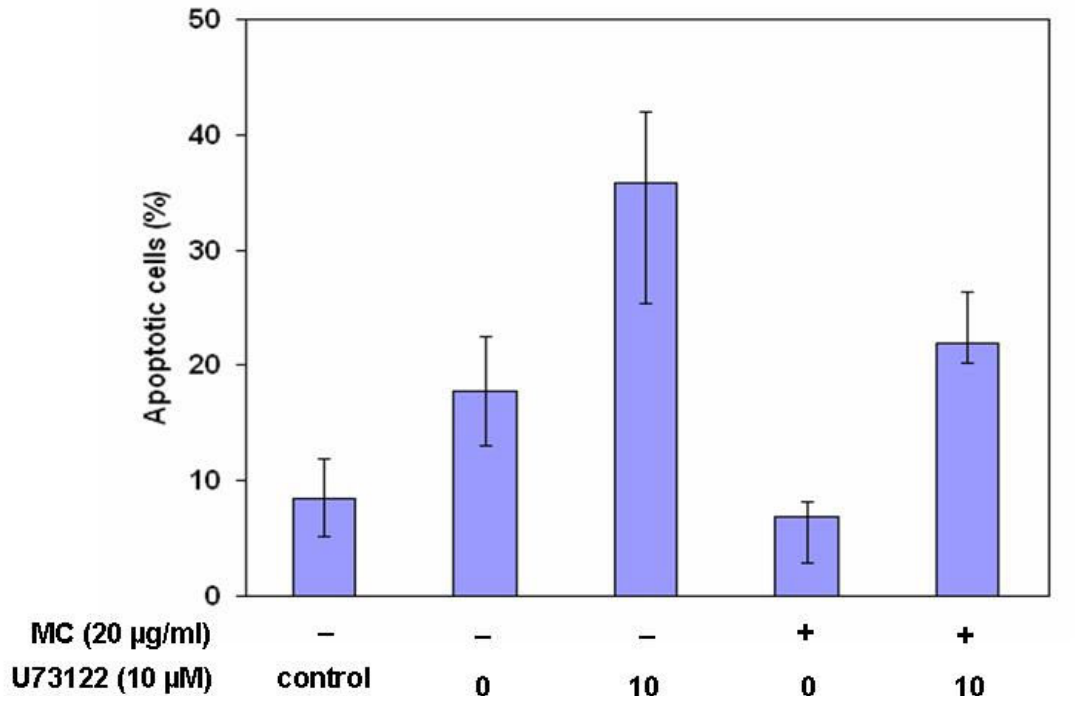

Figure 13. Effect of PLC inhibition on ME-A cell apoptosis under doxorubicin treatment. Apoptosis was induced by addition of 1 µM doxorubicin. ME-A cells were cultivated for 22 h under standard condition (control), or under standard conditions and 1 µM doxorubicin (0) with 10 µM U73122 (10) in the absence (-) or presence (+) of 20 µg/ml ME-C cell MC. Cells were stained with propidium iodide (2 µg/ml) for 10 min. Apoptosis rates (%) were measured by FACS analysis. Numbers given are mean values from three independent experiments with their respective standard deviation.

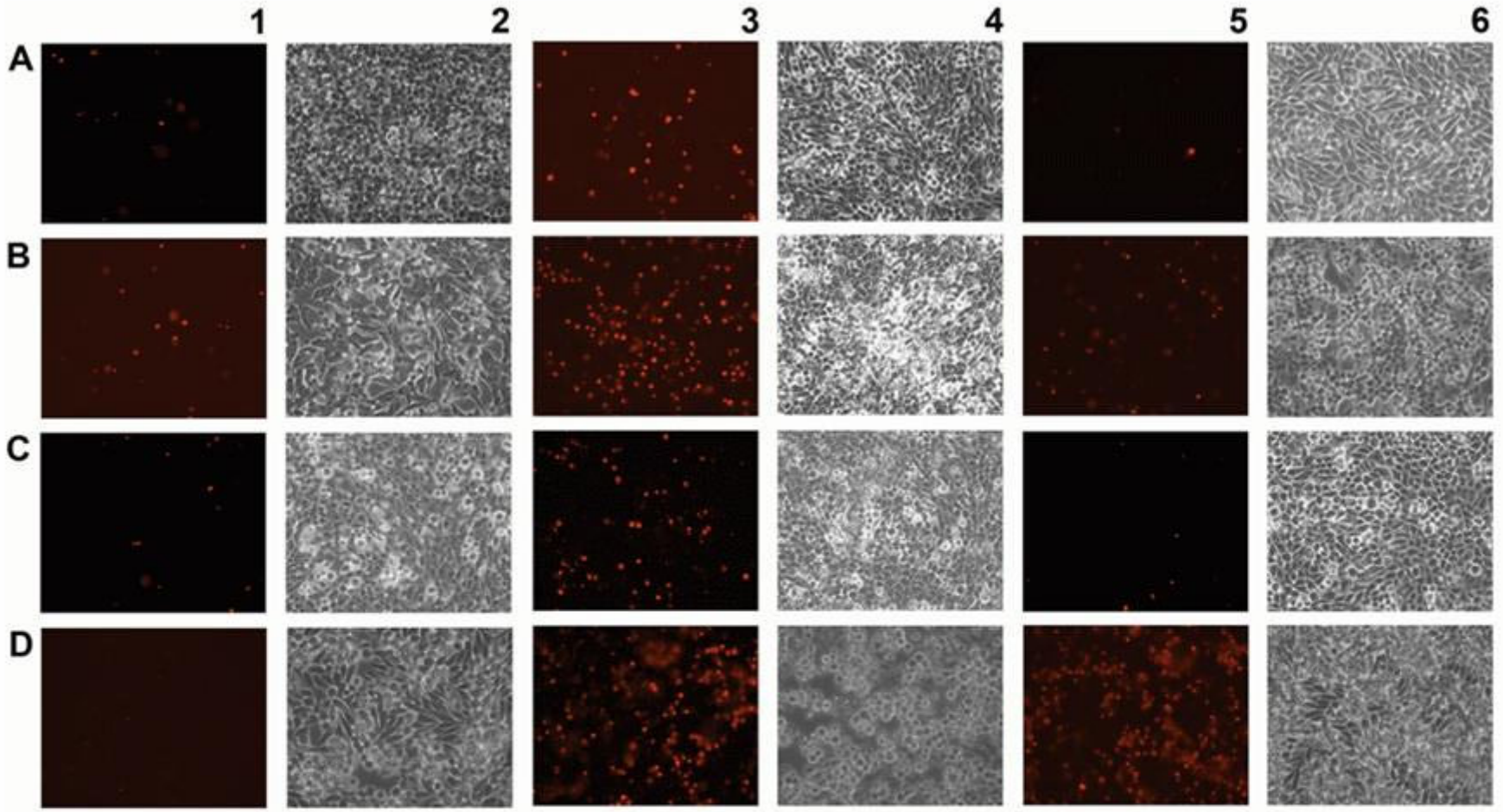

Figure 14. ME-A cell apoptosis under Src kinase, PI3-kinase and PLC inhibition. Fluorescence microscopy was done on of ME-A were cells cultivated for 22 h (1) under standard culture conditions, (3) under serum starvation conditions and (5) serum starvation conditions in the presence of 20 µg/ml ME-C cell MC. To measure apoptosis ME-A cells were stainded 2 µg/ml propidium iodide for 10 min. (2),(4) and (6) are phase-contrast mycroscopy images of propidium iodide stained ME-A cells shown in (1), (3), (5) respectively. ME-A cells were cultivated (A) without inhibitors or (B) in the presence of 10 µM Src inhibitor (PP2), (C) 10 µM PI3-k inhibitor (LY294002), (D) 10 µM PLC inhibitor (U73122). Images were captured with a Zeiss, Axioskop microscope, magnification 40x.

The effect of the Src, PI3-k and PLC inhibitors in ME-A cells growing under serum starvation conditions is shown in Figure 14. Apoptosis was low in ME-A cells cultivated in standard conditions (Figure 14, A1-A2). To test whether the failure of the ME-C cell MC to recover, cells treated with the PLC inhibitor (row D) was dependent on the higher concentration used in previous assays (10 μM) in comparison to the lower concentration used of PI3-k and Src inhibitors (1 and 2.5 μM), the ME-A cells were treated with equal concentrations of each inhibitor (10 μM).

As shown in Figure 14, exposure to Src inhibitior (B3), PI3-k inhibitor (C3) and PLC inhibitor (D3) had dramatic effects on the survival of ME-A cells under serum starvation conditions. After 22 h incubation under serum starvation, apoptotic rates were high as observed by fluorescence microscopy of propidium iodide staining of ME-A cells. The addition of ME-C cell MC (20 μg/ml) rescued ME-A cells cultivated for 22 h under serum starvation conditions (Figure 14, A5) and under serum starvation and exposition to Src inhibitor (Figure 14, B5) or PI3-k inhibitor (Figure 14, C5). However, apoptosis of ME-A cells growing under serum starvation which were exposed to PLC inhibitor (Figure 14, D5) was not prevented by adding ME-C cell MC. As shown in Figure 14, exposure of ME-A cell to PLC inhibitor had strong effects also on the adhesion of these cells (D4) in comparison to the effect of the other inhibitors used (B4, C4). ME-A cells showed a round morphology and the ME-A cell monolayer was severely damaged.

Thus, PLC activity seems to be important for the ME-C cell MC anti-apoptotic signalling in ME-A cells.

4.4. OPN overexpression in ME-A cells

4.4.1. Plasmid pcDNA3-OPN

ME-C cells in comparison to the ME-A cells, overexpressed OPN and were able to secrete it more efficiently than the ME-A cells into culture media. It is not clear whether OPN overexpression and secretion in the culture medium is the only cause for the apoptosis resistance of the ME-C cells to stress conditions. However, strong evidence point to a role of OPN being the major anti-apoptotic factor in the ME-C cell MC. Lowering the levels of OPN in the ME-C cell MC by immunoprecipitation reduced also the ability of the ME-C cell MC to protect ME-A cell from stress-induced apoptosis (Graessmann *et al*., 2006). Therefore, to

overexpress OPN in the ME-A cells, the OPN complete coding region was cloned into a mammalian expression vector and transfected into ME-A cells to test whether the OPN overexpression and secretion into the culture media could confer resistance to these cells against apoptotic stimuli such as cultivation under serum starvation and chemotherapeutic treatment. The pcDNA3-OPN plasmid (Figure 15) contains the complete OPN coding region under the strong regulation of the cytomegalovirus promoter which allows gene overexpression cell type independent. At the C-terminal end of the OPN coding region follows the polyadenylation signal of the bovine growth hormone (BGH PolyA). The plasmid contains two antibiotic-resistant genes. The ampicillin-resistance gene that allows selection in bacteria and the neomycin resistance gene that allows selection in mammalian cells.

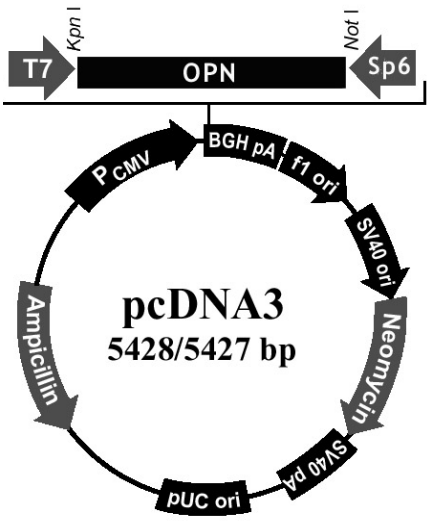

Figure 15. OPN expression vector pcDNA3-OPN. The plasmid pcDNA3-OPN contains the OPN complete coding region under the regulatory control of the cytomegalovirus promoter (Pcmv) and the bovine growth hormone polyadenylation signal (BGHpA). The vector contains resistance genes against ampicillin and neomycin. The size of the vector is indicated in the figure as nucleotide base pairs (bp). The complete OPN coding region comprises 885 bp. Restriction sites for cloning the OPN insert are indicated (KpnI/NotI). T7 and SP6 primers were used for sequencing analysis (for construct information of the vector see material and methods 3.2.3.10.2).

4.4.2. pcDNA3-OPN transfection into ME-A cells and selection of stable cell line overexpressing and secreting osteopontin

Delivery of pcDNA3-OPN into ME-A cells was achieved by transfection using the FuGene HD transfection reagent (Roche) as described in Materials and methods 3.2.2.3 and stable cell lines were obtained after three weeks selection in neomycin (500 µg/ml) containing medium. The pcDNA3-OPN plasmid contains the SV40 polyoma origin (SV40 ori) and can be episomally replicated in cells by the presence of SV40 T-antigen expression such as ME-A cells which express the SV40 T/t antigen (Tzeng *et al*., 1998).

The clonal ME-A cell lines resistant to neomycin were analysed for stable episomal DNA maintenance of the OPN construct. DNA was extracted from each ME-A neomycin-resistant clones by the TRIzol method. A DNA polymerase chain reaction (PCR) using the T7 and OPN specific primers (see Material and methods Table 6) was performed. The T7 primer is in front of the OPN insert in the plasmid and should also be present in the transfected cells. Non-transfected ME-A cell DNA was used as negative control. Non-transfected ME-A cell DNA contains only the endogenous OPN gene. As a positive control, purified plasmid DNA and DNA extracted from a previously transfected cell line in which OPN was inserted in the pTracer plasmid (see Material and methods 3.2.3.10.1.) under the regulatory control of SV40 viral promoter and which also contains the T7 primer forward from OPN was employed (results are shown in Figure 16 under T7-OPN). The pTracer (Invitrogen) plasmid contains the green fluorrescence protein gene (GFP) fused together to the Zeocin resistance gene. Before to the construction of the pCDNA3-OPN plasmid, the OPN complete coding region was cloned into the pTracer plasmid and transfected into the ME-A cells. After selection in zeocin for 4 weeks we obtained clones resistant to the antibiotic, however these clones neither expressed the GFP fusion protein nor could OPN protein overexpression be detected in them. We have no further explanations why OPN overexpression failed in these clones. However, ME-A cells which contained the pTracer-OPN construct after zeocin selection, were used as control for the DNA analysis of the T7-OPN PCR product, as the product was confirmed in the selection for pTracer-OPN positive clones.

Episomal stable maintenance of pcDNA3-OPN was also analysed by performing a PCR using primers specific for the CMV promoter (pCMV) and OPN (see Material and methods Table 6) Purified plasmid DNA was used as positive control. As shown in Figure 16 (under pCMV-OPN), the expected pcDNA3-OPN stable clones were positive for the T7-OPN fragment, in contrast to non-transfected ME-A cells. Clone 1 presents only a weak band as shown in Figure 16. When the cells were analysed for the pCMV-OPN PCR product, clones 2 and 5 were positive. It was possible to observe a weak band for clone 1. Thus, ME-A cell clone 1 episomal maintenance of the pcDNA OPN construct appeared to be weak. Both non-transfected ME-A cells and pTracer transfected cells were negative for the PCR product as expected (see Figure 16).

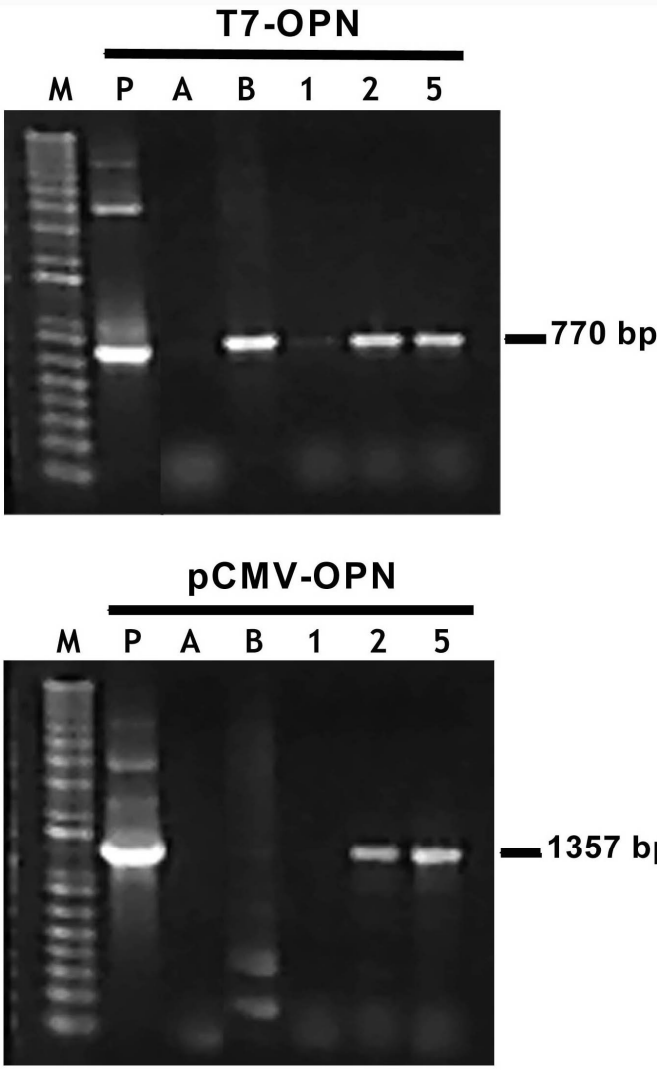

Figure 16. pcDNA3-OPN DNA stable episomal maintenance in ME-A cell neomycin-selected clones. ME-A cells were transfected with the pcDNA3-OPN plasmid. Three clones were selected under neomycin treatment. Total DNA was analysed by PCR and agarose-gel electrophoresis. (P) Plasmid DNA, (A) non-transfected ME-A cells, (B) ME-A cells tranfected with pTracer-OPN plasmids. (1), (2), (5) ME-A neomycin selected clones (containing pcDNA3-OPN plasmid). The PCR products T7-OPN, using primers T7 and OPN 2 (see Material and methods Table 6, product size 770 bp) and pCMV-OPN using primers PCMV and OPN2 (see Material and methods table 6, product size 1357 bp) are indicated in the figure.

The PCR analysis indicated that clones 1, 2 and 5 were positive for the pcDNA3-OPN DNA episomal maintenance in ME-A cells transfected clones. It is not clear whether the integration of DNA occurred at the chromosomal level. However, episomal replication in the cells might be sufficient for OPN protein overexpression in ME-A clones.

To verify that the selected clones overexpressed OPN, mRNA levels were analysed by RT-PCR (20 cycles) from the T7-OPN product using primers T7 and OPN2 (see Materials and methods Table 6). The T7-OPN product should be transcribed from the plasmid in mRNA by the ME-A cell clones. As observed in Figure 17, the clones efficiently expressed the product T7-OPN with the highest expression in clone 5. In contrast, in non-transfected ME-A cells, the PCR T7-OPN product was not present. When OPN mRNA expression was analysed by RT-PCR using OPN specific primers OPN1 and OPN2 (see Materials and methods Table 6),

no differences were apparent in the OPN mRNA expression in ME-A cells in comparison to the OPN mRNA expression of the pcDNA3.0-OPN clones. This result is not surprising as ME-A cells efficiently produce OPN mRNA (as already shown in Results 1.3 and Figure.3). Thus after 20 cycles PCR there were no strong differences in the mRNA expression of the endogenous OPN. However, for T7-OPN product strong differences were observed between the ME-A clones, and ME-A5 clone expressed the product at highest levels in comparison to the other clones selected. ME-A cells, as expected, did not expressed the T7-OPN PCR product. Altogether, the ME-A neomycin selected clones demonstrated to express extra OPN copies originated from the pcDNA3.0-OPN plasmid and the ME-A5 cells were those with the highest expression (Figure 17). As a control for the RT-PCR reaction we also analysed GADPH mRNA expression, and similar levels were observed for the different cell lines (Figure 17).

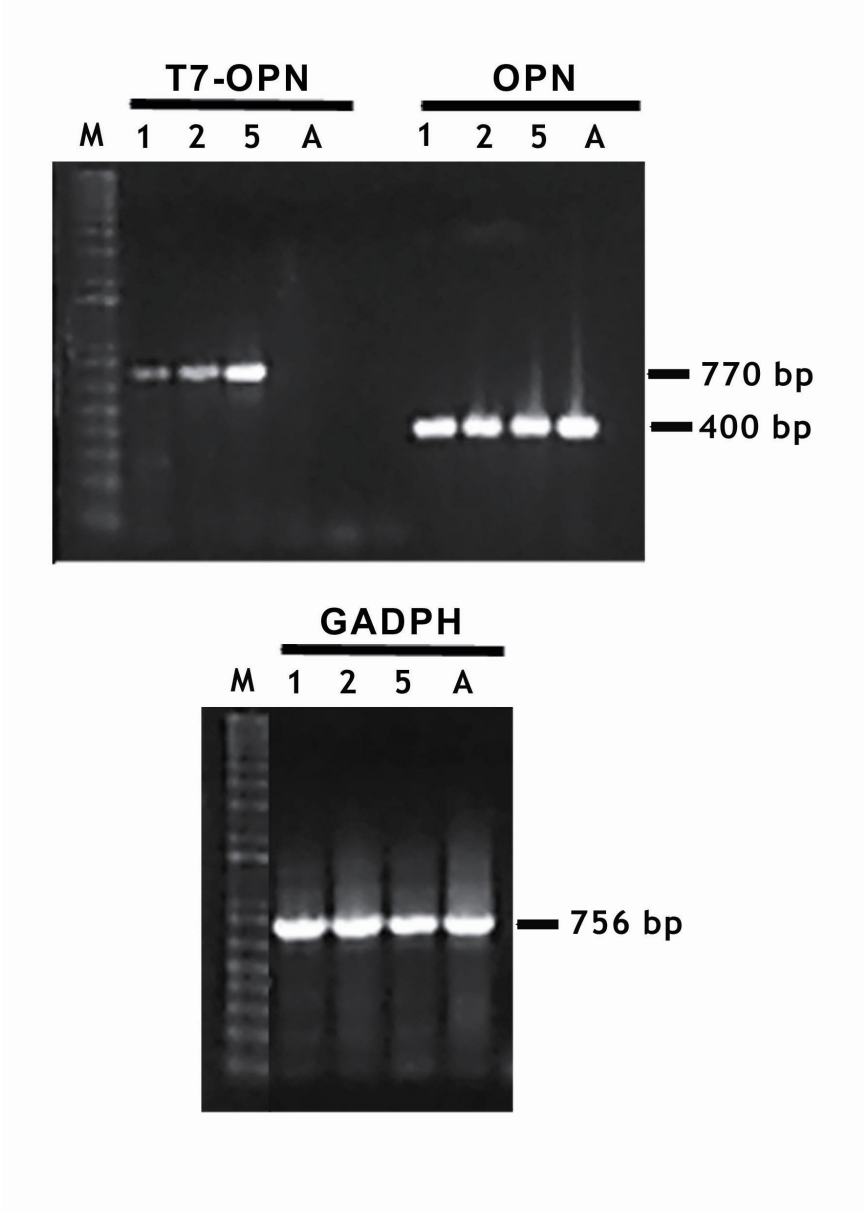

Figure 17. OPN mRNA expression in pcDNA3-OPN ME-A cell neomycin-selected clones. Cells were cultivated for 22 h under standard conditions. mRNA from non-transfected ME-A cells (A), and pcDNA3-OPN-transfected and neomycin-selected clones (1), (2), (5), was extracted by TRIzol reagent method. mRNA was transcribed to cDNA by reverse transcriptase and analysis were done by PCR (20 cycles) and agarose-gel electrophoresis. PCR products T7-OPN (primers T7 and OPN 2), OPN (primers OPN 1 and OPN 2, see Material and methods Table 6.) and as a loading control, GADPH (primers GADPH 1 and GADPH2, see Material and methods Table 6) are indicated in the figure.

4.4.3. OPN overexpression in ME-A cell neomycin-selected clones

To verify the OPN overexpression in ME-A neomycin resistant clones, cells were cultivated under standard conditions for 48 h. Afterward, cells were further incubated under serum starvation conditions for 24 and 48 h and the culture media was collected after 24 h and 48 h incubation. Figure 18 shows the results obtained. Proteins were enriched using columns Ultrafree Biomax 30K (millipore) and were subjected to SDS-PAGE-gel electrophoresis, Western blots and immunodetection with an anti-OPN mouse monoclonal antibody (Santa Cruz) followed by incubation with an anti-mouse HRP-conjugated secondary antibody (Dako) and signal detected by chemiluminescence using the ECL-detection system (Perkin Elmer Life Science). We observed no increase in OPN protein levels in clones 1 and 2. However, the ME-A neomicyn resistant clone 5 secreted higher amounts of OPN in culture media after 24 h serum starvation in comparison to non-transfected ME-A cells (Figure 18, compare lanes A and 5 after 24 h incubation). Using the imageJ software for image analysis the immunoblot bands were quantified and the ME-A5 cell line produced around 3 times more OPN (33.889 arbitrary units) than ME-A cells (11,439 arbitrary units). However, the secreted OPN levels in ME-A neomycin resistant clone 5 did not reach the levels of OPN secretion by ME-C cells (Figure 18, compare lanes ME-C and 5 after 24 hours). The analysis of the immunoblot bands using the imageJ software quantified ME-C cell OPN around 4 times higher than for the ME-A clone 5.

Interestingly, after 48 h incubation under serum starvation conditions the OPN pattern in immunoblots from culture media from ME-A5 differed from that of ME-A cells. As shown in Figure 18 (lane 5 after 48 h incubation), the high molecular OPN modifications were almost not present. There was accumulation of OPN bands in the range 40-42 kDa and the appearance of an OPN modification at about 37 kDa was similar to one found in ME-C cells MC (shown also in Figure 19).

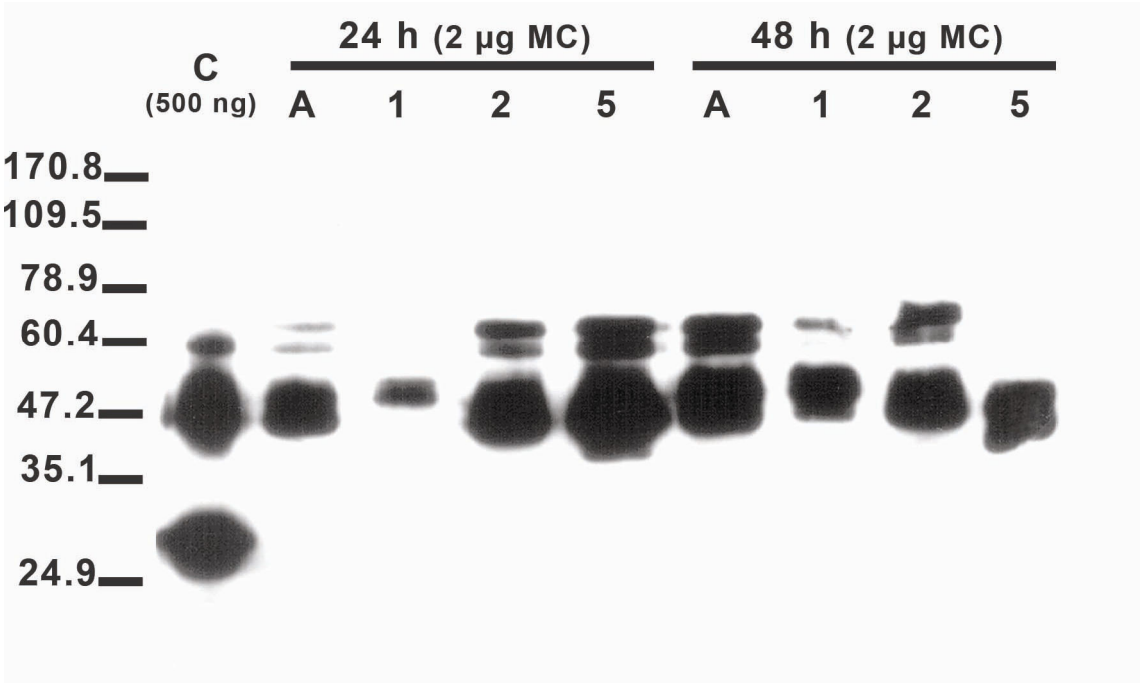

Figure 18. OPN secretion in ME-A cell neomycin-selected clones. 500 ng from ME-C cells MC (C) was used as loading control to compare medium concentrates made from (A) ME-A cells and (1), (2), (5) ME-A cell neomycin-selected clones cultivated under serum starvation conditions after 24 and 48 h incubation. Culture media were collected after the indicated time periods and concentrated using Ultrafree Biomax-30K columns (Millipore). 500 ng of ME-C cells MC, 2 µg of ME-A cells and ME-A cell neomycin-selected clones were loaded in SDS-PAGE-gels and subjected to electrophoresis. Proteins were transferred onto a PVDF membrane (Immobilion P) and hybridised with an mouse monoclonal anti-OPN antibody (Santa Cruz, sc-21742, 1:5000 dilution) followed by incubation with a anti-mouse HRP-conjugated secondary antibody (Dako, 1:5000 dilution) and the signal was detected by chemiluminescence using the ECL-detection system (Perkin Elmer Life Science). The imageJ software for image analysis (Abramoff et al., 2004) was used for quantification of the OPN immunoreactive bands.

4.4.4. Anti-apoptotic activity of ME-A cell neomycin-selected clone 5 (ME-A5) medium concentrates

The results in 4.4.3 demonstrated that ME-A cell neomycin-resistant clone 5 (ME-A5) was able to secrete around 3 times more OPN into culture media after 24 h incubation under serum starvation conditions than the parental ME-A cell line. Furthermore, after 48 h incubation under serum starvation conditions the ME-A5 clones presented an OPN immunoreactive band of around 37 kDa present also in ME-C cell secretions which have not yet been detected in ME-A cell secretions. It is not clear which mechanism produces the appearance of this OPN modification in the ME-A5 clones. However, the accumulation of this modification might have an effect on the anti-apoptotic activity of the ME-A5 cell MC in comparison to the ME-A cells MC, as previous size exclusion chromatographic experiments (see Results 4.2.)

demonstrated that the anti-apoptotic activity of ME-C cell MC accumulated in fractions containing proteins in the 20 – 40 kDa molecular weight range.

Medium concentrates were prepared from both ME-A and ME-A5 cell lines to test their anti-apoptotic properties. Cells were plated at $1x10^5$ cells/ml density, cultivated under standard conditions for 48 h and further incubated for 48 h hours under serum starvation conditions. Cell culture medium was collected, dialysed against 0.1xPBS overnight and lyophilised. Protein concentration was determined and equal amounts of proteins were subjected to SDS-PAGE and Western blot followed by immunodetection using a monoclonal antibody against OPN (Santa Cruz) followed by incubation with an anti-mouse HRP-conjugated secondary antibody (Dako) and signal detected by chemiluminiscence using the ECL-detection system (Perkin Elmer Life Science). ME-C cell MC were compared against ME-A and ME-A5 cells MCs. The differences between the OPN modifications observed in previous experiments (as shown in Figure 18), remained similar (as shown in Figure 19), with the bands of 37 kDa enriched in ME-A5 cells MC and absent from ME-A cell MC (as indicated by the arrow in Figure 19).

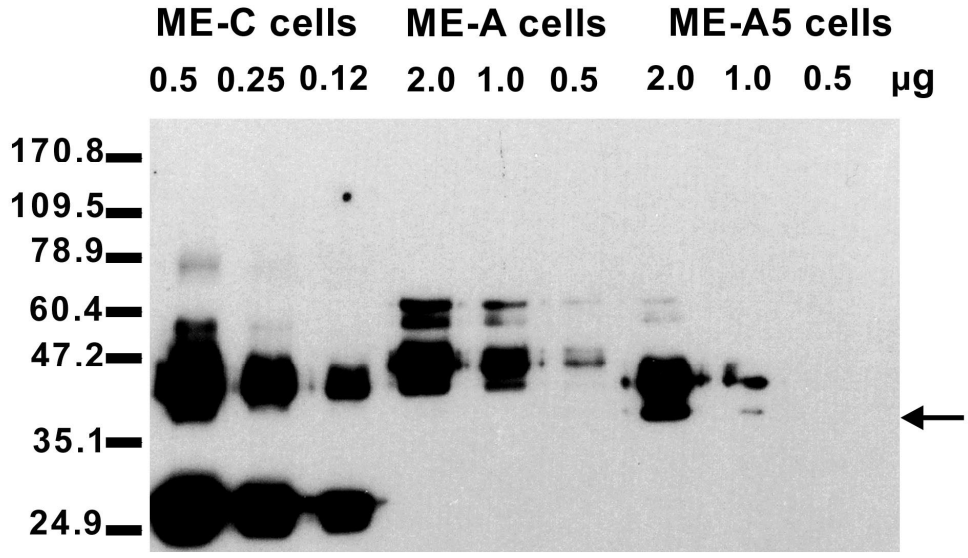

Figure 19. Comparison of OPN protein modifications in ME-C, ME-A and ME-A5 cells medium concentrates. (ME-C) ME-C cells, (ME-A) ME-A cells and (ME-A5) ME-A5 neomycin resistant cells were cultivated under serum starvation conditions for 48 h, culture media collected and dialysed against 0.1xPBS. Proteins were concentrated by lyophilisation to obtain medium concentrates (MCs). As indicated in the figure, different protein amounts of each MC were subjected to SDS-PAGE-gels (10%) electrophoresis and transferred into a PVDF membrane (Immobilion P). Membranes were hybridized with a mouse monoclonal anti-OPN antibody (Santa Cruz, sc-21742, 1:5000 dilution) followed by incubation with an anti-mouse HRP-conjugated secondary antibody (Dako, 1:5000 dilution) and signal detected by chemiluminiscence using the ECL-detection system (Perkin Elmer Life Science). The 37 kDa OPN immunoreactive band secreted by ME-A5 cells is indicated by an arrow.

To compare the anti-apoptotic activity of ME-C cell, ME-A cell and ME-A5 cell medium concentrates, 20 µg/ml of each medium concentrate was added to ME-A cells growing under serum starvation conditions and apoptosis rates were measured by FACS analysis of propidium iodide-stained cells after 22 h incubation. As is shown in Figure 20, ME-A5 cell MC did not show any effect compared to the effect of ME-C cell MC in preventing apoptosis of ME-A cells under serum starvation conditions. Interestingly, ME-A cell MC showed an increase in the apoptotic rate when added to the culture media, which opens the question whether the production of high molecular weight OPN modifications promote apoptosis in contrast to the production of low molecular weight OPN modifications. However, this question was not addressed in this study.

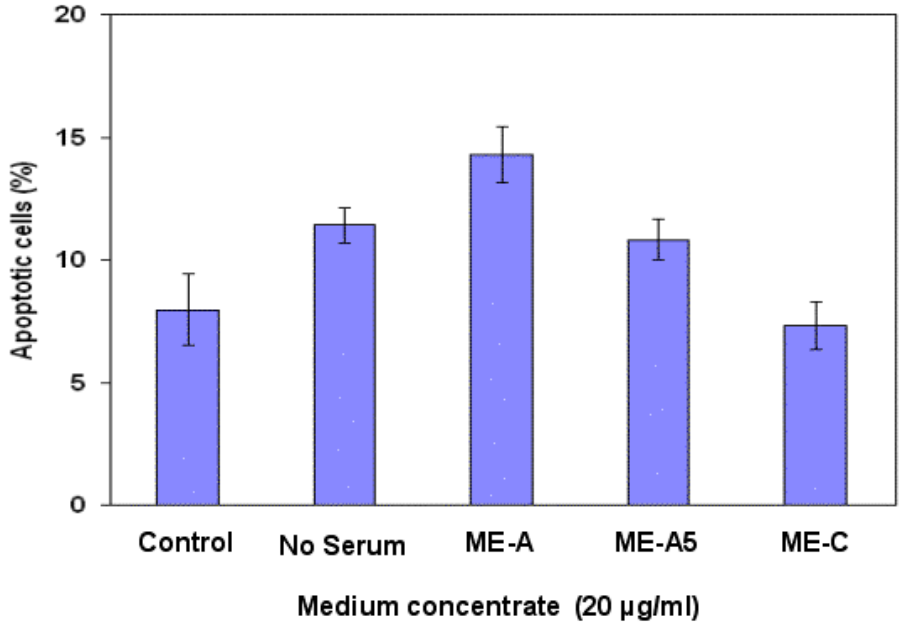

Figure 20. ME-C cell, ME-A cell and ME-C cell medium concentrate anti-apoptotic activity in ME-A cells cultivated under serum starvation. ME-A cells were cultivated under (control) standard conditions, (no serum) serum starvation and serum starvation with (ME-A) 20 µg/ml of ME-A cells MC, with (ME-A5) 20 µg/ml ME-A5 cells MC or with (ME-C) 20µg/ml ME-C cells MC. ME-A cells were stained with propidium iodide (2 µg/ml) and apoptosis was measured by FACS analysis. Numbers given are mean values with their respective standard deviation from three independent experiments.

To answer the question whether the ME-C cell MC anti-apoptotic effect was dependent in the amounts of OPN present we added 3 times higher concentrations of ME-A and ME-A5 cell MCs (60 µg/ml instead of 20 µg/ml) to ME-A cells growing under serum starvation conditions and similar results to those presented in Figure 20 were obtained. ME-A and ME-A5 did not prevent apoptosis of ME-A cells cultivated under serum starvation conditions for

22 h. This result points to a more important role of OPN modifications in preventing apoptosis than the amounts of OPN present in the medium concentrates. This result is also in agreement with previous results from chromatographic protein fractionation experiments (see results 4.2. and Graessmann et al, 2006) where the anti-apoptotic activity concentrated in fractions enriched for proteins in the 20-40 kDa molecular weight range and the highest activity concentrated in those fractions between 20-32 kDa, having a threefold higher anti-apoptotic activity than the whole ME-C cell MC. The higher molecular fractions (>40 kDa) did not contain anti-apoptotic activity (Graesmann et al., 2006).

4.5. Osteopontin receptors in ME-A and ME-C cells

OPN can activate survival pathways by activating cell membrane receptors like integrins and CD44 (see Introduction 1.2.5). Integrin and CD44 activation are an important molecular mechanism to regulate cell adhesion to the extracellular matrix which protects several cell types from apoptosis. It is not known which of these receptors´ are present at the ME-A and the ME-C cells surfaces. To find out wether these receptors are also present in ME-A and ME-C cell membranes, both cell lines were subjected to FACS analysis, immunofluorescence microscopy and Western blot analysis using different specific anti-integrin and anti-CD44 antibodies.

4.5.1. FACS analysis of OPN receptors presented at the surface of ME-A and ME-C cell lines

ME-A and ME-C cells were prepared for FACS analysis of cell membrane receptors as described in Material and methods 3.2.2.7. FACS analysis of the receptors´ surface expression was achieved by incubation of ME-A and ME-C cells with several anti-bodies specific against integrin subunits and CD44 proteins followed by fluorescence immunostaining with secondary antibodies (see Table 1 in Material and methods for antibodies information).

In Figure 21 shows the results of FACS immunofluorescence detection for β1, β3, β5 and αv integrin subunits and CD44 receptors. As described before these integrin subunits are involved in mediating OPN signals in breast cancer cells (see Introduction 1.2.5.2). CD44 is also involved in mediating OPN signals in several types of cells (see Introduction 1.2.5.1).

Apart from using specific primary antibodies followed by immunostaining with secondary fluorescent antibodies (Figure 21, green lines) to detect cell surface receptors in ME-A and

ME-C cells, two negative controls were included in which the ME-A and the ME-C cells were immunostained using only the fluorescent secondary antibodies (Figure 21, red lines) and no antibodies at all (Figure 21, black lines) to avoid fluorescence-unspecific signals from unspecific secondary antibody binding and basal cell fluorescence background, respectively.

The results in Figure 21 demonstrate that ME-A and ME-C cells share a strong presence of β1 integrin and CD44 receptors. Two different rabbit polyclonal antibodies against integrin β1 (Chemicon, ASK3K6 and Chemicon, ASK2A4) were employed for the surface detection of this integrin subunit and both gave similar results with 100% of cells staining positive for the molecule in a single strong fluorescence peak (see Figure 21, ß1 ASK3K6 and ASK2A4) however, with differences in the fluorescence intensities between both antibodies. The fluorescence signal for CD44 was not so evenly distributed as the signal obtained with ß1 antibodies however, around 65% of the ME-A cells and 41% the ME-C cells stained positive for CD44 (see Figure 21, CD44).

FACS analysis using specific antibodies against β3 and αv integrin subunits gave positive fluorescence signals in the ME-A cells (Figure 21, β3 and αv) with well defined fluorescence peaks outside the regions where the respective fluorescence peaks for the negative controls were localized. In contrast to ME-A cells, the ME-C cells were negative for β3 and αv integrin subunit immunostaining.

Both ME cell lines were negative against β5 integrin (as shown in Figure 22, β5)

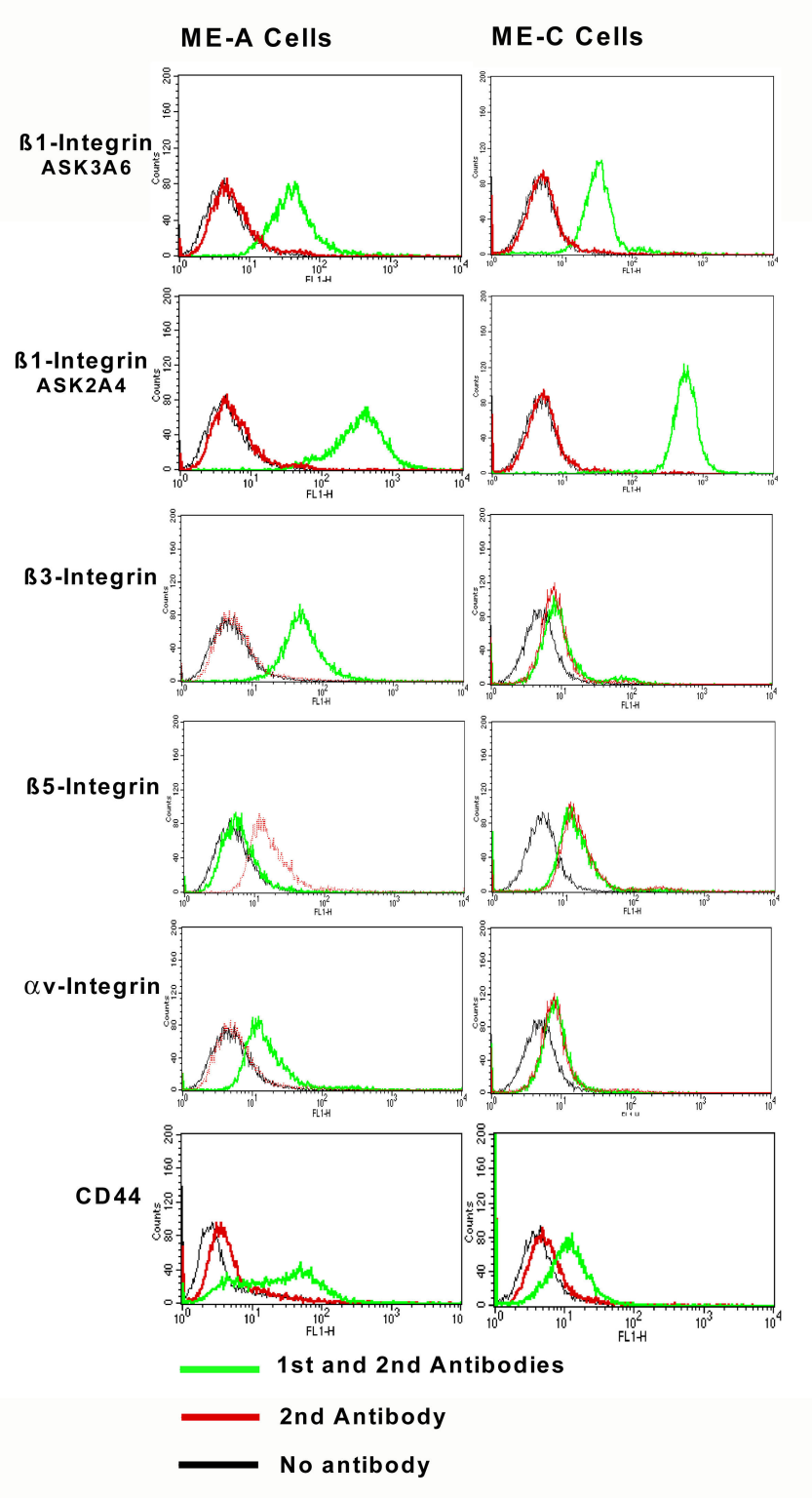

Figure 21. FACS analysis of OPN receptors present in the surface of ME-A and ME-C cells. After handling ME-A and ME-C cells with a trypsin solution for 10 min, $1x10^6$ were counted and cells were incubated with specific primary antibody for 30 min in DMEM medium containing 5%FCS. Cells were incubated separately with each specific primary antibody, rabbit polyclonal anti-ß1 integrin (Chemicon ASK3K6), rabbit polyclonal anti-ß1 integrin (Chemicon ASK2A4), armenian-hamster monoclonal anti-ß3 integrin (Santa Cruz), rabbit polyclonal anti-ß5 integrin (Santa Cruz), armenian-hamster monoclonal anti-αv integrin (Santa Cruz), rat anti-CD44 (BD Pharmigen) in the concentration described in material and methods table 1. Cells were washed two times with PBS followed by 30 min to 1 h incubation at 4°C in absence of light with FITC-conjugated secondary antibodies diluted in PBS (see Material and methods, Table 1, FACS column, for secondary antibodies concentrations). FACS measurements were done with Excallibur FACScan machine and data analysis with CellQuest software. The three lines in each graphic represent cells incubated in the absence of antibodies (black line), cells incubated in the presence of only non-specific fluorescent secondary antibody (negative control, red line) and cells incubated with receptor-specific antibody followed by incubation with fluorescent secondary antibody (green line). In the x-axis the fluorescence intensity is given in logarithmic scale and y-axis represent event (cell) count.

4.5.2. Detection of OPN receptors in ME-A and ME-C cells by immunofluorescence microscopy

ME-A and ME-C cells were plated at a density of $1x10^6$ cells/ml and cultivated 24 h under standard conditions. After incubation, cells were washed in PBS and air-dried, followed by fixation with paraformaldehyde or methanol solutions (see Materials and methods 3.2.2.8). After fixation cells were handled with specific antibodies against the different cell surface receptors for 30-60 min followed by incubation with FITC conjugated secondary antibodies for immunofluorescence detection (for primary and secondary antibodies concentrations used, see material and methods Table 1, under IF column). For nuclear staining, cells were incubated for further 10 min with a DAPI/PBS solution for nuclear staining. Fluorescence detection was done with Zeiss, Axioskop microscope.

As shown in Figure 22, ME-A and ME-C cells presented a specific signal staining at the plasma membrane when the rabbit polyclonal anti-ß1 integrin subunit (Chemicon ASK3K6) and rat anti-CD44 (BD Pharmigen, clone IM7) were employed in agreement with the previous results (see Results 4.5.2.) obtained from fluorescence staining of the cell surface receptors and FACS analysis data.

We were not able to obtain a conclusive staining for β3 and αv integrin receptors in ME-A cells in contrast to the results obtained by FACS data analysis of fluorescence staining (see Results 4.5.2.)

ME-C cells were able to form tumor-like foci-organized structures and as shown in Figure 22 (lower left panel). CD44 was widely distributed at the plasma membrane of ME-C cells in these structures. Similar results were also observed when ME.-C cells were incubated with the specific antibodies for β1 integrin subunit which points to a possible role of these molecules in the organization of multicellular structures in these cells. In ME-A cells these foci-organized structures were not observed.

FACS analysis data and immunofluorescence microscopy demonstrated that CD44 and β1 integrin were strongly expressed at the cell membranes of both ME-A and ME-C cells. The presence of these receptors at the cell surface might be part of a common mechanism to detect OPN-mediated signals both in ME-A and ME-C cell lines.

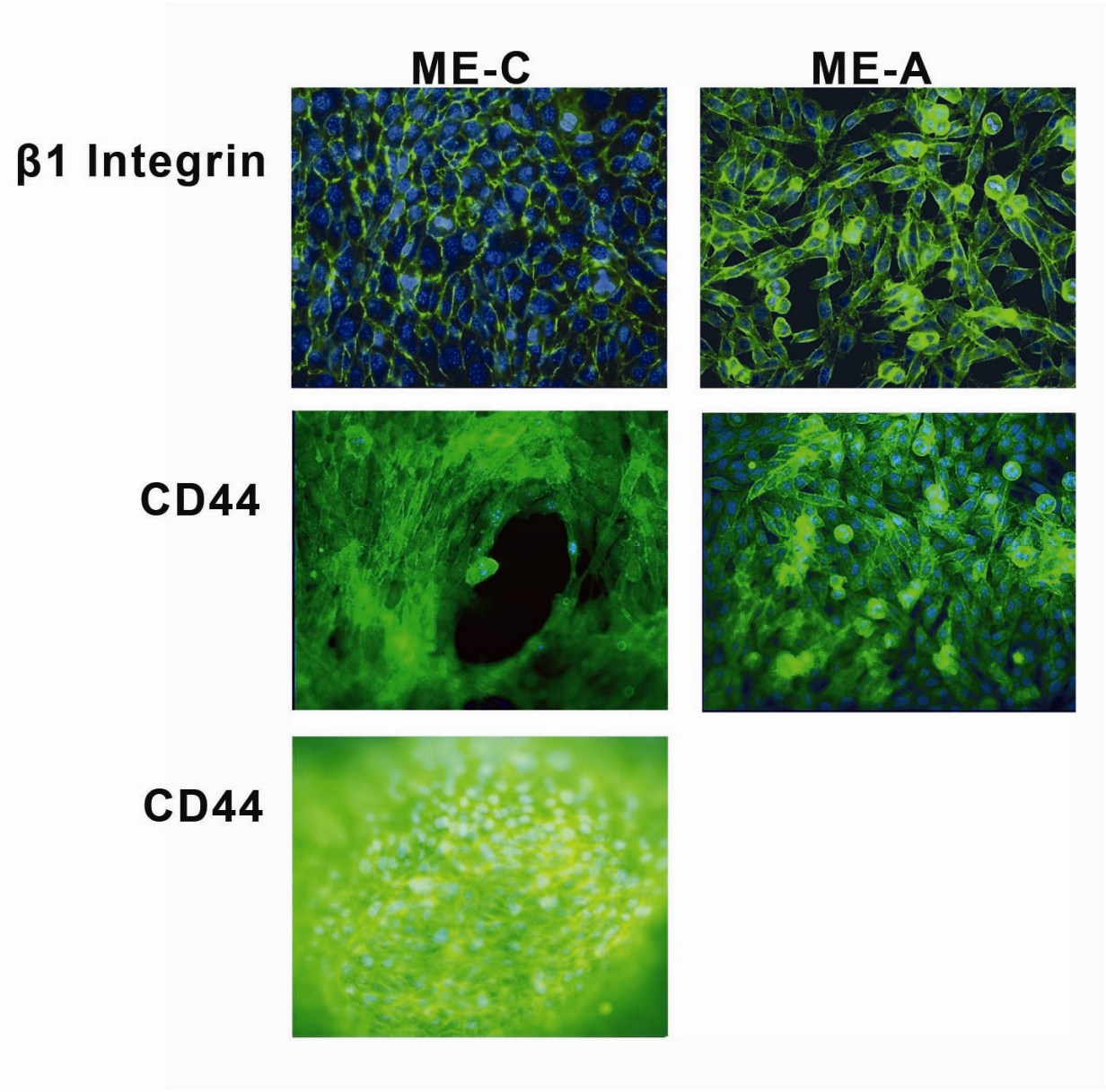

Figure 22. Immunofluorescence microscopy of OPN receptors at the surface of ME-A and ME-C cells. $1x10^6$ ME-A and ME-C cells were cultivated for 24 h under standard culture conditions. Cells were fixed with a 4% paraformaldehyde solution and incubated with specific primary antibodies: rabbit polyclonal anti-integrin β1 (Chemicon ASK3A6) and Rat monoclonal anti-CD44 (BD Pharmigen). Cells were incubated with FITC-conjugated secondary antibodies (see Materials and methods, Table 1 IF column for antibody concentrations used). Cell nuclei were stained with DAPI solution for 10 min. Fluorescence detection was done with Zeiss, Axioskop microscope; magnification 40x. ME-A and ME-C cells are indicated at the top of the figure. Receptors ß1 and CD44 are indicated at the left of the figure.

4.5.3. Western Blot analysis of anti -ß1 integrin subunit receptor in ME-A and ME-C cells

We also tested the presence of receptor molecules in ME-A and ME-C cells by SDS-PAGE-gel electrophoresis and Western Blots. Cells were cultivated for 48 h under standard

conditions, collected and re-suspended in lysis buffer (50 mM Tris/HCl pH 7.8, 150 mM NaCl, 2.5 mM EDTA, 1% NP40, 1 mM PMSF, 1µg/ml Peptastin, Aprotinin, Leupeptin). The protein concentration of cell lysates was measured by the BIORAD reagent method and 20 µg of total protein were loaded into 10% SDS-PAGE-gels. Proteins were transferred to PVDF membranes and the presence of receptors confirmed by immunodetection with a rabbit polyclonal anti-ß1 integrin antibody (Chemicon ASK3K6) followed by incubation with an anti-rabbit HRP-conjugated secondary antibody (Dako, 1:5000 dilution) and signal detected by chemiluminiscence using the ECL-detection system (Perkin Elmer Life Science).

As shown in Figure 23, detection with the anti-β1 integrin antibody showed a similar immunoreactive band pattern for both ME-A and ME-C cells. We detected a strong immunoreactive band at 110 kDa (the calculated molecular weight of β1 integrin is 115 kDa). Thus, Western Blots and immunodetection of β1 integrin subunit confirmed the immunofluorescence staining and FACS data analysis obtained in Results 4.5.1. and 4.5.2. showing that integrin-β1 receptor is present in both cell lines.

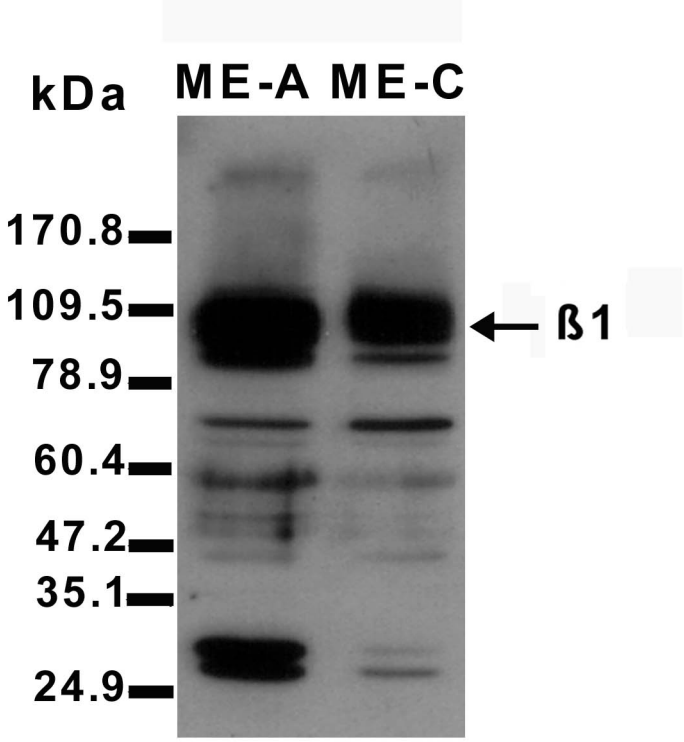

Figure 23. Western Blot analysis of β1 integrin receptor in ME-A and ME-C cells. Cells were cultivated for 24 h under standard culture conditions. 20 µg of cell lysates were loaded in 10% poly-acrylamide-SDS-gels transferred to PVDF membranes and immunodetection was done with an rabbit polyclonal anti-ß1 integrin antibody (Chemicon ASK3K6) followed by incubation with a anti-rabbit HRP-conjugated secondary antibody (Dako) and signal detected by chemiluminiscence using the ECL-detection system (Perkin Elmer Life Science). β1-integrin (~115 kDa) is indicated with an arrow. ME-A and ME-C cells are indicated at the top of the figure.

The results obtained from FACS data analysis, immunofluorescence microscopy and Western Blots analysis allow us to conclude that ME-A and ME-C cells present the OPN receptors β1-integrin and CD44 at the plasma membrane.

5. DISCUSSION

5.1. Chemotherapy resistant ME-C cells overexpressed osteopontin gene and accumulated osteopontin protein in their secretions

ME-A and ME-C cells lines isolated from a WAP SV T/t transgenic mouse breast tumor differ in their resistance to apoptotic stimuli. ME-A cells maintain SV40 T/t antigen transgene expression in tissue culture. ME-C cells have spontaneously lost transgene expression and present a p53 mutation commonly observed in breast cancer (Tzeng *et al*., 1998). ME-A cells exhibited an elevated spontaneous apoptosis rate of 2-5% after 24 h incubation under standard tissue culture conditions (Figures 1A and 1B), while ME-C cell apoptosis was not evident under the same culture conditions (Figures 1K and 1L). ME cell apoptosis was measured by fluorescence microscopy and FACS analysis of propidium iodide stained cells. Cells undergoing apoptosis present changes in phospholipid composition and permeability of the cell membrane. This change in membrane permeability allows the incorporation of propidium iodide into the cells. In healthy cells, propidium iodide is not able to pass through the cell membrane. After incorporating into the cells, propidium iodide intercalates into the cell DNA staining the dying cells (Nicoletti *et al*., 1991; Riccardi *et al*., 2006).

ME-A cells were sensitive to apoptotic stimuli such as serum starvation and chemotherapeutic treatment (e.g. doxorubicin). Under serum starvation conditions ME-A cells underwent extensive apoptosis (Figures 1C and 1D). Apoptotic cells reached levels of 20-30% after 22 h serum starvation (Figure 5). Under treatment with 1 μM doxorubicin ME-A cell apoptosis reached around 15% after 22 h incubation. ME-C cells were insensitive to these stimuli and levels of apoptosis remained low (Figure 1M and 1N).

Co-cultivation experiments demonstrated that ME-C cells protect ME-A cells from apoptosis (Graessmann *et al*., 2006) thus, the anti-apoptotic signal was assumed to act in a paracrine fashion. Microarray analysis of RNA demonstrated that several genes of secreted proteins presented high expression profiles in both cell lines, however, osteopontin (OPN) showed a marked difference in RNA expression between the ME-A and the ME-C cells. OPN has a relatively high expression profile in both ME-A and ME-C cells cultivated under standard and serum starvation conditions (Figure 2 and Graesmann *et al*., 2006). However comparison of OPN expression profiles between ME-A and ME-C cells gave the highest ratio value (ME-C cells/ME-A cells) of differential gene expression, with values of 3.50 and 2.56 under standard and serum starvation culture conditions respectively. This result not only points to a high

OPN expression but also to a marked difference in OPN expression between both ME cell lines. RT-PCR experiments demonstrated that OPN mRNA expression is high in both cell lines as was observed after 20 PCR reaction cycles (shown in Figure 3) in agreement with the microarray data. However, at the protein level marked differences were found between both cell lines in their ability to express and secrete OPN.

Gel electrophoresis and Western blot analysis of OPN protein expression in the ME-A and ME-C cells using a specific monoclonal OPN antibody demonstrated differences in the amounts and OPN isoforms presented by both cell lines (Figure 4). OPN expressed in mouse has a predicted molecular weight of 32.5 kDa. OPN has been described as a highly glycosylated and phosphorylated protein (Denhardt *et al*., 1993; Shanmugam *et al*., 1997; Christensen *et al*., 2005). As shown in Figure 4C, upon SDS-PAGE electrophoresis and Western blot analysis of 10 μg of total protein from ME-C cell lysates, OPN appeared as a pair of immunoreactive bands in the 56 - 58 kDa molecular weight range. In contrast, in ME-A cell lysates OPN was not detected even when 10 times more protein was used for the analysis. Thus, although there is a high OPN mRNA expression levels in ME-A and ME-C cell lines, the differences between OPN mRNA expression levels in both cell lines could be responsible for the remarkable difference in the ability of OPN protein expression between these ME cell lines.

As shown in Figure 4A, ME-C cells secreted OPN more efficiently into the culture medium than ME-A cells. SDS-PAGE electrophoresis and Western blot analysis of ME-C cell medium concentrates (MC) demonstrated that OPN is present in large amounts and revealed multiple bands in the 32-115 kDa molecular weight range, which indicated that OPN is modified extensively in the extracellular compartment. Moreover, an increased amount of OPN was found when cells were incubated under stress conditions, pointing to a functional role of OPN as a stress response protein (Figure 4A and Graessmann *et al*., 2006). In contrast, when ME-A cell medium concentrate was subjected to SDS-PAGE electrophoresis and Western blot analysis, only the 40-42 kDa OPN immunoreactive bands were detected even when 100 times more protein amounts were loaded into gels in comparison to ME-C cell MC (Figure 4B and Graessmann *et al*., 2006).

Thus, ME-A cells not only produced and secreted less OPN, but there were differences in the ability of these cells to produce the diverse isoforms observed in ME-C cells. The causes for

the OPN overexpression in the ME-C cells (in comparison to OPN expression in the ME-A cells) are not known. The SV40 T antigen has been implicated in the activation of c-Ras/Raf pathway (Grammatikakis *et al*., 2001) and OPN is under Ras/Raf transcriptional regulation (Craig *et al*., 1988.; Guo *et al*., 1995; Zhue *et al*., 2005; Hartl *et al*., 2006). As a consequence of SV40 T/t expression in the ME-A cells it could be expected that OPN would be overexpressed in this cell line. However, OPN overexpression is observed in the ME-C cells in which SV40 T/t transgene expression is inactivated as was previously demonstrated (Tzeng *et al*., 1998).

In murine fibroblast p53 is involved in the regulation of OPN expression. The OPN gene promoter region has a p53-responsive element which mediates OPN gene activation (Morimoto *et al*., 2002). In ME-C cells which carry a missense mutation in the p53 gene, which exerts a dominant negative effect on p53 functions (Brachmann *et al*., 1996), it would be expected that OPN expression would be inhibited. However, in ME-C cells OPN gene expression is activated, which indicates that, depending on the cellular context, p53 either activates or suppresses OPN gene expression. Inactivation of p53 by SV40 T/t antigen expression and p53 mutation appear not to be equivalent in respect to OPN expression as observed in the different abilities of ME-A and ME-C to express OPN (as shown in Figure 4). Additional experiments are needed to clarify wether the OPN overexpression observed in ME-C cells is solely dependent on the p53 mutation. p53 wild type expression in ME-C cells may answer this question.

The OPN overexpression in ME-C cells compared to ME-A cells could explain the accumulation of OPN in the cell themselves and in the culture medium from ME-C cells. However, the different OPN modifications observed at the extracellular level cannot be explained solely by the OPN protein overexpression in ME-C cells. Noteworthy is the accumulation of OPN bands in the 20- 40 kDa range in the ME-C cell MC which are not detected in the ME-A cells MC (see Figure 4A). Thus, different proteins other than OPN may have an aberrant expression in the context of a dominant negative p53 mutation. The faster migrating bands in ME-C cells may be a result of the activity of extracellular matrix (ECM) proteins which are able to modify OPN, whose levels of expression are under p53 control and would be affected by the p53 mutation, as is the case for ECM modifying proteins such as heparanase (Baraz *et al*., 2006) and MMP-9 (Liu *et al*., 2006), and which might be still under some p53-dependent regulatory control in ME-A cells.

5.2. Effects of ME-C cell medium concentrate in ME-A cell apoptosis

As discussed above, ME-A and ME-C cells differ in their sensitivity to apoptotic stimuli. Co-cultivation experiments with ME-A and ME-C cells demonstrated that the ME-C cells protect the ME-A cells from stress induced apoptosis. The search for an anti-apoptotic paracrine factor revealed that the ME-C cells in comparison to the ME-A cells overexpress OPN and that ME-C in comparison to ME-A cells accumulate OPN in higher amounts in the culture medium. The expression differences in OPN between the two cell lines went beyond mRNA and protein expression levels. As shown in Figure 4A, OPN immunoblot analysis of medium concentrates (MCs) for both cell lines demonstrated that ME-C cells produced various OPN modifications that were not present in the culture medium from the ME-A cells.

To test wether the ME-C cell secretions could prevent the apoptosis of the ME-A cells, ME-C cell MC was applied to ME-A cells cultivated under stress conditions. Fluorescence microscopy of propidium iodide-stained cells demonstrated that ME-C cell medium concentrates prevented ME-A cell apoptosis under serum starvation conditions (Figures 1D, 1F and Graessmann *et al*., 2006) and 1 µM doxorubicin treatment (Figures 1H, 1J and Graessmann *et al*., 2006) after 22 h incubation, while ME-A cell MC does not prevent apoptosis in ME-A cells cultivated under the same conditions (see Figure 21 for serum starvation conditions). ME-C cell MC prevented ME-A cell apoptosis in a dose dependent manner under serum starvation conditions (Figure 5 and Graessmann *et al*., 2006) and 1 µM doxorubicin treatment (Figure 6 and Graessmann *et al*., 2006). Cultivation of ME-A cells with 20 µg protein/ml of ME-C cell MC was able to reduce the ME-A cell apoptotic rates to control levels after 22 h incubation under stress conditions.

As descrived in the Introduction 1.2.4 (Osteopontin mediated cell survival), OPN overexpression has been implicated in survival mechanisms of normal and tumor cells. Osteopontin is an ECM protein with cell-cell and cell-ECM adhesion and has signalling functions. Aberrant OPN expression has been involved in protection of breast tumor cells from apoptosis (Lopez *et al*., 1996). Thus, the overexpression of OPN by the ME-C cells may act as a protection against cell death in an autocrine manner. Furthermore, in the tumor context, cells overexpressing and secreting OPN may be able to protect neighbouring cells from apoptotic stimuli in a paracrine manner similar to the results obtained by our experiments *in vitro*, where the ME-C cell medium concentrate was able to protect the ME-A cells from stress induced apoptosis (see Figure 1, 5 and 6). These, *in vitro* results are

important because they demonstrated that overexpression of secreted proteins by chemotherapeutic resistant tumor cells might prevent cell death of chemotherapy sensitive cells. Thus, the characterization of secreted protein overexpression in tumors in comparison to normal tissue should be considered in the development of therapies against some cancer types that originate in tissues where extracellular signaling is important for tissue homeostasis, for example in the mammary gland. While chemotherapy is important to target tumor cells for apoptosis, the inactivation of anti-apoptotic proteins using specific antibodies or blocking peptides might be important in the reduction of a pool of chemotherapeutic resistant tumor cells and its paracrine anti-apoptotic signaling.

5.3. ME-C cells and MC anti-apoptotic activity concentrate in proteins between 20-40 kDa molecular weight range

ME-A and ME-C cells MCs differed in their anti-apoptotic activity. The ME-C cell MC had an anti-apoptotic activity which was shown to work in a paracrine manner in ME-A cells and was able to rescue ME-A cells from stress induced apoptosis (see Figure 1). In contrast, ME-A cells MC did not have anti-apoptotic activity. The ME-C cell MC accumulated different OPN immunoreactive bands in the 20-40 kDa molecular weight range that represent different isoforms of this protein, which were not present in ME-A cell MC. However, it is not know whether the accumulation of these OPN modifications was responsible for the anti-apoptotic activity of the ME-C cell MC.

In previous experiments, where the ME-C cell MC was subjected to 2D gel electrophoresis, OPN immunodetection and silver staining of proteins, it was demonstrated that OPN is the most abundant protein in the ME-C cells MC. The ME-A and the ME-C cells MCs were compared using 2D gel electrophoresis and silver staining of proteins. The analyses revealed that there were differences in other less abundant proteins apart from OPN (Graesmann *et al*., 2006). To narrow down the size of the anti-apoptotic inhibitor(s) ME-C cell MC was subjected to size-exclusion gel chromatography using a Superdex 200 column, and protein fractions were tested for their anti-apoptotic activity in ME-A cells cultivated under stress conditions and for the detection of OPN immunoreactive bands. As shown in Figure 7, the anti-apoptotic activity accumulated in fractions where 20-40 kDa proteins were concentrated. Fractions 18 and 19 presented the highest activity. 2 µg protein/ml from fractions 18 to 19 could reduce the stress-induced ME-A cell apoptosis to control levels. This is similar to the effect observed when 20 µg/ml of the unfractionated ME-C cell MC was added to the culture medium. OPN immunoblots demonstrated that in the fractions 18 and 19, the small OPN

isoforms of 20-32 kDa were enriched (Figure 8). However, the anti-apoptotic activity started in fractions where OPN isoforms are detected in the 20-40 kDa range.

When 2D gel electrophoresis of ME-A and ME-C cell MCs was compared in the 20-40 kDa range by silver staining and OPN immunoblots, it was possible to observe that in this region OPN was still the most abundant protein but that less abundant proteins are also present (Graessmann *et al*., 2006). The accumulation of the small OPN immunoreactive bands in the fractions 18 – 19 could be responsible for the functional differences observed between the ME-A and the ME-C cell MCs. Silver staining of proteins separated by 1-dimensional SDS-PAGE electrophoresis demonstrated that there is a strong major band in the 20-32 kDa range (Figure 9). However, it was not determined wether this band corresponds to OPN. Further experiments are necessary to clarify this question.

Protein fractionation by size-exclusion gel chromatography using a Superdex 75 column (Amersham Pharmacia), which allows for a better separation of proteins in the range of 20-30 kDA than the Superdex 200 column, could reduce the number of candidate fractions where proteins responsible for the anti-apoptotic activity accumulate. Peptide-mapping using MALDI-TOF (matrix-assited laser desorption ionisation time-of-flight) could be used in addition to gel chromatography in order to identify the proteins present in the fractions of interest. Identification of proteins would permit further experiments to determine whether OPN modifications and/or other protein(s) are responsible for the anti-apoptotic activity in ME-C cell MC observed in fractionation experiments.

5.4. Osteopontin overexpression does not confer anti-apoptotic properties to ME-A5 cells

One question that arises from previous results, where OPN overexpression was demonstrated to occur in the ME-C cells compared to the ME-A cells is whether OPN overexpression in ME-A cells would be sufficient to make them more resistant to stress conditions.

To answer this question the OPN cDNA sequence was cloned into the mammalian overexpression vector pcDNA3.0. Total mRNAs was extracted from ME-C cells, the mRNAs were reverse transcribed to cDNAs and OPN complete coding region was amplified using OPN specific primers containing in their 5´- and 3´-ends sequences for recognition by specific restriction sites necessary for cloning into the pcDNA3.0 vector. The pcDNA 3.0 vector allowed the cytomegalovirus (CMV) promoter-regulated overexpression of target genes in

mammalian cells and the selection of positive transfected cells through the gain of antibiotic resistance.

ME-A cells were transfected with pcDNA3.0-OPN plasmid (see Figure 15) and several clones were isolated by antibiotic selection. The pcDNA-3.0 contains the SV40 ori which allows episomal DNA replication in SV40 T-antigen-expressing cells. Maintenance of the pcDNA3.0-OPN DNA in ME-A cells after 3 weeks antibiotic selection was verified by PCR for the pCMV-OPN DNA construct (as shown Figure 16) and OPN mRNA expression origining from pcDNA3.0 OPN maintenance in these cells was also verified by RT-PCR (Figure 17). The mRNA analysis demonstrated that after 20 cycles of PCR, ME-A cells and the ME-A cell clones expressed OPN mRNA in similar levels when primers designed to amplify part of the endogenous OPN sequence were employed in the reaction (Figure 17, OPN product). However, when primers designed to amplify sequences originated from the pcDNA3.0-OPN plasmid were employed for PCR (20 cycles), it was possible to demonstrate that ME-A cell clones expressed extra mRNA copies that must have originated from the pcDNA3.0-OPN construct and that the ME-A cell clone 5 (ME-A5) had the most efficient mRNA expression among the clones analysed (see Figure 17, T7-OPN product).

The clones selected where further analysed for OPN protein overexpression by SDS-PAGE electrophoresis and Western blots. In agreement to the RT-PCR experiments, the ME-A5 cell line showed an increased OPN protein expression. ME-A5 cell MC collected after incubation under serum starvation conditions for 24 h showed increased amounts of OPN in comparison with the parental ME-A cell line and the other ME-A cell clones selected (Figure 18). Quantification of OPN overexpression in ME-A5 cells demonstrated that these cells overexpressed around 3 times more OPN than ME-A cells, when immunoblot images were analysed with Image J, an image processing and analysis computer software. However, OPN overexpression levels in ME-A5 cell line remained below the levels of OPN overexpression observed in ME-C cells. 500 ng of ME-C cell MC gave an OPN immunoblot signal stronger than 2 μg (2000 ng) for ME-A5 MCs. Thus, ME-C cell expression is still around 4 times stronger than that of ME-A5 cells, as could be measured with the Image J software (Figure 18). In addition, ME-C cells accumulate fast migrating bands in the range below 32 kDa that were not observed in ME-A or ME-A5 cells. And, as was already discussed for the gel chromatography experiments, this is where the highest anti-apoptotic activity was detected.

Interestingly, after 48 h incubation under serum starvation conditions the ME-A5 cell line showed a different band pattern than the parental ME-A cell line. As shown in Figure 19, ME-A5 cell line produced a 37 kDa OPN band that also was produced by ME-C cells. The presence of this 37 kDa OPN immunoactive band in ME-A5 cell MC could have had an effect of increasing the anti-apoptotic activity of the MC5, as chromatography experiments showed that the anti-apoptotic activity started at fractions around 40 kDa. In addition, ME-A5 accumulated most of the OPN bands in the 40-47 kDa range, and the OPN bands from 56 – 58 kDa range were almost absent in contrast to MC collected from ME-A cells.

These observations prompted us to investigate the biological activity of the ME-A5 cell medium concentrate (MC5). 20 μg/ml of ME-C, ME-A and ME-A5 cell MCs were applied to ME-A cells cultivated under serum starvation conditions. As shown in Figure 20, ME-A5 cell MC was not able to protect ME-A cells from apoptosis under serum starvation conditions. Only the ME-C cell MC was able to rescue cells from apoptosis to control levels again. Thus, even when the ME-A5 cells expressed higher amounts and produced different isoforms of OPN than the ME-A cells, their MC was still not able to rescue ME-A cells from apoptosis induced by serum starvation.

These findings were in agreement with the Superdex 200 column chromatography fractionation experiments and allowed us to speculate that the lower molecular weight OPN isoforms of 20-32 kDa could be responsible for the antiapoptotic activity of the ME-C cell MC. Neither ME-A not ME-A5 cells MC contained any OPN immunoreactive bands of low molecular weight observed in ME-C cells MC. The 37 kDa band that was produced by ME-A5 cells MC and was also present in ME-C cells MC did not contribute to the anti-apoptotic effect as ME-A5 cell MC had no anti-apoptotic properties in ME-A cells grown under serum starvation conditions.

An interesting question that remains open for further investigation is what causes the different OPN patterns observed between ME-A and ME-C cell lines. As discussed above, both cells were able to express and secrete OPN, but only ME-C cells were able to enrich the lower molecular weight OPN modifications, apart from the fact that ME-C cells secrete OPN in higher amounts than ME-A cells. OPN from various cellular sources might have different structural characteristics that allows it to play its various physiological roles (Wai *et al*., 2004; Crawford *et al*., 1998). Small isoforms of OPN might have a different physiological function

than the full length molecule. Recently, it was demonstrated that breast cancer cells express multiple OPN splice variants. A small OPN splice form was defined as a highly specific marker for transformed cells (He *et al*., 2005). The full length OPN aggregates in the presence of calcium at physiological levels, while the small splice variant remained soluble and supported anchorage independent growth thus, promoting metastasis. Crawford and colleagues (1998) found differences in the functional activity of tumor-derived and host-derived OPN. While host-derived OPN functions as a macrophage chemoattractant, OPN produced by tumor cells works to inhibit macrophage function, thus giving to tumor cells an advantage that enables them to escape host immuneresponse mechanisms. The results obtained in this study did not probe that the differences in OPN modifications observed between ME-A and ME-C cell lines were responsible for the anti-apoptotic signal. Nevertheless, it demonstrated that if OPN is involved, the 20-32 kDa OPN modifications present only in the ME-C cells might be responsible for the survival signal.

Apart from gene splicing mechanisms, there are other possible explanations for the appearance of the low molecular weight OPN isoforms observed in the ME-C cell MC as proteinase activity e.g. thrombin cleavage. Thrombin cleaves OPN and renders an amino-terminal osteopontin fragment involved in adhesion and motility processes in different cell types (Senger *et al*., 1996; Yokosaki *et al*., 1999; Helluin *et al*., 2000). The ME-C cell MC was subjected to thrombin cleavage and produced low molecular isoforms from OPN but did not modified the anti-apoptotic properties of ME-C cell MC (M. Graessmann personal observation). One interesting possibility is that thrombin cleavage of ME-A cell MC might promote the production of the low molecular weight OPN isoforms and confer the ME-A cell MC with anti-apoptotic activity. However, thrombin was not expressed in ME-A and ME-C cells as was demonstrated in the microarray data analysis of gene expression in both cell lines. Thus, a mechanism other than thrombin cleavage may be involved in the production of low molecular OPN in ME-C cell lines.

However, thrombin is not the only ECM enzyme acting upon OPN, as functional OPN fragments can be a product of the activity of metalloproteinases MMP-3 and MMP-7 (Agnihotri *et al*., 2001). Cleavage of OPN by MMP-3 and MMP-7 potentiated OPN functions as an adhesive and migratory stimulus associated with cell surface integrins (Agnihotri *et al*., 2001), a biological mechanism used in the wound healing processes and believed to be used aberrantly by tumor cells in metastasis. Thus, differences between ME-A and ME-C cells in

the production and secretion of metalloproteinases could be the reason for the observed differences in secreted OPN isoforms between the two cell lines. However, microarray data analysis does not show gene expression of MMP-7 and MMP-3 in either. OPN is able to influence the activation of MMP-2 (Philip *et al*., 2001) and of CD44, an OPN receptor, activated latent TGF-β2 through a MMP-9 activation mechanism (Yu *et al*., 2004) which influenced tumor growth, metastasis and survival of breast cancer cells. Although these MMPs have not been described as OPN modifying enzymes, these proteins could be involved in the anti-apoptotic effects observed in ME-C cell MC by producing OPN modifications or activating factors in the ME-C cells that remain latent in the ME-A cells and are activated by the 20-32 kDa OPN modifications. More experiments are needed to characterize the differences in the expression of metalloproteinases between the ME-A and the ME-C cells. More knowledge about MMPs composition and its relation to the production of OPN fragments in the normal mammary gland may be important for the development of therapies for breast tumors in which OPN overexpression and eventually production of OPN fragments take place.

5.5. Signaling pathways involved in ME-A cell apoptosis

As previous studies demonstrated, the ME-C cell MC activated an MAPK/MEK 1/2-dependent anti-apoptotic pathway and the MC prevented apoptosis by inhibition of caspase-3 dependent and independent mechanisms. The ME-C cell MC prevented activation of caspase-3 in the ME-A cells cultivated under serum starvation conditions and prevented apoptosis. The MEK 1/2 inhibitor (PD98059) activated caspase activity in the presence of the ME-C cell MC and induced apoptosis of ME-A cells. Doxorubicin increased caspase-3 activity in ME-A cells and promoted apoptosis, however the ME-C cell MC prevented apoptosis even when caspase-3 was active and the MEK 1/2 inhibitor restored apoptosis in ME-A cells. Thus, depending on the apoptotic stimuli, MEK 1/2 activation by the ME-C cell MC prevented apoptosis of ME-A cells by inhibiting caspase-3 or the activity of uncharacterized factor(s) operating downstream of caspase-3 (Graessmann *et al*., 2006). Activation of the MAPK-pathway occurs downstream of various signaling pathways. Activation of non-receptor tyrosine kinase Src family, phospholipase C and PI3-kinase activates the MAPK-pathway after transmembrane receptor activation in different cell systems (reviewed in Katz *et al*., 2007). Specific inhibitors for these molecules were employed to test wether these proteins were involved in the transmission of the signal from the ME-C cell MC to MEK 1/2.

As shown in Figure 12, when the ME-A cells were cultivated under serum starvation conditions in the presence of 10 μM PLC family inhibitor (U73122), the rate of apoptosis increased to more than 60% after 22h incubation. While the addition of 20 μg/ml ME-C cell MC rescued ME-A cells from stress induced apoptosis as demonstrated in Figure 12, the presence of 10 μM PLC inhibitor eliminated the anti-apoptotic activity of the MC. Various phospholipase C isoforms transduce survival signals in different cell types. For example integrin binding promoted PLC-γ activity, regulated by Src activation (reviewed in Vossmeyer *et al*., 2002; Tvogorov *et al*., 2004; Harden *et al*., 2006) and, upon ligation of growth factor receptors, PLC-ε has been involved in Ras/Raf/MAPK/ERK pathway activation, (Reviewed in Wing *et al*., 2003; Harden *et al*., 2006). Thus, PLC plays a role in the anti-apoptotic signal transduction originated by the OPN-containing ME-C cell MC in ME-A cells.

Doxorubicin increases the ME-A cell apoptosis rate to 20% after 22 h incubation. As shown in Figure 13, application of 10μM PLC inhibitor combined with 1 μM doxorubicin increased ME-A cell apoptosis rate to almost 40%. The presence of 10 μM PLC inhibitor prevented the anti-apoptotic effect of the ME-C cell MC in ME-A cells.

Activation of Src is implicated in the protection of cells from apoptosis under growth factor deprivation and anoikis (Frisch *et al*., 1994). When the ME-A cells were cultivated in the presence of 2.5 μM Src family inhibitor (PP2) and incubated under serum starvation conditions for 22h, the ME-A cells apoptosis rate increased to around 40% as shown in figure 10. Thus, inactivation of Src in the ME-A cells had the expected apoptosis rate increment expected from the disruption of Src biological function. However, in contrast to the results obtained with the PLC inhibitor, when 20 μg/ml of the ME-C cell MC was applied to the ME-A cells growing under serum starvation conditions in the presence of Src inhibitor, the ME-C cell MC was still able to reduce apoptosis to control levels. Similar results were obtained in the ME-A cells cultivated under doxorubicin and Src inhibitor treatment. Src activation occurs when growth factor receptors (Frame *et al.* 2004), integrins (Wei *et al*., 2007) and CD44 (Marhabe *et al*., 2004, Ponta *et al*., 2003; Bourguignon *et al.*, 2001) are activated. OPN activates growth factor receptors, CD44 and integrins (reviewed in Weber *et al*., 2001). During development of integrin-dependent adhesion, Src is recruited to cell membranes and activate focal adhesion complexes in which MAPK/MEK molecules are present (Fincham *et al*., 2000; reviewed in Frame *et al*., 2002). From the experiments under Src inhibition (Figure

11) we could conclude that the Src non-recepetor tyrosine kinase family appear not to play a decisive role in the ME-C cell MC anti-apoptotic signal transduction that activates MAPK/MEK 1/2 in the ME-A cells.

ME-A cells incubated under serum starvation conditions in the presence of 2.5 μM PI3-k inhibitor (LY294002) increased the apoptosis rate to 60%, as shown in Figure 12. However, in similar results as those obtained with the Src inhibitor, the ME-C cell MC (20 μg/ml) was able to rescue cells from apoptosis to control levels under PI3-k inhibition. A similar result was observed under treatment with doxorubicin and PI3-k inhibitor. The outcome of these experiments was in agreement with previous results in our group which demonstrated that the anti-apoptotic activity of the ME-C cell MC was independent of Akt activation (Graessmann *et al*., 2006). The PI3-k has been implicated in the activation of a survival signal in different cell types by phosphorylation of its downstream target Akt/PKB (Kulik *et al*., 1997; Johnson *et al*., 2000; Squires *et al.*, 2003; reviewed in Cully *et al.*, 2006), however the ME-C cell MC is able to prevent apoptosis of the ME-A cells under PI3-k inhibition.

The differences observed between the PLC, Src and PI3-k inhibitors were not dependent on the concentrations of inhibitor applied in the assays, as equal concentrations (10 μM) of either Src or PI3-kinase inhibitors did not prevented the anti-apoptotic activity of the ME-C cell MC in ME-A cells growing under serum starvation conditions as is shown in Figure 11. Cells were incubated for 22 h under serum starvation conditions in the presence of 10 μM Src inhibitor (Figure 14 lane B) or 10 μM PI3-k inhibitor (Figure 14 lane C) or 10 μM PLC inhibitor (Figure 14 lane D). Upon addition of 20 μg/ml from ME-C cell MC the apoptosis rate in ME-A cells remained at control levels in the presence of all inhibitors excluding the PLC inhibitor (Figure 14. columns 5 & 6) as observed by fluorescence microscopy of propidium iodide stained cells.

The conclusion from the different inhibitor experiments is that the PLC signal transduction pathway seems to be involved in the activation ME-C cell MC-mediated survival pathway of ME-A cells growing under serum starvation and doxorubicin treatment conditions. However, activation of PLC by the ME-C cell MC in ME-A cell is able to activate a survival signal independent of Src activation and Akt. Further experiments are necessary to discover whether the activation of PLC is involved in the activation of MEK 1/2 in ME-A cells implicated in the anti-apoptotic signalling in previous studies (Graesmann *et al.*, 2006). As PLC activates

the Ras/Raf/MAPK/MEK pathway (Reviewed in Wing *et al.*, 2003; Harden *et al.*, 2006) in some types of cells, inhibition of Ras in ME-A cells should be tested, as Ras might be a good candidate effector molecule in the ME-C cell MC anti-apoptotic signaling pathway.

5.6. ME-A and ME-C cells expressed known osteopontin receptors on their surfaces

OPN can activate various cell membrane receptors and transmembrane adhesion proteins. OPN has been implicated in the activation of integrins and CD44 receptors (see introduction). To investigate which integrin subunits and CD44 receptors are present in the ME-A and on the ME-C cell surfaces, FACS analysis and fluorescence microscopy of immunostained cells and, Western blot and protein immunodetection analysis of cell lysates were performed.

These experiments demonstrated that the ME-A and ME-C cells share some similarities in the presence of OPN receptors on the cell surface. FACS analysis and fluorescence microscopy of immunostained cells demonstrated a strong presence of β1 integrin subunit (100% of the cells) and of CD44. CD44 expression at the cell surface varied among the ME-A and the ME-C cells (65% and 41%, respectively) as demonstrated by FACS analysis where not all cells had the same fluorescence intensity and some cells in the population did not stain positive for the receptor (see Figure 21). CD44 presence at the cell surface has been described as an active process as CD44 extracellular domain can be cleaved during cellular processes rendering a soluble form of CD44 with remodeling activity at the ECM which is able to influence migration and attachment of cancer cells (Cichy *et al*., 2003). In Figure 22, it is possible to observe fluorescence microscopy images of immunostained receptors at the ME-A and ME-C cell surfaces. In agreement with FACS analysis results, β1 integrin subunit showed a strong staining localized at cell surfaces, while the signal of for CD44 seems more diffuse at ME-C cells than for ME-A cell surface. SDS-PAGE electrophoresis and Western blot analysis confirmed the strong presence of integrin β1 subunit in both the ME-A and the ME-C cell lines. Integrin β1 has a calculated molecular weight of 115 kDa. A strong immunoreactive band running at around 110 kDa was present in immunoblots (Figure 23).

OPN activated CD44 and integrin β1-containing receptors. Osteopontin binds to diverse integrin heterodimers containing the β1 subunit (αvβ1, α4β1 , α5β1 , α8β1 , α9β1) and to diverse heterodimers containing αv (αvβ3, αvβ5, αvβ6) (reviewed in Wai *et al*., 2004; Yokosaki *et al*., 2005). Activation by OPN of αvβ3 integrin has been associated with malignancy in breast cancer (Tuck *et al*., 2000; Wong *et al*., 1998), survival of osteoclasts

(Zhao *et al*., 2005) and endothelial cells (Weber *et al*., 2001). Less evidence for integrin β1-containing heterodimers in the OPN-mediated survival of mammary gland epithelial cells is found in the literature. Integrin α9β1 has been linked to lymphatic angiogenesis and lymphatic metastasis. In MCF-7 breast cancer cells overexpressing protein kinase C (PKC) α, ligation of fibronectin to integrin α5β1 rescued cells from apoptosis and allowed anchorage-independent growth under serum starvation conditions (Noti *et al*., 2001). The ME-C cell MC containing OPN may function through a similar mechanism, and activate integrin heterodimer receptors containing the integrin β1 subunit for the activation of the PLC-mediated anti-apoptotic signal cascade in ME-A cells. (PKC) is a known downstream target of PLC (reviewed in Tvogorov *et al.*, 2004; Harden *et al.*, 2006), and we found in this study that PLC is involved in the MC anti-apoptotic signal.

The involvement of PLC in cell survival has been demonstrated in various studies and cell types (reviewed in Wing *et al.*, 2003; Harden *et al*., 2006). Hyaluronan (HA) binding to CD44 activates Ras and PKCζ (Fitzgerald *et al*., 2000). In accordance with Fitzgerald and colleagues (2000) CD44 activation by HA induces association with the leukaemia associated RhoGEF (LARG), Rho/Ras co-activation and activation of PLC-ε-Ca^{+} signaling in head and neck squamous carcinoma cells (Bourguignon et al., 2005). OPN activated CD44 and influenced HA synthesis, which is the most abundant ligand of CD44 and is involved in cancer cell survival (Kosaki *et al.*, 1999; Li *et al.*, 2001; Cook *et al*., 2006) and HA has been found accumulated in human tumors (Horai *et al*., 1981; Yu *et al*., 1985; Bertrand *et al*.,. 1992; Delpech *et al*., 1993; Roponnen *et al*., 1998). OPN was able to influence the expression of CD44 and its splicing isoforms, some of which have been demostrated to be involved in cancer progression and associated with mechanisms of cancer cells survival (Chellaiah *et al*.; 2003a; Chellaiah *et al*., 2003b; Zhu *et al*., 2004; Khan *et al*., 2006; Lee *et al*., 2007). Activation of the Ras pathway induced OPN expression (see introduction 1.2.2). Thus by controlling ligand synthesis and receptor isoform expression OPN might be involved in the activation of a survival positive feedback loop mediated by a PLC/Ras/Raf/MEK pathway in the ME-C cells in an autocrine manner. In ME-A cells the addition of ME-C cell MC might activate the survival signal in paracrine manner.

Inhibition of β1 integrin and OPN might be a good target for breast cancer therapy and in cancers where both proteins are aberrantly upregulated. CD44 antibodies against specific isoforms may also contribute to design therapies that target cancer cells where OPN

overexpression has been detected. Additional experiments are necessary to determine if some of these survival pathways are activated in ME-A cells upon application of the OPN-containing ME-C cell MC. This work gives a new base for the design of experimental settings targeting possible receptors and upstream molecules activated by ME-C cell MC anti-apoptotic signal. Recently, Lee and colleagues (2007) demonstrated in gastrointestinal cancer cells that OPN activation of CD44 functions as an ECM-derived survival signal which is mediated by activation of integrin β1. In breast cancers, integrin heterodimers containing the β1 subunit were involved in mediating resistance to cytotoxic chemotherapies by enhancing cell survival (Aoudjit *et al.*, 2001; Lewis *et al.*, 2002). Aberrant β1 integrin expression has been implicated in human breast carcinomas playing a central role in growth, apoptosis, invasion and metastasis (Zutter *et al.*, 1993; Gui *et al*., 1996; Shaw *et al*., 1999; Berry *et al*., 2004; White *et al*., 2004). β1 integrin blockage using an inhibitory antibody resulted in a significant loss of cancer cells growing in tumor-like structures in a laminin-rich ECM. Cancer cell loss was associated with decrease in proliferation and increase in apoptosis, while non-malignant cells forming tissue-like structures survived treatment with the antibody (Park *et al*., 2006).

6. CONCLUSIONS

The ME-A and the ME-C cell lines were isolated from a breast tumor induced by SV40 T/t antigen expression during pregnancy in the mammary gland of transgenic mice (WAP-SVT/t). The ME-A and ME-C cells differed in their sensitivity to apoptosis. ME-C cells, which spontaneously switched off the SV40 T/t antigen expression and contained a p53 mutation commonly found in breast cancers, were very resistant to apoptotic stimuli such as culture under stress conditions (e.g. serum starvation) or chemotherapy treatment (e.g. doxorubicin). In contrast, ME-A cells which still expressed the SV40 T/t antigen transgene maintained an elevated apoptosis rate in culture similar to that observed during pregnancy in the mammary gland epithelial cells of WAP-SVT/t transgene mice. ME-A cells were sensitive to apoptotic stimuli such as culture under serum starvation conditions and chemotherapy. However, ME-A cells became resistant to these apoptotic stimuli when co-cultivated with the ME-C cell line which suggests the existence of paracrine anti-apoptotic factor(s) secreted by ME-C cells.

Microarray data analysis of differential gene expression between ME-A and ME-C cell lines, with special attention on secreted proteins, demostrated that OPN is overexpressed by ME-C cells in comparison to ME-A cells. This was confirmed by RT-PCR and Western blot analysis of OPN mRNA and protein expression, respectively. ME-C cells secreted OPN in high amounts into the culture medium and the OPN secreted by these cells presented various isoforms with different molecular weights in the 20-115 kDa range. In contrast, ME-A cells are less efficient in OPN expression and secretion. OPN immunoreactive bands were detected in the 40- 42 kDa range in the ME-A cell culture medium. Most of the OPN modifications secreted by ME-C cells were absent in the ME-A cell secretions. Thus, the ME-A and the ME-C cells differed in their ability to express, modify and secret OPN.

The ME-C cell medium concentrates (MC) at a total protein concentration of 20μg/ml were able to prevent apoptosis of ME-A cells cultured under stress and chemotherapy, as measured by flow cytometry (FACS) and immunofluorescence analysis of dying cells after propidium iodide and annexin-V FITC-conjugated staining. The first question that we tried to answer in this study was whether ME-C cell OPN overexpression and secretion protected cells against apoptosis under serum starvation and chemoterapeutical treatment.

To test wether OPN overexpression protect cells against stress induced apoptosis, ME-A cells were transfected with a mammalian expression plasmid containing the complete OPN coding region under the transcriptional control of the CMV promoter (pcDNA3-OPN). This plasmid allowed for episomal replication in SV40 T antigen-expressing cells (such as ME-A cells) as it contains the SV40 origin of replication. Transfected cells were selected for stable episomal maintenance of the plasmid by antibiotic treatment. The ME-A cell clone 5 (ME-A5 cells) obtained from the antibiotic selection (4 weeks) showed OPN overexpression in culture media after 24 h cultivation. ME-A5 cells overexpressed and secreted around 3 times more OPN into culture media than the parental ME-A cell line. Furthermore, an OPN immunoreactive band of 37.5 kDa was produced by the ME-A5 cells that was not produced by the ME-A cells. However, this OPN modification was also produced by the ME-C cells and its production by ME-A5 cells could confer anti-apoptotic properties to their MC. However, when 20 µg/ml ME-A5 cell MC was tested for its antiapoptotic activity in ME-A cells grown under serum starvation conditions, the ME-A5 cell MC was not able to rescue ME-A cell from stress induced apoptosis. This result indicates that the smaller OPN isoforms observed in ME-C cell secretions might be responsible for the anti-apoptotic activity of the ME-C cell MC as neither the ME-A nor the ME-A5 cells produced these isoforms.

ME-C cell MC subjected to size-exclusion gel chromatography experiments using a Superdex 200 column also brought support for the involvement of the small OPN isoforms in the anti-apoptotic activity. Fractions that accumulated the smaller OPN isoforms in the 20-32 kDa range presented the highest anti-apoptotic activity in ME-A cells grown under serum starvation conditions. However, these experiments as well as previous experiments conducted in our group (Graessmann *et al*., 2006) demonstrated that in ME-C cell MC other less abundant proteins, as compared to OPN, are also present in the 20-40 kDa range and might have a role in the anti-apoptotic activity observed.

With regard to the question which signalling molecules are activated upstream of MEK 1/2 by the ME-C cell MC it was demonstrated that the ME-C cell MC activated the phospholipase C (PLC) pathway and that this activation is necessary for the signal transduction of the anti-apoptotic signal in ME-A cells. Previous studies have demonstrated that mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)1/2 is involved in the antiapoptotic signal from ME-C cell MC in ME-A cells (Graessmann *et al*., 2006). Upon OPN ligation to its cell membrane receptors, integrins and CD44, or indirect

activation of growth factor receptors, the MAPK pathway can be activated by upstream signal transduction molecules such as Phosphoinositide-3 kinase (PI3-k), Phospholipase C (PLC) family and Src family kinases. To answer the question wether some of these signaling molecules were involved in the MEK1/2-mediated anti-apoptotic signal from ME-C cell MC, chemical inhibitors were employed to disrupt the activity of PI3-k, PLC family and Src family. While the ME-C cell MC was still able to transmit its anti-apoptotic signal under PI3-k and Src family inhibition to the ME-A cells growing under serum starvation and doxorubicin treatment, PLC family inhibition disrupted this signal promoting ME-A cell death even in the presence of ME-C cell MC. Cell death was measured by FACS and immunofluorescence microscopy of propidium iodide stained cells.

OPN is an extracellular matrix protein involved in several functions including the transmission of survival signals dependent on cell type. These diverse functions depend on receptors present at cell membranes. OPN binds to heterodimers (in general, containing one of the β1, β3, β5 or αv subunits) of the integrin family of receptors involved in cell-ECM adhesion. OPN binds also to the CD44 receptor involved in both cell-cell and cell-ECM adhesion. To characterize the cell membrane receptors that might mediate the anti-apoptotic paracrine signal from the ME-C cell MC in the ME-A cells (and that may mediate the autocrine signal in ME-C cells), ME-A and ME-C cell membranes were subjected to immunofluorescence staining with integrin β1, β3, β5 or αv subunits and CD44 specific antibodies and analysed by FACS and immunofluorescence microscopy. ME-A and ME-C cell express strongly the integrin β1 subunit and the CD44 receptor at their cell surfaces. Western blot analysis using a specific antibody against integrin β1 showed a strong immunoreactive band in both cell lines. Integrin β3, β5 and αv were absent in both cell lines. Thus, integrin heterodimers containing the β1 subunit and CD44 were selected as candidates for the transmission of MC containing OPN-mediated anti-apoptosis signal in both the ME-A and the ME-C cell lines.

7. REFERENCES

Abe, M.; Hiura, K.; Wilde, J.; Shioyasono, A.; Moriyama, K.; Hashimoto, T.; Kido, S.; Oshima, T.; Shibata, H.; Ozaki, S.; Inoue, D.; Matsumoto, T. (2004). Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. Blood. Vol 104. No 8: 2484-2490.

Abramoff, M.D.; Magelhaes, P.J.; Ram, S.J. (2004) Image Processing with ImageJ. Biophotonics International. Volume 11. No. 7:. 36-42.

Agnihotri, R.; Crawford, H. C.; Haro, H.; Matrisian, L. M.; Havrda, M. C.; Liaw, L. (2001). Osteopontin, a novel substrate for matrixmetalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). Journal of Biochemical Chemistry. Vol. 276: 28261-28267.

Agrawal, D.; Chen, T.; Irby, R.; Quackenbush, J.; Chambers, A. F.; Szabo, M.; Cantor, A; Coppola, D.; Yeatman, T. J. (2002). Osteopontin identified as lead marker of colon progression, using pooled sample expression profiling. Journal of National Cancer Institute. Vol 94. No. 7: 513-521.

Allan, A. L.; Georg, R.; Vantyghem, S. A.; Lee, M. W.; Hodgson, N. C.; Engel. C. J.; Holliday, R. L.; Girvan, D. P.; Scott, L. A.; Postenka, C. O.; Al-Katib, W.; Stitt, L. W.; Uede, T.; Chambers, A. F.; Tuck, A. B. (2006). Role of the integrin-binding protein osteopontin in lymphatic metastasis of breast cancer. Tumorigenic and neoplastic progression. Vol. 169. No. 1: 233-246.

Aoudjit, F.; Vouri, K. (2001). Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. Oncogene. 2001. Vol. 20: 4995-5004.

Baraz; L.; Haupt, Y.; Elkin, M.; Peretz, T.; Vlodavsky, I. (2006) Tumor suppressor p53 regulates heparanase gene expression. Oncogene. Vol. 25: 3939-3947.

Berry, M. G.; Gui, G. P.; Wells, C. A.; Carpenter, R. (2004). Integrin expression and survival in human breast cancer. Eur. J. Surg. Oncol. Vol. 30: 484-489.

Bertrand, O.; Girard, N.; Delpech, B.; Duval, C., d´Anjou, J.; Dauce, J.P. (1992). Hyaluronana (hyaluronic acid) and hyaluronectin in the extracellular matrix of human breast carcinomas: comparison between invasive and non-invasive areas. Int. J. Cancer. Vol. 52: 1-6.

Bourguignon, L. Y. (2001). CD44-mediated oncogenic signalling and cytoskeleton activation during mammary tumor progression. Journal of Mammary Gland Biological Neoplasia. Vol. 6: 287-297.

Bourguignon, L. Y. (2002). CD44 mediated oncogenic signaling and cytoskeleton activation during mammary tumor progression. Journal of mammary gland biology and neoplasia. Vol. 6. No. 3: 287-297.

Bourguignon, L. Y. W.; Singleton, P. A.; Zhu, H.; Diedrich, F. (2003). Hyaluronan-mediated CD44 interaction with RhoGEF and Rho kinase promotes Grb2-associated binder-1 phosphorylation and phosphatidyl inositol 3-kinase signalling leading to cytokine (macrophage-colony stimulating factor) production and breast tumor progression. The Journal of Biological Chemistry. Vol. 278. No. 32: 29420-29434.

Bourguignon, L. Y. W.; Gilad, E.; Brightman, A.; Diedrich, F.; Singleton, P. (2005) Hyaluronan-CD44 interaction with leukaemia-associated RhoGEF and epidermal groth factor receptor promotes Rho/Ras co-activation, phospholipase Cε-Ca^{+} signaling, and cytoskeleton modification in head and neck squasmous cell carcinoma. The Journal of Biological Chemistry. Vol. 281, No. 20: 14026-14040.

Brachmann, R. K.; Vidal, M.; Boeke, J. D.(1996).Dominant-Negative p53 Mutations Selected in Yeast Hit Cancer Hot Spots. Proceedings of the National Academy of Sciences of the United States of America. Vol. 93. No.9: 4091-4095

Bramwell, V. H. C.; Doig, G. S.; Tuck, A. B.; Wilson, S. M.; Tonkin, K. S.; Tomiak, A.; Perera, F.; Vandenberg, T. A.; Chambers, A. F. (2006). Serial Plasma Osteopontin levels have prognostic value in metastatic breast cancer. Clinical Cancer Research. Vol. 12. No. 11: 3337-3342.

Chambers A. F.; Behrende, E. I.; Wilson, S. M.; Denhardt, D. T. (1992) Induction of expression of osteopontin (OPN); secreted phosphoprotein) in metastatic, *ras*-transformed NIH3T3 cells. Anticancer Research. Vol 12: 43-48.

Chellaiah, M. A.; Hruska, K. A. (2002). The integrin $\alpha_v\beta_3$ and CD44 regulate the actions of osteopontin on osteoclast motility. Calcif. Tissue Int. Vol. 72: 197-205.

Chellaiah, M. A.; Biswas, R. S.; Rittling, S.R.(2003a) Rho dependent Rho kinase activation increases CD44 surface expression and bone resorption in osteoclasts. Journal of Biological Chemistry. Vol 278: 29086-29097.

Chellaiah, M.A.; Kizer, N.; Biswas, R. (2003b). Osteopontin deficiency produces osteoclast dysfunction due to reduced CD44 surface expression. Mol Biol. Cell. Vol. 14: 173-189.

Chrenek, M. A.; Wong, P.; Weaver, V. M. (2001). Tumor-stromal interactions, integrins and cell adhesions as modulators of mammary cell survival and transformation. Breast Cancer Research. Vol. 3. No. 4: 224-229.

Cichy, J.; Pure, E. (2003). The liberation of CD44. The Journal of Cell Biology. Vol. 161. No. 5: 839-843.

Cook, A. C.; Tuck, A. B.; McCarthy, S.; Turner, J. G.; Irby, R. B.; Bloom, G. C.; Yeatman, T. J.; Chambers, A. F. (2005). Osteopontin induces multiple changes in gene expression that reflect the six “hallmarks of cancer” in a model of breast cancer progression. Molecular Carcinogenesis. Vol 43: 225-236.

Cook , A. C.; Chambers, A. F.; Turley, E. A.; Tuck, A. B. (2006). Osteopontin induction of hyaluronan synthase 2 expression promotes breast cancer malignancy. J. B. C. Vol. 281. No. 34:24381-9.

Coppola, D.; Szabo, M.; Boulware, D.; Muraca, P.; Alsarraj, P.; Chambers A. F.; Yeatman, T. E. (2004).Osteopontin protein expression and pathological stage across a wide variety of tumor histologies. Clinical Cancer Research. Vol. 10: 184-190.

Corso, S:; Comoglio, P. M.; Giordano, S. (2005) Cancer therapy: can the challenge be MET. Trends in molecular medicine. Vol. 11. No. 6: 284-292.

Craig, A. M.; Nemir, M.; Mukherjee, B. B.; Chambers, A. F.; Denhardt, D. T. (1988) Identification of the major phosphoprotein secreted by many rodent cell lines as 2ar/osteopontin: enhanced expression in H-Ras-transformed 3T3 cells. Biochemical and Biophysical Research Communications. Vol. 157. No 1: 166-173.

Craig, A. M.; Denhardt, D.T. (1991). The murine gene encoding secreted phosphoprotein 1 (osteopontin): promoter structure, activity, and induction in vivo by strogen and progesterone. Gene. Vol 100: 163-71.

Crawford, H. C.; Matrisian, L. M.; Liaw, L. (1998). Distinct roles of osteopontin in host defense activity and tumor survival during squamous cell carcinoma progression in vivo. Cancer Research. Vol 58: 5206-5215.

Christensen B.; Nielsen M. S., Haselmann K. F.; Petersen T. E.; Sorensen, E. S. (2005). Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and five O-glycosylation sites and their biological implications. Biochem J 390: 285–292.

Cully, M; You, H.; Levine, A. J.; Mak, T. W. (2006). Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nature Reviews Cancer. Vol. 6: 184-192.

Das, R.; Mahabeleshwar, G. H.; Kundu, G. C. (2004). Osteopontin induces AP-1-mediated secretion of urokinase-type plasminogen activator through c-Src-dependent epidermal growth factor receptor transactivation in breast cancer cells. The Journal of Biological Chemistry. Vol. 279. No. 12: 11051-11064.

Datta, A.; Shi, Q.; Boettinger, D. E. (2001). Transformation of Chicken Embryo Fibroblasts by v-Src Uncouples β1 Integrin-Mediated Outside-in but Not Inside-out Signaling. Molecular and Cellular Biology. Vol. 21 No. 21: 7295-7306.

Delpech, B.; Maingonnat, C.; Girard, N.; Chauzy, C.; Maunoury, R.; Olivier, A.; Tayot, J.; and Ccreissard, P. (1993). Hyaluronan and hyaluronectin in the extracellular matrix of human brain tumor stroma. Eur. Journal of Cancer. Vol. 29A: 1012-1017.

Denda, S.; Reichardt, L. F.; Müller, U. (1998). Identification of osteopontin as a novl ligand for the integrin $\alpha_8\beta_1$ and potential roles for this integrin-ligand interaction in kidney morphogenesis. Vol. 9: 1425-1435.

Denhardt DT, Guo X. (1993). Osteopontin:a protein with diverse functions. FASEB J 7:1475–1482.

El-Tanani, M.; Platt-Higgins, A.; Rundland, P. S.; Campbell, F. C. (2004). Ets gene PEA3 cooperates with β-catenin-lef-1 and c-jun in regulation of osteopontin transcription. The journal of biological chemistry. Vol. 279. No. 20: 20794-20806.

Fedarko, S. N.; Fohr, B.; Robey, P. G.; Young, M. F.; Fisher, L. W. (2000). Factor H binding to bone sialoprotein and osteopontin enables tumor cell evasion of complement-mediated attack. Journal of biological chemistry. Vol 275. No. 22: 16666-16672.

Fincham, V. J.; Brunton, V. G.; Frame, M. C. (2000). The SH3 domain directs acto-myosin-dependent targeting of v-src to focal adhesions via phosphatydilinositol 3-kinase. Mol. Cell Biol. Vol. 20: 6518-6536.

Fitzgerald, K .A.; Bowie, A.G.; Skeffington, B. S.; O´Neill, L. A. J. (1999). Ras, Protein Kinase Cζ, and IκB kinases 1 and 2 are downstream effectors of CD44 during the activation of NF-κB by hyaluronic acid fragments in T-24 carcinoma cells. J Immunol. 2000 Vol.164. No. 4: 2053-2063.

Frame, M. C.; Fincham, V. J.; Carragher, N. O.; Wyke, J. A. (2002). V-src´s hold over actin and cell adhesions. Nature Reviews Molecular Cell Biology. Vol.3: 233-245.

Frame, M. C. (2005). Newest Findings On The Oldest Oncogene; How Activated Src Does it. Journal of Cell Science. Vol 117. No. 7: 989-998.

Franzén, A.; Heinegård, D. (1985). Isolation and characterization of two sialoproteins present only in bone calcified matrix. Biochem. J. Vol. 232: 715-724.
Frey, A. B.; Wali, A.; Pass, H.; Lonardo, F. (2007) Osteopontin is linked to p65 and MMP9-Expression in pulmonary adenocarcinoma but not in malignant pleural mesothelioma. Hystopathology. Vol. 50: 720-726.

Frisch, S. M.; Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. The Journal of Cell biology. Vol. 124. No. 4: 619-626.

Furger, K. A.; Allan, A. L.; Wilson, S. M.; Hota, C.; Vantyghem, S. A.; Postenka, C. O.; Al-Katib, W.; Chambers, A. F.; Tuck, A.B. (2003). β_3 Integrin expression increases breast carcinoma cell responsiveness to the malignancy-enhancing effects of osteopontin.

Fujita, Y.; Kitagawa, M.; Nakamura, S.; Azuma, K.; Ishii, G.; Higashi, M.; Kishi, H.; Hiwasa, T.; Koda., K.; Nakajima, N.; Harigaya., K. (2002). CD44 signaling through focal adhesion kinase and its anti-apoptotic effect. FEBS letters. Vol. 528: 101-108.

Gallet, M.; Sevenet, N.; Dupont, C.; Brazier, M.; Kamel, S. (2004). Breast cancer cell line MDA-MB-231 exerts a potent and direct anti-apoptotic effect on mature osteoclasts. Biochemical and biophysical research communications. Vol 319: 690-696.

Geissinger, E.; Weisser, C.; Fischer, P.; Schartl, M.; Wellbrock, C. (2002). Autocrine stimulation by osteopontin contributes to apoptotic signalling of melanocytes in dermal collagen. Cancer Research. Vol 62: 4820-4828.

Golubovskaya, V. M.; Gross, S.; Kaur, A. S.; Wilson, R. I.; Xu, L. H.; Yang, X. H.; Cance, W. G. (2003) Simultaneous inhibition of focal adhesion kinase and SRC enhances detachment and apoptosis in colon cancer cell lines. Mol. Cancer Res. Vol. 10:755-64.

Gonzales, L.; Agulló-Ortuño, M.T.; García-Martínez, J. M.; Calcabrini, A.; Gamallo, C.; Palacios, J.; Aranda, A.; Martínez-Perez, J. (2006). Role of c-Src in human MCF7 Breast Cancer Cell Tumorigenesis. Journal of Biochemical Chemistry. Vol. 281, No. 30: 20851-20864.

Götte, M.; Yip, G. W. (2006). Heparanase, Hyaluronan, and CD44 in cancers: a breast carcinoma perspective. Cancer Research. Vol. 66. No. 21: 10233-10236.

Graessmann, M.; Berg, B.; Fuchs, B.; Klein, A.; Graessmann, A. (2006). Chemotherapy resistance of mouse WAP-SVT/t breast cancer cells is mediated by osteopontin, inhibiting apoptosis downstream of caspase-3. Oncogene. 1-11.

Grammatikakis, N.; Jaronczyk, K.; Siganou, A.; Vultur, A.; Brownell, H.L.; Benzaquen, M.; Rausch, C.; Lapointe, R.; Gjoerup, O.; Roberts, T.M.; Raptis, L. (2001). Simina virus 40 large tumor antigen modulates the raf signaling pathway. The Journal of Biological Chemistry. Vol. 276. No. 30: 27840-27845.

Gui, G. P.; Wells, C. A.; Yeomas, P.; et al. (1996) Integrin expression in breast cancer cytology: a novel predictor of axillary metastasis. Eur. J. Surg. Oncol. Vol. 22: 254-258.

Guo, X.; Zhang, P.; Mitchell, D. A.; Denhardt, D. T.; Chambers, A. F. (1995). Identification of a ras-activated enhancer in the mouse osteopontin promoter and its interaction with a putative ETS-related transcription factor whose activity correlates with the metastatic potential of the cell. Molecular and Cellular Biology. Vol 15. No. 1: 476-487.

Gunthert, U.; Hofmann, M.; Rudy, W. (1991). A new variant of glycoprotein CD44 confers metastatic potential to ract carcinoma cells. Cell. Vol. 65: 13-24.

Harden, T.K.; Sondek, J. (2006). Regulation of phospholypase C isozymes by Ras superfamily GTPases. Annual Reviews of Pharmacological Toxicology. Vol 46: 335-379.

Hartl, M.; Karagiannidis, A.I.; Bister, K. (2006). Cooperative cell transformation by Myc/Mil(Raf) involves induction of AP-1 and activation of genes implicated in cell motility and metastasis. Oncogene. 1-13.

He, B.; Mirza, M.; Weber, G. F. (2005). An osteopontin splice variant induces anchorage independence in human breast cancer cells. Oncogene. 1-11.

Helluin, O.; Chan, C.; Vilaire, G.; Mousa, S.; DeGrado, W.F.; Bennett, J.S. (2000). The activation state of alphavbeta 3 regulates platelet and lymphocyte adhesion to intact and thrombin-cleaved osteopontin. Journal of Biological Chemistry. Vol. 275: 18337-18343

Hijiya, N.; Setoguchi, M.; Matsuura, K.; Higuchi, Y.; Akizuki, S.; Yamamoto. S. (1994). Cloning and characterization of the human osteopontin gene and its promoter. Biochem. J. Vol 303: 255-262.

Horai, T.; Nakamura, N.; Tateishi, R.; Hattori, S. (1981). Glycosaminoglycans in human lung cancer. Cancer. Vol. 8: 141-146.

Hsieh, Y-H.; Juliana, M. M.; Hicks, P. H.; Feng, G.; Elmets, C.; Liaw, L.; Chang, P-L. (2006). Papilloma development is delayed in Osteopontin-Null mice: Implicating an antiapoptosis role for osteopontin. Cancer research. Vol. 66, No. 14: 7119-7126.

Humphreys, R. C.; Krajewska, M.; Krnacik, S.; Jaeger, R.; Weiher, H.; Krajewski, S.; Reed, J. C.; Rosen, J. M. (1996) Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. Development. Vol. 122: 4013-4022.

Hunter, T.; Selton, B. M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. Vol. 77: 1311-1315.

Hynes, R. O. (2002). Integrins: bi-directional, allosteric signalling machines. Cell. Vol. 110: 673-687.

Ito, T.; Hashimoto, Y.; Tanaka, E.; Kan, T.; Tsunoda, S.; Sato, F.; Higashiyama, M.; Okumura, T.; Shimada, Y. (2006). An inducible short-hairpin RNA vector against osteopontin reduces metastatic potential of human esophageal squasmous cell carcinoma in vitro and in vivo. Clinical Cancer Research. Vol 12 (4). 1308-1316.

Jalkanen S.; Aho, R.; Kallajoki, M.(1989) Lymphocite homing receptors and adhesion molecules in intravascular malignant lymphomatosis. Int. Journal of Cnacer. Vol. 44: 777-782.

Johnson, D.; Agochiya, M.; Samejima, K.; Earnshaw, W.; Frame, M.; Wyke, J. (2000) Regulation of Both Apoptosis And Cell Survival By The v-Src Oncoprotein. Cell Death and Differentiation. Vol 7: 685-696.

Jothy, S. (2003). CD44 and its partners in metastasis. Clinical & Experimental Metastasis. Vol. 20: 195-201.

Kadkol, S. S.; Lin, A. Y.; Barak, V.; Kalickman, I.; Leach, L.; Valyi-Nagy, K.; Majumdar, D.; Setty, S.; Maniotis, A. J.; Folberg, R.; Pe´er, J. (2005). Osteopontin expression and serum levels in metastatic uveal melanoma: a pilot study.

Katz, M.; amit, I.; Yarden, Y. (2007). Regulation of MAPKs by growth factors and receptor tyrosine kinases. Biochimica et Biophysica Acta. 10.

Khan, S. A.; Lopez-Chua, C. A.; Zhang, J.; Fisher, L. W.; Sørensen, E. S.; Denhardt, D. T. (2002). Soluble osteopontin inhibits apoptosis of adherent endothelial cells deprived of growth factors. Journal of Cellular Biochemistry. Vol. 85: 728-736.

Khan, S. A.; Cook, A. C.; Kappil, M.; Günthert, U.; Chamber, A. F.; Tuck, A. B.; Denhardt, D. T. (2006). Enhancement cell surface CD44 variant (v6, v9) expression by osteopontin in breast cancer epithelia cells facilitates tumor cell migration: Novel post-transcriptional, post-translational regulation.

Kim, J. B.; Stein, R.; O´Hare, M. J. (2005) Tumor-stromal interactions in breast cancer. The role of stroma in tumorigenesis. Tumor Biology. Vol. 26: 173-185.

Kim, M. S.; Park, M. J.; Moon, E. J.; Kim, S. J.; Lee, C. H.; Yoo, H.; Shin, S. H.; Song, E. S.; Lee, S. H. (2005). Hyaluronic acid induces osteopontin via the phosphatydilinositol 3-Kinase/Akt to enhance the cell motility of human glioma cells. Cancer Research. Vol. 65. No. 3: 686-691.

Klein, A.; Guhl, E.; Tzeng, Y. J.; Fuhrhop, J.; Levrero, M.; Graessmann, M.; Graessmann, A. (2003). HBX causes cyclin D1 overexpression and development of breast cancer in transgenic animals that are heterozygous for p53. Oncogene Vol. 22: 2910-2919.

Kohlhoff, S.; Ziechmann, C.; Gottlob, K.; Graessmann, M. (2000) SV40 T/t-antigens sensitize mammary gland epithelial cells to oxidative stress and apoptosis. Free. Radic. Boil. Med. Vol. 29. No. 6: 497-506.

Kosaki, R.; Watanabe, K.; and Yamaguchi, Y. (1999). Overproduction of Hyaluronan by expression of the hyaluronan synthase Has2 enhances anchorage-independent growth and tumorigenecity. Cancer Research. Vol. 59: 1141-1145.

Kulik, G.; Klippel, A.; Weber, M. J. (1997). Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Molecular and cellular biology. Vol. 17. No. 3: 1595-1606.

Lakshman, M.; Subramaniam, V.; Wong, S.; Jothy, S. (2005). CD44 promotes resistance to apoptosis in murine colonic epithelium. Journal of cellular physiology. Vol. 203: 583-588.

Larkin, J.; Renukaradhya, G. J.; Sriram, V.; Du, W.; Gervay-Hague, J.; Brutkiewicz, R. R. (2006). CD44 differentially activates mouse NK T cells and conventional T cells. The Journal of Immunology. 268-279.

Lee M.; Kim, J. Y.; Anderson, W. B. (2004). Apoptotic effect of PP2 a Src tyrosine kinase inhibitor, in murine B cell leukemia. J.Cell Biochem. Vol. 93. No. 3: 629-38.

Lee, J. L.; Wang, M. J.; Sudhir, P. R.; Chen, G. D.; Chi, C. W.; Chen, J. Y. (2007). Osteopontin promotes integrin activation through outside-in and inside-out mechanisms: OPN-CD44v interaction enhances survival in gastrointestinal cancer cells. Cancer Research.Vol. 67. No. 5: 2089-2097.

Lesley, J.; Hyman, R. (1992) CD44 can be activated to function as a hyluronic acid receptor in normal murine T cells. Eur. Journal Immunol. Vol. 22: 2719-2723.

Lewis, J. M.; Truong, T. N.; Schwartz, M. A. (2002). Integrins regulate the apoptotic response to DNA damage through modulation of p53. Proc. Natl. Sci. U.S.A. Vol. 99: 3627-3632.

Li, Y.; Heldin, P. (2001) Hyaluronan production increases the malignant properties of mesothelioma cells. British Journal of Cancer. Vol 85. No. 4: 600-607.

Liaw, L.; Birk, D. E.; Ballas, C. B.; Whitsitt, J. S.; Davidson, J. M.; Hogan, B. L. M. (1998). Altered wound healing in mice lacking a functional osteopontin gene (spp1). The Journal of Clinical Investigation. Vol. 101. No. 7: 1468-1478.

Lin, Y. H.; Yang-Yen, H. F. (2001). The osteopontin-CD44 survival signal involves activation of the phosphatidylinositol 3-kinase/Akt signalling pathway. J, Biol. Chem. Vol. 276: 46024-46030.

Liu, X.; Ye, K. (2005) Src homology domains in phospholipase C-gamma1 mediate its anti-apoptotic action through regulating the enzymatic activity. Journal of Neurochemistry. Vol. 3. No. 4: 892- 898.

Liu, J.; Zhan, M.; Hannay, J. A.; Das, P.; Bolshakov, S. V.; Kotilingam, D.; Yu, D.; Lazar, A. F.; Pollock, R. E.; Lev, D. (2006) Wild-type p53 inhibits nuclear factor-kappaB-induced matrix metalloproteinase-9 promoter activation: implications for soft tissue sarcoma growth and metastasis. Mol. Cancer. Res. Vol. 4. No. 11: 803 – 810.

Lopez, C. A.; Carroll, A. G.; Denhardt, D.T.; Lupu, R. (1996). Effect of heregulin on proliferation and expression of apoptosis related genes: interaction with osteopontin. Procedures AACR Ann Mt. Vol. 37:22.

Marhaba, R.; Zöller, M. (2004). CD44 in cancer progression: adhesion, migration and growth regulation. Journal of molecular histology. Vol. 35: 211-231.

Maschler, S.; Wirl, G.; Spring, H.; Bredow, D. v.; Sordat, I.; Beug, H.; Reichmann, E. (2005). Tumor cell invasiveness correlates with changes in integrin expression and localization. Oncogene. Vol. 24: 2032-2041.

Mazzali, M.; Kipari, T.; Ophascharoensuk, V.; Wesson, J.A.; Johnson, R.; Hughes, J. (2002). Osteopontin-a molecule for all seasons. Q. J. Med. Vol. 95: 3-13.

Morimoto, I.; Sasaki, Y.; Ishida, S.; Imai, K.; Tokino, T. (2002) Identification of the osteopontin gene as a direct target of TP53. Genas, Chromosomes & Cancer. Vol 33: 270-278.

Murray, P.; David, E. (2000). Regulation of programmed cell death by basement membranes in embryonic development. The Journal of Cell Biology. Vol. 150. No. 5: 1215-1221.

Nam, J. S.; Ino, Y.; Sakamoto, M.; Hirohashi, S. (2002) Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis.
Clin. Cancer Res. Vol. 7: 2430-6.

Néel, B. D.; Aouacheria, A.; Nouvion, A. L.; Ronot, X.; Gillet, G. (2005). Distinct Protease Pathways Control Cell Shape and Apoptosis in *v-src*-transformed Quail Neuroretina Cells. Experimental Cell Research. Vol. 311: 106-116.

Nemir, M.; Bhattacharyya, D.; Li, X.; Singh, K.; Mukherjee, A. B.; Mukherjee, B. B. (2000). Targeted inhibition of osteopontin expression in the mammary gland causes abnormal morphogenensis and lactation deficiency.The Journal of Biological Chemistry. Vol 275. No. 2: 969-976.

Nicolleti, I.; Migliorati, G.; Pagliacci, M. C.; Grignani, F.; Riccardi, C.A. Rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods. Vol. 139, 271-279.

Noti, J. D.; Johnson, A. K. (2001) Integrin alpha 5 beta 1 suppresses apoptosis triggered by serum starvation but not phorbol ester in MCF-7 breast cancer cells that overexpress protein kinase C-alpha. Int. J. Oncol. Vol. 18. No. 1: 195-201.

Oldberg, Å.; Franzén, A.; Heinegård, D. (1986). Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals Arg-Gly-Asp cell-binding sequence. Biochemistry. Vol. 83: 8819-8823.

Packer, L.; Pavey, S.; Parker, A.; Stark, M; Johansson, P.; Clarke, B.; Pollock, P.; Ringner, M.; Hayward, N. (2006). Osteopontin is a downstream effector of the PI3-kinase pathway in melanomas that is inversely correlated with functional PTEN. Carcinogenesis. Vol 27. No. 9: 1778-1786.

Park, H. B.; Golubovskaya, V.; Xu, L.; Yang, X.; Lee, J. W.; Scully, S. 2nd, Craven, R. J.; Cance, W. G. (2004.) Activated Src increases adhesion, survival and alpha2-integrin expression in human breast cancer cells. Biochem J. Vol. 378: 559-67.

Park, C. C.; Zhang, H.; Pallavicini, M.; Gray, J. W.; Baehner, F.; Park, C. J.; Bissell, M. J. (2006). β1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures *in vivo*. Cancer Researcgh. Vol. 66. No. 3: 1526-1534.

Pereira, P. A.; Rubenthiran, U; Jothy, S. (2001) CD44s expression mitigates the phenotype of human colorectal cancer hepatic metastases. Anticancer research Vol. 21: 2713-2717.

Philip, S.; Bulbule, A.; Kundu, G. C. (2001). Osteopontin stimulates tumor growth and activation of promatrix metalloproteinase-2 through nuclear factor- κß-mediated induction of membrane type 1 matrix metalloproteinase in murine melanoma cells. The journal of biochemical chemistry. Vol. 276. No. 48: 44926-44935.

Ponta, H.; Sherman, L.; Herrlich, P. A. (2003). CD44: From adhesion molecules to signalling regulators. Nature Reviews Molecular Cell Biology. Vol. 4. 33-35

Prols, F.; Loser, B.; Marx, M. (1998). Differential expression of osteopontin, PC4, and CEC5, a novel mRNA species, during in vitro angiogenesis. Exp. Cell Res. Vol. 239: 1-10

Rangaswami, H.; Bulbule, A.; Kundu, G. C. (2005). Osteopontin: role in cell signalling and cancer progression. TRENDS in cell biology. Article in press.

Rangaswami, H.; Anuradha, B.; Kundu, G. C. (2006). Nuclear factor inducing kinase: a key regulator in osteopontin induced MAPK/IκB kinase dependent NF-κB-mediated promatrix metalloproteinase-9 activation. Glycoconj J. Vol. 23: 221-232.

Riccardi, C.; Nicoletti, I. (2006). Analysis of propidium iodide staining and flow cytometry. Nature Protocols. Vol. 1. No. 3: 1458-1461.

Rice, J.; Courter, D. L.; Giachelli, C. M.; Scatena, M. (2006). Molecular mediators of $\alpha_v\beta_3$ endothelial cell survival. Journal of vascular research. Vol. 43: 422-436.

Rittling, R. S.; Novick, K. E. (1997). Osteopontin expression in mammary gland development and tumorigenesis. Cell Growth & Differentiation. Vol. 8: 1061-1069.

Rogers, S.N.; Padanilam, B. J.; Hruska, K. A.; Giachelli, C. M.; Hammermann, M. R. (1997). Metanephric osteopontin regulates nephrogenesis in vitro. American Journal of Physiol. Vol. 272: 469-476.

Ropponen, K.; Tammi, M.; Parkkinen, J.; Eskelinen, M.; Tammi, R.; Lipponen, P.; Agren, U.; Alhava, E.; Kosma, V. M. (1998) Tumor cell-associated hyaluronan as an unfavourable prognostic factor in colorectal cancer. Cancer Research. Vol. 58: 342-347.

Rys, J.; Kruczak, A.; Lackowska, B., et al. (2003). The role of CD44v3 expression in female breast carcinomas. Pol J. pathology. Vol. 54: 243-7.

Sahai, E.; Olson, M. F.; Marshall, C. J. (2001) Cross-talk between Ras and Rho signalling pathways in transformation favours proliferation and increased motility. The EMBO journal. Vol. 20. No. 4: 755-766.

Salih, E.; Ashkar, S.; Gerstenfeld, L. C.; Glimcher, M. J. (1996). Protein kinases of cultured osteoblasts: selectivity for the extracellular matrix proteins of bone and their catalytic competence for osteopontin. J. Bone. Miner. Res. Vol. 11. No. 10: 1461-73.

Samanna, V.; Wei, H.; Ego-Osuala, D.; Chellaiah, M. A. (2006). Alpha-v-dependent outside-in signalling is required for the regulation of CD44 surface expression, MMP-2 secretion, and cell migration by osteopontin in human melanoma cells. Experimental cell research.

Sambrook, J.; Fritsch, E.F; Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press, New York.

Santarelli, R.; Tzeng, Y. J.; Zimmermann, C.; Guhl, E.; Graessmann, A. (1996). SV40 T-antigen induces breast cancer formation with high efficiency in lactating and virgin WAP-SV-T transgenic animals but with a low efficiency in ovariectomized animals. Oncogene Vol. 12: 495-505.

Senger, D. R.; Perruzzi, C. A.; Papadopoulos, A.; Tenen, D. G. (1989). Purification of a human milk protein closely similar to tumor-secreted phosphoproteins and osteopontin. Bichimica et Biophysica Acta. Vol 996: 23-48.

Senger, D.R.; Ledbetter, S.R.; Claffey, K. P.; Papadopoulos-Sergiou, A.; Peruzzi, C. A.; Detmar, M. (1996) Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. American Journal of Pathology. Vol. 149. No. 1: 293-305.

Shanmugam V.; Chackalaparampil I.; Kundu G.C.; Mukherjee A. B.; Mukherjee B. B. (1997). Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells. Biochemistry 36:5729–5738.

Shaw, L. M. (1999). Integrin function in breast carcinoma progression. J. Mammary Gland Biol. Neoplasia. Vol. 4: 367-376.

Smith, J. H.; Denhardt, D. T. (1989). Evidence for two pathways of protein kinase C induction of 2AR expression: Correlation with mitogenesis. Journal of cellular physiology. Vol 139: 189-195.

Smith L. L.; Giachelli, C.M. (1998). Structural requirements for α9β1 mediated adhesion and migration to thrombin-cleaved amino-terminal fragment osteopontin. Exp. Cell. Res. Vol. 242: 351-360.

Smith, L. L.; Greenfield, B. W.; Aruffo, A.; Giachelli, C. M. (1999). CD44 is not an adhesive receptor for osteopontin. Journal of Cellular Biochemistry. Vol. 73: 20- 30.

Squires, M. S.; Hudson, E. A.; Howells, L.; Sale, S.; Houghton, C. E.; Jones, J. L.; Fox, L. H.; Dickens, M.; Prigent, S. A.; Manson, M. M. (2003). Relevance of mitogen activated protein kinase (MAPK) and phosphotidylinositol-3-kinase/protein kinase B (PI3K/PKB) pathways to induction of apoptosis by curcumin in breast cancer cells. Biochemical Pharmacology. Vol. 65: 361-376.

Stier, S.; Ko, Y.; Forkert, R.; Lutz, C.; Neuhaus, T.; Grünewald, E.; Cheng, T.; Dombkowski, D.; Calvi, L. M.; Rittling, S. R.; Scadden, D. T. (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. The journal of Experimental Medicine. Vol 201. No. 11: 1781-1791.

Stoolman, L. M. (1989). Adhesion molecules controlling lymphocyte migration. Cell. Vol. 56: 907-910.

Tuck, A. B.; Elliot, B.; Hota, C.; Tremblay, E. and Chambers, A. F. (2000). Osteopontin-induced, integrin-dependent, migration of human mammary epithelial cells involves activation of the hepatocyte growth factor receptor met (Met). Journal of Cell Biochemistry. Vol. 78: 465-475.

Tuck, A. B.; Hota, C.; Wilson, S. M.; Chambers, A. F. (2003) Osteopontin-induced migration of human mammary epithelial cells involves activation of EGF receptor and multiple signal transduction pathways. Oncogene. Vol. 22: 1198-1205.

Tvorogov, D.; Wang, X-J.; Zent, R.; Carpenter, G. (2004). Integrin-dependent PLC-γ1 Phosphorylation mediates fibronectin-dependent adhesion. Journal of Cell Science. Vol. 118 (3): 601-610.

Tzeng, Y. J.; Guhl, E.; Graessmann, M.; Graessmann, A. (1993). Breast cancer formation in transgenic animals induced by the whey acidic protein SV40 T antigen (WAP-SV-T) hybrid gene. Oncogene 8: 1965-1971.

Tzeng Y.J.; Zimmermann, C.; Guhl, E.; Berg, B.; Avantaggiati, M. L.; Graessmann, M.; Graessmann, A. (1998) SV40 T/t-antigen induces premature mammary gland involution by apoptosis and selects for p53 missense mutation in mammary tumors. Oncogene. Vol. 16: 2103-14.

Tzeng, Y.J.; Gottlob, K.; Santarelli, R.; Graessmann, A. (1996) The SV40 T-antigen induces premature apoptotic mammary gland involution during late pregnancy in transgenic mice. FEBS Lett. 1996 Vol 380. No 3: 215-8.

Tzeng, Y. J.; Guhl, E.; Graessmann, M.; Graessmann, A. (1993). Breast cancer formation in transgenic animals induced by the whey acidic protein SV40 T antigen (WAP-SV-T) hybrid gene. Oncogene. Vol. 8. No. 7: 1965-71.

Urquidi, V.; Sloan, D.; Kawai, K.; Agarwal, D.; Woodman, A. C., Tarin, D.; Goodison, S. (2002). Contrasting expression of Thrombospondin-1 and Osteopontin correlates with absence or prescence of metastatic phenotype in an isogenic model of spontaneous human breast cancer metastasis. Clinical Cancer Research. Vol. 8: 61-74.

Vossmeyer, D.; Hofmann, W.; Loster, K.; Reutter, W.; Danker, K. (2002). Phospholipase C γ Binds α1β1 integrin and modulates α1β1 integrin-specific adhesion.

Wai, P. Y.; Kuo, P. C. (2004). The role of osteopontin in tumor metastasis. Journal of Surgical Research. Vol. 121: 228-241.

Wai, P. Y.; Guo, L.; Gao, C.; Mi, Z.; Guo, H.; Kuo, P. C. (2006). Osteopontin inhibits macrophage nitric oxide síntesis to enhance tumor proliferation. J. Surgery. Vol.140. No. 2: 132.

Wang, S. J.; Bourguignon, L. Y. W. (2006). Hyaluronan-CD44 promotes phospholipase C-mediated Ca2+ signaling and cisplatin resistance in head and neck cancer. Arch. Otolaryngol. Head Neck Surg. Vol. 132: 19-24.

Wang, G.; Platt-Higgings, A.; Carroll, J.; Silva Rundland, S.; Winstanley, J.; Barraclough, R.; Rundland, P. S. (2006). Induction of metastasis by SP100 in a rat mammary model and its association with poor survival of breast cancer patients. Cancer Research. Vol 66 (2): 1199-1207.

Weber, G. F.; Ashkar, S.; Glimcher, M. J.; Cantor, H. (1996) Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science Vol. 271: 509-512.

Weber, G. (2001). The metastasis gene osteopontin: a candidate target for cancer therapy. Biochimica et Biophysica Acta. Vol 1552: 61-85.

Weber, G. F.; Bronson, R.T.; Ilagan, J.; Cantor, H.; Scmits, R.; Mak. T. W. (2002). Absence of the *CD44* gene prevents sarcoma metastasis. Cancer Research. Vol 62: 2281-2286.

Wei, Y.; Tang, C. H.; Kim, Y.; Robillard, L.; Zhang, F.; Kugler, M. C.; Chapman, H. A. (2007). Urokinas receptors are required for α5β1 integrin-mediated signaling in tumor cells. The Journal of Biological Chemistry. Vol. 282. No. 6: 3929-3939.

Wessel, R.; Voos, V:, Aspelmeier, A.; Jurgens, M.; Graesmann, A.; Klein, A. (2006) CorrXpression--identification of significant groups of genes and experiments by means of correspondence analysis and ratio analysis. In Silico. Biol. Vol. 6. No. 1-2: 61-70.

White, D. E.; Kurpios, N. A.; Zuo, D.; et al. (2004). Targeted disruption of β1-integrin in a transgenic mouse model of human breast cancer reveals an essentail roles in mammary tumor induction. Cancer Cell. Vol. 6: 159-70.

Wing, M. R.; Bourdon, M.D.; Harden, T. K. (2003) PLC-ε: a shared effector protein in Ras-, Rho-, and Gαβγ-mediated signalling. Molecular Interventions. Vol 3, No. 5: 274-280.

Wong, N. C.; Mueller, B. M.; Barbas, C. F.; Ruminski, P.; Quaranta, V.; Lin, E. C.; Smith, J. W. (1998). α_v integrins mediate adhesion and migration of breast carcinoma cell lines. Clin. & Exp. Metastasis. Vol 16: 50-61.

Wu, Y.; Denhardt, D.T.; Rittling, S.R. (2000) Osteopontin is required for full expression of the transformed phenotype by the ras oncogene. British Journal of Cancer. Vol 83. No. 1: 156-163.

Wu, G.; Xing, M.; Mambo, E.; Huang, X.; Liu, J.; Guo, Z.; Chatterjee, A.; Goldenberg, D.; Golin, S. M.; Sukumar, S.; Trink, B.; Sydranski, D. (2005). Somatic mutation and gain of copy number of PIK3CA in human breast cancer. Breast Cancer Res. Vol. 7(5):R609-16. Epub 2005 May 31

Ye, B.; Sakates, S.; Mok, S. C.; Horick, N. K.; Rosenberg, H. F.; Vitonis, A.; Edwards, D.; Sluss, P.; Han, W. K.; Berkowitz, R. S.; Cramer, D. W. (2006). Proteomic-based discovery and characterization of glycosylated eosinophil-derived neurotoxin and COOH-terminal osteopontin fragments for ovarian cancer in urine. Clinical Cancer Research. Vol 12. No. 2: 432-441.

Yebra, M.; Parry, G. C. N.; Strömblad, S.; Mackman, N.; Rosenberg, S.; Mueller, B.; Cheresh, D. A. (1996). Requirement of receptor-bound urokinase-type plasminogen activator for integrin $\alpha_v\beta_5$-directed cell migration. The journal of Biological Chemistry. Vol 271. No. 46: 29393-29399.

Yu, Q.; Toole, B. P.; Stamenkovic, I. (1997) Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. Journal of Exp. Med. Vol. 186. No. 12: 1985-1996.

Yu, Q.; Stamenkovic, I. (2000). Cell surface-localized matrix metalloproteinase-9 proteolitically activates TGFβ and promotes tumor invasion and angiogenesis. Genes & Development. Vol. 14: 163-176.

Yu, Q.; Stamenkovic, I. (2004). Transforming growth factor-beta facilitates breast carcinoma metastasis by promoting tumor cell survival. Clinicla and experimental metastasis. Vol. 21: 235-242.

Yuan, R-H.; Jeng, Y-M.; Chen, H-L.; Lai, P-L.; Pan, H-W.; Hsieh, F-J.; Lin, C-Y.; Lee, PH.; Hsu, H-C. (2006) Stahtmin overexpression cooperates with p53 mutation and osteopontin overexpression, and is associated with tumor progression, early recurrence, and poor prognosis in hepato cellular carcinoma. Journal of Pathology.

Yokosaki, Y.; Matsuura, N.; Sasaki, T.; Murakami, I.; Schneider, H.; Higashiyama, S.; Saitoh, Y.; Yamakido, M.; Taooka, Y.; Sheppard, D. (1999) The integrin alpha(9)beta(1) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin.Journal of Biological Chemistry. Vol. 274. No. 51:36328-34.

Yokosaki, Y.; Tanaka, K.; Higashikawa, F.; Keisuke, Y.; Eboshida, Akira. (2005). Distinct structural requirements for binding of the integrins. αvβ6, αvβ3, α5β1 and α9β1 to osteopontin. Matrix Biology. Vol. 24: 418-427.

Zahir, N.; Lakins, J. N.; Russell, A.; Ming, W. Y.; Chatterjee. C.; Rozenberg, G. I.; Marinkovich, M. P.; Weaver, V. M. (2003). Autocrine laminin-5 ligates $\alpha_6\beta_4$ integrin and activates RAC and NF-κB to mediate anchorage-independent survival of mammary tumors.

Zhao, J. J.; Gjoerup, O. V.; Subramanian, R. R.; Cheng, Y.; Chen, W.; Roberts, T. M.; Hahn, W. C. (2003). Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell. Vol. 3: 483-495.

Zhao, H.; Ross, F. P.; Teitelbaum, S. L. (2005). Unnocupied $\alpha_v\beta_3$ integrin regulates osteoclast apoptosis by transmitting a positive death signal. Molecular endocrinology. Vol. 19. No. 3: 771-780.

Zhang, G.; He, B.; Weber, G. F. (2003). Growth factor signalling induces metastasis genes in transformed cells: molecular connection between akt kinase and osteopontin in breast cancer. Molecular and cellular biology. 6507-6519.

Zhu, B.; Suzuki, K.; Goldberg, H. A. (2004). Osteopontin modulates CD44-dependent chemotaxis of peritoneal macrophages through G-protein-coupled receptors: evidence of a role for an intracellular form of Osteopontin. Journal of Cell Physiology. Vol. 198: 155-167.

Zhu, Y.; Denhardt, D. T.; Cao, H.; Sutphin, P. D.; Koong, A. C.; Giaccia, A. J.; Le, Q. T. (2005) Hypoxia regulates osteopontin expression in NIH-3t3 cells via a Ras-activated enhancer. Oncogene. Vol. 24: 6555-63

Zutter, M. M.; Krigman, H. R.; Satoro, S. A. (1993). Altered integrin expression in adenocarcinoma of the breast. Analysis by *in situ* hybridization. Am. J. of Pathol. Vol. 142: 1439-1448.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Selbständigkeitserklärung

Ich, Hugo Ernesto Molina Leddy, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: “20 – 40 kDa OPN isoforms mediate chemotherapy resistance of mouse WAP-SVT/t breast cancer cells and prevent cell death by activation of a phospholipase C regulated mechanism.” selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Hugo Ernesto Molina Leddy

28. September 2007