5.8 ERK1/2 phosphorylation is affected by pharmacological modulation of HB–EGF

Since the ERK1/2 activation in response to TRAP requires the shedding of an EGFR ligand by MMPs and the activation of the ligand–modulated EGFR activation, the objective of the following experiments was to elucidate whether HB–EGF is a key factor of the signaling of the long–lasting ERK1/2 phosphorylation. Therefore, the cells were treated with heparin, a highly–sulfated glycosaminoglycan which is used as anticoagulant and, among other qualities, can bind to HB–EGF and inhibit the HB–EGF–induced EGFR activation. Quiescent VSM cells were pretreated with 100 μg/ml heparin 30 min prior to thrombin

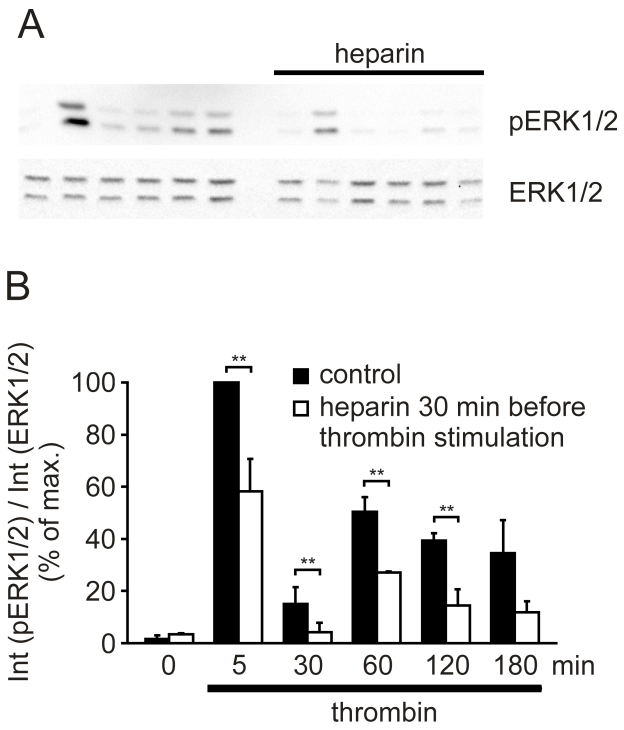

Figure 5.15: Thrombin–induced ERK1/2 activation is inhibited by heparin. A, Quiescent VSM cells were stimulated with 2 U/ml thrombin for up to 180 min in the presence of heparin (100 μg/ml added 30 min prior to thrombin stimulation). Whole–cell lysates were subjected to SDS gel electrophoresis, blotted, and probed with anti–phospho–ERK1/2 and anti–ERK1/2 antibodies. B, Quantitative luminometric analysis of signals obtained in several experiments as shown in (A). Bars represent means ± S.E of 4 independent experiments. Significance was accepted at $p < 0.05$ (*) or $p < 0.01$ (**).

stimulation. Immunoblot analysis of the thrombin–induced biphasic activation pattern of ERK1/2 demonstrated a clear inhibition by extracellular addition of heparin (100 μg/ml), which scavenges the released HB–EGF (Fig. 5.15). Consistent with the findings using the MMP inhibitor, treatment with heparin prior to stimulation also reduced the early ERK1/2 activation to approximately 50%. Since both the early and the delayed second

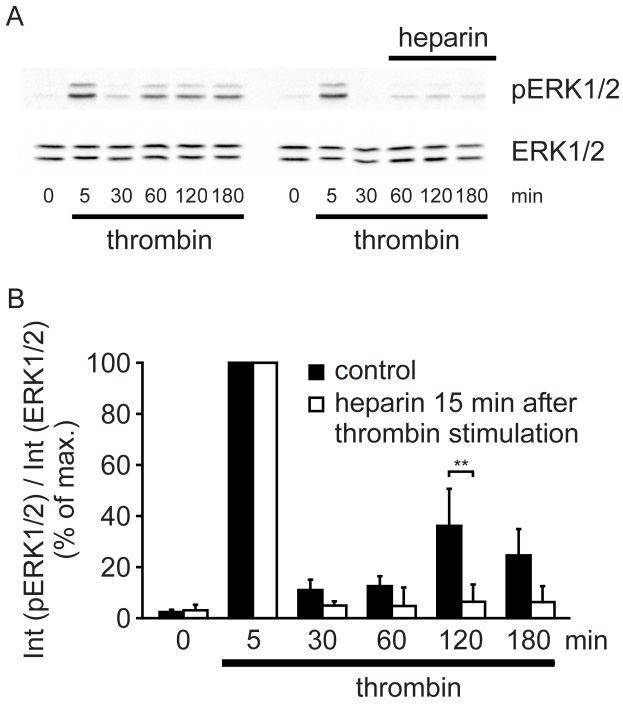

Figure 5.16: Thrombin–induced second phase of ERK1/2 activation requires HB–EGF. A, VSM cells were maintained in serum–free medium for 36 h, stimulated with 2 U/ml thrombin and treated with heparin (100 μg/ml) 30 min after stimulation. Whole–cell lysates were subjected to SDS gel electrophoresis, blotted, and probed with anti–phospho–ERK1/2 and anti–ERK1/2 antibodies. B, Quantitative luminometric analysis of signals obtained in several experiments as shown in (A). Bars represent means ± S.E of 4 independent experiments. Significance was accepted at $p < 0.05$ (*) or $p < 0.01$ (**).

phase of thrombin–induced ERK1/2 phosphorylation were attenuated by heparin, an additional experiment was necessary to demonstrate that, similar to GM6001 and AG1478 treatment, the inhibition of the second phase of the ERK1/2 activation is not an direct effect of the reduction of the early ERK1/2 phosphorylation. The same experiment was repeated, but, in this case, heparin was added 30 min after thrombin stimulation, when

the first phase of the ERK1/2 activation was already finished. Again the second phase of ERK1/2 phosphorylation was blocked by the delayed heparin treatment (Fig. 5.16), indicating that the release and EGFR–binding of HB–EGF are necessary intermediates in the thrombin–induced long–lasting ERK1/2 activation. These results confirm that HB-EGF can be a critical signal transducer during the delayed and long lasting phase of ERK1/2 phosphorylation.

5.9 ERK1/2 phosphorylation is sensitive to siRNA–mediated knockdown of HB–EGF

Pharmacological inhibition of the ERK1/2 activation by heparin–scavenging of HB–EGF may be not a very specific tool. Heparin treatment of VSM cells induces a number of different processes in the cells, which are not wished for a clean result. To increase the specificity of the results by an independent approach and to analyze the influence of HB–

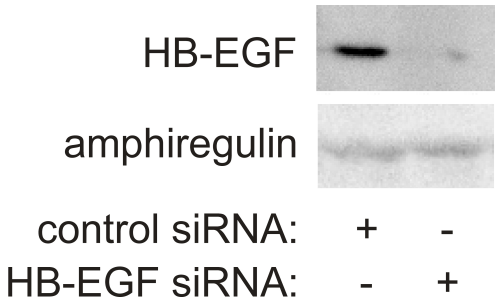

Figure 5.17: Control of the effectivity and selectivity of th HB–EGF siRNA. VSM cells were nucleofected with 100 nM HB–EGF siRNA or 100 nM of a non–silencing control siRNA. After nucleofection, VSM cells were maintained for 24 h in complete medium and for additional 36 h in serum–free medium. Membrane fractions were obtained and probed for HB–EGF and amphiregulin expression using appropriate primary antibodies.

EGF on the late phase of ERK1/2 activation more thoroughly, the expression of HB–EGF was blocked using the siRNA technology. The selectivity and specificity of the HB–EGF siRNA was proved by nucleofecting VSM cells with siRNA directed against the 3–prime

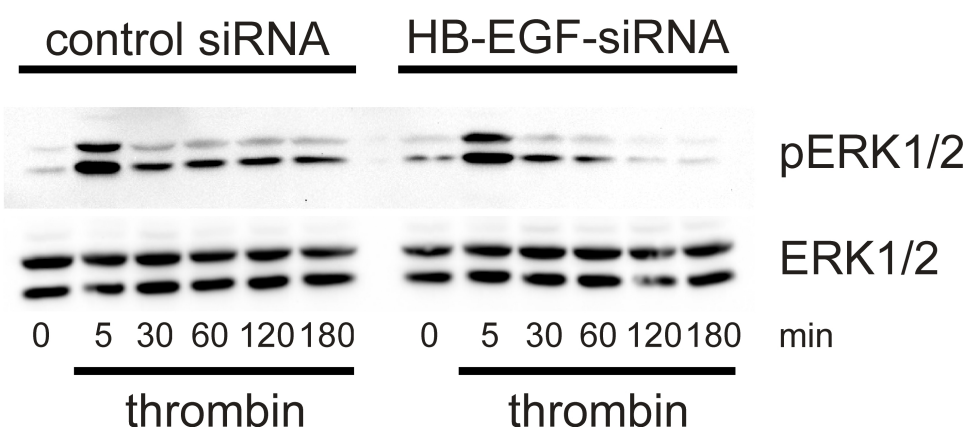

Figure 5.18: Thrombin–induced second phase of ERK1/2 activation requires HB–EGF expression. VSM cells were nucleofected with 100 nM HB–EGF siRNA or 100 nM of a non–silencing control siRNA. Nucleofected VSM cells were maintained for 24 h in complete medium and, for additional 36 h, in serum–free medium. The thrombin–induced phosphorylation of ERK1/2 was assessed in whole cell lysates applying anti–phospho ERK1/2 and anti–ERK1/2 antibodies as indicated.

noncoding region of the rat HB–EGF transcript or with a non–silencing control siRNA. After 2.5 days, a membrane preparation of the cells was performed. Western blot analysis revealed that the expression of HB–EGF was almost completely abolished by the HB–EGF–specific siRNA compared to the control siRNA (Fig. 5.17). The expression of amphiregulin, another member of the EGF family that is endogenously expressed in VSM cells, remained constant after nucleofection of HB–EGF or control siRNA.

Having established the effectivity and selectivity of the HB–EGF siRNA, the effect of the HB–EGF knockdown on the thrombin–induced ERK1/2 activation was analyzed. To this end, VSM cells were nucleofected with the HB–EGF siRNA or with a non–silencing control siRNA, plated in three 35–mm culture dishes, maintained for 24 h in medium, and for additional 36 h in serum–free medium. After stimulation of nucleofected cells with 2 U/ml thrombin for up to 180 min, whole–cell lysates were obteined and probed by Western blot analysis. As shown figure 5.18, HB–EGF knockdown leads to a strong reduction of the thrombin–induced ERK1/2 phosphorylation, whereas the ERK1/2 activation is unaffected by the non–silencing control siRNA nucleofection. These results confirmed that, upon

thrombin stimulation, the signal transmission, which finally leads to the late phase of ERK1/2 activation requires *de novo* expression of HB–EGF.

5.10 Src is implicated in the thrombin–induced ERK1/2 activation

As explained in the introduction, a possible role of non-RTKs in the GPCR–induced EGFR transactivation is discussed. Different studies indicate that, upon GPCR activation, the EGFR transactivation is an intracellular process where Pyk2 and Src, two non–RTKs, play an important role. To elucidate whether Src is involved in the thrombin–induced ERK1/2 activation, VSM cells were pretreated with SU6656, a selective Src inhibitor. Serum-starved VSM cells were pretreated with 5 μM SU6656 (30 min prior to stimulation), stimulated with 2 U/ml thrombin and lysed at the indicated times. As shown figure 5.19, blocking of Src activation almost completely abolished the late-phase of ERK1/2 phosphorylation. This result confirms the possible involvement of the non–RTK in the thrombin–induced VSM cells redifferentiation.

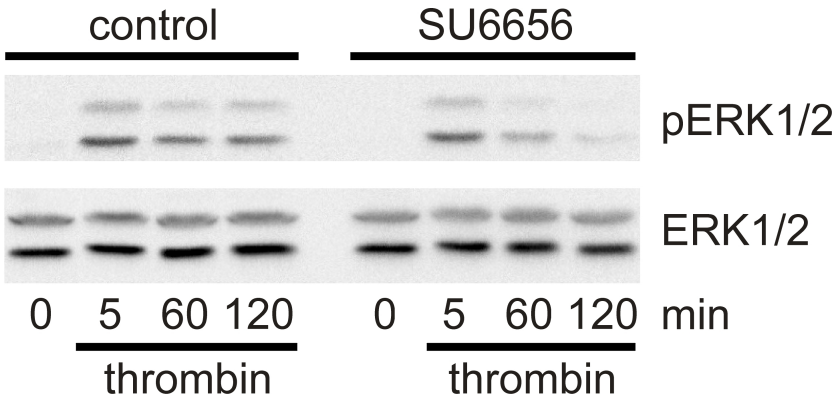

Figure 5.19: Thrombin–induced sustained ERK1/2 activation requires Src activity. VSM cells were maintained in serum–free medium for 36 h, pretreated with 5 μM SU6656 and stimulated with 2 U/ml thrombin. Whole–cell lysates were subjected to SDS gel electrophoresis, blotted, and probed with anti–phospho–ERK1/2 and anti–ERK1/2 antibodies.

5.11 Activated Pyk2 is a necessary intermediate in the EGF–induced Src activation

This study shows that the inhibition of the triple membrane–spanning pathway by inhibition of the MMPs activity, HB–EGF–scavenging or blocking of the EGFR autophosphorylation dramatically reduced the first phase of the ERK1/2 activation in response to thrombin or TRAP. These results, together with other work of our lab [194] demonstrated that both proteins, Pyk2 and Src, are downstream effectors of the transactivated EGFR. These data indicated a sequential arrangement of the non–RTK and the EGFR but the sequence of events, substrates and roles of both proteins in the differentiation signaling pathway remained to be determined. To elucidate whether Pyk2 activation is required to mediate the EGFR–induced activation of Src, VSM cells were nucleofected with Pyk2 siRNA or with a non–silencing control siRNA, plated immediately, maintained 24 h in complete medium, and for additional 36 h in serum–free medium.

Unfortunately, thrombin–induced Src activation was not strong enough to be observed by immunoblot analysis. Therefore and to avoid possible indirect effects downstream of GPCR activation, the EGFR was stimulated directly with 100 ng/ml EGF. After 2.5 days, the expression of Pyk2 was almost completely abrogated by Pyk2 siRNA, whereas it was unaffected by non–specific siRNA (Fig. 5.20). The siRNA–mediated knockdown of Pyk2 expression resulted in approximately 50% inhibition of the EGFR–induced Src activation 1 min after EGF stimulation as detected with the activation site–specific anti–phospho Src (Tyr416) antibody. Meanwhile, Src expression was not influenced by Pyk2 knockdown. As a second control, a non–silencing siRNA construct was used to demonstrate that the Src activation was not affected by siRNA interactions in VSM cells. This result confirmed that, upon EGFR stimulation, Src undergoes an activating phosphorylation downstream of Pyk2 and requires the activation of the non–RTK to open its conformation and start to phosphorylate other proteins. However, further work will be necessary to elucidate the

precise role of Pyk2 and Src in the thrombin-induced ERK1/2 activation in VSM cells.

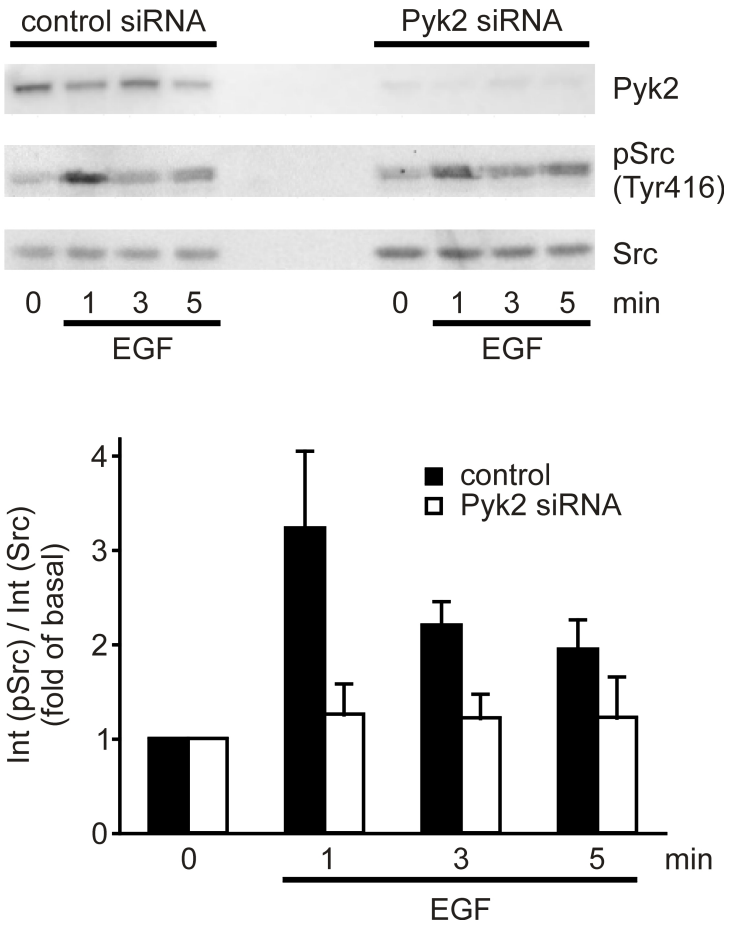

Figure 5.20: Requirement of Pyk2 for EGFR–induced Src activation in VSM cells. A, VSM cells were nucleofected with 100 nM Pyk2 siRNA or 100 nM control siRNA. After nucleofection, VSM cells were maintained for 24 h in complete medium and for additional 36 h in serum–free medium. Upon stimulation with EGF (100 ng/ml) for the indicated times, whole–cell lysates were obtained and separated by 10% SDS–PAGE. The Pyk2 expression was probed with an anti–Pyk2 antibody. The phosphorylation state of Src was assessed applying anti–phospho–Src(Tyr416) and anti–Src antibodies. B, Bars represent means ± S.E of 4 independent experiments.