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BISPHOSPHONATE INHIBITION OF PROSTATE CANCER CELL INVASION, MIGRATION AND CYTOSKELETAL ORGANIZATION

by

Sanna Virtanen

ACADEMIC DISSERTATION

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To my son Samuli
and my family

ABSTRACT

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BISPHOSPHONATE INHIBITION OF PROSTATE CANCER CELL INVASION, MIGRATION AND CYTOSKELETAL ORGANIZATION

Metastatic bone lesions are commonly associated with prostate cancer affecting approximately 60-80% of the patients. The progression of prostate cancer into an advanced stage is a complex process and its molecular mechanisms are poorly understood. So far, no curative treatment is available for advanced stages of prostate cancer. Bisphosphonates (BPs) are synthetic pyrophosphate analogues, which are used as therapeutics for various metabolic bone diseases because of their ability to inhibit osteoclastic bone resorption. Nitrogen-containing bisphosphonates block the function of osteoclasts by disturbing the vesicular traffic and the mevalonate pathway -related enzymes, for example farnesyl diphosphate synthase, which is involved in post-translational isoprenylation of small GTPases. In addition, the anti-proliferative, anti-invasive and pro-apoptotic effects of nitrogen-containing bisphosphonates on various cancer cell lines have been reported.

The aim of this thesis work was to clarify the effects of bisphosphonates on prostate cancer cells, focusing on the mechanisms of adhesion, invasion and migration. Furthermore, the role of the mevalonate pathway and prenylation reactions in invasion and regulation of the cytoskeleton of prostate cancer cells were examined. Finally, the effects of alendronate on cytoskeleton- and actin-related proteins in prostate cancer cells were studied *in vitro* and *in vivo*. The results showed that the nitrogen-containing bisphosphonate alendronate inhibited the adhesion of prostate cancer cells to various extracellular matrix proteins and migration and invasion *in vitro*. Inhibition of invasion and migration was reversed by mevalonate pathway intermediates. The blockage of the prenylation transferases GGTase I and FTase inhibited the invasion, migration and actin organization of prostate cancer cells. The marked decrease of cofilin was observed by the prenylation inhibitors used. Inhibition of GGTase I also disrupted the regulation of focal adhesion kinase and paxillin. In addition, alendronate disrupted the cytoskeletal organization and decreased the level of cofilin *in vitro* and *in vivo*. The decrease of the cofilin level by alendronate could be one of the key mechanisms behind the observed inhibition of migration and invasion. Based on the effects of nitrogen-containing bisphosphonates on tumor cell invasion and cytoskeletal organization, they can be suggested to be developed as therapeutics for inhibiting prostate cancer metastasis.

Keywords: bisphosphonates, cofilin, invasion, mevalonate pathway, migration, prostate cancer cells

YHTEENVETO

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BISFOSFONAATTIEN VAIKUTUS ETURAUHASSYÖPÄSOLUJEN INVAASIOON, LIIKKUMISEEN JA SOLUN TUKIRANKAAN

Edenneessä eturauhassyövässä luuetäpesäkkeitä muodostuu arviolta 60-80 %:lle potilaista. Eturauhassyövän eteneminen on monivaiheinen tapahtumaketju, jonka molekyylitason mekanismeja ei tunneta juuri lainkaan. Tällä hetkellä pitkälle edenneeseen eturauhassyöpään ei ole myöskään olemassa tehokasta hoitoa. Bisfosfonaatit ovat pyrofosfaatin synteettisiä analogeja ja niitä on käytetty luun hajotusta estävinä lääkkeinä erilaisten metabolisten luusairauksien hoidossa. Aminoryhmän sisältävät bisfosfonaatit estävät osteoklastien toimintaa häiritsemällä solunsisäistä rakkulaliikennettä sekä estämällä mevalonaattireitissä toimivien isoprenylaatioentsyymien, kuten farnesyylidifosfaattisyntaasin (FPPS), toimintaa. Aminobisfosfonaatit estävät solujakautumista, solujen liikkumista ja aikaansaavat ohjelmoidun solukuoleman myös monilla syöpäsolulinjoilla.

Tämän väitöskirjatyön tarkoituksena oli tutkia, miten bisfosfonaatit, erityisesti aminobisfosfonaatteihin kuuluva alendronaatti, vaikuttavat eturauhassyöpäsolujen tarttumiseen, invaasioon ja liikkumiseen. Lisäksi selvitettiin, miten mevalonaattireitin ja siinä olevien prenylaatioreittien estäminen vaikuttaa eturauhassyöpäsolujen liikkumiseen, solutukirangan organisaatioon sekä sitä säätelevien rakenneproteiinien määrään. Lopuksi tutkittiin alendronaatin vaikutuksia solun aktiinitukirangan säätelyyn osallistuvien proteiinien toimintaan *in vitro* ja *in vivo*. Tulokset osoittivat, että alendronaatti estää eturauhassyöpäsolujen tarttumista solun ulkoisen matriksin proteiineihin sekä liikkumista ja invaasiota *in vitro*. Mevalonaattireitin yhdisteet palauttivat liikkumisen ja invaasion. Prenylaatiotransferaasien, GGTaasi I:n ja FTaasin inhibiittorit vähensivät eturauhassyöpäsolujen liikkumista ja invaasiota sekä hajottivat aktiinitukirangan järjestäytymisen. Samat inhibiittorit vähensivät myös voimakkaasti kofiliinin määrää. GGTaasi I:n inhibitio vaikutti solujen tarttumista säätelevien fokaaliadheesiokinaasin ja paksilliinin määrään. Tässä yhteydessä alendronaatti hajotti myös solun aktiinitukirangan rakenteen sekä vähensi kofiliinin määrää eturauhassyöpäsoluissa ja kokeellisissa eturauhassyöpäkasvaimissa. Kofiliinin väheneminen saattaa olla yksi avainmekanismeista, jolla alendronaatti vähentää eturauhassyöpäsolujen liikkumista ja invaasiota. Aminobisfosfonaatteja voidaan tämän tutkimuksen perusteella harkita kehitettäviksi eturauhassyövän leviämistä estäviksi lääkkeiksi.

Avainsanat: bisfosfonaatit, eturauhassyöpäsolut, invaasio, kofiliini, liikkuminen, mevalonaattireitti

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ABBREVIATIONS

ADF	**actin depolymerising factor, destrin**
Akt/PKB	**protein kinase B**
Arf6	**ADP-ribosylation factor 6**
AR	**androgen receptor**
ARP2/3	**actin-related proteins2/3**
BMP	**bone morphogenetic protein**
BP	**bisphosphonate**
BPH	**bening prostate hyperplasia**
BSA	**bovine serum albumin**
DHT	**5α-dihydrotestosterone**
DMEM	**Dulbecco's modified Eagle's medium**
ECM	**extracellular matrix**
EMT	**epithelial-mesenchymal transition**
ER	**estrogen receptor**
FA	**focal adhesion**
FAK	**focal adhesion kinase**
FGF	**fibroblast growth factor**
FPP	**farnesyldiphosphate**
FPPS	**farnesyldiphosphate synthase**
FTase	**farnesyltransferase**
FTI	**farnesyltransferase inhibitor**
GAP	**GTPase activating protein**
GDI	**guanosine nucleotide dissociation inhibitor**
GDP	**guanosine diphosphate**
GEF	**guanine nucleotide exhange factor**
GFP	**green fluorescent protein**
GGPP	**geranylgeranyldiphosphate**
GGTase	**geranylgeranyltransferase**
GGTI	**geranylgeranyltransferase inhibitor**
GPCR	**G-protein-coupled receptor**
GTP	**guanosine triphosphate**
HMG-CoA	**hydroxyl-methyl-glutaryl-coenzyme A**
iFBS	**inactivated fetal bovine serum**
ICC	**immunocytochemistry**
IHC	**immunohistochemistry**
IGF	**insulin like growth factor**
ILK	**integrin-linked kinase**
JNK	**c Jun N-terminal kinase**
KDa, kD	**kilodalton**
LIMK	**LIM-kinase**
MAPK	**mitogen activated protein kinase**
MLC2	**myosin light chain 2**
MLCK	**myosin light chain kinase**
MMP	**matrix metalloproteinase**
N-BP	**nitrogen-containing bisphosphonate**
PAK	**p21-activated kinase**
PBS	**phosphate buffered saline**
PDGF	**platelet-derived growth factor**
PDK1	**3-phosphoinositide-dependent protein kinase 1**
PI-3	**phosphoinositide 3-kinase**
PIN	**prostatic intraepithelial neoplasia**
PKA	**protein kinase A**
PKC ε	**protein kinase C epsilon**
PTHrP	**parathyroid hormone -related protein**
RGD	**arginine-glycine-asparticacid**
ROCK	**rho-assosiated kinase**
ROI	**region of interest**
RT	**room temperature**
RTK	**receptor tyrosine kinase**
siRNA	**small interfering RNA**
Src	**proto-oncogenic tyrosine kinase**
SRE	**skeleton related events**
TGF	**transforming growth factor**
TIMP	**tissue inhibitor of metalloproteinase**
TNF	**tumor necrosis factor**
u-PA	**urokinase-type plasminogen activator**

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals I-III:

I Virtanen, S.S., Väänänen, H.K., Härkönen, P.L. and Lakkakorpi, P.T.: Alendronate inhibits PC-3 prostate cancer cell invasion by affecting the mevalonate pathway (Cancer Research 62: 2708-2714, 2002)

II Virtanen, S.S., Sandholm, J., Yegutkin, G., Väänänen, H.K. and Härkönen, P.L.: Inhibition of GGTase I and FTase disrupts cytoskeletal organization of human PC-3 prostate cancer cells (Cell Biology International 34:815-826, 2010)

III Virtanen, S.S., Sandholm, J., Tuomela, J., Väänänen H.K. and Härkönen, P.L.: Alendronate-induced disruption of actin cytoskeleton is associated with decrease of cofilin in PC-3 prostate cancer cells (submitted)

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1 INTRODUCTION

Bone metastases are commonly associated to advanced prostate carcinoma, affecting at least 70% of patients and cause severe clinical problems including hypercalcemia, bone fractures and pain (Green 2002). Within the metastatic lesions, cancer cells and bone cells (osteoclasts and osteoblasts) establish a self-feeding communication circle where cancer cells secrete various growth factors, which further induce the activity of bone cells. Increased bone resorption releases, in turn, various molecules which trigger cancer cell growth and proliferation (Kitazawa and Maeda 1995, Orr et al. 1995, Mundy 1997a, 1997b). Bisphosphonates (BPs), synthetic analogues of the pyrophosphate, are a group of therapeutics used in various metabolic bone diseases because of their ability to inhibit bone resorption. They are water-soluble compounds which bind rapidly into the inorganic bone matrix hydroxyapatite. The potency of BPs to inhibit bone resorption depends on the composition of two side groups. The amino group in the R2 side chain can reduce resorption even as much as 10 000 times compared to a non-aminogroup, including BPs (Russel et al. 2007).

Molecular targets of BPs in osteoclasts have been established. The pyrophosphate–resembling bisphosphonates (e.g. clodronate) are metabolized to toxic ATP analogues, which induce osteoclast apoptosis. Nitrogen-containing bisphosphonates (N-BPs; e.g. alendronate) are more potent inhibitors of bone resorption and their main target in osteoclasts is farnesyl diphosphate synthase (FPPS), an intermediate of the mevalonate pathway (van Beek et al. 1999, Bergstrom et al. 2000, Dunford et al. 2001, Thompson et al. 2002). Geranylgeranyl diphosphate synthase (GGPPS) has recently also been proposed as a novel target of N-BPs in osteoclasts (Goffinet et al. 2006). In addition, N-BPs inhibit the formation and adhesion of osteoclasts and change their morphology (Muhlbauer et al. 1991, Selander et al. 1994). Despite the studies which have demonstrated the effects of N-BPs on various cancer cell lines, their specific targets in cancer cells are so far, mainly undetermined.

Cell spreading is a series of highly regulated phases associated with the normal ontogeny and defence mechanisms of higher living organisms. Cell migration and invasion processes require alterations not only in the expression pattern of adhesion molecules, cell-cell and cell-extracellular matrix contacts, but also in the secretion and activation of proteolytic enzymes, the proliferation pattern and remodelling of the actin cytoskeleton. In general, Rho-family small GTPases (Rho, Rac, and Cdc42) are critical regulators of actin dynamics and contributors of the cell motility (Nobes and Hall 1999, Sequeira et al. 2008). Failures in the regulation patterns of motility can at worst lead into critical changes in normal cell behaviour, uncontrolled growth and formation of malignancies and secondary lesions (Friedl 2004, Sahai 2005). The formation of metastases is a multistep process where malignant cells detach from a primary tumor and invade through the extracellular matrix (ECM), basement membrane and endothelium into blood /lymphatic vessels with the help of matrix metalloproteases. Only a few cancer cells can survive in the blood/lymphatic circulation, ‘escape’ from the immune system, intravasate

into the target tissue and establish secondary leasions (Hart and Saini 1992, Yoneda et al. 1994).

This study was initiated by investigating the effects of bisphosphonates on PC-3 prostate cancer and MDA-MB-231 breast cancer cell invasion, migration and adhesion, and by evaluating the role of the mevalonate pathway in the invasion and migration process. Next, the role of the mevalonate pathway related prenylation reactions in the cytoskeletal organization and invasion of PC-3 prostate cancer cells was studied. Finally, the effects of alendronate on actin organization and levels of various cytoskeletal regulatory proteins in PC-3 prostate cancer cells were evaluated *in vitro* and *in vivo*.

2 REVIEW OF THE LITERATURE

2.1 Prostate cancer

2.1.1 Epidemiology and natural history of prostate cancer

Androgen-regulation has an established role in the development of a normal prostate, but also in malignancies of the prostate. Generally, androgens regulate cell proliferation, development, growth and achieve distinctive secondary sex-characteristics (Huggins 1967). Prostate-specific diseases differ within their clinical outcome. Benign prostatic hyperplasia (BPH) and acute/chronic inflammation are relatively common conditions of the prostate, usually related with unclear etiology (McNeal 1984, Nickel et al. 1999). The association between inflammation and growth of the prostate, promotion of BPH or prostate cancer has been suggested recently, but this data in this concept is highly suggestive (Sciarra et al. 2008). Prostatic intraepithelial neoplasia (PIN) is also proposed as a primary precursor of prostate cancer (Joniau et al. 2005). Histologically, prostate cancer can be divided into epithelial, neuroendocrine, stromal, mesenchymal, hematolymphoid and miscellaneous types, where the epithelial one is the most prevalent. The differentiation status of prostate cancer is graded mainly according to the Gleason Score (grades 1-5), created by Gleason (1966), and at present rarely by the WHO (Tumor, Node, Metastasis) -classification.

Prostate cancer is one of the most common cancer types among men, with 500 000 diagnosed cases/year worldwide, and can be considered in the future as a significant public health problem with constantly increasing incidence rates (Grönberg 2003, Jemal et al. 2008). In Finland prostate cancer is the most prevalent cancer type among the whole population, with over 5 000 new cases per year (Finnish Cancer Registry 2007). The early stage prostate cancer evolves in a multifocal pattern within the prostate gland and treatment of the localized disease has been significantly improved during recent years. More than 90% of prostate cancer patients respond initially to androgen deprivation therapy, but in most cases, an initial responsiveness to androgen withdrawal relapses to an androgen independent (hormonal-refractory) state predicting a poor prognosis. In a relapsed state, cancer cells invade into the periprostatic space of the prostate capsule or the perineural space and lymph nodes, and finally establish secondary lesions nonrandomly into the bone but also into the liver, lungs and brain (Bubendorf et al. 2000, Thalmann et al. 2000). Bone metastases affect approximately 60-90% of patients with advanced prostate carcinoma (Mundy and Yoneda 1995, Green 2002). Bone lesions cause clinical complications, hypercalcemia, bone fractures and pain, and importantly, are the main reason for prostate cancer deaths (Mundy 1997a).

The incidence of prostate cancer increases strikingly with age and is even suggested as a normal phenomenon related to aging (Carter et al. 1990). Also family history, toxic environmental factors, a western type diet, a smoking history and race are related to the risk factors of prostate cancer. However, little is known about the biology and molecular mechanisms behind prostate cancer development and progression, an induction of the androgen-independence, or the reciprocal interactions between prostate epithelial cells

and their surrounding stromal cells (Gleave et al. 1992, Chung 1993). So far, there is no curative therapeutics available for advanced prostate cancer and related bone metastasis.

Approximately 5-10 % of prostate cancer cases have a hederitary background (Langeberg et al. 2007). For example, tumor suppressor ribonuclease L (RNASEL), macrophage-scavenger receptor 1 (MSR 1), and various genes located in chromosome 8 have been proposed as susceptibility gene candidates for the hederitary form of prostate cancer (Carpten et al. 2002, Xu et al. 2002). Also genetic amplification of the androgen receptor is suggested to be associated to advanced prostate cancer (Koivisto et al. 1997, Linja et al. 2004, Dong 2006). The sporadic gene mutations associated to various prostate cancer forms are by far more relevant, accounting for 70-85% of all prostate cancer cases (Langeberg et al. 2007). In addition, mutations in phosphatase and tensin homologue (PTEN) and prostate-specific homeobox (NKX3.1) genes (McMenamin et al. 1999, Bowen et al. 2000), and various bacterial/viral infections also contribute to prostate cancer (DeMarzo et al. 2003).

2.1.2 Growth regulation of prostate cancer

Androgens have an established role in the regulation of prostate development and growth, but also in the development of prostate cancer. The effects of testosterone and more active 5α-dihydrotestosterone (DHT) in the prostate is mediated via the androgen receptor (AR), but it is also possible, that estrogen and estrogen receptors (ERs) might have a role in the regulation of prostate cancer development (Schulze and Barrack 1987, Härkönen and Mäkelä 2004).

Increased growth factor levels or changed growth factor receptor levels are associated to a relapsed form of cancer (Rau et al. 2005, Reddy et al. 2006). Growth factors might also replace the effect of androgens and activate AR in the androgen-depletion state. This is confirmed in studies with insulin-like growth factor 1 (IGF-I), fibroblast growth factor 7 (FGF-7), and epidermal growth factor (EGF) (Culig et al. 1994). On the other hand, epidermal growth factor receptor (EGFR) expression can be regulated by androgens in the normal prostate tissue and also in prostate cancer cells (Brass et al. 1995, Ravenna et al. 1995). Paracrine growth factors secreted by cancer cells/stromal cells can be related to the progression. FGF-family members, transforming growth factor beta (TGF-β), IGF-family, vascular endothelial growth factor- (VEGF) family and EGF-family are suggested to be the most important prostate cancer related growth factors (Kwabi-Addo et al. 2004, Zhu and Kyprianou 2005, Gennigens et al. 2006, Nicholson and Theodorescu 2004, Mimeault et al. 2003). Also the tumor necrosis factor α (TNF-α), angiopoietins, platelet-derived growth factor (PDGF), interleukins and chemokines are associated to angiogenesis essential to the progression of prostate cancer (Presta et al. 2005, Hanahan and Folkman 1996).

2.1.3 Tumor cell metastasis

2.1.3.1 Migration, adhesion and invasion of cancer cells

Directed cell motility is a basis of many physiological processes. During embryogenesis, cells are migrated precisely into the appropriate location in the highly regulated process. In adulthood, neutrophil- / macrophage -mediated chemotaxis, phagocytosis and lymphocyte-mediated immunological responses are enabled by directed cell migration. The massive migration of fibroblasts and vascular endothelial cells is the basic phenomenon in wound healing. On the other hand, the migration of cells contributes also to the underlying mechanisms of life-threathening diseases, such as cancer cell invasion and the formation of fatal metastasis. Cell migration is a complex and strictly controlled process where the cells first initiate a protrusion at the front, which subsequently attaches to the substratum. This is followed by the contraction of the cell body and detachment of the tail, and finally spreading to the direction of the protrusion. An initial event is the sensing of the haptotactic and/or chemotactic signals by cell-surface receptors. Signals can then activate further the complex signaling pathways inside the cell. The final outcome is polymerization of the new actin filaments at the leading edge and protrusions forward. Although the genetic basis of tumorigenesis may vary greatly between various cancer types, the cellular and molecular mechanisms required for the spreading are obviously equal for all cell types. Neoplastic cells and the stromal microenvironment can interact with each other and in carcinomas, the influence of the microenvironment is mediated by bi-directional interactions between epithelial tumor cells and stromal as well as endothelial and immune cells (Bogenrieder and Herlyn 2003).

Invasion as a whole includes the adhesion of cells onto various extracellular matrix proteins, mainly by integrins, and the migration is based on the dynamics of the accurately regulated cytoskeleton. Disruption of cell-cell contacts triggers the detachment of cancer cells from the primary tumor and leads to their migration and invasion into blood or lymphatic vessels through the basal lamina and endothelial cell layer, and extravasation with help of matrix metalloproteinases (MMPs) (Hart and Saini 1992, Yoneda et al. 1994). Tumor cells can migrate as groups with no clear organization, or as single cells in a noncoordinated and in a randomly oriented manner (Friedl 2004, Sahai 2005). Transition from a collective to an individual migration pattern recapitulates the developmental process known as epithelial-to-mesenchymal transition (EMT), which serves as a good indicator of tumor progression (Friedl and Wolf 2003, Guarino 2007). During the EMT, polarized epithelial cells that are tightly connected to each other by intercellular junctions undergo two critical changes. First, they dissociate from neighboring cells and secondly, they acquire the ability to migrate forward from the original tissue.

The altered expression of several critical genes, changes in the molecular mechanisms and cell behaviour patterns that highly polarized and tightly connected epithelial cells need for the transformation into aggressively and randomly migrating cells, are critical in the development and progression of cancer (Sahai 2005). The small G-proteins, Cdc42, Rac and Rho, seem to be at the heart of the initial signals leading to the polarization of

migrating cells and reorganization of the cytoskeleton (Etienne-Manneville and Hall 2002, Charest and Firtel 2007). A simplistic view is that Cdc42 and Rac are gradually activated toward the cell front where they control actin and microtubule rearrangement to promote protrusive activity, whereas Rho is rather active at the cell rear where it induces actomyosin contractility, controls rear-end retraction and allows forward movement. Moreover, Cdc42 not only participates in the protrusive activity, but is also directly involved in the cell orientation during directed migration in response to the exogenous polarity cues, such as chemotactic signals or cell wounding (Etienne-Manneville 2004).

2.1.3.2 Movement patterns of cancer cells

Cells have established several spreading patterns. The best demonstrated mechanism is the ***mesenchymal cell motility model***. It is revealed that 10-40% of carcinomas undergo EMT by using a mesenchymal motility pattern (Thiery 2002). Cells move slowly (0.1-1μm/min), in polar and elongated form with the help of proteolytic enzymes, MMPs and urokinase-type plasminogen activator (uPA), which degrade the extracellular matrix (ECM) (Friedl and Wolf 2003, Friedl 2004). Activation of receptor tyrosine kinase can initiate the mesenchymal motility cascade by the activation of small GTPases Rac, Arp2/3, Scar/WAVE, Ras, Cdc42 and/ or various adaptor proteins, which can promote actin polymerization and the formation of actin-rich protrusions (Pollard and Borisy 2003, Ridley et al. 2003, Zhang and Vande Woude 2003, De Wever et al. 2004). Reconstruction of integrin-dependent focal contacts and focal adhesions (FAs) allow adhesion of the new protrusions into the ECM (Ridley et al. 2003). Also RhoA and its effector proteins Rho-associated coiled-coil-containing kinases 1 and 2 (ROCK1, ROCK2) have a significant, but complex role in the mesenchymal motility model (Ridley et al. 2003, Vial et al. 2003).

Spreading carcinoma cells can utilize also a high-speed (4 μm/min) (rounded-form) ***amoeboid motility model*** (Wyckoff et al. 2000, Friedl and Wolf 2003). Actin polymerization leads to pseudopodal formation in cooperation with the actin-severing protein cofilin. The amoeboid motility model is a fast low-affinity migration pattern driven by a roundish yet flexible cell morphology, dynamic and polarized pseudopod protrusions and retractions, which are independent of the focal contact formation and stress fibers (Aizawa et al. 1997, Ghosh et al. 2004). The amoeboid motility model is similar to the rounded Rho- and ROCK-dependent motility model described by Sahai and Marshall (2003). Cortical actin contraction, driven by Rho/ROCK signalling, promotes the rapid remodelling of the cell cortex characteristics of amoeboid movement. It is likely that much weaker cell–ECM attachments are required for the amoeboid movement model, because it cannot be blocked by inhibition of integrin-β_1 function (Hegerfeldt et al. 2002). Furthermore, cell–ECM adhesions are not organized in large focal adhesions, but are very diffuse (Friedl 2004). A third form of cell motility is the ***collective motility model***. This model involves the movement of whole clusters or sheets

of tumour cells. Mechanistically, this pattern is similar to the collective form of mesenchymal motility, where the cells are located at the front, producing MMPs and generating 'paths' for the following cells (Nabeshima et al. 2002). Sheets of invading carcinoma cells are observed in breast, ovarian and colon carcinomas (Pitts et al. 1991, Nabeshima et al. 1999, Sood et al. 2001). In contrast to single cell movement, which requires the loss of adherens junctions, the maintenance of adherens junctions is important for the collective motility model (Friedl 2004). The molecular mechanisms of this collective form are poorly understood, and it is obvious that mechanisms of the collective cell movement can resemble that of morphogenetic movements of the epithelial sheets during development.

2.1.3.3 Role of adhesion molecules in cancer cell motility

Cell adhesion molecules play an important role in the conformation and shape of the individual cells, and importantly also for the maintenance of tissue organization. They mediate mechanical junctions between cells, but are also important in the cell signalling processes, as scaffold-molecules (Olayioye et al. 2000). In addition, they can cooperate with each other to create functional complexes (Gimond et al. 1999, Sheibani et al. 2000). Adhesion molecules support connections between cells (cell-cell), or between cells and the extracellular matrix (ECM). Cadherins, immunoglobulins and selectins mediate, partly in a calcium dependent manner, homophilic cell-cell connections (Alford and Taylor-Papadimitriou 1996). Integrins mediate, mainly in a heterophilic manner, the cell-ECM connections (Miyasaka 1995). Alterations in the expression/ activity of adhesion molecules promote the suppression of communication and heterogenity between cells. Disruption of cell contacts and the impairment of signal transduction are involved in malignant cell transformation (Takeichi 1993, Mareel et al. 1994, Ruoslahti and Öbrink 1996).

Cadherins (mainly E-, P-, and N-cadherins) are transmembranic glycoproteins which use their cytoplasmic sidechain within the adhesion to the actin cytoskeleton (Fleming and Johnson 1988, Shapiro et al. 1995, Ben-Ze'ev and Geiger 1998). Cadherins participate in the signal transdution pathways regulating the activation of p27, c-Jun, APC/axin and small GTPases (St.Croix et al. 1998, Miller et al. 1999, Pece et al. 1999, Noren et al. 2000, Peifer and Polakis 2000). Increased expression of E-cadherin is demonstrated to rescue cells from the initiation of metastatic transformation (Behrens et al. 1989, Tsuda et al. 1990, Frixen et al. 1991, Katayama et al. 1994, Watabe et al. 1994). Also alterations in the expression level of catenin can affect the ability of cadherins to attach to the actin cytoskeleton (Shibamoto et al. 1994).

Integrins play an important role in maintaining the bidirectional crosstalk between prostate cells and the ECM, and integrin expression is usually altered in advanced prostate cancer. β1-integrin levels are commonly increased, whereas β2 and β3 integrins remain unchanged (Murant et al. 1997). α6β1 integrin is one of the leading members of the adhesion complex, and inhibition of either α6 or β1 integrin can reverse the invasive phenotype of prostate cancer (Cress et al. 1995). The β1-integrin subfamily is proposed

to be affected also in human breast cancer (Gui et al. 1995). Expression levels of the integrins have been altered in various malignant tumors. In metastatic breast cancer cell lines, α5-, αv-, α2-, α3-, β1-, β5- and β3-integrins are downregulated (Zutter et al. 1990, Gui et al. 1997). On the other hand, Kostenuik et al. (1996), Liapis et al. (1996) and Weaver et al. (1997) have demonstrated the overexpression of α2-, β1-, β4- and αvβ3-integrins in various metastatic breast and prostate cancer cell lines. In addition, the expression level of β4 integrin correlates with the tumor size and nuclear grade of early breast cancer (Diaz et al. 2005). Contrary to that, α6β4 integrin can also maintain the viability of breast cancer cells *in vivo* (Lipscomb et al. 2005).

The heterodimeric integrins consist of 18 alpha (α) and 8 beta (β) subunits. They adhere commonly to ECM ligand proteins, which contain RGD (arginin (R), glycin (G), aspartic acid (D)) -sequence (Hynes 1992). 24 combinations of alpha and beta subunits and at least 10 known ligand proteins in ECM have been described so far (Miyasaka 1995, Gui et al. 1997, van der Pluijm et al. 1997, Ruoslahti 1997). Fibronectin (α3β1), vitronectin (αvβ3, αvβ1, αvβ5), laminin (α2β1, α3β1, α6β1) and type I collagen (α2β1, α1β1, α3β1) are the most important RGD-sequence -containing ECM proteins (Hynes 1992, Kostenuik et al. 1996, Gui et al. 1997). Integrins can trigger and transmit signals via intracellular protein kinases, various signalling pathways and the adaptor proteins; for example integrin-linked kinase (ILK), MAP-kinase, FAK, paxillin, talin, and ROCK /Rho GTPase family (Schaller and Parsons 1994, Rosales et al. 1995, Hannigan et al. 1996, Giancotti and Ruoslahti 1999, Welsh and Assoian 2000, Bishop and Hall 2000, Schaller 2001, Juliano 2002, Khyrul et al. 2004).

2.1.3.4 Matrix metalloproteinases (MMPs) in cell invasion

The degradation of ECM in pathological tissue remodeling (inflammation, cancer) is mediated by a variety of hydrolytic enzymes, including MMPs and uPAs. Various proteases appear to work together in this process, but MMPs are rate-limiting molecules in ECM degradation (Birkedal-Hansen et al. 1993, Mignatti and Rifkin 1993, Massova et al. 1998, Stetler-Stevenson 1999). The MMP family consists of at least 25 structurally related zinc-dependent enzymes (Birkedal-Hansen et al. 1993, Basbaum and Werb 1996, Nagase et al. 1996, Park et al. 2000, Lohi et al. 2001). This enzyme-family consists of the type IV collagenases MMP-2 and MMP-9, interstitial collagenase, matrilysin, metalloelastase, stromelysin and membrane-type MMPs. These proteases target the basement membrane constituents; fibronectin, laminin, collagens, proteoglycans and elastin (Matrisian 1990, Bernhard et al. 1994, Murphy and Knauper 1997). The regulation of MMPs is a complex process which occurs at both transcriptional and post-transcriptional levels. MMPs are produced as latent zymogen molecules, which are processed proteolytically into the active, lower molecular weight forms. Under normal conditions, MMPs form complexes with tissue inhibitors of metalloproteinases (TIMPs), which can eventually inactivate MMPs (Birkedal-Hansen et al. 1993, Nagase et al. 1996, Massova et al. 1998).

The activity of MMPs is related to normal and pathological prostate tissue morphology (Wilson 1995, Nelson et al. 2000). The type IV collagenases/gelatinases, particularly MMP-2 and MMP-9, are secreted as 72- and 92-kDa procollagenases, respectively, and they are activated by proteolytic processing (Mazzieri et al. 1997). MMP-2 and MMP-9 cleave not only type IV collagen, but also type I, III, V and XI collagens related to the ECM and the basement membrane (Liotta et al. 1991, Overall et al. 1991, Bernhard et al. 1994, Stearns and Stearns 1996, Murphy and Knauper 1997, Patterson and Sang 1997, Brooks et al. 1998). MMP-2 and MMP-9 are secreted by human prostate cells *in vitro* and *in vivo* (Wilson et al. 1993), and by normal, hyperplastic and neoplastic prostatic tissues in organ cultures together with their inhibitors TIMP-1 and -2 (Lokeshwar et al. 1993).

In the human prostate, MMP-2 is localized mainly to the basal epithelial cell layer, and to a lesser extent to secretory epithelial cells, but not stromal cells of the normal or benign prostatic hyperplastic tissue (Stearns and Wang 1993, Boag and Young 1994, Montironi et al. 1996, Still et al. 2000). Primary human prostatic carcinomas have shown to express higher levels of MMP-2 mRNAs and MMP-2 protein, as compared to normal prostate cells (Stearns and Wang 1993, Knox et al. 1996). Alterations in the MMP-9 and MMP-2 mature/proform ratio are associated with the progression of prostate cancer (Zhang et al. 2004). In addition, increased expression of pro-MMP-2 and its active form in prostate cancer have been reported to correlate with an increased Gleason score (Stearns and Stearns 1996, Wood et al. 1997).

2.1.3.5 Bone metastasis

Each metastatic cancer type (seed) favors its specific target tissues (soil), which have a typical combination of attractants, adhesion molecules and growth factors (Yoneda et al. 1994, Mundy 1997a, 1997b, Hullinger et al. 1998, Lehr and Pienta 1998). This 'Seed and Soil' –hypothesis was created in 1889 by Stephen Paget (Paget 1889). The skeleton is a fertile ground for the growth and survival of the various tumor cell types. Approximately 95-100% of myelomas and 65-80% of breast and prostate cancers metastasize into the skeleton (Cifuentes and Pickren 1979, Yoneda et al. 1994, Mundy and Yoneda 1995). Breast and prostate cancer cells interact with bone-resorbing osteclasts, feeding them with various stimulatory growth factors; TNF-α, PTHrP, IGF-II; and cytokines. This stimulation leads to increased bone resorption/formation by osteoclasts/osteoblasts. The release of stroma-bound growth factors, such as TGF-βs, can in turn stimulate the growth and proliferation of cancer cells and induce EMT (Yoneda et al. 1994, Clohisy et al. 1996, Mundy 1997b, Mundy 1999b, Pederson et al. 1999). Prostate cancer cells can also inhibit the activity of osteoclasts, or stimulate the growth of osteoblasts by TGF-β1, endothelin-1 and FGFs, which can lead to the formation of osteosclerotic lesions. Generally, breast cancer cells establish osteolytic (bone resorbing) metastases and prostate cancer cells generate mainly osteosclerotic (bone forming) metastases (Kitazawa and Maeda 1995, Orr et al. 1995, Adami 1997, Mundy 1997a, Yoneda et al. 1997, 2000, Lehr and Pienta 1998, Festuccia et al. 1999).

2.1.4 Role of cell cytoskeleton in cell motility

Maintenance of the multicellular organism and its ability to recover from injuries and infections, are relying on the cells capacity to respond rapidly to the changes in the external environment by regulating the dynamics/organization of the cytoskeleton. External chemical and mechanical stimuli are transduced via numerous signal transduction receptors. They trigger further complicated networks of the various regulation pathways associated with the remodellation of the actin cytoskeleton and various actin- related regulation proteins (Fig.1) (Horwitz and Parsons 1999, Smilenov et al. 1999, Small et al. 2002). Remodelling of the cytoskeleton strongly correlates with enhanced cell motility and, in some cases, also with the malignant cell phenotype and various other human diseases (Stournaras et al. 1996, Jordan and Wilson 1998, Condeelis et al. 2005, Sahai 2005, Yamaguchi et al. 2005, Carpenter 2000).

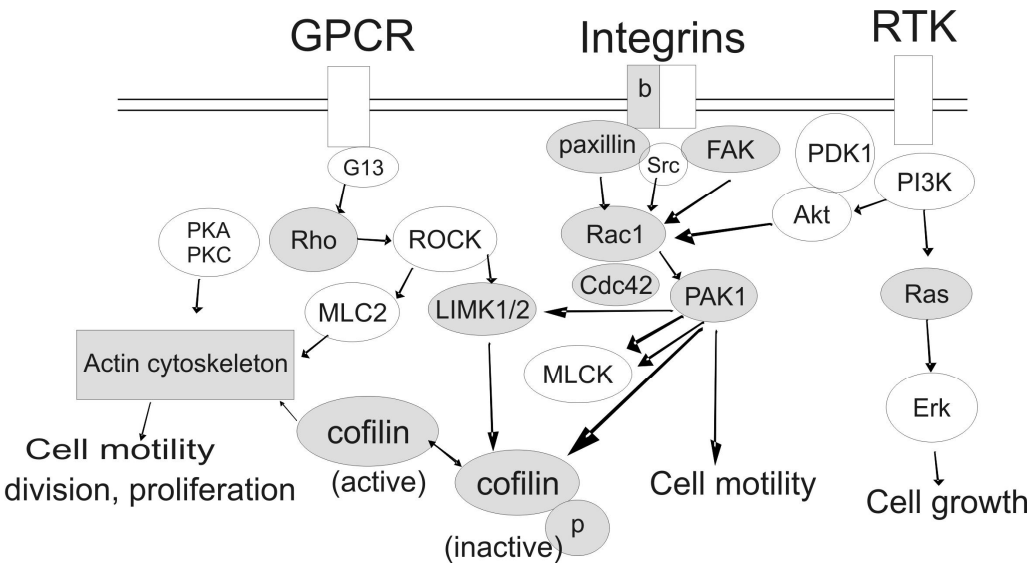

Figure 1. The cytoskeleton-related regulation network in a simplified form. Targets studied in this work are marked with grey color.

2.1.4.1 Actin

Actin is one of the most substantial structure molecules of the cell cytoskeleton, and it appears naturally in two forms; monomeric globular actin (G-actin) and filamentous actin (F-actin). G-actin molecules polymerize into double helical filaments under physiological conditions. Actin-binding proteins can in turn sequester and suppress the assembly of the G-actin into F-actin filaments. Moreover, the G-actin/F-actin equilibrium can be regulated in some cases in an activity-dependent manner. The mechanisms of this regulation pattern depend on the presence of a sequestered G-actin pool, which enables

site-directed F-actin polymerization in response to the appropriate stimuli (Okamoto et al. 2004). F-actin filaments have two ends, the barbed and the pointed end, which lengthen and shorten at different rates. Uncapped actin monomers can incorporate into the barbed end with a higher affinity during polymerization. When actin polymerization occurs adjacent to the plasma membrane, increasing numbers of free barbed ends are generated by three different mechanisms: (1) Uncapping of pre-existing barbed ends, capped by capping protein or gelsolin-related proteins (Hartwig et al. 1995), (2) severing of actin by the actin-binding protein cofilin/actin depolymerizing factor (ADF) (Chan et al. 2000), or (3) *de novo* nucleation of filaments involving the Arp2/3 complex (Pollard et al. 2000) or formins. Furthermore, also the availability of local free monomeric G-actin might be an important factor in the regulation of membrane protrusions. Critical monomer concentration is different at both ends of F-actin and leads to "threadmilling", a special feature of F-actin, consisting of the continuous exchange of actin molecules by assembling at the barbed end by profilin and disassembling at the pointed end by cofilin. When the rate of association at the barbed end is equal to the dissociation rate at the pointed end, the length of F-actin remains constant (steady-state threadmilling) (Fig.2) (Pruyne et al. 2002, Lee and Dominguez 2010).

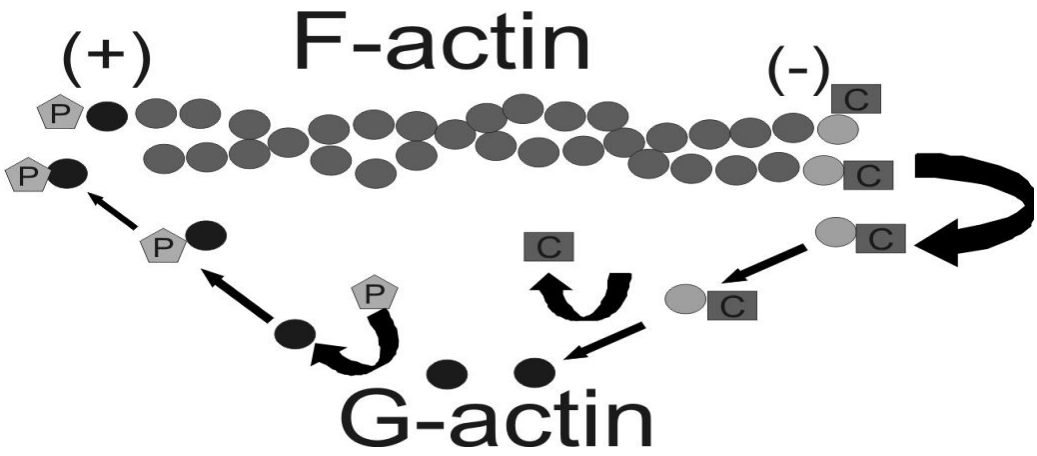

Figure 2. Threadmilling of F-actin regulated with the severing of F-actin by cofilin (C) at the pointed end (-) and the incorporation of actin monomers by profilin (P) to the barber end (+) (Modified from Lee and Dominguez 2010).

2.1.4.2 Actin related proteins

ADF/cofilin, thymosin β4 and profilin are proteins regulating F-actin length (Goldschmidt-Clermont et al. 1992). Also, cross-linking proteins, α-actinin, fascin and Arp2/3 can construct F-actin into bundles or networks, and enhance actin nucleation (Sasaki et al. 1996, Welch et al. 1998). Drebrin and tropomyosin are side-binding proteins which can alter the stability and mechanical properties of actin filaments, and myosin II is an actin-based molecular motor which is regulated by myosin light chain 2

(MLC2) (Ishikawa et al.1994). The dynamics of protrusive structures, filopodia, lamellipodia or podosomes/invadopodia are regulated by the actin polymerization at the leading edge of the migrating cell (Bailly and Condeelis 2002, Pollard and Borisy 2003). The dynamics of F-actin in non-neuronal cells is regulated by Rho GTPases, such as RhoA, Rac1, and Cdc42 (Hall 1994, Vincent and Settleman 1999). These GTPases act as molecular switches, modifying the actin-binding proteins, which can change the conformation of actin. The major regulator of lamellipodial activity in non-neuronal cells is Rac1 (Small et al. 2002, Katoh et al. 2006). Also a glutamate receptor-mediated RhoA-GTPase-dependent signaling pathway can directly control the F-actin reorganization and spine morphology (Schubert et al. 2006).

2.1.4.2.1 Cofilins

Cofilins (1-2, ADF) are a family of highy similar paralogs of small (19-21 kD) proteins which can bind to monomeric and filamentous actin molecules. They are essential proteins in the regulation of actin dynamics at the plasma membrane. Cofilins are characterized in 3 classes. Cofilin 1 is a non-muscle type, cofilin 2 is a muscle type and ADF (also called destrin) is an actin depolymerizing factor (Ono et al. 1994, Vartiainen et al. 2002). Their expression profiles differ in various cell lines. Cofilin 1 is ubiquitous, whereas ADF (destrin) is epithelia-/ endothelia-specific. Cofilins can shorten F-actin length by depolymerizing F-actin in a Ca^{2+} -independent and pH-dependent manner (Nishida et al. 1984, Yonezawa et al. 1985). ADF/cofilins can induce a twist in the F-actin double helix, without changing the filament lenght (McGough et al. 1997). They can both depolymerize F-actin by increasing the off rate in the pointed ends of actin filaments (Carlier and Pantaloni 1997), and directly sever filaments by providing a pool of actin monomers for the reconstruction of new filaments/ free barbed ends and the promotion of lamellipodial assembly (Maciver et al. 1991, Chan et al. 2000, Moon and Drubin 1995, Chen et al. 2000, Hotulainen et al. 2005, Ichetovkin et al. 2002, Ghosh et al. 2004). The depolymerization activity of ADF/cofilin is mainly derived from their ability to increase the rate of dissociation from the pointed end of F-actin (Carlier and Pantaloni 1997).

The activity of ADF/cofilin is regulated reversibly by phosphorylation at a serine-3 by LIM kinase (LIMK) and TES kinase (TESK) on the aminoterminal site (Bamburg and Wiggan 2002). Phosphorylation at serine-3 site abolishes ADF/cofilin binding to F-actin, and thus inhibits its severing ability. Reactivation of cofilin is induced by dephosphorylation of the Ser-3 residue by phosphatises, such as slingshot and chronophin (Agnew et al. 1995, Moriyama et al. 1996, Niwa et al. 2002, Ohta et al. 2003, Gohla et al. 2005, Huang et al. 2006). In addition, Rac, Rho and Cdc42, p21-activated kinase (PAK) and ROCK can inactivate cofilin via activation of LIMK (Mizuno et al. 1994, Okano et al. 1995, Arber et al. 1998, Yang et al. 1998, Sumi et al. 1999, Edwards et al. 1999, Maekawa et al. 1999, Ohashi et al. 2000, Amano et al. 2001). Integrin-linked kinase (ILK)/c-Src is also associated to the regulation of cofilin phosphorylation (Kim et

al. 2008). Moreover, ADF/cofilin accelerates and regulates the treadmilling of F-actin also *in vivo* (Nishida et al. 1984, Yonezawa et al. 1985, Small et al. 1995, Carlier and Pantaloni 1997, Lappalainen and Drubin 1997).

In mouse cell lines, depletion of ADF/cofilin 1 by siRNA impairs actin organization, and cell motility, morphogenesis and cytokinesis (Hotulainen et al. 2005, Condeelis 2001, Vartiainen et al. 2002, Estornes et al. 2007). Overexpression of cofilin has been demonstrated in various invasive cell lines (Sinha et al. 1999, Gunnersen et al. 2000, Keshamouni et al. 2006), and the level of phosphorylated cofilin is reduced in various carcinoma cell lines (Nebl et al. 1996). Overexpression of cofilin is described also *in vivo* in rat mammary tumors (Wang et al. 2004), and in various human tumors (Martoglio et al. 2000, Unwin et al. 2003, Turhani et al. 2006). However, overexpression of cofilin has also been shown to inhibit the invasion of human lung cancer cells (Lee et al. 2005), and downregulation of cofilin has been demonstrated in various human cancers *in vivo* (Ding et al. 2004, Smith-Beckerman et al. 2005).

2.1.4.2.2 LIM kinases (LIMKs) and p21-activated kinases (PAKs)

LIM kinases 1 and 2 (LIMK1 and LIMK2) are serine kinases, and their activity is regulated by phosphorylation on a threonine residue, which enhances their kinase activity. LIMKs are inactivators of actin depolymerising factors cofilin and ADF (Arber et al. 1998, Yang et al. 1998, Stanyon and Bernard 1999). Furthermore, Rho-GTPases Rho, Rac and Cdc42 can regulate LIMK1 activity through their effectors, ROCK and PAKs, which in turn are capable to phosphorylate LIMKs (Edwards et al. 1999, Maekawa et al. 1999, Ohashi et al. 2000, Amano et al. 2001, Dan et al. 2001, Davila et al. 2003). In addition, nischarin can inhibit LIMK activation (Ding et al. 2008), TGFβl signaling can phosphorylate LIMK2, and ROCK1 can block TGFβl-induced LIMK2/cofilin phosphorylation and actin polymerization (Vardouli et al. 2005). Bone morphogenetic protein (BMP) and its type II receptor (BMPRII) are involved in LIMK1 regulation and interactions with the intracellular tail of BMPRII lead to the inhibition of LIMK1 activity (Foletta et al. 2003). LIMK proteins are ubiquitously expressed in all the mouse tissues examined (Foletta et al. 2004), and LIMK1 and LIMK2 seem to have distinct roles and expression profiles in various tissues, and they are found both in the cytoplasm and nucleus of epithelial cells. Furthermore, the subcellular localization of LIMK1 and LIMK2 differs greatly: LIMK1 is localized to focal adhesions in many cultured primary cells and cell lines, while LIMK2 is found in punctae, which resemble endosomes (Acevedo et al. 2006). Overexpression of LIMK1 is observed in various invasive prostate and breast cancer cell lines and in human prostate tumours (Davila et al. 2003, Yoshioka et al. 2003).

PAKs are evolutionarily highly conserved regulators of the actin reorganization and cytoskeleton (Bagrodia and Cerione 1999, Kiosses et al. 1999, Master et al. 2001, Bokoch 2003, Hofmann et al. 2004). Two groups; PAK A (PAK1-PAK3) and PAK B (PAK4-PAK6) have been described in mammals. PAK1 has a well-established role in the regulation of cell growth, cytoskeletal organization, and in the nucleus, it is associated

with the chromatin and modulation of transcription (Singh et al. 2005, Kumar et al. 2006). PAK1-PAK3 can be activated by Rac and Cdc42. PAKs are involved in cell ruffling, lamellipodial extension and the formation/ breakdown of FAs via the PAK/ LIMK/ MLC/ cofilin pathway, and they can also block the reconstruction of stress fibers by the inhibition of a RhoGEF (Manser et al. 1997, Edwards et al. 1999, Alberts et al. 2005, Dharmawardhane et al. 1997, Daniels and Bokoch 1999). In addition, activation of PAK1 via its effector LIMK leads to phosphorylation of cofilin on Ser-3 *in vitro* (Edwards et al. 1999, Maekawa et al. 1999, Ohashi et al. 2000, Amano et al. 2001).

The activity of PAKs is regulated by protein kinase B (PKB/Akt), caspase-3 cleavage, various G-protein coupled receptors, tyrosine kinase receptors, and cytokine receptors (Knaus et al. 1995, Zhang et al. 1995, Kjøller and Hall 1999, Bokoch et al. 1998, Tang et al. 2000, King et al. 2000, Papakonstanti and Stournaras 2002). LIMK (Edwards et al.1999), myosin light chain kinase (MLCK) (Sanders et al. 1999, Goeckeler et al. 2000), MLC (Chew et al. 1998, Zeng et al. 2000), Op18/stathmin (Wittmann et al. 2004), Arp2/3 (Vadlamudi et al. 2004), filamin (Vadlamudi et al. 2002), and cortactin (Webb et al. 2006) are target proteins of PAK1. In addition, PAK1 can stimulate mitogen-activated protein kinase (MAPK), nuclear factor κB (NFκB), and c-Jun N-terminal kinase (JNK) pathways (Kumar et al. 2006). Increased PAK expression has been observed in several human malignancies (Cater et al. 2004, Kumar et al. 2006, Vadlamudi et al. 2000, Adam et al. 2000). In fibroblasts, PAK1 concentrated at the leading edge can regulate lamellipodial extension and directionality (Sells et al. 2000), but also the dynamics of focal adhesions (Manser et al. 1997, Nayal et al. 2006).

2.1.4.2.3 Focal adhesion kinase (FAK)

The rearrangement of the actin cytoskeleton is induced by the modifications of FAK and FAs. FAK, identified in 1992, is a ubiquitous integrin binging non-receptor tyrosine kinase, playing a significant role in integrin/cell adhesion/focal adhesion -related signal transduction pathways (Rodriguez-Fernandez 1999). FAK is regulated by the phosphorylation at the C-terminal domain and complex formation with integrins, paxillin and Src (Burridge et al. 1992, Cary et al. 1996, Gabarra-Niecko et al. 2003, Hsia et al. 2003, Parsons 2003, McLean et al. 2005, Mitra et al. 2005). In cell protrusions, coordinated changes in actin and microtubule structures are regulated by FAK via the Rho-GTPase pathway. Moreover, FAK can bind and phosphorylate GTPase activating proteins (GAPs), and guanine nucleotide-exchange factors (GEFs) for Rho, and neuronal Wiskott–Aldrich syndrome protein (N-WASP). FAK can also exert control over actin crosslinking by phosphorylation of ∝-actinin, and can be associated with the extracellular signal-regulated kinase 2 (ERK2)/MAPK cascade.

On the other hand, the inhibition of FAK can also decrease tyrosine phosphorylation of the p190Rho GTPase activating protein (GAP), and elevate the level of GTP-bound Rho (Playford et al. 2008). The activation of FAK/PI-3 kinase/Cdc42/Rac1 related signaling pathway can trigger actin reorganization, and regulate both cell proliferation and motility

(Kallergi et al. 2007). Activated FAK can interact with various Src-homology (SH2/ SH3)-containing proteins and induce phosphorylation of downstream proteins, for example, paxillin (Burridge et al. 1992, Cary et al. 1996). FAK activation is triggered by a variety of intercellular and intracellular stimuli, including integrins, Src-induced tyrosine phosphorylation, growth factors, steroid hormones, cytokines, and neuropeptides (Koukouritaki et al. 1999, Rodriguez-Fernandez 1999, Wennerberg et al. 2000, Schaller 2001). Overexpression and/or increased activity of phosphorylated FAK is associated with various human cancer cell lines and cancers (Ilic et al. 1995, Tremblay et al. 1996, Jones et al. 2000, Hsia et al. 2003, Lark et al. 2005).

2.1.4.2.4 Paxillin

Paxillin is a 68 kD FA-related integrator protein associated within the platform between the actin cytoskeleton and cytoplasmic integrin domains localized at FAs (Turner et al. 1990, Turner 2000). Paxillin is at the crossroad of the cell adhesion and mechanochemical stimuli in modulating/triggering intracellular signal transduction and consequent changes in FA, actin filament dynamics, cytoskeleton, cell shape, and cell motility (Turner 2000, Brown and Turner 2004, Hu et al 2006). It is activated by the induction of adhesion, integrin ligation, and by the activation of FA, FAK and Src induced phosphorylation at Tyr 118 and Tyr 31 residues (Bellis et al. 1995, Schaller and Parsons 1995), and in some cases, also at Ser/Tyr 188/190 residues (De Nichilo and Yamada 1996, Bellis et al. 1997). Moreover, various growth factors; EGF, IGF-I and VEGF can induce paxillin phosphorylation (Abedi and Zachary 1997, Butler et al. 1997, Tapia et al. 1999) or dephosphorylation (Ishiki et al. 1997, Guvakova and Surmacz 1999). Furthermore, protein kinase A (PKA) can inactivate RhoA which, in turn, dephosphorylates paxillin (Han and Rubin 1996, Lim et al. 1996, Howe and Juliano 2000, Ellerbroek et al. 2003). During the induction of the migration process, paxillin is clustered in FAs with integrins, FAK and talin as a complex responsible for the formation of the leading edge (Turner et al. 1990, Brown and Turner 2004, Tilghman et al. 2005). The paxillin-FA complex remains in the leading edge as the cell migrates, and detaches when it reaches the cell rear (Hu et al. 2006). It is proposed, that paxillin regulates p190RhoGAP activity, and inhibits Rho expression at the leading edge, thus promoting lamellipodial extension (Kulkarni et al. 2000). In addition, paxillin may also play an important role in regulating Cdc42 activity and, hence, cell polarity. Integrin - mediated tyrosine phosphorylation of paxillin can activate Rac via a Crk-Cas-Dock180 signaling pathway leading into the lamelliopdial extension and enhanced migration (Brugnera et al. 2002, Iwasaki et al. 2002).

2.1.5 The mevalonate pathway in cell motility and invasion

2.1.5.1 Structure and importance

The mevalonate pathway is a cholesterol synthesis pathway producing also intermediates and end -products for the post-translational isoprenylation of small GTPases (Ras-, Rho- and Rab-families), nuclear lamins, transducin γ and rhodopsin kinase (Casey 1992). Isoprene units are incorporated into the sterol and nonsterol compounds cholesterol,

dolichol, ubiquinone, isopentenyladenine, geranylgeranyldiphosphate (GGPP, C_{20}) and farnesyldiphosphate (FPP, C_{15}) (Goldstein and Brown 1990, Jackson et al. 1997). FPP is an unsaturated carbon chain, which can be converted from mevalonate by multiple synthesis steps. FPP is a precursor of several end products, such as cholesterol, heme A, dolichols, and ubiquinones, and GGPP can be also synthesized from FPP (Edwards and Ericsson 1999).

Isoprenylation is an essential step in the modification of GTPases and ensures their proper activation and localization into the correct cellular membranes. Isoprenylation of GTPases is catalysed by farnesyltransferase (FTase) and geranylgeranyl-transferases I (GGTase I) and II (GGTase II) (Casey 1992). Small GTPases are crucial in the regulation of cytoskeleton organization/dynamics, membrane vesicle transport and various critical signal transduction pathways (Goldstein and Brown 1990, Casey 1992, van Beek et al. 1999, Shack et al. 1999). The blockade of the rate-limiting step of the mevalonate pathway by the inhibitor of HMG-CoA reductase (HMG-CoA reductase catalyses the conversion of HMG-CoA into mevalonate) suppresses the level of mevalonate and its downstream products, and significantly influences critical cellular functions (Goldstein and Brown 1990, Chan et al. 2003). Statins inhibit the HMG-CoA reductase and block the mevalonate pathway and consequently, the synthesis of cholesterol, but also the isoprenylation of small GTPases.

2.1.5.2 Inhibitors of the mevalonate pathway

The role of the mevalonate pathway as a target of cancer therapy (Andela et al. 2003), and the antitumor properties of statins, inhibitors of farnesyl transferase (FTIs) and geranylgeranyl transferases (GGTIs), have been investigated, and research is focused mostly on statins and FTIs (Brunner 2003). Statins have antimetastatic properties in *in vitro*-experiments and, for example, lovastatin and simvastatin reduce expression of MMP-9 in NIH-3T3 cells (Wang et al. 2000), and in monocytes (Wong et al. 2001). Cerivastatin suppresses expression of u-PA, u-PA receptor (u-PAR), and MMP-9 in monocytes (Ganné et al. 2000). Contrary to that, MMP-2 and MMP-9 activity and uPA secretion are not reduced in F3II mammary carcinoma cells by lovastatin (Alonso et al. 1998). On the other hand, lovastatin reduces the adhesion of lymphoma cells, F3II mammary carcinoma cells and melanoma cells to ECM (Matar et al. 1999, Alonso et al. 1998, Jani et al. 1993), and the invasion of lymphoma cells (Matar et al. 1999), human glioma cells (Prasanna et al. 1996), melanoma cells (Jani et al. 1993) and NIH-3T3 cells (Wang et al. 2000) *in vitro*. Fluvastatin and cerivastatin block the invasion of pancreatic cancer cells, colon cancer cells and breast cancer cells (Kusama et al. 2001, Denoyelle et al. 2001). Moreover, migration of F3II mammary carcinoma cells (Alonso et al. 1998), human monocytic THP-1 cells (Wong et al. 2001), endothelial cells (Vincent et al. 2001) and melanoma cells (Jani et al. 1993) can be reduced by lovastatin, simvastatin and cerivastatin.

In vivo-experiments also suggest that lovastatin decreases the dissemination of F3II mammary carcinoma cells to the lungs (Alonso et al. 1998). Colon-derived MCA-26

cancer cells establish less liver metastasis in lovastatin-treated mice (Jani et al. 1993), and the number of lymph node metastases is decreased significantly by lovastatin treatment in rats with lymphoma (Matar et al. 1999). Similarly, lovastatin and simvastatin treatments decrease experimental lung metastasis in mice inoculated with B16F10 melanoma cells and BN472 mammary adenocarcinoma cells (Kort et al. 1989, Jani et al. 1993). Fluvastatin treatment in mice injected with pancreatic tumour cells can markedly reduce the number and size of metastatic nodules in the liver (Kusama et al. 2002).

The function of Rho and ROCK can also be blocked by the selective commercial inhibitors C3 and Y-27632, respectively. Y-27632 inhibits two isoforms, ROCK I and ROCK II (Uehata et al. 1997). In addition, C3 and Y-27632 suppress the MLC2 phosphorylation, and cell polarization, membrane blebbing and migration of carcinoma cells (Gutjahr et al. 2005). Y-27632 also inhibits the spreading of several animal cancers (Itoh et al. 1999), as well as the migration and *in vivo* dissemination of PC-3 cells (Somlyo et al. 2000), and the invasion of non-small-cell lung cancer cells (Hakuma et al. 2005). C3 reduces the EGF-induced invasion of pancreatic cancer cells (Kusama et al. 2001, Kusama et al. 2002), and migration of THP-1 monocytes (Wong et al. 2001). Another potent ROCK-inhibitor, Fasudil hydrochloride (HA-1077), is also a selective inhibitor of ROCK II (Niggli 1999, Davies et al. 2000).

2.1.5.3 Small GTPases

2.1.5.3.1 Ras GTPase family

The Ras subfamily consists of 18 isoforms of Ras G-proteins (e.g H-Ras, N-Ras, Ki-Ras), which are mutated in more than 10% of human cancers (Coleman et al. 2004). The activation of Ras GTPase links extracellular mitogenic signals with numerous intracellular pathways, which control cell proliferation, growth and differentiation (Goldstein and Brown 1990, Leone et al. 1997, Sridhar et al. 2005). Malignant activity of Ras is induced by mutations supressing GTP-hydrolysing activity and the protein will remain locked in the active state. Ras proteins can be also hyperactivated by the expression/activating mutation of growth factor receptors upstream of the Ras-guanine nucleotide exchange factors (Ras-GEF), or by loss-mutation of the Ras-GAP (Dasgupta and Gutmann 2003). FTIs were initially designed to block Ras farnesylation. Indeed, several observations suggest that FTI-induced effects are mediated obviously by the inhibition of Ras proteins (Cox et al. 1994, Sun et al. 1995, Rose et al. 2001). On the other hand, cells expressing Ras-F are more sensitive to FTI treatment than cells expressing myr-Ras or Ras-GG, which are not affected by FTI treatment (Cox et al. 1994, Reuveni et al. 2000, Rose et al. 2001).

K-Ras and N-Ras, but not H-Ras, are known to be geranylgeranylated in the absence of farnesylation (Whyte et al. 1997).Feldkamp et al. (2001) have reported that the efficacy of FTI treatment can be predicted according to H-, K-, and N-isotype-specific Ras-GTP levels in astrocytoma cells. High levels of H-Ras-GTP and low levels of K-, and N-Ras-GTP were found to be predictive for FTI sensitivity. Despite the possible alternative

geranylgeranylation of both K-Ras and N-Ras, in the absence of farnesylation, malignant growth of cells can be inhibited by FTIs (Sepp-Lorenzino et al. 1995, Sun et al. 1995, Lerner et al. 1997, Whyte et al. 1997, Mangues et al. 1998, Servais et al. 1998).

2.1.5.3.2 Rho GTPase family

Rho GTPases are a group of 20-30 kD size G-proteins. The first Rho coding gene was identified and cloned from *Aplysia* in 1985. Since then, eleven genes encoding mammalian Rho family members and homologue genes have been identified (Bush et al. 1993, Hall 1994, Luo et al. 1994, Hariharan et al. 1995, Foster et al. 1996, Larochelle et al. 1996). The mammalian Rho-family currently consists of 21 members, including Rho (A, B, C), Rac (1,2,3) and Cdc42 (Cdc42Hs, G25K), Rnd1/Rho6, Rnd2/Rho7, Rnd3/RhoE, RhoD, RhoG, RhoBTB (1,2), TC10, TCL, Chp (1, 2), Rif, TTF.

RhoA, Rac-1 and Cdc42 are the best-characterized Rho GTPases so far (Ridley 2001, Coleman 2004) and in the active state, they are able to interact with over 60 target/effector proteins. Generally, all Rho GTPases are prenylated at their C-terminus, but Rnd proteins are the exception; they do not hydrolyse GTP *in vitro*, which is an unusual property of regulatory GTPases. Rho, Rac and Cdc42 regulate three separate signal transduction pathways linking plasma membrane receptors to the variety of critical cellular events and the assembly of distinct filamentous actin structures in a variety of mammalian cell types, as well as in yeast, flies and worms (Hall 1994, Aznar and Lacal 2001, Coxon and Rogers 2003). Not much functional data are currently available from the other 13 members of the mammalian Rho GTPase family (Etienne-Manneville and Hall 2002).

Increasing amount of evidence indicates the important role of Rho family GTPases in epithelial cell migration and cancer cell invasion (Yoshioka et al. 1999, Schmitz et al. 2000). It is already well established that Rho GTPases are the key regulators of actin cytoskeleton/stress fiber assembly/dynamics of actin and cell invasion (Ridley 2001, Raftopoulou and Hall 2004, Riento and Ridley 2003), as well as carcinoma and sarcoma cell polarizarion and migration (Etienne-Manneville and Hall 2002, Raftopoulou and Hall 2004, Gutjahr et al. 2005). In general, RhoA regulates the formation of actin stress fibers and mediates contractility, Rac regulates lamellipodium formation and membrane ruffling, and Cdc42 is involved in the formation of filopodium (Hall 1994, Machesky and Hall 1996, Nobes and Hall 1999). In addition, RhoA is also involved in the formation and maintenance of focal adhesions (Craig and Johnson 1996). Furthermore, Rac-1 and Cdc42 can regulate the organization of smaller focal complexes associated with lamellipodia and filopodia (Machesky and Hall 1996).

A rate-limiting target for protein prenylation necessary for PC-3 cell invasion and migration has not yet been identified, but the invasive PC-3 cells have been reported to express more Rho A than less invasive cancer cell lines (Somlyo et al. 2000, Sahai and Marshall 2002). It has been reported that advanced breast cancers overexpress Rho C (van Golen et al. 2002), and particularly Rho A is overexpressed in various human

tumors (Fritz et al. 1999), while RhoG regulates cell migration in HeLa cells (Katoh et al. 2006). In addition, Rho C has been shown to be critical for the invasive phenotype of PC-3 cells (Yao et al. 2006) and recently, Rho GTPases were proposed to be important in the regulation of PC-3 cell morphology, invasion and tumor cell diapedesis (Sequeira et al. 2008). Moreover, activation of Rac seems to be associated with hepatocellular carcinoma metastasis (Lee et al. 2006).

2.1.5.3.3 Rab GTPase family

The Rab GTPase family is the largest group of membrane trafficking proteins prevalent in eukaryotic cells. 11 Rab isoforms in budding yeasts, 29 in *Caenorhabditis* elegans and in *Drosophila melanogaster*, and more than 60 in humans and mice have been characterized so far (Bock et al. 2001, Pereira-Leal and Seabra 2001, Fukuda 2003). Five Rab isoforms; Rab1/Ypt1, Rab5/Ypt5, Rab6/Ypt6, Rab7/Ypt7, and Rab11/Ypt31 are conserved from yeasts to humans, and 17 Rab isoforms are shared by *Caenorhabditis elegans*, *Drosophila*, and humans. By contrast, other Rab isoforms are mostly vertebrate- or mammalian-specific, with possible unique assignment in the cell-type-specific or tissue-specific membrane trafficking events, but their exact role and specific functions are poorly understood. The active GTP-bound form of Rabs is recruited in the transport of vesicles/organelles, promotion of their trafficking; vesicle budding, vesicle motility, vesicle fusion/docking to specific membranes and interactions with specific effector molecules (Pfeffer 2001, Segev 2001, Zerial and McBride 2001).

The best characterized isoforms are Rab3 subfamily proteins, while their role in vesicle localization (members Rab3 A/B/C/D in humans and mice) and involvement in the regulation of vesicle secretion is established (Takai et al. 1996, Geppert and Südhof 1998), and also other Rab isoforms are proposed to be critical (Riedel et al. 2002, Schlüter et al. 2004, 2006). For instance, the Rab27 subfamily members seem to have a role in association with secretory vesicles, and Rab27-deficient animals display secretion defects in certain secretory cells (Wilson et al. 2000, Gomi et al. 2007, Mizuno et al. 2007, Tolmachova et al. 2007). Rab1, Rab2, Rab5, Rab21 and Rab27A/B proteins are also present in a variety of secretory vesicles in mice (Tolmachova et al. 2004, Takamori et al. 2006, Brunner et al. 2007, Casey et al. 2007, Gomi et al. 2007, Rindler et al. 2007, Tolmachova et al. 2007). Mutations in the RAB27A gene cause a human hereditary disease (type 2 Griscelli syndrome, corresponding murine model ashen), and both type 2 Griscelli syndrome patients and ashen mice have defects in lytic granule exocytosis in cytotoxic T lymphocytes (CTLs) (Wilson et al. 2000). Rab27B knockout mice and Rab27A/B doubleknockout mice exhibit secretion defects in specific secretory cells (Gomi et al. 2007, Mizuno et al. 2007, Tolmachova et al. 2007). In addition, reduced expression of Rab3A and Rab27A seem to be associated with defective insulin release in type 2 diabetes (Abderrahmani et al. 2006).

Interestingly, the Rab11 pathway has been demonstrated to contribute to cell migration, and KO- manipulations disrupt the migration of several cell types (Fan et al. 2004, Powelka et al. 2004, Yoon et al. 2006). Rab11 is also upregulated during skin

carcinogenesis (Gebhardt et al. 2005), and it is connected also to Barrett's dysplasia (Goldenring et al. 1999), and hypoxia-promoted invasive transmigration of carcinoma cells through matrigel (Yoon et al. 2005). Rab25 (or Rab11c), which shows highly restricted expression under normal physiological conditions, is upregulated in invasive breast cancer cell lines and in metastatic tumor cells (Wang et al. 2004). It is also linked to the aggressiveness of breast and ovarian cancer (Cheng et al. 2004a). It is likely that these tumorigenic effects of Rab11 (and obviously also Rab25) are due to their ability to directly control integrin trafficking (Ivaska et al. 2002).

Many integrins accumulate before returning to the cell surface in a Rab11-dependent manner (Roberts et al. 2001, Roberts et al. 2004, Strachan and Condic 2004, Skalski and Coppolino 2005, Tayeb et al. 2005), and the recycling of αvβ3, α5β1 and α6β4 integrins requires interaction with PKB/Akt (Jackson et al. 2000, Roberts et al. 2004, Li et al. 2005, Yoon et al. 2005). Protein kinase C (epsilon) (PKCε) can phosphorylate residues at the N-terminus of vimentin and release the β1-integrin transporting vesicles from the vimentin related intermediate filaments in the perinuclear region. Recycling of integrins to the plasma membrane is triggered, and this also enables β1-integrin -dependent haptotaxis. Furthermore, PKCε/vimentin related mechanisms seem to have selectivity toward integrin transport (Ivaska et al. 2005). Moreover, Rab11 regulated pathways seem to have a role in haptotaxis and the invasion/migration of cancer cells, whereas the Rab4 route contributes specifically to the spatial polarisation of surface αvβ3 during persistent directional cell migration.

2.1.5.4 Prenylation of small GTPases

Newly synthesised small GTPases are soluble cytosolic proteins, which undergo a series of post-translational modifications enabling their association with the appropriate lipid membranes. The mevalonate synthesis pathway includes three specific transferase proteins, which catalyze C-terminal lipidation of over 100 proteins, including the covalent addition of FPP (15-carbon farnesyl), or GGPP (20-carbon geranylgeranyl) isoprenoids to C-terminal cysteine residue ('CAAX' peptide motif) prevalent to GTPases (Schmidt et al. 1984, Casey 1992, Zhang and Casey 1996). Transferases are heterodimeric proteins and composed of α- and β-subunits (Taylor et al. 2003). GGTase-I and FTase share a common α subunit, but have unique β subunits, which dictate their substrate specificity. Inactivation of the gene for the critical β subunit of GGTase-I has been shown to eliminate GGTase-I activity, disrupt the actin cytoskeleton, reduce cell migration, and block the proliferation of fibroblasts expressing oncogenic K-Ras (Sjogren et al. 2007).

FTase catalyzes mainly the prenylation of the Ras GTPase family, GGTase I catalyzes prenylation of the RhoGTPases (Rho, Rac1, Cdc42, Rap1A) and GGTase II catalyzes prenylation of the Rab GTPase family. In addition, four mammalian Ras proteins, H-,N-, KA- and KB-Ras, require farnesylation in order to trigger malignant transformation. On the other hand, Rho-B can be geranylgeranylated or farnesylated and R-Ras, Ki-Ras and

TC-21, which are the promoters of tumorigenesis and/ or metastasis, are all geranyl geranylated (Barbacid 1986, 1987, Casey 1992, Reiss et al. 1990, Zhang and Casey 1996, Zohn et al. 1998) (Fig.3).

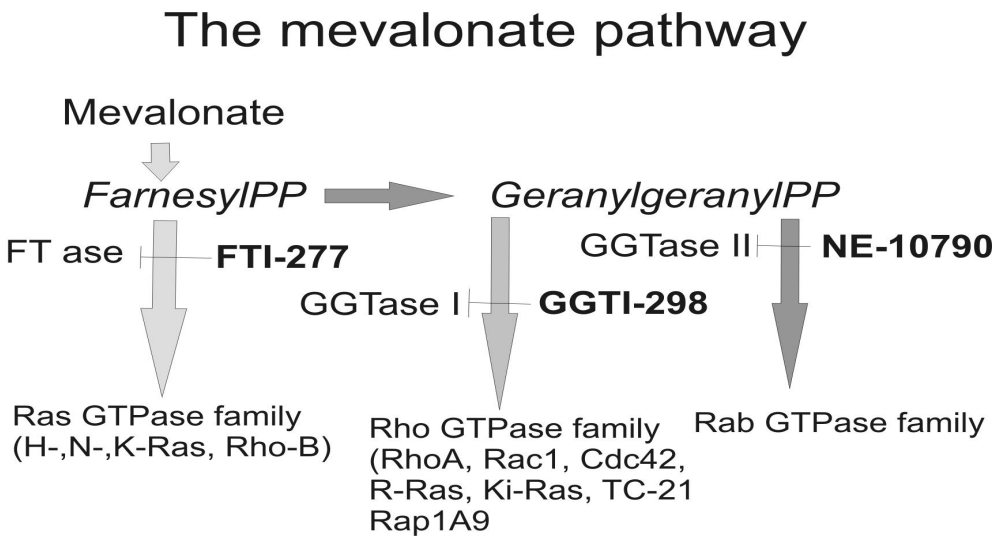

Figure 3. Inhibitors of prenylation in the mevalonate pathway. FTI-277 inhibits the FTase, GGTI-298 inhibits GGTase I and NE10790 inhibits GGTase II (modified from study II, Virtanen et al. 2010).

Prenylation transferases can be blocked by selective inhibitors (Lebowitz et al. 1997). The function of the FTase can be blocked by FTI-277 (Lerner et al. 1995). The GGTase I can be blocked by GGTI-298 (McGuire et al. 1996, Kusama et al. 2003) and the GGTase II can be inhibited by NE10790 (Coxon et al. 2001).

2.1.5.5 Activation of small GTPases

Another way to regulate the activity of small GTP-binding proteins is alternation between an inactive GDP-bound state and an active GTP-bound state. The cycling between inactive and active states allows GTP-binding proteins to act as molecular switches. GTPases are normally in the ‘off’ position until information from upstream signaling pathways promotes a change to the ‘on’ position. GTP is hydrolyzed by the intrinsic GTPase activity of the protein and the switch is returned to the ‘off’ position. Switches between the two stages are regulated by GEFs, which promote the activation of the protein (GDP-GTP exchange) and GAPs, which promote the inactivation of protein by inducing GTP hydrolysis to GDP. In mammalian cells, 60 different GEFs can catalyse the nucleotide exchange and the activation of GTPases. In addition, more than 70 GAPs can in turn stimulate GTP hydrolysis, leading to the inactivation of small GTPases. Four guanine nucleotide exchange inhibitors (GDIs) can, in turn, detach the inactive GTPases from membranes. When the protein is in the active state (GTP-bound), it can interact

with the effector protein and propagate further signaling events, leading to the desired biological responses (Walker and Olson 2005) (Fig. 4).

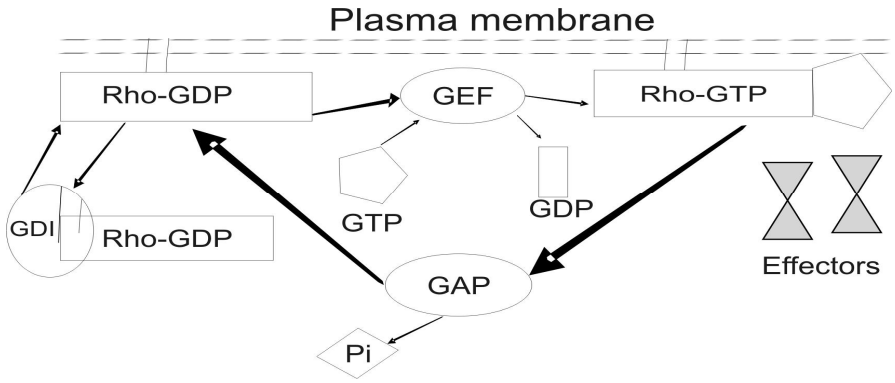

Figure 4. The activation/inactivation process of small GTPases. The GTP-bound form is active and the GDP-bound form is inactive (Modified from Etienne-Manneville and Hall 2002).

2.2 Bisphosphonates (BPs)

2.2.1 Bisphosphonate stucture

The first BP-compounds were initially synthetized in the late 19th century as a raw material for the rubber industry. Since the 1930s, it was realized that the trace amounts of polyphosphates were capable to soften water and inhibit crystallization of calcium salts, such as calcium carbonate (Fleisch and Neuman 1961). Fleisch et al. (1962, 1966) demonstrated that when inorganic pyrophosphate, a naturally occurring polyphosphate and a byproduct of various biosynthetic reactions, was present in serum and urine, it was able to prevent the calcification by binding to the newly synthetized crystals of the hydroxyapatite. Later BPs were also used as additive compounds in toothpastes. Observations of the ability to inhibit bone resorption opened a wide range of possibilities to utilize BPs as therapeutics for various metabolic bone diseases (Mundy 1999a, Rogers et al. 2000).

BPs are synthetic analogues of inorganic pyrophosphates, where the instabile oxygen bridge has been replaced by a carbon atom. The P-C-P backbone structure makes them highly stabile and enables them act as calcium-ion chelators. Interestingly, structure analogues which include more than one carbon atom are inert (Fleisch et al. 1970). The 3-dimensional structure of BPs can bind effectively Ca^{2+}-, Mg^{2+}- and Fe^{2+} -ions which also enables rapid and effective adhesion into the bone hydroxyapatite (Rogers et al. 2000) with only $\leq$1% of BPs remaining in the circulation (Lin 1996). In addition, in rats, BPs can transiently accumulate into the prostate before binding to bone (Fournier et al. 2002). BPs are negatively charged, watersoluble molecules, which are poorly absorbed from the GI-tract and they do not pass through cell membranes. The P-C-P-backbone structure contains two side groups (R1, R2), and the composition of the side groups

dictates their potency to inhibit osteoclastic bone resorption (Rodan and Balena 1993, Hiraga et al. 1996, Rodan 1998) (Fig.5).

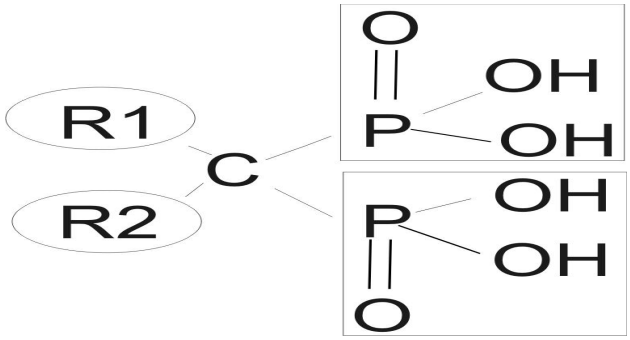

Figure 5. The basic structure of bisphosphonates. Phosphate groups act as 'hooks' which enable binding to hydroxyapatite and biochemical activity. The OH group in R1 enhances binding to bone, and R2 composition determines the antiresorptive potency (Modified from Russell 2007).

In general, two main groups of BPs have been described: **1) pyrophosphate-resembling bisphophonates** are the first generation bisphosphonates, and **2) nitrogen-containing bisphosphonates (N-BPs)** are compounds, which contain the amino group in the R2 side chain (Benford et al. 1999). The amino group in R2 increases the potency to inhibit the resorption even as much as 10 000 times compared to the first generation BPs (Muhlbauer et al. 1991, Russel et al. 1999) (Fig.6).

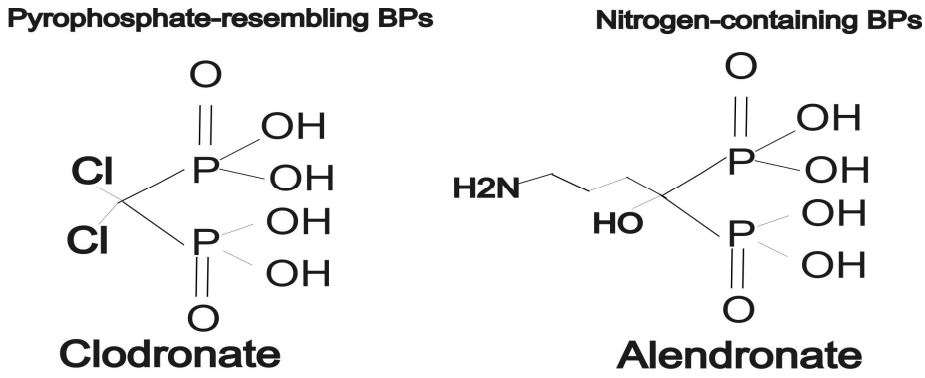

Figure 6. Stuctures of bisphosphonates used in this study (Modified from Russell 2007).

The first synthetized BPs were etidronate and clodronate, which had initially indication to malignant hypercalcemia, myeloma and related hypercalcemia, *fibrodysplasia*

ossificans progressiva and Paget's disease (Ryzen et al. 1985, Siris et al. 1980, Paterson et al. 1983). Later, clodronate was approved as a treatment for osteoporosis in the EU.

2.2.2 Alendronate

Alendronate (4-amino-1-hydroxybutylidene-1,1-bisphosphonate) is one of the most prescribed therapeutics for osteoporosis with established molecular targets. Inhibition of the mevalonate pathway by inducing apoptosis, impairing the activity of Rho and Rab GTPases (Alakangas et al. 2002, Sawada et al. 2002, Denoyelle et al. 2003), and suppressing the membrane localization of Ras in prostate cancer cells have been reported (Oades et al. 2003, Suri et al. 2001). Alendronate has also been shown to inhibit the secretion of MMPs by prostate cancer cells (Stearns et al. 1998), inhibit invasion and MMP-2 secretion by osteosarcoma cells (Cheng et al. 2004b), and the activity of MMPs *in vitro*, but the latter is evident only with high concentrations (Boissier et al. 2000). Alendronate can reduce the primary tumor growth of breast cancer tumors in mice (Sasaki et al 1995), suppress tumor angiogenesis by inhibiting Rho in endothelial cells (Hashimoto et al. 2007), and can inhibit dissemination of ovarian cancer cells *in vivo* (Hashimoto et al. 2005). It can also oppose the growth of orthotopic PC-3 tumors and decrease metastases to prostate-draining lymph nodes, at least partly by decreasing angiogenesis and inducing apoptosis in prostate tumors *in vivo* (Tuomela et al. 2008). More recently, changes in F-actin organization, associated with morphology changes and inhibition of migration of osteosarcoma cells, have been reported (Molinuevo et al. 2007).

2.2.3 Effects of bisphosphonates in osteoclasts

Osteoclasts use acid phosphatases in the resorption process and when the pH reaches 3.5, BPs detach from the bone and local concentrations could rise even to 1 mM at the resorption pit (Sato et al. 1991, Masarachia et al. 1996). Osteoclasts are endocytotic cells and detached BPs are transported into the cell mainly by phagosytosis (Felix et al. 1984). Pyrophosphate-resembling BPs affect glycolysis, the formation of lactate and the oxidation of fatty acids in osteoclasts (Felix and Fleisch 1981). They are metabolically converted into nonhydrolyzable methylene-containing ATP analogs (AppCp) by reversing the reactions of aminoacyltransfer RNA synthase. Active metabolites contain a P-C-P moiety in β,γ-phosphate group, which makes them nonhydrolyzable (Rogers et al. 1994, Frith et al. 1997, Frith et al. 2001). The intracellular accumulation of nonhydrolyzable metabolites in osteoclasts inhibits their function and causes eventually apoptosis (Muhlbauer et al. 1991, Selander et al. 1994, Hughes at al.1995, Selander et al. 1996, Frith et al. 1997). The AppCp-type metabolites are cytotoxic also when they are internalized and they can induce similar changes in cell morphology than clodronate treatment, interferencing with mitochondrial ATP translocases (Lehenkari et al. 2002). Generally, pyrophosphate-resembling BPs seem to act as prodrugs, converted to the active metabolites after intracellular uptake. Accumulation of these metabolites is the main cause of growth inhibition and the cytotoxic effects in osteoclasts (Muhlbauer et al. 1991, Selander et al. 1994, Frith et al. 1997).

At a cellular level, N-BPs disrupt the osteoclastic actin ring structure (Hiroi-Furuya et al. 1999) and inhibit vesicular trafficking, and the formation of the ruffled border characteristic for the active resorbing osteoclasts (Sato et al. 1990, Alakangas et al. 2002). They suppress osteoclast adhesion onto the bone (Colucci et al. 1998), their differentiation and maturation (Boonekamp et al. 1986, Hughes et al.1989), and also proliferation and differentiation of bone-forming osteoblasts (Reinholz et al. 2000). At the molecular level, N-BPs suppress the activity of specific enzymes, for example farnesyl diphosphate-synthase (FPPS), involved in the mevalonate pathway (Benford et al. 1999, van Beek et al. 1999, Bergstrom et al. 2000, Coxon et al. 1999, Dunford et al. 2001, Martin et al. 2001, Thompson et al. 2002). Also, evidence for the geranylgeranyl diphosphate synthase (GGPPS) inhibition by N-BPs has been proposed (Goffinet et al. 2006). In addition, BPs decrease the activity of hydrolytic enzymes in osteoclasts and thus can impair bone resorption and inhibit vacuolar ATPase activity at the ruffled border (Lerner and Larsson 1987, David and Baron 1995). The activation of caspase-3-like proteases via the mevalonate pathway is suggested to be a critical mechanism in the induction of apoptosis in osteoclasts (Hughes et al. 1995, Benford et al. 1999). Moreover, N-BPs can also inhibit the prenylation of geranylgeranyl-groups in osteoclasts (Fisher et al. 1999, van Beek et al. 1999, Bergstrom et al. 2000, Coxon et al. 2000, Dunford et al. 2001, Thompson et al. 2002).

2.2.4 Effects of bisphosphonates in cancer cells

There are relatively few studies concerning the effects of pyrophosphate-resembling BPs on cancer cells. Etidronate has shown cytotoxic effects in breast cancer cells (Zhou et al. 2008) and clodronate, due to the induction of apoptosis, has toxic effects in macrophages (Selander et al. 1996, Frith et al. 1997). In addition, clodronate has pro-apototic effects also in breast cancer cells (Mönkkönen et al. 2008).

During the last decade, more effort has been directed to study the specific effects of N-BPs in cancer cells. The first reports concerning the direct effects on cancer cell lines were published by van der Plujim et al. (1996), and Boissier et al. (1997). They showed that N-BPs can reduce the adhesion of breast and prostate cancer cells *in vitro* on ECM proteins and bone. The inhibition of invasion and migration of breast and prostate cancer cells *in vitro* was repoted later by Boissier et al. (2000). The reduction of primary tumor progression, inhibition of growth and proliferation of breast and prostate tumor cells (Sasaki et al. 1995, Lee et al. 2001) and non-small-cell lung cancer cells (Li et al. 2008) have also been reported. More recent studies have demonstrated that N-BPs can inhibit adhesion of prostate cancer cells to a mineralized matrix and induce apoptosis (Coxon et al. 2004, Matsunaga et al. 2007), decrease migration and invasion of osteosarcoma cells (Cheng et al. 2004b, Molinuevo et al. 2007) and block the dissemination of ovarian cancer cells *in vivo* (Hashimoto et al. 2005). Furthermore, N-BPs induce breast and prostate tumor cell apoptosis *in vitro* (Fromique et al. 2000, Hiraga et al. 2001, Oades et al. 2003, Matsunaga et al. 2007), and inhibit the growth of breast cancer cells (Merrell et al. 2007). Zolendronic acid inhibits visceral metastases in the mouse breast cancer model *in vivo* (Hiraga et al. 2004) and with high concentrations, N-BPs have been reported to

induce apoptosis in macrophages *in vitro* and *in vivo* (Hughes et al.1995, Luckman et al. 1998). Furthermore, a new BP-analogue minodronate has been reported to inhibit osteoblastic prostate cancer tumor formation, proliferation and the progression of prostate tumors (Yonou et al. 2007).

Complex evidence has been provided concerning the molecular effects of N-BPs in various cell lines. Dephosphorylation of FAK and the inhibition of activity/secretion of MMPs and the release of type I collagen in prostate cancer cells have been reported (Stearns and Wang 1998, Boissier et al. 2000, Matsunaga et al. 2007). Also, the inhibition of integrin expression in endothelial cells (Bellahcéne et al. 2007), the reduction of VEGF secretion in squamous cell carcinoma cells (Wypij et al. 2008) and the induction of the p38 pathway in breast cancer cells and macrophage-like J774 cells (Merrell et al. 2007) have been reported. N-BPs can also inhibit neuroblastoma growth by reducing angiogenesis (Bäckman et al. 2008), suppressing MMP-9 expression in macrophages (Tsagozis et al. 2008) and inhibiting the mevalonate pathway in breast cancer cells (Merrell et al. 2007). Inhibitory effects on the activity of Rho GTPases (Denoyelle et al. 2003, Sawada et al. 2002) and Ras membrane localization in prostate cancer cells have also been reported (Oades et al. 2003). The induction of apoptosis is suggested to be mediated by the inhibition of the mevalonate pathway in Caco-2 human epithelial cells (Suri et al. 2001). In addition, the immunomodulating effects of N-BPs have been proposed recently (Galluzzo et al. 2007, Tsagozis et al. 2008, Caccamo et al. 2008). Mönkkönen et al. (2006, 2008) and Räikkönen et al. (2010) have reported that N-BPs can induce the formation of ATP analogs (ApppIs) via the inhibition of the mevalonate pathway, and in addition, also the induction of apoptosis in osteoclasts, and in breast and prostate cancer cells (Mönkkönen et al. 2008). The suppression of uPA (Iguchi et al. 2007) and PKC in prostate cancer cells by pamidronate has recently been reported by Tatsuda et al. (2010).

2.2.5 Bisphosphonates in clinical use

Clodronate and etidronate were the first bisphosphonates approved as a treatment of malignant hypercalcemia and myeloma. During the last decade, bisphosphonates have had a significant contribution to the prevention of osteoporosis with the reduction of the vertebral fractures of 40-50% and the nonvertebral fractures of 20-40% (Sambrook and Cooper 2006, Coleman 2004). The FDA has approved several BPs for clinical use to prevent osteoporosis. They are alendronate, ibandronate, risedronate and zoledronic acid. In addition, zoledronic acid and pamidronate are approved for cancer-related indications in the USA, and clodronate, pamidronate and ibandronate have received regulatory approval in the EU, as a treatment for patients with breast cancer related secondary bone lesions. Only zoledronic acid is approved for the treatment of the bone metastases independent of the primary tumor (Polascik 2009). Clinical trials evaluating the efficiency of various BPs on bone metastasis related to breast cancer revealed a reduced occurrence of skeletal related events (SREs) with patients treated intravenously with pamidronate, ibandronate or zoledronic acid (Rosen et al. 2003, Kohno et al. 2005, Lipton et al. 2000, Body et al. 2004), but also with clodronate (Paterson et al. 1993,

Kristensen et al. 1999). In Finland, according to The Current Care -recommendations (2007) BPs can be used in advanced breast cancer to decrease bone metastasis-associated pain and pathological fractures (Breast cancer: The Current Care -recommendation 2007).

In spite of the fact that prostate cancer is commonly associated with osteoblastic (osteosclerotic) lesions, skeletal metastases can disrupt the balance of bone metabolism and osteoclasts can be activated. In clinical trials with hormone-refractory prostate cancer (HRPC) patients, only zoledronic acid decreased SREs (Saad 2002, Saad et al. 2002). Pamidronate and clodronate were also evaluated for prostate cancer related SREs with no significant efficacy (Small et al. 2003, Dearnaley et al. 2003). The effect of BPs on SREs associated with other cancer types and solid tumors has been evaluated with zoledronic acid, clodronate and ibandronate. Only zoledronic acid and ibandronate had a significant effect on SREs, and zoledronic acid is so far the only compound which has received worldwide regulatory approval in the treatment of bone mestastases related to solid tumors (Polascik 2009). According to The Current care -recommendations, BPs should be considered to be used in advanced prostate cancer to reduce and prevent bone metastasis -associated pain and SREs (Prostate cancer: The Current care -recommendation 2007).

Recently, various reports have described osteonecrosis of the jaw as a severe side-effect associated with the continuous use of N-BPs (Woo et al. 2005, Nastro et al. 2007). Although occurrence of this side-effect is only 0.1%, critical discussion for the therapeutic safety of this quite well tolerated drug-family should be considered. In Finland, The Current Care -recommendations were updated in order to restrict the continuous usage (exceeding 5 years) of BPs in the prevention of osteoporosis (Osteoporosis: The Current Care -recommendation 2006).

3 AIMS OF THE PRESENT STUDY

The purpose of this study was to investigate the effects of bisphosphonates on prostate and breast cancer cells.

The detailed aims were:

1. To study the effects of two different types of bisphosphonates, clodronate and alendronate, on adhesion, invasion and migration of human prostate and breast cancer cell lines.

2. To study the role of the mevalonate pathway and prenylation reactions in the invasion and the organization of the cytoskeleton in prostate cancer cells.

3. To study the effects of alendronate on the cytoskeletal organization of prostate cancer cells in association with the inhibition of invasion.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Cell lines and the production of a conditioned medium

PC-3, an androgen-independent human prostate carcinoma cell line (ATCC, USA), MDA-MB-231 (San Antonio (SA)), a hormone-independent human highly metastatic breast carcinoma cell line (reviewed in Yin et al. 1999), Du-145, a human prostate cancer cell line (ATCC, USA), and MG-63 osteosarcoma cell line (ATCC, USA), were cultured in DMEM (Invitrogen, USA) containing 10 % inactivated fetal bovine serum (iFBS) (Invitrogen, USA), at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed in DMEM containing 1% BSA (bovine serum albumin) (Sigma-Aldrich, USA). For the collection of the conditioned medium, MG-63 cells were cultured for 10 days in DMEM containing 10% iFBS, and ascorbic acid (0.5 μg/ml) (Merck & Co., USA), and then for 2 days in DMEM containing BSA (0.1%), and ascorbic acid (0.5 μg/ml). The conditioned medium was collected from confluent cultures, centrifuged and frozen. It was used as a bone cell-derived attractant in invasion and migration assays, causing a 2-fold increase when compared with fresh DMEM containing 1% BSA.

4.1.2 Chemical compounds

Alendronate (4-amino-1-hydroxybutylidene-1,1-bisphonic acid) was from Merck & Co. (USA), and clodronate (dichloro-methylene bisphosphonic acid) was from Leiras (Finland). Mevastatin, mevalonic acid lactone, geranylgeraniol (an analogue of geranylgeranyl diphosphate) and trans-trans-farnesol (analogue of farnesyl diphosphate) were from Sigma-Aldrich (USA). The concentrations of BPs and other compounds that had no effects on cell growth were used in additional experiments. Geranylgeranyl transferase inhibitor I (GGTI-298) and farnesyl transferase inhibitor (FTI-277) were purchased from Calbiochem-Novabiochem (USA).

4.2 *In vitro*-models

4.2.1 Cell growth and proliferation

PC-3 prostate cancer cells were plated in 24-well plates, 3 000 cells per well, and cultured for 24 hours in DMEM containing iFBS (10%). The cells were treated with various concentrations of the indicated compounds for 24 hours, washed with DMEM containing 10% iFBS, and cultured for an additional 75 hours without the test compounds. The cells were counted with a Coulter Counter (Coulter Electronics Ltd., England) before and after treatment.

4.2.2 Invasion and migration assays

PC-3 cancer cells were pre-treated with the indicated compounds or DMEM + 1% BSA as a control. Commercial cell culture invasion inserts of 8 μm pore size (Becton

Dickinson, USA) were coated for the invasion assay with Matrigel (30 µg/insert = 100 µg/cm^2; Beckton Dickinson, USA) for 24 hours to prepare an *in vitro* basement membrane. For the migration assay, the inserts were coated with laminin (5 µg/cm^2), which was diluted in DMEM according to the manufacturer's protocol (Beckton Dickinson, USA). The assays were started by placing 50 000 cells in 300 µl of DMEM + 1% BSA in the upper chamber and 300 µl of DMEM + 1% BSA and 300 µl of MG-63-conditioned medium in the lower chamber as a chemoattractant to induce invasion. The cells were incubated for 48 hours in the invasion assay, and for 5 hours in the migration assay at 37 °C and 5% CO_2 , and the insert membranes were prepared for microscopy. The cells and membranes were first fixed for 10 minutes in 4% paraformaldehyde in PBS (J.T. Baker, USA) and stained with Mayer's haematoxylin (Zymed, USA) for 24 hours. After washing, the membranes were cut from the inserts, the cells on the upper surface of the membrane were wiped off with a cotton wool pad, and the membranes were mounted in glycerol-PSB (9:1, Merck, USA). The number of cells on the lower surface of the membrane was counted under the microscope (×10 objective) from 10 consecutive fields, representing 40% of the total area of the membrane. The experiments were repeated three times and each treatment was carried out in triplicate.

4.2.3 Adhesion assay

Bacteriological 96-well plates (Greiner, Germany) were coated with various extracellular matrix proteins [fibronectin (1 µg/cm^2); vitronectin (300 ng/cm^2); laminin (5 µg/cm^2); type I collagen (5 µg/cm^2); and type IV collagen (5 µg/cm^2) (Becton Dickinson, USA)] for 24 hours. PC-3 prostate cancer cells were preincubated with 10 µM alendronate or 1% BSA-DMEM (control) for 24 hours. The cells were then suspended in 0.5% BSA-DMEM at a concentration of 200 000 cells/ml, and adhesion to various extracellular matrix proteins was followed for up to 2 hours. Adherent cells were fixed with 4% paraformaldehyde (J. T. Baker, USA) for 10 min and stained with 0.1% crystal violet (Merck, USA) for 20 min. The staining intensity was measured with a spectrophotometric plate reader (Labsystems, Finland) after solubilization of the dye into 10% acetic acid. The proportion of adherent cells was counted as a percentage of the control, which was assessed by allowing 200 000 cells to adhere to the cell culture plates for 4 hours to achieve maximal adhesion.

4.2.4 Fluorescence and immunostainings and microscopic imaging

PC-3 cells were pre-treated with the indicated compounds or DMEM containing 1% BSA (as a control) for 24 hours. After 4 hours adhesion on coverslips coated with Matrigel (Becton Dickinson, USA), the adhered cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 5 min, and stained for 20 min with TRITC (tetramethylrhodamine isothiocyanate)-labelled phalloidin (0.2 µg/ml) (Sigma-Aldrich, USA), which stains F-actin. DNA was visualized using Hoechst 33342 (Sigma-Aldrich, USA). The immunostaining of cofilin was performed by first blocking fixed cells with 0,1% BSA in PBS for 1 hour, and then incubating with the anti-cofilin antibody (Abcam, USA) for an additional 1 hour. After the PBS wash, the cells were

incubated with Alexa Fluor® 488 chicken anti-rabbit IgG (H+L) (Invitrogen, USA) as a secondary antibody for 1 hour. After washing with PBS and H_2O, the mounted coverslips were studied with a Zeiss LSM510 META confocal microscope (Zeiss, Germany). Hoechst 33342, Alexa Fluor® 488 and TRITC-phalloidin were excited with 405nm, 488nm and 543nm laser lines, and the emission data were collected via 420-480nm, 500-550nm and 560 (longpass) LP filters, respectively. The intensity of fluorescence in the immunocytochemical (ICC) staining of cofilin was evaluated with ImageJ 1.42 (Wayne Rasband, USA).

4.2.5 Gelatin substrate zymography

Medium samples for zymography were collected from the upper chambers of the invasion inserts (50 000 cells plated) and lyophilized. 10 µl of each sample was solubilized in the SDS sample buffer. Samples were subjected to electrophoresis in non-reducing conditions in 12% gel co-polymerized with 0.1% gelatin. After electrophoresis, the gel was washed with 50 mM Tris containing 2.5% Triton X-100 for 30 min, then with 50 mM Tris containing 2.5% Triton X-100, 5 mM $CaCl_2$ and 1 µM $ZnCl_2$ for 30 min, and incubated for 24 hours in 50 mM Tris containing 5 mM $CaCl_2$ and 1 µM $ZnCl_2$ at 37 ºC. Finally, the gel was fixed with 50% ethanol/7% acetic acid solution for 30 min and stained with 0.2% Coomassie blue solution. The enzyme-digested regions were identified as white bands against the blue background.

4.2.6 GFP-transfections

PC-3 cells were tranciently transfected with pEGFP-actin (Clontech, USA), GFP-cofilin or GFP-paxillin (kind gifts from Dr Eleanor Goffrey, Turku Centre for Biotechnology, Finland), using 3 µg vector DNA and 7 µl TransFectin Lipid Reagent (Bio-Rad, USA) per 6 cm glass-bottomed culture dishes (MatTek, USA). The indicated amount of vector DNA and TransFectin lipid reagent were first incubated in DMEM for 20 min at room temperature (RT). Then 400 µl of solution was added on the cells in 10% iFBS DMEM. The cells were incubated for 5 hours at 37 °C and 5% CO_2, and washed with 10% iFBS DMEM, and the medium was changed. The cells were cultured in 10% iFBS-DMEM for an additional 48 hours to achieve expression of GFPs.

4.2.7 Fluorescence recovery after photobleaching (FRAP)

GFP-transfected PC-3 cells were treated with either DMEM + 1% BSA (a negative control) or indicated compounds for 2-3 hours. Fluorescence Recovery After Photobleaching (FRAP) experiments were performed as described by Sprague and McNally (2005), with a Zeiss LSM510 META confocal microscope in a humified chamber with 5% CO_2 at 37 ^oC. Cells transiently expressing pEGFP-actin/ -paxillin/ -cofilin-1 were excited with a 488 nm laser line and the emission was collected through a 500–550 nm bandpass filter. Prior to photobleaching, three images were collected. A region of interest (ROI) was chosen and it was photobleached (488 nm with 100%

intensity). The recovery was followed at 2-second intervals. The half time of recovery (T½) and the mobile fraction (Mf) were calculated. The data was measured by means of FCalc® (by Rolf Sara, Turku Centre for Biotechnology, Finland). Briefly, acquired data was corrected for image acquisition-caused photobleaching and the resulting data was fitted to the equation y = (1-exp(kt)).

4.2.8 Rac-1 activity assay

PC-3 cells were grown until semi-confluent and then pretreated with the indicated compounds. The assay was performed using the Rac-1 Activation Assay Kit (Upstate, USA). The cells were first washed with PBS and lysed with an ice-cold lysis buffer provided by the kit. Lysates were prepurified with Glutathione Sepharose 4B beads (GE Healthcare Bio Sciences AB, Sweden) and GTP-bound Rac-1 was then isolated from the lysates with PAK-1 agarose beads, which were washed and boiled in a Laemmli reducing sample buffer for 5 minutes. Aliquots of samples were run on 12 % polyacrylamide gels and the proteins were transferred onto nitrocellulose membranes. GTP-bound Rac-1 was detected in the Western blot with anti-Rac1 antibody (Upstate, USA).

4.2.9 Western blot

The cells were cultured until semi-confluent on 10 cm tissue culture dishes and were treated with the indicated compounds/concentrations or 1% BSA-DMEM (as a control). The cells were lysed in a standard Laemmli sample buffer with β-mercaptoethanol, and the aliquots were boiled for 5 minutes at 100 °C. 30 µl of whole-cell lysate/treatment were separated by SDS-PAGE with molecular weight standards (Bio-Rad, USA), and transferred to nitrocellulose membranes (Millipore, USA). Unspecific binding was blocked with 8% skim milk in Tris-buffered saline with 0.05% Tween 20. After 1 hour, the membranes were incubated with the primary antibodies for total cofilin and phospho-cofilin (Ser-3) (Cell Signaling Technology, USA) total FAK and phospho-FAK (pY397, BD Bioscience, USA), total PAK and phospho-PAK2 (Ser141, Cell Signaling Technology, USA), total LIMK2 and phosphor-LIMK2 (Thr508), total paxillin and phospho-paxillin (Tyr118, Cell Signaling Technology, USA) and β-actin (Sigma-Aldrich, USA). After washings, the membranes were incubated with peroxidase-conjugated AffiniPure goat anti-Rabbit, IgG (H+L) (Jackson ImmunoResearch, USA) as a secondary antibody. The proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Sweden). Quantification of the bands was carried out with an MCID Image Analyzer (Imaging Research, Canada).

4.2.10 RNAi knockdown

PC-3 cells were seeded on a 6-well plate and allowed to grow into 50% confluency in 10% iFBS-DMEM. The cells were then tranfected with 50-200 nM human Cofilin 1 siRNA, GGTase Iβ siRNA, or with control siRNA (all from Santa Cruz Biotechnology, USA), by using Oligofectamine Reagent in OptiMEM (both from Invitrogen, USA).

Transfections were performed according to the instructions of the manufacturer. After 60 hours, the cells were prepared for further experiments.

4.2.11 Flow cytometric analysis

PC-3 cells were treated overnight (o/n) with 10 mM FTI-277, 10 mM GGTI-298, 1 mM NE10790 or 1% BSA-DMEM (as a control), detached and washed twice with 1 ml phosphate-buffered saline (PBS) containing 2% FCS and 0.02% NaN_3 (staining buffer). The cells were stained at 4°C for 30 min in a 100 µl staining buffer containing saturating concentrations of monoclonal antibody (mAb) against integrin-β1, or isotype-specific mAb 3G6 (BD Biosciences, USA). After washing with the staining buffer, the cells were stained with FITC-conjugated anti-mouse Ig (Dako, Denmark). 10 000 living cells were selected by means of a forward and side scatter, and data were collected with FACSCalibur flow cytometer and analysed using CellQuest Pro software (BD Biosciences, USA).

4.2.12 Quantitative real time PCR

Prenylation inhibitor- treated PC-3 cells were lysed and total RNA was purified by using RNeasy Mini Kit (Qiagen, USA).The cDNA was synthesized by using 1 µg of total RNA as the starting material. Quantitation of cofilin 1 mRNA was performed by the QuantiTect SYBR green real time PCR kit (Qiagen, USA), using the DNA Engine Opticon system (BioRad, USA). Amplification conditions were as recommended in the Quantitect SYBR green handbook for two step qRT-PCR (Qiagen, USA). The primers used were as follows: human Cofilin 1: 5'-GATAAGGACTGCCGCTATGC-3', 5'-GCTTGATCCCTGTCAGCTTC-3', human paxillin: 5'-ACTACTGCAACGGCCC CATC-3', 5'-TAGTGCACCTCACAGTAGGG-3' and human FAK: 5'-ATTGCT GCTCGGAATGTTCT-3', 5'-GCTGAGGTAAAACGTCGAAAA-3' and human β-actin:5'-CGTGGGGCGCCCCAGGCACCA-3, 5'-TTGGCCTTGGGGTTCAGGGGG-3'. Annealing temperature at 60°C and 55°C for FAK, and 35 amplification cycles were used. The amounts of cofilin1, paxillin and FAK mRNA were normalized to β-actin expression and each treatment was carried out in triplicate. The results were analysed by the $2^{-deltadelta}$CT –method (Livak and Schmittgen 2001).

4.3 *In vivo*-orthotopic PC-3 prostate cancer cell tumors

4.3.1 Animals

Eight-week-old athymic nu/nu male mice (Harlan Winkelman GmbH, Germany) were maintained in a pathogen-free environment, under controlled conditions (20–21°C, 30–60% relative humidity and 12-hour lighting cycle). They were fed with small-animal food pellets (RM3 ESQC, Special Diet Services, England), and supplied with autoclaved tap water ad libitum. Animal welfare was monitored daily and the animals were weighed twice a week. The animal experiments were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other

Scientific Purposes, and statutes 1076/85 and 1360/90 of The Animal Protection Law in Finland, and EU Directive 86/609. The experimental procedures were reviewed by the local Ethics Committee on Animal Experimentation of the University of Turku and approved by the local Provincial State Office of Western Finland.

4.3.2 Orthotopic inoculation of PC-3 cells into the prostate and alendronate treatments

The mice were treated with an analgesic drug (Temgesic, 0.3 μg/g S.C. before operation, Schering-Plough Nv, Belgium). The mice were anesthetized by isofluran inhalation (1.5–3%, air flow 200 ml/min, Univentor 400 anesthesia unit, Univentor Ltd., Malta). 5×10^5 prostate cancer cells were gently inoculated into the ventral prostate through the dorsal prostate at a 45° angle, avoiding the urethra. Inoculation was performed with a 30 G needle attached to a 25 μl glass syringe (Hamilton Bonaduz AG, Switzerland). After inoculation, the abdominal muscle layer and the skin were closed with a 4-0 nonabsorbable suture (Bondek plus, polyglycolic acid-coated suture and monofilament, polyamide suture both from Genzyme GmbH, Germany). In order to study the effects of alendronate on prostate tumor growth and invasion, the mice were randomized according to weight into 2 groups. The orthotopic experiments were performed using a group of 8 mice. Mice in the alendronate group were treated daily with 0.5 mg/kg *(s.c.)* of alendronate (provided by Merck & Co., USA) in 100 μl PBS, and mice in the control group were injected daily with 100 μl of PBS. The dose of alendronate (0.5mg/kg) was chosen based on preliminary studies. Alendronate and control treatments were started concomitantly with the orthotopic inoculation of PC-3 cells. The mice were sacrificed 4 weeks after inoculation and tissue samples were collected for histological analysis.

4.3.3 Immunohistochemistry

Prostate tumors were formalin-fixed, embedded in paraffin and cut into 5 μm thick sections. The tumor sections were treated with 3 μg/ml polyclonal rabbit anti-cofilin antibody (Abcam, USA) o/n at +4°C followed with 7.5 μg/ml biotinylated anti-rabbit (Ig G) secondary antibody (Vector, USA). The detailed IHC-procedure is provided in publication III. Stainings were imaged by 40 x magnification.

4.4 Statistical analysis

Statistical analyses were carried out using Statistica 6.0, 1997, and SPSS 11.0 for Windows. The normality of the group data was tested by means of the Shapiro–Wilk's *W*-test, and significant differences were tested by means of *t*-tests for independent samples.

5 RESULTS AND DISCUSSION

5.1 Results

5.1.1 Inhibition of prostate cancer cell invasion and migration by bisphosphonates

5.1.1.1 Effect of bisphosphonates on *in vitro* invasion and migration

The study was initiated by evaluating the effect of BPs on the growth rate of PC-3 cells. The effects of various concentrations of alendronate and clodronate were analyzed by counting the cell number in the presence or absence of alendronate or clodronate. Alendronate and clodronate had no effects on the growth rate of PC-3 cells at concentrations less than 10^{-4}M, whereas they inhibited the growth of PC-3 cells at concentrations of 10^{-4}M or higher. Thus a 10^{-5}M concentration of alendronate and clodronate was used in further studies.

We continued our investigations on the putative direct effects of alendronate or clodronate on cancer cell adhesion, migration and invasion *in vitro*. In order to study the individual steps of invasion, cell adhesion, migration and degradation of extracellular matrices, *in vitro* adhesion and migration assays and gelatine substrate zymography were performed. Pretreatment with alendronate inhibited significantly PC-3 cell adhesion to all the used extracellular matrix proteins. Another important component of invasion is migration, which was studied with an *in vitro* migration assay on laminin. Importantly, migration and invasion of PC-3 cells were significantly reduced by the pretreatment of PC-3 cells with mevastatin, alendronate or clodronate when compared to the control treatment.

In further analysis, pretreatment of PC-3 cells for 24 hours with alendronate at concentrations from 10^{-12} M to 10^{-4} M, inhibited PC-3 cell invasion in a dose–dependent manner in the *in vitro* invasion assay. Dramatic and significant inhibition was obtained even with very low alendronate concentrations (IC_{50} was about 1 pM). Alendronate also inhibited cell invasion in a time-dependent manner, with inhibition achieved within 8 hours. Pretreatment of PC-3 cells with clodronate also inhibited cell invasion through matrigel (IC_{50} was about 0.1 μM), but not so effectively as compared to alendronate. The effect of alendronate on the invasion of other cancer cell lines was also evaluated. Alendronate pretreatment inhibited the invasion of DU-145 prostate cancer and MDA-MB-231 breast cancer cells (IC_{50} around 10^{-9} M). Furthermore, the inhibitory effect of alendronate pretreatment was sustained in the absence of BPs during the invasion assay of the cells. Corresponding effects were seen in DU-145 prostate cancer cells and MDA-MB-231 breast cancer cells, suggesting that the effect was not restricted to the PC-3 cancer cell line.

5.1.1.2 Effect of the mevalonate pathway intermediates on bisphosphonate-inhibited invasion and migration

Similar efficacies of mevastatin, alendronate and clodronate in the inhibition of migration and invasion prompted us to investigate the role of the mevalonate pathway in the migration and invasion assays. To estimate the effects of mevalonate pathway intermediates on PC-3 prostate cell motility, we used mevastatin, an inhibitor of the enzyme HMG CoA (β-hydroxy-β-methylglutaryl-CoA) reductase and mevalonate pathway intermediates; mevalonic acid lactone, geranylgeraniol and trans-trans-farnesol; in *in vitro* migration and invasion assays. Although the mevalonate pathway seemed to be important in the migration of PC-3 cells, the addition of trans-trans-farnesol, geranylgeraniol or mevalonate only partially, but statistically significantly, reversed mevastatin inhibition of the migration of PC-3 cells. Similarly, decreased migration of alendronate pretreated cells was partially, but statistically significantly reversed by the addition of geranylgeraniol or trans-trans-farnesol. The addition of mevalonate had no effect in the migration assay.

Pretreatment of prostate cancer cells with mevastatin inhibited invasion significantly, suggesting that the mevalonate pathway is also important for the ability of PC-3 cells to invade. Additionally, the inhibitory effect of mevastatin was effectively opposed by the addition of geranygeraniol, trans-transfarnesol or mevalonate (mevalonic acid lactone), demonstrating that geranylgeranylation and/or farnesylation of proteins is necessary for PC-3 cell invasion. Furthermore, the pretreatment of PC-3 cells with alendronate inhibited invasion significantly, compared to the non-treated control, and the addition of geranylgeraniol or trans-trans-farnesol to alendronate pretreated cells rescued the inhibition of invasion, whereas the addition of mevalonate had no effect on the invasion of alendronate pretreated PC-3 cells.

5.1.2 The role of alendronate and prenylation reactions in the cytoskeletal organization of prostate cancer cells

5.1.2.1 Effect of alendronate on cytoskeletal organization

The general effect of alendronate on the morphology and F-actin organization of PC-3 cells was examined. Morphology and the growth pattern of alendronate treated PC-3 cells were similar, compared to control PC-3 cells. However, the phalloidin staining of F-actin revealed bright clusters of filopodia in the cell membrane, whereas control PC-3 cells lacked such aggregates. Similar F-actin clusters were also found in mevastatin treated PC-3 cells, suggesting that filopodia formation was mediated by the mevalonate pathway. Importantly, we did not find any differences in the nuclear morphology in alendronate treated PC-3 cells, compared to the control treatment. Together with the growth rate experiments, this confirmed that alendronate did not induce apoptosis.

The effect of alendronate on cytoskeletal organization was studied further. The time course and concentration dependency of alendronate induced changes in F-actin

organization were studied in PC-3 cells. The cells were treated for various time periods with alendronate, and F-actin was stained with phalloidin. Strong disruption of F-actin was achieved after 8 hours, but minor effects were seen already within 4 hours. Minor disruption of F-actin was observed with 10^{-10} M alendronate, and total disruption of F-actin organization was achieved with 10^{-9} M alendronate. Disruption of F-actin by alendronate was also seen in DU-145 cells in a concentration-dependent manner.

The effects of alendronate on the dynamics of actin and paxillin were studied using FRAP with GFP-actin-/ GFP-paxillin -transfected PC-3 cells. After transfections, PC-3 cells were treated with 10^{-11} M and 10^{-5} M alendronate for 3 hours. A region of interest (ROI) containing an actin or paxillin structure was photobleached and the recovery of actin/ paxillin fluorescence was calculated. $T_{1/2}$ values indicating the half-time of recovery were assessed. Alendronate at 10^{-5} M but not at 10^{-11} M concentrations inhibited the recovery of actin, compared to the control treatment, but alendronate had no effect on the recovery of paxillin. Moreover, alendronate had no effect on the protein levels of paxillin, FAK or the expression level of β1-integrin.

5.1.2.2 Effect of prenylation inhibitors on cytoskeletal organization

Based on the previous results of mevalonate pathway inhibition by alendronate, we studied further the roles of prenylation reactions on the regulation of the actin cytoskeleton and the *in vitro* invasion and migration of PC-3 prostate cancer cells. The role of different prenylation reactions in PC-3 cell invasion and migration were studied by pre-treating cells with prenylation inhibitors. When the effects of inhibitors were studied on proliferation, no effect on the growth of PC-3 cells could be observed. When the effects of FTI-277 or GGTI-298 were studied on invasion and migration, almost maximal inhibition of invasion and migration was obtained at a 10^{-5} M concentration by both inhibitors. Pre-treatment of PC-3 cells with FTI-277 or GGTI-298 inhibited migration and invasion of PC-3 cells significantly, as compared with the control treatment. Pre-treatment of PC-3 cells with the geranylgeranyltransferase-II-inhibitor NE-10790 had no effect on migration or invasion through matrigel. A statistically significant inhibition of invasion was observed after 4 hours of treatment with GGTI-298 and after 5 hours of treatment with FTI-277.

In order to study the effects of the prenylation inhibitors on cytoskeletal organization, PC-3 and DU-145 cells pre-treated with FTI-277, GGTI-298 or NE-10790 were stained with phalloidin to visualize F-actin. Treatment of PC-3 cells and also DU-145 cells with FTI-277 and GGTI-298 caused a dramatic disruption of stress fibres. In GFP-actin-transfected PC-3 cells disruption of actin fibres was observed within 4 hours with GGTI-298, and around 7 hours with FTI-277. We also studied the effects of GGTI-298, FTI-277 and NE-10790 on the dynamics and organization of GFP-actin in living PC-3 cells using the FRAP technique. FTI-277 and GGTI-289 decreased the recovery of GFP-actin by increasing T½ values significantly compared to the control cells. The inhibitor of GGTase II (NE-10790) had no effect on the cytoskeletal organization or actin recovery.

The effects of prenylation inhibitors on FA-related proteins were investigated. The dynamics of paxillin and the levels of β1-integrin, paxillin and FAK were analyzed after the pretreatment of PC-3 cells with prenylation inhibitors. GGTI-298 inhibited the recovery of GFP-paxillin, while FTI-277 or NE-10790 had no effect. The levels of total and phosphorylated paxillin, as well as that of phosphorylated FAK, were decreased by GGTI-298. A minor effect on the phosphorylation of FAK was observed with FTI-277, while NE-10790 had no effect. Importantly, the prenylation inhibitors had no effect on the relative levels of paxillin or FAK mRNAs. The effects of prenylation inhibitors on the level of β1-integrin were analyzed by means of the flow cytometry. No effects on the expression level of β1-integrin protein were observed by the used inhibitors.

In addition, the effects of FTI-277, GGTI-298 and NE-10790 on Rac-1 activity in PC-3 cells were studied by using a pull-down activity assay for GTP-bound Rac-1. The treatment with GGTI-298 or μM FTI-277 caused an increase in the level of GTP-bound Rac-1, compared with the control or NE-10790 treatment. Similar results were obtained in the activity assay for GTP-bound Cdc42. Finally, the effects of FTI-277, GGTI-298 or NE-10790 pre-treatment on MMP-2 and MMP-9 activity in PC-3 cells were studied by the gelatine-substrate zymography. Pre-treatment of PC-3 cells with FTI-277, NE-10790 or alendronate had no effect on MMP-2 or MMP-9 activities in the invasion assay. In contrast, GGTI-298 treatment led to decreased MMP-9 activity.

5.1.3 The effect of alendronate and prenylation inhibitors on cofilin *in vitro* and *in vivo*

5.1.3.1 Effect of prenylation inhibitors on the level of cofilin in prostate cancer cells

The inhibitory effects of the prenylation inhibitors on invasion and migration prompted us to study further the effects of inhibitors on actin-related proteins. Treatment with GGTI-298 or FTI-277 decreased the protein level of the total and phosphorylated cofilin in PC-3 cells within 5 hours. Almost a total decrease in the protein level of cofilin (phosphorylated and total) was also obtained with GGTI-298 in DU-145 cells, and with GGTI-298 and FTI-277 in MDA-MB-231 cells. Treatment with GGTI-298 reduced also the level of p-PAK-2 in PC-3 cells. None of the prenylation inhibitors had any effect on total PAK, p-PAK-1, p-LIMK, or total LIMK levels. Immunocytochemical staining of cofilin was decreased within 8 hours in GGTI-298-treated PC-3 cells. When the effect of prenylation inhibitors on the relative level of cofilin mRNA was studied by quantitative RT-PCR, no effects were seen. In addition, the role of GGTase Iβ subunit in invasion, F-actin organization and cofilin regulation in PC-3 cells were studied by using specific GGTase Iβ siRNA. First, the effect of GGTase Iβ siRNA on the protein level of GGTase Iβ was studied by Western blot. Silencing of GGTase Iβ mRNA decreased the level of GGTase Iβ protein with the used concentrations. A significant inhibition of invasion and disruption of F-actin organization in PC-3 cells were seen with 5×10^{-8} M to 2×10^{-7} M siRNA concentrations. A marked decrease in the level of cofilin was also achieved with 5×10^{-8} M to 2×10^{-7} M GGTase Iβ siRNA in PC-3 cells.

5.1.3.2 Effect of alendronate on the level of cofilin in prostate cancer cells and prostate tumors

The effects of alendronate on the levels of actin-related cytoskeletal proteins in PC-3, DU-145 and MDA-MB-231 cells were studied by using a Western blot. A decrease in the level of phosphorylated cofilin, as well as total cofilin, was seen in PC-3, DU-145 and MDA-MB-231 cells by alendronate.

In PC-3 cells, a decrease in the levels of phosphorylated and total cofilin with 10^{-5} M alendronate appeared in 4-7 hours, and was obtained with relatively low concentrations (10^{-9} to 10^{-10} M). The effect of alendronate on cofilin mRNA expression was examined with quantitative RT-PCR, but no effects were seen with the alendronate concentrations (10^{-8}-10^{-5} M). The level of cofilin ICC staining in PC-3 cells treated with 10^{-10} M of alendronate was significantly lower as compared to the control cells. The same effect was also seen in alendronate-treated DU-145 cells, and a decrease in cofilin IHC staining was seen in alendronate-treated nude mice PC-3 tumors. Importantly, clodronate had no effect on the level total or phosphorylated cofilin.

5.1.3.3 Effect of RNAi knockdown of cofilin 1 on the invasion and F-actin organization of prostate cancer cells

The role of cofilin in the invasion and F-actin organization was studied further by silencing cofilin mRNA in PC-3 cells. The effects of cofilin 1 siRNA on the levels of phosphorylated cofilin and total cofilin were analyzed by a Western blot. Cofilin siRNA decreased the level of the cofilin protein with 5 x 10^{-8} M to 10^{-7} M concentrations. In addition, cofilin ICC staining was performed to study the specificity of cofilin 1 siRNA in PC-3 cells. The used concentrations decreased the cofilin ICC staining in PC-3 cells effectively. The role of cofilin in F-actin organization and invasion were studied by phalloidin staining and an *in vitro* invasion assay. Cofilin 1 siRNA significantly decreased invasion and disrupted F-actin organization in PC-3 cells.

5.2 Discussion

5.2.1 Bisphosphonate inhibition of prostate cancer cell adhesion, invasion and migration

Bisphosphonates have been used as key therapeutics for the prevention of osteoporosis. In osteoclasts, N-BPs suppress the farnesyl diphosphate synthase (FPPS) related to the mevalonate pathway (Casey 1992, Rogers et al. 2000). The first evidence for the direct effects of N-BPs on metastatic cancer cells were published by van Der Pluijm et al. (1996), Boissier et al. (1997) and Boissier et al. (2000). Due to promising *in vitro* results in various cancer cell lines, BPs and new analogues could be considered as anti-metastatic therapeutics for prostate and breast cancer *in vivo.* According to the existing data, the inhibition of cancer cell growth and the induction of apoptosis *in vitro* have

been shown constantly with high (> 10 μM) BP-concentrations (Fromique et al. 2000, Mackie et al. 2001, Reinholz et al. 2000, Hiraga et al. 2001, Lee et al. 2001, Coxon et al. 2004). Hiraga et al. (2001) reported that ibandronate induces apoptosis of MDA-MB-231 cells associated with bone metastasis, while cells implanted in a mammary fat pad remained resistant to apoptosis. This difference was explained by the elevated concentration of BP within metastatic bone sites as a result of locally increased bone resorption and the release of BPs from the mineralized bone matrix.

In the migration assays performed here, the mevalonate pathway intermediates only partially reversed mevastatin and alendronate -induced inhibition of migration, probably due to the short incubation time (5 hours), which may not have been long enough to replenish the necessary/critical pools of geranylgeranylated and/or farnesylated proteins. Our results also differ from previous observations of Boissier et al. (2000), who did not observe the effects of BPs on MDA-MB-231, or PC-3 cell migration. In our study, the inhibition of PC-3 cell adhesion to fibronectin, type I and IV collagen and laminin was most potent, and our results support the studies of van Der Pluijm et al. (1996), and Boissier et al (1997). The reduced adhesion of alendronate treated PC-3 cells may also reflect a reduction in their migratory phenotype, rather than specific impairment of cell-matrix interaction. This possibility is supported by the fact that the addition of the RGD (Arg-Gly-Asp)-sequence containing peptide further reduced adhesion of alendronate -pretreated PC-3 and MDA-MB-231 cells to vitronectin and fibronectin in our study.

Alendronate pretreatment inhibited the invasion of PC-3, DU-145 and MDA-MB-231 cells in a dose-dependent manner, and our results are in an agreement with the studies of Boissier et al. (2000) and Coxon et al. (2004). Furthermore, the pretreatment of PC-3 cells with alendronate inhibited invasion significantly, as compared to the non-treated control cells. The addition of geranylgeraniol or trans-trans-farnesol to alendronate pretreated cells rescued the inhibitory effect of alendronate, whereas the addition of mevalonate had no effect on the invasion of alendronate pretreated PC-3 cells. These results suggest that the protein geranylgeranylation and/or farnesylation, important for PC-3 cell invasion, are reduced by alendronate, and the alendronate target is downstream of the mevalonate. Pretreatment with clodronate also inhibited PC-3 cell invasion significantly, but the addition of trans-trans-farnesol, geranylgeraniol or mevalonate did not rescue clodronate effects in the PC-3 cell invasion assay. Geranylgeraniol and trans-trans-farnesol further inhibited the invasion of clodronate-pretreated cells when compared to clodronate pretreated cells alone. In addition to PC-3 cells, alendronate inhibited also the invasion of DU-145 prostate cancer cells and MDA-MB-231 breast cancer cells, and the inhibitory effect was reversed by geranylgeraniol and trans-trans-farnesol. Mevastatin blocked PC-3 cell invasion and the mevalonate pathway intermediates downstream of the HMG CoA reductase overcame this inhibition. Similarly, mevastatin prevented the migration of PC-3 cells.

Our results demonstrate that the effect of alendronate on PC-3 cell invasion was mediated by the inhibition of the mevalonate pathway. Compared to mevastatin, alendronate affects the pathway downstream of the HMG CoA reductase step, since mevalonate did not reverse the effect of alendronate. This is in agreement with findings

that alendronate and other nitrogen-containing bisphosphonates inhibit farnesyl diphosphate synthase (FPPS) *in vitro* in osteoclasts (van Beek et al. 1999, Bergstrom et al. 2000). The IC_{50} in the alendronate inhibition of recombinant FPPS *in vitro* is 460 nM (Bergstrom et al. 2000). However, it is difficult to compare IC_{50} values obtained from *in vitro* assays using pure enzymes to those obtained by measuring cellular level functions which seem to be more sensitive. For instance, the IC_{50} of osteoclastic bone resorption is lower than the IC_{50} for inhibition of FPPS *in vitro* by many of the nitrogen-containing bisphosphonates (van Beek et al. 1999). Furthermore, nothing is known about the uptake, accumulation or intracellular concentrations of BPs in tumors or cancer cells. Our results suggest that both protein farnesylation and geranylgeranylation are impaired by alendronate in PC-3 prostate cancer cells, because both trans-trans-farnesol and geranylgeraniol can overcome the inhibitory effects of alendronate. Interestingly, similar results were obtained with DU-145 and MDA-MB-231 cells (unpublished data). In osteoclasts, the effects of nitrogen-containing bisphosphonates seem to be mediated via the inhibition of protein geranylgeranylation (van Beek et al. 1999, Fisher et al. 1999, Russell et al. 1999, Coxon et al. 2000). This suggests that the target of alendronate in cancer cells might be different compared to osteoclasts, and the inhibitory effect of alendronate is not restricted to one cancer cell line.

In contrast to alendronate, the effect of clodronate was not reversed by either trans-trans-farnesol or geranylgeraniol, suggesting a distinct mechanism of action for these two BPs. The mevalonate pathway intermediates have no effects on the cell death induced by pyrophosphate resembling bisphosphonates in osteoclasts (Benford et al. 1999). Previous studies together with our study support the view that pyrophosphate resembling bisphosphonates do not prevent protein prenylation. This is also in agreement with several studies demonstrating the role and accumulation of the nonhydrolyzable ATP analogs in the induction of apoptosis in macrophages and osteoclasts (Rogers et al. 1994, Frith et al. 1997, Frith et al. 2001, Muhlbauer et al. 1991, Selander et al. 1994, Hughes at al. 1995, Selander et al. 1996). It is possible that clodronate, and other pyrophosphate resembling bisphosphonates are metabolized into nonhydrolyzable ATP analog also in cancer cells, which might lead to reduced invasion and migration by so far unknown mechanisms.

The effect of alendronate pretreatment on the activity of invasion -related MMP-2 and MMP-9 in PC-3 cells was also studied. Alendronate had no effect on the level of MMP-2 or MMP-9 activity during the *in vitro* invasion. This suggests that the inhibition of invasion by alendronate was not caused by changes in gelatinase secretion or activation. Boissier et al. (2000) and Stearns and Wang (1998) observed an inhibitory effect of alendronate on MMP-9/-2-activities with relatively high alendronate concentrations ($\geq$50μM) with the different experimental systems. There may also be changes in the accumulation of local or membrane-bound MMPs, or their activities and concentrations in the culture medium, but these were not studied in detail.

5.2.2 The role of the mevalonate pathway and prenylation reactions in the invasion and cytoskeletal organization of prostate cancer cells

Three specific prenylatransferases can catalyze the isoprenylation of small GTPases and their function can be blocked by specific prenyltransferase inhibitors (Lerner et al. 1995, Coxon et al. 2001, Kusama et al. 2003). In our study, FTase inhibitor (FTI-277) and GGTase I inhibitor (GGTI-298), but not the GGTase II inhibitor (NE-10790), blocked the migration and invasion of PC-3 cells almost totally at a concentration of 10 µmol/ L. Further experiments with GGTI-298 and FTI-277 showed the inhibition of invasion in a time- and concentration -dependent manner by both inhibitors. The inhibitory effect of GGTI-298 was achieved earlier (within 4 hours), compared to the inhibition of invasion by FTI-277. After a 5-hour pretreatment, both inhibitors blocked invasion significantly, as compared with the control treatment. After a 6 hour pretreatment, there was no difference between the magnitude of GGTI-298 and FTI-277 inhibition. Kusama and co-workers (2003) have observed the corresponding effects of various concentrations of GGTI-298 and FTI-277 on the invasion of human pancreatic cancer cells, with almost total inhibition of invasion at 10 µmol/ L and 10-15 µmol/ L concentrations, respectively, and our results are in line with this data. GGTI-298 and FTI-277 also induced changes in the F-actin organization in PC-3 cells, and a similar effect was seen in DU-145 prostate cancer cells. GGTI-298 and FTI-277 also reduced the recovery of GFP-actin in PC-3 cells. These effects on GFP-actin were observed at 4 and 7 hours with GGTI-298 and FTI-277, respectively, which coincide with the inhibition of invasion. This suggests that a cascade of events, possibly including synthesis and/or prenylation of new proteins, is required to maintain prenyltransferase function.

A strong decrease in the levels of cofilin (both total and phosphorylated) was seen by GGTI-298 and FTI-277. Cofilin controls the depolymerization/ reorganization of actin filaments and is involved in the regulation of tumour cell migration and invasion (Moon and Drubin 1995, Yang et al. 1998, Chen et al. 2000, Wang et al. 2007), but the interrelationships between the levels of its phosphorylated (inactive) and non-phosphorylated (active) forms and the metastatic capacity of the cells are complex. The strong depletion of cofilin by prenylation inhibitors was associated with the disruption of F-actin organization and a marked decrease of invasion and migration. A decrease in total and phosphorylated cofilin was also seen in GGTI-298– and FTI-277–treated MDA-MB-231 cells, and GGTI-298-treated DU-145 cells. Importantly, prenylation inhibitors had no effect on mRNA levels of cofilin in PC-3 cells.

Time-course studies showed that disruption of the actin cytoskeleton, inhibition of cell migration/invasion and reduction in cofilin levels were all observed within 5 hours after GGTI-298 addition. A strong decrease in the immunostaining of cofilin in GGTI-298-treated PC-3 cells was seen within 8 hours, well in line with the decreased cofilin levels in the Western blot analysis. In the FRAP-analysis, no recovery of cofilin was seen with any treatment. Apparently, the 15 min follow-up period was too short to evaluate the turnover rate of cofilin. This finding is further supported by our studies with cycloheximide. After 12 hours, cycloheximide had no effect on the cofilin protein

expression level (unpublished data). The stability of cofilin confirms its importance in cytoskeletal maintenance. It can be also suggested that the effects of prenylation inhibitors are associated with the increased degradation of the cofilin protein. Although LIMKs and PAK-1 are important regulators of cofilin (Sumi et al. 1999, Amano et al. 2002), GGTI-298 and FTI-277 did not, however, have any effect on the protein levels of PAK-1 or LIMK. It is possible that additional, prenylation-dependent mechanisms, which are distinct from the PAK-1/LIMK pathways, are involved in the regulation of the turnover and degradation of cofilin.

Furthermore, modification of FAs via the activation of integrins and FAK is also needed for the initiation of cell spreading (Rodriguez-Fernandez 1999). Integrins maintain a bidirectional crosstalk between prostate cells and the ECM, and their expression levels have usually changed during the progression of prostate cancer (Murant et al. 1997). Integrins can activate the tyrosine phosphorylation of FAK and paxillin, and initiate various critical signal transduction cascades (Almeida et al. 2000, Parsons et al. 2000). Expression of β_1-integrin is increased in some cases of prostate cancer, as well as in breast cancer (Murant et al. 1997). The GGTase I inhibitor, GGTI-298, suppressed the recovery of GFP-paxillin and levels of p-FAK and paxillin, but had no effect on the expression of the β1-integrin protein. The amount of phosphorylated paxillin was also decreased, which may partly be explained by a decrease in the level of total protein. Importantly, prenylation inhibitors had no effect on the relative mRNA levels of paxillin or FAK. GGTase I -catalyzed reactions can be suggested to be associated with the regulation of focal adhesions in which paxillin, FAK and integrins are clustered as a complex responsible for the formation of the leading edge in actively migrating cells (Turner et al. 1990, Brown and Turner 2004). Treatment with GGTI-298 also decreased the levels of phosphorylated PAK-2. The association of PAK-2 with cytoskeletal organization has not been precisely characterized, but it may have a role in maintaining the cytoskeleton. Decreased levels of paxillin, p-FAK and p-PAK-2, together with disrupted GFP-paxillin dynamics, and F-actin organization, might be 'focal adhesion - specific' effects of GGTI-298, distinct from the integrin regulation and cofilin regulation pathway. It is obvious, however, that the disruption of both FA complexes and cofilin can be involved in the impairment of cytoskeletal organization, and the blockage of migration/invasion of PC-3 prostate cancer cells by the GGTase I inhibitor.

In further experiments, the effect of the RNAi knockdown of GGTase Iβ on invasion and cytoskeletal organization was studied. The RNAi knockdown of GGTase Iβ led to a decreased level of cofilin in PC-3 cells. The inhibition of invasion, disruption of F-actin organization and decrease in cofilin levels by GGTase Iβ siRNA, are in line with the effects of GGTI-298. Sjogren et al. (2007) demonstrated the inhibition of migration, actin organization and proliferation of fibroblasts with a conditional knockout for the β subunit of GGTase-I gene in fibroblasts. Our method differs from that of Sjogren et al., but the effects obtained with the GGTase Iβ siRNA treatment and the conditional knockout of GGTase I were similar. The effects of prenylation inhibitors on cofilin levels might be post-transcriptional, but the possible mechanisms behind the observed decrease of cofilin by prenylation inhibitors remained unclear.

Changes in the expression levels and activity of MMPs in the prostate are related to normal and pathological alterations in tissue morphology (Wilson 1995). MMP-2 and MMP-9 play important roles in cancer invasion and metastasis, and the activity of the mature/proform ratio is associated with the progression of aggressive forms of prostate cancer (Zhang et al. 2004). Pretreatment of PC-3 cells with FTI-277, NE-10790 or alendronate had no effect on MMP-2 or MMP-9 activities in the invasion assay. In contrast, GGTI-298 had an inhibitory effect on MMP-9 activity. This suggests that the inhibition of invasion by GGTI-298 could partly be caused by the inhibition of MMP-9 gelatinase secretion/activation. Wong et al. (2001) have shown that the treatment of THP-1 human monocytic cells with statins or geranylgeranyl transferase-inhibitor L-839,867 inhibited MMP-9 secretion in a dose-dependent manner, while the farnesyl transferase inhibitor had no effect. On the other hand, Wang et al. (2000) demonstrated the inhibition of MMP-9 expression by inhibiting Ras prenylation by lovastatin. Our results support these observations for the positive correlation between MMP-9 inhibition and the inhibition of geranylgeranylation, independently of an inhibitor type.

FTI-277 and GGTI-298 increased the level of GTP-bound Rac-1, which was an unexpected result. Dunford et al. (2006) have shown previously that the inhibition of protein prenylation by bisphosphonate treatment caused Rac, Cdc42 and RhoA GTPase activation in osteoclasts and macrophage-like cells. One explanation for this may be, as discussed by Dunford et al. (2006), that activated but unprenylated GTP-bound GTPases are unable to interact appropriately with the effectors and thus become unable to activate downstream signalling molecules. Although the mechanisms behind the activation of Rac-1 by GGTI-298 and FTI-277 remained unclear in this study, both prenylation and the activation of GTPases are needed for their proper action as regulators of various signalling cascades (Coxon and Rogers 2003, Walker and Olson 2005, Katoh et al. 2006).

5.2.3 The role of cofilin in the alendronate inhibition of cytoskeletal integrity and invasion of prostate cancer cells

Several studies demonstrate that the appropriate dynamics and organization of actin are essential for cell motility (Fenton et al. 1992, Katoh et al. 1998, Yamazaki et al. 2005). A visible disruption of F-actin organization was observed within 4-6 hours after adding alendronate to the PC-3 cell cultures. A time lag required for the induction of disruption suggests that these inhibitory effects are indirect and that *de novo* protein synthesis is probably required. The disruption of F-actin organization preceded the inhibition of invasion and, in fact, as short as 3 hours after treatment with 10^{-5} M alendronate, the dynamic of actin was disrupted, as was shown in FRAP analysis. A visible disruption of F-actin was also seen with relatively low alendronate concentrations (10^{-10} to 10^{-9} M) in PC-3 cells, but also in DU-145 cells. It is possible that alendronate can induce undetectable but critical changes that lead to the inhibition of the actin cytoskeleton and invasion capacity even at very low concentrations. In previous results, the IC_{50} of alendronate in the invasion assay was ~1 pM. An inhibitory effect was achieved after a relatively long treatment period (6-8 hours) in the invasion assay. This also could suggest

that alendronate has indirect effects on modifications of proteins and/or *de novo* protein synthesis.

Cofilin binds to the actin filaments, enhances the severing of filaments and provides actin monomers for the polymerization of new filaments (Chen et al. 2000, Moon and Drubin 1995, Bamburg 1999). The importance of the coflin pathway in the metastasis process is suggested to be critical (Wang et al. 2007). Moreover, the role of cofilin in various cancer cell lines/cancers is complex. Overexpression of cofilin has been demonstrated *in vitro* in various invasive cancer cell lines and tumors (Sinha et al. 1999, Gunnersen et al. 2000, Keshamouni et al. 2006) and *in vivo* tumours (Martoglio et al. 2000, Unwin et al. 2003, Wang et al. 2004, Turhani et al. 2006). On the other hand, downregulation of cofilin has been described in various human cancers (Ding et al. 2004, Smith-Beckerman et al. 2005). Impaired actin organization/ dynamics/ invasion by alendronate were associated with a strong depletion of cofilin in PC-3 cells. Corresponding effects were also seen in DU-145 prostate cancer cells and MDA-MB-231 breast cancer cells. Importantly, although alendronate strongly decreased the level of cofilin, it had no effect on the relative level of cofilin mRNA in PC-3 cells, which suggests that alendronate regulates cofilin at the posttranscriptional level. In addition, ICC staining of cofilin was decreased by a 6-8 hour alendronate treatment of PC-3 cells with relatively low alendronate concentrations, and a similar effect was seen in DU-145 cells. Depletion of cofilin was also seen in IHC staining of alendronate-treated PC-3 nude mouse tumors. Adequate administration and achievement of a therapeutic concentration inside the target tumor tissue are important questions in the potential use of BPs as a prostate cancer therapy. They rapidly bind to bone and less than 1% of administred BPs remain in the blood circulation (Rodan and Balena 1993). In our *in vivo* studies, mice were daily treated with 0.5 mg/kg of alendronate for 4 weeks. Although the alendronate concentration in tumors might be quite low, our previous study demonstrated that the inhibition of PC-3 cell invasion is achieved *in vitro* at picomolar concentrations. Additionally, Fournier et al. (2002) have demonstrated that zoledronic acid accumulates rapidly in bone, but transiently also in the prostate. This could be one mechanism underlying the prostate-related effects and decrease of cofilin level in PC-3 tumors by alendronate. Importantly, clodronate had no effect on the level of cofilin in PC-3 cells with concentrations which were shown to inhibit the invasion of PC-3 cells in our previous study. The depletion of cofilin may thus be primarily associated with the inhibitory effects of N-BPs on the mevalonate pathway -related proteins. In addition, the effects of alendronate on the PNT1A prostate cell line are studied. Our unpublished preliminary data reveals that alendronate has no inhibitory effects on cofilin levels or actin organization in PNT1A cells. The inhibition of cytoskeletal organization by alendronate might thus be ‘cancer cell specific’, but these results remain elusive.

The RNAi knockdown of cofilin 1 effectively disrupted F-actin stress fibers and inhibited the invasion of PC-3 cells. Hotulainen et al (2005) have demonstrated that the suppression of cofilin by siRNA disrupts motility and actin organization in mammalian non-muscle cells. Our results are in line with this data. Cofilin can be considered as a critical regulator of the invasion process and F-actin organization. The mechanisms

underlying the decrease of cofilin in alendronate treated PC-3 cells and PC-3 tumors remained unclear. It is obvious, however, that the observed decrease of cofilin was induced by alendronate and cofilin siRNA, and both compounds have similar effects on invasion and actin organization in PC-3 cells. These results also suggest the view that an alendronate-induced decrease of cofilin precedes the disruption of F-actin organization and the inhibition of invasion. A decrease of cofilin may thus have a critical role in alendronate interference with actin organization and cancer cell invasion.

N-BPs have been demonstrated to inhibit integrin expression in endothelial cells (Bellahcène et al. 2007), and to induce detachment of prostate cancer cells in association with dephosphorylation of FAK (Matsunaga et al. 2007). In this study, alendronate had no effect on paxillin dynamics or the protein levels of paxillin or FAK, or β1-integrin expression levels. Our results differ from those of Bellahcène et al. (2007) and Matsunaga et al. (2007), which could be due to a cell line specificity or different experimental setup. FAK, paxillin and integrins are key proteins which are strictly associated with the regulation of actin dynamics (Burridge et al. 1992, Schaller and Parsons 1994, Parsons et al. 2000, Mitra et al. 2005), but in this study, the inhibitory effect of alendronate seemed to be associated more or less with actin-/ cofilin -related regulatory pathways.

6 CONCLUSIONS

I

Bisphosphonates alendronate and clodronate inhibit the adhesion, invasion and migration of PC-3 prostate cancer and MDA-MB-231 breast cancer cells. Inhibition of motility by alendronate is mediated via the inhibition of the mevalonate pathway (Fig.7). This data suggests that nitrogen-containing bisphosphonates may have therapeutic potential, even at very low concentrations in the prevention of prostate cancer metastasis

II

Inhibition of the mevalonate pathway by blocking the GGTase I and FTase catalyzed reactions interfere not only with the activation/dynamics of cytoskeletal key molecules, but also with the invasion/migration of prostate cancer cells by mechanisms that are associated with the regulation of the organization of actin and/or cofilin and focal adhesion complex (Fig.7). The results suggest that FTase- and GGTase I-catalyzed prenylation reactions can be suggested as interesting targets for the inhibition of prostate cancer metastasis.

III

Alendronate inhibition of invasion in prostate cancer cells is associated with the interference of actin dynamics and F-actin organization, and a particularly strong decrease in the level of cofilin in *in vitro* cell cultures and in *in vivo* PC-3 prostate tumors of nude mouse. A decreased level of cofilin and the disruption of the actin cytoskeleton seem to precede the alendronate effects on cell invasion. The cofilin knockdown experiments with inhibitory siRNA suggest that alendronate regulation of the cofilin pathway is essentially associated with alendronate-induced disruption of the cytoskeleton. Our results emphasize the potential of alendronate in the inhibition of prostate cancer metastasis by disturbing the regulation of cofilin and the cytoskeletal organization/integrity (Fig.7).

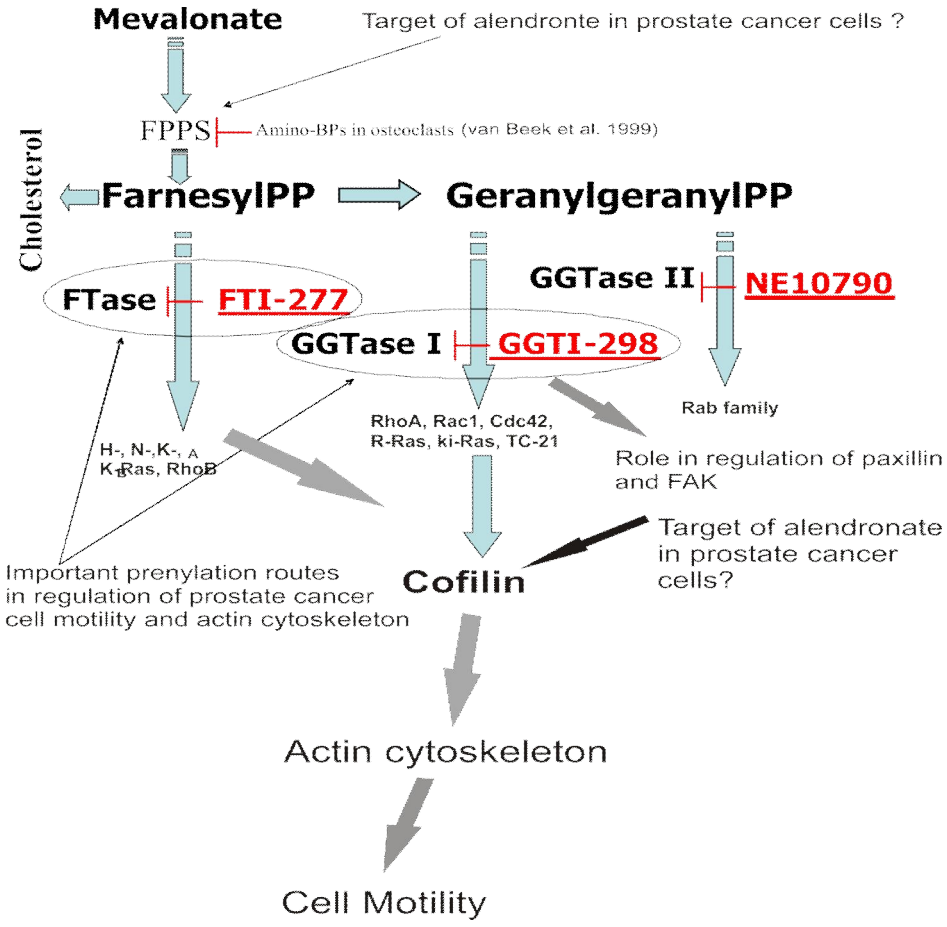

Figure 7. Scheme for the targets and mechanisms of alendronate inhibition of cytoskeletal organization, invasion and migration of prostate cancer cells. Alendronate inhibits the invasion and migration of prostate cancer cells via the inhibition of the mevalonate pathway. Based on our results, alendronate can be suggested to affect a step located upstream of farnesylPP, but downstream of mevalonate in prostate cancer cells. The prenylation routes catalyzed by FTase and GGTase I can be considered to be essential in the invasion, migration and cytoskeletal organization of prostate cancer cells. The GGTase I -catalyzed prenylation route is also associated with the regulation of focal adhesion -related proteins, FAK and paxillin. Alendronate also decreases the level of cofilin *in vitro* and *in vivo* prostate cancer cells and prostate cancer tumors. Cofilin decrease can be considered to be a critical mechanism in the alendronate regulation of actin organization and cancer cell motility.

7 ACKNOWLEDGEMENTS

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