Chapter 6

Discussion

The aim of this thesis was to investigate molecular mechanisms that contribute to the thrombin–induced redifferentation of VSM cell and, more specifically, to elucidate the mechanisms underlying the cessation and reappearance of ERK1/2 phosphorylation. In this study it was demonstrated that the thrombin–induced second phase of ERK1/2 activation requires *de novo* RNA and protein synthesis. Since the long–lasting ERK1/2 phosphorylation was inhibited by disruption of membrane protein transport, a newly synthesized plasma membrane protein seemed to play a critical role in the transmission of the signal. Analysis of transcription levels of several genes revealed that HB–EGF is upregulated after stimulation of PAR1, PAR2 and PAR4 with thrombin and that the expression of this growth factor correlated with the long–lasting ERK1/2 phosphorylation. Since inhibition of MMPs as well as inhibition of EGFR autophosphorylation leads to the diminishment of the late phase of ERK1/2 activation, the shedding of EGFR ligands and EGFR activation are necessary intermediates in the TRAP–induced ERK1/2 activation. Treatment of VSM cells with heparin or siRNA-mediated knockdown of HB-EGF expression resulted in the inhibition of the thrombin–induced long–lasting ERK1/2 phosphorylation confirming that the second phase of ERK1/2 activation requires *de novo* protein expression and activity of HB–EGF.

6.1 Thrombin induces *de novo* protein expression of HB–EGF

The importance of a biphasic ERK1/2 activation in the promotion of cellular differentation is well described. Stimulation of β–adrenergic receptors (β–AR) in astrocytes is involved in the differentiation and maturation of these cells [195, 196]. When primary cultures are stimulated with isoproterenol, a β–AR agonist, a biphasic ERK1/2 activation and differentiation of the cells is induced. The sustained activation of ERK1/2 begins 12 h after stimulation, with a maximal activity after 24 h. In addition, an initial activity of PKA appears to be a prerequisite for the isoproterenol–induced differentiation signal [197]. The same effect was described later for thyroid hormone stimulation [198]. Likewise, IGF–I induces a biphasic ERK1/2 activation and, subsequently, differentiation of skeletal muscle cells [199]. Interestingly, the stimulatory effect of IGF–I on muscle cell differentiation has not yet been clearly defined. Additionally, TGF–β1 has been described to induce the phenotypic modulation of mesenchymal cells to SM cells promoting the expression of SM–α actin, SM–MHC, smooth muscle protein 22–α (SM22α), and calponin [200]. However, another study suggested that it is the TGF–β1–activated PI3K/Akt pathway rather than the TGF–β1-induced biphasic ERK1/2 activation that regulates this phenotypic modulation [201]. These findings contradict the results of Reusch et al. obtained in thrombin–stimulated aortic smooth muscle cells. In that study, the PI3K/Akt activation induces Raf phosphorylation and inactivation and, thus, the subsequent inhibition of the late phase of ERK1/2 phosphorylation. Moreover, the expression of SM–α actin and SM–MHC was blocked [202].

Whilst the general mechanisms particular characteristics of the thrombin–induced ERK1/2 activation have already been described, it is more difficult to explain how the ERK1/2 activation vanishes and re–appears after about one hour of delay. A possible explanation for the termination and reappearance of a signal on the observed timescale would be a feedback

loop that utilizes protein inactivation or degradation, transcriptional regulation or trafficking of preformed proteins such as activated receptors to the plasma membrane. Since the thrombin–induced second phase of the ERK1/2 phosphorylation was inhibited by actinomycin D, which blocks transcription by DNA binding, or by cycloheximide, an inhibitor of protein biosynthesis, transcriptional activity and protein translation were required for triggering the second phase of thrombin–induced ERK1/2 phosphorylation. These data indicate that *de novo* protein expression is a key intermediate of processes, which subsequently lead to a second and long–lived ERK1/2 phosphorylation.

Several studies attempted to elucidate the molecular mechanisms that regulate the cessation and reappearance of the ERK1/2 phosphorylation. The necessity of *de novo* RNA and protein synthesis for the sustained ERK1/2 activation introduced in this study is a new concept. However, leukotactin–1 (Lkn–1) stimulation of human osteogenic sarcoma (HOS) cells induces a biphasic ERK1/2 activation that depends on G_i/G_o proteins, and PLC activation [203]. Since the expression of the immediate early response genes c–fos, c–myc, egr–1 an c–jun is upregulated 30 min after Lkn–1 stimulation, *de novo* protein expression might be a key factor in the Lkn–induced second phase of ERK1/2 activation in HOS cells. Additionally, the endothelin B (ET_B) receptor induces a biphasic ERK1/2 activation upon IRL1620 treatment in VSM cells. The inhibition of this long–lasting ERK1/2 activation by MMP inactivation and by inhibition of the EGFR autophosphorylation suggests that the second phase of ERK1/2 activation depends on the transactivation of the EGFR by HB–EGF [204], but the precise molecular mechanisms involved remain to be elucidated.

Here we demonstrate that, upon stimulation of VSM cells with thrombin, *de novo* expression of HB–EGF is upregulated and promotes the appearance of a long–lasting second phase of ERK1/2 phosphorylation. A thrombin–induced upregulation of HB–EGF transcription has been shown at RNA levels, whereas the EGFR transcription was only slightly upregulated and PAR1 transcription was unaffected by thrombin stimulation. These results were confirmed on protein levels, which revealed a maximum of HB–EGF expression

at about 1–2 h after thrombin stimulation. A functional implication of HB–EGF in the sustained ERK1/2 signaling was confirmed using pharmacological and RNA–interference–based approaches.

Since PAR1 couples to the G_q, G_i and $G_{12/13}$ families of heterotrimeric G proteins, activation of multiple signal transduction pathways may drive numerous gene expression programs in VSM cells. A fundamental response of quiescent cells to GPCR stimulation with thrombin is the activation of gene expression of immediate early growth–response genes such as c–jun, c–fos or myc, which constitute the first wave of gene expression [205]. C–fos, fosB, c–jun, junB, junD, fra–1, fra–2 and egr–1 belong to the so–called immediate–early genes, whose expression increases rapidly and transiently when quiescent cells are stimulated. c–fos, fosB, and fra–1 are nuclear proteins that form homo- or heterodimers with the jun protein family, increasing their binding affinity for the activator protein 1 (AP–1) promoter site at the DNA [206]. The gene products of the immediate–early genes are nuclear intermediate of signal transduction in cellular growth and differentiation. In the case of thrombin, the immediate–early gene response depends on extracellular Ca^{2+}, Na^+/H^+ exchange and PKC activation. Another stimulant in VSM cells, arginine vasopressin (AVP), induces the expression of the immediate–early genes c–fos and egr–1. Moreover, upon activating the V1 receptor, it also requires the activation of PKC and the release of intracellular Ca^{2+} to transactivate the EGFR indicatin that PKC as well as Ca^{2+} are critical intermediate for the stimulation of the expression. Interestingly, the expression of c–Fos is modulated via an ERK1/2–independent signaling pathway, which requires an HB–EGF–induced EGFR activation, whereas the expression of Egr–1 is regulated via the Ras/MEK/MAPK pathway [207]. Additionally, the critical role of the MAPK signaling pathway in the activation of c–fos and c–jun transcription has been shown in VSM cells [208] and in primary cultured rat astrocytes [209] upon Ag–II stimulation.

It has to be mentioned that, the products encoded by these ubiquitous genes have no direct effect on the GPCR–induced EGFR transactivation or the subsequent signal-

ing through the Ras/Raf/MEK/ERK pathway [209]. Another work affirmed that, upon thrombin stimulation, the immediate–early gene expression was increased 15 min after stimulation with a maximal response at 30–60 min in rat pulmonary arterial smooth muscle cells. However, an analysis of c–Jun and c–Fos expression rates in thrombin stimulated VSM cells revealed an upregulation of protein expression 5 min after stimulation followed by a rapid decrease in expression levels. Since both, the onset and the peak of HB–EGF expression in thrombin–stimulated VSM cells, are delayed compared to immediate early genes, HB–EGF expression may be either regulated through gene products of immediate early genes or by independent mechanisms.

The gene products of c–jun and c–fos comprise a part of the AP–1 transcription factor complex which can form many different combinations of heterodimers and homodimers. Each combination determines the genes that are regulated [210]. The AP–1 complex dimers recognize either 12–O–tetradecanoylphorbol–13–acetate (TPA) response elements or camp response elements (CRE) [211]. In several cell lines, it has been demonstrated that, under specific conditions, HB–EGF can be one of the targets of AP–1. This affirmation is supported by Pascall et al. who reported that the rat HB–EGF promoter contains two AP–1 sites [212]. Moreover, in chicken embryo fibroblasts, the expression of HB–EGF is tightly correlated with the activity of Jun [213]. Another study demonstrated that the upregulation of HB–EGF expression during chemotherapy conditions in human colorectal cancer cells, pancreatic cancer cells and cervical cancer cell is induced by AP–1 complex [214]. In rat aortic SM cells, HB–EGF is upregulated by glucose–induced hyperosmotic stress. Under these conditions, the c–jun amino terminal kinase (JNK) is activated via Ca^{2+}–dependent Pyk2 signaling cascades and induces HB–EGF expression upon AP–1 activation [215]. Furthermore, in bladder smooth muscle cells could be shown that AP–1 mechanically activates HB–EGF expression [216]. All these results obtained in different cell lines and under several conditions indicate that HB–EGF is part of the second wave of gene expression and may be regulated by transcriptional activity of the AP–1 complex.

Other AP–1–independent mechanisms that regulate HB–EGF expression may include E26 transformation–specific sequence (Ets) transcription factors . The c–ets–1, ets–2, erg, elk–1 and elk–2 genes belong to a family of transcription factors and are implicated in the regulation of cell proliferation and differentiation [217]. It is well established that their function can be controlled by phosphorylation–mediated effects on DNA binding, protein–protein interaction, transcriptional activation, and subcellular localization [218]. The best characterized Ets modulators are the MAPKs. Additionally, one of the best–studied mechanisms through which ERK1/2 modulates gene expression involves the phosphorylation of Elk–1. In VSM cells, this ERK1/2–induced Elk–1 phosphorylation is a critical intermediate the in serum–induced c–fos transcriptional activation [190]. Interestingly, the Ets components are induced in VSM cells in response to various stimuli and play a critical role in regulation of vascular specific gene expression, vascular inflammation, and remodeling, and cell migration [219, 220, 221]. In addition, the c–ets–1 gene products can cooperate with the c–Fos/c–Jun complex (AP–1 complex) to activate the expression of certain promoters [222]. In NIH 3T3 cells transformed by oncogenic Ras and Raf, the HB–EGF expression is upregulatated by activation of an AP1/Ets–site on the HB–EGF promoter [223]. In bladder smooth muscle cells, mutational inactivation of the Ets component of the AP–1/Ets site abolished the transcriptional activation of the HB–EGF gene by mechanical signal, suggesting that an Ets family transcription factor cooperates with AP–1 to mediate the stretch response [216].

Other mechanisms to regulate HB–EGF expression utilizes Sp1 and nuclear factor–kappa B (NFkB). Sp1 is the first identified member of a family of zinc finger transcription factors and has been implicated in multiple physiological processes including cell cycle regulation, hormonal activation, apoptosis, angiogenesis, etc [224]. Similar to Ets transcription factors, activation of Sp1 can be induced by ERK1/2 phosphorylation [225]. Since the HB–EGF promoter exhibits specific binding sites for Sp1, an influence of the transcription factor on the expression of HB–EGF is expected. This effect was demonstrated in hu-

man keratinocytes [226]. Additionally, the HB–EGF promoter contains two NFkB binding sites. NFkB is a protein complex which acts as transcription factor and is involved in responses to cytokines, stress, free radicals and others [227]. The implication of NFkB in the HB–EGF gene expression is demonstrated in human peripheral blood monocytes, where the platelet–activating factor (PAF)–induced HB–EGF expression is inhibited by blocking NFkB activation [228].

One should also mention that, besides transcriptional regulation, the abundance of HB–EGF mRNA may be controlled by mRNA–stabilization [229]. The control of the mRNA decay is associated with specific sequence elements situated in the 3´ untranslated end of the mRNA molecule. The AU–rich (ARE) elements are a well–characterized group of these sequence elements and consist of several copies of the AUUUA sequence. These mRNA elements can control the decay of mRNA by binding to proteins that either stabilize or destabilize the mRNA molecule [230]. A characterization of the 3´–UTR sequence of HB–EGF mRNA revealed that it contains four ARE sequence elements [229] suggesting an important role for ARE in the regulation of HB-EGF expression.

6.2 Effects of brefeldin A or cytochalsin D treatment in thrombin–induced VSM cells

The appearance of a second phase of PAR1–, PAR2– and PAR4–induced long-lasting ERK1/2 phosphorylation was blocked by brefeldin A (see Fig. 5.3). In mammalian cells, the target of brefeldin A are guanine nucleotide–exchange factors (GEFs) which are characterized by a conserved catalytic domain called the Sec7 domain [231]. GEFs catalyze the activation of a small GTPase called ADP ribosylation factor (Arf) 1p [232]. Arf1, in turn, is responsible for the recruitment of coat proteins (coatomer, also called COPI as well as clathrin via an adaptor complex to membranes, resulting in the formation of transport

vesicles [233] and the activation of membrane–modifying enzymes such as PLC and PIK [234]. Arf1p and brefeldin A–sensitive GEFs are primarily localized to the Golgi apparatus of mammalian and yeast cells [235]. Brefeldin A binds at the interface between Arf–GTP and the Sec7 domain and inhibits the interaction of the proteins [236]. Thus, one effect of brefeldin A is the loss of COPI coats from the Golgi apparatus and the inhibition of the anterograde vesicle transport to the plasma membrane [237]. Therefore, the protein transport from the ER to the Golgi apparatus is inhibited, leading to protein accumulation in the ER and abrogation of forward trafficking to the plasma membrane.

After translocation into the ER, plasma membrane–resident proteins and proteins dedicated for secretion must pass through the Golgi apparatus before reaching their final destination in the plasma membrane. Since the second phase of ERK1/2 phosphorylation starts at about 1 h after stimulation with thrombin, the maturation and intracellular trafficking of newly synthesized HB–EGF must, thus, proceed rather quickly. It is demonstrated that inhibition of the intracellular trafficking of the growth factor by brefeldin A treatment prevents the necessary HB–EGF localization into the plasma membrane by retention of the protein in the ER (Fig. 5.8, B). Thus it is likely that, the inhibition of HB–EGF trafficking by blocking the Sec7/Arf–dependent vesicular transport leads to an inhibition of the thrombin–induced long–lasting ERK1/2 phosphorylation.

Interestingly, the Arf complex does not only play a role in the anterograde vesicle transport but also in receptor endocytosis. It has been demonstrated that Arf1 is required for the recruitment of all adaptor proteins onto intracellular membranes, and that it is needed for COPI recruitment, too. Arf1 can directly interact with coat components or modulate the vesicular trafficking by controlling phosphoinositide levels. Upon stimulation with thrombin, PAR1, PAR3, PAR4 and the EGFR are removed from the plasma membrane by endocytosis via a vesicular transport. This contributes not only to desensitization by reducing the number of surface receptors, but also to the signaling cascades of the receptors, which may continue after internalization. An ongoing association between GPCRs and the

MAPK signaling pathway has been reported in endosomes. Pathways regulating growth and survival, including MAPKs and phosphoinositide–3 kinase can be activated by the internalised EGFR [238]. The EGFR internalisation is mediated by clathrin–coated vesicles, the best–identified pathway of receptor endocytosis. Activated PAR is initially desensitized by rapid phosphorylation by GRKs, followed by binding to βarrestin and internalization via a clathrin– and dynamin–depending pathway [239, 240]. Although a recycling of some receptors has been demonstrated, EGFR and PAR1 are predominantly targeted toward lysosomes for terminal degradation [241].

It is possible that the signals involved in initiating HB–EGF transcription require PAR and EGFR endocytosis. Thus, the inhibition of the thrombin–induced second phase of ERK1/2 phosphorylation could be caused by blocking the retrograde vesicle trafficking by brefeldin A treatment but not by inhibition of the anterograde vesicle transport. Activation of the EGFR results in binding of clathrin adaptor protein adaptor complex 2 to the receptor and this interaction regulates the selective recruitment of the EGFR into coated pits during endocytosis [242]. As explained before, Arf1–induced adaptor complex activation is inhibited by brefeldin A. However, other studies suggest alternative internalization pathways, which are brefeldin A insensitive. In human epidermoid carcinoma A–431, the binding of adaptor complex 1 to the endosomes and the association with the EGFR is brefeldin A–independent as is the adaptor complex 2 binding to the EGFR at the plasma membrane [243]. Additionally, a recent report of Kumari & Mayor demonstrated that Arf1 is involved in a dynamin–independent endocytic pathway, but that this pathway is triggered via a brefeldin A–insensitive GEF [244].

In line with the assumption that transactivation of the EGFR critically depends on the expression and surface localization of HB–EGF, we found that the membrane–localized HB–EGF pool is rapidly depleted upon thrombin stimulation and is replenished 60 min later in the plasma membrane of VSM cells. In brefeldin A–treated cells, this depletion was further enhanced, and the subsequent replenishment was missing. These results suggest

that the induction of HB–EGF expression depends on an intact anterograde signaling via Sec7/Arf.

The interpretation of various experiments applying cytochalasin D treatment is more complicated. Cytochalasin D is a fungal metabolite which binds to actin filaments and blocks their polymerization and elongation. As a result of the inhibition of actin polymerization, cytochalasin D can inhibit cellular processes such as cell division and vesicular trafficking. Similar to treatment with brefeldin A, cytochalsin D blocked the thrombin–induced second phase of ERK1/2 activation. Theoretically, inhibition of the vesicular transport from the Golgi apparatus to the plasma membrane prevents the plasma membrane localization of HB–EGF, and, subsequently, the shedding of the growth factor by MMPs, and the EGFR–induced ERK1/2 activation. In VSM cells nucleofected with a cDNA plasmid encoding a C–terminally YFP–tagged HB–EGF construct, treatment of cytochalasin D 1 h after nucleofection induces an accumulation of the fluorescent fusion protein in vesicular structures (Fig. 5.8, C). The inhibition of the actin polymerization by cytochalasin D blocks the vesicular trafficking and, therefore, the fluorescent fusion protein can be visualized only in vesicular structures whereas in untreated VSM cells, HB–EGF–YFP is rapidly integrated into the plasma membrane.

Since cell–biological, genetic and biochemical studies support the hypothesis that the actin cytoskeleton is coupled to endocytic proccesses [245, 246, 247], the inhbition of the thrombin–induced second phase of ERK1/2 activation by cytochalasin D treatment could not only be caused by disruption of the anterograde trafficking. However, although actin filaments facilitate the constitutive receptor–mediated endocytosis under some conditions [248, 249], disruption of actin filaments did not always pertube the endocytic clathrin coated vesicle formation in mammalian cells [250].

In agrement with the results of the cytochalsin D treatment in thrombin–induced VSM cells and considering that different modulators could act towards different pathways to abolish the thrombin–induced late–phase of ERK1/2 phosphorylation, the second phase

of MEK/ERK–activation can be synergistically driven by several mechanisms. The anterograde trafficking of HB–EGF probably represents only one of the mechanisms that are required for the late phase by maintaining or even re–wakening the signal input that is provided by continuous PAR1 activation and extracellular EGFR transactivation. However, further work will be necessary to elucidate the role of endocytic and anterograde trafficking in thrombin–induced VSM cells.

6.3 Trafficking of HB–EGF

Since the beginning of the second phase of ERK1/2 phosphorylation occurs at about 1 h after stimulation, the required protein must be expressed, processed and transported rather quickly to the plasma membrane. Although it has been clearly demonstrated that in human VSM cells HB–EGF mRNA induction typically is a rapid process [133], the duration of HB–EGF processing and trafficking to the plasma membrane remains unknown. To investigate whether HB–EGF processing and transporting are fast enough for the HB–EGF localization into the plasma membrane to occur before the beginning of the second phase of ERK1/2 phosphorylation, a YFP–tagged autofluorescent fusion construct was generated, and its subcellular localization of HB–EGF–YFP was followed in living VSM cells. An almost complete plasma membrane localization of the fusion protein was evident as early as 5 h after nucleofection, which was the earliest time point after which the expression level and autooxidative maturation of the fluorescent protein allowed a confocal imaging analysis (see Fig. 5.7). Since endomembrane compartments did not show a significant accumulation of the protein, it can be concluded that HB–EGF can rapidly exit the ER and Golgi complex to get inserted into the plasma membrane. The localization of nascent HB–EGF in endomembrane compartments or transport vesicles became only unmasked if the anterograde trafficking was blocked by cytochalasin D or brefeldin A. A comparative analysis in HEK293 fibroblasts showed that the plasma membrane insertion of HB–EGF–YFP

was less efficient than in primary cultures of VSM cells, indicating a cell type–specificity in the rapid anterograde trafficking of this protein. One might argue that preformed and intracellularly stored HB–EGF–bearing vesicles can be inserted into the plasma membrane in a secretion–coupling model. However, since brefeldin A blocks the export pathway at the transition between the ER and the Golgi complex, the effective blockade of the late phase ERK1/2 phosphorylation by this compound proves the requirement of the whole export pathway.

6.4 ERK1/2 activation requires the triple membrane–spanning pathway

In thrombin–stimulated VSM cells, the biochemical processes that triggered the early and the delayed ERK1/2 phosphorylation seemed to be almost identical, presumably the triple membrane–spanning pathway, which has been worked out by Prenzel and colleagues [98]. In VSM cells, both waves of TRAP–induced ERK1/2 phosphorylation required an MMP–mediated shedding of heparin–sensitive EGFR ligands and EGFR transactivation. Since the first ERK1/2 activation is reduced by blocking of the MMP activity or EGFR autophosphorylation, the inhibition of the second wave of ERK1/2 phosphorylation might be a consequence of the reduction of the first ERK1/2 activation. Under these conditions, the PAR1–induced EGFR transactivation during the early phase may be required to induce the expression of HB–EGF which is a necessary prerequisite for the transmission of the differentiation signal. This idea is supported by other groups which affirm that the HB–EGF expression is part of an autocrine positive feedback loop [251, 252]. However, the inhibition of the second phase of ERK1/2 activation without blocking of the early phase (treatment of VSM cells 30 min after stimulation) demonstrated that the long–lasting ERK1/2 phosphorylation depends on the continuous shedding of newly synthesized

HB–EGF. The requirement of HB–EGF shedding for the signaling of the second phase of ERK1/2 activation is already described for other receptors. In atherosclerotic plaques, for example, ET_B receptor expression is upregulated in VSM cells. ET–1–induced ET_B activation leads to a biphasic ERK1/2 phosphorylation and cell redifferentiation which requires HB–EGF shedding by MMPs and EGFR transactivation [204].

6.5 Possible implication of the HB–EGF–Ct in the signaling pathway

The HB–EGF shedding by MMP–2, –3, –7, ADAM 9, 10, 12 and 17 to yield sHB–EGF, which binds and activates the EGFR is well understood [105, 123, 253]. However, the possible implication of the cytosolic C–terminus of cleaved HB–EGF in the signaling cascade remains unknown. Previous studies demonstrated that the HB–EGF cytoplasmic domain interacts with different signaling pathways. The multi–functional protein Bcl–2–associated athanogene-1 (BAG–1) has been reported to bind the cytoplasmic domain of HB–EGF and increase the secretion of the protein [130]. Additionally, in Vero cells, phosphorylation of a serine residue within the cytoplasmic domain of HB–EGF induces ectodomain shedding of the growth factor and is involved in HB–EGF–dependent tumorigenesis [131]. The cytosolic terminus of HB–EGF has also been implicated in different processes. HB–EGF–Ct generated by shedding of pro–HB–EGF after stimulation of the cells contains a transmembrane region and is translocated from the plasma membrane into the ER and the nuclear membrane by retrograde membrane trafficking [253]. The localization of HB–EGF–Ct in the nuclear membrane is supported by electron microscopy. Promyelocytic leukemia zinc finger (PLZF) and B–cell leukemia 6 (Bcl6) proteins were identified as protein that interact with the cytoplasmic domain of HB–EGF [134, 254]. Both proteins are transcriptional repressors localized in the nucleus [255, 256] which cause transcriptional repression through

recruitment of a repressor complex. Interaction of HB–EGF–Ct with PLZF or Bcl6 decreases the expression of their target genes [134, 254]. Additionally, in mouse embryonic fibroblasts, HB–EGF–Ct is involved in the signaling, which induces c–Myc expression and cell cycle progression by EGFR and FGFR stimulation [257]. In HEK293 cells, the trafficking of HB–EGF–YFP by thrombin stimulation was analyzed by confocal microscopy but we were not able to resolve the retrograde trafficking of HB–EGF–Ct–YFP with this method. Therefore, it remains to be investigated whether HB–EGF–Ct is implicated in the signaling which leads to the VSM cell differentiation.

6.6 Effects of the pharmacological or siRNA mediated HB–EGF modulation on the ERK1/2 activation

In this work, it was demonstrated that upon thrombin stimulation, the *de novo* expression of HB–EGF promotes the appearance of a long–lasting second phase of ERK1/2 phosphorylation in VSM cells. Different experiments described in the previous chapter focused our investigations to elucidate the role of HB–EGF in the transmission of the redifferentiation signal. A functional role of HB–EGF in the long–lasting ERK1/2 activation signaling was demonstrated by pharmacological and RNA–interference–based approaches.

The ability of HB–EGF to bind heparin sets HB–EGF apart from other members of the EGF family such as EGF and TGF–α, and facilitates the pharmacological blocking of the protein. The heparin–binding domain consists in 21 AA mapped to AA 93–113 [258]. The highly conserved primary structure of the domain between human, pig and rodents indicates evolutionary pressure and, thus, a biological significance of the domain [259]. In VSM cells, the cell surface HSPG modulates HB–EGF activity by binding to the HB–EGF heparin–binding domain, which is required for efficient EGFR activation [113]. The addition of heparin to the cells competes with the cell surface HSPGs, blocks its

binding to HB–EGF and aborts the HB–EGF–induced EGFR activation. As with GM6001 and AG1478 treatment, the inhibition of both waves of ERK1/2 activation by heparin support the theory that both phases require the triple membrane–spanning pathway to the EGFR transactivation. The inhibition of the thrombin–induced EGFR transactivation and the subsequently early ERK1/2 activation by heparin treatment was demonstrated by Kalmes in aortic SM cells [260]. However, the effect of the heparin scavenging of HB–EGF in the long–lasting ERK1/2 activation was not investigated. According to the results obtained applying modulators of MMPs activity and EGFR autophosphorylation, the inhibition of the long–lasting ERK1/2 phosphorylation without blocking of the early phase (treatment of VSM cells 30 min after stimulation) indicated that the long–lasting ERK1/2 phosphorylation critically depends on the HB–EGF–induced EGFR activation.

A problem of the heparin scavenging of HB–EGF is that the effects may be poorly specific. The binding of heparin to HB–EGF is only one effect of the glycosaminoglycan, whose principal pharmacological effect is its anticoagulant activity. The requirement of *de novo* HB–EGF expression for triggering a long–lasting ERK1/2 activation in response to thrombin was confirmed by RNA–interference. Since VSM cells could not be efficiently transfected by lipofection methods, the HB–EGF siRNA and non–silence control siRNA was integrated into the cells by nucleofection. The main problem of this method is still the low efficiency of nucleofected cells, which is about 20% as determined with a cDNA plasmid encoding enhanced yellow fluorescent protein (pEYFP-C1). Since siRNA constructs are much smaller than plasmid cDNA, their nucleofection efficiency is expected to be markedly higher. The effectivity and selectivity of the HB–EGF targeting siRNA was first probed comparing the effect of the HB–EGF siRNA on the amphiregulin and HB–EGF expression. Amphiregulin is a growth factor which belongs to the EGF family, is endogenously expressed in VSM cells, and its structure is similar to HB–EGF. Immunoblot analysis demonstrated that the expression of amphiregulin was unaffected by HB–EGF siRNA nucleofection, whereas the expression of HB–EGF was completely abolished. Since

the inhibition of the HB–EGF expression by siRNA treatment was successful, the effect of the HB–EGF knockout in the ERK1/2 activation in response to thrombin stimulation was analyzed. The inhibition of the thrombin–induced long–lasting ERK1/2 activation by RNA interference–mediated modulation of HB–EGF indicates the functional role and the requirement of *de novo* expression of the growth factor in the redifferentiation signal transmission. However, in other cell types, the HB–EGF knockout by RNA interference gave other results. In renal proximal tubular cells (RPTC), for example, inhibition of the HB–EGF expression by siRNA treatment leads to an inhibition of proliferation [261].

As an outlook, it would be very interesting to observe the effects of HB–EGF knockout in the development of the atherosclerosis *in vivo*. At the moment it is only known that HB–EGF–deficient mice revealed a crucial role of the growth factor for proper heart development and function [262, 263], for skin wound healing [264] and for eyelid development by promoting epithelial sheet migration through activation of the EGFR–ERK signaling cascade [265]. Future investigations should elucidate the implication of HB–EGF in the redifferentiation of other cell types and the functional role of the growth factor in the development of vascular diseases such as atherosclerosis *in vivo*.

6.7 Role of Pyk2 and Src in the thrombin-induced ERK1/2 activation

The functional implication of the non–RTK Pyk2 and Src in the GPCR–induced receptor tyrosine kinase transactivation remains elusive. In this study it was demonstrated that thrombin–induced long-lasting ERK1/2 phosphorylation is inhibited in presence of SU6656, an small–molecule inhibitor which selectively blocks Src and other members of the Src family. This result indicates the functional role of Src in the transmission of the differentiation pathway. Nevertheless, the function of the proteins in the thrombin–induced signaling

cascade is not clear. In VSM cells, angiotensin II (AT–II) stimulation Ca^{2+}–dependently activates Pyk2 to regulate Src and, subsequently, to recruit Grb2 by EGFR phosphorylation, triggering the Ras/Raf/MEK/ERK cascade [266]. These findings contradict with Touyz et al., who showed that upon Ang–II stimulation of VSM cells, AG1478 treatment inhibits Src phosphorylation and localizes the non–RTK downstream of the EGFR [267]. In agreement with this theory, the results of this work and other investigations in our laboratory confirm that EGFR activation is a prerequisite to Src and Pyk2 activation. Since the thrombin–induced early phase of ERK1/2 phosphorylation is reduced by MMP inactivation, heparin–scavenging of HB–EGF and EGFR autophosphorylation blocking, we can conclude that the TMS pathway is implicated in the thrombin–induced EGFR transactivation. These findings are supported by Schauwienold et al., showing not only the abrogation of the Pyk2 and Src activation by disruption of the HB–EGF–mediated EGFR transactivation, but also the contribution of both proteins to the expression of contractile proteins and cell differentiation [194].

Since a role of Src and Pyk2 downstream of the EGFR in the thrombin–induced ERK1/2 activation has been suggested by previous work in the lab, the importance of these proteins in the signaling cascade was investigated. It is known that the Pyk2/Src complex can contribute to the signaling transmission by phosphorylating Shc [268] or by directly recruiting the Grb2/Sos complex to initiate guanine nucleotide exchange of Ras and activate the MAPK pathway [269]. Although several studies suggested that Src is a downstream mediator of Pyk2 in the EGFR–induced Src activation, the position of the non–RTK in the signaling cascade was not clear. Inhibition of the Pyk2 expression by RNA interference reduces Tyr416 phosphorylation of Src by approximately 50% indicating a function of Pyk2 in amplifying Src activation (Fig. 5.20).

6.8 Overview of the thrombin–induced ERK1/2 activation signaling pathway

Generally, thrombin is a serine proteinase and the principal effector protease of the coagulation cascade. Thrombin stimulation of VSM cells activates the PAR1, PAR2 and PAR4, all members of a small family of GPCRs [270]. However, TRAP stimulation activates only PAR1 [271]. PAR1, the prototype of this family, is activated by cleaving its amino–terminus at a specific site [272] and the new N terminus acts as a ligand to activate the receptor. PAR1 can couple to members of the $G_{12/13}$, $G_{q/11}$, and G_i families. Activation of the receptor induces a conformational change that causes the binding of the G proteins and its activation. Activated G proteins can initiate different signaling pathways. $G_{q/11}$ and G_i activate PLCβ [273], initiating phosphoinositide hydrolysis to yield $InsP_3$ and DAG, which leads to calcium mobilization and activation of the PKC. Additionally, G$\beta\gamma$ subunits can activate PI3K [274], PLCβ, K^+ channels, GPCR kinases and non–RTK.

The thrombin–induced activation of the PAR–family receptors initiates a signaling cascade, which leads to MMP–2, –3, –7, ADAM 9, 10, 12 and 17 activation that cleave the pro–form of HB–EGF to yield sHB–EGF that activates the EGFR. The activation mechanism, which regulates MMP/ADAM proteins, is not clear. PKC is a candidate mediator for MMP activation. Although stimulation of cells with PKC activators induces a rapid processing of pro–HB–EGF [106] and PKC is directly implicated in ADAM9–induced HB–EGF shedding [107], the GPCR–induced EGFR transactivation is only partially sensitive to PKC inhibition. This suggests that different MMPs may cleave pro–HB–EGF through PKC–dependent and PKC–independent pathways in response to different stimuli or in a cell type–specific manner.

There was a considerable dispute about the role of the non–RTK Pyk2 and Src in the GPCR–induced EGFR transactivation. It is described that in fibroblasts G$\beta\gamma$ induces Src activation which couples to Ras/Raf/MEK/ERK via docking a Shc–Grb2 complex

[275]. Other work affirms that mobilization of Ca^{2+} induces Pyk2 activation, which leads to EGFR phosphorylation [266]. These results are supported by Andreev et al., assuring that, upon GPCR stimulation, activated Pyk2 forms a complex with Src that phosphorylates EGFR directly [276]. However, Prenzel et al. demonstrated the requirement of HB–EGF for the thrombin–induced EGFR transactivation [98]. Additionally, new findings demonstrated that Pyk2 and Src form a complex at the transactivated EGFR and interdependently support tyrosine phosphorylation [194].

Following pro–HB–EGF shedding, sHB–EGF binds the extracellular domain of EGFR leading to receptor dimerization [95] and activation of the intrinsic kinase activity by autophosphorylation of various tyrosine residues in positions 992, 1068, 1086 1148 and 1173 [96, 277]. These phosphorylated tyrosines are docking sites for SH2 domain–containing proteins like p120–GAP, Shc, Grb2 or Src. Pyk2–induced Src activation is necessary for Shc phosphorylation and its association with Grb2 and the subsequent ERK1/2 activation [269]. Additionally, it is described that the EGFR–induced Src activation can lead to additional tyrosine phosphorylation of the EGFR at positions Tyr845 and Tyr1102 by Src [165], which is required for EGFR signaling and mitogenesis [166, 167]. However, our experiments demonstrated that phosphorylation of the EGFR at Tyr845 could be demonstrated after EGF treatment, but not upon thrombin stimulation [194]. Since Tyr845 of the EGFR is not phosphorylated by Src, the elucidation of the EGFR phosphorylation sites in response to thrombin requires further investigation. After Grb2 activation, the docking protein form a complex with Sos, induces the exchange of GTP for GDP of Ras and activates the Ras/Raf/MEK/ERK signaling pathway. ERK1/2 is translocated into the nucleus and phosphorylates different transcription factors which activate the expression of the immediate early growth–response genes. As explained before, the transcription of c–jun and c–fos is upregulated 5 min after stimulation followed by a rapid decreasing of the expression levels. Possibly, the protein products of the immediate early growth–response genes are involved in the stimulation of HB–EGF transcription, which is upregulated 30 min

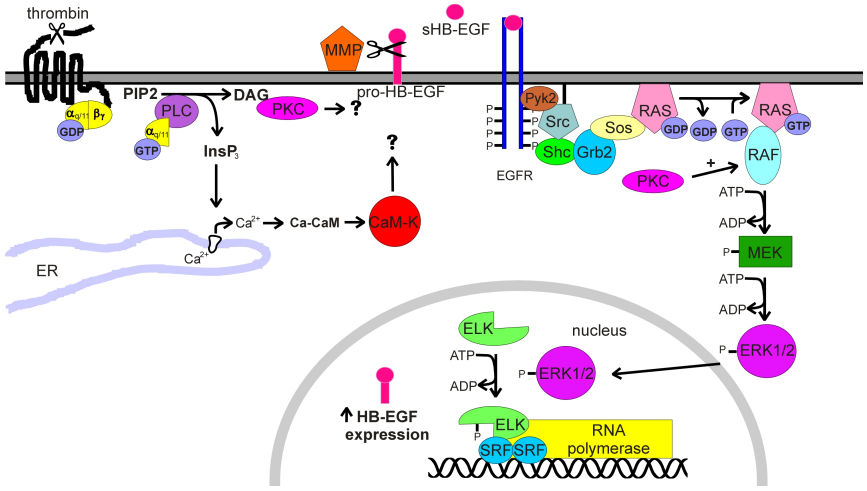

Figure 6.1: Thrombin-induced EGFR transactivation and ERK1/2 phosphorylation. Protease-activated receptor (PAR) is activated by cleavage in the N-terminal region. The new N-terminus in turn binds to the extracellular loop-2 of the receptor and activates the signaling cascade. After stimulation, PAR activates different G proteins, which induce the PLCβ activation. $InsP_3$ and DAG, produced by activated PLCβ, induce Ca^{2+} mobilization and activation of the PKC and calcium/calmodulin-depending kinase. The details of the thrombin-induced shedding of HB-EGF and EGFR transactivation remain unknown. EGFR dimerization and autophosphorylation induces ERK1/2 phosphorylation by activation of the MAPK signaling pathway. Activated ERK1/2 is translocated into the nucleus, where it phosphorylates several transcription factors such ELK and promotes the transcription of specific target genes.

after thrombin stimulation. A possible implication of the EGFR or/and PAR1 endocytosis in the upregulation of HB–EGF expression, as well as the role of HB–EGF–Ct in the signaling pathway must be investigated in the future. The newly synthesized pro–HB–EGF is transported to the plasma membrane after protein modification in the ER and Golgi apparatus and cleaved by MMPs to generate sHB–EGF that continuosly activates the EGFR to trigger the second phase of ERK1/2 activation and, subsequently, the expression of contractile proteins and cell differentiation [53].

Summary

Vascular smooth muscle (VSM) cells are highly specialized cells whose primary function is the contraction of blood vessels and the regulation of vascular tone. They respond to a variety of stimuli in the vessel wall. These stimuli can be of biochemical or mechanical nature and can lead to changes in cell function and phenotype, both, under physiological and pathological conditions. Differentiated VSM cells express a unique set of contractile proteins and signaling molecules. They exhibit a low rate of proliferation and a low synthetic activity. Under pathological conditions, VSM cells change from the normal contractile, differentiated state to a proliferative, synthetic phenotype. Pathologically altered VSM cells then migrate to the intima region and contribute to the progression of vascular diseases. However, redifferentiated VSM cells may also be important in maintaining the stability of the plaque through the formation of a firm fibrous cap, which prevents plaque rupture, which could otherwise cause thromboembolic complications.

It is well accepted that the ERK/MAPK signaling pathway is involved in the phenotypic modulation of several cell types. However, the signaling cascades and transcriptional regulators, which lead to cell differentiation, are still poorly defined. Various studies have demonstrated that a thrombin–induced redifferentation of VSM cells and the increased expression of smooth muscle α–actin and smooth muscle–specific myosin heavy chain require a biphasic activation of ERK1/2 via EGFR transactivation. However, the mechanisms of the cessation and delayed reappearance of the ERK1/2 phosphorylation remain elusive.

The findings presented here demonstrate the necessity of *de novo* RNA and protein

synthesis for the long–lasting ERK1/2 activation. Inhibition of the second phase of the ERK1/2 phosphorylation by blocking vesicle trafficking, or by brefeldin–A–induced disruption of the structure and function of the Golgi apparatus, indicated that the transport of plasma membrane–resident or of secretory proteins may play a critical role in this signalling cascade. Analysis of the thrombin–induced transcription and expression rates of plasma membrane–resident proteins revealed an upregulation of heparin–binding EGF–like growth factor (HB–EGF) with a temporal pattern that closely matches the second phase of ERK1/2 activation. Using confocal laser scanning microscopy, it could be demonstrated that HB–EGF is rapidly processed and transported to the plasma membrane. This result was supported by HB–EGF immunoblot analysis in VSM cell membrane fractions. Inhibition of both phases of ERK1/2 activation by MMP inactivation and blocking of the EGFR autophosphorylation demonstrated the requirement of the extracellular shedding and receptor tyrosine kinase activation for the PAR1–induced ERK1/2 phosphorylation. Since the ERK1/2 activation requires de novo protein synthesis and since HB–EGF expression coincides with the second phase of ERK1/2 phosphorylation, the role of the growth factor in this signaling pathway was analyzed. Pharmacological and RNA interference–mediated modulation of HB–EGF expression abolished the late phase of ERK1/2 phosphorylation, demonstrating that *de novo* HB–EGF expression and HB–EGF–induced EGFR transactivation are necessary intermediates in the thrombin–induced long–lasting ERK1/2 activation in VSM cells. Future investigations should elucidate the implication of HB–EGF in the redifferentiation of other cell types.

Although it is suggested, that Src and Pyk2 contribute to the thrombin–induced ERK1/2 activation, there is a controversy over the role of both proteins in the GPCR–induced ERK1/2 phosphorylation. The results of this work demonstrated that, upon thrombin stimulation, the long–lasting ERK1/2 activation requires Src activation. Moreover, reduction of the first phase of ERK1/2 phosphorylation by MMP inactivation, HB–EGF–scavenging, or blocking of the EGFR autophosphorylation indicated that Src and Pyk2 are

downstream effectors of the transactivated EGFR. Surprisingly, analysis of EGFR phosphorylation sites in response to thrombin showed that the Tyr845 of the EGFR was not phosphorylated by Src after EGFR transactivation. Finally, the critical role of Pyk2 in the EGFR–induced Src activation in VSM cells was demonstrated by siRNA–mediated Pyk2 knockdown. Further work will be necessary to elucidate the particular role of Src and Pyk2 in the thrombin–induced ERK1/2 activation.

Zusammenfassung

VSM-Zellen sind hochspezialisierte Zellen deren Hauptfunktionen die Kontraktion von Blutgefäßen sowie die Regulation des Blutdruckes sind. Sie reagieren auf eine Anzahl von Stimuli, die auf die Blutgefäßwand wirken. Diese Stimuli können biochemischer oder mechanischer Natur sein und können zu Veränderungen der Zellfunktion und des Phänotyps führen. Diese Veränderungen können sowohl unter physiologischen als auch unter pathologischen Zuständen entstehen. Differenzierte VSM-Zellen kennzeichnen sich durch die Expression kontraktiler Proteine sowie Signalmoleküle. Zusätzlich zeichnen sie sich durch eine verringerte Proliferation und eine geringe synthetische Aktivität aus. Unter pathologischen Bedingungen können ausgereifte Gefäßzellen einen Wechsel von einem normalen kontraktilen und differenzierten zu einem proliferierenden, synthetisierenden Zustand vollziehen. Diese Pathologisch veränderten VSM-Zellen migrieren zur Intima-Region und der Entwicklung kardiovaskulärer Krankheiten beteiligt. Jedoch können Redifferenzierte Zellen auch beim Aufrechterhalten der Stabilität eines Plaques von Bedeutung sein, da sie die Bildung einer stabilen fibrösen Kappe begünstigen und somit einen Zerfall des Plaques verhindern.

Es ist weitgehend bekannt, dass der ERK/MAPK Signalweg an der phänotypischen Modulation von verschiedenen Zelltypen beteiligt ist. Jedoch sind die Signalkaskaden und deren transkriptionalen Regulatoren, die zur Zelldifferenzierung führen noch nicht vollständig geklärt. Verschiedene Studien haben gezeigt, dass eine Thrombin-induzierte "Redifferenzierung" von VSM-Zellen und die damit einhergehende steigende Expression

der Proteine smooth muscle α-actin und die smooth muscle myosin heavy chain eine zweiphasige Aktivierung von ERK1/2 mittels EGFR-Transaktivierung erfordert. Jedoch ist der Mechanismus des Beginns und des Wiedererscheinens des Differenzierungs-Signalwegs unklar.

Die in dieser Arbeit präsentierten Ergebnisse zeigen die Notwendigkeit einer *de novo* RNA- und Protein-Synthese von HB-EGF für die lang anhaltende Aktivierung von ERK1/2. Eine Inhibierung der zweite Phase einer ERK1/2 Phosphorylierung durch Blocken des Vesikel-Verkehrs oder durch Zerstören der Struktur sowie Funktion des Golgi-Apparates mittels Brefeldin A-Induktion, weisen daraufhin, dass der Sekretionsprotein-Transport oder der Transport an der Plasmamembran-lokalisierter Proteine eine entscheidende Rolle in der Thrombin-induzierten Signalkaskade der VSM-Zell Differienzierung spielen. Es konnte gezeigt werden, dass die Stimulation glatter Gefäßmuskelzellen mit Thrombin zu einer erhöhten Transkription und Proteinexpression von HB-EGF führt. Zusätzlich konnte gezeigt werden, dass diese Veränderungen mit der zweiten Phase der ERK1/2 Aktivierung korrelieren. Mittels konfokaler Laser Scanning Mikroskopie wurde ermittelt, dass HB-EGF rasch prozessiert und zu Plasmamembran transportiert wird. Dieses Ergebnis konnte durch eine Immunoblot Analyse von HB-EGF aus VSM-Zell-membranen unterstützt werden. Die Inhibierung beider ERK1/2 Phasen durch MMP-Inaktivierung, und die Blockade der EGFR Autophosphorylierung zeigen, dass die PAR1-induzierte EGFR Transaktivierung den "triple membrane-spanning" Signalweg erfordert. Da die ERK1/2 Aktivierung eine de novo Protein Synthese erfordert und eine erhöhte HB-EGF Expression während der zweiten Phase der ERK1/2 Phosphorylierung beobachtet werden konnte, wurde der Einfluss von HB-EGF in diesem Signalweg untersucht. Nach pharmakologische Modulation und den Einsatz von RNA-Interferenz, konnte gezeigt werden, dass die späte Phase der ERK1/2 Phosphorylierung verhindert wurde und dass die *de novo* Expression von HB-EGF und dessen induzierte EGFR Transaktivierung notwendige Schritte bei der Thrombin-induzierten Redifferenzierung von VSM-Zellen sind. Weitere Untersuchungen

sind notwendig, um den Einfluss von HB-EGF auf die Redifferenzierng anderer Zellen zu verstehen.

Obwohl angenommen wird, dass Src und Pyk2 zur ERK1/2 Phosphorylierung beitragen, so sind die Meinungen über ihre Rolle in der GPCR-vermittelten Thrombin-abhängigen ERK1/2 Aktivierung verschieden. Die Ergebnisse dieser Arbeit zeigen, dass eine Stimulation glatter Gefäßmuskelzellen mit Thrombin, eine Src Aktivierung benötigt, um ERK1/2 zu phosphorylieren. Da sowohl die Inhibition der MMP Aktivität, als auch die Bindung von HB-EGF durch Heparin oder durch Blockade der EGFR Autophosphorylierung die zweite Phase der ERK1/2 Aktivierung drastisch reduzieren, konnte gezeigt werden, dass Pyk2 und Src nachgeschaltete Effektoren der Transaktivierung des EGFR sind. Untersuchungen der EGFR Phosphorylierung nach Stimulation von glatten Muskelzellen mit Thrombin zeigte, dass das Tyr845 des EGFR nicht durch Src phosphoryliert wird. Schließlich konnte durch Pyk2 Knockout mittels siRNA gezeigt werden, dass Pyk2 ein Zwischenprodukt der EGFR-induzierten Src Aktivierung in VSM-Zellen ist. Untersuchungen müssen vorgenommen werden, um die genaue Rolle von Src und Pyk2 in der Thrombin-induzierten Phosphorylierung von ERK1/2 zu verstehen.

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Abbreviations

AA	amino acid(s)
ADAM	a disintegrin and metalloproteinase protein
AP-1	activator protein 1
APS	ammoniumpersulfate
ARE	AU-rich
Arf	ADP ribosylation factors
AT-II	angiotensin II
ATP	adenosine triphosphate
AVP	arginine vasopressin
β-AR	β-adrenergic receptors
β-ME	β-mercaptoethanol
BAG-1	bcl-2-associated athanogene 1
Bc16	b-cell leukemia 6
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CFP	cyant fluorescent protein, color variant of GFP
COPI	coat proteins
CRE	camp response elements

Csk	COOH-terminal Src kinase
DAG	diacylglycerol
dNTP	mix of deoxyribonucleotides
cLSM	confocal laser scanning microscope
ECM	extracellular matrix
EDTA	ethylendiaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Elk1	ets-like transcription factor-1
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
ET_B	endothelin B
Ets	E26 transformation-specific sequence
FAK	focal adhesion kinase
FBS	foetal bovine serum
FGFβ	fibroblast growth factor β
FGFR	fibroblast growth factor receptor
FRS2α	fibroblast growth factor receptor substrate 2α
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GEFs	guanine nucleotide-exchange factors
GPCR	G protein-coupled receptor
G protein	guanine nucleotide binding protein
Grb2	growth factor receptor-bound protein 2
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate

GTPase	GTP hydrolase
HB-EGF	heparin-binding EGF-like growth factor
HEK293	human embryonic kidney (clone293)
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HERP1	HES-related repressor protein
HOS	human osteogenic sarcoma
HSPGs	heparan sulfate proteoglycans
IGF-1	insulin-like growth factor 1
IGFR	insulin-like growth factor receptor
IL-1	interleukin-1
IRS1	insulin receptor substrate-1
ISP	isoproterenol
JNK	c-jun amino terminal kinase
KLF4	kruppel-like factor 4
Lkn-1	leukotactin-1
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase pathway
mRNA	messenger ribonucleic acid
MEK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
NES	nuclear export signal
NFkB	nuclear factor-kappa B
NGF	nerve growth factor
non-RTK	nonreceptor tyrosine kinase
ORF	open reading frame
PAF	platelet-activation factor
PAGE	polyacrylamide gel electrophoresis

PAR	protease-activated receptor
PC12	pheochromocytoma cell
PH domain	pleckstrin homology domain
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3 kinase
PI_2P	phosphatidylinositol-2 phosphate
PI(3)P	phosphatidylinositol 3-phosphate
$PI(3,4)P_2$	phosphatidylinositol 3,4-biphosphate
PI(4)P	phosphatidylinositol 4-phosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PKA	protein kinase A
PKB	protein kinase B
PLC	phospholipase C
PLZF	promyelocytic leukemia zinc finger
PMSF	phenylmethylsulfonyl fluoride
PTB	phosphotyrosine-binding
PTP	protein tyrosine phosphatase
Pyk2	proline rich tyrosine kinase 2
Ras	rat sarcoma
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rotations per minute
RPTC	renal proximal tubular cells

RT	room temperature
RTK	receptor tyrosine kinase
SCFR	stem cell factor receptor
SDS	sodiumdodecyle sulfate
SDS-PAGE	sodiumdodecyle sulfate- polyacrylamide gel electrophoresis
S.E.	standard error
siRNA	small interfering RNA
SFKs	Src family kinases
SH	Src homology
Shc	Src homology 2 domain containing
SM	smooth muscle
SM-α actin	smooth muscle α actin
SM-MHC	smooth muscle myosin heavy chain
Sos	son of sevenless
Sp1	specificity protein 1
SPARC	secreted protein, acidic and rich in cysteine
Src	v-src sarcoma
Taq	*Thermophilus aquaticus*
TBS	Tris-buffered saline
TEMED	N,N,N´,N‘- Tetramethylethylene diamine
TGFα	transforming growth factor-α
TGFβ	transforming growth factor β
TH	thyroid hormone
TM	transmembrane
TMS	triple membrane-spanning
TNFα	tumor necrosis factor α
TPA	12-O-tetradecanoylphorbol-13-acetate

TRAP	thrombin receptor agonist peptide
Tris	tris (hydroxymethyl-)aminomethan
Triton X-100	octylphenylpoly (ethylene glycol ether)
VSMC	vascular smooth muscle cell(s)
YFP	yellow fluorescent protein, color variant of GFP

List of own publications

Publications in peer-reviewed journals

Pérez Sastre A., Grossmann S., Reusch H.P., and Schaefer M. Requirement of an intermediate gene expression for biphasic ERK1/2 activation in thrombin-stimulated vascular smooth muscle cells. *J. Biol. Chem.* 283: 25871-25878.

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Congress abstracts

Pérez Sastre A., Reusch H.P., and Schaefer M. Role of HB-EGF in the differentiation of smooth muscle cells. *Frühjahrstagung der Deutschen Gesellschaft für Pharmakologie und Toxicologie (DGPT)*, Mainz, 2007.

Pérez Sastre A., Reusch H.P., and Schaefer M. Role of HB-EGF in the thrombin-induced second phase of ERK1/2 activation. *Annual Workshop of the Graduiertenkolleg 865 (Mechanism in vascular regulation)*, Berlin, 2006.

Pérez Sastre A., Reusch H.P., and Schaefer M. Role of HB-EGF expression in the thrombin-induced redifferentation of vascular smooth muscle cells. Poster presentation at the *KININ 2007 Berlin: 2nd International Conference on Exploring the Future of Vascular and Inflammatory Mediators*, Berlin, 2007.

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