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DISSERTATION

Titel der Dissertation

The role of resveratrol on growth factor-induced signalling events in vascular smooth muscle cells

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Abstract

Resveratrol (RV), a polyphenolic compound found in grapes and berries, has been shown to exert various health-beneficial effects that may help to prevent cardiovascular diseases and cancer. In atherosclerosis, vascular smooth muscle cells (VSMC) play a crucial role in most steps of disease progression. Upon growth factor attraction, VSMC start to migrate towards the site of blood vessel injury, start to proliferate and therefore contribute to the narrowing of the artery. Until now, the molecular mechanisms of the putative vasoprotective properties of RV are insufficiently understood. In this study, we therefore tried to unravel some of the molecular actions of RV in growth factor-activated VSMC.

By using epidermal growth factor (EGF) as activating stimulus we found that binding of RV to integrins, which play a mayor role in cell adhesion and survival, accounted only to a minor extend for the strong inhibiting properties of RV on Akt phosphorylation shown previously. EGF-mediated dephosphorylation of focal adhesion kinase (FAK), a key protein in processing integrin and growth factor signals, was completely restored by RV, which was mainly due to impaired binding of FAK to the phosphatase Shp-2. Moreover, single phosphorylation sites within FAK were very differently regulated by stimulation with EGF and RV. We furthermore excluded a major contribution of the estrogen receptor α (ERα) to EGF-mediated VSMC activation and were able to show that RV acted as a potent antagonist of ER in estrogen-stimulated cells. Extracellularly produced H_2O_2 was strongly affected by RV. Both, Angiotensin II- and EGF-induced production of extracellular H_2O_2 were completely blocked. Additionally, unexpected high basal levels of H_2O_2 in unstimulated VSMC were diminished by 60% underlining RVs very potent antioxidative properties. Migration studies illustrated that RV was able to selectively abrogate EGF- but not PDGF-triggered chemotaxis of VSMC. Long-term treatment with the growth factors revealed that enhanced FAK phosphorylation was not affected by RV. By conducting cytoskeleton staining experiments, we could observe that especially lamellipodia formation was impaired in EGF-, but not PDGF-stimulated cells pretreated with RV. The molecular basis for this phenotypic limitation could be specifically attributed to the small GTPase Rac1. Pull-down experiments revealed that Rac1 activity of EGF- but not PDGF-stimulated VSMC

was strongly inhibited by additional treatment with RV, whereas activity of cdc42 was not changed.

In summary, our study contributes new knowledge to understand the molecular mechanisms by which RV interferes with signalling pathways induced by growth factors in vascular smooth muscle cells. Moreover, we could identify a novel anti-migratory effect of RV on EGF-stimulated VSMC.

Zusammenfassung

Dem polyphenolischen Molekül Resveratrol (RV), das vor allem in Trauben und Beerenfrüchten vorkommt, werden präventive Wirkungen bei Herzkreislauf-Erkrankungen und Krebs zugeschrieben. Vaskuläre glatte Muskelzellen (VSMC) spielen bei der Entstehung von Arteriosklerose eine entscheidende Rolle. Durch Ausschüttung von Wachstumsfaktoren wandern VSMC in Richtung der Intima und beginnen sich dort zu teilen, was zur Verengung des Blutgefäßes beiträgt. Bis heute sind die molekularen kardiovasoprotektiven Mechanismen von RV ungenügend verstanden. Aus diesem Grund versuchten wir in dieser Studie mögliche molekulare Mechanismen von RV aufzuklären.

In mit *epidermal growth factor* (EGF) stimulierten VSMC konnten wir zeigen, dass das Binden von RV an Integrine, welche wichtige Funktionen für das Anhaften und das Überleben der Zelle übernehmen, nur einen geringen Anteil an der stark inhibierenden Wirkung von RV auf die Phosphorylierung von Akt, welche bereits früher von unserer Arbeitsgruppe gezeigt wurde, hat. Die EGF-induzierte Dephosphorylierung von *focal adhesion kinase* (FAK), die ein zentrales Protein bei der Integrin-, und Wachstumsfaktor-vermittelten Reizweiterleitung ist, wurde durch RV-Behandlung vollständig verhindert. Dies war auf eine verminderte Bindung von FAK an die Phosphatase Shp-2 zurückzuführen. Zudem waren einzelne Phosphorylierungsstellen innerhalb von FAK durch EGF-, und RV-Behandlung sehr unterschiedlich reguliert. Eine Beteiligung des Östrogenrezeptors α (ERα) an der EGF-vermittelten Aktivierung von VSMC konnten wir ausschließen, obwohl RV eine antagonistische Wirkung auf ER in Östrogen-stimulierten VSMC zeigte. Extrazellulär produziertes H_2O_2 war sehr stark von RV beeinflusst. Sowohl die Angiotensin II-, als auch die EGF-induzierte H_2O_2-Produktion wurden durch Vorinkubation mit RV inhibiert. Darüber hinaus konnten wir beobachten, dass die unerwartet hohen basalen H_2O_2-Werte von RV zu 60% inhibiert werden konnten, was die stark antioxidative Wirkung von RV unterstreicht. Migrations-Studien zeigten uns, dass RV selektiv EGF-, aber nicht *platelet-derived growth factor* (PDGF)-vermittelte Migration vermindern konnte. Langzeit-Stimulation mit den Wachstumsfaktoren zeigte, dass die erhöhte FAK-Phosphorylierung durch Vorinkubation mit RV nicht verändert war. Durch das Färben des Cytoskeletts von VSMC zeigte sich, dass die EGF-induzierte aber nicht die PDGF-vermittelte Formierung

von Lamellipodien durch RV stark beeinflusst war. Auf molekularer Ebene konnte die kleine GTPase Rac1 als Grund für die verminderte Lamellipodien-Ausbildung ausfindig gemacht werden. Präzipitations-Experimente zeigten, dass die Aktivität von Rac1 ausschließlich in EGF-stimulierten jedoch nicht in PDGF-behandelten VSMC durch RV inhibiert wurde. Die Aktivität von cdc42 hingegen wurde durch keinen Stimulus beeinflusst.

Zusammengefasst können wir sagen, dass diese Studie dazu beiträgt, die molekularen Mechanismen von RV in Wachstumsfaktor-induzierten VSMC besser zu verstehen. Zusätzlich konnten wir einen neuartigen anti-migratorischen Effekt von RV in EGF-stimulierten VSMC identifizieren.

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INTRODUCTION

B Introduction

1. The cardiovascular system

In vertebrates, supplementation and distribution of nutrients, oxygen and collection of waste products is carried out by a closed circulating system, which comprises a pump (the heart), a distribution network (the blood vessels) and liquid (the blood itself), which is titled “the cardiovascular system” [1]. Diseases connected to the cardiovascular system include stenosis of blood vessels (atherosclerosis, coronary heart disease [CHD]), heart-associated diseases (heart failure) and systemic diseases, which often arise from aforementioned maladies (hypertension, arrhythmia, stroke, heart attack) [2]. The risk factors to develop a cardiovascular disease (CVD) are as manifold as the resulting illnesses and include: smoking, high cholesterol diet, minor physical exercise and genetic predisposition [3, 4]. The world-wide frequency of CVD rose steadily over the last decades. In the U.S., over one-third of the population suffers from one or more diseases concerning the cardiovascular system and therefore such illnesses are nowadays the major cause of death, followed by cancer. The resulting direct and indirect costs for 2009 will exceed 475 Billions of Dollars in the U.S. [5]. Therefore, huge effort is made all over the world to cope with and effectively combat various CVD.

The human blood vessel consists of three distinct layers, each separated from the other by an elastic lamina. The intima is the most inner and thinnest region consisting of one single layer of endothelial cells, which forms the barrier to the circulating blood stream. The intima is surrounded by the media, the thickest layer within the vessel, mainly built up by vascular smooth muscle cells (VSMC) and connective tissue. VSMC within the media are mainly responsible for constriction and dilatation of the blood vessel. The very outside region is the adventitia, which consists almost exclusively of connective tissue and which is strongly innervated to supply the blood vessel with neuronal inputs [1].

1.1. Molecular events of atherosclerosis

Atherosclerosis, blood vessel narrowing in response to inflammation and lipid accumulation, is a multi-step process and involves diverse subtypes of cells and tissues [6]. Risk factors which might lead to atherosclerosis can be subsumed in two groups: genetic background and environmental factors. Whereas genetic predispositions include hypercholesterolaemia causing high levels of low-density lipoprotein (LDL) [7], reduced concentrations of high-density lipoprotein (HDL) [8], mutations in the gene encoding LDL receptor-related protein 6 (LRP6) [9], gender [10] and family history [11], environmental factors include high-fat diet, smoking [7], or infections [12]. In most cases, however, a combination of increased life span, unhealthy eating habits and genetic susceptibility lead to atherosclerosis or other types of CVD.

The initial steps of atherosclerosis include the accumulation of LDL in the sub-endothelial matrix, which often takes place at arterial branching points, where endothelial cells (EC) are exposed to enhanced fluid shear stress [13]. In these areas, the EC monolayer is not as tight as in tubular regions of arteries and therefore lipoproteins can diffuse through the endothelium [14]. Together with LDL, other lipoproteins as lipoprotein A accumulate in the intima of the vessels and activate EC to produce pro-inflammatory molecules, as macrophage-stimulating growth factor (M-CSF) [15]. By upregulating adhesion molecules on their surface, EC attract monocytes and lymphocytes to bind and “roll” along the endothelial monolayer until they migrate into the intima of the vessel to become macrophages (Fig. 1). To be taken up by invading macrophages, LDL has to be highly oxidized, which is triggered by reactive oxygen species (ROS) and the activity of different enzymes, such as myeloperoxidase, sphingolipase and 12/15-lipoxigenase [16, 17]. In general, macrophages play a pivotal role in many stages of atherosclerosis from the initiation to more progressive states and exert many effects within the lesion, like infiltration, LDL uptake and cytokine production [18]. Macrophages express toll-like receptors (TLR) which have been shown to act as receptors for heat-shock proteins and modified lipids and lead to enhanced macrophage activation upon binding of their ligands [19]. On the other hand, macrophages upregulate a set of scavenger receptors as CD36 (cluster of differentiation 36) or scavenger receptor-A (SR-A), which enables them to bind oxidized LDL to become foam cells [20].

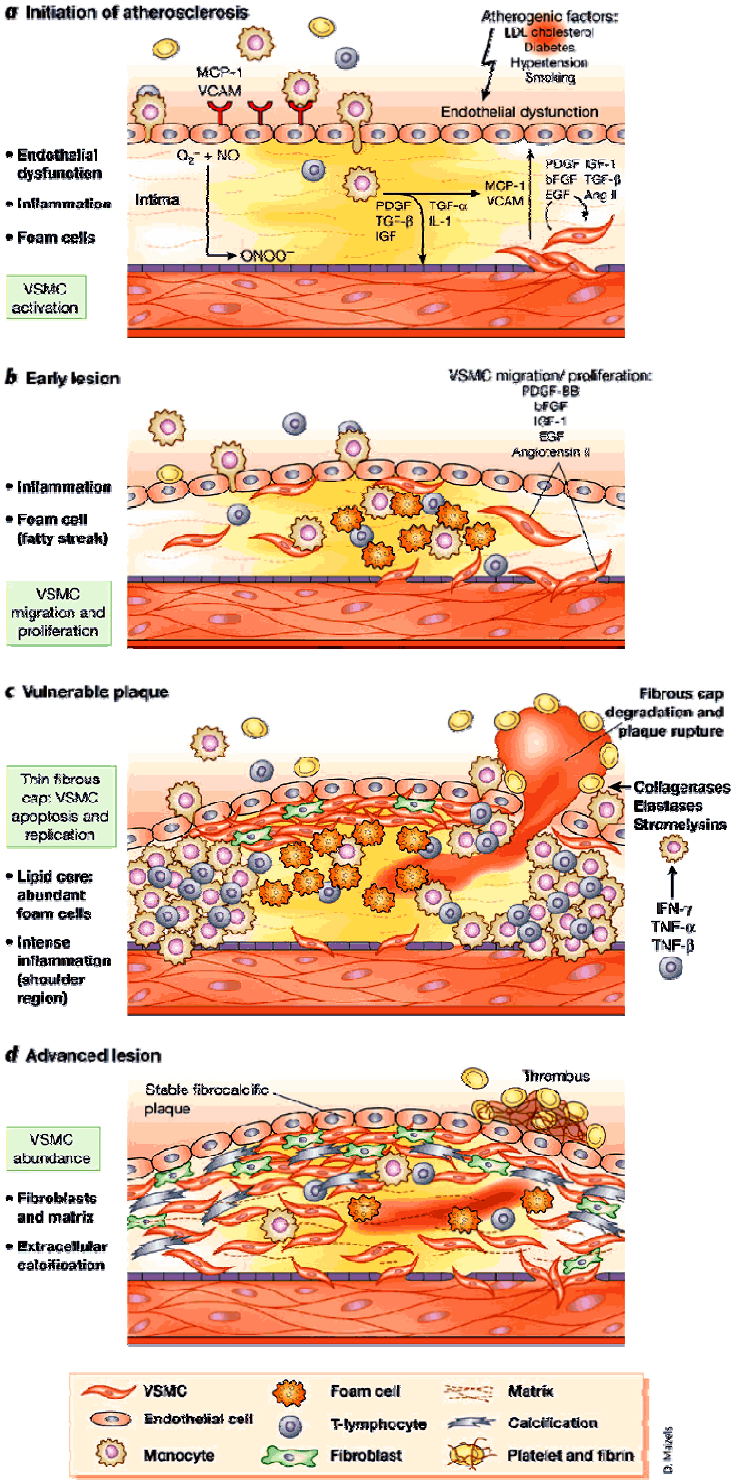

Figure 1: Four main steps in the progression of atherosclerosis

Atherogenic factors lead to endothelial dysfunction which, in turn, results in the upregulation of adhesion molecules. Immune cells (predominantly monocytes and T-cells) bind to receptors on endothelial cells and invade the intima of the vessel. Upon oxidative stress, LDL becomes oxidized and is subsequently taken up by macrophages which develop into foam cells. Enhanced growth factor production leads to the attraction and proliferation of VSMC which can result in the formation of a fibrotic cap. Cap rupture can happen in response to enzymatic activity, apoptosis of VSMC and shear stress and is followed by thrombosis. Sophisticated lesions can calicify and therefore prevent further fibrotic events. For abbreviations, see section G. Figure from Dzau et al. 2002.

A recent study revealed that the interaction of CD40 with CD40L, which is a central event in the induction of immune responses in lymph nodes, is also taking place in the atherosclerotic lesion. Since CD40 is expressed on macrophages, the interaction between T-cells and macrophages contributes to enhanced cytokine production [21]. High growth factor concentrations within the area of the lesion finally leads to the attraction of VSMC which migrate from the media into the intima of the vessel [22].

As a next step, VSMC start to proliferate and to produce a tight meshwork of extracellular matrix (ECM) which leads to both the thickening of the lesion and the building of a new layer of cells within the atherosclerotic plaque, the neointima [23]. VSMC then start to produce growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) or Angiotensin II (Ang II) which act both in an autocrine and paracrine way. It is still a matter of debate, whether neointima formation arises from some few VSMC, which expand in a clonal fashion, or if there is steady-state transmigration from the media into the atherosclerotic lesion [24, 25]. Furthermore, there is evidence that EC are able to trans-differentiate into VSMC in stages during embryogenesis and additionally give rise to neointima formation [26]. Some studies highlighted that bone marrow-derived progenitor cells of VSMC can account for the overall pool of VSMC within neointimal regions [27].

Highly proliferative VSMC produce an augmented mass of ECM, and together with cholesterol and its esters a fibrous cap can develop (Fig. 1). The incidence of fibrous lesions is enhanced by some parameters like hypertension or elevated homocysteine levels [28]. In response to shear stress and/or activity of certain enzymes within the lesion, such as matrix metalloproteinases (MMP) and collagenases, the fibrous cap can rupture [29, 30]. Furthermore, necrotic and apoptotic cells in the lesion can enhance rupture events, too [31]. Since the rupture of the cap directly leads to platelet aggregation at the site of injury, obviation of thrombosis is an important field of today´s research. Atherosclerosis-mediated thrombosis accounts for the highest number of atherosclerosis-related deaths due to stroke or heart attack. There are, however, some scientific problems to cope with, since no reliable and widely accepted animal models for atherosclerosis-associated thrombosis exist [32]. Calcification and neovascularization on the other hand are able to counteract thrombus origination, since both phenomena often lead to the stabilisation of the fibrous caps [33].

1.2. The role of VSMC in atherosclerosis

As already discussed above, VSMC play a crucial role in many stages of atherosclerosis [22], whereas their most predominant impact is considered to be on more advanced stages of the disease. However, VSMC can be found in the pre-atherosclerotic intima as well, where they build up so-called “intimal thickenings”. They are predominantly found in regions of arterial branches and occur nearly in all infants and can even develop by age [34]. Moreover, studies revealed that arterial regions which are likely to develop atherosclerotic lesions contain more intimal VSMC as other zones of the blood vessels [35]. Like in the research field of thrombosis, there is still a lack of appropriate animal models in the scientific research area of intimal thickenings.

The phenotype of intimal VSMC clearly differs from those of the media. In comparison to medial VSMC, intimal VSMC have a higher capacity to produce ECM and cytokines, whereas they express lower amounts of α-smooth muscle actin and smooth muscle myosin heavy chain (SM-MHC) [36]. Additionally, several studies have pointed out that VSMC are able to undergo switches from a contractile to a more “synthetic” phenotype, which is characterized by a higher ability to migrate and to take up lipids. Therefore VSMC give rise to the pool of lipid-loaded cells within the lesion [37]. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are well studied transmembrane proteins predominantly found on endothelial cells, where they are responsible for the binding and “rolling” of monocytes and leukocytes along the EC monolayer. The existence of VCAM-1 on VSMC opens new perspectives for cell-cell interactions in atherosclerotic lesions, as it was reported that VSMC could directly interact with both, macrophages and T-cells and therefore contribute to the inflammatory reaction within lesions [38]. VCAM-1-mediated interaction of VSMC with macrophages even prevented the latter from apoptosis, which might account for monocyte accumulation during early stages of atherosclerosis [39].

Cytokines are produced by nearly all cell types found in atherosclerotic lesions. VSMC predominantly release PDGF, interferon gamma (IFNγ), monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor beta (TGF-β). The contribution of VSMC-produced cytokines to the entire cytokine pool in the lesions is not exactly known, since all aforementioned molecules can be supplied by other cell types as well [40]. A

key event during atherosclerosis involving VSMC is the production of ECM. The network consists of proteoglycans, fibronectin and type I collagen. Moreover, lipoprotein integration into the meshwork is one hallmark of atherosclerotic ECM [41]. Once integrated, LDL becomes more rapidly oxidized which in turn enhances proteoglycan production of VSMC resulting in a positive feedback loop accelerating lesion progression [42]. The composition of the ECM has furthermore influence on VSMC proliferation, since laminin- or collagen-bound VSMC predominantly found in healthy vessels are arrested in G_1 phase, whereas proteoglycan-bound smooth muscle cells re-enter the cell cycle by downregulation of inhibitors of cyclin-dependent kinases (CDK) [43].

Hypertrophy is a unique feature of muscle cells and contributes to the disease pattern of hypertension and atherosclerosis [44]. It is defined as an increase in cell size without duplication of DNA and is a reversible process which plays an important role in arterial wall thickening [45, 46]. The main molecules to induce hypertrophy are ligands of G protein-coupled receptors (GPCR), such as Ang II, the major effector of the renin-angiotensin system (RAS), which is highly involved in a plethora of CVD. It acts mainly in an autocrine fashion to induce protein synthesis, ROS production and water uptake [47], which will be discussed later in detail.

2. VSMC activation

2.1. Activation by EGF

One of the most abundant receptors found on cells contributing to atherosclerosis is the EGF receptor (EGFR). It is part of the receptor tyrosine kinase (RTK) family and has been detected on rat and human VSMC [48, 49], endothelial cells and macrophages [50]. Moreover, EGFR overexpression is often an incidence for the development of solid tumours [51]. The receptor is contributing to different stages of mammalian embryonic development and EGFR knock-out mice display epithelial immaturity and dysfunction and die 15-18 days after birth [52, 53].

EGFR ligands are small peptides sharing a motif of six cysteine residues within a region of 35-40 amino acids, which build up a three-loop structure to bind to their cognate receptors. The family consists of: EGF, heparin-binding EGF-like growth factor (HB-EGF), TGF-β, epiregulin (EPR), betacellulin (BTC), amphiregulin (AR) and four neuregulins (NRG-1-4) [54]. To become biologically active, all members of the family have to be cleaved by a disintegrin and metalloproteinase (ADAM) proteins. The inactive precursor is a transmembrane protein which contains nine extracellular domains of which only the membrane-closest is defined as the later active molecule [55]. Whereas EGF is only secreted by platelets in sufficient amounts, HB-EGF can act both autocrine and paracrine and is released by nearly all cells located at atherosclerotic lesions. Moreover, it has become recognized that HB-EGF is elevated in adipose tissue, and obese mice and humans show increased plasma levels of HB-EGF, which may give rise to coronary diseases [56]. Nevertheless, all members of EGFR ligands trigger transformation of VSMC to a migratory and proliferative phenotype and are involved in the progression of atherosclerosis [57]. Since platelets are not able to synthesize EGF on their own, it is speculated that EGF is produced by megakaryocytes and stored by platelets in high amounts for later growth factor release [58].

The 200 kbp gene encoding for the EGFR protein is located on the short arm of chromosome 7 and gives rise to four members of the protein family (named ErbB1-4) [59]. Whereas VSMC express all four types of EGF receptors, other cells involved in atherosclerosis only display ErbB1 (also termed as the “classical” EGFR) on their surfaces. In addition, binding specificities underline the importance of ErbB1, since it is the only receptor type to bind EGF, HB-EGF, TGF-β, EPR and BTC [60]. The receptor comprises a 621 amino acid extracellular, a 23 amino acid transmembrane and a 542 amino acid cytoplasmatic domain, whereas the extracellular region can be dissected into four subdomains. Two cysteine-rich domains are interacting with two identical domains of other EGFR proteins to build homo- or heterodimers, whereas the two remaining domains interact with the ligand and thus account for the specificity of the receptor [61]. The most conserved region within the EGFR protein family encodes for the cytoplasmatic kinase domain which was reported not to affect the expression pattern or membrane trafficking of EGFR but is pivotal for signal transduction and cellular responses [62].

The potency of a growth factor to induce a cellular response is not only determined by the concentration of the ligand, but also by the surface abundance of the receptor. Therefore receptor shuttling, internalization and degradation are appropriate means for cells to influence signalling strength and duration [63]. After protein translation, EGFR are directly shuttled to the membrane, where they can be integrated into caveolae (cholesterol-rich membrane regions with high amounts of the protein caveolin), which is, however still a matter of debate [64, 65]. Upon ligand binding EGFR are shuttled to lipid rafts, which are cholersterol-rich membrane regions where several proteins are concentrated and therefore reflect “hot spots” of signalling [66]. Moreover, it was observed that EGF stimulation leads to downregulation of EGFR on the surface, but it is still debated whether this fact is due to enhanced receptor endocytosis or a decrease in EGFR backhaul to the surface [63]. In addition, the dimerization status of the receptor is another variable in the internalization efficiency, since EGFR homodimers are more rapidly endocytosed as heterodimers of EGFR plus ErbB2 or ErbB3 [67].

The initial steps of EGFR-mediated signalling pathways are incompletely understood. Two current models exist: (a) one ligand-bound EGFR monomer binds asymmetrically to an unligated EGFR before a second EGF molecule binds [68]; (b) symmetric interaction of two EGFR monomers which are both already coupled to EGF molecules [69]. However, receptor dimerization is the crucial step to bring the two cytoplasmatic kinase domains in proximity to stabilize and autophosphorylate each other at the very C-terminal region. This part of the receptor is the most variable within the receptor family and contains five important phosphorylation-sensitive tyrosine residues, which can be both autophosphorylated and transphosphorylated, for example by Src [70, 71]. Thereafter, src homology 2 (SH2) domain-containing proteins can bind and initiate the assembly of signalling complexes. Among others, direct interaction partners of EGFR at distinct tyrosine residues at the C-terminus are: phospholipase C gamma (PLCγ), phospholipase D (PLD), Src, growth factor receptor-bound protein 2 (GRB2), GRB2-associated binder-1 (Gab-1), , SH2-containing phosphatase-1 (Shp-1) and Ras [66, 72-74]. The fact that various EGFR homo- and heterodimers can emerge potentiates the number of possible C-terminal signalling complexes and is therefore an important basis for the diversity of the signalling output [75]. Phosphoinositide-3 kinase (PI-3K), for example was shown to interact either in direct fashion or indirectly via adapter proteins, depending on the type of the EGFR dimer [76]. Additionally, Xia and colleagues have shown that signal transducer and activator of transcription (STAT) proteins were found in direct interaction with unphosphorylated EGFR, meaning that also inactive EGFR is able to bind proteins [77]. Given the fact that many signalling complexes can form as a result of EGFR activation, it is unsurprising that multiple pathways, which are often cross linked, are brought into action upon EGF stimulation. In the following section, only the most important cascades will be discussed.

Raised attention has been drawn to the Ras activation pathway over the last decades. EGFR-mediated Ras activation starts with the recruitment of GRB2 (Fig. 2). It is still not fully clear if in this particular case, GRB2 binds directly to the activated EGFR or needs the assistance of the adapter SH2-containing collagen-related protein (Shc) [78]. Nevertheless, key player of Ras activation is GRB2, which is constitutively bound to the guanine nucleotide exchange factor son of sevenless (SOS). By bringing SOS in proximity to membrane-anchored Ras, GDP is exchanged for GTP leading to Ras activation. Next, Raf gets activated by the kinase domain of Ras. MAPK/ERK kinase-1

(MEK-1) is subsequently phosphorylated and finally activates ERK-1 and -2, which translocate to the nucleus where they phosphorylate specific transcription factors, as Ets or c-fos [79]. The activation of MAPK provides a negative feedback loop, since once phosphorylated, the GRB2-SOS complex dissociates and abrogates the signal cascade [80]. The adapter protein GRB2 consists of two SH2 domains and one SH3 domain and is therefore able to interact with a multitude of different proteins. In this respect, the question arises, how specificity and selectivity are achieved. First, compartmentalization plays an important role. In one small area of the cell, for example at the membrane, the concentration of certain proteins is higher than elsewhere. Second, the concentration of individual binding partners has impact on the protein-protein interactions. At low concentrations interactions of highly affine proteins are preferred, whereas low-affinity interactions can take place when protein concentrations are high.

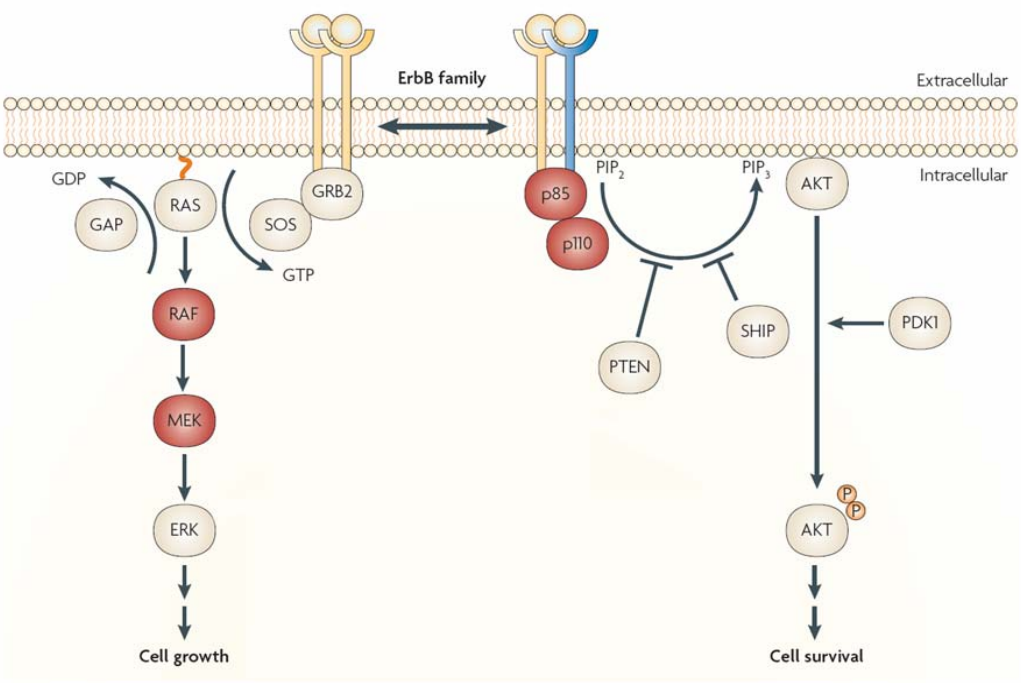

Figure 2: EGF-mediated signalling

Upon ligand-binding, EGF -monomers form homo- or heterodimers to transphosphorylate their C-terminal domains. Either adapter proteins (GRB2) or kinases (PI-3K) can bind and initiate signal transduction towards the Ras or the Akt pathway. For abbreviations, see section G. Figure from Sharma et al. 2007, adopted.

A second major interaction partner of great importance is Src, though it is not fully understood to which extent EGFR and Src need each other to be activated. On the one hand, overexpression of Src clearly enhanced proliferation of EGF-triggered cells, but contrariwise interaction of Src with the EGFR was difficult to prove. The two kinases share many substrate molecules making it impossible to distinguish between Src- and EGFR-mediated activation [81, 82]. Hence, it is controversial if Src accounts for EGFR activation or if Src activity is the result of an upstream EGFR activation.

Another class of proteins activated by EGFR signalling is connected to phospholipid metabolism. Phospholipids often act as important second messengers in the cell and therefore their production has to be regulated in a stringent fashion [83]. Phosphatidic acid (PA) is generated by PLD from phosphatidylcholine and PLD can be directly activated by the EGFR [84]. PLCγ on the other hand catalyzes 1,2-diacylglycerol (DAG) plus 1,3,5-trisphosphate (IP_3) from its precursor phosphatidylinositol 4,5-bisphosphate (PIP_2). DAG is an important cofactor for protein kinase C (PKC) and IP_3 triggers Ca^{2+} release from intracellular stores and thus affects Ca^{2+}-dependent signalling pathways [85]. By affecting survival, proliferation and migration PI-3K is inevitable in intracellular signal responses [86]. PI-3K is a heterodimer consisting of a regulatory domain (p85) and a catalytic subunit (p110). Of the three family members only class IA-enzymes of PI-3K are activated by the EGFR [87]. In the cytoplasm, p110 remains in an inactive state tightly bound to p85. By binding directly or via adapter proteins to EGFR, p85´s function is to bring p110 in vicinity to its membrane-anchored substrate PIP_2 where PI-3K changes its conformation and p110 becomes activated (Fig. 2) [88]. PI-3K catalyzes the production of membrane-bound PIP_3 from PIP_2 which serves as docking site for pleckstrin homology domain (PH)-containing molecules.

One of the best characterized PIP_3-binding molecules is Akt (also named protein kinase B [PKB]). Akt signalling plays a pivotal role in cell survival and cell cycle events. It was first detected as a homologue of viral *v-akt* and was shown to display protein homology with protein kinase A and C (PKA, PKC) leading to its surrogate name PKB [89, 90]. Until today, three members of the Akt family have been described (Akt1-3). Although they are transcribed from different genes, they share high sequence similarity and consist of an N-terminal PH domain followed by a kinase domain and a C-terminal regulatory domain [91]. Akt1 and Akt2 display a broad tissue distribution pattern,

whereas Akt3 is predominantly found in neuronal cells [92]. In vascular tissues, Akt1 has a central role [93]. Two phosphorylation sites are crucial for activation of Akt: Thr^{308} and Ser^{473}, whereas the latter amino acid is required for full kinase activity and might be autophosphorylated by Akt itself or by the mTOR complex 2 (mTORC2) [94]. As already mentioned above, by formation of PIP_3 Akt is recruited to the membrane, where 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates Akt at Thr^{308} (Fig. 2) [95]. Active Akt is then able to phosphorylate a broad spectrum of substrates, including prosurvival proteins and molecules promoting cell cycle progression, for example cAMP response element-binding protein (CREB), inhibitor of NF-κB (Iκ-B), $p21^{WAF1}$ or endothelial nitric oxide synthase (eNOS). Furthermore it can inactivate proapoptotic molecules like BAD [96]. Akt is therefore a central molecule in EGF-mediated VSMC activation.

It has also been reported that the estrogen receptor (ER) is able to take part in EGFR signalling [97]. Truncated versions of ERα were shown to be located at the cell membrane within caveolae, where they collaborate with PI-3K and Src in signalling [98]. Furthermore, a potential contribution of ER to Akt signalling has been documented [97].

2.2. Activation by PDGF

As the EGFR, the platelet-derived growth factor receptor (PDGFR) is a member of the RTK family. The fully functional PDGFR is a dimeric molecule consisting of either two α- two β- or one α- and one β-monomer(s) [99]. The PDGFR can be found on various cell types, including VSMC, endothelial cells, fibroblasts and neurons [100]. The great importance of the PDGFR has been demonstrated with the help of mouse knock-out studies, in which animals displayed severe kidney and blood vessel deficiencies and exhibited embryonic lethality [101]. The different cell types which contribute to atherosclerosis mainly express PDGFR-α and -β homodimers. The ligands for PDGFR appear in three isoforms, as do the receptors: PDGF-AA, PDGF-BB and PDGF-AB, each of them favouring its cognate receptor. PDGF-BB, however, has the ability to bind to all three receptor dimers and is therefore often used as the experimental stimulus of choice [102]. Platelet-released PDGF is a very potent chemoattractant and mitogenic molecule, whose binding to the receptor triggers similar intracellular signalling events as

EGF does at the EGFR and for VSMC, PDGF represents even the strongest pro-migratory molecule. Dimerization of the PDGFR leads to autophosphorylation at several tyrosine residues within the 459 amino acids-containing C-terminus and subsequent binding of mainly SH2 domain-containing binding partners, among others: PI-3K, Src, PLCγ, Shp-2, GRB2, Ras and GTPase activating protein (GAP) [103]. By activation of the Ras, PI-3K and PKC pathways PDGF signalling leads to cell proliferation, enhanced motility and reduced apoptosis, while diverse pathways are able to cross-talk with each other. As in EGF signalling, PDGF-triggered cell activation is influenced by both, ligand concentration and receptor abundance [104]. Diverse negative feedback loops enable the shut-off of PDGF signalling, e.g. activation of certain phosphatases (Shp-2, low molecular weight phosphotyrosine phosphatase [LMW-PTP] or CD45), induction of cyclooxygenases or receptor internalization [105-107].

Taken together, PDGF signalling has many molecular similarities with EGF signalling, since both stimuli lead to enhanced cell viability, motility and proliferation. Moreover, the same adapter proteins can be recruited to the activated receptors leading to the induction of aforementioned signalling pathways. Evident cell-specific differences between EGF and PDGF signalling are due to unequal receptor expression levels, ligand concentration and the duration of the signalling events [108].

2.3. Activation by Ang II

Ang II is the end product of a cleavage cascade involving several enzymes. Angiotensinogen, produced in the liver, is cleaved by renin to form angiotensin I (Ang I). By subsequent activation of angiotensin converting enzyme (ACE), Ang I is further cleaved to form the active octopeptide Ang II [109]. Since Ang II controls systemic blood circulation effects such as vasoconstriction or water and salt homeostasis, severe complications can occur if levels are enhanced. One of the most prominent negative effects is hypertension in response to enhanced and sustained vasoconstriction and therefore a lot of scientific effort is made to find accurate ways to counteract enhanced Ang II levels [110]. Until now, ACE inhibitors and Ang II type-1 receptor (AT-1R) antagonists are used to medicate hypertension [111].

At the molecular level Ang II mainly acts via binding to AT-1R, a member of the seven-transmembrane family of GPCR. GPCR represent the biggest family of transmembrane receptors in many species, including mouse and human [112]. They comprise seven transmembrane α-helices which covalently bind to G proteins at their C-terminal cytoplasmatic domain. The superfamily of G proteins can be divided into two classes. The first group of G proteins consists of only one subunit and these molecules are therefore called small GTPases. They will be discussed in another section later in detail. The other class of G proteins are heterotrimeric G proteins (also called large G proteins), which comprise three subunits (α, β, γ) and are coupled to GPCR. They are divided into four subclasses according to their α-subunit. Large G proteins get activated by ligand-induced conformational changes of the receptor which in turn leads to the exchange of GDP for GTP and subsequent dissociation of the α-subunit from the $\beta\gamma$-subunit [113]. Both subunits act as second messengers and can separately activate different signalling pathways [114].

AT-1R are expressed on a wide variety of cells, including vascular and coronary tissues [115]. The receptor expression can be influenced by Ang II itself, since enhanced Ang II levels can lead to upregulation of AT-1R [116]. Furthermore other agonists, as EGF or PDGF can influence AT-1R expression [117]. Like in the case of RTK, AT-1R signalling can be abrogated by receptor internalization after ligand binding. Another negative feedback loop is receptor phosphorylation by G protein receptor kinases (GRK), which leads to the release of the G protein from the receptor [118].

Ang II signalling in VSMC leads to enhanced vasoconstriction, hypertrophy, proliferation and cell survival. G protein-triggered activation of signalling molecules include PLC and PLD, which produce second messengers such as DAG or IP_3, in turn leading to Ca^{2+} efflux and cell contraction [119]. Moreover by activating PKC, DAG production leads to the activation of the growth-promoting Ras pathway [120]. Besides the “classical” G protein-mediated activation of signalling cascades, production of reactive oxygen species (ROS) plays an essential role in Ang II-triggered VSMC activation. ROS are mainly produced by NAD(P)H oxidases (NOX) and comprise several oxygen products including superoxide (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2) [121]. Especially hydrogen peroxide and superoxide have emerged not to be only oxidizing agents but being important second messengers participating in diverse signalling

cascades [122]. In addition to p38 activation, ROS are able to trans-activate RTK like EGFR and PDGFR in different ways. At least three possible mechanisms have been unravelled: (a) cysteine-containing, oxidation-sensitive phosphatases, for example Shp-2, are directly inhibited by ROS; (b) ROS can directly modify protein-protein interactions by altering the occurrence of disulfide bonds; (c) ROS enhance proteolysis of suppressor-proteins [123, 124]. All these actions prolong or even enhance RTK signalling cascades and therefore contribute to a more activated cell phenotype.

In feedback regulation processes of signalling cascades, phosphatases in general and protein tyrosine phosphatases (PTP) in particular, act in a coordinated manner together with kinases to control both, strength and duration of signalling events in a very fine-tuned manner [125]. For the last few years it has become more and more evident how important and complex the activation, action and interplay between kinases and phosphatases are. Shp-2 is a non-receptor PTP consisting of two SH2 domains and a catalytical domain [126]. Shp-2 can be held in a self-inactivating conformation, where one of the SH2 domains physically interacts with the catalytical phosphatase domain. Upon binding to either phosphorylated tyrosine residues within the C-terminal domain of Shp-2 itself or to phosphorylated tyrosine residues on other proteins, Shp-2 opens up its conformation and enables the catalytic domain [127]. Another distinctive property of PTP is the presence of an oxidation-sensitive cysteine residue within the catalytic domain, which can lead to reversible inhibition of the phosphatase in the presence of ROS [128]. This additional inhibitory effect of ROS on PTP activity can result in sustained signal durations and is therefore an important regulatory mechanism in several pathways [129]. Moreover, Shp-2 was shown to be influenced in growth factor signalling in both, VSMC and fibroblasts and is involved in cell migration [130-132].

2.4. Activation via integrins

Integrins are a huge family of transmembrane proteins which major functions are the anchorage of cells to the surrounding ECM, the intracellular fixation of the cytoskeleton and the transmission of survival signals [133]. The family comprises 18 α- and 8 β-subunits, which can form at least 24 different non-covalently bound heterodimers [134]. Each of the monomers consists of a large extracellular, a transmembrane and a quite

short intracellular domain, of which only the β-subunit contains two tyrosine residues which are prone for phosphorylation [135]. Integrins were first discovered to solely act as adhesion molecules but for several years, it is getting clearer that they take part in various aspects of cell behaviour, like proliferation, survival and motility [136]. Integrin knock-out studies support the broad expression spectrum and the varying duties of the diverse integrin subtypes. Ablation of integrin genes can either lead to embryonic lethality (β_1 knock-out) or give rise to normal mouse development (β_5 knock-out) (reviewed in [137]).

The list of possible extracellular binding proteins is long and comprises, among others, laminins, collagens, fibronectin, vitronectin and osteopontin. [138]. All ligands share the crucial receptor recognition sequence Arg-Gly-Asp (RGD) which directly interacts with both subunits of the integrin heterodimer [139]. Whereas the positively charged arginine interacts with negatively charged side chains on the α-subunit, aspartic acid couples to a region on the β integrin, called MIDAS (metal ion-dependent adhesion site) [140]. It is still not fully understood, which structural rearrangements occur after ligand binding. A recent model suggests that after binding to ECM proteins, the two very flexible subunits undergo a scissor-like movement followed by a switchblade-like opening of the two extracellular parts of the integrin, which ends up in a highly accessible cytoplasmatic β-subunit [141]. It is now more obvious that integrin activation is a multi-step process and that several conformational states can be achieved, which results in diverse ligand affinity levels.

After ligand binding, integrins often cluster in caveolin-enriched regions within the membrane which are called focal adhesions [142]. These regions often comprise other molecules involved in signalling, as RTK and intracellular kinases (see above). Within the last decade it emerged as a fact that integrin and growth factor signalling are tightly interconnected. First evidence, however, was given nearly 30 years ago, when Src was found to be localized within focal adhesions and scientists were able to immunostain these sites with phosphotyrosine-specific antibodies [143, 144]. Direct proof was gained when physical interaction of integrins with growth factors was verified and integrin agonists were proven to phosphorylate growth factor target proteins [145, 146]. Additionally, several growth factor stimuli as EGF or PDGF but also the hormone insulin were shown to optimally activate cells only when bound to ECM [147]. Furthermore,

both RTK and integrins can act as mechanosensors in response to shear stress, where they translate mechanical stimuli into intracellular chemical signals [148].

In vascular biology, several integrins were shown to play essential roles in adhesion and signalling: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ respectively. On VSMC, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_V\beta_3$ are found to be expressed. [149]. In vascular tissues $\alpha_V\beta_3$ is of particular interest since both, α_V and β_3 single knock-out mice display severe defects concerning intestinal vasculature and show inhibited platelet aggregation [150, 151]. Furthermore, several independent studies discovered major implications of $\alpha_V\beta_3$ in diverse signalling pathways. Co-immunoprecipitation studies revealed that $\alpha_V\beta_3$ interacted with PDGFR and influenced PDGF-triggered migration, $\alpha_V\beta_3$ ligands enhanced survival in an EGF-dependent manner and furthermore prolonged EGFR phosphorylation. $\alpha_V\beta_3$ antagonists on the other hand induced apoptosis in proliferating endothelial cells, suggesting a survival-specific role for $\alpha_V\beta_3$ [152-154]. Additionally, various studies examined the role of $\alpha_V\beta_3$ in different diseases. Antagonists to $\alpha_V\beta_3$ inhibited the progression of breast cancer and neovascular diseases as rheumatoid arthritis and even diminished the infection rate of hanta-, and rotaviruses [155-157]. $\alpha_V\beta_3$ was furthermore shown to be involved in the migration of VSMC during restenosis (renarrowing of blood vessels after angioplasty) [158].

As mentioned above, the cytoplasmatic domain of the β integrin contains two tyrosine residues which can be phosphorylated and serve as recognition sequences for SH2-containing proteins like Shc. Since integrins do not contain a catalytic domain, other kinases have the ability to trans-phosphorylate β-subunits of integrins. However, phosphorylation is not mandatory since proteins expressing SH3 domains are able to bind to the cytoplasmatic tail of integrins in a phosphorylation-independent manner, which represents even the bigger pool of integrin interaction partners [159, 160]. Enzymatically inactive talin and vinculin are both linking integrins to the intracellular actin network [161]. Contrarily, kinases, phosphatases and adapter proteins actively contributing to several signalling events also bind directly to integrins, as integrin-linked kinase (ILK), Src, GRB2, paxillin or Shp-2 [160]. Of exceptional importance is focal adhesion kinase (FAK) which is linked to integrins either via paxillin or Src and influences both integrin and growth factor signalling.

2.5. Focal adhesion kinase, the main link from integrins to growth factors

FAK was first discovered 1992 independently by two groups as a non-receptor kinase mainly found within focal adhesions [162, 163]. The family of FAK comprises only two members: FAK and proline-rich tyrosine kinase 2 (PYK2). FAK is widely expressed in nearly all tissues, whereas PYK2 is predominantly found in brain tissue and to a low extent in liver, spleen, kidney, lung and the hematopoietic system [164]. Moreover, truncated versions of FAK and PYK2, FAK-related non-kinase (FRNK) and PYK2-related non-kinase (PRNK), are expressed in certain tissues and act as dominant-negative regulators of FAK and PYK2 signalling [165]. Due to its wide expression pattern, FAK knock-out mice die early in embryogenesis but mesodermal cells obtained from these embryos at day 8 showed a reduced migration and spreading capacity, indicating the important role of FAK in cell motility. However, these cells exhibited normal focal contact formation, which means that FAK is not obligatory in these processes [166]. Interestingly, FAK knock-out embryos exhibit severe vascular defects, as they fail to develop blood vessels which highlights a crucial role for FAK in vasculogenesis [167].

FAK is a 125 kDa protein that contains an N-terminal FERM (protein 4.1, ezrin, radixin and moesin homology) domain, which serves as docking site for growth factor receptors [168]. Moreover, it can interact with actin-binding proteins like ezrin and mediates recruitment of non-receptor tyrosine kinases to RTK or integrins [169]. The FERM domain can furthermore be sumoylated leading to enhanced FAK activity and nuclear translocation [170]. The centrally located catalytic domain is followed by a C-terminal focal adhesion targeting (FAT) domain, which serves as docking site for GRB2, paxillin and talin, all adapter proteins which target FAK to integrins within focal contacts [171]. Between the kinase domain and the FAT domain, two proline-rich regions can be found, which provide binding sites for SH3-containing proteins, such as GTPase regulator associated with FAK (GRAF) and p130Cas (p130 Crk-associated substrate), the latter important for promotion of migration (Fig. 3) [172].

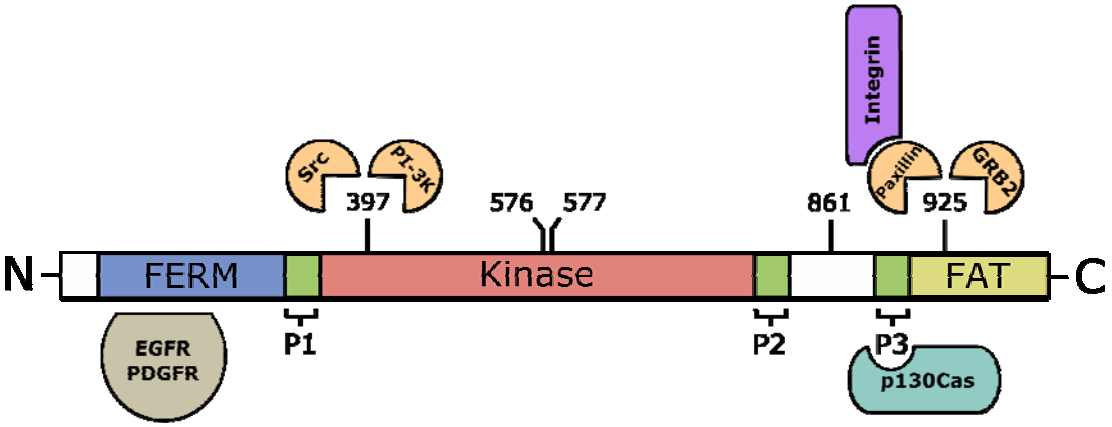

Figure 3: Schematic representation of FAK with selected interaction partners

FAK is able to autophosphorylate itself at Tyr^{397}, which serves as docking site for Src and PI-3K. Src is able to further phosphorylate FAK at Tyr$^{576/577}$, Tyr^{861} and Tyr^{925}. The latter is an important binding region for structural or adapter proteins such as GRB2. Furthermore, paxillin binds and connects FAK to integrins. Proline-rich motifs (P1-P3) display anchoring sites for SH3-containing proteins such as p130Cas. The N-terminal FERM-domain couples FAK to RTK. For abbreviations, see section G.

Of particular importance is binding of Src to phosphorylated Tyr^{397} near the proline-rich region (P1, Fig. 3), since Src is able to phosphorylate FAK at Tyr$^{576/577}$, which together with the autophosphorylation site Tyr^{397} facilitates full FAK activity [173]. FAK-bound Src on the other hand undergoes conformational changes resulting in an even higher activation status of Src. Moreover, phosphorylated Tyr^{397} can be recognized by several other SH2-containing proteins, as PI-3K, PLC, Shp-2 and other members of the Src family [174]. Beside Tyr$^{576/577}$, residues Tyr^{861} and Tyr^{925} can be phosphorylated by Src, the latter becoming a recognition site for the adapter GRB2, linking FAK signalling to the Ras pathway [175]. Tyr^{861} on the other hand, is very poorly characterized and it is still unknown which consequences emerge when FAK becomes phosphorylated at this specific residue. After growth factor stimulation or during mitosis, one serine residue within the FAT domain can get susceptible for phosphorylation, too [176]. However, the molecular effects of serine phosphorylation of FAK are still under investigation.

The main substrates of FAK are paxillin and p130Cas, which can be phosphorylated by the active Src-FAK complex. Crk (CT10 sarcoma oncogene cellular homologue) can then bind to phosphorylated paxillin and p130Cas, which leads to enhanced migration potential upon activation of small GTPases. Inhibition of FAK activity can occur either via

dephosphorylation or via binding of inhibitor proteins to the catalytic domain of FAK, such as FAK-inhibitory protein 200 kDa (FIP200). Phosphatases reported to be able to bind to and dephosphorylate FAK are LMW-PTP or Shp-2 [177].

The activity of FAK can be influenced by diverse input signals, from integrins to growth factors. Thereby, the multiple tyrosine residues of FAK act as a platform for many different interaction proteins and because of this, FAK plays an indispensable role in multiple signal transduction pathways. It has been clearly shown, that FAK is necessary for cells to survive and resist anoikis, a term describing apoptosis as a result of loss of adherence [178]. Moreover, FAK is indispensable in various aspects of cell motility. Due to its very upstream position in signalling, it is still unknown in which way certain signal inputs lead to specific and well-timed arrangements of FAK-protein complexes and to very distinct downstream effects. Future work will contribute new knowledge to current open questions of FAK activation.

2.6. Molecular basics of VSMC migration

The term migration describes the orchestrated and directed movement of cells towards a target area. Chemotaxis includes migration towards a gradient of a chemotactic stimulus, for example a growth factor [179]. At the molecular level, migration starts with the transduction of a chemoattractant stimulus from the cell surface to the cytoplasm, where coordinated cytoskeleton rearrangements lead to the polarization of the cell. At the leading edge, actin polymerization induces the formation of extensions, which show high adhesiveness to the substratum due to newly formed focal contacts [180]. Simultaneously at the rear end, focal contacts disperse and the myosin machinery is responsible for the trailing of the cell's back side towards the stimulus. Moreover, it has to be ensured that all organelles move with the cytoskeleton towards the leading edge. By attaching the subcellular structures to motor proteins, they can move along the microtubules [181]. Of particular importance is the microtubule organizing centre (MTOC), where polymerization events of α and β tubulin take place. MTOC is located in front of the nucleus near the leading edge and is also involved in cell polarization events [182]. In general, actin filaments are considered to be responsible for the maintenance

of the cell shape and microtubules are stronger involved in trafficking duties, like organelle and protein shuttling.

Two types of transmembrane proteins play the central role in cell movement: integrins, which loosen or tighten the connection to the ECM and growth factor receptors (RTK and GPCR). As already mentioned above, PDGF is the most important pro-migratory stimulus for VSMC [183]. Additionally, Ang II, TGF-β, thrombin, HB-EGF, EGF but also collagens, ATP, glucose or serotonin are reported to act as migration stimuli for VSMC (reviewed in [184]). On the other hand, different agents inhibit VSMC migration: hypoxia-inducible factor-1 (HIF-1), interleukin-1β, interleukin-10, epinephrine, dopamine or cAMP (also reviewed in [184]). Moreover, physical factors can affect the migration potential of VSMC, like shear stress or blood flow [185, 186]. PDGF-BB is furthermore able to indirectly enhance and prolong migration by influencing the expression of EGF and fibroblast growth factor (FGF), which are both able to act in an autocrine fashion [187]. *In vivo*, migration is not triggered by a single stimulus which is often used *in vitro* but is rather a result of a diverse cocktail of chemoattractants and anti-migratory stimuli. At the molecular level, *in vivo* migration is orchestrated by several signalling cascades which probably cross-talk to each other [188]. One example is the proven migration-enhancing cross-talk between PDGFR and sphingosine-1 phosphate (S1P) receptor [189].

At the intracellular site, actin polymerization at the plus end and disassembly at the minus end of filaments is a central step for cells to move forward. Actin assembly is orchestrated by formins with the help of small GTPases. This leads to the activation of Wiskott-Aldrich syndrome protein (WASP) and WASP family verprolin-homologous protein (WAVE), which in turn induces the actin-related protein 2/3 (ARP2/3) complex [190]. Other concentrated areas are focal contacts at the cell membrane, where both signalling proteins, such as Src and FAK, and structural proteins, as paxillin, vinculin or tensin, are located [191, 192]. The important development of new focal contacts at the leading edge of a migrating cell depends on the following steps: ligation and clustering of integrins, intracellular phosphorylation events of target proteins and the resulting assembly of filamentous actin (F-actin) structures [193]. Beside protein phosphorylation (for example FAK and paxillin), additional activity of calpains and MMP, which are able to cleave proteins within focal contacts, has been described to be an inevitable event in VSMC migration [194, 195]. Increase in intracellular Ca^{2+} furthermore induces migration,

as elevated Ca^{2+} activates myosin light chain kinase (MLCK), a direct activator of the motor protein myosin II [196].

The small GTPases Rac1, RhoA and cdc42, which build up the subgroup of Rho family GTPases, share 80-90% homology with each other [197]. As the trimeric GPCR-coupled large G proteins, they belong to the superfamily of G proteins. In contrast to the trimeric molecules, they comprise only one subunit which is similar to the α-subunit of the large G proteins. Small GTPases get activated by exchanging GDP for GTP with the help of guanine nucleotide exchange factors (GEF). GTPase activating proteins (GAP) on the other hand accelerate hydrolysis of GTP to GDP and act as negative regulators of the small GTPases (Fig. 4). Furthermore, guanine dissociation inhibitors (GDI) minimize the spontaneous GDP-GTP exchange and hold GTPases in an inactive state [198]. Proteins of the Rho family do not only contribute to all stages of migration (Fig. 4), but are also involved in other cellular events such as transcription, survival or cell cycle progression [197, 199, 200]. Several stimuli have been demonstrated to activate Rho GTPases in VSMC, among others EGF and PDGF [201, 202]. Although induced very early in migration, studies report that FAK is situated upstream of Rho A and is able to activate it [203]. There are, however, reports which put FAK rather downstream of the Rho GTPases [204].

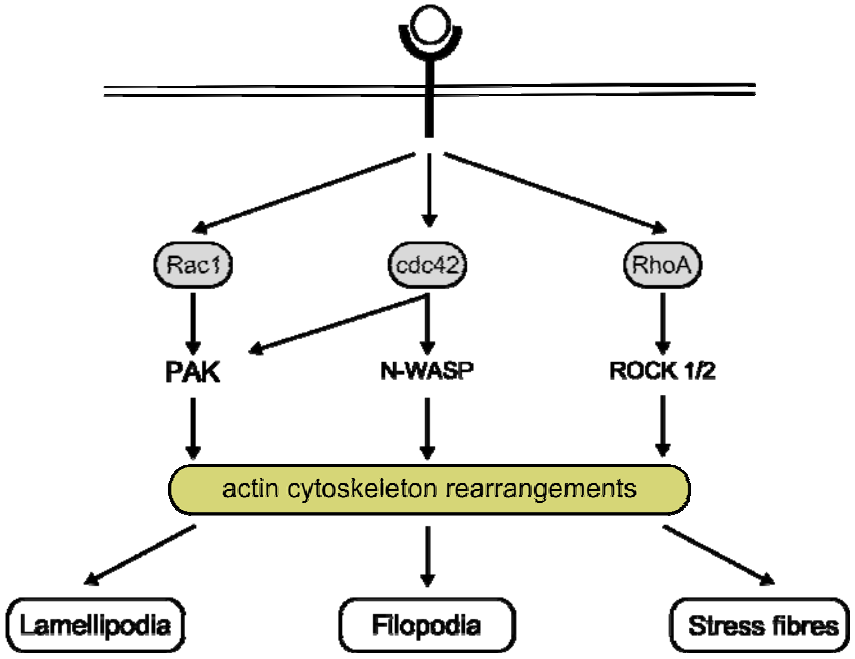

Figure 4: Signalling proteins involved in migration

Upon growth factor binding, small GTPases Rac1, cdc42 and RhoA become activated and initiate distinct signalling pathways to form accurately defined actin substructures. Figure from Chelly et al. 2001, adopted.

In VSMC, the number of direct downstream effector proteins of Rho GTPases is big. Rho A is directly activating Rho-associated coiled-coil-containing protein kinases (ROCK1/2) which phosphorylate and inactivate myosin light chain phosphatase (MLCP), leading to enhanced myosin contractility [205]. Furthermore, Rho A activates LIM kinase which enhances stress fibre formation (parallel microfilament bundles containing F-actin) [206]. The central downstream effector of both cdc42 and Rac1 is p21-activated kinase (PAK). PAK has been shown to exert many different effects on cytoskeleton proteins. On the one hand, it phosphorylates LIM kinase and MLCP, both promoting migration [207] and on the other hand it is able to phosphorylate cortactin and caldesmon, which enhances actin depolymerization and stress fibre disassembly [208, 209]. Thus, it is still unclear how PAK activation leads to coordinated cell movement. Rac1 is moreover involved in lamellipodia formation (branched protrusions at the front edge of a migrating cell) where it facilitates the formation of the ARP 2/3 complex, responsible for actin-branching [210]. Cdc42 on the other hand was shown to be mainly responsible for filopodia development. These spike-like actin bundles are tightly engaged with structural proteins as fimbrin and emerge from the very front of the moving cell. These “tentacles”

are the first regions where new adhesion sites are formed and focal contacts emerge [211].

Cell migration is a tightly orchestrated rearrangement of the whole cytoskeleton of a cell. Although many molecular key players are well known it is still astonishing, how certain stimuli lead to either cell growth or migration. Intracellularly, it is also very interesting that master regulators such as GTPases are able to process one signal input to specific downstream effects. Future work will hopefully provide more insight, how signal specificity is guaranteed within a cell.

3. Resveratrol

The polyphenolic compound resveratrol (RV, Fig. 5) is a phytoalexin, a molecule which certain plants produce in response to injury, stress, UV light or infection, and which is predominantly found in berries, nuts and grapes [212]. It was first discovered to be the active compound in a Japanese plant (*Polygonum cuspidatum*), used against diseases such as dermatitis and gonorrhoea [213]. In 1976 it was first detected to be produced in grapevines (*Vinis vitifera*) and was later on also quantified in red wine [214, 215]. RV is discussed to play a major role in explaining the French paradox, which is the fact that in certain regions of France the risks to develop cardiovascular diseases are very low, although the traditional diet is rich in saturated fatty acids. Additionally, in France the consumption of red wine is highest in Europe and therefore this is discussed to counteract the unhealthy eating habits [216].

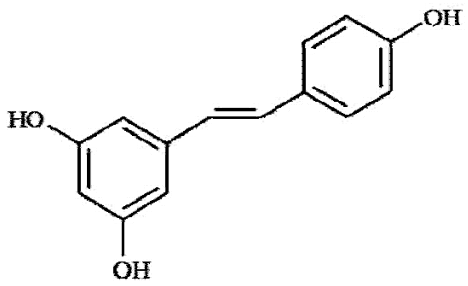

Figure 5: Chemical structure of RV

The amounts of RV detected in red wine range from 1 to 26 µM [217]. The concentrations can vary, since many parameters influence RV production in grapes: sun exposure, cultivar or previous fungal infections. White wine contains only little amounts of RV because it is, unlike red wine, fermented without the fruits and RV is not found in the juice but in high concentrations in the skins [215].

In the last decade, a lot of effort was made to scientifically proof the health-beneficial effects of RV. Indeed, it has become clear that RV can affect a plethora of different molecular targets and can act also systemically. A major molecular effect of RV is its antioxidative property. It was shown, that RV can counteract oxidation of LDL, an important step in the initiation of atherosclerosis [218]. Furthermore, it also inhibited the cellular uptake of oxidized LDL [219]. Intracellularly, RV was shown to influence the activity of certain endogenous antioxidants, as catalase, glutathione reductase or glutathione-S-transferase [220]. Neuro- and cardioprotective heme oxygenase-1 was shown to be induced by RV, too [221]. In ApoE-deficient mice, an animal model for inducible hypercholesterolemic atherosclerosis, RV enhanced ischemia-induced neovascularization [222]. RV also inhibited the aggregation of platelets, which can occur in late stages of atherosclerosis [223]. In humans, red wine intake went along with enhanced antioxidant activity measured in serum [224]. When using cultured VSMC, RV was shown to inhibit proliferation and ROS production [225, 226]. It blocked moreover both Ang II- and EGF-induced Akt phosphorylation [227].

Among the diverse effects of RV, its anti-inflammatory and immunomodulatory capacity should be mentioned. It was shown, that RV affects the activity of cyclooxygenases (COX) both *in vitro* and *in vivo* [228, 229]. In macrophages, LPS-induced iNOS activation was reduced by RV and the expression of adhesion molecules on polymorphonuclear (PMN) cells was diminished by addition of RV [230, 231]. At the transcriptional level, RV was documented to affect the activity of NF-κB by either inhibiting I-κB phosphorylation or by abolishing nuclear translocation of p65 [232, 233]. Since NF-κB is a transcription factor involved in diverse cellular processes ranging from apoptosis to inflammation [234], molecules as RV are of particular interest for clinicians and researchers. Furthermore, RV inhibits the activity of another important transcription factor, activator protein-1 (AP-1) and was shown to be able to act both as agonist or antagonist for the estrogen receptor (ER) mainly by its structural similarity to estrogen [233, 235, 236]. It is also proven that RV can act as an anti-invasive compound, since it diminishes migration of certain cancer cells in part by affecting the phosphorylation status of FAK [237, 238]. Direct interactions of RV with distinct cell components have been observed, ranging from surface receptors as integrins to cytoplasmatic proteins or even nuclear molecules including DNA [239-241].

Remarkable effects have been documented when administering RV systemically. Baur and colleagues demonstrated that RV supplementation (22.4 mg/kg/d) in mice counteracted the physiological parameters of a high-calorie diet, as elevated body weight, decreased motor function or low insulin sensitivity [242]. Similarly, another study revealed that RV (4 g/kg food/d) enhanced mitochondrial function, muscle strength and endurance of both standard- and high fat diet-fed mice [243]. Experiments using a short-lived fish showed that supplementation of RV (24-600 µg/g food/d) to the standard food enhanced lifespan and diminished age-related immobility and loss of cognitive competence [244]. In this context, RV is often referred to as caloric restriction (CR) mimetic, because an attenuation of food intake by 30% is a well-known possibility to prolong the life-span of model organisms [245]. In both models for life-span extension activation of sirtuin 1 (SIRT1), a histone deacetylase, has been shown to be crucial [243, 246].

Due to its apoptosis-inducing and anti-proliferative effects RV has been discussed not only to exert beneficial effects, since the specificity of RV to target cancer cells is not given [247]. Additionally, RV was documented to induce senescence (permanent cell cycle arrest, in which cells do not respond to any stimulus) in human endothelial cells [248]. Moreover, *in vivo* bioavailability of RV is very low as a result of diminished intestinal uptake and short half-life due to rapid metabolism (8-14 minutes) [249]. Until now, it is not fully understood if metabolites of RV can act as active substances, since such molecules are considered to exert reduced cell permeability [250].

Previous studies in our lab documented that 50 µM RV inhibited both, Ang II- and EGF-mediated Akt phosphorylation, whereas p38 phosphorylation remained unaffected by the polyphenol. While both, EGF-triggered and Ang II-induced activation and phosphorylation of the EGFR was not influenced by RV, the interaction between EGFR-associated Gab1 and p85 was inhibited by the polyphenol [227, 251]. However, the direct molecular target of RV in this specific context was not found so far.

4. Rationale and aims of the study

Today, it is well known that RV exerts many health-beneficial qualities, especially in the area of cardiovascular diseases. However, beside the versatile phenotypic cardioprotective properties, the cellular and molecular actions of RV are poorly characterized. We therefore wanted to gain more insight into the anti-migratory and anti-hypertrophic actions of RV in growth factor-induced VSMC.

Integrins and the direct downstream kinase FAK are not only important key players in adhesion and migration, they play also important roles in growth factor signalling and survival. We wanted to unravel, if integrin $\alpha_V\beta_3$ or FAK are involved in the potent inhibitory actions of RV on Akt phosphorylation via possible direct receptor binding, changes in the phosphorylation levels or alterations in the interaction capacity with target molecules. Moreover we tried to elucidate, if estrogen signalling is contributing to EGF-mediated Akt phosphorylation and if RV could therefore exert its inhibiting abilities through a possible antagonism to the estrogen receptor. As the antioxidative properties of RV are well-studied, we furthermore tried to unravel if VSMC are able to produce extracellular ROS after growth factor stimulation and if RV can affect this, which might contribute to RVs inhibitory effects on VSMC. Migration of VSMC towards the site of inflammation is one initial event in the early phases of atherosclerosis. We therefore wanted to know, if RV can negatively affect migration of VSMC and if so, what the molecular key players are.

MATERIALS AND EXPERIMENTAL PROCEDURES

C Materials and experimental procedures

1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma Aldrich (including RV; St. Louis, MO, USA) Carl Roth (Karlsruhe, Germany) or Fluka (Buchs, Switzerland). EGF was purchased from Millipore (Temecula, CA, USA), Beriglobin was obtained from Behring (Vienna, Austria), PDGF was purchased from Bachem (Weil am Rhein, Germany) and Complete™ was bought from Roche (Penzberg, Germany).

RV was diluted in DMSO yielding a 50 mM stock solution, which was stored at -80°C for further usage. EGF was resolved in PBS to a concentration of 100 µg/ml and preserved at -20°C. Ang II and PDGF were dissolved in PBS containing 0.25% BSA. Ang II was stored in 2 mM aliquots at -80°C, PDGF stocks (10 ng/µl) were kept at -20°C, respectively. Estrogen (β-estradiol) was dissolved in 96% ethanol and kept at -80°C in 10 mM aliquots.

2. Buffers

PBS pH 7.4	NaCl	7.2 g
autoclaved	Na_2HPO_4	1.48 g
	KH_2PO_4	0.43 g
	ddH_2O	ad 1000 ml
PBS^+	NaCl	8.0 g
sterile-filtered (0.22 µm)	KCl	0.2 g
	Na_2HPO_4	1.15 g
	KH_2PO_4	0.2 g
	$MgCl_2$ x 6 H_2O	0.1 g
	$CaCl_2$ x 2 H_2O	0.1 g
	ddH_2O	ad 1000 ml
FACS buffer pH 7.4	NaCl	8.12 g
sterile-filtered (0.22 µm)	KH_2PO_4	0.26 g
	Na_2HPO_4	2.35 g
	KCl	0.28 g
	LiCl	0.43 g
	NaN_3	0.20 g
	Na_2EDTA	0.36 g
	ddH_2O	ad 1000 ml
Krebs-Ringer phosphate glucose	NaCl	145 mM
buffer (KRPG)	Na_2HPO_4	5.70 mM
pH 7.4	KCl	4.86 mM
stored at 4°C	$CaCl_2$ x H_2O	0.54 mM
	$MgSO_4$ x 7 H_2O	1.22 mM
	Glucose	5.50 mM
Amplex Red™ reagent	KRPG	3.64 ml
protected from light	HRP 1 U/ml	75 µl
	10 mM Amplex Red™	37.5 µl
10x Electrophoresis buffer	Tris-base	30 g
stored at 4°C	Glycine	144 g
	SDS	10 g
	ddH_2O	ad 1000 ml

5x Blotting buffer	Tris-base	15.2 g
stored at 4°C	Glycine	72.9 g
	ddH_2O	ad 1000 ml
1x Blotting buffer	5x blotting buffer	200 ml
stored at 4°C	Methanol	200 ml
	ddH_2O	ad 1000 ml
TBS-T pH 8.0	Tris-base	3.0 g
stored at 4°C	NaCl	11.1 g
	Tween-20	1 ml
	ddH_2O	ad 1000 ml
Cytoskeleton buffer (CB)	NaCl	150 mM
pH 6.1	EGTA	5 mM
	$MgCl_2$	5 mM
	Glucose	5 mM
	MES (2-(*N*-morpholino) -	10 mM
	ethanesulfonic acid	
CB-1	CB	4.75 ml
	Triton-X (10%)	250 µl
	Glutaraldehyde	5 ml
CB-2	CB	9.90 ml
	Glutaraldehyde	100 µl
Digestion buffer	Collagenase (246 U/mg)	0.1 g
	HEPES	0.24 g
	Ascorbic acid	0.005 g
	Gentamycin sulfate	0.005 g
	BSA	0.1 g
	Ham´s F12 medium	ad 100 ml
	(PAN biotech, Germany)	

Table 1: Buffers used for various experiments

3. Cell culture

Rat VSMC were isolated from thoracic aortas of male Sprague-Dawley rats by enzymatic digestion [252]. Aortic tubes were collected from three to four sacrificed animals and transferred to PBS^+-containing dishes. Connective tissue was removed by scratching and aortas were subsequently transferred to dishes containing prewarmed digestion buffer. After an incubation phase of approximately 15 minutes, the tubes were cut longitudinally and the inner surfaces of the aortas were disposed from endothelial tissue by scraping. Aortas were then chopped into small pieces and seeded in 75 cm^2 flasks containing prewarmed cell culture medium. After a few days of incubation, VSMC emigrated from the aortic tubes to the bottom of the flask where they started to proliferate. When confluence was reached, VSMC were frozen in aliquots of 1 x 10^6 cells/ml and then stored in liquid nitrogen. For all experiments, VSMC isolated by the same method and kindly provided by Kathy K. Griendling (Emory University, Atlanta, USA) were used.

VSMC between passage 7 and 15 were used for all experiments. Cells were cultured in Dulbecco´s Modified Eagles Medium (DMEM) at 37°C and 5% CO_2. Cells were passaged when 90% confluence was reached by detaching with Trypsin-EDTA. For experimental approaches, cells were seeded either in 6-well or 24-well plates or in 6 cm or 10 cm dishes for two to three days, respectively. Before stimulation VSMC were serum-starved by incubation with DMEM containing 0.1% CS, antibiotics and L-glutamine for 24 to 48 hours.

Mouse fibroblasts expressing an exon 3-deleted version of Shp-2 (a gift from Benjamin Neel, Yale University, Connecticut, USA) were cultivated in cell culture medium containing 10% CS, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2. Cells were seeded in gelatine-coated 10 cm dishes and serum-starved in DMEM containing 0.2% CS for 24 hours before stimulation.

Cell culture medium	DMEM (Lonza, Switzerland)	500 ml
	Calf serum (CS) (Lonza)	55 ml
	Penicillin (Lonza)	10.000 U/ml
	Streptomycin (Lonza)	10.000 U/ml
	L-glutamine (Lonza)	200 mM
Starving medium	DMEM	500 ml
	Calf serum	500 µl
	Penicillin (Lonza)	10.000 U/ml
	Streptomycin (Lonza)	10.000 U/ml
	L-glutamine (Lonza)	200 mM
Trypsin/EDTA in PBS	Trypsin	0.05%
sterile-filtered (0.22 µm)	EDTA	0.02%
Freeze medium	DMEM	8.0 ml
	CS	2.0 ml
	DMSO	1.1 ml

Table 2: Cell culture material utilized for experiments

4. Whole cell lysates

After stimulation, VSMC were lysed as described elsewhere [227]. Briefly, cells were washed twice with ice-cold PBS and incubated with an appropriate volume of cold lysis buffer containing protease and phosphatase inhibitors for 15 minutes at 4°C. VSMC were scraped off, transferred to pre-cooled 1.5 ml tubes and subsequently spun in a table-top centrifuge for 10 minutes at 4°C with full speed to pellet insoluble material. Supernatants were aspirated, transferred to new tubes, frozen in liquid nitrogen and stored at -80°C until further usage. For protein quantification, Bradford method was used like previously described and recommended in the manufacturer's guidelines [253]. For Western Blot, an adequate amount of protein lysate was mixed with 3 x SDS-PAGE dye and heated for 5 minutes at 95°C before loading samples onto gels.

5. Western Blot

Western Blot method was used to determine changes in protein expression levels and was applied as previously described [254]. 20 to 30 µg of protein was loaded onto 7.5-15% SDS-PAGE gels and electrophoresis was carried out at 55-125 V for 60 to 90 minutes (Biorad, CA, USA). Thereafter, gels were blotted at 120 mA per blot for 90 minutes (Biorad, CA, USA) and membranes were blocked for 1 hour at room temperature with TBS-T containing 5% non-fat milk. Prior to incubation with appropriately diluted primary antibody at 4°C over night, the membranes were washed three times with TBS-T. Secondary antibody was incubated for 1 hour at room temperature and membranes were analyzed using a densitometer (LAS-3000™, Fujifilm, Japan).

Target	*Conjugated*	*Source*	*Dilution or Concentration*	*Application*	*Provider*
α-actin	FITC	mouse, mc	1:250	IF	Sigma Aldrich
Akt		rabbit, pc	1:1000	WB	Cell Signaling
phospho-Akt-Ser^{473}		rabbit, pc	1:1000	WB	Cell Signaling
α_V integrin		rabbit, pc	3 ng/µl	FACS	Santa Cruz
β_3 integrin	PE	hamster, mc	1.5 ng/µl	FACS	Invitrogen
β_3 integrin		rabbit, pc	1:1000	WB	Cell Signaling
β_3 integrin		mouse, mc	20 µg/ml	blocking	BD Pharmingen
cdc42		rabbit, mc	1:1000	WB	Cell Signaling
DNA-Polymerase		goat, pc	1:200	WB	Santa Cruz
ERα		mouse, mc	1:500	WB	Thermo
FAK		rabbit, pc	1:1000	WB	Cell Signaling
phospho-FAK-Tyr^{397}		mouse, mc	1:1000	WB	BD Transduction
phospho-FAK-Tyr$^{576/577}$		rabbit, pc	1:1000	WB	Cell Signaling
phospho-FAK-Tyr^{861}		goat, pc	1:500	WB	Santa Cruz
phospho-FAK-Tyr^{925}		rabbit, pc	1:1000	WB	Cell Signaling
goat IgG	HRP	bovine, pc	1:2500	WB	Santa Cruz
mouse IgG	HRP	goat, pc	1:1000	WB	Upstate
rabbit IgG	HRP	goat, pc	1:2500	WB	Cell Signaling
rabbit IgG	FITC	goat, pc	3 ng/µl	FACS	Invitrogen
Rac1		mouse, mc	1:1000	WB	Cell Biolabs
Shp-2		rabbit, pc	1:1000	WB	Santa Cruz
tubulin		mouse, mc	1:500	WB	Santa Cruz

Table 3: Antibodies used in various experiments: *HRP, horseradish peroxidase; IF, immunofluorescence; IgG, immunoglobulin G; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; mc, monoclonal; pc, polyclonal; PE, phycoerythrin; WB, Western Blot*

6. Immunoprecipitation

In order to confirm protein-protein interactions or the phosphorylation status of a specific protein, immunoprecipitation experiments were conducted as explained elsewhere [227]. Cells were lysed as specified above and 250-500 µg of protein was adjusted to 1 µg/µl with lysis buffer containing protease, and phosphatase inhibitors. 600 ng-2 µg of antibody was added and samples were incubated over night at 4°C in a rotator (LTF, Wasserburg, Germany). Next, 25 µl of prewashed protein A/G agarose beads (Santa

Cruz, CA, USA; 1:1 in PBS) per sample were added and incubated for additional 2 hours. After three times of washing, immunoprecipitated proteins were mixed with 15 µl of 3 x SDS-PAGE dye and heated for 5 minutes at 95°C before applying to SDS-PAGE.

7. FACS

For the determination of the surface expression pattern of proteins on VSMC, FACS (fluorescence-activated sell sorting) was utilized. The protocol was mainly used as stated elsewhere [255]. 5 x 10^5 VSMC were harvested, washed and transferred into FACS tubes and subsequently resuspended in 50 µl PBS containing 0.5% BSA and 0.1% azide. 5 µl Beriglobin (1:8 working solution in PBS/BSA/azide) was added and the cells were incubated on ice for 5 minutes. Then, cells were incubated with 10 µl of the proper diluted, directly fluorescence-conjugated or unconjugated primary antibody for 20-30 minutes at 4°C in the dark. In case of using an unconjugated first-step antibody, samples were washed twice in FACS buffer and incubated for additional 30 minutes with a fluorescence-labelled secondary antibody at 4°C in the dark. The suspension was washed once more with FACS buffer and finally once with PBS. Cells were immediately analyzed with the FACSCalibur™ cytometer (BD Biosciences, Mountain View, CA, USA)

8. Amplex Red Assay

The non-fluorescent compound Amplex Red™ (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen, CA, USA) is a substrate of horseradish peroxidase (HRP) and is able to be converted into resorufin in the presence of H_2O_2 in a 1:1 stoichiometry [256]. Since Amplex Red™ is not entering living cells, it is highly suitable to measure H_2O_2 which is released by cells [257]. VSMC were grown in 24-well plates and serum-starved for 24 hours. Cells were washed once with prewarmed PBS before the KRPG buffer containing the Amplex Red™ reagent was applied to the cells. Plates were incubated for 15 minutes at 37°C before VSMC were stimulated with different compounds for 5-45

minutes. 80 µl of the supernatant was transferred into 96-well plates to measure produced resorufin in triplicate with a fluorometer (Genios Pro, Tecan, Switzerland).

9. Cytoplasmatic versus nuclear extracts

To obtain nuclear extracts, VSMC were grown in 10 cm dishes. After starvation, cells were stimulated and washed twice with ice-cold PBS. Adherent cells were gently detached in 1 ml of PBS by scraping, transferred into precooled 1.5 ml tubes and centrifuged for 5 minutes with 3.500 rpm at 4°C. After aspirating the PBS, pelleted cells were resuspended in 300 µl of ice-cold buffer A and incubated for 15 minutes on ice. Before vigorous vortexing, 15 µl of 10% NP-40 was added and lysates were spun for 30 seconds with full speed at 4°C in a table-top centrifuge. Supernatants containing the cytoplasmatic fractions were transferred into new tubes for further usage. The pellets containing the nuclear fractions were aspirated in 50 µl ice-cold buffer B. Extracts were incubated for 15 minutes of reciprocal shaking and insoluble material was separated from the nuclear protein fraction by centrifugation with full speed for 4 minutes at 4°C. The protein concentration of both fractions was quantified by Bradford method before loading on SDS-PAGE gels.

Lysis buffer (stock solution)	HEPES	50 mM
for whole cell extracts	NaCl	50 mM
pH 7.5	NaF	50 mM
stored at 4°C	$Na_4P_2O_7$ x 10 H_2O	10 mM
	EDTA	5 mM
	Na_3VO_4	1 mM
→ prior to use	stock solution	850 µl
	Triton X-100 (10%)	100 µl
	PMSF (100 µM)	10 µl
	Complete™	40 µl
Nuclear extracts (Buffer A)	HEPES (100 µM)	1000 µl
cytoplasmatic fraction	KCl (100 µM)	1000 µl
stored at 4°C	EGTA (10 µM)	100 µl
	EDTA (10 µM)	100 µl
	ddH_2O	ad 10 ml
→ prior to use	Buffer A	1988 µl
	DTT (1 M)	2 µl
	PMSF (100 µM)	10 µl
Nuclear extracts (Buffer B)	HEPES (100 µM)	1000 µl
nuclear fraction	NaCl (5 M)	400 µl
stored at 4°C	EGTA (500 µM)	10 µl
	EDTA (500 µM)	10 µl
	Glycerol	1250 µl
	ddH_2O	ad 5 ml
→ prior to use	Buffer B	98 µl
	DTT (100 µM)	1 µl
	PMSF (100 µM)	1 µl

Table 4: Different cell lysis buffers

10. Wound healing assay

For the quantification of VSMC migration, wound healing assays were performed. VSMC were grown in 6-well plates to 95% confluence and serum-starved for 24 hours. For each well, two scratches were made using a sterile 100 µl-1000 µl tip and detached cells were washed away subsequently. Thereafter, starving of VSMC was continued for additional 24 hours. Before induction of migration, scratches were photographed with 200-fold magnification (Light Microscope Olympus CKX31; Olympus Live View Digital SLR Camera E-330, Hamburg, Germany). After 21 hours of migration, pictures of the same scratch area were taken and paired images were analyzed to quantify the narrowing of scratches (cell profiler software, Broad Institute Imaging Platform).

11. Fluorescence microscopy

2 x 10^5 VSMCs were seeded on coverslips in 12-well plates over night, washed with PBS and fixed with ice-cold Aceton/Methanol (1:1) for 15 minutes at 4°C. After washing with PBS, coverslips were placed onto droplets of diluted α-smooth muscle actin-FITC antibody (1:250 in PBS containing 1% BSA) and incubated for 1 hour at 4°C in the dark. Coverslips were washed twice with PBS, mounted onto glass slides and left over night to dry in the dark at 4°C. Samples were then analyzed with a fluorescence microscope (BX51 microscope, Olympus, Hamburg, Germany).

To visualize the actin cytoskeleton of VSMC, 5 x 10^4 cells were seeded on coverslips as mentioned above. Cells were serum-starved for 24 to 48 hours and then treated with different stimuli. After washing with PBS, cells were incubated for 1 minute with prewarmed CB-1 buffer. After aspirating the supernatant, VSMC were subsequently covered with a layer of prewarmed CB-2 buffer for 15 minutes at room temperature. Samples were washed three times with PBS and incubated with a 1:200 dilution of phalloidin-FITC for 30 minutes at room temperature in the dark. Plates were rinsed three times with PBS before mounting on glass slides. Samples were dried over night at room temperature and then acquired with a fluorescence microscope (BX51 microscope, Olympus, Hamburg, Germany).

12. GTPase assay

Agarose beads exclusively binding to GTP-bound GTPases can be used to easily pull down only the active fraction of a pool of proteins. For our purpose, we used PAK1 PBD Agarose beads (Cell Biolabs, CA, USA) which specifically bind and precipitate active Rac1 and cdc42 [207]. VSMC were seeded in 10 cm dishes and grown to 90% confluence. After 48 hours of serum starvation cells were stimulated and lysed by scraping (lysis buffer from Cell Biolabs, CA, USA; 1:5 in ddH_2O). Before IP, crude cell lysates were precleared and insoluble material was pelleted (4 minutes, full speed, 4°C). Afterwards, 20 µl of the lysate was taken away for loading control and the remaining volume was mixed with 20 µl of bead slurry. Immunoprecipitations were incubated for 45 minutes at 4°C in a rotator and then washed three times with lysis buffer. Beads were mixed with 15 µl of 3 x SDS-PAGE Dye and cooked for 5 minutes at 95°C before loading on 15% SDS-PAGE gels.

13. Computer software

Name	*Version*	*Provider*
AIDA (Advanced Image Data Analyzer)	4.06	Raytest Inc
CellProfiler	1.0.5122	Broad Institute Imaging Platform
Cell Quest Pro	5.2	BD Biosciences
GraphPad PRISM™	4.03	GraphPad Software Inc
Image Reader LAS 3000™	2.0	Fujifilm
Magellan	5.03	Tecan
ViCell™ XR	2.03	Beckman Coulter

Table 5: Computer software used for evaluation of raw data

14. Statistical Analysis

All data are expressed as means ± SEM. Statistical analyses were performed using two-tailed, paired t-tests unless otherwise noted. $P < 0.05$ was considered significant. All tests were evaluated using GraphPad PRISM™ software. Statistical comparisons were always done to vehicle-treated cells unless otherwise mentioned.

RESULTS

D Results

1. Isolation and identification of vascular smooth muscle cells

Rat aortic vascular smooth muscle cells were isolated as described in the chapter materials and experimental procedures. Five days after isolation, VSMC were seeded onto cover slips and stained with an antibody specific for rat α-smooth muscle actin. Fig. 6 shows that all detected cells were stained positive and the isolated batch of VSMC was not contaminated with other cell types of aorta-surrounding tissue.

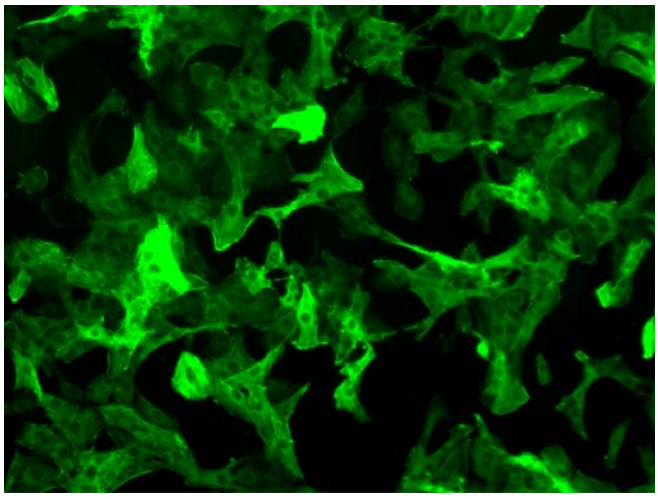

Figure 6: α-smooth muscle actin staining of VSMC

Fluorescence microscope picture showing rat vascular smooth muscle cells in passage one after isolation from the aorta. Cells were stained with a monoclonal and FITC-labelled rat-specific α-smooth muscle actin antibody (200-fold magnification).

2. Influence of RV on EGF- and Ang II-induced Akt phosphorylation

Phosphorylation of Akt is one central molecular event in cell survival, hypertrophy and proliferation. RV was shown to potently prevent both EGF- and Ang II-triggered phosphorylation of Akt [227]. After 24 hours of serum starvation, VSMC were stimulated with either 100 ng/ml EGF for 5 minutes or 100 nM Ang II for 10 minutes, respectively. RV was added 30 minutes before growth factor stimulation. Cells were subsequently washed with ice cold PBS and lysed. Fig. 7 shows that Akt was strongly phosphorylated when treated with EGF. Sustained Akt phosphorylation was also documented when stimulated with Ang II. In both cases of stimulation, RV was potently preventing the phosphorylation of Akt (75% inhibition in EGF-treated cells and complete abrogation of Akt phosphorylation in Ang II stimulated VSMC), whereas total Akt amounts remained unaffected. These results go along with previous published data of our lab [227].

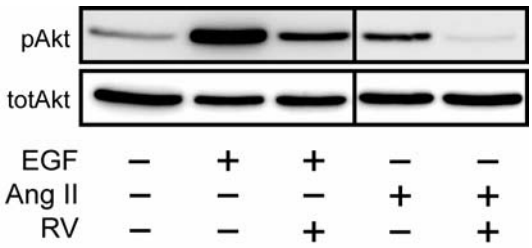

Figure 7: RV interferes with EGF- and Ang II-induced Akt phosphorylation

VSMC were preincubated with 50 µM RV for 30 minutes before inducing Akt phosphorylation with 100 ng/ml EGF for 5 minutes or 100 nM Ang II for 10 minutes, respectively. 20 µg of protein was loaded onto SDS-PAGE gels and Western Blot membranes were blotted against pAkt and total Akt. One representative blot is shown.

Based on these findings, we wanted to elucidate the molecular mechanisms underlying the potent inhibitory actions of RV on Akt phosphorylation and tried to find putative molecular target molecules which are affected by RV.

3. Possible targets for RV to inhibit the phosphorylation of Akt

3.1. The role of the estrogen receptor α in the process of EGF-mediated Akt phosphorylation

Since RV has been shown to act as a selective estrogen receptor mediator (SERM) in certain cell systems *in vitro* [235, 236], we wanted to know if estrogen receptor (ER) signalling is playing a role in our experimental setting of rapid Akt activation. We first determined the protein expression of ERα in VSMC by Western Blot and detected, beside the ERα full length version of 67 kDa also an unknown ~55 kDa protein, which was highly enriched in the nuclear fraction, but was not further investigated (Fig. 9A). As a next step, we asked whether estrogen stimulation could lead to Akt phosphorylation in a similar manner as EGF did. We set up a time-kinetic experiment, stimulating serum-starved VSMC for 2 to 30 minutes with 100 nM estrogen (Fig. 8A). Indeed, estrogen was able to significantly increase Akt phosphorylation with a maximal 2.5-fold induction after 2 minutes of stimulation and a 2.1-fold enhancement after 5 minutes of estrogen stimulation. However, compared to EGF-mediated Akt phosphorylation (20- to 30-fold induction above basal levels), the capacity of estrogen to induce Akt phosphorylation was much lower. By preincubating VSMC for one hour with 10 μM of fulvestrant, a selective ER antagonist, estrogen-triggered Akt phosphorylation was abrogated indicating that Akt phosphorylation through estrogen stimulation was mediated via a direct involvement of the ER (Fig. 8B).

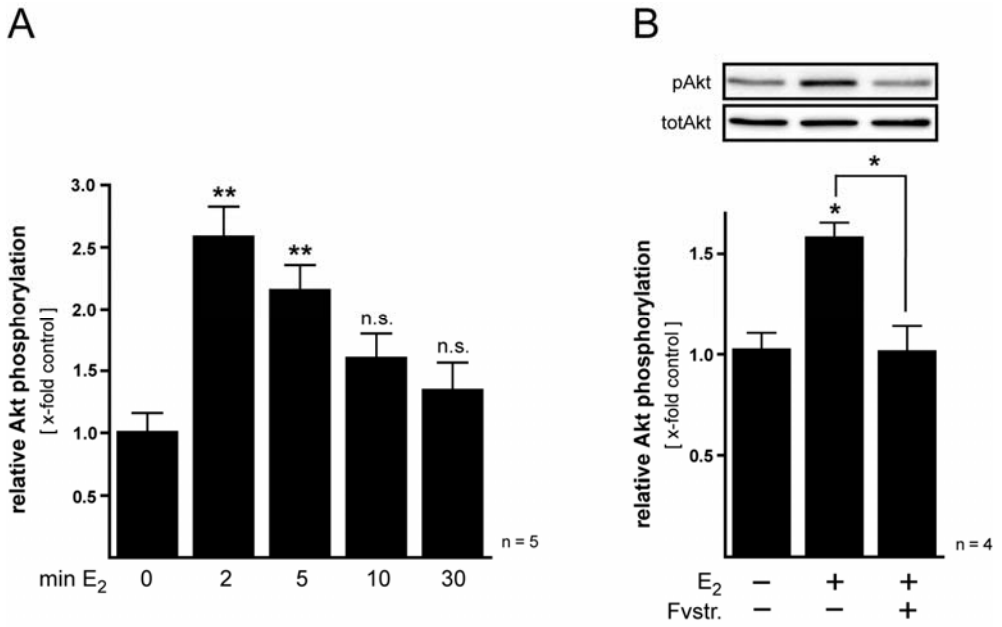

Figure 8: Estrogen stimulation leads to enhanced pAkt levels via direct ERα contribution

(A) *100 nM estrogen was used to treat VSMC for the indicated time periods. Western Blot analyses of (phospho) Akt were performed to quantify the relative phosphorylation status of Akt. Bar graph shows the summary of five independent experiments, where densitometric analyses are normalized to vehicle control (E_2, estrogen; **, $p < 0.01$; ns, not significant; mean ± SEM; one-way ANOVA).* ***(B)*** *VSMC were preincubated with 10 µM fulvestrant for 1 hour and subsequently stimulated with estrogen for 5 minutes. Western Blot analyses of four experiments are summarized in the graph with one representative blot above (Fvstr, fulvestrant; E_2, estrogen; *, $p < 0.05$; mean ± SEM).*

Due to its chemical structure, estrogen passes the cell membrane and binds to ER in the cytoplasm. ER then dimerizes and translocates to the nucleus to bind to its specific ER response elements on DNA [258]. Beside this “classical” genomic activation pathway, rapid signalling events including membrane-bound ER also take place in some cell systems [259]. To exclude the genomic pathway of ER signalling, we extracted cytoplasmatic and nuclear proteins after stimulating VSMC with different combinations of 50 µM RV (30 minutes preincubation) and 100 ng/ml EGF or 100 nM estrogen (5 minutes each). In none of the stimulation conditions, ER was shuttled to the nucleus demonstrating that nuclear translocation and ER-mediated initiation of transcription did not occur in our chosen experimental setting of rapid signalling (Fig. 9B).

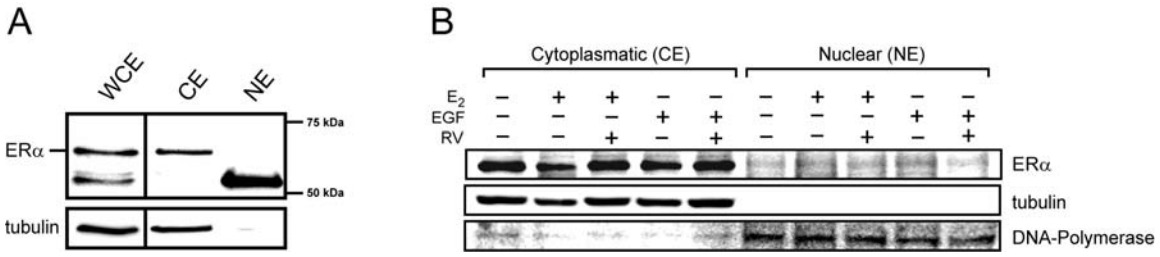

Figure 9: Estrogen-induced Akt phosphorylation is triggered via a non-genomic pathway

(A) *Unstimulated VSMC were used to obtain whole cell extracts (WCE), cytoplasmatic extracts (CE) and nuclear extracts (NE) Western Blot membranes were incubated with antibodies against ERα and tubulin.* ***(B)*** *VSMC were incubated with the indicated stimuli (preincubation with RV for 30 minutes followed by 5 minutes stimulation with either estrogen or EGF) and cytosolic versus nuclear extract were made. Proteins were separated by SDS-PAGE and membranes were blotted against ERα, tubulin (cytoplasmatic fraction purity control) and DNA-polymerase (nuclear fraction purity control). One representative blot is depicted.*

It has been demonstrated that ERα can be located at membranes in proximity to EGFR and furthermore is able to contribute to Akt signalling [97]. To test if RV could inhibit estrogen-induced Akt phosphorylation as well, VSMC were treated with 100 nM estrogen after 30 minutes of RV incubation (50 µM). RV pretreatment strongly inhibited estrogen-mediated Akt phosphorylation. Moreover, additional preincubation with fulvestrant 1 hour before RV treatment did not influence the potent phosphorylation-inhibiting properties of RV on Akt (Fig. 10A).

Furthermore, fulvestrant was utilized in EGF-treated VSMC as well, where we treated smooth muscle cells for 1 hour with fulvestrant before adding RV for additional 30 minutes. Finally, EGF was added to the cells at concentrations of 100 ng/ml for 5 minutes. Fig. 10B shows, that fulvestrant neither changed EGF-mediated Akt phosphorylation nor RV´s dephosphorylating effects on Akt. Taken together, these results indicate that RV can act as a potent antagonist of ERα but a contribution of ERα to EGF-induced Akt activation could not be documented.

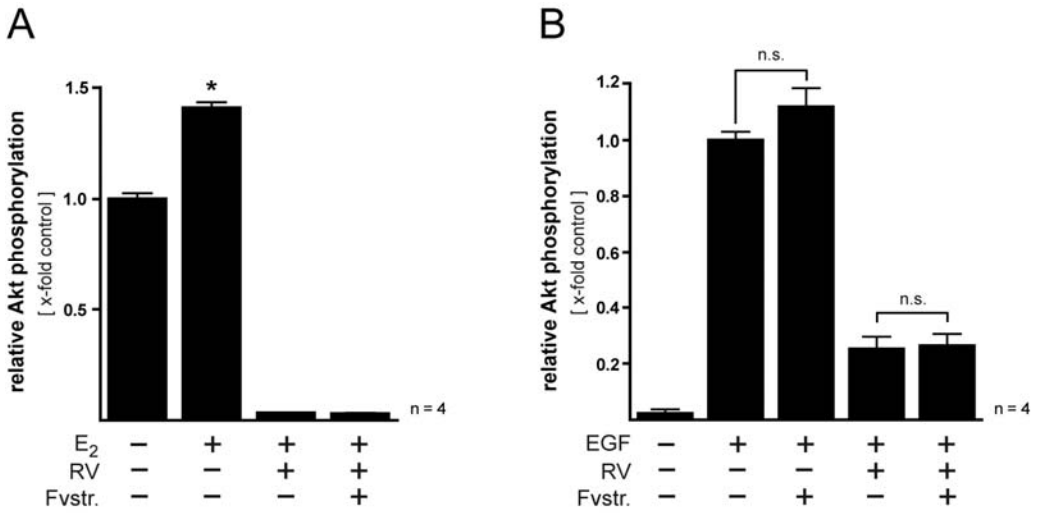

Figure 10: ER signalling does not contribute to RV effects on EGF-induced pAkt

(A) *VSMC were incubated with 50 µM RV (30 minutes) with or without pretreatment of fulvestrant for 30 minutes. Cells were then stimulated with 100 nM estrogen for 5 minutes, lysates were separated by SDS-Page and blotted. Densitometric values of phospho-Akt were correlated to total Akt protein amounts (*, $p < 0.05$; mean ± SEM, n = 4).* ***(B)*** *Cells were pretreated with 10 µM fulvestrant for 30 minutes before administration of 50 µM RV for additional 30 minutes. Cells were subsequently stimulated with 100 ng/ml EGF for 5 minutes. Western blot analyses were quantified densitometrically (ns, not significant; mean ± SEM, n = 4).*

3.2. Integrins as possible target of RV

One very important role of integrins is to anchor single cells with each other and to the meshwork of extracellular matrix (ECM) and therefore stabilizing the cells within the tissue [133]. Furthermore integrins play an important role in growth factor-mediated signalling events [146]. Previous studies revealed that RV was able to bind to integrin $\alpha_V\beta_3$ in cancer cells, thereby triggering ERK 1/2 and p53 phosphorylation [241]. We therefore wanted to examine a putative role of the cell adhesion molecule integrin $\alpha_V\beta_3$ in VSMC either as possible binding site for RV or as candidate molecule which contributes to the inhibiting effects of RV on Akt phosphorylation. Firstly, the surface expression pattern of integrin $\alpha_V\beta_3$ on VSMC was determined. Unstimulated cells were incubated with different fluorescence-labelled antibodies specific for either subunit of the integrin $\alpha_V\beta_3$ and then analyzed by FACS. As shown in Fig. 11, α_V as well as β_3 integrin monomers were weakly expressed on the surface of untreated rat VSMC. Furthermore,

pretreatment with RV or EGF for 2 to 48 hours did not change the surface expression levels of α_V and β_3 (not shown).

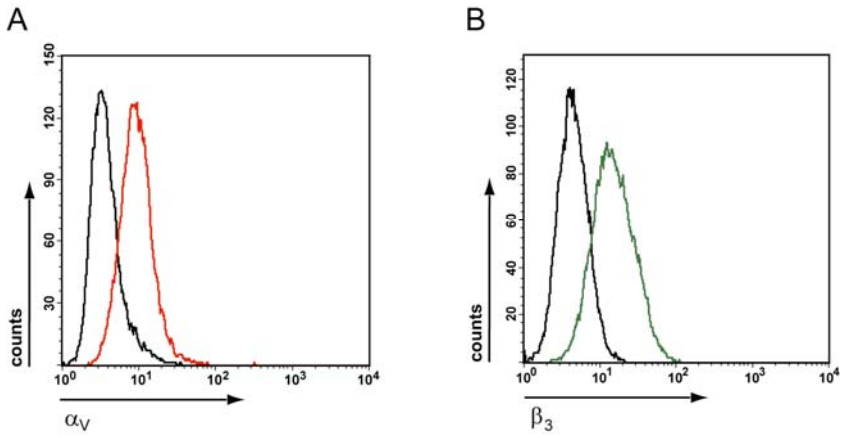

Figure 11: Surface expression pattern of $\alpha_V\beta_3$ integrins on VSMC

(A) *FACS histogram showing integrin α_V surface expression. Cells were first incubated for 1 hour with unconjugated rabbit anti-α_V antibody, washed and a secondary, FITC-labelled anti-rabbit antibody was added for another hour. As negative control, rabbit-IgG isotype control treated cells were used. Logarithmic fluorescence intensity is plotted against cell number. Black line indicates control-stained cells, red line α_V-stained cells.* ***(B)*** *VSMC were stained with directly conjugated β_3-PE antibody. As negative control, unstained cells were utilized. Histogram plot depicts logarithmic fluorescence intensity against cell number. Black line shows unstained cells, green line β_3-PE stained VSMC.*

Next we wanted to know if, and to what extent integrin $\alpha_V\beta_3$ accounts for the inhibiting actions of RV on Akt phosphorylation. VSMC were preincubated with 50 or 500 µM of a small peptide Arg-Gly-Asp (RGD), which is the recognition motif of many integrin-binding proteins [138]. In a second experimental approach, cells were preincubated with 20 µg/ml of a monoclonal blocking antibody against β_3 integrin. After 2 hours of either RGD or β_3 antibody incubation, cells were treated with RV for additional 30 minutes before stimulation with 100 ng/ml EGF for 5 minutes. Western Blot analyses showed, that pretreatment with either of the integrin blocking agents, 500 µM RGD and 20 µg/ml anti-β_3 antibody, could counteract the inhibiting actions of RV on Akt phosphorylation to a low, but significant extend (Fig. 12A and B). Pretreatment with either 50 µM of RGD or control antibody did not change the inhibiting effects of RV on pAkt.

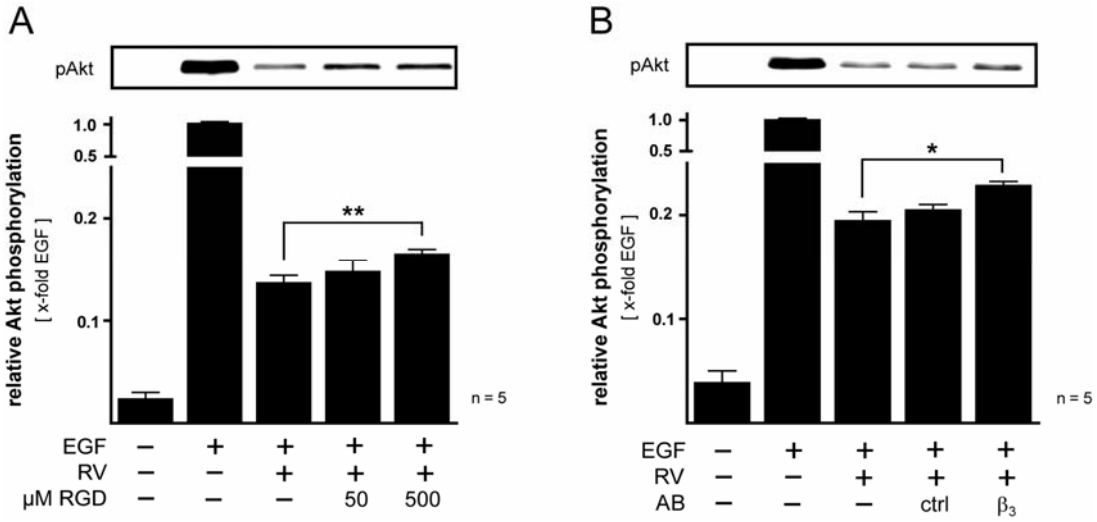

Figure 12: Effects of blocking molecules on RV-mediated dephosphorylation of Akt

VSMC were treated with 100 ng/ml EGF for 5 minutes. 50 µM RV was added 30 minutes before EGF stimulation. ***(A)*** *RGD blocking peptides at indicated concentrations were preincubated 2 hours before RV addition. Bar graph shows densitometric analysis of phospho-Akt band intensities correlated to total Akt amounts. One representative blot is shown above (**, $p < 0.01$; mean ± SEM; n = 5).* ***(B)*** *20 µg/ml of a monoclonal anti-β_3 blocking antibody was preincubated 2 hours before RV treatment. As negative control, an unspecific IgG isotype-ident antibody was used at same concentrations. Bar graph shows phospho-Akt band intensities related to total Akt amounts. One representative blot is shown (ctrl, control antibody; β_3, monoclonal anti-β_3 integrin antibody; *, $p < 0.05$; mean ± SEM; n = 5).*

Moreover, we could observe by Western Blot analysis that β_3 expression levels were downregulated by 20% only in the case of 2 hours preincubation with anti-β_3 antibody and subsequent stimulation with both RV and EGF (Fig. 13). In experiments using RGD blocking peptides, no significant changes in β_3 expression could be observed (data not shown).

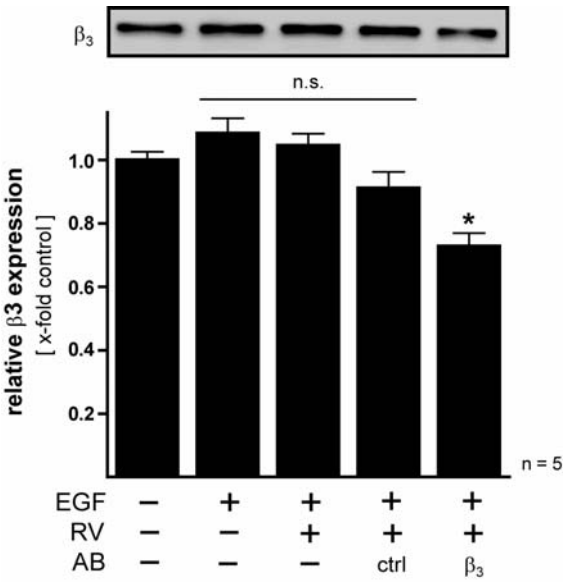

Figure 13: Downregulation of β_3-integrin expression after incubation with anti-β3 blocking antibody

*VSMC were pretreated with 20 µg/ml anti-β_3 antibody for 2 hours. 50 µM RV was added 30 minutes before EGF stimulation (100 ng/ml, 5 minutes). After stimulation, cell lysates were subjected to Western Blot. Bar graph is expressed as x-fold control of β_3 integrin expression correlated to tubulin levels. One representative blot is shown (ctrl, control antibody; β_3, monoclonal anti-β_3 antibody; *, $p < 0.05$; ns, not significant; mean ± SEM; n = 5).*

β_3 integrins contain two important phosphorylation-sensitive tyrosine residues at the cytoplasmatic C-terminus [135]. We tried to unravel, whether RV could affect the phosphorylation status of β_3 integrin and therefore influences integrin signalling. Unfortunately, we were not able to immunoprecipitate β_3 integrin to check possible phosphorylation changes within the molecule and therefore cannot exclude that RV potentially influences integrin phosphorylation. Furthermore, we speculated that integrins expressed on adherent and highly confluent VSMC would mostly be bound to target structures on neighbouring cells or connected to ECM. If RV acted via binding to integrins, multiple free and unbound integrins would positively influence the impact of RV´s inhibiting properties. However, the adherence and confluence status of VSMC had no influence on diverse stimuli since detached cells reacted equally to EGF and RV administration as attached VSMC (Fig. 14).

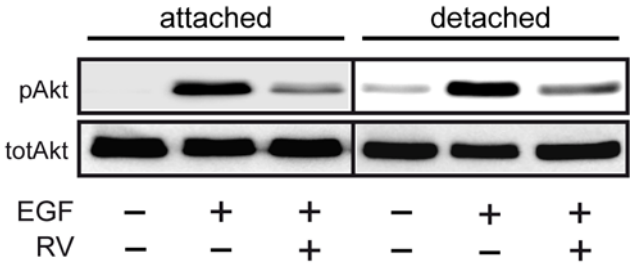

Figure 14: Detachment has no influence on the inhibiting actions of RV

100% confluent VSMC were trypsinized, spun and reseeded into new dishes containing starving medium ("detached") or left adherent ("attached"). RV was pretreated for 30 minutes and cells were stimulated with 100 ng/ml EGF for 5 minutes. Western Blot shows one representative blot of pAkt and totat Akt amounts.

In summary, we conclude that integrin $\alpha_V\beta_3$ is clearly not the main target molecule of RV concerning its inhibitory properties on Akt phosphorylation, although a minor participation of this specific integrin might take place.

3.3. Influence of RV on FAK phosphorylation

Since FAK is one crucial and central molecule in mediating both RTK and integrin signals downstream of the referred receptors [168], we wanted to know if the activity and phosphorylation status of FAK was affected by RV, which possibly accounted for the impaired Akt phosphorylation. First experiments with EGF-stimulated cells revealed, that 100 ng/ml EGF led to a significant FAK dephosphorylation by approximately 20% ($p < 0.05$) which could be completely counteracted by preincubation with RV (Fig. 15A). Since EGF-triggered dephosphorylation of FAK could be due to inhibition of a kinase or activation of a phosphatase, we preincubated VSMC with 50 µM of the phosphatase inhibitor sodium ortho-vanadate (Na_3VO_4). Preincubation with Na_3VO_4 for 30 minutes completely restored the phosphorylation status of FAK, indicating that EGF was able to activate one or more phosphatases which in turn led to dephosphorylation of FAK (Fig. 15B)

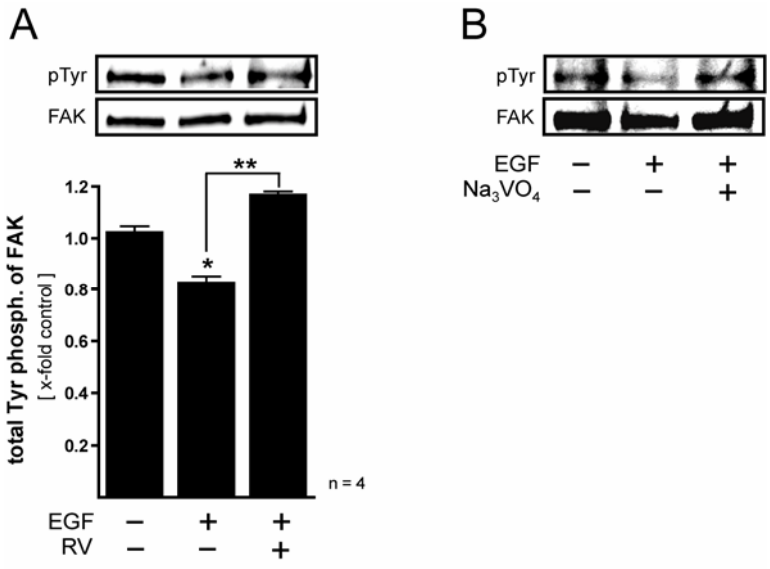

Figure 15: EGF-initiated FAK dephosphorylation is reversed by RV and mediated by phosphatases

(A) *After preincubation with 50 µM RV or vehicle for 30 minutes, VSMC were stimulated with 100 ng/ml EGF for additional 5 minutes. Cell lysates were obtained and 250 µg of protein was immunoprecipitated with anti-FAK antibody overnight. Western blot membranes were first probed with anti-phospho tyrosine antibody. For loading control, membranes were stripped and reincubated with anti-FAK antibody (*, $p < 0.05$; **, $p < 0.01$; mean ± SEM, n = 4).* ***(B)*** *50 µM of Na_3VO_4 was added to VSMC for 30 minutes followed by 5 minutes stimulation with 100 ng/ml EGF. After lysis of cells, immunoprecipitations with anti-FAK antibody were carried out overnight. Precipitates were subjected to Western Blot and membranes were probed with anti-phospho tyrosine antibody and anti-FAK antibody, respectively. One representative blot is shown.*

Since the involvement of Shp-2 in growth factor-mediated FAK signalling is well described [177], we checked whether Shp-2 was involved in the EGF-mediated dephosphorylation process of FAK. We conducted co-immunoprecipitation experiments stimulating VSMC for 5 minutes with EGF with or without pretreatment with RV. In comparison to unstimulated or RV-pretreated cells, a significant higher amount of Shp-2 could be co-immunoprecipitated with FAK in EGF-stimulated cell lysates (20% enhancement), indicating a direct link between FAK dephosphorylation and elevated FAK-Shp-2 interaction (Fig. 16).

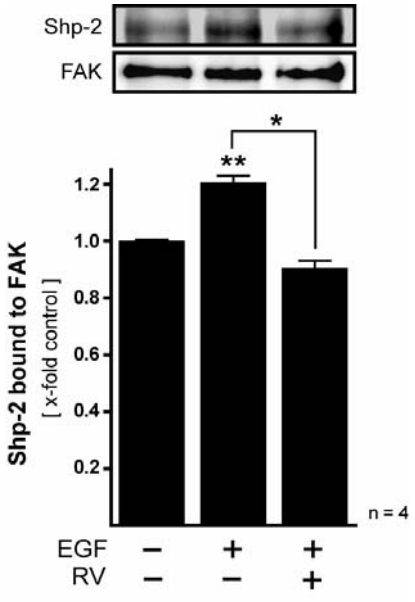

Fig. 16: Enhanced Shp-2-FAK interaction is counteracted by RV

Prior to stimulation with 100 ng/ml EGF for 5 minutes, VSMC were incubated with vehicle or 50 µM RV for 30 minutes. Lysates were immunoprecipitated with anti-FAK antibody and Western Blot membranes were blotted against Shp-2 and FAK, respectively. One representative blot is shown. Bar graph shows summary of four independent experiments (, $p < 0.05$; **, $p < 0.01$; mean ± SEM).*

Another proof for this hypothesis was gained when aforementioned experiments were repeated in cells expressing a truncated version of Shp-2 (Shp-$2^{\Delta 46\text{-}110}$). These cells lack the N-terminally located exon 3 of Shp-2 and therefore the protein lost the ability to interact properly with possible target molecules [131]. Moreover, Shp-$2^{\Delta 46\text{-}110}$ mouse fibroblasts express very little amounts of truncated Shp-2 compared to the expression pattern of wild-type Shp-2 in VSMC (Fig. 17A). In these cells, EGF and RV stimulation did not cause dephosphorylation of FAK but rather enhanced FAK´s phosphorylation status by 20-50 % (Fig. 17B). Moreover, no interaction between FAK and Shp-$2^{\Delta 46\text{-}110}$ could be observed (data not shown). In sum, these experiments underpin a direct influence of Shp-2 in the EGF-induced dephosphorylation process of FAK and a possible inhibiting role of RV regarding a FAK-Shp-2 interaction.

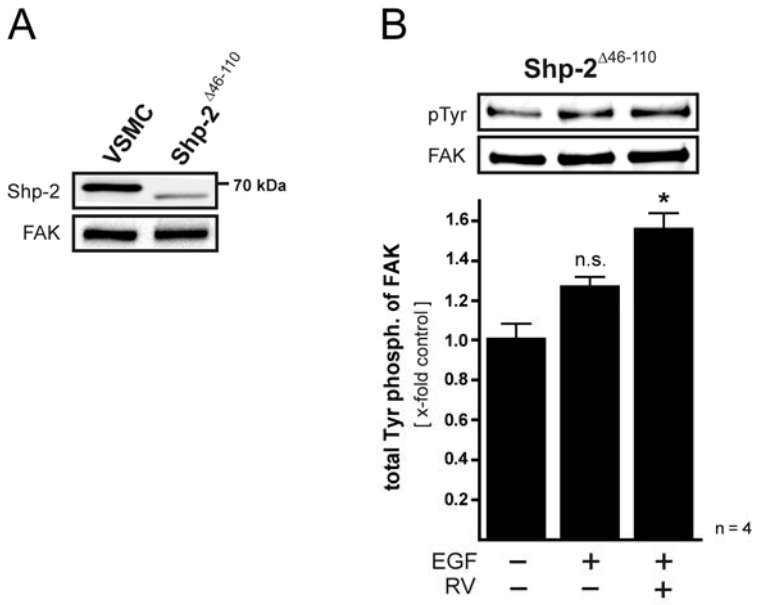

Figure 17: Shp-2$^{\Delta 46\text{-}110}$ fibroblasts do not show FAK dephosphorylation upon EGF treatment

(A) *Comparison of Shp-2 expression between VSMC and Shp-2$^{\Delta 46\text{-}110}$ mouse fibroblasts. Unstimulated cells were lysed and 30 µg of protein was applied to SDS-PAGE. Membranes were incubated with anti-Shp-2 and anti-FAK antibody, respectively.* ***(B)*** *Shp-2$^{\Delta 46\text{-}110}$ mouse fibroblasts were pretreated with 50 µM RV or vehicle for 30 minutes and then stimulated with 100 ng/ml EGF (5 minutes). 250 µg of cell lysate was used for immunoprecipitation of FAK. Phosphotyrosine bands were normalized to total FAK amounts. One representative blot is shown, bar graph represents four independent experiments (*, $p < 0.05$; ns, not significant; mean ± SEM).*

The 125 kDa protein FAK is strongly regulated via phosphorylation of several distinct tyrosine residues within the molecule [173]. To unravel the involvement of the different phosphorylation sites of FAK in EGF signalling, we used four phospho-specific antibodies covering the most important and well described phosphorylation sites of FAK. Whereas phosphorylation of Tyr^{397}, which serves as hallmark for FAK activity, was significantly downregulated by 20% by both EGF and additional RV treatment, the residues Tyr$^{576/577}$ were hyperphosphorylated by EGF and additional RV stimulation (Fig. 18). Furthermore, no significant changes could be observed when investigating tyrosine residues Tyr^{861} and Tyr^{925}. The size of FAK on the one hand and its several phosphorylation sites on the other hand, makes it a perfect molecule to interact with a big number of different proteins. After verifying clear differences between the distinct phosphorylation sites of FAK, we wanted to take a deeper look into possible protein-protein interaction changes as a result of phosphorylation alterations.

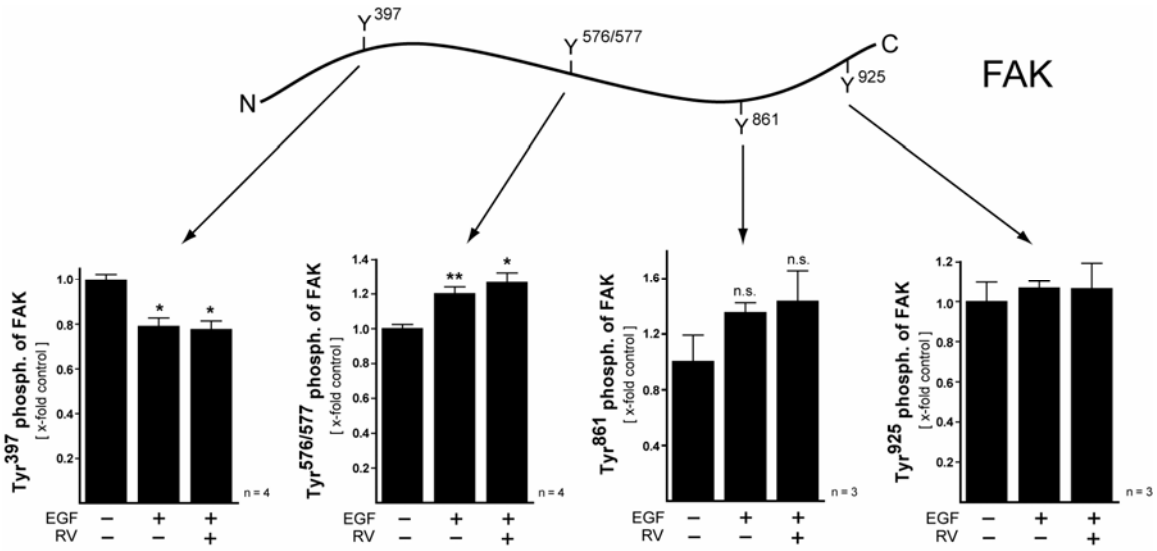

Figure 18: Single phosphorylation sites of FAK are differently regulated by EGF and RV

Schematic representation of the FAK molecule showing all phosphorylation-prone tyrosine residues. Prior to EGF stimulation (100 ng/ml for 5 minutes) smooth muscle cells were pretreated with 50 µM RV or vehicle for 30 minutes. For immunoprecipitation, 250-500 µg of protein lysate was incubated over night with anti-FAK antibody. Different phospho-specific antibodies were used for Western blot and band intensities were normalized to total FAK levels. Bar graphs represent three to four independent experiments (, $p < 0.05$; **, $p < 0.01$; ns, not significant; mean ± SEM).*

Unfortunately, all proteins which we investigated were not co-immunoprecipitated with FAK. These proteins include Src, PI3K, Akt and Gab-1 (data not shown). Our results reveal that different phosphorylation sites of FAK are very unequally affected by EGF and RV. However, changes in interaction patterns between FAK and target proteins could not be observed. We therefore cannot exclude a putative role of RV in these processes of direct or indirect protein-FAK interactions.

3.4. Extracellular H_2O_2 production is vigorously decreased by RV

RV is well known to act as an antioxidative molecule and both, intracellular and extracellular produced ROS are indispensable second messengers in cell signalling [218, 220, 260]. By utilizing Amplex Red™, which exclusively detects extracellularly produced H_2O_2, we observed that unstimulated VSMC steadily generated H_2O_2 which led to high basal levels of extracellular hydroxide peroxide. Both Ang II and EGF were able to trigger enhanced production of extracellular H_2O_2 in a time-dependent manner (Fig. 19). Whereas EGF stimulation led to increasing accumulation of H_2O_2 peaking after 15 minutes of stimulation (20% above basal levels, Fig. 19A), Ang II treatment revealed that H_2O_2 production was highest after 10 minutes (15% above basal levels) descending again at later time points (Fig. 19B).

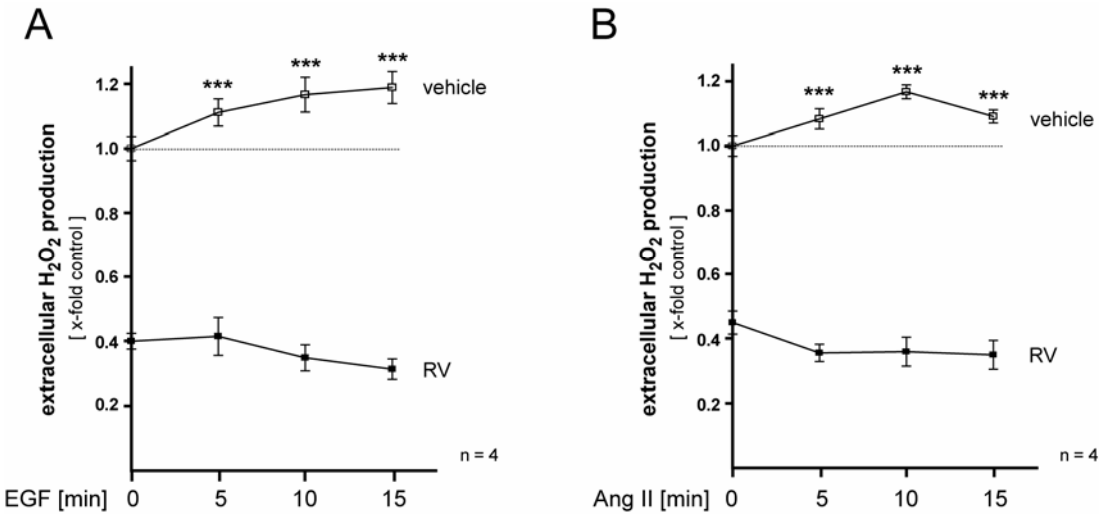

Figure 19: RV extenuates both basal and Ang II- or EGF-induced extracellular H_2O_2 levels

(A) *VSMC were stimulated in Amplex Red buffer with EGF (100 ng/ml) for 5 to 15 minutes after preincuabtion with RV or vehicle control for 30 minutes. Produced H_2O_2 was quantified using a fluorometer. Absolute values were correlated to catalase-treated cells used as negative control. Graph shows relative extracellular H_2O_2 production expressed as x-fold vehicle-treated cells (–□–, vehicle-treated cells; –■–, RV-treated cells; ***, $p < 0.001$; mean ± SEM; n = 4).* ***(B)*** *Cells were treated and analyzed as indicated in (A) except the usage of Ang II (100 nM) as stimulant. (–□–, vehicle-treated cells; –■–, RV-treated cells; ***, $p < 0.001$; mean ± SEM; n = 4). For all statistical analyses, vehicle-treated cells were correlated to RV-treated cells for each time point separately*

However, pretreatment with RV for 30 minutes strongy reduced both, basal H_2O_2 levels and growth factor-induced H_2O_2 production. RV decreased H_2O_2 levels down to 40% of basal levels, no matter if EGF or Ang II were applied or not.

In summary, we found that RV acts as a very potent neutralizing molecule for extracellular H_2O_2 and is able to compensate basal and growth-factor induced levels of H_2O_2 in VSMC, therefore possibly influencing signalling mediated by extracellularly produced H_2O_2.

In the context of EGF-mediated rapid Akt phosphorylation, we investigated possible target molecules of RV, which could explain the very potent inhibitory actions of RV. We were able to demonstrate, that ERα does not contribute to EGF-mediated phosphorylation of Akt, although RV showed anti-estrogenic capacity. Furthermore, we could show that integrin $\alpha_V\beta_3$ has only minor influence on the inhibitory actions of RV on pAkt. FAK may contribute to the effects of RV, but most likely does not completely explain the inhibitory actions of RV. Moreover, RV acts as H_2O_2 neutralizer, which possibly influences also intracellular signalling events.

4. Anti-migratory effects of RV on growth factor-induced VSMC

4.1. EGF induces migration of VSMC

As RV is a potent anti-invasive compound already described in different cancer cell lines and VSMC migration plays a pivotal role especially in the early stages of atherosclerosis [22, 237], we asked whether RV is able to inhibit migration of VSMC. We conducted wound healing assays, where a scratch was applied to a confluent monolayer of serum-starved cells. After 24 hours of serum deprivation, smooth muscle cells were stimulated with different concentrations of EGF for 21 hours to initiate migration. The rate of cells which migrated within 21 hours was microscopically evaluated and quantified (see materials and methods section for details). While 1 ng/ml EGF only slightly induced migration of VSMC, stimulation with 10-100 ng/ml EGF resulted in a dose-dependent increase of migration (Fig. 20A). 20 ng/ml EGF led to a 50% increase, which could not be enhanced by applying higher concentrations of EGF. We therefore decided to use 20 ng/ml of EGF for all subsequent experiments to initiate migration. Furthermore we made sure that observed effects were indeed due to VSMC migration and not mediated via EGF-induced proliferation or cell hypertrophy. By using mitomycin C, a potent and well-known inhibitor of proliferation, migration rates initiated by EGF did not change significantly (Fig. 20B).

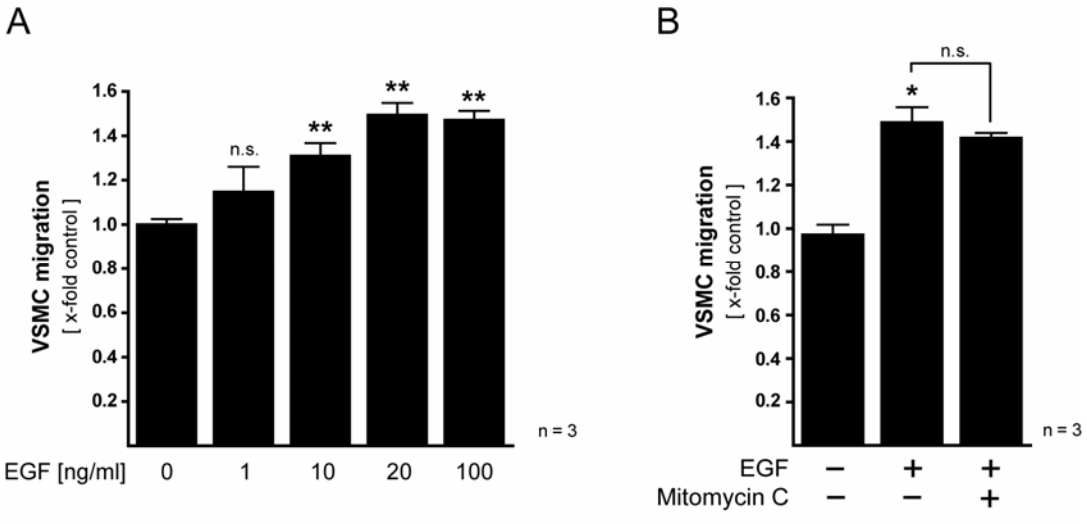

Figure 20: EGF induces migration of VSMC in a dose-dependent manner

(A) *Different concentrations of EGF have been used to induce migration in wound healing assays. Photos were made before and after 21 hours of stimulation with indicated concentrations of EGF and were then evaluated. Bar graph shows summary of three independent experiments (**, $p < 0.01$; ns, not significant; mean ± SEM; one-way ANOVA).* ***(B)*** *Before induction of migration (20 ng/ml EGF for 21 hours) VSMC were incubated with 100 nM mitomycin C or vehicle for 30 minutes. Wound healing assay photos were evaluated as described in materials and experimental procedures. Graph depicts summary of three experiments (*, $p < 0.05$; ns, not significant; mean ± SEM).*

4.2. RV abrogates EGF-induced but not PDGF-induced migration

Next we wanted to know if RV was able to block EGF- or PDGF-induced migration. PDGF is even a stronger migration stimulus as EGF and is normally used in concentrations of 2-10 ng/ml ([261] and data not shown). Wound healing assays were performed as described before using 20 ng/ml of EGF and 2 ng/ml PDGF. Prior to growth factor stimulation, 50 µM of RV was added for 30 minutes. Fig. 21A shows representative pictures of wound healing assays before and after an incubation period of 21 hours where different stimuli were applied. While RV was able to completely block EGF-induced migration, it could not reduce PDGF-triggered migration to a significant extent.

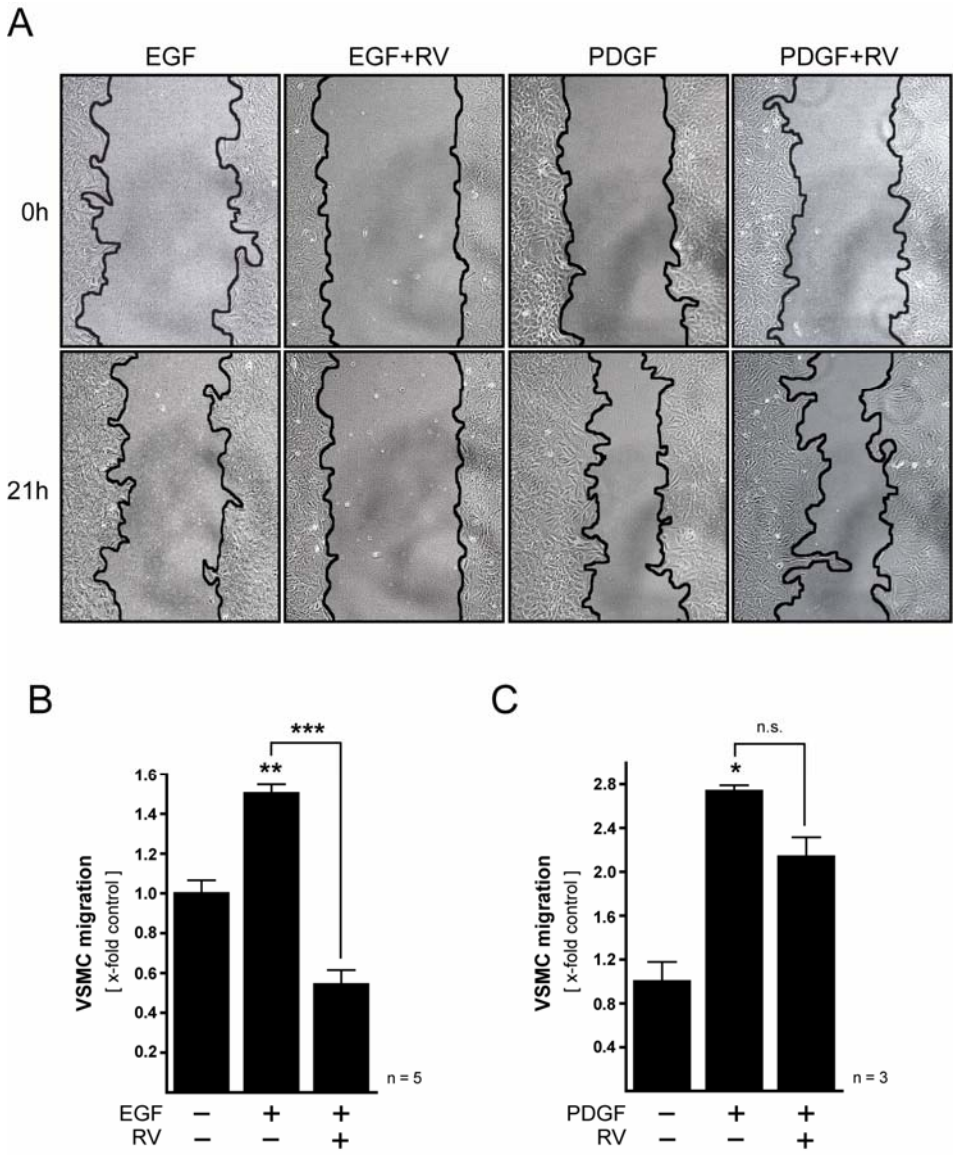

Figure 21: RV blocks EGF-induced but not PDGF-mediated migration

(A) *Representative photos from scratch assays before and after a stimulation time of 21 hours. RV was preincubated for 30 minutes and EGF (20 ng/ml) or PDGF (2 ng/ml) was added for 21 hours (200-fold magnification).* ***(B)*** *and* ***(C)*** *Bar graphs represent changes in the migration capacity of VSMC after 21 hours of stimulation with indicated compounds. Arbitrary units were normalized to vehicle-treated cells and are expressed as x-fold control (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant; mean ± SEM; n = 3-5).*

Fig 21B and C summarize evaluations of three to five independent wound healing assays. Whereas RV reduced EGF-mediated migration by 67%, a slight but non-significant migration decrease in PDGF-triggered VSMC could be observed. RV´s diverse effects were not due to the chosen growth factor concentrations, since RV was still able to block EGF-, but not PDGF-induced migration, when even higher concentrations of the growth factors were used (not shown).

To unravel possible differences of action of RV when using either EGF or PDGF as stimuli, we employed cytoskeleton stainings of VSMC using phalloidin-FITC which binds to actin bundles within the cell. Fig. 22 shows representative photographs of phalloidin-stained cells after 2 hours of EGF or PDGF stimulation (20 ng/ml and 2 ng/ml, respectively) with or without RV pretreatment (50 µM for 30 minutes), where a clear morphological difference could be observed.

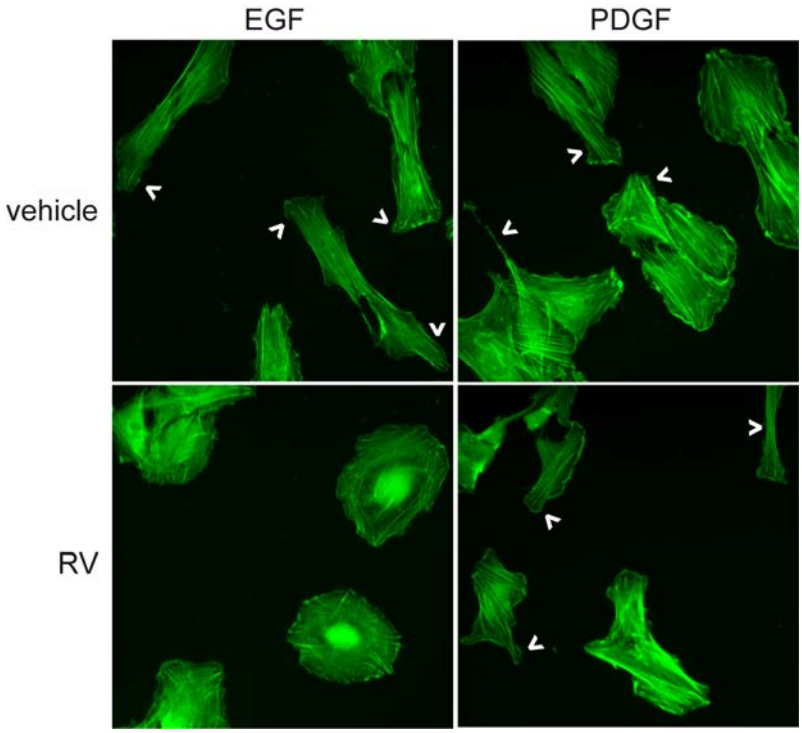

Figure 22: RV reduces lamellipodia formation of EGF- but not of PDGF-treated VSMC

RV-preincubated cells (50 µM for 30 minutes) were stimulated with EGF (20 ng/ml) or PDGF (2 ng/ml) for 2 hours, respectively. After washing and fixation, cells were stained with FITC-conjugated phalloidin for 30 minutes to visualize the actin structures of the cytoskeleton. Dried samples were monitored using a fluorescence microscope. Arrows indicate developed lamellipodia (400-fold magnification).

Both, EGF and PDGF led to evident cytoskeleton rearrangements after 2 hours indicating the activation of migration pathways. Sustained formations of lamellipodia (indicated with arrows) and stress fibres could be monitored. Pretreatment with RV abrogated lamellipodia formation in EGF- but not in PDGF-stimulated VSMC leading to a more round-shaped morphology in the case of EGF-treated cells. On the contrary, addition of RV had no clear effect on stress fibre formation in either EGF- or PDGF-stimulated VSMC.

4.3. RV does not influence hyperphosphorylation of FAK

FAK plays a pivotal role in both, rapid signalling cascades distributing incoming integrin or growth factor signals, and long term actions referred to chemotaxis, like cytoskeleton rearrangement or actin stabilization [166]. We therefore wanted to know, if FAK phosphorylation is changed over a prolonged time frame of EGF stimulation. For this purpose, we established time course experiments monitoring the overall phosphorylation status of FAK by immunoprecipitation. Fig. 23A clearly shows that a dynamic process of phosphorylation and dephosphorylation events takes place, where a first 20% increase of FAK phosphorylation could be observed after 2 hours of EGF stimulation. However, FAK reached an even higher phosphorylation level after 8 hours of stimulation (60% induction) which was followed by a subsequent drop to basal levels until the end of the time course experiments after 17 hours.

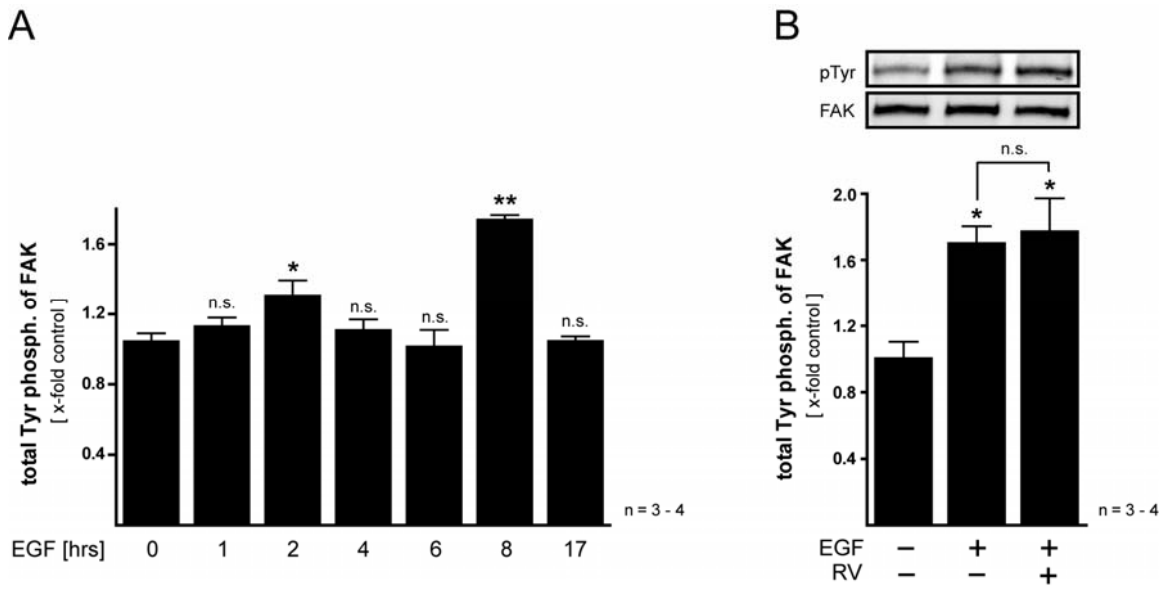

Figure 23: Long-term stimulation with EGF induces hyperphosphorylation of FAK after 8 hours which is not affected by RV

(A) *Smooth muscle cells were stimulated with 100 ng/ml EGF for the indicated time periods. 250 µg of protein was used for immunoprecipitation with anti-FAK antibody. Immunoblots were first probed with anti-phospho tyrosine antibody, then incubated with anti-FAK antibody. Graph shows summary of three to four individual experiments (*, $p < 0.05$; **, $p < 0.01$; ns, not significant; mean ± SEM; one-way ANOVA).* ***(B)*** *VSMC were pretreated with vehicle or 50 µM RV and then challenged with 100 ng/ml EGF for 8 hours. Immunoprecipitations (250 µg protein with anti-FAK antibody over night) were loaded onto PAA-gels and separated by electrophoresis. Phosphotyrosine band intensities were calculated against total FAK amounts (*, $p < 0.05$; ns, not significant; mean ± SEM; n = 3-4).*

Thereafter we asked the question, whether RV can influence hyperphosphorylation of FAK after 8 hours of EGF administration. VSMC were preincubated with 50 µM of RV and then stimulated with 100 ng/ml EGF for 8 hours. Pretreatment with RV, however, had no significant effect on the elevated phosphorylation status of FAK (Fig. 23B). Long term experiments using PDGF as stimulus gained equal results. PDGF slightly induced FAK phosphorylation which was not affected by preincubation with RV (data not shown).

4.4. Rac1 is the molecular reason for the anti-migratory activity of RV

The molecular requirement for lamellipodia formation is mainly activation of the small GTPase Rac1, which we next investigated. By conducting pull-down assays where only GTP-bound Rac1 was immunoprecipitated, we could determine the molecular effects of RV on EGF- and PDGF-induced lamellipodia outgrowth. Fig. 24A depicts, that 20 ng/ml of EGF caused a 2.4-fold Rac1 activation after 5 minutes which was completely inhibited by preincubation of 50 µM RV. PDGF at 2 ng/ml induced a comparable Rac1 activation (2.5-fold), which was almost unaffected by RV (Fig. 24B).

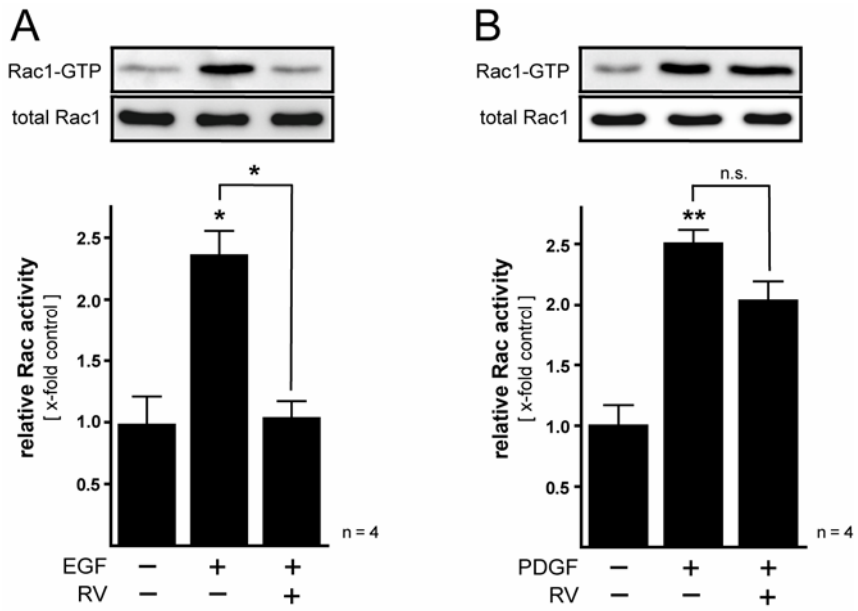

Figure 24: EGF- but not PDGF-activated Rac1 is strongly inhibited by RV

*For immunoprecipitating the active form of Rac1, VSMC were pretreated with RV for 30 minutes and then stimulated with 20 ng/ml EGF **(A)** or 2 ng/ml PDGF **(B)** for 5 minutes. Cells were subsequently lysed and an aliquot of each whole cell lysate was taken away for determination of total Rac1 amounts. The remaining lysate was incubated with PAK Agarose beads for 45 minutes. Immunoprecipitated active Rac1 was correlated to the whole Rac1 amounts. Bar graphs show summaries of three to four independent experiments with representative blots above (*, $p < 0.05$; **, $p < 0.01$; ns, not significant; mean ± SEM).*

On the other hand cdc42 activity, which is responsible for filopodia development was not affected by either EGF or PDGF stimulation (Fig. 25). Moreover, RV had no effect on cdc42 activity, either.

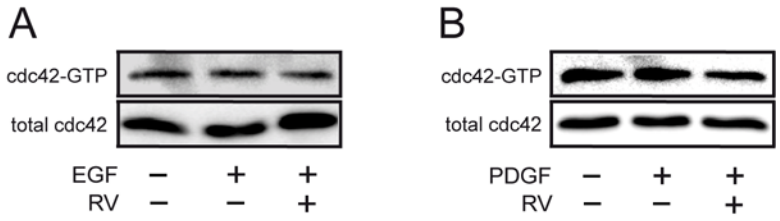

Figure 25: cdc42 activity is not affected by EGF, PDGF and RV

*Active cdc42 was immunoprecipitated for 45 minutes with PAK-agarose beads after stimulation with 20 ng/ml EGF **(A)** or 2 ng/ml PDGF **(B)** for 2 minutes. RV was added 30 minutes before growth factor stimulation. Before IP, whole cell lysate aliquots were drawn off. Representative Western Blots are shown.*

RhoA, molecular prerequisite for stress fibre formation, could not be precipitated and therefore we cannot definitely exclude that RhoA activity is influenced by RV. However, phalloidin stainings revealed no obvious changes of stress fibre formation (Fig. 22). With these experiments we could clearly show, that RV selectively affects lamellipodia formation in EGF-triggered VSMC, and therefore inhibits exclusively migration of EGF-stimulated, but not PDGF-treated cells, which we moreover could document by Rac1 pull-down experiments.

Taken together, we could show that EGF induces migration in VSMC which is completely blocked by RV in a stimulus-dependent manner, since PDGF-mediated migration and lamellipodia formation is not affected by pretreatment with the polyphenol. At the molecular level, hyperphosphorylation of FAK is not affected by RV. Enhanced activity of Rac1 on the other hand, is strongly inhibited in EGF- but not in PDGF-stimulated VSMC, which at least in part explains the growth factor-dependent anti-migratory modes of action of RV.

DISCUSSION

E Discussion

The polyphenol RV has been described to be one main active compound which accounts for the health-beneficial effects of red wine and may be responsible, at least in part, to explain the French paradox [216]. It has already been shown, that RV exerts advantageous properties to diminish certain parameters of cardiovascular risk factors, as lowering LDL oxidation [218]. Since CVD are today's most abundant illnesses in the world, much effort is made to find potent compounds to fight these diseases [5]. VSMC play a central role in many aspects of atherosclerosis, from the initiation to more progressive states of the disease [22]. The migration of VSMC into and subsequent proliferation within the intima of the atherosclerotic lesion mainly accounts for the narrowing of the atherosclerotic blood vessel [22]. Therefore, prevention of VSMC migration, proliferation and hypertrophy may improve the chances to delay or even circumvent the progression of atherosclerosis. It has been already shown, that RV influences Ang II- or EGF-induced Akt activation in VSMC and acts as antioxidative molecule to diminish ROS production [226, 227]. Until now it is not clear how RV exerts its manifold beneficial properties and which molecules are directly or indirectly affected by the molecular actions of RV.

In search of the molecular target that accounts for RV-mediated inhibition of Akt phosphorylation upon EGF, we investigated a series of possible candidate proteins in this respect.

RV can act as SERM, which means that it is able to bind to ER in an antagonistic or agonistic way, depending on the cellular system and concentration [235, 236]. Beside the "classical" genomic actions of ER, it has been demonstrated that the nuclear receptor can contribute also to rapid signalling cascades, where it is located near the membrane and can influence among others Akt signalling [97]. We wanted to determine if ERα contributes to EGF signalling and if RV was able to act as a SERM. We could clearly show that ERα was able to contribute to Akt phosphorylation in a rapid and non-genomic way (Fig. 8A and 9B). In EGF-stimulated cells, however, participation of ERα was not taking place, since the selective ER antagonist fulvestrant neither changed

EGF-induced Akt phosphorylation nor the inhibiting effects of RV on pAkt. It seems that RV is able to bind to ERα at the ligand binding domain in an antagonistic fashion, since pretreatment of VSMC with RV followed by subsequent addition of estrogen strongly inhibited phosphorylation of Akt (Fig. 10A). Since these effects were even stronger than the influence of fulvestrant, we assume that RV is able to potently bind to ERα in an antagonistic way. Due to the structural similarity of estrogen and RV, it is likely that RV fits into the ligand binding domain of VSMC ERα , which has already been mentioned by others [262]. These results do not directly proof that RV acts as antagonist for ERα and results from co-incubation experiments with fulvestrant and RV implicate the possibility that RV either binds to another area within ERα which causes a synergistically inhibition of the receptor. However, it can be speculated that RV binds to the ligand binding domain of ERα with a higher affinity than fulvestrant, which may cause the latter to dissociate from its receptor.

Furthermore possible effects on ERβ, which is also expressed in VSMC but only poorly described [263], were not determined. The molecular basics of rapid signalling of membrane-bound ER are not well studied and it is still not clear, whether a certain isoform of ER is responsible for the rapid signalling effects or if all ER subtypes are able to do so [264]. Moreover, several truncated versions of ERα have been discovered to be expressed in various tissues. One N-terminally deleted 46 kDa variant has raised the attention of several groups, because of its broad expression pattern and full functionality [265]. Moreover, it has been shown that the 46 kDa version of ERα was contributing to rapid signalling events in vascular endothelial cells and was located and membrane-anchored in caveolae, which represent hotspots of signalling [265]. Although we used an antibody targeting the C-terminus of ERα, which means that also N-terminally deleted, short versions of ERα should be recognized by this antibody, we could not discover a 46 kDa version in VSMC. However, a protein with approximately 55 kDa size was detected (Fig. 9A), which we solely found to be expressed in the nucleus of VSMC. Whether this protein is really a short ERα version or only an artefact of the antibody remains elusive.

Taken together, we could show that rapid and non-genomic ER-mediated signalling towards Akt is occurring in our experimental setting of estrogen-stimulated VSMC. Although RV potently inhibited estrogen-mediated Akt phosphorylation, no contribution of ERα in EGF-mediated signalling could be monitored.

Integrins, the big family of transmembrane adhesion molecules, are pivotal sensors to control cell attachment to the surrounding ECM [133]. Integrins contribute to intracellular signalling mainly by binding adapter proteins and/or by phosphorylation of the β-subunit and therefore transmitting survival signals into the cell [138]. Since integrin signalling is able to contribute to different aspects of RTK signal transduction [146], we asked the question if the inhibiting properties of RV on Akt phosphorylation are mainly or at least in part transmitted via integrins. Recently, it has been demonstrated that RV can directly bind to a particular integrin, $\alpha_V\beta_3$ [241]. By using two cancer cell lines (MCF-7 and MDA-MB) and purified integrin monomers, the group showed that especially the β_3-subunit had the ability to bind RV. Furthermore, the interaction of RV with the integrin had also impact on the signalling, since blocking molecules inhibited the RV-dependent hyperphosphorylation of ERK 1/2 and p53. This report was the first one to show that integrins contain a receptor site for RV, which is moreover involved in signalling. Furthermore, it has already been shown that the $\alpha_V\beta_3$ integrins are expressed on rat VSMC [266], which was also confirmed by our group (Fig. 11).

By preincubating VSMC with blocking molecules, as the small peptide RGD or β_3-blocking antibodies, we wanted to determine if the inhibiting effects of RV on EGF-induced Akt phosphorylation could be influenced. However, preincubation of VSMC with both blocking molecules had only minor effects on the potential of RV to inhibit Akt phosphorylation (Fig. 12). In the case of using RGD peptides, it is still reasonable to assume that RV binds to a region within the integrin, other than the classical ligand binding site occupied by the RGD peptide. Although Lin and colleagues argue that RV binds at least to a region near the ligand binding domain [241], direct proof for this hypothesis remains elusive. By using the, in comparison to the RGD peptide, much bigger blocking antibody against β_3 integrins we tried to occupy a large region within the integrin monomer. However, experiments using anti-β_3 integrin antibodies yielded comparable results. This means that RV is mainly not acting via integrin $\alpha_V\beta_3$ to exert its inhibitory actions on Akt phosphorylation. It is still possible, however, that also the antibody binds to its target structure and leaves unoccupied regions for RV-binding, which we cannot exclude with these experiments. The concentrations of both blocking molecules were in accordance with other *in vitro* studies [241, 267]. Since the antibody is widely used as a rat-specific blocking molecule to prevent integrin-igand interaction, we can exclude that the antibody was not targeting the integrin on the surface of VSMC.

Since integrins are able to transduce signals via phosphorylation of the β-subunit and it was already shown that EGF affects integrin phosphorylation [268], we wanted to examine the phosphorylation status of β_3 integrins when using EGF and RV as stimuli. On the one hand it was not possible to detect changes in the phosphorylation status of β_3 integrin utilizing whole cell lysates. On the other hand we were not lucky to immunoprecipitate the β-subunit from the crude cell lysate, although different lysis buffers and antibodies were used. Since IP of β_3 did not work at all, we cannot rule out possible changes in the phosphorylation status of β_3 and furthermore of integrin-protein interactions when stimulating VSMC with EGF and RV. It is feasible that RV possibly acts intracellularly to selectively influence interactions between the β_3 integrin and one or more interacting protein(s). In our experimental setting it would be interesting to examine especially the phosphatase Shp-2 or the kinases FAK and Src, since they were shown to directly interact with integrins and moreover play significant roles in growth factor signalling [66, 160, 269]. Interestingly, we observed that the β_3 expression was significantly reduced when VSMC were incubated with a combination of blocking antibody, RV and EGF. It can be speculated, that the entry of RV into the cell may occur via receptor internalization, which is a well known phenomenon [270]. Unfortunately, this effect was not seen when RGD peptide or RV plus EGF alone were applied, which means that this result may be an artefact and β_3 downregulation is due to the action of the blocking antibody per se.

Another hint that integrins might not be the main target or entry site for RV was given, when we detached VSMC from their substratum and left them in suspension. We thought that if RV was able to bind integrins, detached VSMC which were not bound to ECM and therefore displaying solely unligated integrins on their surfaces would even amplify the inhibiting effects of RV on Akt signaling. Since suspended VSMC reacted exactly in the same way as adherent cells, this was another evidence that integrins might not be the primary target molecules of RV. Nevertheless, VSMC express at least four different integrin heterodimers (see introduction) and we therefore cannot definitely exclude that perhaps other integrin subtypes are more susceptible to RV-binding and may contribute to a bigger extent to the inhibiting effects of RV on Akt phosphorylation. Moreover, it was pretty difficult to find appropriate tools to detect species-specific integrin subunits, as this research field is quite new and very diverse.

In summary we could show, that integrin $\alpha_V\beta_3$, although expressed on VSMC, contributes only to a very small extent to the inhibiting properties of RV on Akt phosphorylation and presumably can be excluded as the main molecular target of RV in VSMC.

FAK, although first discovered just 15 years ago, is a very well studied molecule playing important roles in both growth factor- and migration-dependent signalling processes [168]. Due to its size and multiple phosphorylation sites, it is a very suitable molecule to properly distribute incoming growth factor and integrin signals by interacting with different adapter proteins [173]. Since we were not able to determine possible changes in the interaction pattern of integrins with FAK (see above) we had a deeper look into FAK phosphorylation, which is a crucial parameter of its activity. We first discovered that FAK was dephosphorylated when stimulated with EGF for five minutes. Given the fact, that EGF stimulation is a rather activating stimulus and FAK phosphorylation is a criterion for its activity, it was quite unexpected that total FAK phosphorylation was diminished upon stimulation with a mitogen. However, our group was not the only one observing such results [271]. It is in fact plausible, that after applying an activating stimulus, the cell switches to a more motile phenotype and therefore has to destruct focal contacts, which starts with FAK dephosphorylation and subsequent focal complex disassembly [271]. Further addition of RV to EGF-stimulated cells completely restored the phosphorylation status of FAK, indicating that RV most likely changed parameters in the focal complex formation giving us a first indication that RV might act in an anti-migratory way. FAK dephosphorylation did not change the recruitment of FAK to the EGFR, although it has been shown that these molecules are able to interact with each other [271, 272].

By stimulating VSMC with a phosphatase inhibitor, we could show that FAK dephosphorylation was phosphatase-related and mainly a result of either activation of a phosphatase or a prolonged interaction of FAK with a target phosphatase, but probably not due to inhibition of a kinase. The phosphatase Shp-2 has been well described to be part of the focal adhesion complex and it is known that FAK can be a direct substrate of Shp-2 [273, 274]. Enhanced interaction of Shp-2 with FAK was observed also by us

when carrying out co-immunoprecipitation experiments, presuming a direct interaction between these two proteins. However, we cannot eliminate the possibility that the interaction between FAK and Shp-2 is mediated by one or more adapter proteins. It is even likely that more proteins are involved given the fact that focal complexes comprise of several molecules. The importance of Shp-2 in FAK dephosphorylation was further proved when using fibroblasts expressing a truncated and functionally inappropriate acting Shp-2 [131]. Both, EGF and RV stimulation in these cells led to a slight induction of FAK phosphorylation above basal level, which itself was augmented compared to the FAK phosphorylation status in VSMC. Moreover according to the literature [275], truncated Shp-2 failed to physically interact with FAK and therefore could not exert its phosphatase activity.

Shp-2 has drawn the attention of our group even before, since we speculated that the highly redox-regulated phosphatase could be the primary target of the antioxidant RV. However, EGF stimulation experiments using a single-methylated derivative of RV (3,5-dihydroxy-4´-methylstilbene) gained results comparable to those using the hydroxylated molecule when determining Akt phosphorylation, although the derivative showed no antioxidative capacity (Schreiner Cornelia, PhD thesis 2009). This would mean that RV exerts its inhibitory activity not only due to its antioxidant properties, but potentially via deregulating protein recruitment processes.

Taken together, our results assume that RV diminishes the interaction of FAK with Shp-2 and therefore counteracts EGF-mediated FAK dephosphorylation by the proteintyrosine phosphatase Shp-2.

Since the phosphorylation levels of single tyrosine sites within FAK can be regulated independently, we wanted to know if the single tyrosine residues are stronger influenced by either EGF, RV or both than the total phosphorylation of FAK. Surprisingly, although the auto-phosphorylation site Tyr^{397} was affected in an equal way as the overall phosphorylation status of FAK upon EGF stimulation, addition of RV did not restore phosphorylation of this particular site to normal levels as expected. Since this residue is thought to be a suitable parameter for FAK activity, this would mean that both, EGF

alone and in combination with RV diminished the kinase activity of FAK within a short incubation time of five minutes. These observations were also made by others using either EGF [271] or RV [237] as stimuli, although in both studies cancer cell lines were used. It is feasible, that FAK activity is downregulated after EGF-stimulation because of the aforementioned actions of focal complex disassembly. It would be interesting to investigate EGF- and RV-mediated time kinetics of FAK Tyr^{397} phosphorylation. It is possible that RV keeps Tyr^{397} phosphorylation reduced for a prolonged time frame, which would inhibit FAK activity whereas EGF stimulation would lead only to a short Tyr^{397} dephosphorylation phase which would be restored or even enhanced to reactivate FAK for migration.

Other important phosphorylation sites are $Tyr^{576/577}$, which are phosphorylated by members of the Src family [173]. Our observations revealed that these tyrosine residues were differently regulated as Tyr^{397}, since EGF with or without RV enhanced the phosphorylation of $Tyr^{576/577}$. Although it is well established that Tyr^{576} and Tyr^{577} are additional markers for FAK activity, recent data dealing with those residues are rare. In most cases, researchers look exclusively at Tyr^{397} especially when determining the activity of FAK. Since phosphorylation of $Tyr^{576/577}$ within the kinase domain of FAK leads to conformational changes which go along with enhanced activity but do not additionally represent binding sites for adapter proteins, these results would mean that FAK activity is enhanced upon EGF alone or in combination with RV, counteracting previous results of diminished Tyr^{397} phosphorylation. Moreover, it is undisputed that Tyr^{397} phosphorylation is followed by binding of Src and subsequent $Tyr^{576/577}$ phosphorylation [172]. We, however, failed to co-immunoprecipitate Src with FAK. One explanation for these apparently contradictory results might be the time kinetics of FAK activation. Since we stimulated VSMC only for five minutes with EGF, we might have missed the hyperphosphorylating phase of Tyr^{397}, which is an important requirement for Src binding and subsequent $Tyr^{576/577}$ phosphorylation. This means, that Y^{397} phosphorylation and binding of Src might have occurred during the first four minutes of EGF stimulation and for this reason we were only able to detect Src-mediated $Tyr^{576/577}$ hyperphosphorylation at our five minutes time point. Indeed, it has been already shown by others that Tyr^{397} phosphorylation was rapidly induced after two minutes of growth factor stimulation [276]. Further time-course experiments would be reasonable to elucidate this possibility. Moreover the Src family comprises not only Src, but also Fyn, Yes, Lyn and other

members [277]. It is well known that FAK can physically interact with and be phosphorylated by different members of the Src family [278], which we were not investigating in our studies.

Since phosphorylation of Tyr^{861} and Tyr^{925} did not change significantly after five minutes of EGF +/- RV stimulation, we could exclude any effects of the two stimuli on adapter protein binding to Tyr^{925} of FAK in this time range. However, the phosphorylation kinetics of these two particular tyrosine residues might be different, which has to be taken into account. Possible changes in the binding affinities between PI-3K and FAK would have been interesting to study, since it was shown that FAK is able to link signalling to Akt via contribution of PI-3K [279]. However, we were not able to detect an interaction of FAK with PI-3K in our cellular setting.

We might have had also technical problems when carrying out co-immunoprecipitation experiments, since we failed to detect several target proteins which should interact with FAK. This fact is most likely due to the composition of the lysis buffer, although we made several adoptions. Especially salt concentrations play a stringent role when monitoring protein-protein interactions, which should be taken into consideration for future experiments. Additionally as already mentioned above, we might just have missed the right time points after EGF stimulation to detect changes in protein-protein interactions. Moreover a putative role of FRNK, the N-terminally deleted negative regulator, has not been determined. It was shown, that FRNK is expressed in embryonic rat VSMC at high levels [165], but it is still not completely clear how FRNK expression is regulated and what the molecular and phenotypic consequences of FRNK overexpression are [191]. It is speculated, that FRNK gets expressed when the composition of the ECM changes, for example within sites of vascular injury, and it was clearly demonstrated that FRNK upregulation led to FAK dephosphorylation [280].

We could show that rapid dephosphorylation of FAK is due to enhanced interaction with Shp-2, but there are still open questions regarding the kinetics of single phosphorylation sites of FAK when stimulated with EGF and the role of RV in this context.

RV is a well-known antioxidative compound and has been shown to inhibit ROS production in many cell systems [281, 282]. In VSMC, RV was reported to diminish ROS-dependent DNA damage [226]. ROS are not only produced intracellularly, but are also generated in the microenvironment of ligand-bound growth factor receptors. At the EGFR, extracellular ROS are generated and act as important second messengers to trigger growth factor phosphorylation and facilitate signalling [260, 283]. By using Amplex Red™ we wanted to know, if growth factor stimulation can lead to enhanced amounts of extracellularly produced H_2O_2 in VSMC and if RV could reduce them.

Surprisingly, we found that VSMC were producing quite high amounts of extracellular H_2O_2 even when left unstimulated. The measured values were approximately three times higher compared to cells treated with catalase used as negative control. Whether these high levels were stress-induced due to the handling of the cells (VSMC were transferred to another room, washed with PBS and incubated in a special buffer) or if this state should be considered as “normal”, cannot be answered at present. However, a recent study using VSMC and Amplex Red™ observed comparable high background H_2O_2 production [284]. RV was reducing the basal H_2O_2 levels by 60%. Addition of either Ang II or EGF enhanced extracellular H_2O_2 by 20% in a time-dependent manner, which could be also counteracted by pretreatment with RV. These rather low amounts of growth factor-induced H_2O_2, although going along with the results of aforementioned paper [284], might be due to limitations of the assay system. One can imagine that generated H_2O_2 which is released to the supernatant might increase vigorously but very locally around the receptor but cannot be measured to this extent in the whole supernatant due to a dilution effect. The source of generated H_2O_2 still remains to be determined, although Dikalov and colleagues identified NOX1 as the producer of Ang II-mediated extracellular H_2O_2 [284]. EGF-triggered H_2O_2 production might be the result of receptor-ligand interaction itself, since DeYulia et al. demonstrated that H_2O_2 can even be generated when using isolated EGFR plus EGF in a cell-free system [260]. Since H_2O_2 has been shown to both diffuse through membranes to activate intracellular signals and to be able to phosphorylate EGFR [283, 285], it is very likely that a 60% inhibition of H_2O_2 production by RV may cause implications also in intracellular signalling cascades. Additional studies in our group revealed, that RV not only affected extracellular but also intracellular ROS levels (Schreiner Cornelia, PhD thesis 2009) underlining the very potent antioxidative properties of the polyphenol.

With these experiments we could verify the potent antioxidant capacity of RV and furthermore could show that RV is able to diminish extracellularly produced ROS levels. Whether this effect is due to inhibition of H_2O_2 production at the protein level or via a possible scavenger potential of RV, awaits further investigations.

The main goal, to find the specific target of RV which accounts for the inhibiting effects of RV on Akt phosphorylation, was unfortunately not reached. We investigated several possible target molecules which we found contributing not or only poorly to the effects of RV (integrin $\alpha_V\beta_3$, ERα) and found FAK, which is maybe involved to a bigger extent in these processes. Within the scope of this project, other putative molecules could also be excluded as the main targets of RV: SIRT and redox-regulation of Shp-2 (Schreiner Cornelia, PhD thesis 2009), which leads us to the assumption that one single target protein might not exist. The effects of RV can be explained by the fact that multiple proteins are involved, which in sum might lead to the vigorous effects of RV. Based on this presumption, it is possible that several signalling events upstream of Akt are moderately affected by RV, which all end up in decreased Akt phosphorylation. On the other hand, the seen effects of RV are very specific for the PI-3K/Akt pathway, since previous results of our lab showed no or very little inhibiting properties of RV on other signalling pathways [251]. One way to solve this problem would be to carry out affinity studies attaching RV to solid phase columns and “fishing” possible target proteins out of whole cell lysates. By following mass spectrometry, new putative target proteins could be detected which can be followed up *in vitro* and *in vivo*.

RV has been documented to be an anti-invasive molecule in different cancer cell studies [237, 238]. VSMC migration is one very crucial step in the early phases of atherosclerosis, when cells migrate from their natural surrounding of the media into the intima of the vessel [22]. We therefore wanted to investigate, if RV can act as anti-migratory compound in VSMC, which would make it a putative promising agent for counteracting early steps of atherosclerosis. For this purpose, we performed wound healing experiments, which is a well-established method for the quantification of migration [286]. Beside the wound healing assay, another method to quantify migration is the Boyden chamber assay, where a pore-comprising insert is put into a well

containing the stimulus of choice. Cells are then seeded into the upper chamber of the insert and migration through the pores towards the lower well takes place. Afterwards, migrated cells are stained and counted [287]. Supporters of this method highlight, that the Boyden chamber assay is more convenient to measure chemotaxis towards a gradient and cells have to actively migrate through the small pores. There are, however, also disadvantages. Beside the high costs, the stimulus gradient is not maintained for a long time, since it distributes throughout the complete volume of medium.

To induce migration, we decided to establish dose-response experiments using 1-100 ng/ml EGF. We discovered that 20 ng/ml EGF was inducing migration to 50% above basal level which could not be enhanced further, even when five times more EGF was used. Indeed, it is well known that low concentrations of a growth factor would preferentially initiate migration, whereas higher amounts tend to induce proliferation [288]. For PDGF, we established migration assays ranging from a concentration of 500 pg/ml to 10 ng/ml (not shown). In this case we used 2 ng/ml for subsequent experiments, since it induced migration robustly (2.5 fold) and was in accordance with other publications, where VSMC have been utilized for migration studies either [261]. The proliferation cycle of cultured rat VSMC lasts approximately 26-28 hours (Sroka Irene, PhD thesis 2009) and since we stimulated our cells for maximal 21 hours, we excluded that cell proliferation could perhaps falsify our results of migration induction. Moreover we used in preliminary experiments mitomycin C, a potent proliferation blocker [289], to obviate proliferation by a second method.

By preincubating VSMC with 50 µM RV, we could clearly show that RV specifically affects EGF-, but not PDGF-induced migration (Fig. 21). This effect was not due to the usage of different concentrations of growth factors, since very similar patterns of action were discovered when using higher amounts of either growth factor, presuming a substantial growth factor-specific anti-migratory effect of RV.

Since the molecular basics of migration are very complex, we focussed first on a few central key players of cell chemotaxis: FAK and the small GTPases RhoA, cdc42 and Rac1. First experiments targeting FAK in a time-kinetics approach revealed that FAK was hyperphosphorylated upon EGF stimulation after 8 hours but RV was not able to compensate this effect. Given the fact, that activation of small GTPases, especially

Rac1 and cdc42, occurs very rapidly [201, 202] and the assumption that FAK would act upstream of these molecules, it would be feasible to furthermore look at FAK phosphorylation after one to two minutes of growth factor stimulation. Such short incubation times are, in fact, not easy to reproduce and results are therefore often unstable. It remains therefore elusive if total phosphorylation or phosphorylation of certain tyrosine residues within FAK might be affected by very short growth factor stimulation times. Furthermore it is until now not fully understood, whether FAK acts upstream or downstream of the small GTPases and FAK is also discussed to be even not absolutely necessary for activation of small GTPases [203, 204].

Fluorescence-labelled phalloidin is widely used as a molecule to specifically stain actin-bundles in the cell [290] providing qualitative evidence, which actin structures might be affected by a migration-inhibitory stimulus. Phalloidin stainings of EGF-activated VSMC revealed, that mainly lamellipodia formation was affected by RV (Fig. 22). We furthermore wanted to confirm the microscopically observed changes in the actin architecture by more quantitative methods. We therefore conducted pull-down assays targeting GTP-bound small GTPases which are accountable for the development of specific actin substructures. The time-kinetics of growth factor-induced GTPase-activation is very cell-type specific and can also vary between the single GTPases. Whereas cdc42 and Rac1 get activated within minutes [201, 202, 291], RhoA is often shown to bind GTP after 30 to 90 minutes [292, 293], which makes it very tricky especially for RhoA to catch the right activation time point.

We could clearly show that impaired lamellipodia-formation is indeed due to inhibition of Rac1 in EGF- but not in PDGF-stimulated VSMC after five minutes (Fig. 23). These observations go along with the wide accepted model, that Rac1 is mainly responsible for the constitution of lamellipodia. Since PDGF-activated Rac1 was not influenced by RV, it is not likely that RV acts as a direct Rac1 inhibitor. Interestingly it was shown recently, that EGF-mediated Rac1 activation is mediated by the MAPK MEK1, which acted directly upstream of Rac1 [294]. Moreover, our group could show that RV was significantly inhibiting ERK1/2 phosphorylation in VSMC when using Ang II [251, 295]. These data could lead to the assumption, that RV possibly targets the upstream of Rac1 and ERK 1/2 located kinase MEK1, which awaits further investigation. Another putative target of RV might be Dock4, recently identified as specific GEF for Rac1 [296].

On the contrary, formation of filopodia was hardly seen microscopically nor did we determine any changes in cdc42 activation upon EGF, PDGF or RV stimulation. Since the formation of filopodia is one important step in cell migration and has to occur [211], we therefore speculate that we did not choose the right time point after growth factor stimulation to monitor the development of filopodia. Although we were able to detect single filopodia under the microscope, their capillary and needle-like structure made it difficult to detect changes in the number per cell or even the structure. At the molecular level, cdc42 activity was monitored after two minutes of growth factor stimulation, which was in accordance with other publications [202, 297]. Initial experiments revealed, that a slight but not significant activation of cdc42 (15% above basal level) could be monitored after two minutes. This effect, however, was very unstable and not reproducible. Moreover Czuchra and colleagues could observe that cdc42 is not essential for filopodia formation, since cdc42-deficient fibroblasts did not show significant changes in migration speed or filopodia number per cell and therefore speculated that other members of the Rho family could compensate for the loss of cdc42 [298]. Indeed, another study revealed Rho in filopodia (RIF) as a novel and important protein involved in filopodia formation [299]. Furthermore, technical issues might have played a role that we did not have the ability to determine changes in cdc42 activity. PAK Agarose beads comprise a small amino acid sequence of PAK, a direct downstream target of both Rac1 and cdc42, which means that both GTPases are able to bind to the beads. Rac1 amounts in VSMC are quite high, and therefore active Rac1 could rapidly occupy the beads-attached PAK recognition sites which might have the consequence that weakly expressed cdc42 has no chance to bind.

The third member of small GTPases we were interested in was RhoA, a protein strongly involved in stress fibre formation. Microscopically, we could clearly observe the development of stress fibres after stimulation with either EGF or PDGF for one to four hours. Additional treatment with RV, however, had apparently no effects on the generation of stress fibres visualized by phalloidin-FITC. Unfortunately, pull-down assays targeting activated RhoA did not work at all, which was most likely due to non-binding of VSMC RhoA to the beads-linked binding site. For positive control, we loaded 1 mg of lysate with GTP to gain high amounts of GTP-bound RhoA. Even with this high GTP-RhoA-containing lysate, we were not successful to immunoprecipitate RhoA, which prompts us to speculate that rat RhoA was not binding to the beads supplied by the

company. Furthermore, the possible wide time-frame of RhoA activation could still be a problem in future approaches to effectively precipitate active RhoA. One possibility to circumvent these technical problems is to apply another and more sensitive method, like approaches which are based on changes in the emission wave length when the substrate is been converted.

Taken together, we could highlight a stimulus-specific anti-migratory action of RV in VSMC, which is mainly a result of impaired Rac1 activation followed by reduced lamellipodia development.

All experiments in this study were conducted using 50 µM of RV. This is a quite high concentration when compared to the maximal *in vivo* levels which can be achieved in target tissues of humans either drinking red wine or eating RV-containing tablets [300]. In addition, it has been clearly shown that blood half-life of RV is short and that RV metabolites occur in plasma and urine. It is still not fully understood if certain metabolites of RV possess equal or even higher activity as unmetabolized RV [301, 302]. In some experiments, we tried to investigate the role of integrins as potential receptors for RV. Until now, it is completely unknown if RV can act via receptor binding or not. Due to its structure, RV is able to pass the cell membrane without the need of transport proteins, which has been shown to occur *in vitro* [303] and is also likely *in vivo*. However, some of the effects exerted by RV are still explainable via a direct receptor interaction. Moreover, biodistribution of RV and its shuttling to tissues remain open questions. It has been shown that ^{18}F-labelled RV was predominantly found to be enriched in liver and kidneys of rats, and was excreted rapidly and to a high extent [304]. Since we tried to unravel possible new molecular targets of RV in a mechanistic *in vitro* study it is, as part of the fundamental research, plausible to use 50 µM RV as a tool to find new molecular targets to fight cell hypertrophy and which might provide insight into the molecular actions of the polyphenol. Moreover, this study is based on previous results gained in our lab using 50 µM RV and therefore it was feasible not to change the experimental setting when continuing to search for the molecular targets of RV in VSMC.

Isolated VSMC from rat aortas were used in this study to mimic the *in vivo* situation within atherosclerotic lesions. It should be taken into account that VSMC grown *in vitro*, definitely react differently as VSMC embedded in the arterial wall or the atherosclerotic lesion. The multiple interactions which take place between VSMC and ECM or VSMC and cells of other tissues cannot be imitated *in vitro*, where they are grown in Petri-dishes and without co-cultivation of other cell types. Results from such *in vitro*-based approaches should therefore always be confirmed *in vivo* as well, since several studies highlighted evident differences between these two models [305]. One good example is the fact, that mature contractile VSMC isolated from the media of animals and grown in cell culture undergo a “phenotype switch” and gain characteristics of intimal VSMC [306]. Our *in vitro* cell culture model is therefore an appropriate tool to study dedifferentiated, non-contractile VSMC in an activated manner similar to intima-invading VSMC *in vivo*. However, by comparing the gene expression profile of freshly isolated medial VSMC and cultivated late-passage VSMC, Shanahan and colleagues found 12 markers differentially expressed mainly involving proteins of the cytoskeleton network and ECM [307]. *In vitro* research is nevertheless a cruical requirement to elucidate mechanistic principles for successive investigations *in vivo*.

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APPENDIX

G Appendix

1. Abbrevations

A

ACE	angiotensin converting enzyme
ADAM	a disintegrin and metalloproteinase
Ang I	angiotensin I
Ang II	angiotensin II
AP-1	activator protein-1
AR	amphiregulin
ARP 2/3	actin-related protein 2/3
AT-1R	Ang II-type 1 receptor

B

bFGF	basic fibroblast growth factor
BSA	bovine serum albumine
BTC	betacellulin

C

Cas	Crk-associated substrate
CB	cytoskeleton buffer
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CE	cytoplasmatic extract
CHD	coronary heart disease
COX	cyclooxygenase
CR	caloric restriction
CREB	cAMP response element-binding protein
Crk	CT10 sarcoma oncogene cellular homologue
CVD	cardiovascular disease

D

DAG	1,2-diacylglycerol
DMSO	dimethylsulfoxide

E

EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	EGF receptor
eNOS	endothelial nitric oxide synthase
EPR	epiregulin
ER	estrogen receptor
ERK	extracellular signal-regulated kinase

F

FACS	fluorescence-activated cell sorting
F-actin	filamentous actin
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FERM	protein 4.1, ezrin, radixin and moesin homology
FIP200	FAK-inhibitory protein 200 kDa
FGF	fibroblast growth factor
FRNK	FAK-related non-kinase
Fvstr	fulvestrant

G

Gab-1	GRB2-associated binder-1
GAP	GTPase activating protein
GDI	guanine dissociation inhibitor
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GRAF	GTPase regulator associated with FAK
GRB2	growth factor receptor-bound protein
GRK	G protein receptor kinases

H

HB-EGF	heparin-binding EGF-like growth factor
HIF-1	hypoxia-inducible factor-1
HDL	high density lipoprotein
HRP	horseradish peroxidase

I

ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon gamma
IGF	insulin-like growth factor
IL	Interleukin
ILK	integrin-linked kinase
Iκ-B	inhibitor of NF-κB
iNOS	inducible nitric oxide synthase
IP_3	1,3,5-trisphosphate

K

KRPG	Krebs-Ringer glucose buffer

L

LDL	low density lipoprotein
LMW-PTP	low molecular weight tyrosine phosphatase
LRP6	LDL receptor-related protein 6

M

MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage-stimulating growth factor
MEK	MAPK/ERK kinase
MIDAS	metal ion-dependent adhesion site
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MMP	matrix metalloproteinase
MTOC	microtubule organizing centre

mTOR	mammalian target of rapamycin
mTORC	mTOR complex

N

NE	nuclear extract
NF-κB	nuclear factor kappa B
NOX	NAD(P)H oxidase
NRG	neuregulin

P

p130Cas	p130 Crk-associated substrate
PA	phosphatidic acid
PAK	p21-activated kinase
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	pleckstrin homology
PI-3K	phosphoinositide-3 kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PLCγ	phospholipase C gamma
PLD	phospholipase D
PKC	protein kinase C
PMN	polymorphonuclear cell
PRNK	PYK2-related non-kinase
PTP	protein tyrosine phosphatase
PYK2	proline-rich tyrosine kinase 2

R

RAS	renin-angiotensin system
RGD	arginine–glycine–aspartic acid (Arg-Gly-Asp)
RIF	Rho in filopodia
ROCK 1/2	Rho-associated coiled-coil-containing protein kinase 1/2
ROS	reactive oxygen species

RTK	receptor tyrosine kinase
RV	resveratrol

S

S1P	sphingosine-1 phosphate
SH2	src homology 2
Shc	SH2-containing collagen-related protein
Shp-1	SH2-containing phosphatase 1
Shp-2	SH2-containing phosphatase 2
SIRT1	sirtuin 1
SM-MHC	smooth muscle myosin heavy chain
SOS	son of sevenless
Src	Rous sarcoma oncogene cellular homolog
STAT	signal transducer and activator of transcription

T

TGF-α/β	transforming growth factor alpha/beta
TLR	toll-like receptor
TNF α/β	tumour necrosis factor alpha/beta

V

VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cell

W

WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin-homologous protein

2. Publications

2.1. Publications

Kumerz M, Heiss EH, Atanasov AG, Dirsch VM. The anti-migratory potential of resveratrol on EGF-activated vascular smooth muscle cells. *In preparation*

Schreiner CE, Kumerz M, Gesslbauer J, Erker T, Atanasov AG, Heiss EH, Dirsch VM. Resveratol-mediated inhibition of Akt phosphorylation in Ang II- or EGF-activated VSMC: role of ROS and NADPH oxidases 1 and 4. *In preparation*

Taschner S, Göbel F, Kumerz M, Richter S, Jörgl A, Strobl H. Functional association of GATA-1/GATA-1s-mediated repression of the vitamin-D-receptor with inhibition of granulo-monocytopoiesis – results from a functional screen. *Manuscript under revision*

2.2. Poster presentations

Kumerz M, Heiss EH, Atanosov A, Dirsch VM. Distinct anti-migratory effects of Resveratrol on EGF- versus PDGF-stimulated vascular smooth muscle cells. 21st scientific congress of the ÖPhG, Vienna (Austria), 2009. *Poster prize award winner*

Schreiner C, Kumerz M, Gesslbauer J, Atanosov A, Heiss EH, Erker T, Dirsch VM. Resveratrol-mediated inhibition of Ang II –induced Akt phosphorylation in VSMC – is it an antioxidant activity? 21st scientific congress of the ÖPhG, Vienna (Austria), 2009.

Kumerz M, Schreiner C, Heiss EH, Dirsch VM. Resveratrol inhibits EGF-induced Akt-phosphorylation in vascular smooth muscle cells: role of integrin αVβ3, focal adhesion kinase and Shp-2. 5th international EDHF Symposium, Tampere (Finland), 2008.

Schreiner C, Kumerz M, Heiss EH, Dirsch VM. The role of Shp2 and ROS in resveratrol-mediated inhibition of Akt phosphorylation in vascular smooth muscle cells. 5th international EDHF Symposium, Tampere (Finland), 2008.

Kumerz M, Taschner S, Göbel F, Strobl H. Inhibition of myeloid cell differentiation versus proliferation is differentially regulated by separate functional domains of GATA-1 - results from a functional genetic screen. European Congress of Immunology, Paris (France), 2006.

3. Curriculum vitae

Personal Data

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Scientific education

10/2006 – 09/2009	PhD Thesis at the Department of Pharmacognosy, University of Vienna, Austria **Supervisor:** Dr. Verena Dirsch **Project Title:** The role of resveratrol on growth factor-induced signalling events in vascular smooth muscle cells
10/2004 – 04/2006	Diploma Work at the Institute of Immunology, Medical University of Vienna, Austria **Supervisor:** Dr. Herbert Strobl **Project Title:** Functional and molecular analysis of the transcription factor GATA-1 in monopoiesis
10/2001 – 04/2006	Studies of Genetics and Microbiology University of Vienna, Austria
09/1999 – 06/2001	Studies of Biology University of Graz, Austria
09/1990 – 06/1998	Bundesrealgymnasium Klagenfurt-Viktring

Work Experience

07/2004 – 08/2004	Novartis Forschungsinstitut Vienna, Austria **Supervisor:** Dr. Tamas Schweighoffer
07/2002 – 08/2002	Federal Bacteriological and Serological Research Institute, Klagenfurt, Austria

Other activities

03/2005 – 07/2005	Student Supervision at the Department of Molecular and Cellular Genetics Medical University of Vienna, Austria

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