The Role of the SHB Adapter Protein in Cell Differentiation and Development

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Abstract

The present study was conducted in order to assess a role of the SH2 domain-containing adapter protein SHB in development and cell differentiation.

Embryonic stem (ES) cells overexpressing SHB and SHB with an inactive SH2 domain (R522K-SHB) were obtained. Microarray analysis in the SHB clone revealed altered expression of genes connected with neural cell function. The R522K-SHB clone exhibited altered expression of several transcription factors related to development. ES cells were differentiated by forming aggregates named embryoid bodies (EBs). The morphology of EBs was altered in the R522K-SHB clones, which showed fewer cavities. Expression of endodermal markers was decreased in the R522K-SHB EBs.

To further investigate the role of SHB in differentiation, murine ES cell lines deficient for one (SHB+/−) or both SHB alleles (SHB−/−) were generated. SHB deficient clones increased the expression of mesendodermal and endodermal markers and decreased expression of two receptors, VEGFR2 and FGFR1, connected with blood vessel differentiation. Similarly, blood vessels showed an altered morphology in SHB+/− and SHB−/− EBs after VEGF stimulation. SHB−/− ES cells also formed fewer blood colonies than control ES cells.

Finally, the role of the SHB adapter protein in vivo was analyzed by generating a SHB deficient mouse (SHB−/−). SHB−/− animals are viable, fertile, but suffer from leukopenia and anemia. SHB−/− animals demonstrate an abnormal morphology of blood vessels in the liver and kidney. Breeding of SHB+/− animals revealed an abnormal segregation of the mutant allele with an increased number of SHB+/− animals and a decreased number of SHB−/− and SHB+/+ animals. Backcross analysis of SHB−/− females with SHB+/+ males displayed an increased number of SHB−/− offspring already at the blastocyst level. Simultaneously, embryos from SHB−/− mothers show an increased malformation rate in comparison to embryos from SHB+/+ mothers.

In summary, the study suggests a role of SHB in reproduction and development and in mesodermal and endodermal specification.

Keywords: SHB, transgene, ES cells, cavitation, knockout, mouse, vasculogenesis, hematopoiesis, endoderm, mesoderm, malformation

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Aj dobrému kocúrovi niekedy myš utečie.
(slovenské písloví)

Even from the best tomcat a mouse can escape
(Slovak proverb)
Reports constituting the thesis

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


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Abbreviations

ES  Embryonic stem
LIF  Leukemia inhibitory factor
SH2  Src homology 2 domain
PTB  Phospho-tyrosine binding domain
JAK  Janus tyrosine kinase
FGF  Fibroblast growth factor
BMP  Bone morphogenic protein
VEGF  Vascular endothelial growth factor
EPO  Erythropoietin
IL-6  Interleukin-6
PDGF  Platelet-derived growth factor
TGF-β  Transforming growth factor-β
SHB  Src homology 2 protein of beta cells
SHB^+/+  Wild type
SHB^+/-  One SHB allele is inactivated
SHB^-/-  Both SHB alleles are inactivated
NO  Nitric oxide
EB  Embryoid body
shRNA  Short hairpin RNA
R522K-SHB  R522K-SHB mutant with inactive SH2 domain
GFP  Green fluorescent protein
LacZ  lacZ reporter gene encoding ß-galactosidase
DMEM  Dulbecco’s Modified Eagle Medium
IMDM  Iscove’s Modified Dulbecco’s Medium
FGFR  FGF receptor
VEGFR  VEGF receptor
PDGFR  PDGF receptor
STAT  Signal transducer and activator of transcription
PDX1  pancreatic duodenal homeobox transcription factor
Introduction

The murine genome contains more than 20 000 genes, a figure similar to that of the human genome, and the function of most of these is unknown. Analysis of cDNA shows that orthologous mouse and human coding exons are typically conserved at 85% (1). Studies employing manipulation of murine genes could suggest similar roles in the human. Introduction of a new gene or a genetic element (transgene) into the genome or inactivation of both endogenous copies of a specific gene (gene knockout) are two methods commonly used for such studies.

Embryonic stem (ES) cells are pluripotent cells and can contribute to any embryonic tissue. Moreover, they are suitable for genetic manipulations and enable the study of gene function in vivo and in vitro.

Extensive studies on the genetically manipulated organism or cell can produce ideas about gene performance and the relevance of its absence, mutation or over-abundance for disease.

In the present work I have used both transgene expression and gene knockout to evaluate the impact of the adapter protein SHB for cell differentiation and mouse development.
Background

ES cells
ES cells and pluripotency
ES cells present an important tool for performing genomic manipulation. ES cells are derived from the pluripotent cells of the inner cell mass of pre-implanted blastocysts (2; 3). When injected back into blastocysts they can contribute to both somatic and germline tissue (4). Moreover ES cells are suitable for targeting mutagenesis (5; 6) and creation of mutated mice.

Pluripotency of ES cells can be maintained by culture of these on mitotically inactivated feeder cells, which serve as matrix for ES adherence, in the presence of leukemia inhibitory factor (LIF). LIF belongs to the family of interleukin 6 (IL-6) type cytokines. LIF engages a heterodimeric receptor complex consisting of gp130 and LIF receptor α-chain. This complex activates the Janus family of tyrosine kinases (JAKs). STAT proteins, signal transducers and transcription activators, are key substrates for JAKs. STAT3 (7; 8) together with Src-family kinases (9) and myc are essential for self-renewal of ES cells.

Formation of embryoid bodies
Upon withdrawal of feeders and LIF, ES cells differentiate. Usually the methodology for ES cell differentiation involves the formation of spherical structures called embryoid bodies (EBs). There are several techniques, that make possible the generation of EBs. The most common is EB formation in suspension (10), by culture in hanging drops (11) or by cultivation in methyl-cellulose (12). Around day 2 of differentiation outer cells in EBs differentiate into extraembryonic endoderm which is later on specified into parietal and visceral endoderm. At the same time the process of forming the EB counterpart to the proamniotic cavity starts together with the specification of primitive ectoderm (13).

The process of proamniotic cavity formation involves extraembryonic endoderm and extracellular matrix together with the polarization of primitive ectoderm. Both fibroblast growth factor (FGF) and bone morphogenic protein (BMP) signaling are important for successful “cavitation” (14; 15) (Fig.1).
Primitive ectoderm gives rise to definitive endoderm, mesoderm and ectoderm. Different culture conditions can expand predominantly certain cell types.

Hematopoietic and vascular cell differentiation

One of the first events in embryogenesis subsequent to gastrulation is the formation of blood islands in the yolk sac. Blood islands contain primitive hematopoietic blood cells surrounded by endothelial cells. Both these lineages are thought to originate from a common precursor named the hemangioblast (17). Subsequent to primitive hematopoiesis, definitive hematopoiesis occurs.

EBs are used to study in vitro hematopoietic differentiation. These are subsequently dispersed and grown in semisolid media, methyl-cellulose. Several factors like BMP4, vascular endothelial growth factor (VEGF) and basic FGF have been found to be essential for hematopoietic differentiation (18; 19). First bipotential precursors for primitive hematopoiesis and endothelium are formed. From these the former precursors for the myeloid and erythroid lineages evolve.

In early protocols to study blood vessel differentiation in vitro EBs were grown in methyl-cellulose (20) but subsequently protocols utilizing attachment cultures on glass or plastic have been developed (17; 21). Blood vessels arise from the EB core and grow towards the periphery. Addition of angiogenic factors like erythropoietin (EPO), IL-6, FGF2, platelet-derived growth factor (PDGF) and VEGF2 significantly increase the formation of blood vessels-like structures in vitro (20; 22).

Neural differentiation

Several protocols have been described to study in vitro differentiation of ES cells into neural cells. In one of these, 4 day old EBs are grown in the presence of retinoic acid (23; 24). Some of the differentiated cells showed fea-
tures of glial cells and GABAergic neurons. More recent data confirm the importance of retinoic acid for differentiation of spinal motoneurons (25).

Another protocol is based on the fact that FGF2 promotes survival and proliferation of early neural precursors. EBs are grown here in serum free media in the presence of FGF2 supplemented with insulin, transferrin, selenium and fibronectin. Under these condition most of the cells die, but those that survive are at least 80% nestin positive neural precursor cells (26).

There is also a protocol in which ES cells are differentiated as single cells growing on feeders of mesodermal cells (27). That strategy is based on the fact that signals from mesoderm promote differentiation of ectoderm into neuroectoderm. More than 90% of the differentiated cells are under these conditions nestin positive. Moreover, these neural precursors seem to have the potential to differentiate into the full dorsal-ventral range of neuroectodermal derivates (28).

Attempts to recapitulate embryogenesis during ES cell differentiation by a sequential process including the formation of primitive ectoderm, neural plate and neural tube have been made (29).

**Differentiation of pancreatic β cells and hepatocytes**

In vivo, the pancreas and liver derive from duodenal endoderm. The liver cords and ventral pancreas arise from the ventral endoderm, whereas the dorsal pancreas arises from the dorsal foregut. Later on the ventral and dorsal pancreatic buds fuse and form one organ. Blocking hedgehog signaling plays an essential role for the formation of both liver and pancreas. Activins and FGF2 are candidates for mediating those blocking signals (30). Signals from cardiac mesoderm seem to be crucial for divergence between forming liver or ventral pancreatic bud. (31).

In vitro differentiation of ES cells into pancreatic β cells has so far not been successful (32-34). Pancreatic differentiation is a complex process, depending on temporally and spatially restricted interactions between endoderm and mesoderm. Recently some attention was paid to a multi-step differentiation process reproducing certain steps of early gut endoderm differentiation which is more in resemblance of the in vivo situation (35). Of crucial importance in this approach is to find markers that can distinguish definitive endoderm from extraembryonic endoderm (36).

During EB formation hepatocyte-like cells arise (37-39). The number of hepatocyte-like cells could be enriched by limited exposure to serum, addition growth factors, hormones and constitutive expression of hepatic transcription factors (36; 40; 41).
Differentiation of cardiomyocytes

After seven days of EB differentiation in an adhesive dish distinct beating areas of cardiomyocytes start to appear. Analyses of these beating areas have revealed that cardiomyocytes differentiated from EBs exhibit features of the early myocardium (42). Several proteins like transforming growth factor-β (TGF-β) (43), BMP2 (44), BMP antagonist noggin (45) and FGF2 (44) have been reported to increased the percentage of cardiomyocytes per EB. Other factors like nitric oxide (NO) (46) and retinoic acid (11) showed the same effect. Moreover, pre-transplantation treatment of ES cells with TGF-β increased the restored area after ischemic myocardial injury (47).

Creating mouse models

Reverse genetics represents a powerful technique drawing a direct line between a specific phenotype and a genetic modification. Different species can be used to study gene function, but small rodents especially mice, are among the most popular. The advantages of mouse usage are its small size, short reproductive cycle, high homology between mouse and human on protein level and ability to introduce a mutation into the murine genome. Several mouse models quite faithfully mimic human diseases (48-51). On the other hand there are some transgenic and knockout mice representing truly extreme phenotypes (52), which hardly could give proper information about the gene function in humans. There are also genetically manipulated mice with milder phenotypes than was predicted. The explanation for that can be selection of an inappropriate mouse strain (53), genome compensation (54) or the short life span in rodents.

Anyway, despite many drawbacks, creating of mouse models represents an irreplaceable method for genome analysis.

Transgene expression

ES cells or oocytes can be targets for genetic manipulation and gene silencing (55; 56). Transgene expression is the method where a transgene (often a novel gene, the dominant negative copy or the third wild type copy of the endogenous gene) is introduced into the cell and randomly integrated into the genome. Manipulated cells can give rise to transgenic animal (Fig. 2). The transgene can be under the control of a constitutive promoter which thus renders the transgene always active (57); under the control of tissue specific promoter which is active only in the specific tissue at a specific time (58) and under the control an inducible promoter where the activity depends on induction (59). The consequences of transgene expression enables us to specify the function of endogenous genes.
A transgene can be also short inverted repeats separated by a spacer of different length. Such repeats correspond to a specific mRNA and these are driven by RNA polymerase III promoter. As a result from transgene expression is a short hairpin RNA (shRNA), which can bind to the mRNA. mRNA is degraded by a cellular mechanism and thus protein expression is aborted (56; 60; 61).

Figure 2. Transgene expression (55), in vitro (A) and in vivo procedures (B) are shown.
Gene knockout

The knockout technique inactivates the endogenous copy of a specific gene. The targeting construct includes an unfunctional copy of the gene and a selective marker. The linearized targeting vector is introduced into ES cells by electroporation and the DNA construct is integrated into the chromosome. A small percentage of the integration occurs site-specific by homologous recombination; and the endogenous allele is replaced by the inactivated gene. ES cells carrying the inactive allele can be injected into blastocyst to produce chimera. If the chimera generates germline the mutation can be transmitted to the next generation (62) (Fig.3). Knockout constructs differ in their specificity considering time and place of their action. Constitutive knockout inactivates the gene in every tissue. In some cases this leads to early embryonic lethality (63).

More sophisticated methods employ tissue specific knockouts of the gene (64; 65). The specific enzyme (Cre recombinase) is able to excise (flox) the sequence between two direct repeats (loxp sites). The targeting vector contains loxp sites in intronic sequences before and after the exon of interest. The expression of the gene is not affected and normal animals will be born. In case the animal carrying loxp will be mated with an animal carrying the gene for the Cre recombinase, offspring will excise the exon in the cells that express Cre. When tissue specific promoters drive Cre gene expression, gene knockout will occur in a tissue specific manner. If the Cre recombinase is under the control of an inducible promoter the activity depends on the presence of inducer (repressor or activator) in the system (66).

A knockin enables the mapping of the actual gene activity in the tissue (67). A reporter gene (GFP, LacZ) is integrated in the same reading frame as the disturbed gene. All tissues, where the targeted gene is normally active, express the product of the reporter gene (tissues turn green or blue).
Figure 3. Constitutive gene knockout (55), homologous recombination screened by PCR, in vitro (A) and in vivo procedures (B) are shown.
The SHB adapter protein

The term “adapter proteins” stands for proteins involved in cell signaling that do not contain any intrinsic enzymatic activity. These proteins just contain specific motifs in their tertiary structure called binding domains, which enable protein-protein interactions.

The Src homology 2 (SH2) domain containing adapter protein (SHB) was originally identified as a serum-induced gene expressed in β-cells (68). SHB contains at least four different domains: N-terminal proline-rich motifs (69), central the phospho-tyrosine binding domain (PTB) (70; 71), the potential tyrosine phosphorylation sites (70; 72) and C-terminal SH2 domain (69). Based on homology of tyrosine phosphorylation sites and SH2 domain, SHB is a member of a family of adapter proteins. The protein family is composed at least of five members: SHB, SHD, SHE, SHF, SHG (68; 73-75).

The SHB SH2 domain was found to interact with several receptors like the VEGF receptor 2 (VEGFR-2) (76), the FGFR receptor 1 (FGFR1) (69; 77), PDGF receptor (PDGFR) (69) and T-cell receptor (78; 79). Other SHB domains interact with different cytosolic proteins and transduce thus the signal downstream from the receptor. Mutation in SHB SH2 domain (SHB-R522K) confers SH2 inactivation and abolished interaction with receptors (Fig. 4).

Previous studies uncovered the role of SHB in quite a few biological processes. SHB is involved in apoptosis of several cell types (80-82), the SHB protein was also found to participate in T-cell signaling after CD3 and IL-2 stimulation (78; 79) and to be involved in blood vessel formation after FGF stimulation (83). Recently a role of SHB for blood vessel formation in differentiating EBs was described (22).

In the present work we have analyzed the role of SHB in cell differentiation and development. Both transgene expression and knockout models were used in this context.
Figure 4. Structure of the SHB adapter protein
Specific aims

To create ES cell lines overexpressing SHB or the R522K-SHB transgene and to monitor their gene expression profile and evaluate their potential for differentiation.

To create a knockout ES cell line for the SHB adapter protein and determine the role of SHB for differentiation of endoderm and mesoderm.

To create a conditional knockout mouse for the SHB adapter protein and assess the SHB function in vivo.
Methodology

Genomic manipulation

Gene targeting (II,III)
The ES cells line GSI-1/129SvJ was inactivated at both (SHB\(^{+/−}\)) or one (SHB\(^{+/-}\)) of the SHB alleles. These were inactivated in ES cells in vitro in two subsequent steps. The first allele was inactivated by deletion of the first exon, the second allele was inactivated by insertion of neomycin resistance gene in the 1\(^{st}\) exon. When knockout mouse was generated, only one allele was targeted, germline chimera were created and homozygotes for deletion of the 1\(^{st}\) exon were produced.

Transgene expression (I)
The ES cells line R1/129Sv containing a PDX1-lacZ knockin were stably transfected with wild type SHB cDNA (SHB), with SHB cDNA carrying an inactive SH2 domain (R522K-SHB, R522K-SHB-2) or with empty vector (C, C2). The SHB inserts were in the pCAGGS expression vector and selected for hygromycin resistance.

Cell lines and culture condition
ES cells were cultured in the presence of murine embryonic fibroblasts in in Dulbecco’s Modified Eagle’s Medium (DMEM)/glutamax (Invitrogen), penicillin/streptomycin, 15% heat-inactivated fetal bovine serum (Invitrogen), 1x non essential amino acid (Invitrogen), sodium pyruvate (Invitrogen), monothioglycerol (Sigma) and LIF (Chemicon International). Cells were grown at 37\(^{°}\)C in 5% CO\(_2\).

EB differentiation

EB formation in suspension (I)
For ES cell differentiation, fibroblasts and LIF were omitted. ES cells were trypsinized (5 min, 37\(^{°}\)C) and kept in suspension in Petri dishes (Day 0) at a concentration of 1-1.25\(\times\)10\(^5\) cells and further maintained as EBs incubated
for 4 or 20 days. Medium was changed every second day. In some experiments Matrigel was added at 4% (vol/vol) on Day 0.

**EB formation by hanging drops (II)**

For ES cell differentiation, fibroblasts and LIF was omitted. ES cells were trypsinized (5 min., 37°C) and aggregated by the hanging drop technique at 1200 cells/20µl for 2 days. EBs were then grown in suspension one more day and then attached on tissue culture dishes. EBs were harvested at 8, 12 and 21 days after the initiation of differentiation.

**Animals (III)**

All the animal handling was approved by the Uppsala animal ethics committee. The weight of the mice was measured from the second week after birth when the mice were ear marked. Mice were weaned at three weeks after birth. Three different wild type strains (mix between FVB/N, C57BL/6 and 129/SvJ or C57BL/6 or CBA) were used for testing SHB⁺ and SHB⁻ maternal allele ratio in the offspring.

For blastocysts analysis, C57BL/6 SHB⁺/+ males were mated with SHB⁺/⁻ females. Females were sacrificed 3 days after appearance of vaginal plugs (3,5dpc). The genital tract was removed and put into a dish with PBS. All blastocysts were washed out from the uterus by PBS into the Petri dish. Blastocysts were transferred into a new Petri dish with PBS and put into PCR tubes, one blastocyst per tube. Blastocysts were scored for size prior to PCR. PBS from the dish was used as a negative control for PCR. PCR tubes were spun down and supernatants were removed. The same procedure was performed for analysis of oocytes.

For blood analysis, seven week old animals were bled retroorbitally (84). Absolute number of thrombocytes, erythrocytes and leukocytes were counted in a Coulter counter. Relative numbers of different white blood cells were counted under the microscope.

For embryonic analysis, pregnant females were sacrificed at 10.5dpc. Embryos were subjected to basic morphological examination (the position in the uterus, the crown-rump length and the somite number and the presence of heart beat). The number of corpora lutea and the number of resorptions were indicated for each litter. One limb, yolk sac or all embryonic tissue (in case of resorptions) was used for genotyping by PCR or by Southern blot.
Microarray analysis (I)
ES cells from clones SHB, R522K SHB-1 and control (C) were collected for mRNA preparation (RNeasy kit and Oligotex kit). 1μg high-quality poly(A)+ RNA was used for cDNA synthesis using an oligo (dT) primer with the T7 RNA polymerase promoter sequence incorporated followed by first and second – strand cDNA synthesis using standard procedures. The biotinylated RNA probes were then transcribed using T7 RNA polymerase. The yield of this reaction was approximately 50μg biotinylated RNA for hybridization. The generated probes were then hybridized to the Affymetrix U74A chips. The A chip was composed of spotted sequences that at that time were derived from 6500 known murine cDNA and 6500 ESTs. The intensity of the probes binding to the spots was assessed by scanning and computerized storage. The files containing the comparisons were then analyzed with Resolver software or Affyware and differences comparing SHB or SHB-R522K-1 with the control ES clone were determined. Fold changes and significance values after comparison with internal controls were calculated using Resolver software.

RT-PCR analysis (I,II)
RNA was isolated using RNAeasy mini kit (Qiagen) or the Direct mRNA Micro Kit (Qiagen), according to the manufacturer’s instructions. For semi-quantitative RT-PCR, the One Step RT-PCR Kit (Qiagen) was used. Real time RT-PCR was done in one step reaction using SYBR® Green RT-PCR Kit (Qiagen) or alternatively by a two step reaction. M-MuLV Reverse Transcriptase (Finnzymes) converted 1μg of total RNA into cDNA. For PCR reactions SYBR® Green Taq Ready Mix™ (Sigma) was used. The PCR reactions were run on a Light Cycler (Roche). Transcription levels were then normalized against β-actin or glyceraldehyde 3-phosphate dehydrogenase. Specific pairs of RT-PCR primers were designed by program: http://www.broad.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi. Each primer was also tested for presence of palindroms and hairpin loops: http://www.cybergene.se.

Histology
General histology was performed on tissue that had been fixed in 10 % formaldehyde or 4 % paraformaldehyde overnight. Tissue was embedded in paraffin before hematoxylin-eosin staining. Tissue for cryosectioning was quickly frozen in dry ice cooled 2-isopentane, 5μm sections were immunostained with primary antibody, rat
anti-mouse CD31 (BD Bioscience), washed with TBS-tween and incubated with biotinylated secondary antibody goat anti-rat (Vector Laboratories Inc.), and again washed with TBS-tween. Tissue was incubated with strepavidin-HRP (Perkin Elmer), washed with TBS-tween and H₂O and the chromogen substance was added (AEC-kit, rat (Vector Laboratories Inc). Hematoxylin-eosin staining was used as a background staining.

For electron microscopy pieces of tissue were fixed in 2 % (vol/vol) glutaraldehyde and 1 % (w/vol) paraformaldehyde in PBS, and embedded in Agar 100. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Electron microscopy was carried out with a Hitachi H-7100 transmission electron microscope at an accelerating voltage of 75 kV.

For immunohistochemical staining, EBs grown on glass chambers were washed with Tris-buffered saline (TBS) and fixed with Zincfix over night. Next day, EBs were washed with TBS and treated with H₂O₂ in methanol to block endogenous peroxidases. EBs were washed with TBS and unspecific interactions were blocked. The tissue was incubated with primary antibody, rat anti-mouse CD31 or CD41 (BD Bioscience), washed with TBS-tween and incubated with biotin or immunofluorescence labeled secondary antibody and again washed with TBS-tween. EBs were incubated with strepavidin-HRP (Perkin Elmer), washed with TBS-tween and H₂O and the chromogen substance was added (AEC-kit, rat (Vector Laboratories Inc).

In vitro Hematopoietic differentiation

ES cells were differentiated according to a modified protocol from Stem Cell Technologies Inc. Two days prior to differentiation, ES cells were split and cultured in IMDM ES media: Iscove’s Modified Dulbecco’s Medium /L-glutamine/HEPES (Invitrogen), penicillin/streptomycin, 15% heat-inactivated fetal bovine serum (Invitrogen), 1x non essential amino acids (Invitrogen), sodium pyruvate (Invitrogen), monothioglycerol (Sigma), recombinant leukemia inhibitory factor (LIF; Chemicon International). ES cells were trypsinized when reached 25-50% confluence and transferred to Petri dish in IMDM differentiation media. Cells were grown at 37°C, 5% CO₂ for 7 days. Next, media containing EBs were collected in Falcon tubes and allowed to settle. Supernatants were removed and EBs were trypsinized and dispersed several times by needle. ES cells were counted and mixed by vortexing with methylcellulose medium (MethoCult®, StemCell Technologies Inc) and plated by a syringe. Cell colonies were scored after 7-10 days.
Statistical analysis

Statistical analysis was performed using PractiStat software. The weight of the animals and the distribution of blood cells were evaluated by 2-tailed Student’s t-test. Distribution of maternal and paternal allele and the malformation rate of the embryos were evaluated by the chi-square test.
Results

Paper I

R522K-SHB ES cells exhibit an altered gene expression profile

Microarray analysis of R522K-SHB revealed 128 changes of which 107 were decreased gene expression. Notably, the expression of several transcription factors involved in development were decreased (Sox17, GLI2, Retinoic acid receptor, GBX2, Kruppel-like factor 7). Expression levels of several signaling and extracellular proteins, such as laminin α 1, collagen IV, Shc, lysophospholipase, phosphatidylinositol transfer protein β were decreased. Five genes (GLI2, SOX17, Kruppel-like factor 7, collagen type IV and laminin α 1) from both groups were tested by RT-PCR (clone R522K SHB-1) and real time RT-PCR (clone R522K-SHB-2). All genes but GLI2 were decreased, validating the major part of the microarray data.

SHB transfection alters the expression of genes involved in neural function and early development

Microarray analysis of wild-type SHB caused 16 changes in gene expression. From these 5 genes (G-utrophin, plated-activating factor acetylhydrolase, lysine-ketoglutarate reductase, PEG1/MEST and Twist gene) were tested by RT-PCR. All of them except the Twist gene revealed the same expression pattern as in the microarray. Many of the SHB-induced changes in gene expression are related to neural function, two genes belong to the two cells stage gene family and two genes are connected to mesodermal development.

Impaired cavity formation in R522K-SHB and R522K-SHB-2 EBs

Subsequently ES cells were differentiated into EBs. When the morphology was inspected on 4 day old EBs, both control and SHB clones developed cystic cavities (42 and 59% respectively). The proamniotic cavity is smooth and regular and covered with at least one layer of ectodermal cells. The cavitation almost completely failed in the R522K-SHB clone. The R522K-SHB-2 clone exhibited fewer (20%) regular cavities than the control clone. The cavitation in clone R522K SHB was restored by adding 4 % Matrigel at day 0 of EBs formation.
Downregulation of endodermal markers in R522K-SHB and R522K-SHB-2

EBs were differentiated further in suspension culture and gene expression was assessed at day 20. The ectodermal marker heavy neurofilament, the mesodermal marker (cardiac actin and β-globin) and the endodermal markers albumin and amylase were tested. Overexpression of R522K-SHB negatively affected expression of albumin and amylase in comparison to control or SHB cells in both R522K-SHB clones. The impaired differentiation into cells expressing these genes suggests that SHB is required for differentiation in these pathways since R522K-SHB may function in a dominant-negative fashion (71; 77; 79). Expression of β-globin was also decreased in R522K-SHB (data not shown). Expression of other markers was not altered.

Addition of 4% Matrigel at day 0 of EB formation does not restore gene expression of albumin at day 20. These data suggest that SHB is not only of importance for the production of extracellular matrix components at the first days of development but also at later stages of development independently of the cavitation process.

It was previously reported that a transgenic mouse expressing SHB under the insulin promoter had an increased β-cell mass. For this reason we studied the expression of endocrine pancreatic markers (insulin, glucagon and PDX1) in 20 day old EBs. Expression of all three genes was decreased in both R522K-SHB clones in comparison to control. We conclude that SHB is important for appropriate expression of these endocrine markers in differentiating EBs. Indeed, a recent publication shows that SHB overexpression promotes PDX-1 and insulin gene expression in differentiation EBs. This effect could relate to VEGF signaling.

Paper II

Targeted inactivation of the SHB gene

The correct targeting of the first allele occurred only in one clone out of 408. This clone contained loxP sites flanking both sides of the 1st exon. Subsequent treatment with Cre recombinase floxed the 1st exon.

The inactivation of the second allele occurred in 1 clone out of 480. As SHB+/− cells two sister clones with random insertion of the targeting construct were chosen. The mutation resulted in loss of SHB protein expression.

Increased expression of mesendodermal markers in SHB−/− EBs

Our previous results from the paper I suggested a role of SHB in endodermal differentiation. Moreover SHB was found to be involved in vascular formation (22). We wanted to analyze markers for early endoderm and mesoderm
after 12 days of differentiation. The markers studied were Cxcr4, Sox17, E-cadherin, Brachyury, Mix1 and m-Twist.

In differentiating mouse ES cells, Cxcr4 is expressed in definite endoderm and mesoderm (85), Sox17 in visceral and definite endoderm (86), E-cadherin in visceral and definite endoderm and mesendoderm (85), whereas both Brachyury and Mix1 are expressed in mesoderm, mesendoderm and endoderm (85). Twist is expressed in mesoderm (87). Cxcr4 and Brachyury showed increased expression in SHB−/− clone in comparison to SHB+/- clone (P<0.05 respectively P<0.001). In case of Cxcr4, the SHB +/- clone also displayed increased expression. All other genes exhibited a similar pattern of expression, although the effects failed to reach statistical significance.

Expression of hematopoietic and vascular markers in SHB−/− EBs

Our next goal was to test if mRNA levels of markers for hematopoietic and vascular differentiation were altered in the SHB−/− clone. The markers studied were VEGFR-2, FGFR-1, Tal1, CD41, β-globin and CD45.

VEGFR-2 has been shown to be essential for hematopoietic and vascular development (88). The RNA content of VEGFR-2 in SHB−/− and SHB+/- EBs after 12 days of differentiation was decreased to 75% and 55% respectively, of the RNA level in the corresponding SHB+/- control (P<0.01).

FGFR-1 is a receptor with a role in hematopoietic and vascular differentiation (89). The SHB−/− clone exhibited decreased FGFR-1 expression at day 12 of EB differentiation (P<0.01).

CD41 is commonly expressed in cells appointed to differentiation to hematopoietic cells (90). On the mRNA level, CD41 was increased in SHB−/− clone (P<0.05). The SHB−/− clone exhibited a similar, non-significant tendency. The increased expression of CD41 was confirmed on the protein level by immunofluorescence microscopy, when EBs were grown as an adherent cell culture. CD41 positive cells (CD41+) were stained more intensively in SHB−/− and SHB+/- clones than in the corresponding SHB+/- control cells. Moreover CD41+ cells derived from SHB−/− and SHB+/- EBs made clusters of cells staining strongly for CD41+ whereas those from SHB+/- EBs exhibited weaker staining and were more equally distributed. This suggests that CD41+ cells derived from the SHB−/− and SHB+/- clones represent a different cell type than the CD41+ cells derived from the SHB+/- clone.

Tal-1, another marker for hematopoietic cells precursors, was also upregulated in the clones deficient for SHB expression, although the variability was too large for the effect to reach statistical significance.

The expression of β-globin and CD45 were not affected at any time point.
Impaired blood vessel formation in SHB\(^{-/-}\) EBs

EBs were grown as adherent cell cultures to study blood vessel formation. At the basal conditions EBs from SHB\(^{-/-}\) and SHB\(^{+/-}\) clones did not show any different CD31-staining pattern compared with the SHB\(^{+/+}\) clone. However after VEGF-A stimulation when typical peripheral vascular plexa develop, both the SHB\(^{-/-}\) and SHB\(^{+/-}\) clones demonstrated decreased capillary density in these plexa compared with the SHB\(^{+/+}\) clone.

SHB\(^{-/-}\) ES cells are impaired in hematopoietic colony forming assay

ES cells from the SHB\(^{+/-}\), SHB\(^{+/-}\) and SHB\(^{-/-}\) formed EBs and these were tested for their capacity to differentiate into blood cells. Blood colonies were scored after day 7 to 11 of differentiation in methyl-cellulose. SHB\(^{-/-}\) ES cells displayed a significantly decreased capacity to form blood colonies in comparison to the SHB\(^{+/+}\) clone (P<0.001). The SHB\(^{+/-}\) ES cells also exhibited a decreased potential to form blood colonies.

Gene expression of endodermal markers at 21 days

SHB deficient cells showed altered expression after 12 days of differentiation of FGFR-1 mRNA, which is involved in endodermal differentiation and patterning. Moreover, the early endodermal markers Cxcr4 and Sox17 were increased in the SHB\(^{-/-}\) and SHB\(^{+/-}\) EBs at same time point. To test if endodermal differentiation is affected also at a later time point, we have analyzed the expression of several endodermal markers at 21 days of differentiation. Gene expression of amylase, α-fetoprotein (AFP), hepatocyte nuclear factor 1α (HNF1α) and pancreatic duodenal homeobox factor-1 (PDX-1) were tested. Expression of amylase, AFP and HNF1α was significantly increased on day 21 of differentiation in clone SHB\(^{+/-}\) in comparison to control clone SHB\(^{+/+}\). The trend to increase expression of endodermal markers was obvious also in the SHB\(^{-/-}\) EBs, but the effects were smaller and less consistent.

Paper III

Generation of knockout mice

Correctly targeted ES cells, containing loxP sites flanking the 1\(^{st}\) exon, were injected into blastocysts. After blastocyst implantation two germline chimeric males were born. To delete the first exon, one SHB\(^{loxP/loxP}\) male from the second generation of offspring was mated with a female expressing Cre recombinase under the control of the β-actin promoter. Eight animals were born. Six of them (3 males and 3 females) had the 1\(^{st}\) exon completely floxed in one allele (SHB\(^{+/-}\)).
Abnormal segregation ratio of SHB\(^{-}\) allele

When SHB\(^{+/-}\) animals were mated, the offspring demonstrated an abnormal segregation ratio of the knockout allele. Both SHB\(^{+/+}\) and SHB\(^{-/-}\) animals were born at a lower frequency than expected from Mendelian genetics. There were 16\% of SHB\(^{+/-}\) and 16\% of SHB\(^{-/-}\) animals instead of 25\% each. A decreased number of SHB\(^{-/-}\) animals could be explained by a higher embryonic mortality of the knockout animals, whereas the explanation to the relative loss of SHB\(^{+/-}\) animals is less apparent.

We wanted to test the hypotheses that loss of the SHB allele (SHB\(^{-}\)) presents an advantage for blastocyst formation, survival or implantation in comparison to the wild type allele (SHB\(^{+}\)). We have mated SHB\(^{+/-}\) and SHB\(^{+/-}\) or SHB\(^{+/-}\) and SHB\(^{-/-}\) in both sex combinations. The genotyping of pups or embryos revealed that animals possessing the maternal SHB\(^{-}\) allele are more common than those possessing the maternal wild type allele. On the other hand, the frequency of newborns possessing the SHB\(^{+}\) or SHB\(^{-}\) paternal allele was similar.

By PCR analysis we have demonstrated that the unequal distribution of the maternal SHB\(^{+}\) and SHB\(^{-}\) allele is already present at the newly ovulated oocyte and blastocyst level.

SHB\(^{-}\) animals are leukopenic and anemic

SHB is involved in T-cell signaling (79) and ES cell differentiation to hematopoietic cells in vitro ((22) and Paper II). Our intention was to evaluate the impact of SHB on blood cell differentiation in vivo.

Analysis of peripheral blood revealed that the number of leukocytes in SHB\(^{-/-}\) animals dropped to 57\% of that in SHB\(^{+/-}\) and SHB\(^{++}\) animals (P<0.001). The volume fraction of erythrocytes in SHB\(^{-/-}\) animals was 94\% of that of the control animals (P<0.05).

Differential counting of white blood cells revealed a non-significant trend towards a decreased number of monocytes and eosinophils in the SHB\(^{-/-}\) animals.

SHB\(^{-/-}\) and SHB\(^{+/-}\) embryos exhibit an increased malformation rate

Our hypothesis was that SHB\(^{-/-}\) animals have an increased malformation rate, which would explain the decreased number of SHB\(^{-/-}\) animals per litter. We mated SHB\(^{+/-}\) males against SHB\(^{+/-}\) females, or SHB\(^{+/-}\) and SHB\(^{-/-}\) males again SHB\(^{+/-}\) females. Females were sacrificed 10.5 dpc. and embryos were analyzed. We detected an increased malformation rate in both the SHB\(^{-/-}\) and SHB\(^{-/-}\) embryos in comparison to wild type embryos. Resorptions, gross malformations with loss of embryo structure, neural tube defects, tail rotation defects, malrotation and hemorrhages were observed. The total number
of malformations was 34% in embryos deficient for the SHB maternal allele, compared with 10% of malformations occurring in SHB^{+/+} embryos (P<0.01).

**Altered vascular morphology in SHB^{−/−} animals**

There is evidence from our previous studies that the SHB protein is involved in blood vessel formation ([22; 83] and Paper II). We have stained sections of frozen tissue from different organs with the antibody against the CD31 glycoprotein, an endothelial cell marker.

Interestingly, blood vessels in SHB^{−/−} animals exhibited different morphology than SHB^{+/+} animals. While liver from SHB^{+/+} animals exhibited diffuse staining following the discontinuous sinusoid capillary, the staining in the liver tissue from SHB^{−/−} animals displayed a much stronger and more distinct staining pattern resembling classical capillaries rather than sinusoidal structures. The altered morphology was apparent also using electron microscopy, commonly showing liver capillaries with continuous and thickened endothelial cytoplasm completely surrounding a red blood cell. Liver capillaries from SHB^{+/+} animals are made from discontinuous endothelium having thin cytoplasm.

Kidney capillaries in SHB^{−/−} animals also exhibited a different morphology than those of controls. The glomeruli were compact in SHB^{−/−} kidneys with stronger CD31 staining. Electron microscopy revealed the presence of fenestrations in both SHB^{+/+} and SHB^{−/−} glomerular endothelium. However, the SHB^{−/−} glomerular endothelium showed hypertrophic cytoplasm with an increased thickness.
Discussion

The present study investigates the role of the SHB adapter protein in cell differentiation and development using transgenic and knockout approaches.

In the transgenic study, ES cells overexpressing wild-type SHB or SHB containing an SH2 domain mutation that renders it inactive were employed. Microarray analysis of ES cells overexpressing wild-type SHB revealed changes in expression of genes related to early development and neural function. In this context it may be of importance that embryos deficient for the SHB adapter protein sometimes carry neural tube defects.

The undifferentiated R522K-SHB ES cells exhibited numerous changes with respect to ES cell characteristics. More than 100 genes exhibited changed expression levels and most of these changes were down-regulation. Among the genes that were expressed at lower levels in the R522K-SHB cells were those corresponding to the transcription factors (retinoic acid receptors, GLI2, Kruppel-like factor 7, and SOX17) and extracellular matrix proteins (laminin α 1 and collagen IV). The altered gene expression pattern has an immediate impact on the differentiation of the ES cells; the early cavitation is impaired and the epithelial lining morphologically altered. The process of EB cavitation has been shown to depend on FGF (15; 91; 92) and BMP (14) signaling. Moreover, cavitation in FGFR-2 deficient EBs is connected with the absence of laminin α 1 and collagen IV which could be restored by the addition of Matrigel, containing components of basement membrane (91). Similarly cavitation was restored in R522K-SHB EBs by Matrigel addition. Although we have shown that SHB binds to FGFR-1, an interaction between SHB and FGFR-2 has not been tested. However, there is probably a connection between impaired cavitation in R522K-SHB and FGF signaling.

R522K-SHB EBs show severe defects in the expression of the endodermal markers albumin, amylase, PDX1, insulin and glucagon after 21 days of differentiation. In this context it is interesting that Sox17, an endodermal transcription factor is expressed at a lower level in undifferentiated R522K-SHB ES cells.

The inactivation of the 1st exon in the SHB gene causes loss of SHB protein expression. Since SHB in previous studies has been shown to influence both mesodermal and endodermal specification in EBs, we focused on the study of differentiation towards these two lineages. Several markers (Cxcr4,
Brachyury, Sox17, E-cadherin, Twist, Mix1) for mesendodermal specification were studied. Two of them, Brachyury and Cxcr4, showed significantly increased expression in SHB−/− EBs after 8 and 12 days of differentiation. Other mesendodermal markers showed the same tendency, but the effects were not significant. Subsequently four endodermal markers (amylase, AFP, PDX1 and HNF1α) were studied. All but PDX1 were significantly increased in the SHB+/− clone and the SHB−/− clone exhibited the same tendency. These results are contradictory to the expression profile of R522K-SHB EBs, where all the endodermal markers tested were down regulated (Paper I).

SHB is known to be involved in blood vessel differentiation. The RNA contents of two receptor, VEGFR-2 (93) and FGFR-1 (89), involved in blood vessel differentiation was analyzed. Both SHB+/− and SHB−/+ EBs showed reduce expression of VEGFR-2 after 12 days of differentiation. At the same time point, the RNA content of FGFR-1 was reduced in the SHB−/− EBs. On the other hand when examining the SHB+/− and SHB−/+ EBs for their ability to form blood vessels, it was noted that both clones were able to form blood vessels under basal conditions. These results are different from those of the R522K-SHB EBs (22). Both SHB+/− and SHB−/− EBs formed less developed vascular plexa after VEGF stimulation. SHB plays a role in the differentiation endothelial IBE cells and is involved in VEGF-induced migration of porcine endothelial cells (76; 83). The combined data suggest impaired signaling downstream of VEGFR-2 in SHB deficient cells during blood vessel formation.

The RNA content of two markers for hematopoietic progenitors, CD41 and Tall, was increased in SHB−/− EBs. An increased mRNA content of CD41 in SHB−/− EBs would suggest a higher potential of SHB deficient ES cells to differentiate into blood cells. However, this notion was not confirmed when performing a colony formation assay on these cells. This discrepancy could be explained by existence two populations of CD41 positive (CD41+) cells, CD41 bright and CD41 dim (94). It is possible that SHB−/− promotes primarily the expansion of the CD41+ “bright” population of cells that fails to differentiate into hematopoietic progenitors.

The differentiation potential of SHB−/− cells suggests a role of SHB in mesodermal specification. The expression profile of different mesendodermal specification genes suggests, that SHB promotes the differentiation to both mesoderm and endoderm. Alternatively, SHB could have role in the correct tuning in subsequent specification and patterning of these two lineages.

Certain aspects of the SHB−/− phenotype resemble those of FGFR-1 deficient mice (95). FGFR-1 deficient cells contribute to both mesodermal and endodermal tissue, but with poor migration and inappropriate patterning. FGFR−/− cells were occasionally observed in blood islands, but only situated close to the posterior midline. Similarly, SHB deficient ES cells contribute to a lower extent to hematopoiesis than control cells. In addition, signals origi-
nating from visceral endoderm, such as Ihh are important for blood island formation (96) Aberrant expression of mesendodermal and endodermal markers in SHB deficient cells could have a consequence for blood island formation.

The action of R522K-SHB differs according to the cell type or signaling pathway in which it operates. R522K-SHB acts in a dominant negative manner in T cell receptor signaling and in FGF-dependent formation of blood vessels (22; 79; 83). During stress fiber formation after PDGF stimulation R522K-SHB operates in a silent manner (97). In EB differentiation R522K-SHB could either exert dominant-negative effects or cause a completely novel response independently of blocking receptor signaling (Paper I). R522K-SHB may behave differently in different systems depending on whether the signaling process involves the SH2 domain or involves only other SHB domains. In the latter case, overexpression of R522K-SHB may amplify other non-receptor events, causing a completely novel response. If the signaling pathway involves the SH2 domain, the R522K mutation could be either silent, allowing endogenously expressed SHB to function, or dominantly negative if the response requires an assembly of components that fails due to inclusion of the SHB mutant in this complex.

In general the R522K-SHB ES cells exhibited a more prominent phenotype than the SHB+/− ES cells. This could have several explanations: 1) R522K-SHB competes not only with the SHB protein but also with other members of this protein family (SHD, SHE, SHF, SHG) blocking their action. 2) In the SHB−/− cells, these other members may compensate for certain SHB functions. 3) R522K-SHB interferes with or activates another pathway, in which the wt SHB protein is not involved.

The in vivo knockout study revealed no major abnormality. However more scrutinious analysis revealed several differences from normality. The most obvious difference was recorded in the number of SHB+/+ and SHB−/− offspring in the first filial (F1) generation from SHB+/− parents. These deviated significantly from the expected number of 25% each. One possible explanation can be found in intrauterine embryonic death. Accordingly, the SHB+/− or SHB−/− embryos showed increased rates of resorptions and malformations compared with wild type embryos. We were not able to determine the reason for the elevated malformation rate in SHB+/− and SHB−/− embryos. It could be due to the maternal SHB− allele in the embryo, the maternal SHB− allele in the mother or a combination of both factors.

To explain the relative decrease in the number of SHB−/+ animals per litter we have mated SHB−/+ males with SHB+/− females. Analysis of newborns and embryos revealed that the maternal SHB− allele is present nearly twice as often as the maternal SHB+ allele. This increased ratio was observed at the blastocyst and ovulated oocyte stages. It could be explained by a non-
random distribution of each allele between 1st polar body and oocytes during meiosis or some advantage for SHB- oocytes to ovulate preferentially.

Blood analysis of SHB−/− animals revealed these to be mildly anemic. The finding is in the line with our experiments in vitro in which SHB−/− deficient ES cells were less efficient in differentiating to blood cells in vitro (Paper II). SHB interacts with VEGFR-2 and so the SHB deficiency can have a negative impact on VEGFR-2 signaling at the hemangioblast level, at which it is important for blood cell differentiation (88). Consequently, other blood cell types should be reduced if the effect of SHB resides at the hematopoietic stem cell level. We observed leukopenia but not thrombocytopenia making the mode of action of SHB in this context uncertain.

We could see increased thickness of endothelial cytoplasm in liver and kidney. Similarly, SHB deficient EBs exhibited impaired formation of blood vessels after VEGF stimulation in vitro. This may suggest a role of SHB in blood vessel formation after VEGF stimulation also in vivo.

Interestingly, kidney, thymus and spleen are organs which lack expression of other SHB family proteins. Thus, glomeruli formation and hematopoiesis could not be rescued by the action of the SHD, SHE, SHF and SHG proteins. The role of the SHB adapter protein is summarized in Fig. 5.

Figure 5. Possible involvement of SHB at multiple stages of development. Absence of SHB does not affect development of early mesoderm, but SHB is involved (SHB+) in hemangioblast differentiation and probably also functions at later stages in blood vessel and blood differentiation. When considering endoderm differentiation, SHB could have a blocking role (SHB ┴) for the expression of early endodermal markers. The role of SHB in pancreatic and liver development is presently not clear (SHB?) since the observed in vitro effects could be secondary to the effects on early endoderm, or direct effects at these later stages. In addition, the effect in vivo of the SHB knockout has not been elucidated in these organs.
Conclusions

R522K-SHB ES cells exhibit an altered gene expression profile

The R522K-SHB transgene impairs cavitation in EBs

The R522K-SHB transgene exerts a negative influence on expression of endodermal markers

SHB is involved in mesodermal and endodermal specification

SHB is involved in in vitro and in vivo hematopoiesis

SHB is involved in blood vessel formation

SHB deficiency disrupts equal distribution of maternal alleles in offspring

SHB deficiency is connected with an increased malformation rate among offspring
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