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„Analysis of the role of β-Catenin in Bcr/Abl + CML and B-ALL“

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*...meiner Familie und*

*meinen Freunden gewidmet...*

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## *List of Abbreviations*

| | |
|---|---|
| ABL | abelson kinase |
| AML | Acute Myelogenous Leukemia |
| APC | Adenomatous Polyposis Coli |
| B-ALL | B-cell acute lymphocytic leukemia |
| BCR | breakpoint cluster region |
| BSA | bovine serum albumin |
| CBP | CREB-binding Protein |
| CLL | Chronic Lymphocytic Leukemia |
| CML | chronic myelogenous leukemia |
| DMEM | Dulbecco's Modified Eagle Medium |
| Dsh | Dishevelled |
| EDTA | ethylendiamin tetraacetic acid |
| EGTA | ethylene glycol tetraacetic acid |
| FACS | fluorescence activated cell sorting |
| FCS | fetal calf serum |
| FL | Fetal Liver |
| GFP | Green Fluorescent Protein |
| GSK3β | Glycogen Synthase Kinase 3 β |
| HRP | horseradish peroxidase |

| | |
|---|---|
| HSC | Hematopoietic Stem Cell |
| IL | Interleukine |
| IL7Rα | Interleukine 7 receptor alpha |
| IRES | Internal Ribosomal Entry Site |
| JAK | Janus Kinase |
| LB medium | Luria broth |
| LEF | Lymphocyte Enhancer Factor |
| LRP | Low Density Lipoprotein Receptor-Related Protein |
| MAPK | Mitogen-Activated Protein Kinase |
| NTP | Nucleotidetriphosphate |
| OD | Optical Density |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PI | Propidium Iodide |
| PI3K | Phosphoinositol-3-Kinase |
| PKA | Protein Kinase A |
| PKB | Protein Kinase B |
| pMSCV | Plasmidal Murine Stem Cell Virus |
| RIPA buffer | RadioImmunoPrecipitation Assay buffer |

| | |
|---|---|
| RPMI medium | Roswell Park Memorial Institute medium |
| RTK | Receptor Tyrosine Kinase |
| SCF | Stem Cell Factor |
| SDS-PAGE | Sodiumdodecylsulfate polyacrylamide gel electrophoresis |
| STAT | Signal Transducer and Activator of Transcription |
| TBS | Tris-buffered Saline |
| TCF | T-Cell Factor |
| Wnt | Wingless Integration 1 |
| βTrCP | Beta-transducing repeat-containing protein |

## Introduction

Leukemia is a form of cancer that affects blood cells. It is characterized by an overproduction of a specific blood cell type, leading to e.g. anemia, bleedings, fatigue or bad wound healing, because of the deficit in all other blood constituting cells. Finally this can lead to the death of the patient, in case the disease is not treated.

There are two major forms of leukemia, which are chronic and acute leukemia. However, for both types of the disease, there is a lymphocytic and a myeloid form. There are 4 major types of leukemia- chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL) and acute lymphocytic leukemia (ALL). Incidence rates of all four forms vary between different age groups. Whereas ALL is mainly found in children and AML in young adults, CLL and CML are predominantly found in elderly patients [1].

Although ALL, AML, CLL and CML all belong to the same type of cancer, they evolve differently depending on the specific fusion gene, which is generated after the breakage of chromosomes. One of the thereby formed oncoproteins, which has been shown to be associated with B-ALL and CML, is the fusion protein BCR/ABL [2,3,4].

This protein is a fusion of a sequence in the breakpoint cluster region on chromosome 22 and the Abelson kinase (c-abl), which lies on chromosome 9 (see *Figure 1*) [5] and is also often called Philadelphia chromosome. Together, these two pieces result in a constitutively active tyrosine kinase, which can vary in protein length.

There are three different isoforms of BCR/ABL, which all cause a different clinical phenotype. p185, is the shortest form of the protein, and causes B-cell acute lymphoblastic leukemia (B-ALL), which is characterized by a proliferation of immature B-cells and is mostly the cause for childhood leukemia. The p210-form in contrast, is the cause for chronic myelogenous leukemia (CML), which is

a myeloproliferative disorder and is often found in older patients. The longest protein form, p230 causes an indolent myeloproliferative disorder, called chronic neutrophilic leukemia [6].

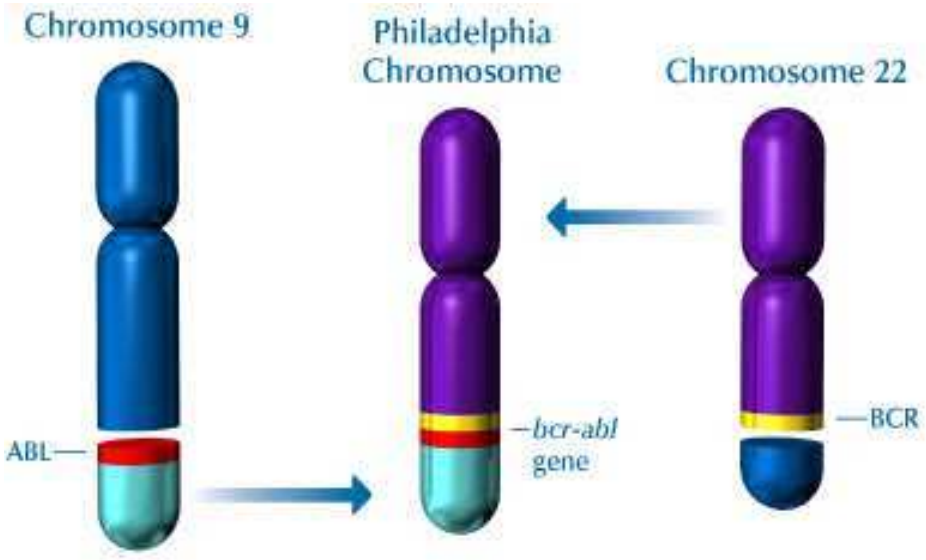


***Figure 1*** *The BCR/ABL fusion gene [7]*

To be able to study the different forms of BCR/ABL-induced leukemia in more detail, mouse models have been developed. As diseases in animals do often not resemble the situation in humans, mouse bone marrow cells were transduced with specifically designed retroviral BCR/ABL-constructs. Those were accordingly injected into lethally irradiated mice, where they are able to induce the different forms of leukemia mimicking the human *in vivo* situations very closely [8,9].

Here, we concentrate on the two shorter isoforms of the BCR/ABL fusion protein, p185 and p210 that have already been described in many different studies [2,3,4].

β-catenin is the key player of the canonical Wnt-signaling pathway. This pathway is of major importance during development (e.g. cell polarity or axis specification) and does also play an important role in tissue homeostasis (e.g. proliferation and differentiation). The whole Wnt- pathway is about β-catenin stabilization. If no Wnt- signal is available, Glycogen synthase kinase- 3β (GSK3β) is unhamperedly able to phosphorylate β-catenin and mark it thereby for degradation in the proteasome (see *Figure 2 A*). To activate the signaling cascade, a Wnt-protein has to bind to the receptor Frizzled and its co-receptor Low Density Lipoprotein Receptor-Related Protein (LRP). Dishevelled binds to this complex as well, whereby it is now able to inhibit GSK3β activity. β-catenin is thereby stabilized, accumulates in the cytoplasm and can now be further modified to either go into the nucleus in order to enable transcription of β-

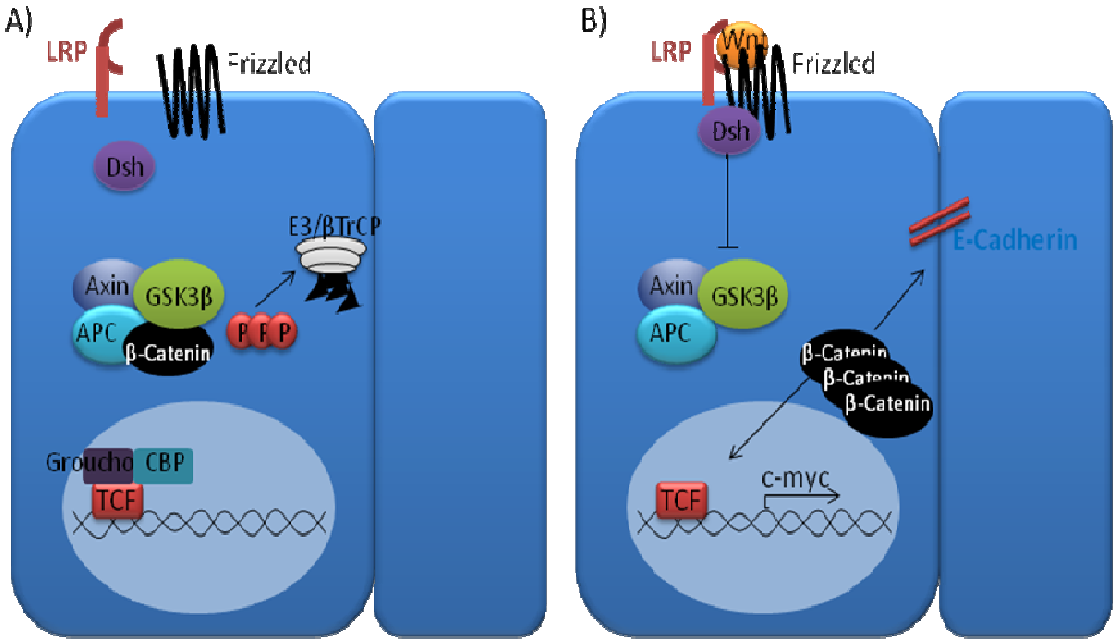


***Figure 2*** *Wnt-Signaling Pathway: A) inactive Wnt-Pathway; B) activated Wnt-Pathway; LRP- Low Density Lipoprotein Receptor-Related Protein, Dsh- Dishevelled; APC- Adenomatous Polyposis Coli; GSK3β- Glycogen Synthase Kinase- 3β; CBP- CREB-binding protein; TCF- T-cell factor; βTrCP- Beta-transducing repeat-containing protein; Wnt- Wingless Integration 1*

catenin target genes, like c-myc or cyclin D1, together with T-Cell Factor/ Lymphocyte enhancer factor (TCF/LEF), or it can travel to the cell membrane and act in cell adhesion by binding E-cadherin and α-catenin (see *Figure 2 B*) [11,12,13].

Normally, there is a balance between those two above mentioned functions of β-catenin. But this equilibrium can easily be destabilized by hyperactive signaling pathways.

β-catenin has already been shown to play an active role in the formation of some adenocarcinomas [14]. But it can also contribute to diverse other solid cancers like ovarian cancer [15] or thyroid carcinoma [16], and it is also able to influence hematopoietic cells [17]. Therefore it is very likely, that similar signaling pathways apply in the case of leukemia.

Evidence for the importance of β-catenin in human CML was found by Jamieson et al. in 2004, where they could show that there is a more than 10-fold increase of β-catenin activity in CML diseased GMPs in blast crisis compared to CML hematopoietic stem cells (HSCs) or normal granulocyte-macrophage progenitors (GMPs) and HSCs. Blast crisis is the last of three stages in CML development. First, the patient passes through chronic phase, which can last up to several years. At this stage, increased numbers of progenitor cells and more mature cells can be measured, but those cells still look normal concerning their phenotypes. The overproduction of cells leads to a myeloproliferative syndrome. From accelerated phase to blast crisis, several further mutations occur, which make the cells look blast-like. Those GMP-like cells are present in overwhelming numbers and proliferate rapidly. Once arrived at this stage, leukemia is not treatable anymore [18].

When inhibiting β-catenin, in *in vitro* experiments, the proliferation and self-renewal of CML cells was reduced, which might indicate a dependence on this protein for leukemia progression [17]. Whether this situation is also resembled *in vivo,* was investigated in 2007 by the group of Tannishtha Reya, who created a conditional β-catenin knock-out mouse. Interestingly, the mouse was not able

to develop CML, which most likely originates in hematopoietic stem cells (HSCs), but could still sicken from ALL, which is thought to emanate from committed progenitors. The conclusion they drew was that it might be highly dependent on the cell type from which the disease develops, whether a CML or a B-ALL develops [19].

The group of Dr. Ron Smits could show that the phosphorylation of tyrosine 654 of the β-catenin molecule by a receptor tyrosine kinase (RTK), which could be the constitutively active tyrosine kinase BCR/ABL, leads to a decreased binding affinity for E-cadherin, which in turn makes it more available for protein kinase A (PKA). PKA is now able to phosphorylate the molecule at serine 675, which leads to hyperactive transcriptional activity of β-catenin responsive genes and thereby leads to a distorted balance in protein function [20].

All together, it could be shown that the p210 isoform of BCR/ABL, which is able to induce CML, physically interacts with β-catenin by phosphorylating tyrosine 654 (Y654) [10]. If the same is true for p185 could not be identified.

To investigate the role of β-catenin in BCR/ABL p210 and BCR/ABL p185 induced leukemia and to see whether there is a difference in the interaction of β-catenin with the two BCR/ABL isoforms, the following study was conducted. With the help of a mouse model, derived from the Lab of Dr. Ron Smits (Erasmus MC, Rotterdam), different experiments could be performed. The mice were designed to carry a mutation, which substitutes a tyrosine (Y) by a glutamate (E) in the residue 654 of β-catenin. The consequent protein acts by increasing the transcriptional activity and by decreasing the affinity for cadherin adhesion molecules. Homozygous mice are embryonically lethal, probably due to an anterior truncation, whereas heterozygous mice are viable but easily develop intestinal tumors [20].

To find out about the role of β-catenin in BCR/ABL induced leukemias, several different *in vitro* assays were performed, which should help to find out about the leukemia induction capability of both oncoproteins, when there is increased β-catenin activity involved.

# Methods

## Heat Shock Transformation of DH5α

Heat shock competent E.coli of the strain DH5α and plasmid DNA (diluted 1:1 000 000), were thawed on ice for 10-15 min. Following plasmids were used for transformation: pMSCV-p210-IRES-GFP, pMSCV-p185-IRES-GFP and pMSCV-IRES-GFP. After the incubation on ice, 1µl of each of the above mentioned plasmids was added to one of the three prepared tubes containing 50µl of bacteria. The tubes were put on ice for further 10min. Afterwards, the bacteria-plasmid mixtures were placed in the heating block for 1min at 42℃. Immediately afterwards, the tubes were put on ice for 2min. Thereafter, 1ml of LB medium (10g Bacto-tryptone, 5g yeast extract, 10g NaCl, 800ml H2O added, pH adjusted to 7,5; volume adjusted to 1L with H2O; autoclaving) was added to the fragile bacteria. The bacteria were put into the heating block for 30min at 37℃ and 450rpm to allow 1,5 cycles of replication of DH5α bacteria. The bacteria were centrifuged for 5min at 3200rpm. Accordingly, the supernatant was removed until approximately 200µl. The bacteria were resuspended with a 200µl pipette and pipetted onto LB agar plates (see LB medium; additionally 15g Bacto-agar added before pH adjustment) containing Ampicillin (50mg/ml Ampicillin added after autoclaving, shortly before plates were poured). With the help of 2mm glass balls, bacteria were spread. The glass balls were removed when the plates were dry. All three plated were incubated over night at 37℃.

On the next day no colonies could be observed due to unidentified reasons, therefore several alterations were made in temperature, dilution of plasmids or incubation times. All together the experiment was repeated 5 times, with no positive result. Therefore it was decided on borrowing the plasmids for the transfection of producer cell lines from the group of Dr.Veronkia Sexl.

## Growing of the producer cell lines

For the first experimental approach, it was decided on using GP+E86 producer cells. Those were already producing the viruses of interest, which were either GFP, p210 or p185. The advantage of these cells is, that they have good adherence to the dish, which makes culturing and later isolation of viral supernatant much easier. Disadvantage of this cell line is, that the infection rate of cells is rather low, which was eventually the reason, why it was decided on using Phoenix producers after the first experimental approach. Phoenix producers are more effective in infecting cells, but also dissolve much easier from culture dishes, which requests rather gentle handling during culturing. Furthermore those cells tend more easily to transform into an oncogenic phenotype, therefore cell density has to be kept much lower compared to GP+E86 cells.

## Transfection of plasmids into retroviral producer cell line

As Phoenix cells do not stably integrate plasmid DNA, producer cells had to be transfected with the vectors of interest and Lipofectamine® 2000 (Invitrogen) two days before viral supernatant could be collected.

For infection of Phoenix cells with pMSCV-IRES-GFP, pMSCV-p210-IRES-GFP or pMSCV-p185-IRES-GFP, complete DMEM medium (+10% FCS, 5ml L-Glutamine, 5ml Penicillin/Streptomycin) was exchanged with plain DMEM. In the meantime 3x 1,5ml of plain DMEM were mixed with 10µg of the corresponding plasmids (GFP stock: 1µg/ml; p210 stock: 1,357µg/ml; p185 stock: 0,45µg/ml). Besides, 3x 1,440µl of plain DMEM were mixed with 60µl of Lipofectamine® 2000, all was incubated at room temperature for 5min until the DMEM-DNA mix could be united with the DMEM-Lipofectamine ® 2000 mix. The mixture was dropped carefully on the serum-starving Phoenix cells. After 3 hours, plain DMEM was exchanged with complete DMEM. Cells were allowed to produce viral supernatant for 48 hours.

## Isolation of fetal liver cells

Fetal livers were isolated from E.12.5- E.17.5 old mouse embryos with the help of a pair of scissors and tweezers. Tissue samples were taken from each of the isolated embryos for later genotyping. The fetal livers were put into prepared 1,5ml centrifugation tubes and provided with 500µl of RPMI (500ml complete RPMI: 10% FCS, 5ml L-Glutamine, 5ml Penicillin/Streptomycin, 500µl β-Mercaptoethanol). Accordingly, they were homogenized with a 22xG needle and centrifuged for 5min at 1400rpm.

In the meantime viral supernatants of GFP, p210 and p185 GP+E86 producers were collected. Those were filtered with 45µm filters before infection, to get rid of producer cells and cell debris. For an effective infection, 1:1000 diluted Polybrene, 1:20 diluted SCF (supernatant 1:40),1:100 diluted IL-3, 1:100 diluted IL-6 and 1:1000 diluted IL-7 were additionally added to each 15ml tube.

As the infection of fetal liver cells with GP+E86 producer cells did not work properly, it was decided on using Phoenix producer cell line for all further experimental approaches.

Fetal liver cells were provided with 600µl of fresh RPMI and each of them was split equally into three 15ml Falcon tubes. Viral supernatants were also split equally to provide every single fetal liver with equal amounts of virus.

## Infection of fetal livers with viral supernatant

For infection of fetal livers with viral supernatant, two different approaches were chosen. For the first 2 litters, fetal livers were infected by mixing the cells with equal amounts of virus. The cells were then incubated in Falcon tubes over night in the incubator and turned upside-down several times during this incubation period.

For the third litter, the infection was done by plating equal numbers of cells on 6-well plates and mixing them with even amounts of viral supernatant. The virus was then centrifuged onto the cells for 1,5 hours at 2000rpm (spin-down infection for improved transduction efficiency).

Further proceedings of infected fetal livers were the same for all three litters.

## Precipitation of DNA and Genotyping of fetal livers

For genotyping of the isolated fetal livers, tissue samples were taken from each of the embryos and put into separate tubes. The tissues were digested with 500µl Proteinase buffer containing 6µl of Proteinase K. The lysates were put on 56℃ over night. To get full lysis of proteins, the samples were vortexed from time to time. When there were no visible tissue fragments left, DNA could be precipitated. Therefore 700µl of Chloroform were added to the digested embryos together with 200µl of 5M NaCl. The tubes were shaken for 10min over-head. Afterwards they were centrifuged for 10min at 13 200rpm at room temperature. 500µl of Isopropanol were pipetted into fresh tubes. 400µl of the upper phase of each digested tissue, were pipetted on top of the Isopropanol in the freshly prepared tubes. Each of the tubes was shaken vigorously and let stand for further 10min until the foam had dissolved. The precipitated DNA was centrifuged for 10min at 13 200rpm at room temperature. The supernatant was removed until 40-50µl and replaced with 800µl of 70% EtOH. Again, the tubes were centrifuged for 10min at 13 200rpm at room temperature. Now EtOH was removed, the pellets were dryed and DNA could be gently resolved in 200µl of $H_2O$ or TE buffer.

For genotyping, freshly isolated DNA was used. A master-mix containing 10x Buffer (final concentration: 1x), $MgCl_2$ (final concentration: 1,35mM), dNTPs (final concentration: 200µM), primer forward (bcatY654Efor: GATTGGAGACCAGAAGCCTTG; final concentration: 0,3µM), primer reverse (bcatY654Erev: ACCCAATGTAAAGCATGACGTG; final concentration: 0,2µM), $H_2O$ and Taq Polymerase (0,1 units) was prepared.

18µl of the master-mix were pipetted into each of the prepared PCR tubes. 2µl of DNA were added to each tube. For the negative control, $H_2O$ was used instead of DNA.

PCR was run under following conditions: 1) 95℃ for 5min; 2) 95℃ for 30 sec; 3) 55℃ for 30 sec; 4) 72℃ for 40 sec; steps 2-4 w ere repeated 35 times; 5) 72℃ for 5 min; 6) 15℃ until stop of PCR.

5µl of Orange G were mixed with the 20µl DNA of the PCR reaction. DNA was loaded on a 2% Agarose gel. Electrophoresis was run with 100-150V. Bands were observed with the help of a UV-Transilluminator.

## Colony Formation Assay

For colony formation assay, infected fetal liver cells were brought to a total cell number of 120 000 cells in 300µl RPMI. Those cells were then intermingled with 3ml of MethoCult® (methylcellulose medium for mouse cells; STEMCELL Technologies) until a homogenous mixture was obtained. 3ml of this viscous mixture, were then plated equally on two 3cm petri- dishes. Thereby it was taken care to avoid bubble formation. The plates were put on 37℃ and 5% CO2.

When the colonies on the dishes were about 1mm in size, they were counted under the microscope.

## Liquid cell culture

Fetal liver cells that were not needed for clonal assay, were plated on 6-well plates and provided with RPMI. When cells were able to proliferate, this was an indication for successful infection of cells. Furthermore some of those cells could be used for Western Blot analysis, proliferation assay and apoptosis assay.

Cell lines, which were established from wild-type and heterozygous fetal livers cells of litter 3 were additionally analyzed with FACS, by staining the cells with IL7R Pacific Blue, Ter119 PE, CD3e PerCP, Mac1 PECy7, Gr1 APC and CD19 APC-Cy7 (BD Biosciences). Each antibody was diluted 1:100 in a single tube containing 100µl of PBS. Additionally 1:100 diluted FcγR was added to block the Fcγ receptor. 50µl of cells were incubated on ice in the dark with 50µl of

antibody mixture for 20min. Then, the cells were washed twice with PBS. The cells were now ready for FACS analysis.

## Proliferation Assay

For proliferation assay, p185 infected blood derived cells and p185 infected EE/EE fetal liver cells (FL3, litter 2) were used. Those were allowed to grow in several passages until a homogenous phenotype was derived. p185-infected bone marrow cells were not the optimal control for this experiment, but none of the YY/YY fetal liver cells from the first two litters grew any further, when kept in liquid culture. Therefore, these cells served as p185 wt control.

3 000 000 cells per well (1 000 000 cells/ml) were seeded in triplicates on a 6-well plate. Cell numbers were adjusted with CASY cell counter (Innovatis). To get all p185 infected cells into G1 phase, they were provided with serum-free medium for 24h. After this interval, 10% FCS were added to each well to induce cell cycling. 50µl cell sample were taken for 3 days to be in pursuit of cell proliferation. Cell numbers were determined with CASY cell counter.

## Apoptosis Assay

For apoptosis assay, the same cells were used as for proliferation assay.

Again, p185 infected wt cells and p185 infected double mutant cells were seeded in triplicates on a 6-well plate. The 3 000 000 cells per well (1 000 000 cells/ml) were provided with serum-free medium. Cell numbers were adjusted with CASY cell counter and determined every day in course of the experiment. Beside the 50µl samples taken for cell counting, 500µl cells were taken for FACS analysis every day.

To determine how many of the seeded cells are already in an apoptotic state, cells were stained with PI (Molecular Probes) and Annexin V APC (BD Biosciences). Therefore, the 500µl cells were centrifuged for 5min at 1200rpm in FACS tubes. Accordingly, the cells were washed once with PBS to get rid of cell debris and most of the dead cells. The cells were now incubated in the dark at room temperature with PBS containing 1:100 FcγR (BD Biosciences), 1:100

diluted PI and 1:100 diluted Annexin V APC. The cells were washed again with PBS before they could be analyzed with FACS Canto II.

## Western Blot

To analyze differences in protein levels of p185 wt cells compared to p185 EE/EE cells, Western Blot analysis was performed.

Cell lysates of the above mentioned cell lines were prepared by freezing cell pellets, after washing with PBS, in liquid nitrogen in order to destroy cell membranes. Shortly before SDS-PAGE was performed, the cell pellets were thawed and resuspended in 200µl of RIPA buffer [150mM NaCl, 50mM Tris (pH 7.4), 1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 1mM Na3VO4, 25mM NaF, 1mM PMSF, 1:100 β- Glycerol-phosphate (0,5M stock) 1:100 DTT (1M stock)] and stored on ice for 10-15min. Afterwards, the tubes containing the lysed cells, were centrifuged for 5min at 13 200rpm and 4℃. Supernatants were collected and transferred into fresh 1,5ml tubes. To determine protein concentrations of the two lysates, OD values were determined with Bradford Assay. For calculation of protein concentrations from OD values, a BSA-standard curve was used.

Bradford solution (Biorad) was diluted 1:5 with $H_2O$. 1ml of this solution was pipetted into 3 separate cuvettes. One should serve as blank, to the other 2 cuvettes 1µl of the corresponding cell lysate was added. The cuvettes were vortexed and accordingly placed in a spectrophotometer for OD measurement. Protein levels were adjusted to either 15µg or 20µg for SDS-PAGE with RIPA buffer.

A 6% gel and a 10% gel were prepared and loaded with 15µg or 20µg of protein from the above mentioned lysates. The gels were run with 150V. Afterwards the proteins were transferred onto a nitrocellulose membrane for 1,5h at 120mA in a semi-dry transfer.

After the transfer, the membranes were stained with Ponceau and cut into pieces for later antibody treatment. The membranes were destained with TBST

and blocked with 5%BSA in TBST for ½ hour. Afterwards, they were washed 3 times for 5min with TBST and incubated in 5ml of diluted primary antibody in TBST over night at 4℃. The membranes were again wa shed 3x 5min with TBST, before they were incubated in the corresponding horseradish peroxidase (HRP) secondary antibody solution (1:5000 diluted HRP antibody in TBST). for ½ hour. The membranes were again washed 3x 5min with TBST.

Detection reagent A (GE Healthcare) was mixed with detection reagent B (GE Healthcare) in a 1:1 ratio. The solution was incubated at room temperature for a few minutes, until the temperature had increased to approximately 25℃.

The membranes were now covered with the detection solution for almost a minute before they were dipped into $H_2O$. The membrane pieces were put together and covered with foil. Then they were fixed in a cassette for film development. X-ray films (Mediphot X-90 RP, Colenta) were now exposed to the membranes in the dark for different time intervals. After exposure, the films were developed in the developing machine (Optimax, Protec).

# Results

## Culturing of producer cells and transfection with plasmids

### *GP+E86*

Initially, we intended to infect fetal liver cells from β-catenin transgenic mice using stable producer cell lines. The producer cell line, which was chosen, was the GP+E86 cell line. Those are fibroblast-like cells with suitable adherence capabilities. GP+E86 cells used in this experiment, had already one of the 3 plasmids –pMSCV-IRES-GFP, pMSCV-p210-IRES-GFP or pMSCV-p185-IRES-GFP- incorporated and required no transfection beforehand.

The cells were grown to about 70% confluence on 15cm dishes, until they could be used for infection of fetal liver cells.

As infection efficiencies of packaging cells are rather low, a different transient high titer producer cell line, called Phoenix cells, was used for further repetitions of the infection experiment.

### *Phoenix*

The cell line Phoenix, which has an epithelial phenotype was used after GP+E86 cell failed to infect fetal liver cells efficiently. But before infection, the producer cells had to be transfected with Lipofectamine® 2000 and the plasmids derived from SexI group. The cells were seeded at a confluency of around 50% at day 0 and subsequently transfected with 10µg of DNA on day 1.

| Plasmid | Plasmid Concentration (µg/µl) | Amount of Plasmid used (µl) |
|---|---|---|
| pMSCV-IRES-GFP | 1 | 10 |
| pMSCV-p210-IRES-GFP | 1,357 | 7,4 |
| pMSCV-p185-IRES-.GFP | 0,45 | 22,2 |

***Table 1*** *pMSCV plasmids with corresponding concentrations and amounts used for transfection*

Calculations can be observed in *Table 1*. To get the Liposome-DNA structures into the cells, the cells had to suffer from serum starvation for several hours.

After transfection, serum-free medium was replaced with DMEM medium containing 10% FCS. Phoenix producers were allowed to produce virus for 48 hours. Afterwards, fresh supernatant was collected and immediately used for further infection of fetal liver derived cells.

## Transformation of plasmid DNA in E.coli bacteria

In order to amplify the BCR/ABL-constructs for subsequent transformation and production of high-titer viral particles, 3 different plasmids (pMSCV-IRES-GFP, pMSCV-p210-IRES-GFP and pMSCV-p185-IRES-GFP) with Ampicillin resistance were used. pMSCV-IRES-GFP represents the empty-vector backbone that should serve as a control during later infection steps. The method of choice was heat-shock transformation.

As no colonies grew on the Ampicillin containing agar plates, alterations were made in amount of bacteria, amount of plasmid, incubation temperatures as well as incubation times. In total, this experiment was repeated five times, with no positive result. However, we received sufficient amounts of the required plasmids from the lab of Dr. Veronika Sexl. Those were later needed for infection of producer cells.

## Isolation and genotyping of fetal liver cells

Heterozygous β-catenin mutant mice (β-catenin$_{Y654E}$) with C57/B6 background were interbred to receive all three possible genotypes- wild-type (YY/YY), heterozygous (YY/EE) and double mutant (EE/EE). Plug checks were done to ensure pregnancy of the mice. Embryos were then isolated in the time span of E12.5 to E17.5. In total, 23 embryos from 3 different litters were isolated. All embryos were infected with the three plasmids mentioned above.

To be able to draw conclusions from later experiments, the embryos had to be genotyped. For genotyping of β-catenin mice, a specific protocol has been established in our lab. Tissue samples were taken from each embryo and

digested with Proteinase K at 56℃ over night. Acco rdingly DNA was isolated from the digested samples and amplified with PCR.

On Agarose gel, 3 different band patterns could be observed, depending on the genotype of the embryos: wild-type embryos showed a single band around 200bp, whereby double mutant embryos had a single band at 300bp. Heterogeneous embryos are supposed to show 2 bands, one at 200bp and the second one at 300bp. Nevertheless, also a third band could be observed, which was probably due to unspecific binding of the primers. As can be seen in *Figure 3* to *Figure 5*, all 3 genotypes are represented. Unfortunately, we obtained all 3 genotypes only in litter 2, probably due to a wrong set-up of breedings. For the other 2, the Mendelian rule of inheritance cannot be applied. The genotypings of litter 1 are shown in *Figure 3*. Here, all of the embryos were wild-type for the β-catenin allele. From litter 2, depicted in *Figure 4*, 4 embryos were heterogeneous for the mutation (#1,# 5,# 8 #,9), 3 were double mutants (#2, #3, #6) and 2 wild-type (#4,#7). In litter 3 (see *Figure 5*), none of the embryos had a double mutation in the β-catenin gene. Here, the embryos #1 to #4 were wild-type and embryos #5- #7 were heterogeneous. It seems like a heterozygous mouse was interbred with a wild-type mouse.

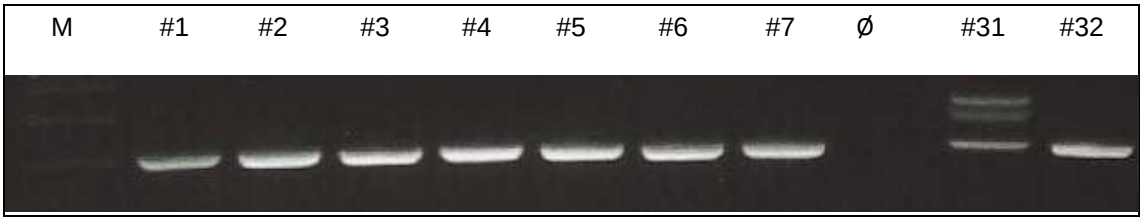


***Figure 3*** *Genotypings of embryos from litter 1; M = marker; #1- #7 = wild-type embryos (YY/YY); Ø = negative control; #31 and #32 = positive controls*

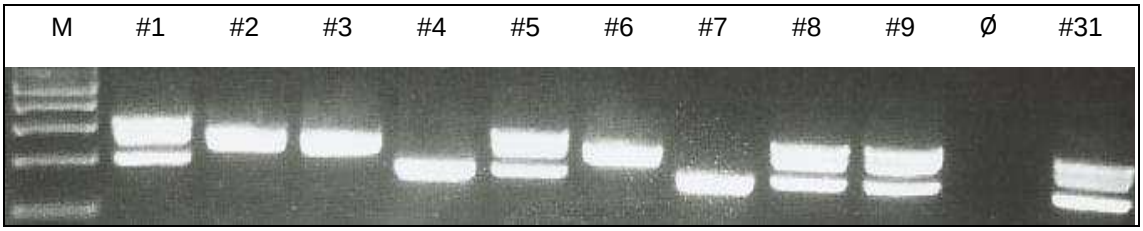


***Figure 4*** *Genotypings of embryos from litter 2; M= marker; #1,5,8,9= heterozygous embryos (YY/EE); #2,3,6= double mutant embryos (EE/EE); #4 and 7= wild-type embryos (YY/YY); Ø = negative control; #31= positive control*

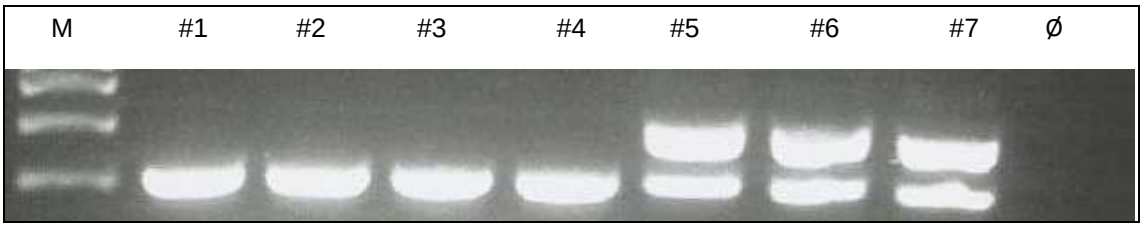


***Figure 5*** *Genotypings of embryos from litter 3; M= marker; #1-4= wild-type embryos (YY/YY); #5-7= heterozygous embryos (YY/EE); Ø = negative control*

## Colony Formation Assay

Fetal livers were infected with the three respective viruses produced by the packaging cell line. They were seeded at a total number of 120 000 cells per 300µl and mixed with Methylcellulose for mouse cells.

Only colonies from litter 2 could be counted for this colony formation assay, as no colonies evolved from the plated cells of litter 1 or 3.

All cells were plated on two 3cm petri-dishes (duplicates) and incubated on 37℃ and 5% CO2 for at least 14 days. Then, the exp eriment was evaluated by counting the colonies which had formed. The fetal liver cells of the embryos from litter 1 and 3 did not form any colonies. Therefore, the experiments could not be evaluated in those two cases.

In *Table 2*, colony numbers of the infected fetal livers from litter 2 can be seen. Those counts were divided in small, medium and large colonies. It was also differentiated between scattered and spherical colonies. Spherical colonies appeared to have a compact core with just few scattered surrounding cells. However, scattered colonies did often have just a small compact core with many scattered cells surrounding them.

As can be noted, most colonies were formed by p185-infected EE/EE fetal liver cells, when only considering medium and large colonies. GFP-infected EE/EE cells also formed all kinds of colonies, but however less than p185 cells. Interestingly, p210-infected cells did hardly form any colonies. This result could be due to an accidental interchange of plasmids.

Wild-type as well as heterozygous cells of all three differently infected fetal livers cells –GFP, p210 and p185- did just form few or no colonies at all.

| GFP | YY/YY | | | | YY/EE | | | | | | | | EE/EE | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FL4(1) | FL4(2) | FL7(1) | FL7(2) | FL1(1) | FL1(2) | FL5(1) | FL5(2) | FL8(1) | FL8(2) | FL9(1) | FL9(2) | FL2(1) | FL2(2) | FL3(1) | FL3(2) | FL6(1) | FL6(2) |
| Spherical Colony | | | | | | | | | | | | | | | | | | |
| large | | | | | 1 | 1 | | | | | | | 2 | | 1 | | | |
| medium | 2 | 2 | 1 | 1 | | 10 | 2 | | 1 | | | | 4 | 3 | 6 | 3 | 1 | |
| small | 13 | 8 | 7 | 6 | 4 | 14 | 7 | 8 | 5 | 5 | 5 | 1 | 11 | 6 | | 3 | 11 | 6 |
| Scattered Colony | | | | | | | | | | | | | | | | | | |
| large | | | | | 1 | | | | | | | | 2 | | 3 | | | |
| medium | | 1 | | | 1 | 5 | 1 | | | | | | 7 | | 9 | 3 | | 1 |
| small | 3 | 4 | 2 | 1 | | 6 | 4 | 3 | 4 | 2 | | | 19 | 5 | 10 | 3 | 2 | |
| **p185** | YY/YY | | | | YY/EE | | | | | | | | EE/EE | | | | | |
| | FL4(1) | FL4(2) | FL7(1) | FL7(2) | FL1(1) | FL1(2) | FL5(1) | FL5(2) | FL8(1) | FL8(2) | FL9(1) | FL9(2) | FL2(1) | FL2(2) | FL3(1) | FL3(2) | FL6(1) | FL6(2) |
| Spherical Colony | | | | | | | | | | | | | | | | | | |
| large | | | | | | 1 | 1 | | | | | | 1 | | 1 | | | |
| medium | | | | | 1 | 1 | 1 | 1 | | | | | 3 | 7 | 5 | 1 | | |
| small | 1 | 5 | 7 | 7 | | 1 | 8 | 6 | 16 | 12 | 16 | 14 | 20 | 5 | 4 | 5 | | |
| Scattered Colony | | | | | | | | | | | | | | | | | | |
| large | | | | | | | | | | | | | 2 | 5 | 4 | | | |
| medium | | | | | | | 2 | | 1 | | | | 10 | 16 | 6 | 4 | | |
| small | 4 | 3 | 1 | 1 | | | 6 | 1 | 3 | 1 | 2 | 5 | 21 | 10 | 13 | 7 | | 3 |
| **p210** | YY/YY | | | | YY/EE | | | | | | | | EE/EE | | | | | |
| | FL4(1) | FL4(2) | FL7(1) | FL7(2) | FL1(1) | FL1(2) | FL5(1) | FL5(2) | FL8(1) | FL8(2) | FL9(1) | FL9(2) | FL2(1) | FL2(2) | FL3(1) | FL3(2) | FL6(1) | FL6(2) |
| Spherical Colony | | | | | | | | | | | | | | | | | | |
| large | | | | | | | | | | | | | | | | | | |
| medium | 2 | 1 | | | | | | | | | | 1 | | | | | | |
| small | 19 | 10 | | | 18 | 14 | 9 | 14 | 12 | 7 | 7 | 11 | 13 | 12 | 14 | 6 | 4 | 4 |
| Scattered Colony | | | | | | | | | | | | | | | | | | |
| large | | | | | | | | | | | | | | | | | | |
| medium | | | | | | | | | | | | | | | | | | |
| small | 1 | | | | 1 | | | | | | | 1 | 2 | | | | | |

***Table 2*** *Colony Formation Assay; colony counts of virus-infected fetal liver cells (GFP, p185, p210) plated in Methylcellulose; colonies were discriminated regarding their size (small, medium and large) and form (scattered and spherical); numbers in brackets indicate dishes with cells of the same genotype and treatment; FL= fetal liver; YY/YY= wild type cells; YY/EE= cells heterozygous for β-catenin mutation; EE/EE=cells homozygous for β-catenin mutation*

*Figure 6* visualizes the cell colony counts in a bar graph. Here, we have evaluated only medium and large colonies, since small colonies consisted of only few cells and thus might have formed as a consequence of transient growth factors rather than from introduced constructs.

Depicted in the graph, are all three genotypes of litter 2, namely YY/YY (n= 4), YY/EE (EE/YY) (n= 8) and EE/EE (n= 6). The colony numbers were presented as the average number of colonies/plate and were grouped by the same genotypes. All GFP-infected fetal liver cells are shown in green, p185-infected cells in red and p210-infected cells in blue. The error bars respresenting standard deviation are relatively large because some plates did not give rise to colonies *in vitro* and thus were valued zero.

As depicted in *Figure 6*, EE/EE fetal liver cells transduced with the pMSCV-vector containing only GFP formed more colonies than their YY/YY and YY/EE counterparts. This result was very interesting, as GFP-infected cells should not be able to proliferate in methylcellulose.

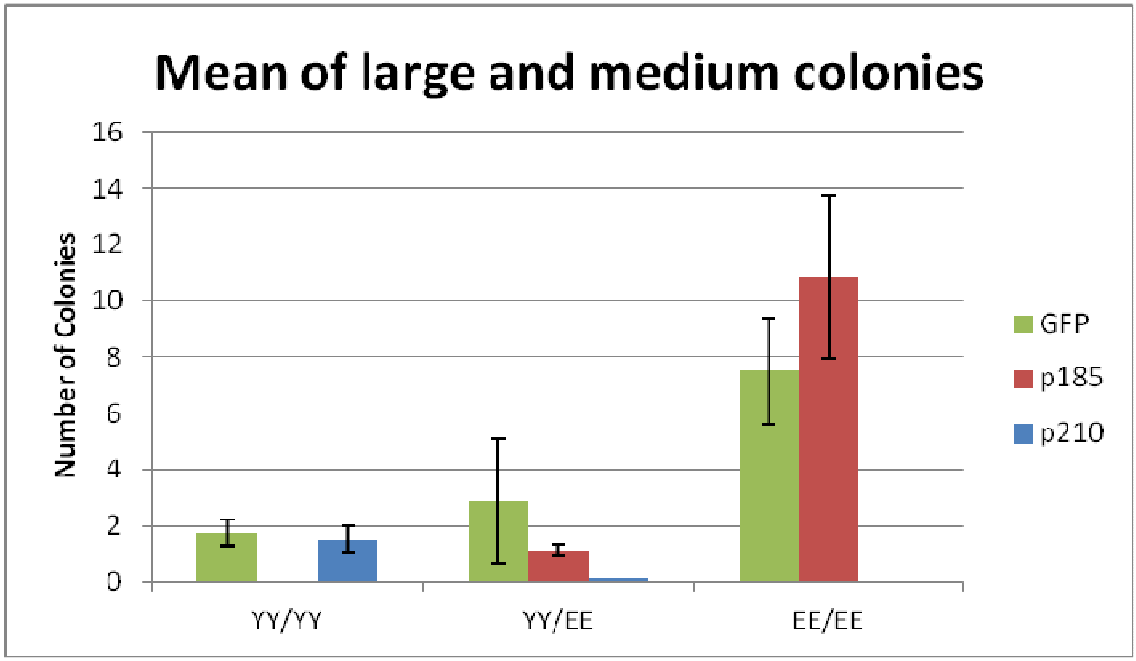


***Figure 6*** *Colony formation: The average numbers of colonies formed by infected fetal liver cells; cells of the same genotypes (YY/YY, YY/EE, EE/EE) were clustered; green bars indicate GFP-infected cells, red bars p185-infected cells and blue bars p210-infected cells; error bars indicate the standard error of the mean (SEM)*

β-catenin knockout mice do not form CML, but succumb to a B-ALL later in age [19]. Thus, we have initially speculated that our gain-of-function mutant (EE/EE) might exert a more aggrestive form of CML as compared to control cells. When comparing now these cells, containing no oncogene, to p210-transduced cells, latter formed only very few colonies in YY/YY and YY/EE cells and none in EE/EE cells. We would have expected those cells to form most colonies and none in GFP-infected cells.

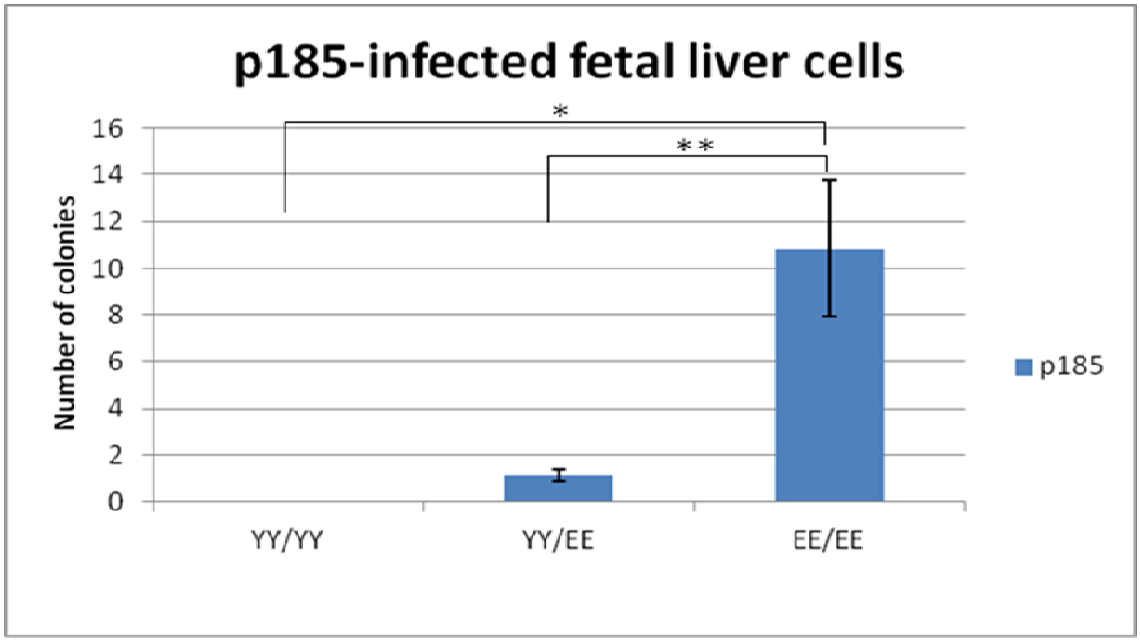


**Figure 7** *The average numbers of colonies formed by p185-infected fetal liver cells of all three genotypes (YY/YY, YY/EE, EE/EE); p<0.05 = *; p<0.01= ***

p185-infected fetal liver cells showed significant differences in the number of colonies, when comparing YY/YY to EE/EE cells (p<0.05) in *Figure 7*. The difference was even more significant when comparing YY/EE to EE/EE fetal liver cells (p<0.01).

It is very unlikely that GFP-infected fetal liver cells have a higher tumorigenic potential in Y654E cells than p210-infected cells, therefore we suggest an accidental interchange of vectors at the beginning of the experiment. Nevertheless, it can be seen from *Figure 6* that there is a difference in

tumorigenicity of cells infected with p185 compared to p210-infected ones which would indicate a difference in function of β-catenin Y654E in those cells.

*Figure 7* compares the average numbers of colonies formed by the three different genotypes of p185-infected fetal liver cells. As can be seen, there is a significant difference ($p<0.05$) between the numbers of colonies formed by wild-type cells compared to double mutant cells and even a very significant difference ($p<0.01$) between heterozygous and double mutant cells. BCR/ABLp185- transformed EE/EE fetal liver cells formed around five- fold more colonies than the other genotypes.

These results show that β-catenin contributes to B-ALL leukemia development. Nevertheless error bars of *Figure 6* and *Figure 7* are relativley high to draw clear conclusions from this experiment, which is the result of the small sample size available.

## FACS analysis

Fetal liver cells, which were not needed for Colony Formation Assay, were seeded in liquid medium. Those cells were grown until cell lines were established.

From litter 1, no cell line could be established, as the virus titer was rather low. From the seeded cells of litter 2 just one single p185 EE/EE cell line grew out. Cells from litter 3, infected with either p185 or p210, were all able to proliferate in 10% FCS in RPMI medium *in vitro* and readily formed factor independent cell lines. Interestingly, infection with pMSCV-IRES-GFP did not lead to outgrowth of any cell lines. Thus, it is possible that GFP and p210 vectors were mixed up in the first place (litter2).

These cell lines were analyzed by FACS to determine the phenotype of the cells. *Figure 8* shows FACS blots of p185 and p210 infected YY/YY and YY/EE fetal liver cells stained for IL7Rα and CD19. It can be seen that p210 as well as p185 infected cells are $CD19^{high}$ and $IL7R^{medium}$.

Cells were furthermore identified to be negative for Mac1 (macrophage marker), Gr1 (granulocyte marker), Ter119 (erythrocytic marker) and CD3e (T-cell marker) (data not shown).

When comparing FACS blots of wild-type and heterozygous fetal liver cells infected with either p185 or p210, no significant differences in receptor expression could be observed. All seemed to be B-cell lineages, when only considering the tested surface receptors.

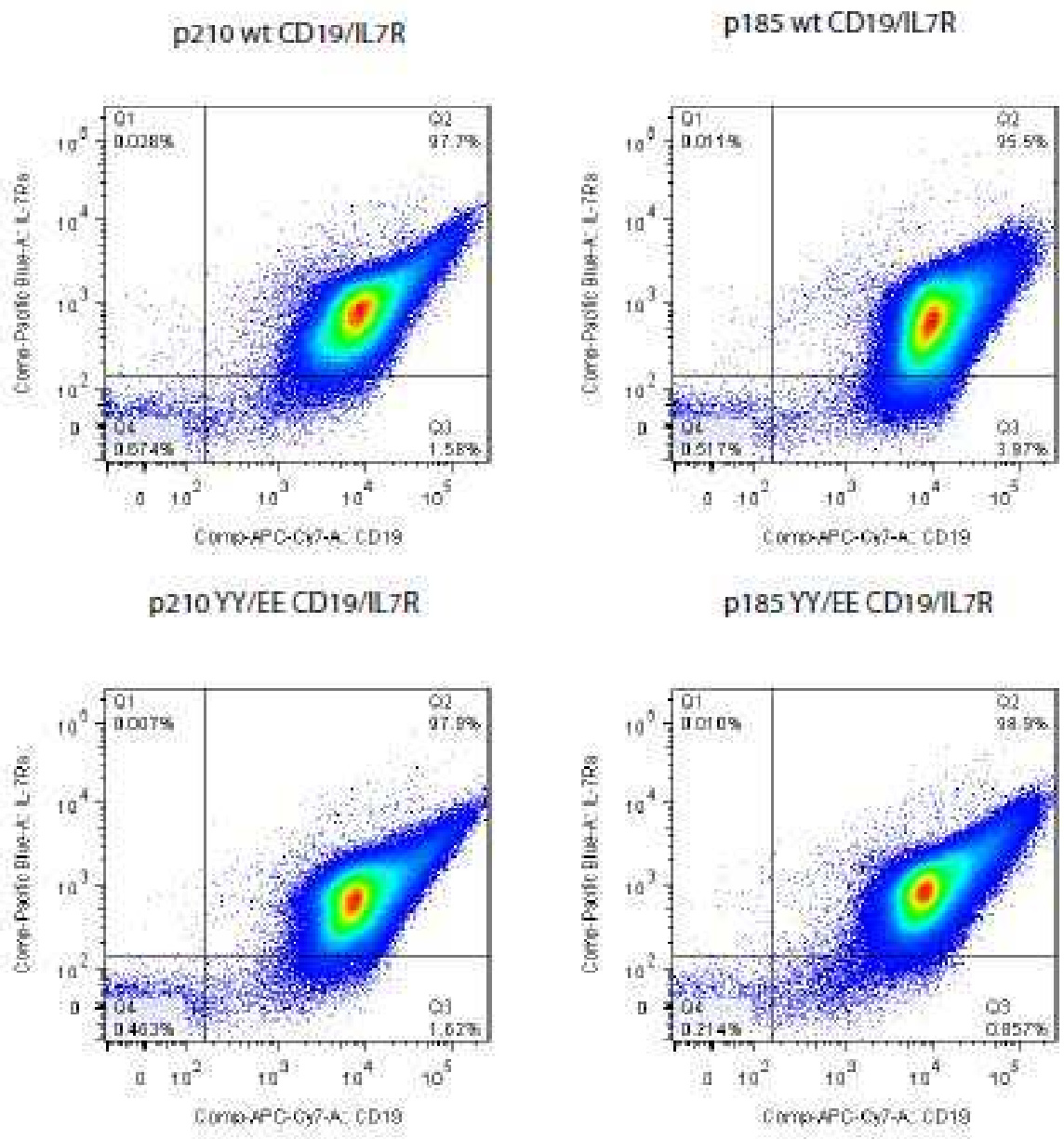


***Figure 8*** *FACS blots of p210 or p185 infected fetal liver cells stained for IL7Rα and CD19; one exemplified blot was taken for heterozygous (YY/EE) and one for wild-type (wt) fetal liver cells infected with either p185 or p210*

## Proliferation Assay

To analyze whether the differences observed in colony formation assays were cell-autonomous, we performed apoptosis and proliferation assays with previously described cell lines.

For proliferation assay, p185- infected double mutant fetal liver cells from litter 2 and p185 infected wild-type bone marrow cells were compared side-by-side. The bone marrow cells should serve as wild-type controls, as none of the wild-type fetal liver cells from this litter were able to proliferate. From litter 1, none of the cells survived. However, all of the p185- or p210-infected cells from litter 3 did propagate. But due to time issues, those cells could only be used for FACS analysis.

The seeded cells, were first brought into G1, to allow concurrent cycling of the cells. This was done by removing serum from the medium for 24 hours. After this interval, serum was again added and cells were allowed to progress in their cell cycle. The cell numbers were then measured for 3 days. The cell counts are shown in *Table 3* and *Table 4*. The cell numbers were just adjusted on day 0, which was the day of seeding.

| p185 progenitors (wt) | | | | |
|---|---|---|---|---|
| | ***day 0*** | ***day1*** | ***day 2*** | ***day3*** |
| ***1*** | 1000000 | 3876000 | 1322000 | 45690000 |
| ***2*** | 1000000 | 4680000 | 2591000 | 59470000 |
| ***3*** | 1000000 | 5490000 | 3960000 | 75750000 |

***Table 3*** *Proliferation assay of p185-infected wild-type bone marrow cells; cells were seeded in triplicates to get three independent values for cell counts from day 0 to day 3*

| FL3 double mutant (EE/EE) | | | | |
|---|---|---|---|---|
| | ***day 0*** | ***day 1*** | ***day 2*** | ***day3*** |
| ***1*** | 1000000 | 5895000 | 4888000 | 87110000 |
| ***2*** | 1000000 | 6251000 | 5768000 | 98730000 |
| ***3*** | 1000000 | 6628000 | 6714000 | 110700000 |

***Table 4*** *Proliferation assay of p185-infected double mutant fetal liver cells; cells were seeded in triplicates to get three independent values for cell counts from day 0 to day 3*

From day 1 to day 2, we observed an increased growth of p185 EE/EE cells, as can be seen in *Figure 9*. The day after, cell numbers of p185-infeced wt cells decreased again, probably due to adaptation issues. However, from day 2 on, cell numbers were increasing again.

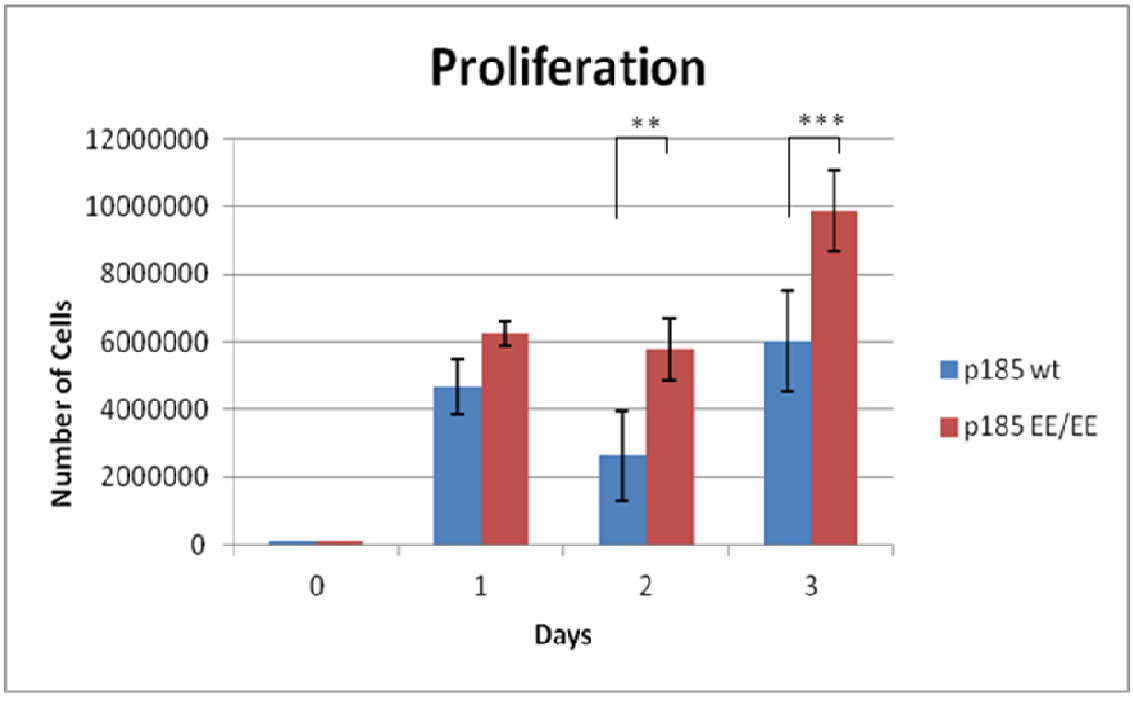


***Figure 9*** *Cell numbers of p185 wild-type cells (blue) compared to p185 double mutant cells (red); cell numbers were determined from day 1 to day 3; serum was added after day 1*

When comparing cell numbers of p185 wt bone marrow cells to p185 EE/EE fetal liver cells in the course of the experiment, it can be recognized that there is

continuous increase in growth rate of the latter, whereas wild-type cell numbers did not change significantly.

## Apoptosis Assay

For apoptosis assay, 1 000 000 cells/ml of p185 wt bone marrow cells and p185 EE/EE fetal liver cells were seeded on a 6-well plate in triplicates. Cells were provided with serum-free medium to force the cells into apoptosis. Cell numbers were measured every day with CASY cell counter. Additionally cells were analyzed with FACS Canto II from day 1 to 3, to determine how many of the cells had already gone or are already on their way into apoptosis. Therefore cells were stained with PI and Annexin V.

In *Figure 10* and *Figure 11* such FACS plots can be observed. Cells are first negative for both, PI and Annexin V (live cells). But the more the cells get apoptotic, the more Annexin V is expressed. Cells, which only have high Annexin V expression, but are not yet positive for PI (early apoptotic), are still living cells, as PI just stains DNA and DNA is only accessible, when the plasma membrane of the cell is not intact anymore. Cells, which have already entered the apoptotic pathway and show already high expression of Annexin V, begin to shrink and the membrane is degraded. Thereby, PI is able to stain DNA inside the cells. Those cells are already dead, but have not been completely decomposed (late apoptotic). In the last quadrant (dead cells), Annexin V expression is already lost and PI levels are very high. Here, the cells are already dead and the only thing that is left are defragmented cells with DNA inclusions.

Cells used for the FACS blots in *Figure 10*, are p185 infected wild-type cells. As can be seen, there were no differences in the distribution of live cells, early apoptotic cells, late apoptotic cells and dead cells, when comparing triplicates (FACS blots from left to right). Cells were seeded in triplicates to minimize the probability for outliers. But also when looking at day 2 or day 3 of the experiment, there is no major change in distribution of the cells, just a few more percent of cells have undergone apoptosis after 3 days of starvation.

FACS blots in *Figure 11* show p185 infected β-catenin double mutant fetal liver cells going into apoptosis. Those seem to undergo the single steps of apoptosis

concerning Annexin V and PI levels, which are described above. Interestingly, the level of Annexin V in apoptotic p185 EE/EE cells is much higher as compared to wt p185 cells. p185 wt cells do not seem to express much Annexin V, when they are going into apoptosis, but immediately increase the levels of PI. There are hardly any cells in the early and late apoptotic sector present. But when looking now at p185 EE/EE cells, many cells can be observed which express those high levels of Annexin V, which are typical for apoptotic cells.

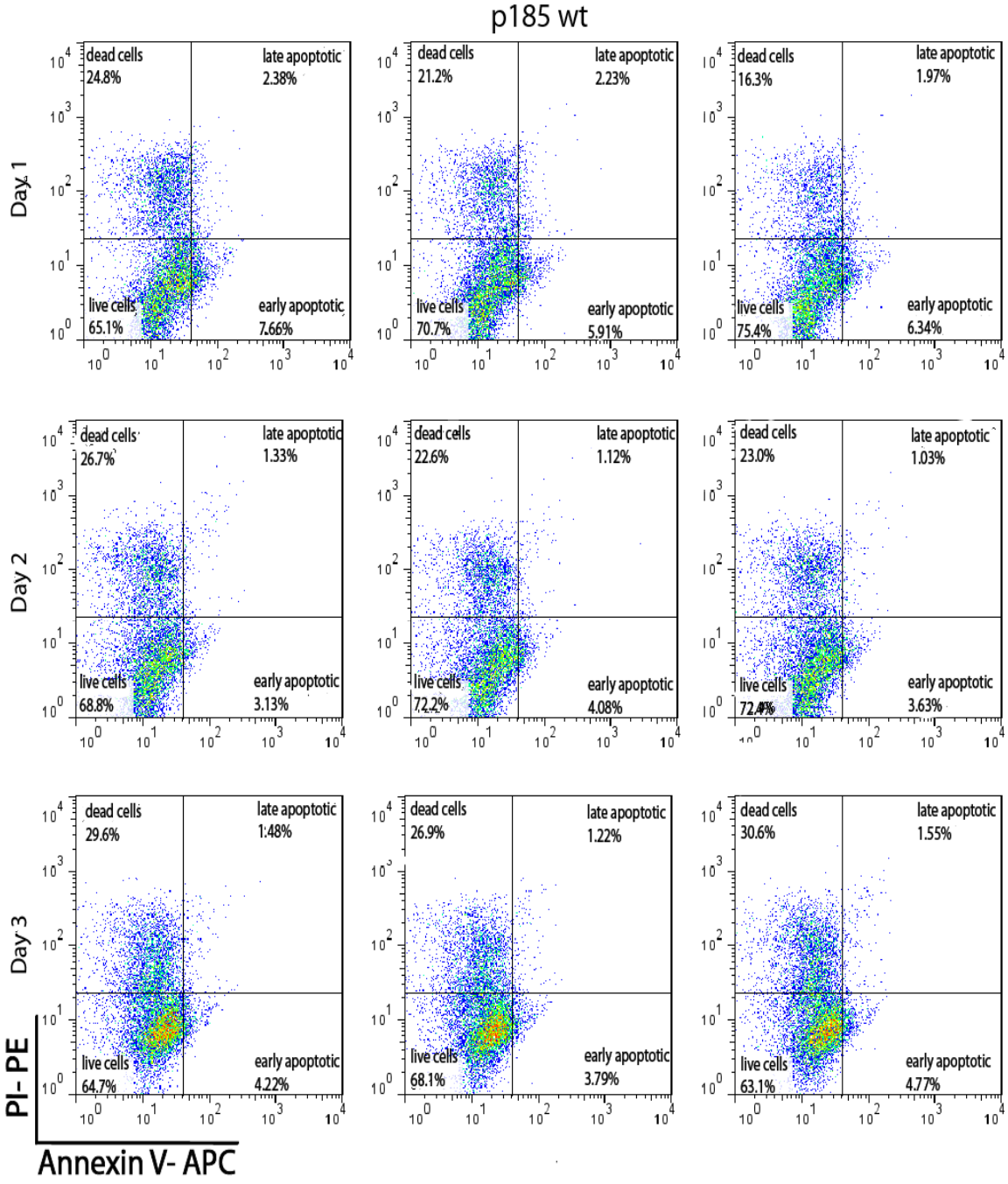


***Figure 10*** *FACS blots of p185 wt bone marrow cells going through the different stages of apoptosis; cells were seeded in triplicates (FACS blots from left to right) and kept under starvation conditions for 3 days to push them into apoptosis; live cells- quadrant: cells are negative for Annexin V and PI; early apoptotic- quadrant: cells are positive for Annexin V, but negative for PI; late apoptotic- quadrant: cells are positive for Annexin V and PI; dead cells- quadrant: cells are negative for Annexin V, but positive for PI*

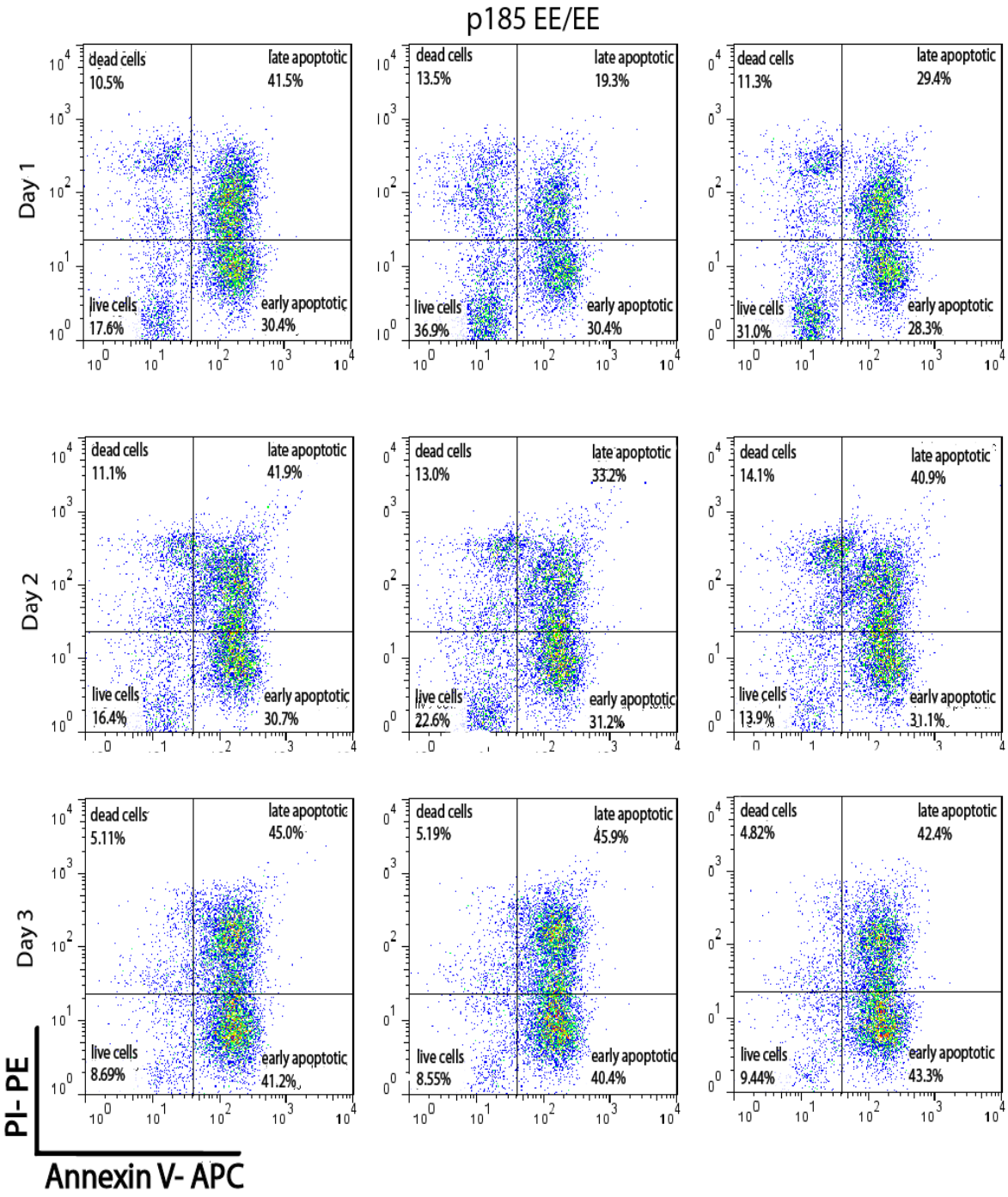


***Figure 11*** *FACS blots of p185 EE/EE fetal liver cells going through the different stages of apoptosis; cells were seeded in triplicates (FACS blots from left to right) and kept under starvation conditions for 3 days to push them into apoptosis; live cells- quadrant: cells are negative for Annexin V and PI; early apoptotic- quadrant: cells are positive for Annexin V, but negative for PI; late apoptotic- quadrant: cells are positive for Annexin V and PI; dead cells- quadrant: cells are negative for Annexin V, but positive for PI*

## Determination of Protein levels

To see whether an increased tumorigenicity of β-catenin Y654E in B-ALL has any influence on other downstream signaling pathways, Western Blot analysis was performed. Therefore, cell pellets were frozen in liquid nitrogen and lysed with RIPA buffer. Protein concentrations were calculated (Standard Curve: OD 0,264 ≙ 2,5µg) and brought to 15µg or 20µg with RIPA buffer, before they were separated according to their size on a polyacrylamide gel (see *Table 5*).

**Western Blot 1 (20µg protein):**

- ***p185 wt:*** 6,1µl lysate 1 + 13,9µl RIPA buffer; + 5µl SDS loading dye
- ***p185 EE/EE:*** 15,9µl lysate 1 + 4,1µl RIPA buffer; + 5µl SDS loading dye

**Western Blot 2 (15 µg protein):**

- ***p185 wt:*** 4,56µl lysate1 + 15,44µl RIPA buffer; + 5µl SDS loading dye
- ***p185 EE/EE:*** 11,9µl lysate 1+ 8,1µl RIPA buffer; + 5µl SDS loading dye

**Western Blot 3 (20µg protein):**

- ***p185 wt:*** 4,54µl lysate 2 + 15,46µl RIPA buffer; + 5µl SDS loading dye
- ***p185 EE/EE:*** 10,89µl lysate 2 + 9,11µl RIPA buffer; + 5µl SDS loading dye

Afterwards the proteins were transferred to a nitrocellulose membrane, which was treated with antibodies for proteins from different signaling pathways. Thereby the phosphorylated as well as the unphosphorylated forms of the proteins were analyzed. Those were phospho-STAT1 (p-STAT1), STAT1, phospho-STAT3 (p-STAT3), STAT3, phospho-STAT5 (p-STAT5), STAT5, phospho-p38 (p-p38), p38, phospho-Akt (p-Akt) and PI3K. β-actin was always used as loading control.

| | | Standard Curve | | Determined Values | |
|---|---|---|---|---|---|
| | | ***OD*** | ***Protein Concentration (µg/µl)*** | ***OD*** | ***Protein Concentration (µg/µl)*** |
| **Lysates for Western Blot 1 and 2** | ***P185 wt*** | 0,264 | 2,5 | 0,347 | 3,286 |
| | ***P185 EE/EE*** | 0,264 | 2,5 | 0,133 | 1,259 |
| **Lysates for Western Blot 3** | ***P185 wt*** | 0,264 | 2,5 | 0,465 | 4,403 |
| | ***P185 EE/EE*** | 0,264 | 2,5 | 0,194 | 1,837 |

***Table 5*** *Calculations of protein concentrations with the help of Bradford Assay; reference values were derived from a standard curve*

*Figure 12* shows the differences in protein levels of p185 infected wild-type bone marrow (p185 wt) and transgenic (p185 EE/EE) fetal liver cells. As can be observed from *Figure 12*, STAT1, p-STAT3, STAT5, p38 and PI3K levels seem to be unchanged, when comparing the two cell lines. But when looking at the phosphorylated forms of STAT1 (p-STAT1) and p38 (p-p38) and the unphosphorylated form of STAT3, protein levels seem to be lower in double mutant cells. The only protein which was up-regulated, was the phosphorylated Akt (p-Akt), which is known to act pro-proliferative and anti-apoptotic. β-actin was used as control as its protein levels should be the same for all cells.

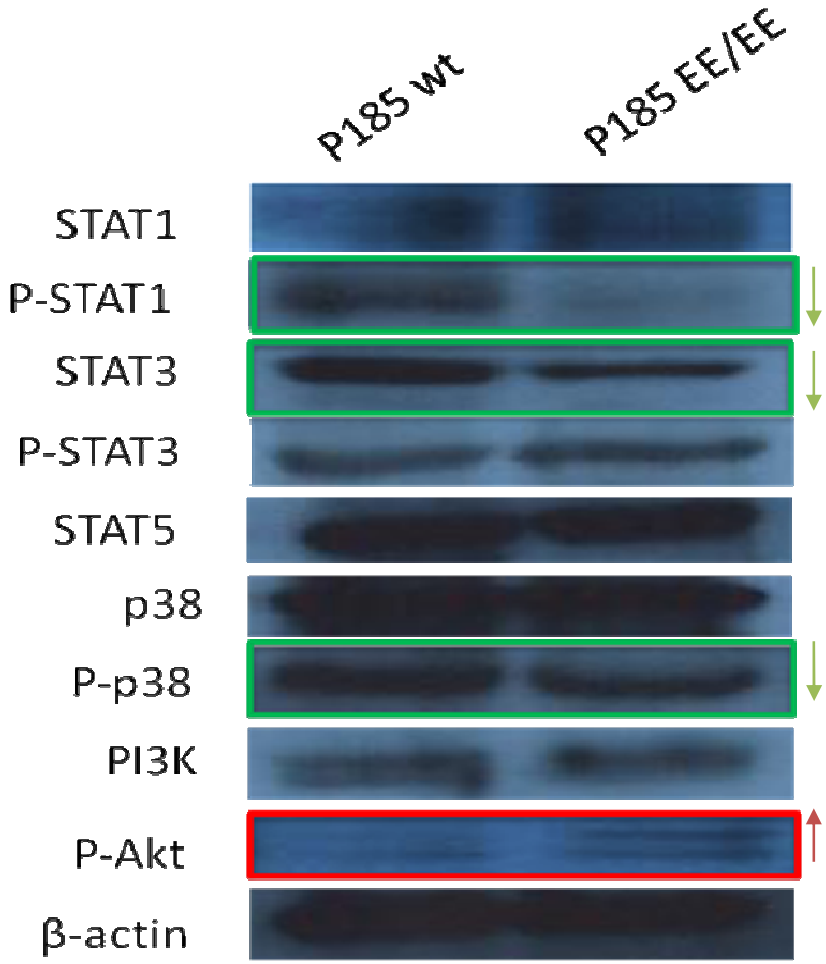


***Figure 12*** *Comparison of protein levels of p185 wt and p185 EE/EE cells; STAT1, p-STAT3, STAT5, p38 and PI3K levels are steady in both cell lines; increase in protein levels of p-STAT1, STAT3 and p-p38 are indicated with a green box; decrease in protein levels of p-Akt is indicated with a red box; β-actin is used as control*

## Conclusion

Although the hematopoietic system has been studied intensively since many years and although many mysteries concerning blood cells have already been solved, there are still various issues unclear concerning the origin of hematologic diseases. This is also the case for leukemia. There are several different forms of leukemia, all differing from each other through mutations or the cell type from which the disease evolves. It has already been shown that several types of leukemia, e.g. CML or B-ALL, are caused by a protein called BCR/ABL [2,3,4]. This fusion protein, which is caused by a chromosomal translocation, is also known under the name Philadelphia chromosome. It results in a constitutively active tyrosine kinase, which continuously phosphorylates itself and other proteins. Thereby, several signaling pathways are hyperactivated, leading to an imbalance in signaling pathways and resulting in a hyper-proliferation of specific cell types.

In case of BCR/ABL- induced leukemia, there are different possibilities how the disease evolves, as there are different forms of the fusion protein. The p230 version causes chronic neutrophilic leukemia [6], whereas BCR/ABL p210 causes a chronic myelogenous leukemia, which is characterized by immense increase in myeloid cells [2]. The third isoform, p185, is implicated as a cause of childhood leukemia and it leads to an increase of immature B-cells. Therefore, this disease is called acute B-lymphocytic leukemia or B-ALL [4].

The main focus in this study lies on the two shorter versions of BCR/ABL, namely p185 and p210. For p210-induced leukemia, the cell of origin could already be identified, which is most likely to be a stem cell [19 and Kovacic et al 2012]. In contrast, p185-induced leukemia has been shown to arise in a particular cell type, which are immature B-cells. Interestingly, it has recently been identified that BCR/ABL-induced B-ALL already manifests in the hematopoietic stem cells [21 and Kovacic et al 2012].

For an effective therapy, it would be of major importance to possess this knowledge. Until now, there are only few possibilities to treat leukemia, but

none of those are perfect. Identifying the interacting proteins of BCR/ABL could be one way to find a promising therapy.

In 2007, a paper was published, showing that the Wnt-signaling protein β-catenin is an interaction partner of BCR/ABL p210. Furthermore, β-catenin has been identified to be of major importance for the progression of CML, as the development of this form of leukemia is not possible anymore, when the protein is knocked out *in vivo*. However, the knock-out mice were still able to develop B-ALL, which may indicate an independent role of β-catenin in B-ALL [10]. However, the researchers did not show whether or not the interaction between β-catenin and BCR/ABL p185 is essential for the development of B-ALL.

To investigate this issue, genetically modified mice were received from the lab of Dr. Ron Smits. The mice were designed to carry a point-mutation in their endogenous β-catenin. This mutation is a Y to E mutation at the residue 654, which resembles a phosphorylated tyrosine, thus resulting in a hyperactive transcriptional activity of the protein. Homozygous mice are embryonically lethal, whereas the heterozygous ones are prone to develop intestinal tumors [20].

To circumvent the lack of adult homozygous β-catenin EE/EE mice, fetal liver cells were isolated from embryos and infected with BCR/ABL p185 or BCR/ABL p210. Thereby we wanted to elucidate the role of a gain-of-function mutation of β-catenin in CML and B-ALL.

To see which of the three genotypes, YY/YY, YY/EE or EE/EE, are more prone to develop tumors, a colony formation assay was established. In parallel, the embryos had to be genotyped by PCR. We observed that only the genotypes of litter 2 were distributed according to Mendelian rules (see *Figure 4*). A possible explanation for this could be that the elder generations of the mice were not correctly genotyped and hence, the crossings were not set up correctly, as it is very unlikely to receive only wild-type embryos when a YY/EE mouse is crossed with a YY/EE mouse.

For colony formation assay, BCR/ABL-infected cells were seeded in methylcellulose, which should provide a surrounding, where only leukemic cells are able to survive and form colonies, as growth factors for cell growth of normal cells are very scarce.

The seeded cells were incubated until the colonies were big enough to be counted under the microscope. However, some of the experiments did not progress as expected and hence, colonies developed in only one out of three trials.

The reason for the non-functioning of the other trials could be that infection efficiencies of the packaging cell lines were simply too low. Another cause could have been that the density of the seeded cells was too low, as for litter 3 at least the liquid cultures were able to grow and form cell lines (data not shown), which is very unlikely to happen with non-transformed cells.

It is important to mention that we observed colonies in all cases of GFP-infected fetal liver cells. Normally, GFP-infected fetal liver cells are not able to proliferate in methylcellulose. As can be seen from *Table 2*, GFP-infected cells formed more colonies than p210- infected ones, which was expected to be exactly the other way round. p210- infected fetal liver cells in contrast did only form few colonies, independent of the genotypes. One explanation for this strange trend is that the plasmids used for transfection were accidentally interchanged. If so, the generated data would be consistent with the study performed by Reya et al., who could show that β-catenin knock-out mice were not able to develop CML, however, they could still sicken from B-ALL, which would indicate a difference in interaction of p185 and p210 with β-catenin.

p185- infected EE/EE fetal liver cells formed the most colonies, comparing them to YY/YY and YY/EE cells infected with the same virus, which might be an evidence for improved interaction of the oncoprotein with the mutated β-catenin or for enhanced signaling downstream of the BCR/ABL/β-catenin complex.

When comparing now p210- or GFP- infected fetal liver cells to p185- infected cells, it can be seen that there is quite a difference in colony formation capacity. Even if the vectors were interchanged, we can show that there is discrepancy in the mode of interaction of β-catenin with BCR/ABL p185 and BCR/ABL p210.

The rest of the infected fetal liver cells that were not needed for Colony Formation Assay, were plated in liquid medium, where they were allowed to grow until cell lines were established. These cell lines are characterized by a homogeneous culture of immortalized cells. They were further grown to density from where they could be used for subsequent FACS analysis.

FACS analysis was performed to learn more about the expressed surface receptors, which are indicative of a certain cell type. Cells expressing high levels of Mac1 and Gr1 are of myeloid origin [22] and contain monocytes, granulocytes and macrophages. In contrast, CD19 positive cells represent B-cells [23]. When analyzing p210- infected fetal liver cells, we could observe that these cells were CD19+ and IL7R+ -just like p185-infected fetal liver cells (see *Figure 8*).

This result contradicts other studies, showing that p210 induces CML, which is characterized through an over-production of myeloid cells that are Mac1+ and Gr1+, but not CD19+ [22]. Again, there is the possibility for an interchange of vectors used for transduction. Most likely, the borrowed plasmids were already wrongly labeled, but this has not been investigated yet.

To see whether or not cell lines derived from various β-catenin fetal liver cells (YY/YY, YY/EE or EE/EE) have a modified proliferation rate, a proliferation assay was established. At the time point when this experiment was performed and before the experiments with litter 3 were started, the only cell line to which the p185 EE/EE cells could be compared, were p185 wild-type bone marrow cells that had already been in culture for several passages. Similarly to p185-infected fetal liver-derived cell lines, this cell line is composed of CD19+/IL7Rα+ cells albeit derived from bone marrow cells. These cells were not the perfect cell type control, but they have represented the only choice at the time point of

assay performance. When looking at the growth curves in *Figure 9*, it can be seen that the cells are stagnant from day 1 to day 2, which is probably due to adaptation issues. Then, cell numbers increase on the next two days.

This early decrease of cell numbers was very unexpected, as there should still be nutrients for the cells in the medium. Furthermore, also cell numbers for apoptosis assay did not decrease that fast. One possibility is that the cells were simply too dense and thereby inhibited the growth of each other.

When comparing now proliferation capability of p185 wt and p185 double mutant cells with each other, p185 EE/EE fetal liver cells seem to grow faster than p185 wild-type bone marrow cells (*Figure 9*). Differences in growth already become significant at day 2, where double mutant cells are already much higher in number. At day 3, the distinction becomes even more significant. Nevertheless, this experiment should be repeated with smaller cell numbers to avoid the stagnation of proliferation on day 2. In addition, wild-type and heterogeneous p185 infected fetal liver cells should also be included to compare all three genotypes.

The same cells, which were used for proliferation assay, were also used for apoptosis assay. The cells were seeded in triplicates with equal cell numbers and deprived of medium for three days to induce apoptosis. To see which cells first undergo apoptosis, samples were taken from both cell lines and stained every day with PI and Annexin V. Those were accordingly analyzed with FACS.

When looking at p185 wt FACS blots from day 1 to 3, it can be seen that the numbers of apoptotic cells are not augmented (see *Figure 10*). There was no shift in distribution of living, early apoptotic, late apoptotic and dead cells. However, we observed a gradual progression of apoptosis from day 1 to day 3 in p185 EE/EE cells (see *Figure 11*). But there is another prominent difference, when comparing wild-type to double mutant cells. Normally, apoptosis can be distinguished from necrosis by an increased expression of Annexin V, which binds phosphatidylserine and thereby destabilizes the cell membrane. In course of regulated cell death, the cell is gradually degraded (see *Figure 13*) [24].

In the experiment described here, cells were stained with Annexin V and PI. Thereby the different phases of apoptosis should be visualized. Cells in the live cell gate, express low levels of Annexin V and PI is also hardly present. When the cells progress in apoptosis, the expression of Annexin V is increased, but PI levels are still low. Those cells are still living but early apoptotic. In the late apoptotic phase, many cells are already dead, but still not degraded. Here, PI levels as well as Annexin V levels are high. When the cells have finally undergone apoptosis, the cells are destroyed and PI is fully able to stain DNA, therefore its expression is high. Annexin V is now nearly gone.

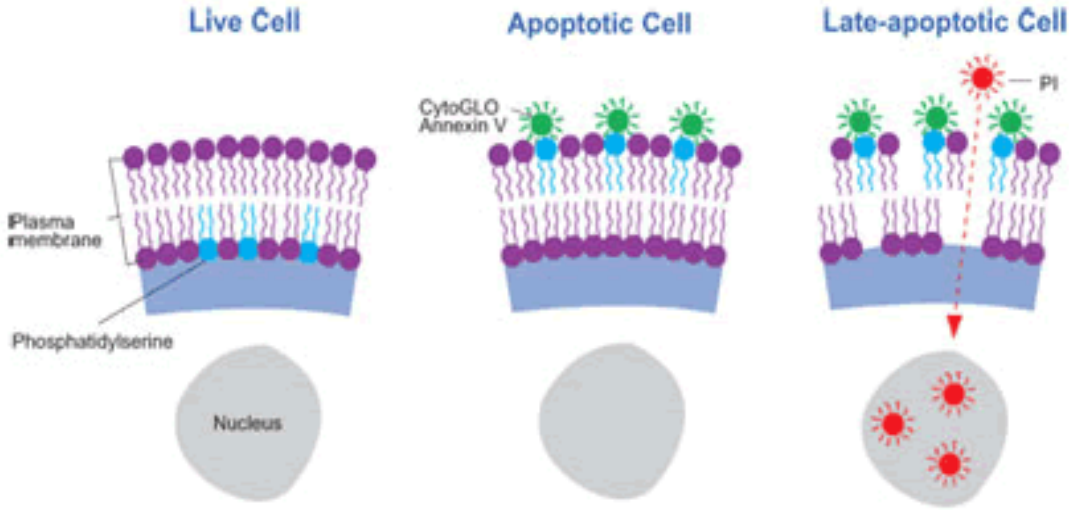


***Figure 13*** *Stages of Apoptosis; Live Cells: Phosphatidylserine is on the inner membrane, the membrane lipids are still in balance; Apoptotic Cell: Annexin V integrates into the membrane and induces flipping of Phosphatidylserine to the outer membrane; Late-apoptotic Cell: the cell membrane is destabilized through shift of lipids and PI is able to enter the nucleus and stain the DNA* [25]

This process can be easily followed when looking at the FACS blots in *Figure 11*, where cells traverse through the stages of early and late apoptosis. But when comparing now those FACS blots to the FACS blots in *Figure 10*, they seem to miss early and late apoptotic phase and immediately show high levels of PI. Annexin V is hardly present. This lack of apoptotic markers might indicate that p185 wt cells did not gradually undergo apoptosis, but instead directly underwent necrosis.

For molecular analysis of differences between the β-catenin genotypes, the cell line from litter 2, namely p185 EE/EE, was used. As a control, the cell line established from p185 infected wild-type bone marrow (p185 wt) was used. This cell line was already in culture for several passages and was the only available control cell line at the time of the experiment.

Equal protein amounts were loaded on a polyacrylamide gel and separated according to their size. The nitrocellulose membranes were stained with antibodies for proteins from different signaling pathways. Among those were proteins from the JAK-STAT (STAT1, STAT3, STAT5), MAPK (p38) and PI3K (PI3K, Akt) signaling pathway. We have analyzed the levels of unphosphorylated as well as phosphorylated proteins.

When comparing protein levels of p185 wt cells to protein levels of p185 EE/EE cells, some major differences could be observed. Proteins which were down-regulated in double mutant cells were p-STAT1, total STAT3 and p-p38 (see *Figure 12*). The only protein, which was up-regulated in p185EE/EE cells, compared to wild-type cells, was the phosphorylated form of Akt, which is known to have anti-apoptotic and pro-proliferative effects [26]. β-actin was used as control, as this protein is known to be a housekeeping gene, which should have equal protein levels throughout all cells.

However, differences in protein levels could also be due to the different cell types which were used. There might be more active signaling in bone marrow cells than in fetal liver cells in general.

All in all, some interesting data were obtained, which give some indications for the importance of β-catenin during B-ALL formation, as can be seen from colony formation assay. However, whether this protein drives the B-ALL formation more actively than the CML formation has to be carefully investigated in future experiments.

Furthermore, it could be shown that β-catenin EE/EE cells infected with BCR/ABL p185 have a proliferative advantage compared to cells with normal

levels of this protein. However, the difference in Annexin V levels between wild-type and double mutant cells, cannot be explained yet. Whether this effect is indeed due to apoptosis or to some other deregulated up-stream signals, should also be elucidated in follow-up experiments.

The B-cell phenotypes from FACS analysis were also very surprising, as p210 cells were expected to express myeloid receptors and therefore show a myeloid phenotype in general. Also here, a plausible explanation is a possible interchange of vectors at the very beginning.

Why of all those tested proteins, which are known to be deregulated in certain cancers, p-Akt was the only up-regulated protein, can also not be explained until now. It is feasible that leukemic cells acquire the ability to evade apoptosis and become more active in proliferating. However, Akt is one of the major proteins that regulate apoptosis by exerting anti-apoptotic and pro-proliferative signals. It is possible that strong AKT signaling that is active in EE/EE p185-infected cells, can also explain the increased proliferation of these cells. This conclusion is not consistent with the data from the apoptotic assay. However, a block at the early stage of apoptosis could also explain the higher proliferation rate. In order to find out about the exact signaling cascade in which Akt is involved, up- and down-stream targets, like Bad, Mad or Caspases have to be looked at in the future. Working on the signaling cascade could also give evidence on whether wild-type cells tested here, were necrotic or not.

Of course, all experiments have to be repeated several times with higher sample numbers to be able to receive significant results. Furthermore, a better control should be used for further biochemical analysis and experimental set-ups have to be improved.

In the future, many other experiments, like *in vivo* studies, should also be performed. p185- or p210-infected β-catenin fetal liver cells of all three genotypes can be sorted according to their cell types and are then injected into mice to evaluate their leukemia induction capacity *in vivo*. Also the cell lines grown from the infected fetal livers could be injected into the mice to see if they

are able to induce leukemia. So there are still many options for further processing of this study.

In case that the above-mentioned experiments provide results, which give an indication that β-catenin is a significant factor during CML or B-ALL formation, this might represent a big step forward in the treatment of leukemia.

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## Summary

The fusion oncogene BCR/ABL is the cause of about 90% of all chronic myeloid leukemia (CML) and of about 30% of all acute B-cell lymphoid leukemia (B-ALL) in patients. The 210kDa isoform of BCR/ABL has been associated with CML, whereas the 185kDa isoform induces B-ALL in humans and mice.

Recently, BCR/ABL p210 oncoprotein has been suggested to directly interact with the tyrosine 654 of β-catenin, hereby implicating the Wnt-signaling in the progression of BCR/ABL+ CML to blast crisis. However, it is still unclear whether this interaction may be accounted to an aggravated phenotype in BCR/ABL+ CML. In addition, it has not yet been clarified whether this interaction is essential for the development of BCR/ABL p185-induced B-ALL.

Using a novel transgenic mouse model mimicking the phosphorylated tyrosine 654 residue of β-catenin (Y to E-mutation), we show that fetal liver cells carrying a double mutant genotype exert a highly increased transformating capacity after infection with BCR/ABL p185. Also the proliferation ability of these cells is increased compared to wild-type cell lines. Interestingly, we further show that EE/EE leukemic cells react with increased Annexin V levels upon serum-withdrawal, whereas the necrotic cells are strongly reduced. Lastly, we suggest that the AKT- pathway –among others- might be a key player downstream of the BCR/ABL/β-catenin complex and essential for the regulation of cell survival in B-ALL. While phospho-AKT levels are increased in BCR/ABL p185+ EE/EE leukemic cells, we observed a down-regulation of STAT1-, STAT3- and p38-dependent signaling.

Taken together, the constitutively active β-catenin might be essential for an increased survival and proliferation of leukemic cells in BCR/ABL p185+ B-ALL and in blast crisis CML. Hence, our data provide an insight into the molecular biology of BCR/ABL+ leukemia and could also establish a basic concept for a potential drug interference with progressing leukemia.

## Zusammenfassung

Das Fusionsonkogen BCR/ABL ist die Ursache von zirka 90% aller chronisch myeloischen Leukämien (CML) und von zirka 30% aller akuten lymphatischen B-Zell-Leukämien (B-ALL) in Patienten. Die 210kDa Isoform von BCR/ABL wurde mit CML in Verbindung gesetzt, wobei die 185kDa Isoform B-ALL in Menschen und Mäusen auslöst. Erst kürzlich wurde darauf hingewiesen, dass das BCR/ABL p210 Onkoprotein direkt mit dem Tyrosin 654 von β-Catenin interagiert -was die Bedeutung des Wnt-Signalwegs mit dem Fortschreiten von BCR/ABL + CML zur Blastenkrise impliziert. Nichtsdestotrotz ist es noch immer unklar, ob diese Interaktion zu einem verstärktem Phänotyp in BCR/ABL+ CML beiträgt. Darüber hinaus konnte noch nicht geklärt werden, ob diese Interaktion für die Entwicklung von BCR/ABL p185- induzierter B-ALL essentiell ist.

Unter Verwendung eines neuen transgenen Mausmodells, welches das phosphorylierte Tyrosin 654 von β-Catenin nachahmt (Y zu E Mutation), konnten wir zeigen, dass fötale Leberzellen mit einem Doppelmutante-Genotyp ein erhöhtes Transformationspotential aufweisen, nachdem sie mit BCR/ABL p185 infiziert wurden.

Ebenso war das Proliferationsvermögen dieser Zellen erhöht verglichen mit den Wildtyp-Zelllinien. Interessanterweise konnten wir weiters zeigen, dass EE/EE leukämische Zellen mit erhöhten Annexin V-Levels auf Serumentzug reagieren, wohingegen die nekrotischen Zellen stark reduziert sind.

Zuletzt schreiben wir dem PI3K/AKT-Signalweg unterhalb vom BCR/ABL- β-Catenin-Komplex eine Hauptrolle in der Regulation der erhöhten Überlebensrate der Krebszellen zu.

Während phospho-Akt Levels in BCR/ABL+ EE/EE leukämischen Zellen erhöht sind, konnten wir eine verminderte Expression von STAT1-, STAT3- und p38-abhängiger Signaltransduktion feststellen.

Zusammengefasst könnte das konstitutiv-aktive β-Catenin eine essentielle Rolle bei einer erhöhten Überlebens- und Proliferationsrate von leukämischen Zellen

in einer BCR/ABL+ B-ALL und einer Blastenkrise- CML spielen. Somit liefern unsere Daten einen Einblick in die Molekularbiologie von BCR/ABL+ Leukämien und könnten ebenfalls ein grundlegendes Konzept für eine mögliche Interferenz von Medikamenten in einer fortschreitenden Leukämie etablieren.

# Curriculum Vitae

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