Chapter 3

Materials

3.1 Chemicals

Acrylamide solution	Roth, Karlsruhe, Germany
Agar–Agar	Roth, Karlsruhe, Germany
Agarose	Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	Sigma, Schnelldorf, Germany
Ampicillin	PAA, Pasching, Austria
Aprotinin	Sigma, Schnelldorf, Germany
Benzamidine	Sigma, Schnelldorf, Germany
Boric acid	Merck, Darmstadt, Germany
Bovine serum albumin (BSA)	Sigma, Schnelldorf, Germany
Bromphenol blue	Sigma, Schnelldorf, Germany
Calcium chloride	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Sigma, Schnelldorf, Germany
Dodecyl sulfate sodium salt (SDS)	Merck, Darmstadt, Germany
Ethanol	J.T. Baker Deventer, Netherlands
Ethidium bromide solution (1%)	Roth, Karlsruhe, Germany

Ethylenedinitrilotetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Foetal bovine serum	Gibco BRL, Eggenstein, Germany
Formaldehyde	Merck, Darmstadt, Germany
Glucose	Merck, Darmstadt, Germany
Glutamine	Biochrom, Berlin, Germany
Glycerine	Merck, Darmastadt, Germany
Glycine	Applichem, Darmstadt, Germany
4–(2–hydroxyethyl)–1–piperazineethanesulfonic acid (HEPES)	Merck, Darmstadt, Germany
Hydrochloric acid (HCl)	Roth, Karlsruhe, Germany
Isopropanol	J.T. Baker, Deventer, Netherlands
Magnesium chloride	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
N,N,N',N'–Tetramethylethylenediamine (TEMED)	Sigma, Schnelldorf, Germany
Peptone	Roth, Karlsruhe, Germany
Phosphate buffered saline (PBS)	PAA, Pasching, Austria
Phenylmethylsulfonylfluorid (PMSF)	Sigma, Schnelldorf, Germany
Ponceau S	Merck, Darmstadt, Germany
Potassium chloride	Merck, Darmstadt, Germany
Saponin Sodium chloride	Merck, Darmstadt, Germany
Streptomycin	PAA, Pasching, Austria
Saccharose	Roth, Karlsruhe, Germany
Tris(hydroxymethyl–)aminomethane (Tris)	Applichem, Darmstadt, Germany
Trizol reagent	Invitrogen, Leek, Netherlands
Trypsin/EDTA solution	Biochrom, Berlin, Germany
Tryptose phosphate broth solution	Sigma, Deisenhofen, Germany

Tween–20	Applichem, Darmstadt, Germany
Yeast extract	Roth, Karlsruhe, Germany

3.2 Modulators

Thrombin from bovine plasma, 500 U	Calbiochem, Darmstadt, Germany
AG1478	Sigma, Schnelldorf, Germany
Brefeldin A	Sigma, Schnelldorf, Germany
Cytochalasin D	Sigma, Schnelldorf, Germany
Epidermal growth factor (EGF)	Sigma, Schnelldorf, Germany
GM6001	Biomol, Hamburg, Germany
Heparin	Sigma, Schnelldorf, Germany
Nocodazole	Calbiochem, Darmstadt, Germany
SU6656	Biaffin GmbH & Co KG, Kassel, Germany
TRAP (FLLRN)	Biozol, Eching, Germany

3.3 Antibodies

Anti–HB–EGF	Santa Cruz Biotechnology, Santa Cruz, USA
Anti–Goat IgG peroxidase conjugate	Sigma, Schnelldorf, Germany
Anti–Rabbit IgG peroxidase conjugate	Sigma, Schnelldorf, Germany
Anti–Pyk2	Becton Dickinson, Franklin Lakes, USA
Anti–Src	Cell Signaling, Danvers, USA
Anti–EGFR	Cell Signaling, Danvers, USA
Anti–phospho EGFR (Tyr845)	Cell Signaling, Danvers, USA

Monoclonal anti–Myosin (smooth) clone HSM–V	Sigma, Schnelldorf, Germany
Monoclonal anti–α smooth muscle actin	Sigma, Schnelldorf, Germany
Anti–phospho–p44/42 MAP Kinase (Thr202/Tyr204)	Cell Signaling, Danvers, USA
Anti–p44/42 MAP Kinase	Cell Signaling, Danvers, USA
Anti–phospho–Src Family (Tyr416)	Cell Signaling, Danvers, USA
151–IgG	Developmental Studies Hybridoma Bank, Iowa City, USA
Anti–amphiregulin	Santa Cruz Biotechnology, Santa Cruz, USA

3.4 siRNA constructs

A rat HB–EGF–, Pyk2–specific siRNA and Allstars negative control constructs were obtained from Qiagen.

	siRNA sequence
HB–EGF	5'–r(CGC UGG AUU UGA UGA GCU A)dTdT
Pyk2 1	5'–r(GGA CAA GUA UGA AUG UCU A)dTdT
Pyk2 2	5'–r(GGA UCG UCA UGG AAC UGU A)dTdT
Pyk2 3	5'–r(CAG UGU ACA UUG GAA UUU A)dTdT
Pyk2 4	5'–r(GGG AUA UUG CUG UCC GGA A)dTdT

3.5 Vectors

pcDNA3	Invitrogen, Leek, Netherlands
pcDNA3.1–V5/His–TOPO	Invitrogen, Leek, Netherlands

pcDNA3–C terminal cyant fluorescent protein (CFP)	AG Michael Schaefer, Berlin, Germany
pcDNA3–C terminal yellow fluorescent protein (YFP)	AG Michael Schaefer, Berlin, Germany

3.6 Cells

Competent bacteria:

Top 10	Invitrogen, Karlsruhe, Germany

Eukaryotic cells:

HEK293 (human embryonic kidney cells)	American Type Culture Collection, Rockville, USA
Primary cultures of newborn rat aortic smooth muscle (VSM cells)	AG Peter Reusch, Bochum, Germany

3.7 Enzymes and Kits

EndoFree Plasmid Maxi–Kit	Qiagen, Hilden, Germany
Expand High Fidelity PCR System	Roche, Mannheim, Germany
GoTaq Flexi DNA Polymerase	Promega, Madison, USA
M–MLV–RT	Invitogen, Leek, Netherlands
RNeasy Mini Kit	Qiagen, Hilden, Germany
Qiagen Spin Miniprep–Kit	Qiagen, Hilden, Germany
TOPO–TA–Cloning–Kit	Invitrogen, Leek, Netherlands
T4–DNA–Ligase	New England Biolabs, Beverly, USA
RNase H	New England Biolabs, Beverly, USA
Chemiluminescence Detection kit	Applichem, Darmstadt, Germany

Fugene 6 transfection reagent	Roche, Mannheim, Germany
Nucleofector Kit 4837	Amaxa Biosystems, Bonn, Germany

3.8 Other materials and devices

Nucleofection device	Amaxa Biosystems, Bonn, Germany
Hybond ECL nitrocellulose membranes	Amersham Pharmacia, Freiburg, Germany
Nonfat dried milk powder	Applichem, Darmstadt, Germany
Precision Plus Dual Color Protein Standard	Bio–Rad, Munich, Germany
RNAse–Inhibitor–Cocktail	Applichem,Darmstadt, Germany
1 kb DNA Standard	Gibco BRL, Rockville, USA

3.9 Media, buffers and solutions

3.9.1 Cell culture

MEM medium for VSM cells:

MEM–EBSS (Minimum Eagle's Medium, Earle's salt) with

Foetal bovine serum (FBS)	10%
Glutamine	4 mM
Penicillin	100 U/ml
Streptomycin	100 μg/ml
Tryptose phosphate broth solution	2%

Serum–free MEM medium for VSM cells:

MEM–EBSS (Minimum Eagle's Medium, Earle's salt) with

BSA	1% (w/v)

Glutamine	4 mM
Penicillin	100 U/ml
Streptomycin	100 μg/ml
Tryptose phosphate broth solution	2%

3.9.2 Bacterial cultures

LB medium:

Peptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
in H_2O; pH 7.4	

LB agar plates:

Agar-agar	1.5%
in LB medium	

SOB medium:

Peptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l
KCl	2.6 mM
in H_2O; pH 7.0	

SOC medium:

$MgCl_2$	20 mM
Glucose	20 mM
in SOB medium	

3.9.3 SDS–PAGE and Western–Blot solutions

3x Lysis buffer:

Bromphenol blue	0.3%
DDT	150 mM
Glycerine	30%
SDS	6%
Tris HCl; pH 6.8	90 mM

10x TBS–Buffer:

Tris	20 mM
NaCl	137 mM
in H_2O; pH 7.6 with HCl	

TBS–T–Buffer:

Tween–20	0.1%
in 1x TBS	

4x TrisCl/SDS, pH 6.8:

Tris	125 mM
SDS	0.4%
in H_2O; pH 6.8 with HCl	

4x TrisCl/SDS, pH 8.8:

Tris	375 mM
SDS	0.4%
in H_2O; pH 8.8 with HCl	

Running buffer:

Tris	25 mM
Glycine	192 mM

SDS	0.1%
in H_2O	

Transfer buffer:

Tris	25 mM
Glycine	0.2 M
Methanol or ethanol	20%
in H_2O	

Blocking buffer and antibody dilution buffer:

Nonfat dried milk powder	5% (w/v)
in TBS–T	

3.9.4 Lysis buffer for preparation of membrane fractions

HEPES	50 mM
EDTA	1 mM
Aprotinin	10 μg/ml
PMSF	200 μM
Benzamidine	1 mM

3.9.5 Confocal microscopy buffer

HBS buffer:

BSA	0.2%
$CaCl_2$	1 mM
Glucose	5.5 mM
HEPES	10 mM
KCl	6 mM

$MgCl_2$	1 mM
NaCl	128 mM
in H_2O; pH 7.4	

Chapter 4

Methods

4.1 Cell culture

Primary cultures of newborn rat aortic smooth muscle cells were maintained at 37° C and 5% CO_2 in a humidified atmosphere. Cells were passaged every 2 days by washing with PBS without Ca^{2+}/Mg^{2+} and incubating with trypsin/EDTA solution to release the cells from the flask. After 2–10 min, cells were resuspended in a small volume of fresh serum–containing medium and transferred to a new flask at a dilution of 1:2–1:5. For the experiments, cells were seeded onto 35–mm or 66–mm culture dishes. Unless otherwise stated, VSM cells were maintained in serum–free medium for 36 hours prior to the experiment.

4.1.1 Transient transfection of HEK293 cells

HEK293 cells were plated one day before transfection. 100 μl serum–free medium and 4 μl Fugene 6 were mixed for 35–mm culture dish and incubated for 2–3 min. 1–2 μg plasmid DNA were added to the prediluted Fugene 6 and the complex was allowed to proceed for 15 min at room temperature. Subsequently, the transfection solution was carefully added to the cells. The confluence of the cells was 50–80% on the day of the experiment.

4.1.2 Nucleofection of vascular smooth muscle cells

The Nucleofector technology is a non–viral transfection method designed for primary cells and hard–to–transfect cell lines like vascular smooth muscle cells. This system combines electrical parameters and cell type–specific solutions to drive plasmid DNA, oligonucleotides as well as siRNA into the cell. VSM cells were detached from the bottom of the flask with trypsin/EDTA and counted. 2 x 10^6 cells were centrifuged for 10 min at 800 rpm and resuspended in 100 μl of the nucleofection solution (test solution #4837 for rat VSM cells) containing 10 μg of DNA. Nucleofection was performed with the program T–28. Transfected cells were plated immediately after the procedure in three 35–mm culture dishes. For the small interfering RNA nucleofection, VSM cells were treated with a 100 nM siRNA or 100 nM Allstars negative control siRNA instead of DNA. After nucleofection, cells were grown in the presence of 10% FBS for 24 h and additionally for 36 h in a serum–free quiescent medium containing 1% (w/v) bovine serum albumin instead of FBS prior to agonist stimulation and cell lysis.

4.2 Molecular biology

Standard techniques used in this section are described in the manual "Molecular Cloning" of Sambrook and Russel [191].

The expression plasmids pcDNA3, altered version of the pcDNA3 containing fluorescent protein cDNA for 5´ or 3´ fusion with a PCR product, and pcDNA3.1–V5/His–TOPO vector were used for the heterologous expression in mammalian cells. These vectors contain a CMV promotor and a polyadenylation signal necessary for the optimal expression in eucaryotic cells. The pcDNA3.1–V5/His–TOPO vector was linearized and supplemented with a single 3´ thymidine overhang and a topoisomerase covalently bound to the vector. The PCR products were ligated efficiently with the vector by the topoisomerase, which links the single deoxyadenosine of the 3´ ends of the PCR products with the 3´ thymidine

overhangs of the vector. Once the PCR product was cloned into the pcDNA3.1/V5–His–TOPO vector, the correct orientation within the sequence of the PCR insert was analysed by restriction endonuclease digests.

The design of PCR primers is critical for the efficiency of the PCR and the expression of the heterologous protein. To this end, forward primers for PCR cloning contained an optimised ribosomal binding site (Kozak consensus sequence GCCACC) upstream of the ATG start codon. In addition, reverse primers incorporated appropriate restriction sites or stop codons.

4.2.1 Cloning of the HB–EGF cDNA into pcDNA3–YFP/CFP

For the cloning of the sequence encoding pre–pro–HB–EGF, the cDNA was amplified by PCR. The forward primer was 5'–GCC ACC ATG AAG CTG CTG CCG and the reverse primer was 5'–TCT AGA CAG TGG GAG CTA GCC AT. The PCR product was subcloned into pcDNA3.1–V5/His–TOPO vector. The sequences and correct orientation of the insert were controlled and the insert was subcloned into pcDNA3–YFP and pcDNA–CFP via Hind III and Xba I.

4.2.2 RNA extraction

Total RNA of primary cultures of VSM cells was isolated with Trizol reagent. VSM cells were scraped off and centrifuged for 10 min at 800 rpm. The supernatant was sucked off and cells were resuspended in Trizol. After incubation for 5 min at room temperature, 0.2 ml chloroform per 1 ml of Trizol was added and the samples were shaked vigorously for 15 s. The resulting samples were stored at room temperature for 2–15 min and centrifuged at 12,000 g for 15 min at 4° C. Following centrifugation, the colorless upper aqueous phase was collected. RNA remained exclusively in the aqueous phase whereas DNA and proteins distributed to the interphase and organic phase. The aqueous phase was transferred to a

fresh tube. The RNA from the aqueous phase was precipitated by mixing with isopropanol (0.5 ml isopropanol per 1 ml Trizol) and the samples were incubated at room temperature for 5–10 min and centrifuged at 12,000 g for 8 min at 4° C. The supernatant was removed and the RNA pellet was washed (by vortexing) with 75% ethanol and centrifuged at 7,500 g for 5 min at 4° C. The ethanol was removed and the RNA pellet was briefly air–dried. The RNA was dissolved in RNAse–free water, incubated for 10 min at 50–60° C and stored by –80° C.

4.2.3 Multiplex RT–PCR

To analyse the transcription of the HB–EGF, PAR1, EGFR and GAPDH, a multiplex RT–PCR was performed. Total RNA (1 μg) of rat VSM cells was reversely transcribed using MMLV reverse transcriptase and an oligo–(dT) primer.

Multiplex PCR was performed with Taq polymerase in the supplied magnesium–free buffer supplemented with 1.5 mM $MgCl_2$. The intron–spanning PCR primer pairs were selected based on published gene sequences for rattus norvegicus HB–EGF (NM_012945), PAR1 (NM_012950), EGFR (NM_031507) and GAPDH (NM_017008). A 605– nucleotide (nt) fragment of the HB–EGF transcript, was amplified using a sense 5´–GCT GCC GTC GGT GGT GCT GAA–3´ and antisense 5´–GCC ATG CCC AAC TTC ACT TTC T–3´ primer pair. A 354–nt PAR1 fragment was amplified using a sense 5´–CCT ATG AGA CAG CCA GAA TC–3´ and antisense 5´–GCT TCT TGA CCT TCA TCC G–3´ primer pair. A 439–nt EGFR fragment was amplified using a sense 5´–GGG AGC TGC CGT GTC AAA GA–3´ and antisense 5´–GGT GCC ACC CGC AGG TAC T–3´ primer pair. A 541–nt fragment of GAPDH was co–amplified as an internal control, using a sense 5´–TTA GCC CCC CTG GCC AAG G–3´ and antisense 5´–CTT ACT CCT TGG AGG CCA TG–3´ primer pair.

The parameters for the PCR included the following steps: initial denaturation at 94° C

for 2 min, 26 amplification cycles (denaturation at 94° C for 20 s, annealing at 60° C for HB–EGF, 56° C for PAR1 and 58° C for EGFR for 45 s, extension 72° C for 1 min), extension at 72° C for 7 min and storage at –20° C.

The PCR amplicons were analysed and stained on 1.5% agarose gel containing 0.002% ethidium bromide and the quantitative analysis of signals was performed with a fluorescence imaging system (Fujifilm LAS–1000). Intensities were integrated, corrected for background signals and evaluated in a TINA 2.09 software (Raytest).

4.2.4 Preparation of membrane fractions

VSM cells were washed with ice–cold PBS and scraped off into lysis buffer. The lysates were passed 6 times through a 26 gauge needle and centrifuged (12,000xg) for 20 min at 4° C. The pellet was resuspended in Laemmli buffer. The samples were boiled at 95° C for 5 min to denature the proteins and sonicated for 3 s at 30 W (Sonifier B–12 with microtip, Branson, Danbury, USA) to reduce the viscosity.

4.3 Immunochemical assays

4.3.1 SDS–PAGE and immunoblot

SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, is a commonly used method to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding, posttranslational modifications and other factors). The polyacrylamide gel is a crosslinking of acrylamide monomers with the use of catalysts. Once the gel has set, protein samples can be loaded into the gel and separated along an electric field.

Cells were lysed directly with lysis buffer (100 μl per 35–mm culture dish). The samples were boiled at 95° C for 5 min and sonicated for 3 s at 30 W. 10–25 μl of the samples were

loaded into wells in the stacking gel. SDS–PAGE was performed with 1.5 mm spacers in mini gel systems (Mini–protean Tetra Cell system, Bio–Rad, Munich) at a constant voltage of 200 V.

In order to make the proteins accessible to antibody detection, they were transferred from the gel onto a nitrocellulose membrane by tank–blotting for 120 min at a maximum voltage of 120 V and 100 mA per gel (Mini Trans–Blot Cell system, Bio–Rad, Munich).

To prevent interactions between the membrane and the antibody used for detection of the target protein and to avoid false positives, the nitrocellulose membranes were incubated for 1 h with blocking puffer. After 5 min washing in TBS–T, the membrane was incubated overnight with a dilute solution of primary antibody under gentle agitation at 4° C. Blots were subsequently washed 3 times with TBS–T for 5 min and incubated with a dilute solution of horseradish peroxidase–coupled second antibody for 60 min. Blots were washed thrice with TBS–T and detected using chemiluminescence. The western blots were incubated with a substrate that emits photons when converted by a photochemical reaction through the peroxidase enzyme activity of the secondary antibody. Light was detected by a CCD camera system (LAS–1000, Fujifilm, Raytest, Straubenhardt). Intensities were integrated, corrected for background signals and evaluated in TINA 2.09 software (Raytest).

4.4 Imaging techniques

4.4.1 Confocal microscopy

A confocal laser scanning microscope (cLSM) was used to assess the subcellular localization of HB–EGF. CLSM is a technique to obtaining high–resolution optical sectioning images. The key feature of confocal microscopy is its ability to produce in–focus images of thick samples. Because the images are acquired point–by–point and out of focus light is rejected

by a pinhole, the technique enables visualization deep within both living and fixed cells and tissues and affords the ability to collect sharply defined optical sections from which three–dimensional renderings can be created.

VSM cells were nucleofected with an expression plasmid encoding HB–EGF–YFP. One hour after nucleofection and seeding onto poly–L–lysine –coated glass coverslips, cells were treated with the modulators and incubated for another 4 h. Glass coverslips were mounted in a custom–made chamber and placed onto the stage of an inverted cLSM (LSM 510 Meta; Carl Zeiss). The cells were visualised using an a–Plan–Fluar 100x/1.45 oil immersion objective (Carl Zeiss). YFP was excited at 488 nm and detected through a 505–nm long pass filter. All experiments were performed at room temperature in HBS buffer.

4.4.2 TIRF microscopy

Total internal reflection fluorescence (TIRF) microscopy is an optical technique that allows the observation of thin optical sections (about 150 to 150 nm) at the bottom of cells. Using TIRF microscopy, fluorophores are excited through an evanescent wave that originates from the interface between two media with different diffractive indices (glass cover slip and media/cells). This phenomenon enables the study of cellular membrane activities, movements of cells, protein tracking and other processes.

After nucleofection with plasmid encoding HB–EGF–YFP, VSM cells were grown on glass coverslips (24 mm diameter) in 35–mm culture dishes until confluency. Prismless TIRFM was carried out with a system as described previously (Axelrod, 2003). For excitation of YFP, the 488–nm line of an Argon–ion–laser (Lasos GmbH, Ebersberg, Germany) was selected. Fluorescence light was detected using a 514–nm long pass filter (Razor Edge, Semrock, Rochester, NY). Image acquisition was performed with a back–illuminated EM–CCD camera (iXon DV887, Andor, Belfast, UK) in combination with TillVision software (TILL Photonics, Gräfelfing, Germany).