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Direktor: Prof. Dr. med. Jan-Henning Klusmann

Expression of Endogenous Retrovirus 3 (ERV3) in Tumor Cells

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von Yomara Yardén Bustamante Rivera

geboren am 18.07.1984 in Guatemala

Betreuer: apl. Prof. Dr. rer. nat., rer. medic. habil. Martin Sebastian Staege

Gutachter:
1) Apl. Prof. Dr. Malte Kornhuber, Sangerhausen/Halle(Saale)

2) PD Dr. Benno Weigmann, Erlangen

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Referat

Objectives: The human endogenous retrovirus 3 (ERV3) might be involved in the biology of various diseases including cancer. Read-through transcript of ERV3 and the neighbouring gene zinc finger protein 117 (ZNF117) exists and ZNF117 seems to contribute in the biological effects of ERV3. In this study the aim was to characterize the expression and the function of the ERV3/ZNF117 locus in tumor cells.

Methods: In order to characterize the consequences of expression of ERV3 and ZNF117, an inducible vector system was used. The plasmids were transfected into the well characterized Ewing sarcoma cell line A673. Expression of the transgenes was induced with doxycycline and the expression of ERV3, ZNF117 and the fusion transcript ERV3-ZNF117 was investigated by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). In addition, expression of the mentioned transcripts in different tissues was investigated by qRT-PCR. Finally, mixed lymphocyte-tumor cell cultures were used for characterization of the immunostimulatory activity of transgenic cells.

Results: Expression of ERV3 in normal tissues and tumor cells was as reported in the literature with exceptional high expression in placenta. ERV3 was induced in cell cycle arrested B cells. The expression of ZNF117 in tissues and tumor cells was not correlated with the presence of ERV3 and *vice versa*. Transgenic over-expression of ERV3 had no effect on the expression of ZNF117 or the fusion transcript. Similarly, transgenic over-expression of ZNF117 had no effect on expression of ERV3. Expression of Ewing sarcoma associated genes was not altered in ERV over-expressing cells and no effect of ERV3 expression on the immunostimulatory activity of the cells was observed. As an interesting side aspect of the study, it was observed that doxycycline might be able to down-regulate Ewing sarcoma associated genes like cyclin D1 with concomitant reduction of cell proliferation.

Conclusions: ERV3 upregulation does not induce expression of ZNF117 or the fusion transcript ERV3-ZNF117 and *vice versa*. Further studies are needed to understand better the function of ERV3 in pathologies and its relation with ZNF117.

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Abbreviations

bp	base pair
BC	buffy coats
°C	degree Celsius
CCND1	Cyclin D1
cDNA	complementary DNA
CHL	Classical Hodgkin lymphoma
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ERV	endogenous retrovirus
ES	Ewing sarcoma
EVE	endogenous viral elements
EWS-FLI1	oncogenic fusion protein
FACS	fluorescence activated cell scanning
FCS	fetal calf serum
FLI1	Friend leukemia virus integration site 1
FOXP3	forkhead box P3
FSC	forward scatter
g	g-force
GATA3	GATA-Bindingprotein 3
GFP	green fluorescent protein
HERV	human endogenous retrovirus
HL	Hodgkin Lymphoma
HPLC	high performance liquid chromatography
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
IL-2	Interleukin-2
LIPI	Lipase I
min	minute
mL	milliliter
mM	millimole

ng	nanogram
nM	nanomole
ORF	open reading frame
OS	overall survival
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein complex
PI	propidium iodide
qRT-PCR	real-time quantitative reverse transcription-polymerase chain reaction
RNA	ribonucleic acid
RORC	retinoic acid receptor-related orphan receptor C
RPMI	Roswell Park Memorial Institute Medium
TBX21	T-box transcription factor TBX21
TAE	Tris-acetate-EDTA
U	units
UV	Ultraviolet
µg	microgram
µL	microliter

1. Introduction

The following introduction was partially published (Bustamante et al., 2018).

1.1 Endogenous viral elements (EVE)

Several virus species like parvovirus B19 or Epstein Barr virus have the ability of lifelong latent infections in humans (Norja et al., 2006; Thorley-Lawson et al., 2013). Sometimes this persistence is associated with the integration of the virus in the host genome (Wang et al., 2015). If this integration happens in the germ line, there can be a vertical transmission of the virus like an ordinary gene (Feschotte and Gilbert, 2012). Endogenous viral elements (EVE) can become stable genetic elements of the host species if their presence causes no detrimental effects in the host (Villesen et al., 2004). This is how several virus families have entered eukaryotic genomes, among them for example: borna-, bunya-, circo-, filo-, flavi- hepadna-, herpes-, orthomyxo-, parvo-, reo-, rhabdoviruses, (Belyi et al., 2010; Katzourakis and Gifford, 2010; Gilbert et al., 2014; Thézé et al., 2014; Sofuku et al., 2015; Tweedy et al., 2016), as well as a large number of retroviruses (Benveniste et al., 1974; Janeway et al., 1980; Vargiu et al., 2016).

Endogenous retroviruses (ERV) are the largest group of EVE constituting more than 8% of the human genome (Griffiths, 2001). Millions of years ago, ERV appeared as an integral part of the genome of nearly all higher eukaryotes (Heidmann et al., 2009). Human ERV (HERV) integrated in the genome up to 100 million years ago (Belshaw et al., 2004; Escalera-Zamudio and Greenwood, 2016). ERV can be either a complete or a partial retroviral sequence integrated as a normal component of the DNA of all cells. They are transmitted in a Mendelian way (Muir et al., 2004). The infectious retrovirus integration of HERV in the germ line DNA and therefore in the offspring, has played an important role in the evolution (Deininger et al., 2003). ERV has a preferential location on the Y chromosome of humans, chimpanzees and orang-utans (Sin et al., 2010). Probably this preference is caused by reduced recombination events on the Y chromosome (Kjellman et al., 1995).

Various genetic and epigenetic mechanisms inactivate EVE (Escalera-Zamudio and Greenwood, 2016). Among the genetic mechanisms are deletions, inversions and point mutations in the open reading frames of the viral proteins. Therefore, most EVEs are no longer capable to replicate and to form virus particles. However, a release of EVE virus particles has been observed in certain cancer types as well as in other diseases (Wang-Johanning et al., 2007; Volkman and Stetson, 2014). Apart from this, epigenetic mechanisms play an important role

for EVE regulation. They are reversible and, consequently, reactivation of EVE can occur under physiological and pathological conditions (Blazkova et al., 2009; Lee et al., 2012).

Representing the largest number of EVE in the human genome, HERV and HERV like elements (HERVLE) have shown to be reactivated in certain types of cancer (Bannert and Kurt, 2004). Reactivated HERVLE modulate expression of adjacent genes and they also act as alternate promoters for varying cellular genes in Hodgkin lymphoma and Non-Hodgkin lymphoma cells (Huff et al., 2005; Lamprecht et al., 2010; Lock et al., 2014; Babaian et al., 2016). HERVLE associated promoters or enhancers have been found in many other human genes including midline 1 (Landry et al., 2002), endothelin receptor type B (Medstrand et al., 2001), apolipoprotein C1 (Medstrand et al., 2001), beta-1,3-galactosyltransferase 5 (Dunn et al., 2003), insulin like 4 (Bièche et al., 2003), interleukin-2 receptor beta chain (Cohen et al., 2011), CD5, (Renaudineau et al., 2005), and prolin dehydrogenase 1 (Suntsova et al., 2013).

A physiological function is only known for a few ERVs, for example, the preserved open reading frame of the envelope gene of ERVW-1 (de Parseval et al., 2003). The ERVW-1 envelope, also known as syncytin 1, is expressed almost exclusively in the placenta and it was demonstrated to be involved in trophoblast fusion during placental development (Mi et al., 2000; Muir et al., 2004). There is also a syncytin 2, encoded by the envelope gene from HERV-FRD-1 and likewise involved in human placenta development (Esnault et al., 2008). It seems probable that other preserved EVE-related open reading frames in eukaryotic genomes have additional, yet unknown, physiological or patho-physiological functions.

Until now there is no universally accepted classification of ERV. They have been grouped into classes based on sequence similarities (Blomberg et al., 2009). ERV contain over 200 distinct groups and subgroups. They have been divided into 3 broad classes: Class I ERV is related to gammaretroviruses and include human ERVE and ERV3; Class II ERV is related to betaretroviruses and include human ERVK and mouse mammary tumor virus; Class III ERV is related to *Spumaretrovirinae* and include ERVL. Human ERV groups are predominantly named as HERV or ERV, as in HERVK and ERV3 (Katzourakis and Tristem, 2005).

1.1.1 Endogenous retrovirus 3 (ERV3)

ERV3 (also known as HERV-R) is unique to the great apes and Old World monkeys. It has been inserted into the primate genome 30-40 million years ago. In this respect, ERV3 is located in great apes, monkeys and humans at an identical genomic position, with the exception of *Gorilla* where it has not been found. Notwithstanding, some Gorilla sequences seem to have a

similarity to human ERV3 (Bustamante et al., 2018; Kim et al., 2006). As a result of being the third identified human endogenous retrovirus locus (after ERV1 and virus 51-1), it was named ERV3. In the mid-80s, ERV3 was isolated from human DNA and cDNA libraries (O'Connell et al., 1984; Cohen et al., 1985). Human ERV3 is located on chromosome 7, at 7q11. It has sequence similarities with mammalian type C retroviruses which qualify it as a class I ERV (Kim et al., 2000).

Some of the transcripts from the ERV3 locus contain sequences from a zinc finger protein (ZNF117) with an unknown function (Kato et al., 1990). It is interesting that these read-trough transcripts were more abundant in peripheral blood mononuclear cells (PBMC) from patients with multiple sclerosis than in PBMC from healthy individuals. Nevertheless, a link between the ERV3 locus and multiple sclerosis has not yet been established (Clausen, 2003). Approximately 40 ERV3-like elements are in the human genome but only the copy on chromosome 7q11 has a complete open reading frame for a viral envelope protein. The other open reading frames are inactivated by non-sense mutations (Kannan et al., 1991; Kjellman et al., 1995; Andersson et al., 2005). There has not been any observation regarding associations between diseases and the presence of polymorphisms in the long terminal repeat (LTR). In addition, there has not been an association concerning the non-sense mutations in the ERV3 open reading frame that lead to truncated proteins (Rasmussen et al., 1996; Rasmussen and Clausen, 1998).

In normal placenta, ERV3 is highly expressed in the first trimester. An immunosuppressive function in the context of mother-fetus interaction has been proposed (Venables et al., 1995; Holder et al., 2012). During embryogenesis, ERV3 is expressed in an organ-specific way suggesting that it plays a role in the development and differentiation of human tissues (Andersson et al., 2002). In addition to placenta, several normal tissues have detectable ERV3 transcripts (summarized in Bustamante et al., 2018) such as:

- Lymphoid organs (spleen, lymph nodes, thymus)
- The gastro-intestinal tract (stomach, duodenum, small bowel, appendix, colon, rectum)
- The endocrine system (adrenal glands, thyroid)
- The urinary system (kidney, urinary bladder)
- Male and female reproductive system (testis, corpus luteum, Fallopian tubes)
- The respiratory system (lung bronchial epithelium)
- Astrocytes, sebaceous glands, and salivary glands

1.1.2 ERV3 and pathologies

ERV encoded antigens can be recognized by cytotoxic T cells and this immune system stimulation might be involved in autoimmunity (Haist et al., 1992). ERV3 is upregulated by cytokines in endothelial cells (Sasaki et al., 2009). In addition, antibody cross-reactivity between exogenous retroviruses and ERV3 peptides have been described (Katsumata et al., 1999). The possible involvement of ERV3 in autoimmunity requires further investigation. Until now it has been suggested as an auto-antigen involved in different immunopathologies. It was found upregulated in blood cells but downregulated in skin biopsies with morphea. Moreover, it was not only detected in synovial tissues from patients with rheumatoid arthritis and osteoarthritis but also in synovial tissues of healthy individuals (Takeuchi et al., 1995; Li et al., 1996; de Parseval et al., 1999; Blank et al., 2009; Nelson et al., 2010; Kowalczyk et al., 2012; Nelson et al., 2014).

Even though ERV3 was considered to be a tumor suppressor, an involvement in the pathogenesis of many diseases is possible based on its upregulation in different cancer types (Matsuda et al., 1997; Lin et al., 1999; Lin et al., 2000). ERV3 is highly expressed in normal placenta but is absent in choriocarcinoma (Cohen et al., 1988; Kato et al., 1988). Together with syncytin 1 and syncytin 2, ERV3 is downregulated in hydatidiform moles and malignant gestational trophoblastic tumors compared to normal placenta (Bolze et al., 2016). ERV3 is upregulated together with other ERV in endometrial carcinoma samples with low differentiation state (Strissel et al., 2012).

In ovarian cancer, ERV3 is co-expressed with other ERV species. In effect, 30% of ovarian cancer patients have antibodies against ERV3 whereas such antibodies are not detectable in healthy controls (Wang-Johanning et al., 2007). In breast cancer, a recent study has indicated an increased level of ERV3 in the blood of untreated patients. After therapy, these levels of ERV3 decreased (Rhyu et al., 2014). Elevated presence of ERV3 has been detected in colorectal, lung and liver cancer as well as in prostate cancer cells (Wang-Johanning et al., 2003; Ahn and Kim, 2009; Lee et al., 2014). An upregulation of ERV3 was detected after irradiation of head and neck squamous cell carcinoma cells (Michna et al., 2016), during monocytic differentiation of acute myelogenous leukemia cells (Larsson et al., 1997; Abrink et al., 1998) as well as during differentiation of normal squamous cells (Otsuka et al., 2006). Expression of ERV3 is high in cell cycle arrested leukemia cells (Abrink et al., 1998). Proliferating lymphoma cells have less levels of ERV3 RNA compared to the levels found in

growth inhibited lymphoma cells (Kewitz and Staege, 2013). ERV3 seems to be expressed preferentially in differentiated or growth inhibited cells compared to proliferating tumor cells.

1.2 Ewing sarcoma

In 1921, James Ewing described a tumor as a "diffuse endothelioma of bone" (Ewing, 1921). He reported a round cell sarcoma of the radius in a 14-year-old girl. Today, many of the described characteristics in his original report are still very important (Ozaki, 2015). Ewing sarcoma (ES) is a highly malignant tumor. It is the second most common primary malignant bone tumor in children and adolescents (Tirode et al., 2014; Ozaki, 2015; Tu et al., 2017). The maximum incidence is during adolescence and young adulthood, where the mean of diagnosis is at the age of 15 years (Tirode et al., 2014; Gaspar et al., 2015). The annual incidence of Ewing sarcoma is approximately 3 per million. Males are affected more frequently than females (with a ratio of 3:2) (Tirode et al., 2014; Ozaki, 2015). The incidence of ES differs markedly among populations. It is rare among black children and the Chinese population, versus a 6 times higher incidence among Caucasian children (Ozaki, 2015).

Histologically, Ewing sarcoma is composed of uniformly undifferentiated small round basophilic cells (Ozaki, 2015; Tu et al., 2017). The tumor cells often have round nuclei, little cytoplasm but abundant cytoplasmic glycogen and express CD99 on the plasma membrane (Tirode et al., 2014; Ozaki, 2015).

In his first reports, James Ewing proposed an endothelial origin for the sarcoma (Ewing, 1921). There is supporting evidence of a mesenchymal stem cell origin of Ewing sarcoma (Tu et al., 2017). On the other hand, there is the hypothesis of neural crest origin supported by morphological features like rosette formation (Ozaki, 2015). Moreover, the genome expression profile is more similar to that of neural crest stem cells. In presence of various differentiation-inducing agents, ES cell lines undergo neural differentiation (Ozaki, 2015; Tu et al., 2017).

Ewing sarcoma is characterized by a specific t (11; 22) (q12; q11.2). This translocation fuses in most cases the exon 7 of EWSR1 gene on chromosome 22 with the exon 6 of FLI1 gene on chromosome 11 (Tirode et al., 2014: Gaspar et al., 2015; Ozaki, 2015). It was detected for the first time in the 1980s. Furthermore, it is considered a hallmark of Ewing sarcoma, being present in around 85% of the cases (Ozaki, 2015; Tu et al., 2017). The remaining 15% of the patients have EWSR1 fused to genes encoding other members of the ETS family of transcription factors (Tirode et al., 2014; Ozaki, 2015). The fusion gene EWS-FLI1 acts as an

aberrant transcription factor encoding chimeric proteins. These proteins modulate transformation, differentiation, cell growth and signal transduction (Ozaki, 2015; Tu et al., 2017).

Most of Ewing sarcoma cases are seen in the bone, but 15-20% of the cases originate in the soft tissue surrounding bones (Tu et al., 2017). Any bone can be affected, but the most commonly affected sites are the pelvis, the femur and the tibia (Tirode et al., 2014; Ozaki, 2015). Furthermore, the extraskeletal ES affects most frequently the thigh, the gluteal region, the back, the lower leg, and the retroperitoneal region (Tirode et al., 2014; Ozaki, 2015). Patients can show local symptoms such as tumor mass formation, induration, pain, swelling, venous dilation, and hyperemia (Ozaki, 2015). ES is often accompanied by anemia and leukocytosis, elevation of blood sedimentation rate, and the serum levels of lactate dehydrogenase (LDH). The rise of LDH is associated with a poor prognosis (Ozaki, 2015). In skeletal ES, plain radiographs exhibit infiltrative destruction of the affected bone. In the extraskeletal soft tissue cases, computed tomography (CT) is useful. Pathological fractures sometimes occur due to bone metastasis (Ozaki, 2015).

Initial tumor volume or size is considered an important prognostic factor in localized ES. Patients with large tumors have a poor prognosis. If a tumor has a maximal diameter of more than 8 cm or a volume bigger than 200 mL, it is regarded as a large tumor (Gaspar et al., 2015). The success in treatment has been increased since the 1970s, being currently multidisciplinary involving chemotherapy, surgery, and radiotherapy/postoperative radiotherapy (Gaspar et al., 2015; Ozaki, 2015). Local ES lesions are usually treated via surgical excision or radiotherapy, or a combination of both (Ozaki, 2015). Overall survival (OS) for localized disease is 65-75% (Gaspar et al., 2015). Metastatic status at diagnosis is the strongest prognostic factor, a total of 34% of cases have metastasis during the diagnosis. The five-year OS remains 30%. Patients with lung metastasis alone have a better prognosis than patients with bone or bone marrow metastasis (Gaspar et al., 2015; Ozaki, 2015).

In spite of the improvement in the survival rate of ES, due to improvements in intensive chemotherapy, the prognosis remains unsatisfactory, and acute and long-term toxicities of therapy are substantial (Gaspar et al., 2015; Ozaki, 2015).

1.3 Hodgkin lymphoma

Hodgkin lymphoma (HL) is named after the English doctor Thomas Hodgkin who first described the clinical features of this disease in 1832 (Hodgkin, 1832). This is one of the most curable pediatric and adult cancers. In addition, it is one of the most frequent lymphomas in the western world. The annual incidence is about 3 cases per 100,000 persons. Besides, the long-term survival rates exceed 90% after treatment (Küppers et al., 2012; Terezakis et al., 2014; Mauz-Körholz et al., 2015). HL, a B cell-derived cancer, comprises 6% of pediatric cancers. Epidemiologically, it is distinct from adult HL but both share identical clinic-pathologic characteristics. The classification is the following (Küppers et al., 2012; Terezakis et al., 2014):

- Classical HL (CHL): the malignant cells are referred to as Reed-Sternberg (R-S) cells
    - Nodular sclerosis (NSHL): R-S cells are found in nodules formed by collagenous bands dividing lymph nodes. It is the most common subtype in all age groups, but is more frequent in adolescents - 77% - and adults - 72% - than in children- 44%.
    - Mixed-cellularity (MCHL): CD15+ R-S cells are present in a background of abundant normal reactive cells. It is more common in younger children- 33% - than in adolescents - 11% - or adults - 17%.
    - Lymphocyte-rich: CD15+ R-S cells are identifiable in a background of lymphocytes.
    - Lymphocyte-depleted (LDHL): rare.
- Nodular lymphocyte-predominant HL (NLPHL): relatively more common in young children, CD20+ (B-lymphocyte marker). The patients with this type of HL are biologically different, showed by improved disease-free survival.

Tumor cells usually retain key phenotypic features of the normal cells from which they originate. In CHL, the Reed-Sternberg cells show deregulated activation of multiple signaling pathways and transcription factors. They can show a very unusual phenotype with the co-expression of various hematopoietic lineages like markers of T cells, cytotoxic cells, B cells, dendritic cells, NK cells, myeloid cells and granulocytes. The tumor cells always express the activation marker CD30 (Küppers et al., 2012). The malignant cells of NLPHL are lymphocyte-predominant (LP) cells. These cells show various B cell markers indicating their B cell derivation. They exhibit antigen-activated mature B cells involved in T cell–dependent immune responses (Küppers et al., 2012).

There is a genetic predisposition to HL. The role of Epstein-Barr virus (EBV) in the pathogenesis of HL is well known. This DNA herpesvirus establishes a life-long persistent infection preferentially in human B-cells, suggesting that EBV infection might play a major role as an initial event in HL pathogenesis (Küppers et al., 2012; Terezakis et al., 2014). More than 90% of the world´s population is infected with EBV. Most of the related cases of EBV-positive and HL are associated with the host´s geographic origin and immune status, being more common in developing countries. The relation between CHL and EBV-positivity is reported in the following continents, Africa with cases of 80%, in Latin America with cases ranging from 70-80%, and in Asia with cases of 65%. Classical Hodgkin lymphoma has frequent presence of EBV in the neoplastic cells (predominantly of mixed cellularity type). In contrast, NLPHL is usually not associated with EBV (Huppmann et al., 2014).

The clinical presentation includes typically the involvement of peripheral lymph nodes, with a dominant nodal mass. Painless cervical adenopathy is present in 80% of the cases. It can also affect organs such as the liver, lungs, and bone marrow. One-third of the patients will have systemic ¨B¨ symptoms (fever-temperature higher than 38°C; night sweats and loss of more than 10% of body weight within 6 months). The systemic ¨B¨ symptoms result from cytokine secretion and give a worse prognosis (Küppers et al., 2012; Terezakis et al., 2014).

Patients with advanced-stage disease at the moment of diagnosis have a poorer outcome (Terezakis et al., 2014). Since the late 1970s pediatric HL has been treated successfully. Unfortunately, survivors are at risk of having a long-term-toxicity including life-limiting second cancers, infertility, and toxicity to the heart and lungs (Küppers et al., 2012; Mauz-Körholz et al., 2015). The current aim is to maintain efficacy, but to reduce toxicity. This is being accomplished by combining modality therapy with reduced dose radiation in an effort to cure children with minimal side effects (Küppers et al., 2012; Terezakis et al., 2014).

2. Objectives

The up-regulation of ERV3 in different cancer types might suggest an involvement in the pathogenesis of it. The reactivated ERV could be a new target of therapy. The zinc finger protein 117 (ZNF117) forms a genetic unit with ERV3. The physiological function of ZNF117 has not been clearly identified but it seems to contribute in the biological effects of ERV3. This study will characterize the ERV3/ZNF117 locus in tumor cells. For this end, the expression profile of ERV3, ZNF117 and ERV3-ZNF117 read-through transcripts in varying tumor cell lines and normal tissues will be analyzed. Furthermore, ERV3 and ZNF117 will be transgenically over-expressed in tumor cells and the consequences of this overexpression will be studied.

3. Materials and Methods

3.1 Materials

3.1.1 Cell lines

3.1.1.1 Tumor cells

Two main cell lines were used, A673 and P493-6.

a) A673: The main cell line used was the Ewing sarcoma cell line A673 which comes from the American Type Culture Collection (ATCC). These adherent cells were generated from the Ewing Sarcoma from a 15 year old girl (Giard et al., 1973).

b) P493-6: This B cell line carries a conditional tetracycline-regulated myc and it was established as a model for cell cycle activation by myc in Burkitt lymphoma cells (Schuhmacher et al., 2001).

In addition to these cells lines, RNA from the following cell lines was used (Table 1).

Table 1. Cell lines used to screen the expression of ERV3 and ZNF117. The cell lines NALM-6, SK-N-MC, SH-SY5Y, SiMa, and all Hodgkin lymphoma cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (DSMZ). IMR32 cells were kindly provided by F. Berthold, Cologne. RNA from all other cell lines was kindly provided by I. Volkmer, Halle.

Cell type	Cell line	Reference
Acute myeloid leukemia	THP-1	Tsuchiya et al.,1980
	HL-60	Collins et al.,1977
B-cell acute lymphoblastic leukemia	NALM-6	Hurwitz et al., 1979
Breast cancer	MCF-7	Soule et al., 1973
Burkitt lymphoma	Daudi	Klein et al., 1968
	Raji	Pulvertaft, 1964
Cervical cancer	HeLa	Gey, 1951
Ewing sarcoma	SK-N-MC	Biedler et al., 1973
	RD-ES	Sano et al., 1990[1]
Fibrosarcoma	HT-1080	Rasheed et al., 1974
Hodgkin lymphoma	L-1236	Wolf et al., 1996
	L-428	Schaadt et al., 1979
	L-540	Diehl et al., 1981
	KM-H2	Kamesaki et al., 1986
	HDLM-2	Drexler et al., 1986
Lymphoblastoid cell lines	LCL73	Hoennscheidt et al.,2009
	NM IIb	Hoennscheidt et al.,2009
	A2+2310	Hoennscheidt et al.,2009
Neuroblastoma	SH-SY5Y	Biedler et al., 1973
	SiMa	Marini et al., 1999
	IMR32	Tumilowicz et al., 1970
T-cell acute lymphoblastic leukemia	Jurkat	Schneider et al., 1977

[1] No original description of this cell line is published.

In addition, RNA from the murine cell line LB.27.4 (Kappler et al., 1982) was used as negative control.

3.1.1.2 Normal cells and tissues

The peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of HLA-A1, A2. The samples were from healthy donators from the blood bank of the University Hospital, Halle (Saale). This process was done with the previous authorization of the Ethical Commission of The Medicine Faculty from the Martin-Luther University Halle Wittenberg. A total of 5 anonymous blood samples were used. Furthermore, RNA from the following tissues was used:

Table 2. **RNA from normal tissues used for screening of ERV3 and ZNF117**. The BC (buffy coats) came from the blood bank of the University Hospital of Halle (UKH-Universitätsklinikum Halle). The RNA was already prepared by Ines Volkmer, Halle.

Samples	**Distributor**
BC536	Blood bank UKH
BC204	Blood bank UKH
BC426	Blood bank UKH
BCA1	Blood bank UKH
BCA1A2670	Blood bank UKH
Brain	Agilent, Santa Clara, California, United States
Breast	Agilent
Cerebellum	Agilent
Kidney	Agilent
Lung	Agilent
Lymph node	Agilent
Ovary	Agilent
Pancreas	Agilent
Placenta	Agilent
Skeletal muscle	Agilent
Testes	Agilent
Thymus	Agilent

3.1.2 Primers

All primers used for Real Time-PCR (qRT-PCR) and sequencing are listed below. The base pairs (bp) stand for the size of the product amplified by the primers. Sense and antisense are the sequence directions in the gene. The primers were purchased from INVITROGEN (Carlsbad, California, United States), MWG (Ebersberg) or from SIGMA (München).

Table 3. Primers for qRT-PCR. See the explanation in the text.

Target	Sequence	Product size
ADRB1	sense: GGGGAAGGGAGAAGCATTAG antisense: GGTTTGCCCTACACAAGGAA	289 bp
CCND1	sense: GATCAAGTGTGACCCGGACT antisense: GGAGAGGAGGGACTGTCAGG	264 bp
c-myc	sense: GGCTCCTGGCAAAAGGTCA antisense: CTGCGTAGTTGTGCTGATGT	119 bp
ERV3	sense: TGTGGGGGAATGAACATGGG antisense: AGAGAATGGGCTTGGGTGTG	297 bp
EWS-FLI1	sense: GTGATACAGCTGGCGTTGGCG antisense: CCACTAGTTACCCACCCCAAAC	330 bp
FOXP3	sense: CATGATCAGCCTCACACCAC antisense: CCACTTGCAGACACCATTTG	223 bp
GATA3	sense: GTCCTGTGCGAACTGTCAGA antisense: CTGCAAAAATGCAAGTCGAA	458 bp
HPRT1	sense: ACCAGTCAACAGGGGACATAA antisense: CTTCGTGGGGTCCTTTTCACC	190 bp
HygB[1]	sense: ACATTGTTGGAGCCGAAATC antisense: GAATTCAGCGAGAGCCTGAC	376 bp
KCNAB3	sense: TCAGAGGGAGAAGGTGGAGA antisense: GGTCCATGACTTTGGCTTGT	215 bp
LIPI	sense: TCCGAGAATAGAGACCATTCTGA antisense: GCTCTCTGGTGGTTGCATTT	654 bp
pAcGFP1-N3[2]	sense: CACATGAAGCAGCACGACTT antisense: TTGCCATCCTCCTTGAAATC	176 bp
pRTS1-EGFP[2]	sense: GCTGTTTTGACCTCCATAGAAGA antisense: CACTGCATTCTAGTTGTGGTTTG	894 bp
RORC	sense: ACCAAAAATGGATGGGATGA antisense: GACTGGAGCACCATGGAAAT	356 bp
TBX21	sense: TTGAGGTGAACGACGGAGAG antisense: CCAAGGAATTGACAGTTGGGT	235 bp
XIST	sense: CTCCAGGCCAATGAGAAGAA antisense: TGGCACAGTCCACCAAATTA	235 bp
ZNF117	sense: TTATTTTGCCCAACACCTTTG antisense: GGACCAGTTAAAGGCTCTTCC	403 bp

1 aminoglycoside phosphotransferase from E. coli

2 primers used for vector detection

Table 4. Cloning and sequencing primers. See the explanation in the text.

Target	Sequence
ZNF117	sense: ACCTGTCTGGAGCAAGGAAA antisense: CAATGAGTTTTGAGGATCAGGTA
ZNF117	sense: GCCTTTAACCAGTCCTCAGC

3.1.3 FACS antibodies

All antibodies used in the flow cytometry during the investigation are listed in the following table.

Table 5. FACS antibodies. The antibodies are provided by Becton Dickinson (Heidelberg).

Specificity	Label	Catalog number	Clone	Isotype
CD3	PE	345765	SK7	IgG1
CD4	PerCP	345770	SK3	IgG1
CD8	PerCP	345774	SK1	IgG1
CD25	PE	341009	2A3	IgG1

PE: Phycoerythrin

PerCP: Peridinin Chlorophyll

3.1.4 Reagents

All the chemical products, enzymes and vectors used during the experiments are listed in the table below with the name of the different distributors.

Table 6. Chemicals, enzymes and vectors.

Reagent	Distributor
β-Mercaptoethanol	Roth, Karlsruhe
2 x SYBR Green PCR Master Mix	Thermo Scientific, Waltham, Massachusetts, United States
5 x GoTaq Buffer	Promega, Madison, Wisconsin, United States
6 x DNA loading dye	Thermo Scientific
10 x FastAP buffer	Thermo Scientific
10 x Green buffer	Thermo Scientific
10 x Orange buffer	Thermo Scientific
10 x T4 DNA Ligase buffer	Thermo Scientific
10 x Tango buffer	Thermo Scientific
Ampicillin	SIGMA, Darmstadt
Aqua B. Braun	Braun AG, Melsungen
Biocoll Separating Solution	Biochrom GmbH, Berlin
DEPC-Water	Thermo Scientific
DMSO	Roth, Karlsruhe
dNTP Mix 10mM	Thermo Scientific
EcoRI	Thermo Scientific
EcoRV	Thermo Scientific
E. coli XL Blue	Stratagene, San Diego, California, United States
EDTA	SIGMA
Erythrocyte Lysis buffer	c.c.pro.GmbH, Oberdorla
Ethanol (96%)	SIGMA
Ethidium bromide solution 10mg/mL	ICN Biomedicals, Irvine, California, United States
FastAP Thermosensitive alkaline phosphatase	Thermo Scientific
Generuler 1 kb Plus DNA Ladder	Thermo Scientific
Generuler 100 bp Plus DNA Ladder	Thermo Scientific

GoTaq Polymerase	Promega
HPLC-Water	SIGMA
Nuclease free water	Thermo Scientific
Opti-MEM	Gibco, Carlsbad, California, Unites States
peqGOLD Universal Agarose	VWR, Darmstadt, Germany
pGEM®-T- Easy	Promega
PromoFectin transfection reagent	PromoKine, Heidelberg, Germany
Propidium iodide	Roth
qScript cDNA SuperMix	Quanta Biosciences, Beverly, Massachusetts, United States
SfiI	Thermo Scientific
Sodium acetate 3M	Merck
TAE Buffer 50 x pH 8	2M Tris-HCl, 1M acetate, 0.05M EDTA
T4 DNA Ligase	Thermo Scientific
Trypan Blue dye	Invitrogen, Carlsbad, California, United States
Trypsin-EDTA	Biochrom GmbH
XbaI	Thermo Scientific
XhoI	Thermo Scientific

All the vectors used during the experiments are listed in the table below.

Table 7. Vectors used in this investigation.

Reagent	**Source**
HaloTagxERV3[1]	Promega
pRTS1[2]	Kindly provided by G.W. Bornkamm, München
pUC19-SfiI	Kindly provided by G.W. Bornkamm, München

[1]Halotag ORF Clone pFN21AE1261
[2]Bornkamm et al., 2005

All the kits used during the experiments are listed in the table below with the name of the distributors.

Table 8. Kits.

Reagent	**Distributor**
BigDye Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Foster City, California, United States
DNA, RNA and protein purification Nucleospin RNA	Macherey-Nagel, Düren
DNA, RNA and protein purification Nucleospin Gel and PCR Clean-up	Macherey-Nagel
GeneJET Gel Extraction Kit	Thermo Scientific
GeneJET Genomic DNA Purification Kit	Thermo Scientific
GeneJET Plasmid Midiprep Kit	Thermo Scientific
GeneJET Plasmid Miniprep Kit	Thermo Scientific
High Pure RNA Isolation Kit	Roche, Mannheim
Universal RNA Purification Kit	Roboklon GmbH Berlin

All the cell culture mediums and buffers used during the experiments are listed in the table below with the name of the distributors**.**

Table 9. Cell culture medium and buffer

Reagent	Composition/Distributor
Dulbecco´s MEM	Biochrom GmbH, Berlin
LB-Medium	Roth, Karlsruhe
PBS	Lonza, Basel, Switzerland
RPMI 1640	Biochrom GmbH
SOB Medium	Roth

In the following table is presented the list of all the supplements or antibiotics used for the cell culture medium.

Table 10. Supplements for cell culture medium.

Reagent	Distributor
Doxycycline 5 mg/mL	SIGMA
Fetal Calf Serum (FCS)	Biochrom GmbH
Hygromycin B 50 mg/mL	Pan, Aidenbach
Interleukin-2 5µg/mL	Biomol GmbH, Hamburg
Penicillin/Streptomycin	Biochrom GmbH
Tetracycline	SIGMA

3.1.5 Plastic material

The plastic material used for the experiments was distributed by Eppendorf, Hamburg (qRT-PCR reaction tubes); Biozym, Hessisch Oldendorf (pipette tips); Greiner bio-one, Kremsmüster, Austria (reaction tubes, serological pipettes, culture flasks); Sarstedt, Nümbrecht (FACS reaction tubes); Nalgene, Darmstadt (cryopreservation tubes) and TPP, Trasadingen, Switzerland (cell culture flasks and plates). Leucosep tubes for the isolation of peripheral blood mononuclear cells were purchased from Greiner bio-one, Kremsmünster, Austria. The glass Pasteur pipettes were purchased from Laborfachhandel Dr. Ilona Schubert, Leipzig.

3.1.6 Devices and equipment

Below is the list of all the machines and devices used in the laboratory.

Table 11. Devices and equipment.

Device/Equipment	Marking/Designation	Distributor
Cell counter chamber	Neubauer-Zählkammer	VWR
Centrifuge	Multifuge 1S-R Megafuge 1.0	Thermo Scientific
Cryo Freezing Container	Nalgene TM Cryo1C	VWR
Flow Cytometer	FACScan FACSCalibur	Becton Dickinson
Fluorescence microscope	Axiovert 25	Zeiss, Oberkochen
Fume hood	AirflowControl	Bense, Hardegsen
Gel electrophoresis chambers	Sub-Cell GT	BIO RAD, Hercules, California, United States
Gel electrophoresis equipment	PowerPac 2000	BIO RAD
Incubator, gassed	BBD 6220	Heraeus, Hanau
Microscope camera	AxioCam MRm	Zeiss, Jena
Microwave	SS-804H	Bosch, Stuttgart
Optical microscope	Axiovert25 Axiovert25 HBO50	Zeiss
Pipette controller	Accu-jet	Brand, Wertheim
Real Time PCR Machine	Rotor-Gene Q	Qiagen, Venlo, Holland
Real Time PCR Machine	Rotor-Gene RG-3000	Corbett Research, Cambridge,United Kingdom
Shaking incubator	SM-30 C	Edmund Bühler, Hechingen
Spectrophotometer	DU 800	Beckman Coulter, Brea, California, United States
SpeedVac	Concentrator 5301	Eppendorf
Sterile bench	HeraSafe	Heraeus
Table centrifuge	Biofuge fresco	Heraeus
Thermocycler	Mastercycler gradient order Mastercycler personal	Eppendorf
ThermoMixer	Thermomixer comfort	Eppendorf
ThermoMixer	Thermomixer compact	Eppendorf
UV Table	PH1um20W/M	Biotec-Fischer, Reiskirchen
UV transilluminator	Quantum	Vilber Lourmat, Eberhardzell
Vacuum pump	BVC21	Vacuubrand, Wertheim
Vortexer	Vortex-Genie 2	Scientific Industries, Bohemia, New York, United States
Water bath	GFL 1083	Thermolab, Burgwedel

3.1.7 Software and Internet programs

For the analysis of the results the following programs were used: CellQuest Pro, BD Biosciences (measure and interpretation of FACS probes); Rotor Gene Q Series Software, QIAGEN, Venlo, Holland (the interpretation of the qRT-PCR); Microsoft Excel, Microsoft (evaluation, tabulation and presentation of the information in graphics); Microsoft

PowerPoint, Microsoft (working with graphics) and Microsoft Word, Microsoft (presentation of the final work). Furthermore, the following internet information platforms and internet programs were used.

Table 12. Internet programs.

Name/Reference	Use	Internet Address
Blast (Altschul et.al. 1997)	Comparison of sequences	www.ncbi.nlm.nhi.gov/blast
DNA Molecular Weight	Calculation of Molecular Weight	www.bioinformatics.org/sms2/dna_mw.html
GeneDoc	Edit and arrange sequences	http://www.softpedia.com/get/Science-CAD/GeneDoc.shtml
pDRAW32	Analysis for DNA cloning	www.acaclone.com
Primer3Plus	Selection of primers for a DNA sequence	www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
Reverse Complement	Conversion of a DNA sequence into its reverse-complement counterpart	www.bioinformatics.org/sms/rev_comp.html
SnapGene	Analysis for DNA cloning and determination of enzyme cutting sites	www.snapgene.com/products/snapgene_viewer/

3.2 Methods

3.2.1 Molecular biological methods

3.2.1.1 Transformation of Escherichia coli

During the transformation, an aliquot of competent Escherichia coli XL1 blue has been thawed on ice. The plasmid-DNA was then mixed with the bacteria. The amount of plasmid-DNA could be 10 µL (1-5 ng) or the complete ligation probe. The probe was then incubated for 30 minutes on ice followed by a heat shock of 45 seconds in a 42°C water bath. After this heat shock the probe was incubated on ice for 2 minutes. 1 mL of warm LB medium was then added and the probe was left for 60 minutes in the shaking incubator. The mixture was afterwards centrifuged for 10 minutes at 500 g and the supernatant was removed. The remaining pellet was resuspended and added to a LB-agar plate with the specific antibiotic (ampicillin). The agar plate was left for incubation in 37°C overnight. Next, colonies were picked up with a pipette tip and introduced in a 50 mL tubes. These tubes had 10 mL LB medium and 10 µL of

ampicillin at a concentration of 50 µg/mL. Finally, they were left overnight in the shaking incubator at 37°C.

3.2.1.2 Plasmid preparation

After the overnight incubation the colonies from the transformation were isolated with the GeneJET Plasmid Miniprep Kit. The procedure from the kit was followed and the final concentration was measured in the spectrophotometer.

3.2.1.3 Restriction digestion

During the present work, the following enzymes provided by Thermo Scientific were used: EcoRI, EcoRV, SfiI, XbaI and XhoI. The protocol of each enzyme was closely followed and the enzymes were usually inactivated after digestion. The cloning was done with the pUC19SfiI vector which was cut with the restriction enzymes XhoI and XbaI. A commercially available vector containing ERV3 was used as source for ERV3 (Promega). This ERV3 containing vector was also cut with XhoI and XbaI. After gel electrophoresis, the ERV3 fragment was isolated and ligated into the isolated linearized pUC19SfiI vector. Finally, ERV3 was isolated from the pUC19SfiIxERV3 vector by digestion with SfiI. The plasmid pRTS1 was cut with this restriction enzyme also and after gel electrophoresis, the ERV3 fragment was isolated and ligated into the isolated pRTS1 vector. This procedure replaced the luciferase insert from the pRTS1 vector with the ERV3 open reading frame.

3.2.1.4 Dephosphorylation

After the digestion, the plasmid was dephosphorylated, as a preparation step during cloning. This process was done with FastAp Thermosensitive Alkaline Phosphatase. The mixture included the plasmid-DNA, 10 x reaction buffer, 1 U of the alkaline phosphatase and water. It was thoroughly mixed, spun briefly, and incubated for 10 minutes at a temperature of 37°C. To stop the reaction, the probe was heated for 5 minutes at a temperature of 75°C.

3.2.1.5 Transgene regulation with doxycycline/tetracycline

The vector pRTS1 is an EBV-derived plasmid that is characterized by high inducibility in the presence of doxycycline (Bornkamm et al., 2005). A green fluorescence could be seen in the transfected cells when they were successfully induced with doxycycline. See the paragraph of Fluorescence Microscopy for more information.

Tetracycline was used for downregulation of myc in P493-6 cells. P493-6 cells are cells which carry a tetracycline-regulated myc (Pajic et al., 2000). In presence of tetracycline, myc is downregulated in this cell line.

3.2.1.6 Agarose gel electrophoresis

The separation of DNA and the visualization of the DNA products were made with the gel electrophoresis. To prepare the agarose gel, peqGOLD Universal Agarose (1.5%) was mixed with TAE buffer. Next, the mixture was heated for the time lapse of 2 minutes in the microwave. The evaporated water was replaced and then ethidium bromide (0.7 µg/mL gel) was added to the mixture. Finally, the agarose gel was poured into a gel chamber. When it was dry and solid the DNA samples were poured in it with the 6 x loading dye. The DNA was separated with electrophoresis and was then observed under the UV Transilluminator.

3.2.1.7 DNA gel extraction

First, the gel was placed in the UV table cautiously and the desired DNA band was cut with a scalpel. Next, the piece of gel was transferred to a reaction tube. The reaction tube with the gel inside was weighed and the GeneJET Gel Extraction instructions were followed. Finally the concentration was measured in the spectrophotometer.

3.2.1.8 Ligation

To calculate the amount of insert in the ligation probe, the following formula was used:

$$\text{Insert (ng)} = \frac{10 \text{ x } 50 \text{ ng vector x length of the insert (bp)}}{\text{length of the vector (bp)}}$$

The reaction mixture consisted of a 50 ng of vector; the amount obtained with the above showed formula from insert; 2 µL 10 x T4 DNA Ligase Buffer; 1 Weiss U (0.2 µL) T4 DNA Ligase and the rest of water for a total amount of 20 µL. The sample was then incubated for an hour at room temperature and finally it was used for the transformation.

3.2.1.9 RNA isolation

First, the cells were taken from the medium culture and centrifuged for 10 minutes at 250 g. Next, the supernatant was aspirated and the RNA isolation with the pellet proceeded. For this process, the Universal RNA Purification Kit (Roboklon) was used for up to 1 x 10^9 cells. Also, the High Pure RNA Isolation Kit of Roche was used for lower amounts of cells, up to 1x10^6

cells. The instructions were exactly followed with both kits. At the end, the concentration of the samples was measured with the spectrophotometer.

3.2.1.10 cDNA synthesis

For this process, the following chemicals were used: qScript cDNA SuperMix (4 µL), 1 µg of RNA and DEPC water to make a sample with a total amount of 20 µL. The qScript cDNA SuperMix contains 5 x reaction buffer made up optimized concentrations of $MgCl_2$, dNTPs, recombinant RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo (dT) primers and stabilizers. Finally, the sample was incubated in the Thermocycler with the following program procedure: 5 minutes in 25 °C, 30 minutes in 42 °C and 5 minutes at 85 °C.

3.2.1.11 Polymerase chain reaction (PCR)

This process was done to amplify a DNA fragment. Therefore, specific primers were used to amplify only the segment of interest. The sample had a total volume of 25 µL and consisted of 5 µL Go Taq Buffer; 0.5 µL 10 mM dNTP Mix; 0.25 µL of sense primer and 0.25 µL of antisense primer; 0.2 µL of Taq enzyme; 16.8 µL of water and 2 µL of cDNA. The concentrations were 1 x GoTaq Buffer; 0.2 nM dNTP Mix; 0.25 µM of each primer and 1 U of polymerase. The incubation was done in the Thermocycler. The procedure was as follows:

95°C for 5 minutes
95°C for 30 seconds ⎫
60°C for 30 seconds ⎬ 35 cycles
72°C for 30 seconds ⎭
72°C for 5 minutes

3.2.1.12 Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

This method confirms the expression of certain genes in a quantitative level. It was done with the fluorescent dye SYBR® which attaches itself in the new synthesized DNA. The reaction sample consists of 10 µL of 2x SYBR® Green PCR Master Mix, 7 µL of water, 1 µL of each primer (sense and antisense 25 µM each) and 1 µL of cDNA for a total amount of 20 µL. The process was always done on ice and in the sterile bench. The SYBR® Green PCR Master Mix was always protected from light. The Real Time PCR was performed in the Rotor Gene with the following process:

Initial Denaturalization:	95°C for 2 minutes	
Denaturalization:	95°C for 30 seconds	40 cycles
Annealing:	60°C for 30 seconds	
Elongation:	72°C for 45 seconds	
Final Elongation:	72°C for 5 minutes	

The expression level of the gene was determined with the comparative CT method (Livak and Schmittgen, 2001) and the HPRT1 gene was used as a housekeeping control. The same number of cycles was used in all experiments done, such as cell screening, molar calibration curves and the characterization of gene expressions.

3.2.1.13 Mycoplasma PCR

The Mycoplasma contamination of cell lines is a problem in the laboratories (Uphoff et al., 2013). Therefore, it is important to establish a method in every cell culture laboratory to detect such contamination. The PCR method is a fast technique to detect it. Therefore, a Mycoplasma PCR was set to verify the absence of infection of the A673 cells. The A673 cells were analyzed together with the transgene A673 cells, with empty vector and with vector with insert. None of the analyzed cell lines were positive. In this way, the experiments were run without concern of contamination. A sample from contaminated HEK cells was used as a positive control. The mixture was made as in a usual PCR described before. The primers were a mix of oligonucleotides with a concentration of 5 µmol/L (Methodensammlung der Bund/ Länder-Arbeitsgemeinschaft Gentechnik, 2011). Their sequences are mentioned in the figure legend. The positive control was provided by I. Volkmer, Halle. PCR was done with the following program:

1)	Denaturalization 92°C x 2 minutes	1 cycle
2)	Annealing 65°C x 1 minute	
3)	Elongation 72°C x 1 minute	
4)	Denaturalization 94°C for 4-15 seconds	35 cycles
5)	Annealing 65°C for 8-20 seconds	
6)	Elongation 72°C for 16 seconds with 2 seconds extension in each cycle	

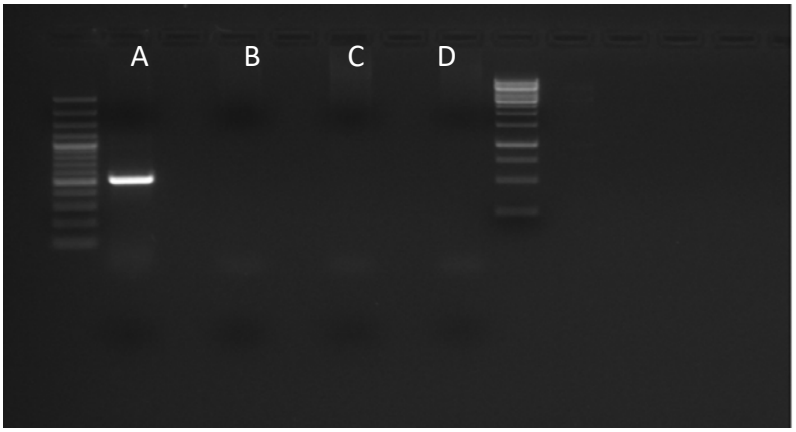


Figure 1. PCR agarose gel for detection of mycoplasma contamination. A: Positive control; B: A673 cells; C: A673 with empty pRTS1 and D: A673 with pRTS1 and ERV3. The only band is seen in the positive control (A). The primers sequences used were (5´-3´): 5' Primer: cgcctgagtagtacgttcgc/cgcctgagtagtacgtacgc/tgcctgggtagtacattcgc/tgcctgagtagtacattcgc/cgcctgagtagtatgctcgc/cacctgagtagtatgctcgc/cgcctgggtagtacattcgc and 3'Primer:gcggtgtgtacaagacccga/gcggtgtgtacaaaacccga/gcggtgtgtacaaaccccga. Each primer had a concentration of 5 µmol/L.

3.2.1.14 Sequencing of DNA fragments

The sequencing of DNA fragments was done with the BigDye® Terminator v1.1 Cycle Sequencing Kit. The following mixture was used: 0.2 µL of 25 µM primer (for each sample one primer); 2 µL 5x Big Dye Buffer; 1 µL Big Dye Mix; 10-30 ng of DNA or maximal 300 ng of vector or plasmid, and the rest was filled with HPLC water to reach a total amount of 10 µL. The sample was mixed and then it was incubated in the Thermocycler. The incubation lasted for 10 seconds under the temperature of 96°C and for 4 minutes under the temperature of 60°C, the cycle was repeated 30 times.

The chemical precipitation was started after the 30 cycles were finished. First, 1 µL of 3M sodium acetate was added to give the PCR product a pH of 4.6. Second, 25 µL of 100% ethanol was added and mixed. Next, the sample was incubated for 15 minutes in room temperature. After the incubation, the centrifugation was done for 15 minutes at 2500 g. By then, the pellet was not visible anymore. Therefore, the supernatant was carefully aspirated and 25 µL of 70% ethanol was added. The sample was centrifuged again, with the time lapse of 5 minutes at 2500 g. Then, the supernatant was removed and the probe was dried in the SpeedVac for 20 minutes. Finally, the sample was sent to the Sequencing Service of the University Hospital, Halle (Saale). When the results were received, the information could be read and analyzed with SnapGene Viewer and GeneDoc.

3.2.2 Biological cell methods

3.2.2.1 Cell culture

All cell culture usually began in a 6 well plate or a 25cm^2 flask. For longer periods of time, 25 or 75cm^2 flasks were used.

a) A673 cells

The cell culture experiment was done in sterile culture flasks or plates depending on the experiment. The cells were kept in the incubator with the following conditions: 37°C, 5% CO_2 and 95% humidity. In the experiment with adherent cells like A673, Dulbecco´s Medium was used. The medium was mixed previously with 10% of Fetal Calf Serum and 1% Penicillin/Streptomycin. The cell culture procedures were always done under a sterile bench. Every three to four days the cells were splitted, depending on their growth it was usually having a relation 1:10. To remove adherent cells, the old medium was aspirated; the cells were washed with PBS and then trypsin was added covering the entire cell surface of the flask and incubated for 4 minutes. Finally the effect of trypsin was neutralized with medium and the cells were transfered into the new flasks. The transfected cells with pRTS1 vector have always received Hygromycin B, in concentrations ranging from 100 and 400 µg/mL. The expression of the transfected vector pRTS1 was then induced by adding 1 µg/mL doxycycline.

b) B cells

Three different cell lines were cultivated: KM-H2, L-428 and P493-6. The cell culture experiment was done in sterile culture flasks under a sterile bench. The cells were kept in the incubator under the same conditions mentioned above. These cells were kept in RPMI medium, previously mixed with 10% of Fetal Calf Serum and 1% Penicillin/Streptomycin. Every three to four days the cells were splitted, depending on their growth it was usually having a relation 1:3. The B cells are suspension cells. Therefore, they can be harvested without the addition of reagents like trypsin. For treatment of cells with tetracycline, 1 µg/mL tetracycline was added to the culture medium.

3.2.2.2 Counting cells

The cells were counted in Neubauer cell chambers for experiments where the exact number of cells was needed. Under a coverslip, 10 µL of sample were pipetted (10 µL cells: 90 µL Trypan blue dye). All the living cells between the four big quadrants were counted, taking out the amount of cells pro milliliter with the following formula:

$$\text{cells per mililiter} = \frac{\text{cell number}}{\text{number of included quadrants}} \text{ x } 10 \text{ x } 10{,}000$$

10 stand for the dilution factor always used with Trypan blue dye.
10,000 stand for the chamber constant.

3.2.2.3 Transfection

The transfection of A673 cells was done with PromoFectin Transfection Reagent. During the first day, 400,000 A673 cells were added to each well of a 6 well plate. The wells contained 4 mL of Dulbecco´s Medium. During the second day, when transfection was done, two solutions were prepared. For each well, 6 µL of PromoFectin were mixed with 100 µL of Opti-MEM (PromoFectin solution) and 3 µg of DNA were diluted in 100 µL of Opti-MEM (DNA solution). Both solutions were diluted, gently vortex and spun. The PromoFectin solution was added all at once to the DNA solution, vortex-mixed and spun. Then, the sample was incubated at room temperature for 20-30 minutes. Finally, the PromoFectin/DNA solution was dropped into the well containing the A673 cells and gently homogenized by swirling the plate. After 24 hours, each well was harvested with trypsin and splitted into two parts. One was a negative control and the other one was a positive control with 1 µg/mL doxycycline. 48 hours after the transfection, with doxycycline being added one day before, the cells were observed under the fluorescent microscope to verify if they turned green in color.

Hygromycin B was added to the cells with induced transgene expression as well as into the negative controls, while doxycycline was only added into the induced ones. Hygromycin B is a selection antibiotic that allows getting stable transgenic cells. Every time the cells were splitted both antibiotics were used.

3.2.2.4 Fluorescence microscopy

The UV lamp was turned on for 15 minutes before using the microscopy. The 6 well plates or flasks were observed using the conventional light and when the filter was set, the blue light from the UV lamp illuminated the cells. With the excitation stimulus of the blue light, it was possible to see the green fluorescence color of the reporter protein GFP. With this, the success of the transfection and expression of the plasmid could be proved (Kain et.al., 1995). Pictures of the cells were taken with the adapted microscope camera.

3.2.2.5 Isolation of peripheral blood mononuclear cells (PBMC)

The leucosep tubes were used to isolate the buffy coats from the blood sample provided by the blood bank. The mentioned tubes were filled with 15 mL Biocoll separation solution and centrifuged for 30 seconds at 1000 x g. As a result, the solution was sent below the porous barrier. Next, the blood sample was poured into de Leucosep tube and centrifuged for 10 minutes at 1000 x g. After this, the instructions from the kit were followed. Finally, the cells were ready to be counted and used either in a cell culture or were frozen for future experiments.

3.2.2.6 Cell co-culture

The co-culture was done with adherent A673 cells and PBMCs using 75cm^2 flasks and RPMI medium. This medium is favorable for PBMC and A673 cells can also grow in it. 2.5 million A673 cells with 10 million PBMC were used. This experiment was done with A673 cells with empty pRTS1 vector and vector pRTS1xERV3. A HLA-typed blood sample was received from the blood bank, and it was then prepared to isolate the PBMC. Four flasks were divided from each of the samples with empty pRTS1 and with pRTS1xERV3. The first day, the cells were counted and added to the flasks. Only the cells without doxycycline were used because doxycycline was first added for this experiment. In total 10 million cells were used, each flask with 2.5 million cells. Two flasks with empty pRTS1 were left without doxycycline and to the other two flasks, doxycycline was added. The same was done with the four flasks with pRTS1xERV3.

On the second day, the four flasks with doxycycline were observed with the fluorescence microscope to prove if they were green. The medium was changed to eliminate the dead cells and new doxycycline was added. The PBMC were counted to add 10 million cells to the A673 cells in each of the 8 flasks. When all the flasks contained the A673 cells and PBMC, half of them received IL-2. The dose used was 25 Units/mL. The total amount of medium was 20 mL.

The cells were continuously examined to see differences in growth during the time. On day 4, the 20 mL of medium having the PBMC were transferred into a 50 mL tube. They were stored in the incubator while harvesting the A673 cells with trypsin. The cells were again counted to use the same amount of 2.5 million cells in the new flask. This time only 5 mL medium were poured in the flask. The remaining cells were centrifuged and the pellet stored for future analysis. After one hour of having the recently splitted A673 in the new flasks, the 20 mL of medium with PBMC was added, having a total of 25 mL of medium.

On day 7, the medium having the PBMC was taken aside in a tube and the cells were counted. Some milliliters of the sample were used for FACS analysis and the rest was used for RNA isolation. The A673 cells were also counted and the same division was done, for FACS and RNA isolation. To get an overview, the flasks of the experiment are listed below:

- A673 cells, empty pRTS1 vector, PBMC, no doxycycline
- A673 cells, empty pRTS1 vector, PBMC, no doxycycline, with IL2
- A673 cells, pRTS1 vector with ERV3, B PBMC CA0102, no doxycycline
- A673 cells, pRTS1 vector with ERV3, PBMC, no doxycycline, with IL2
- A673 cells, empty pRTS1 vector, PBMC, with doxycycline
- A673 cells, empty pRTS1 vector, PBMC, with doxycycline, with IL2
- A673 cells, pRTS1 vector with ERV3, PBMC, with doxycycline
- A673 cells, pRTS1 vector with ERV3, PBMC, with doxycycline, with IL2

The analysis in the co-culture samples was done separately. First, the A673 co-culture samples were analyzed. These cells were taken from the bottom of the flasks and they contain A673 cells in contact with PBMC. Second, the PBMC which were the cells in the supernatant that had been in contact with A673 cells were analyzed.

In order to see the effect of doxycycline on PBMC without having contact with tumor cells, a similar experiment was done as a control. There were two groups, one with no changes during the week. The second group had the same split on day 4 as if having A673 cells. The flasks were 75cm^2 with 20 mL of RPMI and 10 million cells. The samples used in both versions of the experiment were the following ones:

- PBMC without doxycycline
- PBMC without doxycycline with IL2
- PBMC with doxycycline
- PBMC with doxycycline with IL2

These samples were also analyzed with a qRT-PCR on day 7^{th} using four primers and HPRT1 as a housekeeping gene.

3.2.2.7 Cryopreservation and re-culture of Cells

To freeze the cells, they were counted and centrifuged for 10 minutes at 250 g. The medium was aspirated and the pellet was resuspended with culture medium and 10% DMSO. The sample was poured in special cryopreservation tubes, using no more than 1 mL per tube. All the tubes were stored in a cryopreservation container which slowly froze the sample (approximately 1°C/min) up to -80°C. After this period of time, the samples could be stored in liquid nitrogen.

To unfreeze the samples, they were taken out from the nitrogen tanks and quickly defrosted on ice. Then, they were washed by adding 10 mL of medium. Next, they were centrifuged for 10 minutes at 250 g. The medium was aspirated and the pellet was resuspended with culture medium and placed on the flask to be kept in culture.

3.2.3 Immunological methods

3.2.3.1 Flow cytometry

The fluorescence activated cell scanning (FACS) analyzes the different characteristics of the cells, like cell size with the FSC (Forward Scatter) and granularity with the SSC (Side Scatter). It is also possible to determine the different cell populations by marking the cells with specific antibodies. Two conjugated antibody types were used, fluorescent dye PE and PerCP. For the FACS analysis with the co-culture, two antibody combinations were used. The first combination had CD3-PE and CD8-PerCP. The second group had CD25-PE and CD4-PerCP. In this experiment, cells were harvested and 1 x 10^6 cells were poured into a FACS tube. Then, the cells were centrifuged at 250 g for 10 minutes and the medium was aspirated. The pellet was resuspended with 50 µL PBS and 10 µL of the specific antibody. Next, the mixed sample was incubated in a dark environment at 4°C for 20 minutes. After the incubation, the cells were washed with 1 mL of PBS and centrifuged for 10 minutes at 250 g. Finally, the medium was aspirated and the pellet was resuspended with 500 µL of PBS. The cells were ready for analysis. See Figure 2 for an example.

3.2.3.2 Cell viability test with propidium iodide (PI)

FACS can also be used to determine the viability of cells. First, the cells were harvested and poured into a FACS tube. Second, the cells were centrifuged at 250 g for 10 minutes and the medium was aspirated. Next, the pellet was resuspended with 500 µL PBS and 5 µL of propidium iodide. The concentration of PI was 2 mg/mL and 1 µL PI was used for each 100 µL

PBS. The analysis was done immediately after the PI was mixed with the cells. See Figure 2 for an example.

The usual gating strategy is shown in Figure 2. In the Dot Plot of Figure 2 A, the X axis was FSC (Forward Scatter) and the Y axis was SSC (Side Scatter). The relation between FSC and SSC shows the size and granularity of the cells, respectively. It also allows creating a ¨living gate¨ since the cells with disrupted membrane have more granularities and can be distinguish from the living cells this way. Also, the debris is counted as event because of their small size. Therefore, they can also be recognized and discarded from the ¨living gate¨.

The cells (events) were also presented in the X axis as FSC and in the Y axis with PI to see the difference between living and dead cells (Figure 2 B). The last relation made between the events was X axis GFP and Y axis FSC (Figure 2 C). It shows the amount of cells that were successfully transfected and it shows the green fluorescence in the presence of doxycycline.

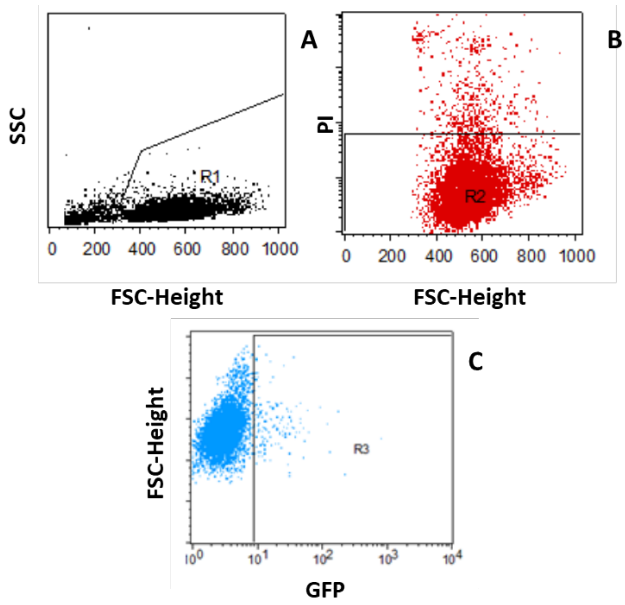


Figure 2. FACS analysis from transgenic cells. This is an example of how the gates and axis were used for FACS analysis. This data is from the empty pRTS1 vector without doxycycline. A shows the relation between FSC (size of the cells) and SSC (cell granularity). B shows the relation between FSC (size of the cells) and PI (dead cells). C shows the relation between GFP (green fluorescence) and FSC (size of the cells). See the text for more information.

4. Results

4.1 Analysis of B cell lines

In presence of tetracycline, myc is downregulated in P493-6 cells and their growth stops. In this condition of arrested cells, ERV3 is upregulated (Kewitz and Staege, 2013). Three different cell lines (KM-H2, L-428 and P493-6) were analyzed in the presence or absence of tetracycline to search if this effect with ERV3 was caused only by tetracycline. Two different flasks were split from each cell line, one negative control having only medium and one sample with 1 µg/mL tetracycline. After one week, RNA was isolated to run a qRT-PCR. The relative expression of three target genes was investigated (Figures 3 to 5). The results showed that tetracycline alone does not upregulate ERV3 or ZNF117 in proliferating B cells (KM-H2 cells and L-428 cells). To prove if tetracycline was active, the expression of myc was measured. Interestingly, ZNF117 was upregulated in tetracycline treated P493-6 cells. P493-6 cells were especially used because they carry a tetracycline-regulated myc (Pajic et al., 2000). In presence of tetracycline, myc is downregulated in this cell line. The results proved the same downregulation here (Figure 5).

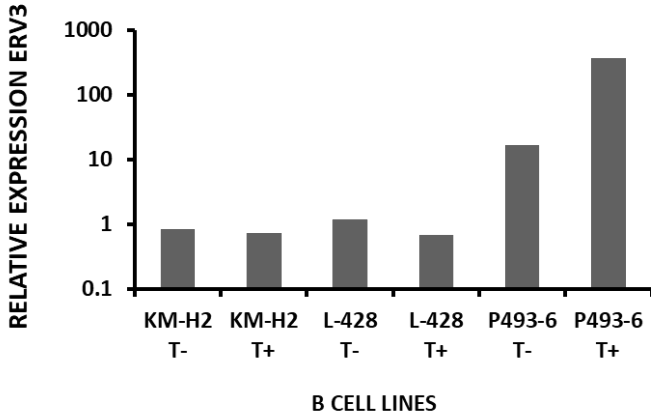


Figure 3. Relative expression of ERV3 in three different B cell lines. T- stands for the negative control in the presence of only medium (KM-H2 T-, L-428 T- and P493-6 T-). T+ stands for cells having tetracycline (KM-H2 T+, L-428 T+ and P493-6 T+). For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The $2^{-\Delta\Delta Ct}$method was performed for the determination of the gene expression.

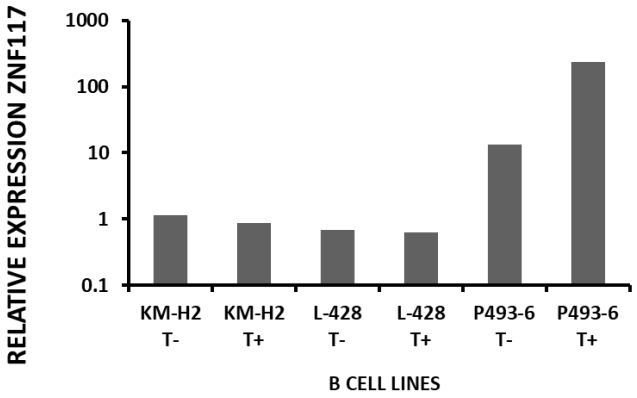


Figure 4. Relative expression of ZNF117 in three different B cell lines. T- stands for the negative control in the presence of only medium (KM-H2 T-, L-428 T- and P493-6 T-). T+ stands for cells having tetracycline (KM-H2 T+, L-428 T+ and P493-6 T+). For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression.

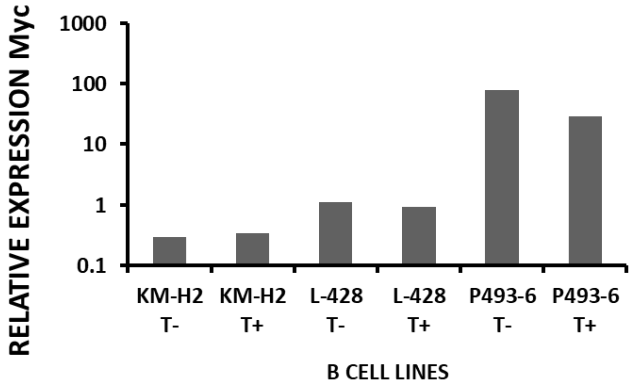


Figure 5. Relative expression of Myc in three different B cell lines. T- stands for the negative control in the presence of only medium (KM-H2 T-, L-428 T- and P493-6 T-). T+ stands for cells having tetracycline (KM-H2 T+, L-428 T+ and P493-6 T+)-6-6. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression.

4.2 Expression screening of ERV3, ZNF117, and ERV3-ZNF117

4.2.1 Expression of ERV3

The cell screening was done to determine the variability of expression of ERV3 in different samples. Two different groups of cells were used, tumor cells and normal tissue cells. Material from all the cell lines was present either as cDNA or RNA in the laboratory. In total, 42 different cell types were analyzed with a qRT-PCR (repeated three times). In the qRT-PCR, the ERV3 primers and the housekeeping gene HPRT1 primers were always used. The results are shown in Figure 6. Placenta had the highest expression. In the cell screening, the RNA from LB.27.4, a murine cell line (Kappler et al., 1982) was used as a negative control.

4.2.2 Expression of ZNF117

Only 22 cell lines were chosen for this experiment according to the results from the cell screening of ERV3. The groups were again tumor cells and normal tissue cells. The conditions of the experiment were the same as with ERV3 cell screening. The housekeeping gene was HPRT1. The qRT-PCR was repeated three times. Figure 7 shows the results of ZNF117 expression in the different cell lines. The expression of ZFN117 was not upregulated in placenta.

4.2.3 Expression of ERV3-ZNF117

A third experiment was made to see the expression of the fusion transcript ERV3-ZNF117. For this last screening, 10 cell lines were chosen according to the results of the ERV3 and ZFN117 screenings. The qRT-PCR was made just one time and the housekeeping gene was HPRT1. Figure 8 shows the results. The expression was not upregulated in placenta.

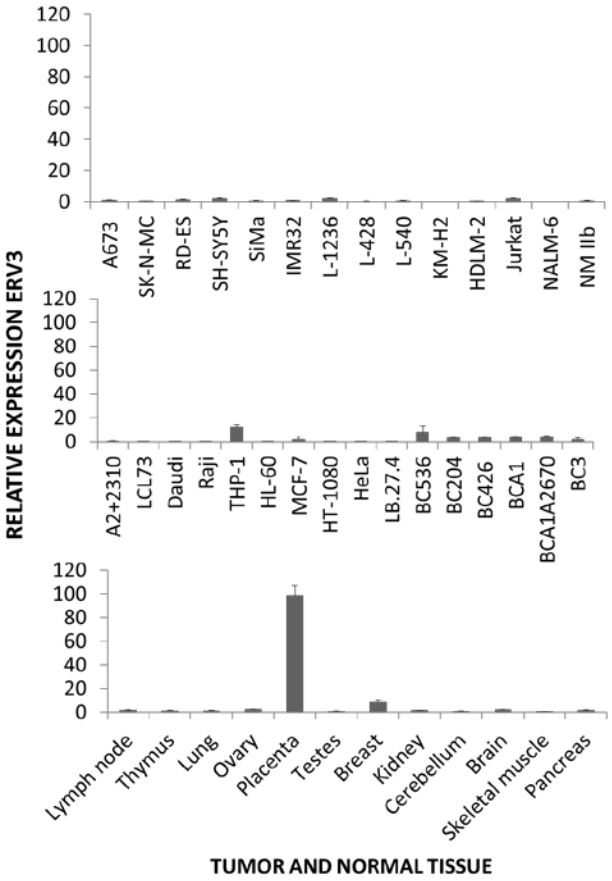


Figure 6. Relative expression of ERV3 in the screening. The relative expression in the 42 samples screened for expression of ERV3. For comparative analysis, HPRT1 was used as housekeeping control and the $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. The median of all samples (from the three qRT-PCRs) was set as 1. Presented are means and standard deviations from these experiments.

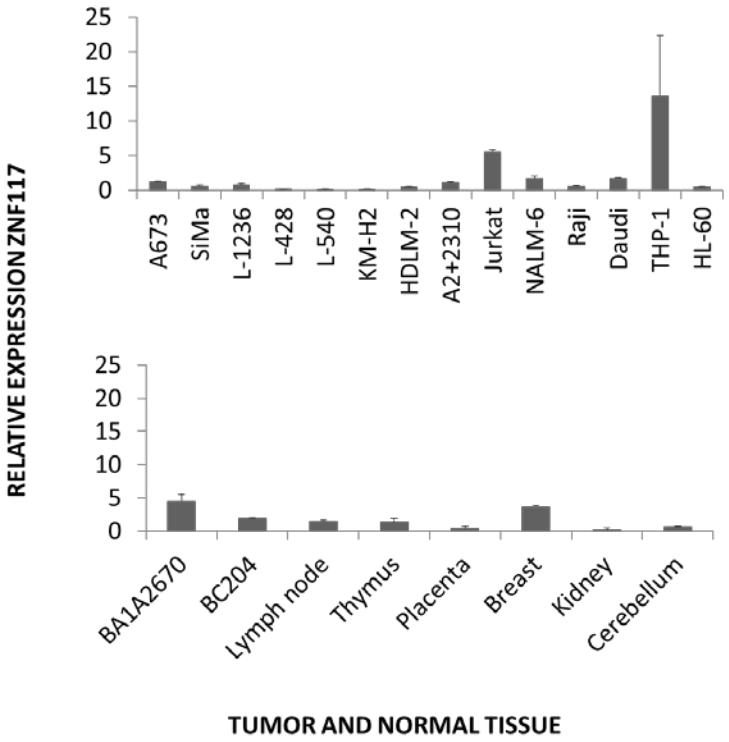


Figure 7. Relative expression of ZNF117 in the screening. The relative expression in the 22 samples screened for expression of ZNF117. For comparative analysis, HPRT1 was used as housekeeping control and the $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. The median of all samples (from the three qRT-PCRs) was set as 1. Presented are means and standard deviations from these experiments.

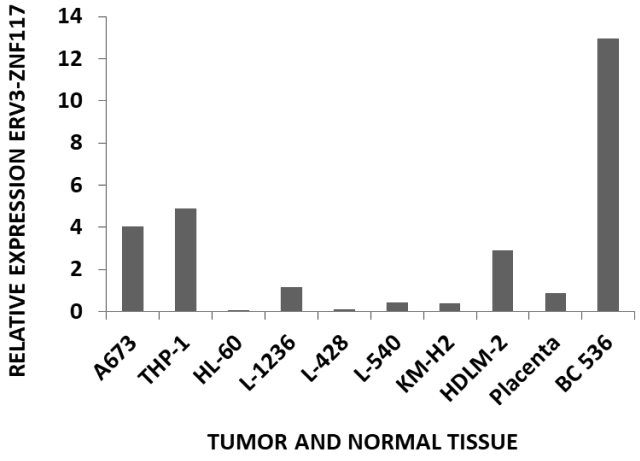


Figure 8. Relative expression of ERV3-ZNF117 in the screening. The relative expression in the 10 samples screened for expression of ERV3-ZNF117. For comparative analysis, HPRT1 was used as housekeeping control and the $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. The median of all samples was set as 1. One qRT-PCR was done.

In order to analyze the relation between the fusion transcript and each gene, a second analysis was done. The relative expression of the fusion transcript ERV3-ZNF117 was compared with the relative expression of ERV3 (Figure 9). Only the cell lines that were analyzed with both primers are shown. Placenta shows a high relative expression of ERV3 but a relative low one of ERV3-ZNF117. On the other hand THP1 cells present a high relative expression of ERV3-ZNF117 but low with ERV3.

The relative expression of the fusion transcript ERV3-ZNF117 was also compared with the relative expression of ZNF117 (Figure 10). Only the cell lines that were analyzed with both primers are shown. THP1 cells show a high relative expression of ZNF117 and of ERV3-ZNF117.

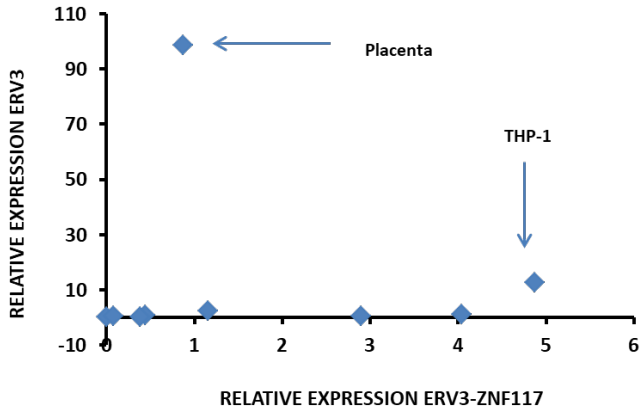


Figure 9. Relative expression of ERV-ZNF117 vs. relative expression of ERV3 in the screening. The cells shown here are from the screening as described above. X axis presents the relative expression of the fusion transcript ERV3-ZNF117 and Y axis presents the relative expression of ERV3.

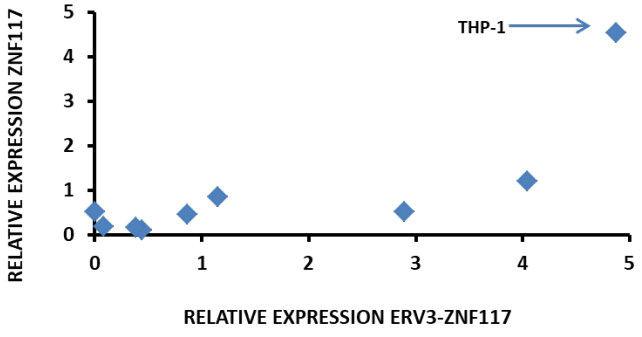


Figure 10. Relative expression of ERV3-ZNF117 vs. relative expression of ZNF117 in the screening. The cells shown here are from the screening as described above. X axis presents the relative expression of the fusion transcript ERV3-ZNF117 and Y axis presents the relative expression of ZNF117.

4.3 Molar calibration curves for ERV3 and ZNF117

4.3.1 ERV3

The mole calculation gives information about the exact amount of mole ERV3 per mole HPRT1 in each sample. For the preparation of the ERV3 calibration curve, the commercial vector was used. The molar calculation of HPRT1 was available from previous experiments in the laboratory. Several dilutions of this vector were used as templates in a qRT-PCR and the obtained Ct values were used for calculation of the regression line. Based on these data, CT values from different samples were re-calculated and molar concentrations were determined as shown in Figure 11.

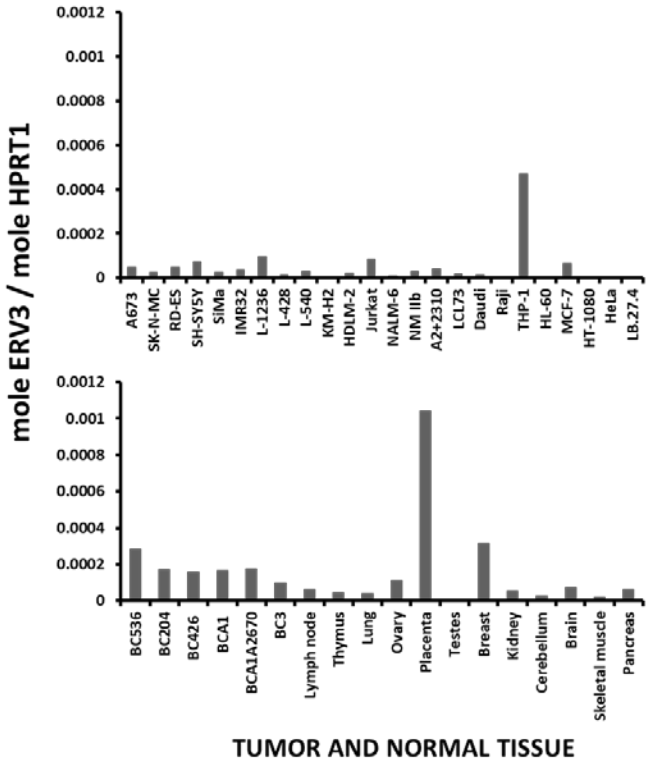


Figure 11. Molar calculation of ERV3 expression. The graphic shows the amount of mole ERV3 per mole HPRT1 from the 42 cell lines screened for ERV3.

4.3.2 ZNF117

The mole calculation of ZNF117 was started differently to the mole calculation from ERV3 because the ZNF117 sample came from a PCR product. The PCR product was isolated and the concentration of the product was determined. After preparing a calibration curve the Ct values were used for calculation of molar amounts of ZNF117 in the different samples. The results of the analysis are shown in Figure 12.

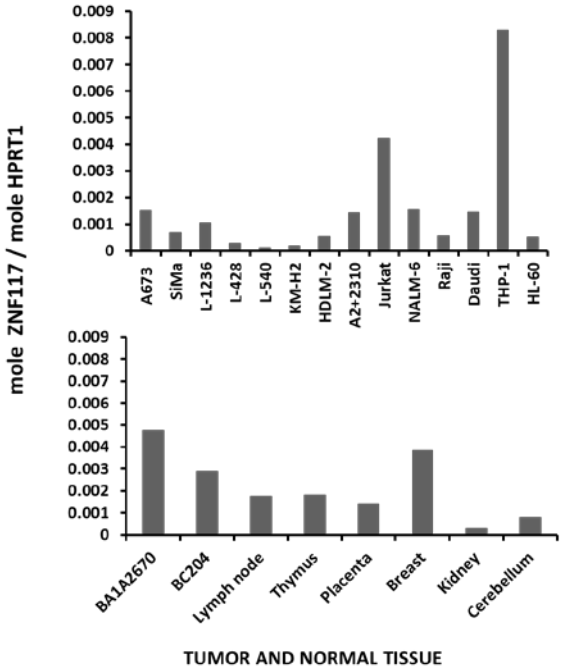


Figure 12. Molar calculation of ZNF117 expression. The graphic shows the amount of mole ZNF117 per mole HPRT1 of the 22 cell samples screened for ZNF117 expression.

4.3.3 Comparison between ERV3 and ZNF117 expression

The results from ERV3 mole calculation were compared with the results from ZNF117 mole calculation in a scatter diagram to see the relation of both in several normal and tumor tissues (Figure 13). Placenta and THP-1 showed the highest values, indicated by blue arrows on the figure.

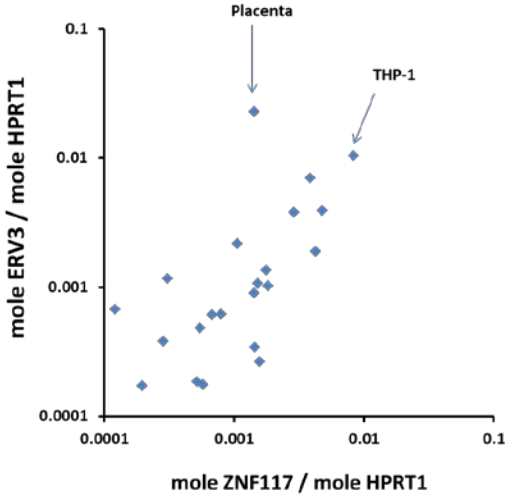


Figure 13. Relation between ERV3 and ZNF117 expression in tumor and normal tissues. The 22 tissues analyzed in the mole calculation with ZNF117 (Figure 12) have been compared with ERV3 results. Two tissues are pointed here, placenta and THP1 because of their high values.

4.4 Preparation of plasmids pUC19SfiI and pRTS1

To start the characterization of ERV3, it was necessary to prepare recombinant plasmids. The cloning was done with the pUC19SfiI vector which was cut with the restriction enzymes XhoI and XbaI. As source for ERV3 a commercially available vector containing ERV3 was used (see material and methods). The ERV3 containing vector was cut with XhoI and XbaI. The ERV3 insert as well as the linearized vector pUC19SfiI were isolated from agarose gels. After ligation of vector and insert, the transformation in Escherichia coli (E.coli) XL Blue was performed. The next day, four colonies were selected from the agarose plate and transferred to LB medium with ampicillin for incubation. The DNA was then purified with the DNA Plasmid Purification Kit and the DNAs where cut with XhoI and XbaI to verify the presence of the correct insertion of ERV3 in the vector. One from the correct clones was chosen for the next steps of expression plasmid preparation.

In this step, the insert ERV3, taken from the pUCSfiI vector, was inserted in the final vector pRTS1. The plasmid pRTS1 was cut with the restriction enzyme SfiI and dephosphorylated. Before the ligation, ERV3 was also isolated from the pUC19SfiIxERV3 vector by digestion with SfiI. After ligation of vector and insert, the transformation in E.coli XL Blue was done and four colonies from the agarose plate were chosen for vector preparation. Verification of correct insert was made by digestion with EcoRI and SfiI restriction enzymes (Figure 14). One of the four clones had the correct insert in the vector, marked with a blue arrow. For further experiments this recombinant DNA was multiplied and isolated with the GeneJET Plasmid Midiprep Kit to assure enough amount of DNA.

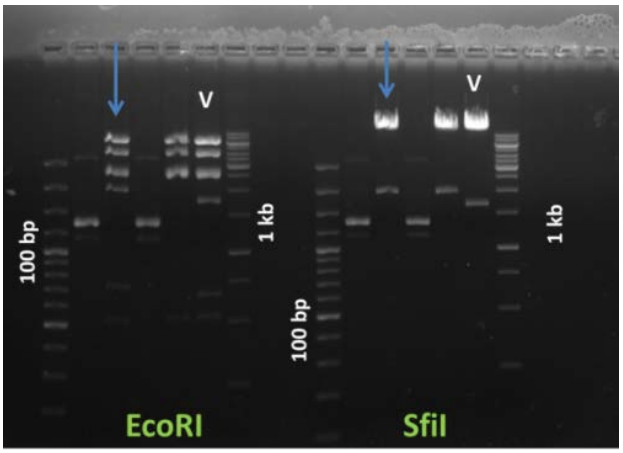


Figure 14. Agarose gel from vector pRTS1 with insert ERV3 digested with the restriction enzymes EcoRI and SfiI. Four samples of recombinant DNA and a negative control of empty vector pRTS1 (marked as V) were digested with each enzyme. The gene rulers are shown (100bp DNA ladder and 1kb DNA ladder). The correct sample is marked with a blue arrow.

4.5 Transfection of ERV3 in A673 cells

In order to have cells with the recombinant DNA, a transfection was done. The following experiments were done with A673 cells (Ewing sarcoma cells) because these cells can be easily transfected and are immunologically well characterized. The cells were cultured in a 6 well plate and the transfection was started a day after plating, when the cells already adhered at the surface of the well. During the first 24 hours, the experiments had only two wells, one with empty vector pRTS1 and the other one with pRTS1 with ERV3 insert (pRTS1xERV3). After the first day, these two wells were harvested with trypsin and split into two wells. In other words, two wells were having empty vector and two wells were having vector with insert. The division was made to have cells with induced transgene expression with doxycycline (1 µg/mL) and a negative control without doxycycline from each sample. Next, doxycycline was added to induce the expression of the transgene. After 24 hours the cells were analyzed with the fluorescence microscopy. The cells having doxycycline showed green fluorescence (Figure 15).

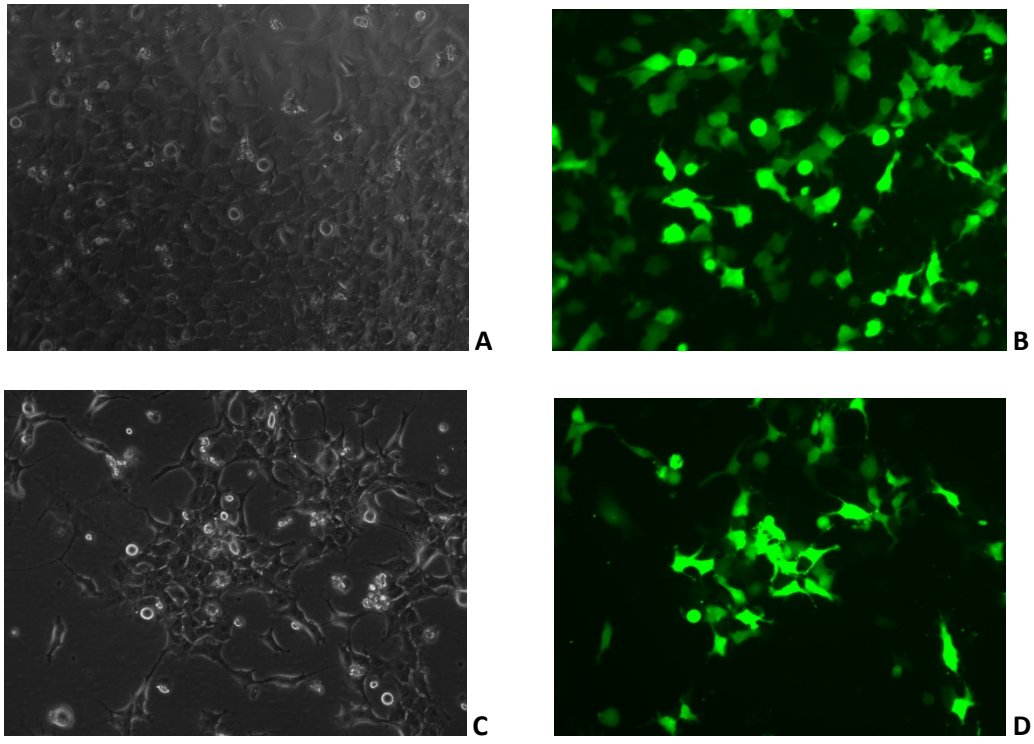


Figure 15. Microscope photographs from transfected cells. Cells with empty pRTS1 vector are (A and B) cells with pRTS1xERV3 are (C and D). The induction with doxycycline was successful, shown by green fluorescence in pictures B and D. 20x objective, magnified.

4.6 Characterization of ERV3 transgenic A673 cells

After the transfection, the cells were cultivated and split every three to four days depending how full the plate or the flask was. When the cells were harvested with trypsin, at least one third of the cells were taken for RNA isolation and posterior reverse transcription to get cDNA. Next, the cDNA was used for further analyses with a qRT-PCR to characterize the transgenic cells. During the experiments, there were always four samples, empty vector pRTS1 with and without doxycycline and pRTS1 with insert with and without doxycycline.

One of the first experiments was to measure the relative expression of ERV3 in the presence and absence of doxycycline with and without insert (Figure 16).

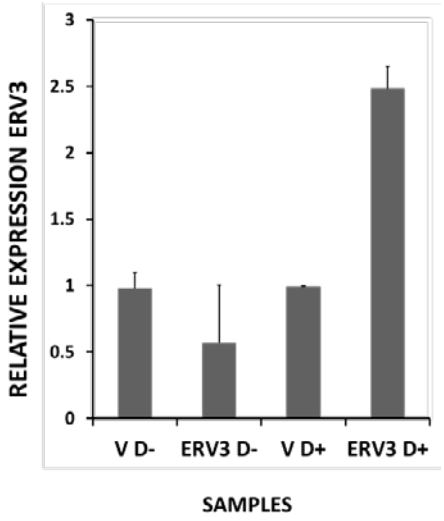


Figure 16. Relative expression of ERV3. This figure shows the relative expression of ERV3 in the transgenic A673 cells. The abbreviations stand for: Cells transfected with empty vector pRTS1 without doxycycline (V D-), pRTS1xERV3 without doxycycline (ERV3 D-), and empty vector pRTS1 with doxycycline (V D+). (ERV3 D+): pRTS1xERV3 with doxycycline. Two qRT-PCRs were done. The $2^{-\Delta\Delta Ct}$method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Presented are means and standard deviations from these experiments.

In an additional experiment the cells without doxycycline were splitted into 6 well plates. The expression of ERV3 was followed over one week after adding doxycycline. During the experiment, a negative control always remained without doxycycline. Doxycycline (1 µg/mL) was added to the other wells. It is important to note that the doxycycline was just added during the start of the experiment. Wells were harvested after varying time points and analyzed by a qRT-PCR (Figure 17).

The same samples were then used to test the expression of various genes. A qRT-PCR with the same kinetics was done to search the relative expression of the neighbor gene ZNF117 (Figure 18). The fusion transcript ERV3-ZNF117 (Figure 19) was likewise investigated along with the expression of EGFP (Figure 20). Even if the cells already covered 100% of the space, the cells were not divided to maintain the same conditions during the whole experiment.

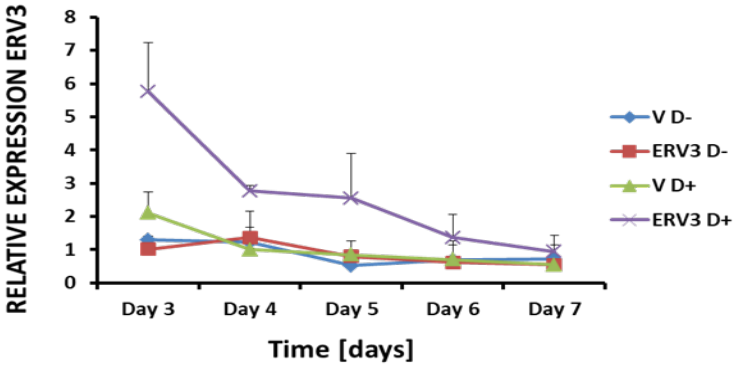


Figure 17. Relative expression of ERV3 in the transfected A673 cells; kinetics. The samples were: empty pRTS1 vector without doxycycline (V D-); pRTS1 with ERV3 without doxycycline (ERV3 D-); empty pRTS1 vector with doxycycline (V D+) and pRTS1 with ERV3 with doxycycline (ERV3 D+). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done. Presented are means and standard deviations from these experiments.

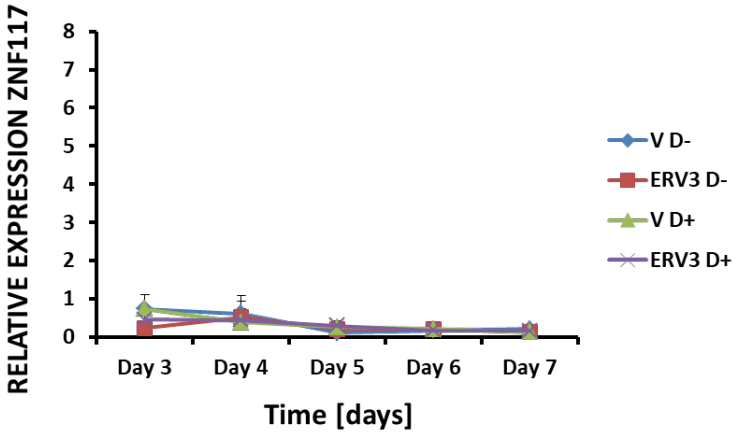


Figure 18. Relative expression of ZNF117 in the transfected A673 cells; kinetics. The samples were: empty pRTS1 vector without doxycycline (V D-); pRTS1 with ERV3 without doxycycline (ERV3 D-); empty pRTS1 vector with doxycycline (V D+) and pRTS1 with ERV3 with doxycycline (ERV3 D+). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done. Presented are means and standard deviations from these experiments.

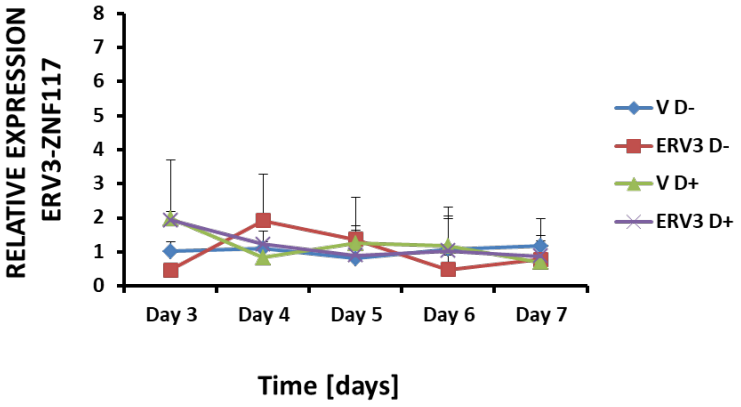


Figure 19. Relative expression of ERV3-ZNF117-fusion transcripts in the transfected A673 cells; kinetics. The samples were: empty pRTS1 vector without doxycycline (V D-); pRTS1 with ERV3 without doxycycline (ERV3 D-); empty pRTS1 vector with doxycycline (V D+) and pRTS1 with ERV3 with doxycycline (ERV3 D+). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done. Presented are means and standard deviations from these experiments.

The expression of EGFP was also measured in the kinetics to see its expression during the days. Especially because the cells were green with the empty vector and the vector with insert. The results of day 3 showed the highest expression of almost all genes measured during the kinetics. Therefore, only day 3 is shown in Table 13. A representative experiment is shown in Figure 20.

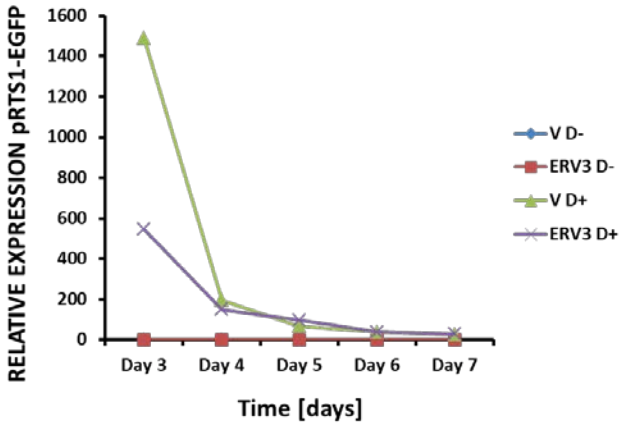


Figure 20. Relative expression of EGFP in the transfected A673 cells; kinetics. The samples were: A673 cells transfected with empty pRTS1 vector without doxycycline (V D-); pRTS1xERV3 without doxycycline (ERV3 D-); empty pRTS1 vector with doxycycline (V D+) and pRTS1xERV3 with doxycycline (ERV3 D+). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Presented are means and standard deviations from these experiments. See text for further information.

Three representative experiments are shown here where EGFP was measured. Empty pRTS1 D+ means A673 cells transfected with empty pRTS1 vector with doxycycline, pRTS1xERV3 D+ means A673 cells transfected with pRTS1xERV3 with doxycycline. The $2^{-\Delta\Delta Ct}$ method was used for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1.

Table 13. Relative expression of pRTS1-EGFP on day 3 in three different experiments. D+ stands for: with doxycycline.

	Experiment 1	**Experiment 2**	**Experiment 3**
Empty pRTS1 D+	380.04	1488.87	154.34
pRTS1xERV3 D+	101.83	544.96	82.71

In support of the results from the qRT-PCR, a FACS analysis was done (Figure 21). The FACS analysis showed a similar effect. The empty vector showed always a higher expression than the vector with insert when GFP was measured. From the cells with empty pRTS1 vector, 10% were green. In contrast, only 5.34% of the cells with pRTS1xRV3 insert were green.

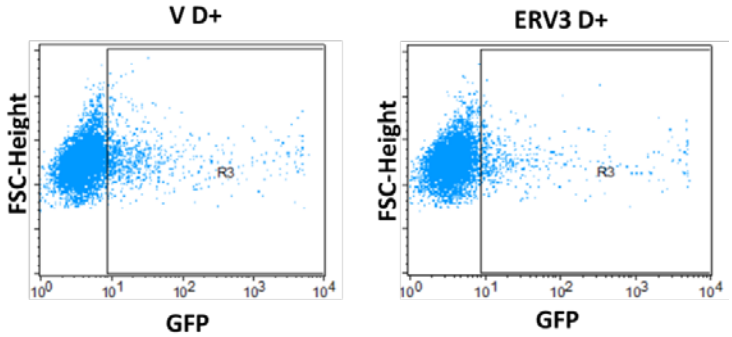


Figure 21. FACS analysis from doxycycline treated transgenic cells. V D+ stands for empty pRTS1 vector with doxycycline and ERV3 D+ stands for vector with ERV3 with doxycycline. The results seen are the comparison between FSC-Height and GFP, the gate R3 shows the green cells.

To test whether the difference in GFP expression between the different vectors was a consequence of different transfection efficacy, expression of the resistance gene hygromycin phosphotransferase was analyzed. A qRT-PCR was done using only the samples of day 3 from the ERV3 kinetics shown in Figure 17. Relative expression of hygromycin phosphotransferase is shown in Figure 22 supporting the hypothesis that the differences might be due to transfection efficacy.

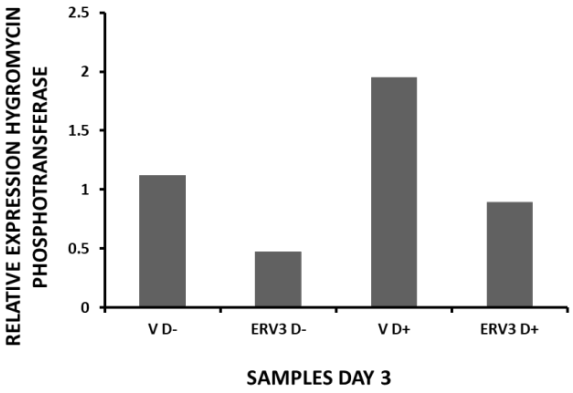


Figure 22. Relative expression of hygromycin phosphotransferase in the transfected A673 cells. This figure shows the empty pRTS1 vector without doxycycline (V D-); pRTS1 with ERV3 without doxycycline (ERV3 D-); empty pRTS1 vector with doxycycline (V D+) and pRTS1 with ERV3 with doxycycline (ERV3 D+). The $2^{-\Delta\Delta Ct}$method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. One qRT-PCR was done.

4.7 Gene expression in the transgenic cells

The samples from the ERV3-transgenic A673 cells were analyzed to compare the relative expression of various genes. These investigated genes are usually expressed in Ewing sarcoma. This experiment was done to analyze if their expression was still present in the transgenic Ewing sarcoma A673 cells. The analyzed genes were ADRB1, LIPI, EWS-FLI1, CCND1, KCNAB3 and XIST. The samples were: empty vector pRTS1 with and without doxycycline and pRTS1xERV3 vector with and without doxycycline. The results are shown in Figure 23 to Figure 28. Overall, the gene expression profile suggested clonal variation with low impact of ERV3 expression.

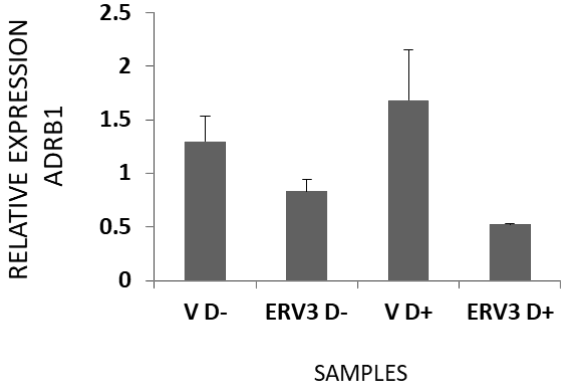


Figure 23. Relative expression of ADRB1 in transfected A673 cells. V D- (empty vector without doxycycline); ERV3 D- (vector with ERV3 without doxycycline); V D+ (empty vector with doxycycline); ERV3 D+ (vector with ERV3 with doxycycline). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done. Presented are means and standard deviations from these experiments.

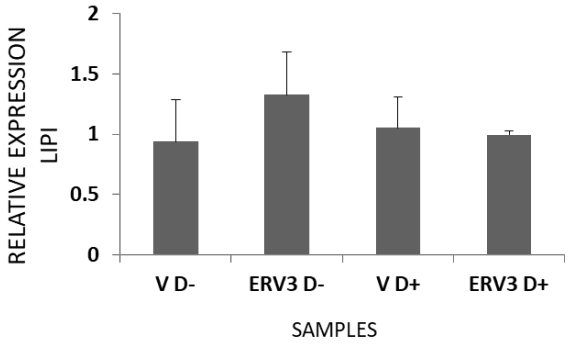


Figure 24. Relative expression of LIPI in transfected A673 cells. V D- (empty vector without doxycycline); ERV3 D- (vector with ERV3 without doxycycline); V D+ (empty vector with doxycycline); ERV3 D+ (vector with ERV3 with doxycycline). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done. Presented are means and standard deviations from these experiments.

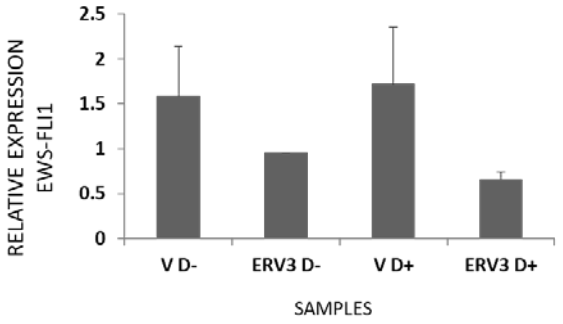


Figure 25. Relative expression of EWS-FLI1 in transfected A673 transfected cells. V D- (empty vector without doxycycline); ERV3 D- (vector with ERV3 without doxycycline); V D+ (empty vector with doxycycline); ERV3 D+ (vector with ERV3 with doxycycline). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done. Presented are means and standard deviations from these experiments.

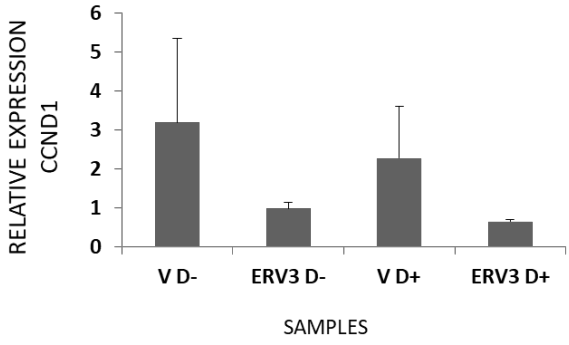


Figure 26. Relative expression of CCND1 in transfected A673 cells. V D- (empty vector without doxycycline); ERV3 D- (vector with ERV3 without doxycycline); V D+ (empty vector with doxycycline); ERV3 D+ (vector with ERV3 with doxycycline). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done.

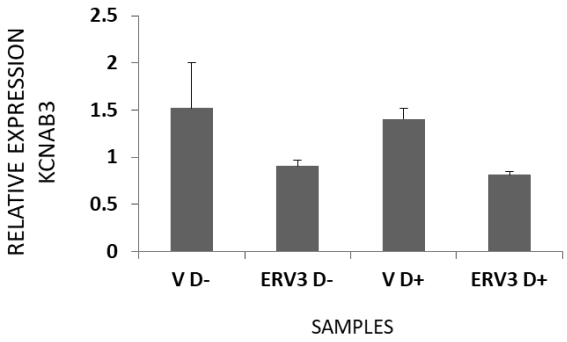


Figure 27. Relative expression of KCNAB3 in transfected A673 cells. V D- (empty vector without doxycycline); ERV3 D- (vector with ERV3 without doxycycline); V D+ (empty vector with doxycycline); ERV3 D+ (vector with ERV3 with doxycycline). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done. Presented are means and standard deviations from these experiments.

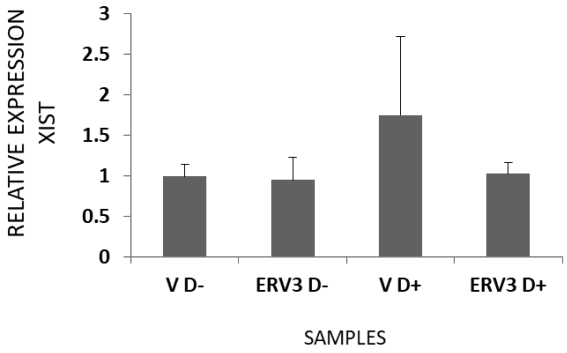


Figure 28. Relative expression of XIST in transfected A673 cells. V D- (empty vector without doxycycline); ERV3 D– (vector with ERV3 without doxycycline); V D+ (empty vector with doxycycline); ERV3 D+ (vector with ERV3 with doxycycline). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Two qRT-PCRs were done. Presented are means and standard deviations from these experiments.

4.8. CCND1 expression in A673 cells treated with doxycycline

In consideration of the downregulation seen with CCND1 in the presence of doxycycline (in the experiment described in paragraph 4.7) a new experiment was done. In four 25cm^2 flasks, A673 cells were cultured in medium with different concentration of doxycycline. The first flask was the negative control, without doxycycline. The second flask had 1 µg/mL of doxycycline, the same concentration used in the transfected cells. The third flask had 4 µg/mL and the fourth flask had 8 µg/mL. This experiment was repeated three times. The cells were split on

day 4 and the experiment was finished on day 9. The cells were counted and analyzed in a qRT-PCR on both days.

The results are shown in the following Figures 29 and 30, showing the relation between cell number and relative expression of CCND1. Figure 31 shows the expression in the presence of different concentrations of doxycycline. A high variation among experiments was found even though they had the same conditions. The cells having the highest dose of doxycycline especially in day 9 had less number of cells and relative expression of CCND1.

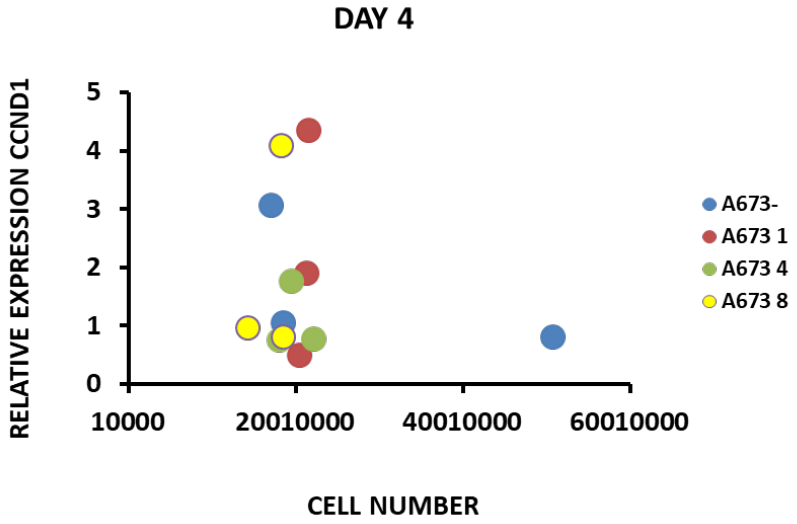


Figure 29. Relative expression of CCND1 vs. cell number on the 4th day. The experiment was repeated three times, as shown with three results per doses. In the X axis the cell number is shown in relation with the relative expression of CCND1 in the Y axis. The samples are described as followed: cells without doxycycline (A673-), the cells having 1 µg/mL (A673 1); the cells having 4 µg/mL (A673 4) and the cells having 8 µg/mL (A673 8). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1.

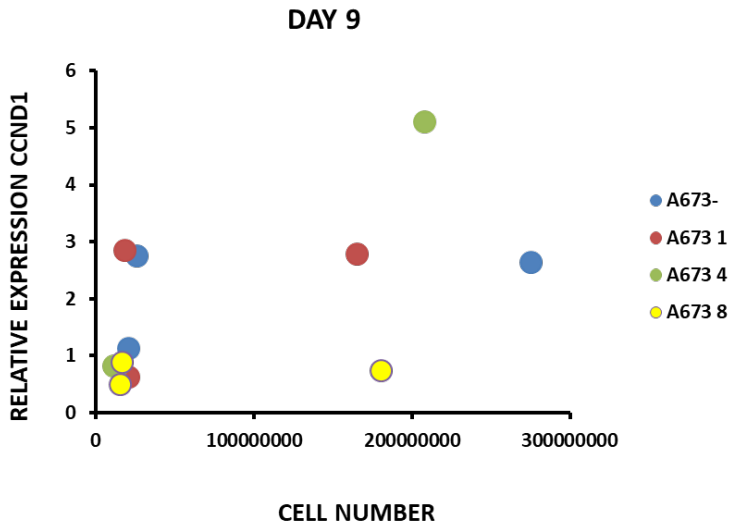


Figure 30. Relative expression of CCND1 vs. cell number on the 9th day. The experiment was repeated three times, as shown with three results per doses. In the X axis the cell number is shown in relation with the relative expression of CCND1 in the Y axis. The samples are described as followed: cells without doxycycline (A673-), the cells having 1 µg/mL (A673 1); the cells having 4 µg/mL (A673 4) and the cells having 8 µg/mL (A673 8). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1.

The following figure shows the relative expression of CCND1 in the presence of different concentrations of doxycycline on day 9. The experiment with the highest dose of doxycycline showed the lowest relative expression of CCND1.

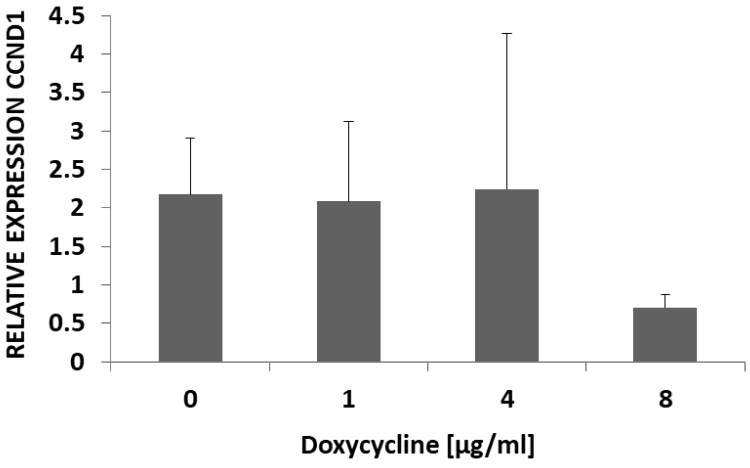


Figure 31. Relative expression of CCND1 in A673 cells after treatment with doxycycline. This figure shows the results of three qRT-PCRs. The experiment is from day 9. A673- stands for the cells without doxycycline. A673 1 stands for the cells with 1 µg/µL doxycycline. A673 4 stands for the cells with 4 µg/µL doxycycline. A673 8 stands for the cells with 8 µg/µL doxycycline. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Presented are means and standard deviations from these experiments.The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression.

4.9 Transfection of pRTS1xZNF117 plasmids in A673 cells

The neighboring gene ZNF117 is of interest because of the possible relation in the biological effects of ERV3. Therefore, in addition to ERV3, a transfection of ZNF117 was performed in A673 cells. A vector containing ZNF117 (pRTS1xZNF117) was available from preliminary studies in the laboratory. The vector was amplified in E.coli XL Blue and 8 colonies were picked from the agar plate for analysis. The DNA of these colonies was extracted with the Plasmid DNA Purification kit and digested with restriction enzymes EcoRV and SfiI (Figure 32). The restriction pattern of all clones was proven to be correct. The transfection was done with PromoFectin in a 6 well plate with A673 cells, empty vector pRTS1 and pRTS1xZNF117. After 24 hours, the two wells were divided to induce two of them with doxycycline, leaving two wells as negative control. At last, the cells were analyzed by fluorescence microscopy to prove the success of the transfection (Figure 33).

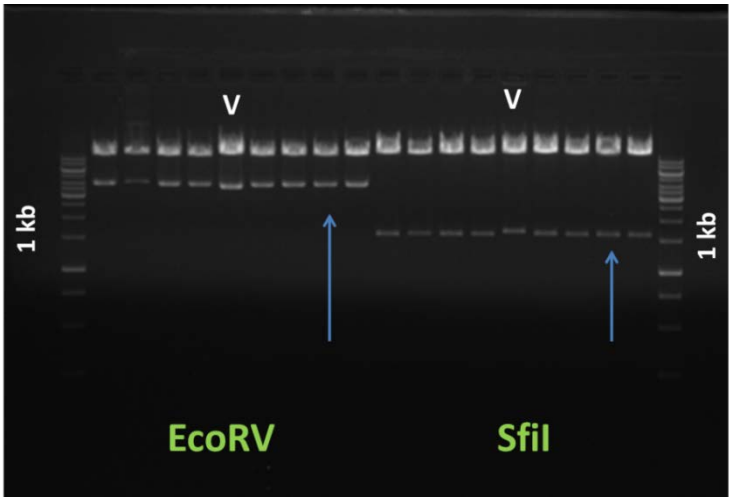
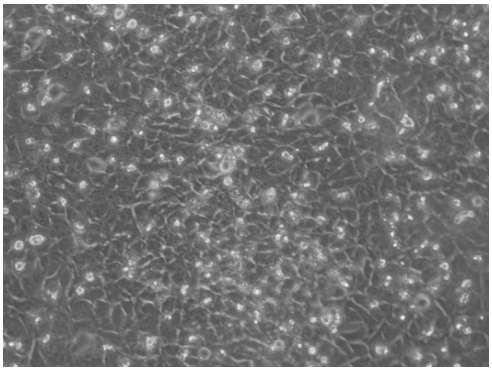


Figure 32. Agaroses gel from vector pRTS1 with insert ZNF117 digested with the restriction enzymes EcoRV and SfiI. 8 samples of recombinant DNA and a negative control of empty vector pRTS1 (marked as V) were used with each enzyme. The 1kb DNA ladder was used. The colony that was used is marked with a blue arrow.


A

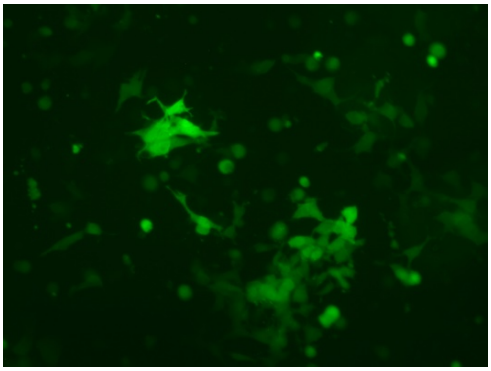

B

Figure 33. Microscope photographs from transfected cells. The transfected cells with pRTS1 vector and ZNF117 insert (A and B) are shown. After the induction with doxycycline the green fluorescence is shown. 20x objective, magnified.

The plasmid pRTS1xZNF117 was sequenced. The result had 100% identity with the sequence provided by NCBI (National Center for Biotechnology Information) identified as NM_015852.

4.10 Characterization of ZNF117 transgenic A673 cells

Once the transfection was concluded, the characterization of the cells was done to get more information of ZNF117 gene. The cells were divided into empty vector and vector with insert. From each sample a negative control was left without antibiotic. The other cells were induced with 1 µl/mL doxycycline. The relative expression of ZNF117 was measured in the cells transfected with pRTS1xZNF117. The results are shown in Figure 34. Two other gene expressions were analyzed: the relative expression of ERV3 (Figure 35) and the fusion transcript ERV3-ZNF117 (Figure 36). The experiment showed an upregulation of ZNF117 when induced with doxycycline. ERV3 and the fusion protein ERV3-ZNF117 were not upregulated in presence of doxycycline.

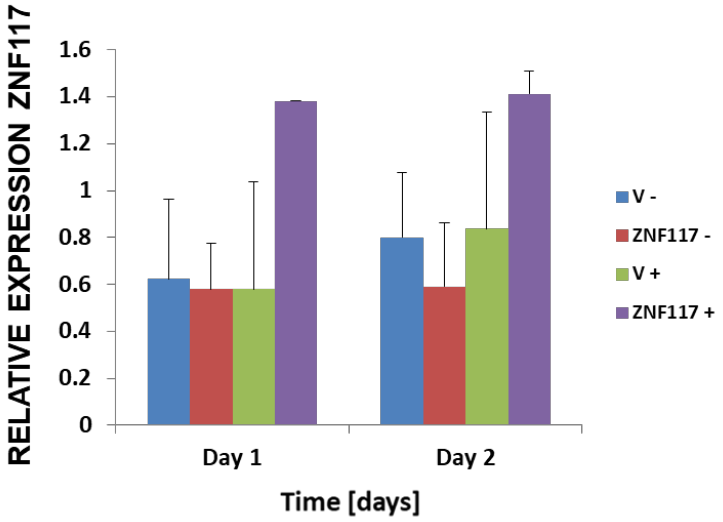


Figure 34. Relative expression of ZNF117 in the transfected A673 cells; kinetics. The samples were: empty pRTS1 vector without doxycycline (V D-); pRTS1 with ZNF117 without doxycycline (ZNF117 D-); empty pRTS1 vector with doxycycline (V D+) and pRTS1 with ZNF117 with doxycycline (ZNF117 D+). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Presented are means and standard deviations from these experiments.

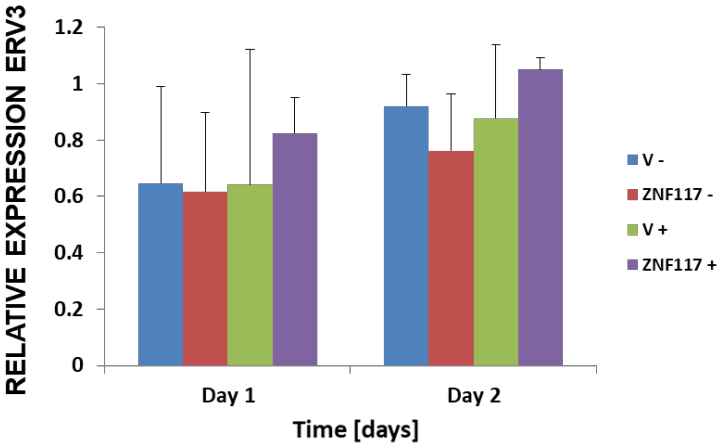


Figure 35. Relative Expression of ERV3 in the transfected A673 cells; kinetics. The samples were: empty pRTS1 vector without doxycycline (V D-); pRTS1 with ZNF117 without doxycycline (ZNF117 D-); empty pRTS1 vector with doxycycline (V D+) and pRTS1 with ZNF117 with doxycycline (ZNF117 D+). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Presented are means and standard deviations from these experiments.

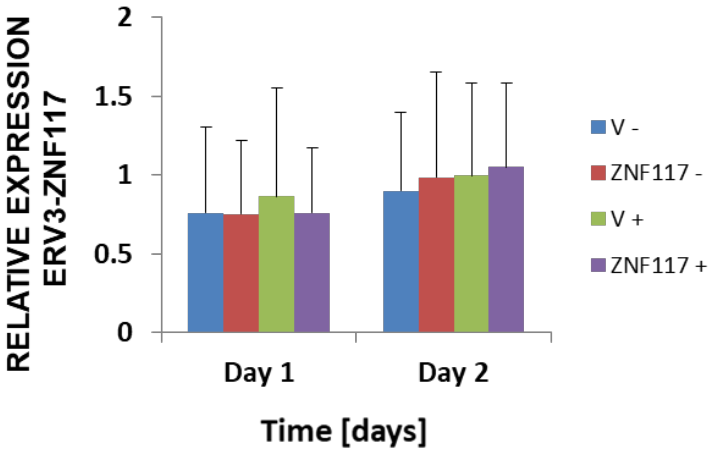


Figure 36. Relative expression of ERV3-ZNF117 fusion transcript in the transfected A673; kinetics. The samples were: empty pRTS1 vector without doxycycline (V D-); pRTS1 with ZNF117 without doxycycline (ZNF117 D-); empty pRTS1 vector with doxycycline (V D+) and pRTS1 with ZNF117 with doxycycline (ZNF117 D+). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. Presented are means and standard deviations from these experiments.

4.11 Co-culture of A673 cells and PBMC

To illustrate the immunological relation between transgenic cells and PBMCs a co-culture experiment was done. It was performed two times using A673 cells with empty pRTS1 vector and vector pRTS1xERV3. In addition PBMC isolated from HLA-typed blood sample were added. On the first day the A673 cells were counted and added to the flasks. On the second day, the PBMC were counted and added to the A673 cells in each of the 8 flasks. Half of the samples received IL-2, 25 Units/mL. The cells were continuously observed to notice differences in growth during the time (Figure 37).

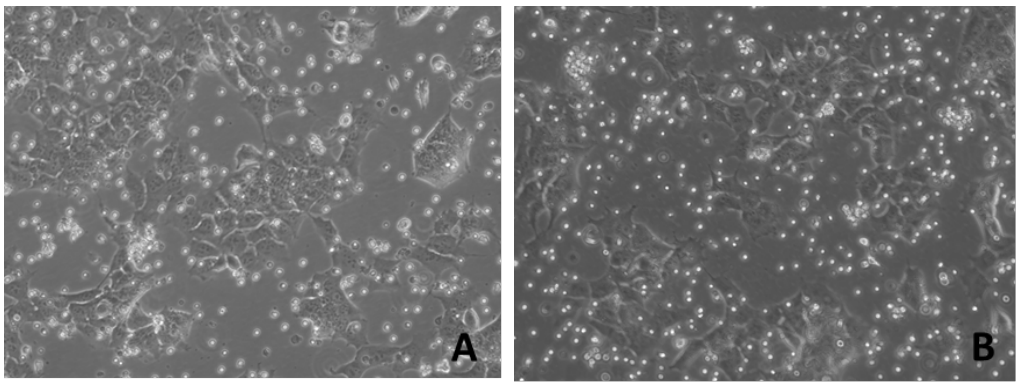


Figure 37. Co-culture of transfected A673 cells and PBMC. A673 cells with empty pRTS1 vector in co-culture with PBMC (A). A673 cells with pRTS1xERV3 vector with PBMC (B). 20x objective, magnified.

The co-culture was divided at day 4 and totally harvested at day 7 for analysis. Posterior analysis was made with FACS and qRT-PCR. Table 14 shows the cell number of the two different experiments. The total numbers were obtained on day 7. No clear effects of ERV3 on the co-culture system were observed.

Table 14. Cell numbers in the co-culture of A673 cells with PBMC. The results show the number of cells counted when the flasks were harvested on day 7. Two experiments were done, shown as #1 and #2. A673 stands for all the cells taken from the bottom of the flasks that were mainly A673 cells in contact with PBMC. PBMC stands for the cells from the supernatant of the flasks that were mainly PBMC in contact with A673 cells. V (empty pRTS1); ERV3 (pRTS1 with ERV3); IL2+ (presence of IL2); IL2-(absence of IL2); D- (without doxycycline); D+(with doxycycline).

Samples	#1	#2
A673 V D- IL2-	10250000	15500000
A673 V D- IL2+	11650000	9750000
A673 ERV3 D- IL2-	9850000	10000000
A673 ERV3 D- IL2+	9100000	7000000
A673 V D+ IL2-	9700000	8750000
A673 V D+ IL2+	10750000	8000000
A673 ERV3 D+ IL2-	7350000	6000000
A673 ERV3 D+IL2+	8050000	9750000
PBMC V D- IL2-	4375000	9375000
PBMC V D- IL2+	2750000	9375000
PBMC ERV3 D- IL2-	5000000	10625000
PBMC ERV3 D- IL2+	4250000	8125000
PBMC V D+ IL2-	3875000	10625000
PBMC V D+ IL2+	3000000	5000000
PBMC ERV3 D+ IL2-	4000000	8125000
PBMC ERV3 D+ IL2+	5875000	10000000

In order to analyze the relation of the co-culture with antibodies, a FACS analysis was performed. The experiment was done twice: experiment #1 and experiment #2 shown in the columns of each table. The table 15 shows the expression of CD3+ and CD8+ in the co-culture cells. The table 16 shows the expression of the other antibodies, CD4+ and CD25+. Again, no clear effects of ERV3 on the system were observed. A673 stands for all the cells taken from the bottom of the flasks that were mainly A673 cells in contact with PBMC. PBMC stands for the cells from the supernatant of the flasks that were mainly PBMC in contact with A673 cells.

Table 15. FACS results from the co-culture of A673 cells with PBMC. Two independent experiments show the percentages of CD3 and CD8 positive cells in the lymphocyte gate. A673 stands for all the cells taken from the bottom of the flasks and PBMC stands for the cells from the supernatant. The abbreviations are: V (empty pRTS1 vector); ERV3 (pRTS1 with ERV3); IL2+ (presence of IL2); IL2- (absence of IL2); D- (without doxycycline); D+ (with doxycycline).

CD3+		
Samples	#1	#2
A673 V D- IL2-	47.11	45.70
A673 V D- IL2+	56.84	55.40
A673 ERV3 D- IL2-	60.52	60.00
A673 ERV3 D- IL2+	61.89	48.57
A673 V D+ IL2-	61.18	46.25
A673 V D+ IL2+	66.78	39.93
A673 ERV3 D+ IL2-	42.54	51.30
A673 ERV3 D+IL2+	60.85	51.51
PBMC V D- IL2-	95.65	88.87
PBMC V D- IL2+	93.28	86.60
PBMC ERV3 D- IL2-	96.46	87.49
PBMC ERV3 D- IL2+	94.21	86.37
PBMC V D+ IL2-	95.45	89.79
PBMC V D+ IL2+	95.31	86.50
PBMC ERV3 D+ IL2-	96.33	88.10
PBMC ERV3 D+ IL2+	94.31	84.70

CD8+		
Samples	#1	#2
A673 V D- IL2-	22.22	14.14
A673 V D- IL2+	27.92	17.2
A673 ERV3 D- IL2-	31.37	17.88
A673 ERV3 D- IL2+	30.18	13.37
A673 V D+ IL2-	27	11.43
A673 V D+ IL2+	36.27	12.49
A673 ERV3 D+ IL2-	23.51	14.47
A673 ERV3 D+IL2+	42.72	15.15
PBMC V D- IL2-	46.41	21.36
PBMC V D- IL2+	45.99	21.46
PBMC ERV3 D- IL2-	44.16	20.98
PBMC ERV3 D- IL2+	46.6	21.03
PBMC V D+ IL2-	39.77	23.51
PBMC V D+ IL2+	42.18	21.14
PBMC ERV3 D+ IL2-	40.65	21.12
PBMC ERV3 D+ IL2+	43.96	21.62

Table 16. FACS results from the co-culture of A673 cells with PBMC. Two independent experiments show the percentages of CD4 and CD25 positive cells in the lymphocyte gate. A673 stands for all the cells taken from the bottom of the flasks and PBMC stands for the cells from the supernatant of the flask. The abbreviations are: V (empty pRTS1 vector); ERV3 (pRTS1 with ERV3); IL2+ (presence of IL2); IL2- (absence of IL2); D- (without doxycycline); D+ (with doxycycline).

CD4+		
Samples	#1	#2
A673 V D- IL2-	12.548	23.94
A673 V D- IL2+	12.96	26.25
A673 ERV3 D- IL2-	13.22	33.8
A673 ERV3 D- IL2+	8.09	22.68
A673 V D+ IL2-	21.19	35.13
A673 V D+ IL2+	11.67	20.31
A673 ERV3 D+ IL2-	8.47	30.2
A673 ERV3 D+IL2+	9.15	32.35
PBMC V D- IL2-	30.957	60.48
PBMC V D- IL2+	32.44	57
PBMC ERV3 D- IL2-	34.5	55.85
PBMC ERV3 D- IL2+	34.76	57.02
PBMC V D+ IL2-	38.13	60.15
PBMC V D+ IL2+	37.21	58.42
PBMC ERV3 D+ IL2-	40.98	60.82
PBMC ERV3 D+ IL2+	5.4	57.94

CD25+		
Samples	#1	#2
A673 V D- IL2-	3.148	6.68
A673 V D- IL2+	7.2	6.2
A673 ERV3 D- IL2-	1.02	8.73
A673 ERV3 D- IL2+	4.5	8.66
A673 V D+ IL2-	3.1	6.1
A673 V D+ IL2+	7.36	13.46
A673 ERV3 D+ IL2-	1.98	8.27
A673 ERV3 D+IL2+	6.03	11.7
PBMC V D- IL2-	2.227	9.41
PBMC V D- IL2+	4.35	9.68
PBMC ERV3 D- IL2-	2.19	12.61
PBMC ERV3 D- IL2+	3.62	9.46
PBMC V D+ IL2-	2.49	12.94
PBMC V D+ IL2+	5.22	9.19
PBMC ERV3 D+ IL2-	2.7	11.19
PBMC ERV3 D+ IL2+	2.7	9.23

The samples were also analyzed in a qRT-PCR to compare the relative expression of various genes. The experiment was repeated twice with different samples of donators for PBMC. Four different genes were analyzed: TBX21, GATA3, FOXP3 and RORC. The housekeeping gene was HPRT1. The results are from the samples of day 7. Two different figures are shown for each gene. One figure shows the cells that were taken from the bottom of the flasks. They are mainly A673 cells in contact with PBMC. The second figure shows the PBMC from the supernatant of the flasks that were mainly PBMC in contact with A673 cells. They are shown in a separate figure for a better appreciation of the results. Figures 38 to 41 show the relative expression in the cells that were at the bottom, mainly A673. Figures 42 to 45 show the relative expression in the cells that were in the supernatant, mainly PBMC. The values were different analyzing cells in the supernatant or harvested from the bottom. However, no clear effects of ERV3 expression were observed.

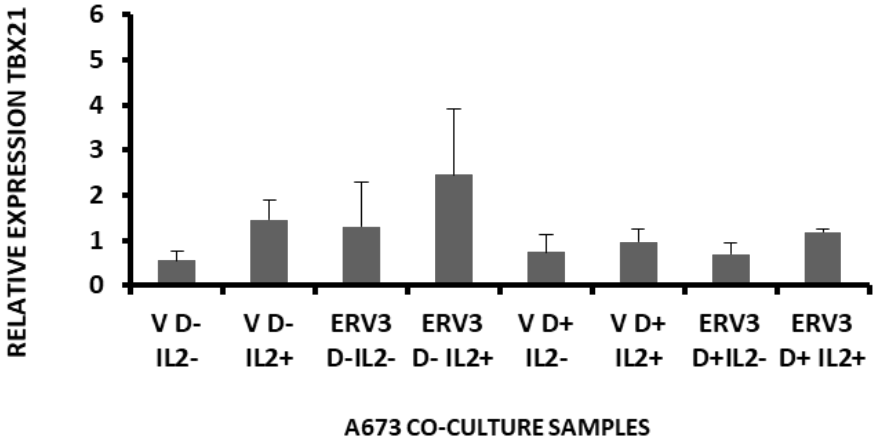


Figure 38. Relative expression of TBX21 in the co-culture samples (A673 cells in contact with PBMC). The abbreviations are: V (empty vector), ERV3 (vector with insert), D- (without doxycycline), D+ (with doxycycline), IL2- (without IL2), and IL2+ (with IL2). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

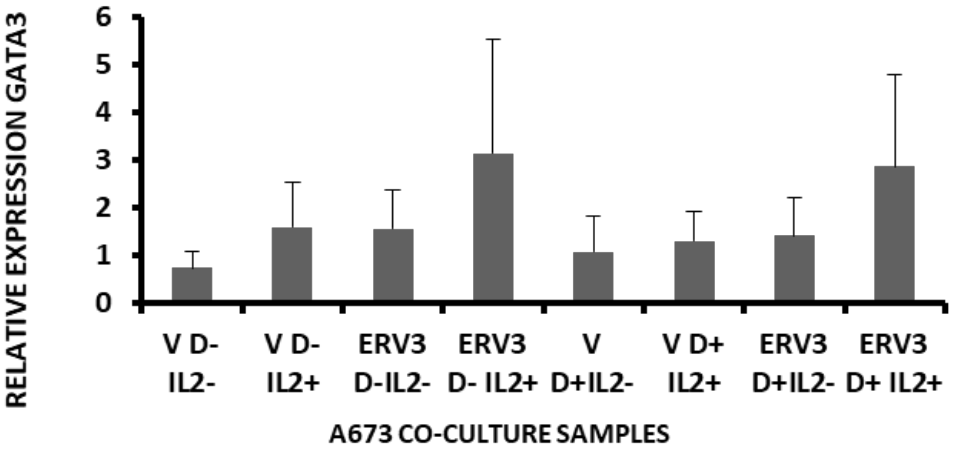


Figure 39. Relative expression of GATA3 in the co-culture samples (A673 cells in contact with PBMC). The abbreviations are: V (empty vector), ERV3 (vector with insert), D- (without doxycycline), D+ (with doxycycline), IL2- (without IL2), and IL2+ (with IL2). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

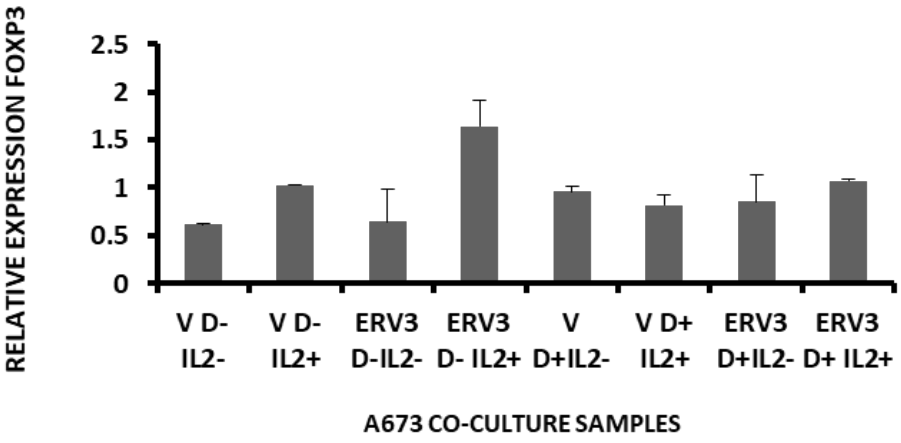


Figure 40. Relative expression of FOXP3 in the co-culture samples (A673 cells in contact with PBMC). The abbreviations are: V (empty vector), ERV3 (vector with insert), D- (without doxycycline), D+ (with doxycycline), IL2- (without IL2), and IL2+ (with IL2). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

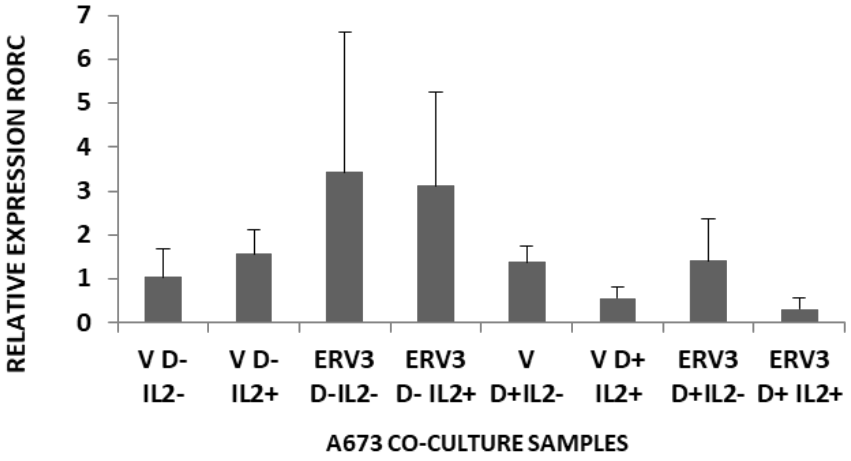


Figure 41. Relative expression of RORC in the co-culture samples (A673 cells in contact with PBMC). The abbreviations are: V (empty vector), ERV3 (vector with insert), D- (without doxycycline), D+ (with doxycycline), IL2- (without IL2), and IL2+ (with IL2). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

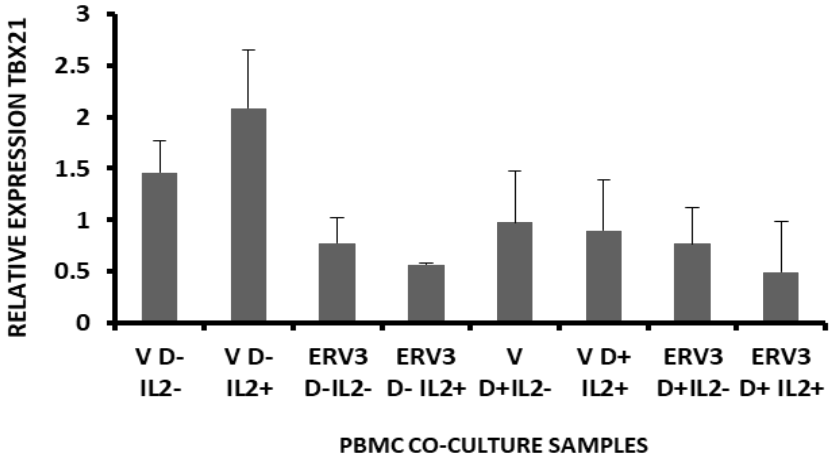


Figure 42. Relative expression of TBX21 in the co-culture samples (PBMC in contact with A673 cells). The abbreviations are: V (empty vector), ERV3 (vector with insert), D- (without doxycycline), D+ (with doxycycline), IL2- (without IL2), and IL2+ (with IL2). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

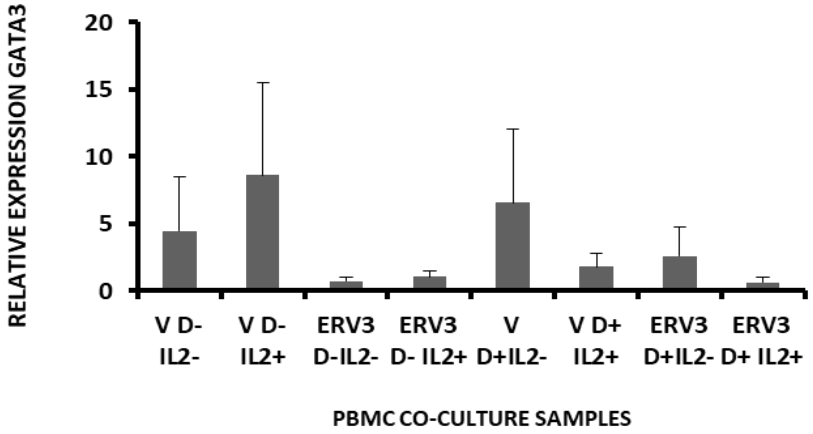


Figure 43. Relative expression of GATA3 in the co-culture samples (PBMC cells in contact with A673 cells). The abbreviations are: V (empty vector), ERV3 (vector with insert), D- (without doxycycline), D+ (with doxycycline), IL2- (without IL2), and IL2+ (with IL2). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

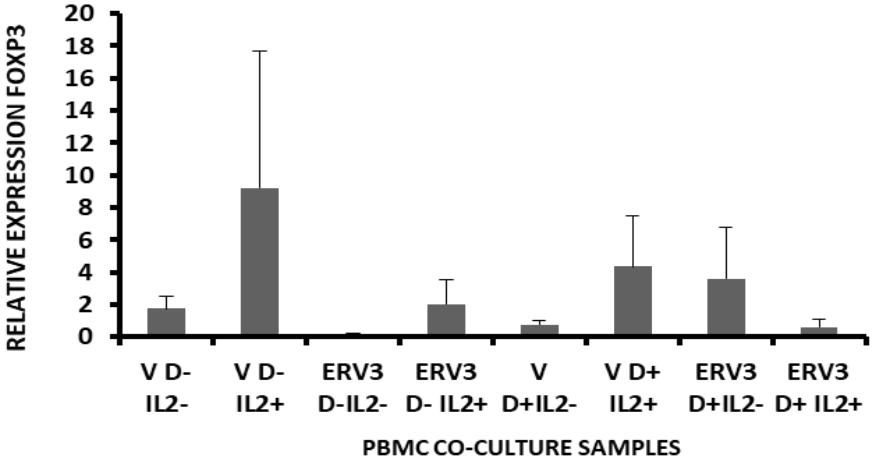


Figure 44. Relative expression of FOXP3 in the co-culture samples (PBMC cells in contact with A673 cells). The abbreviations are: V (empty vector), ERV3 (vector with insert), D- (without doxycycline), D+ (with doxycycline), IL2- (without IL2), and IL2+ (with IL2). The $2^{-\Delta\Delta Ct}$ method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

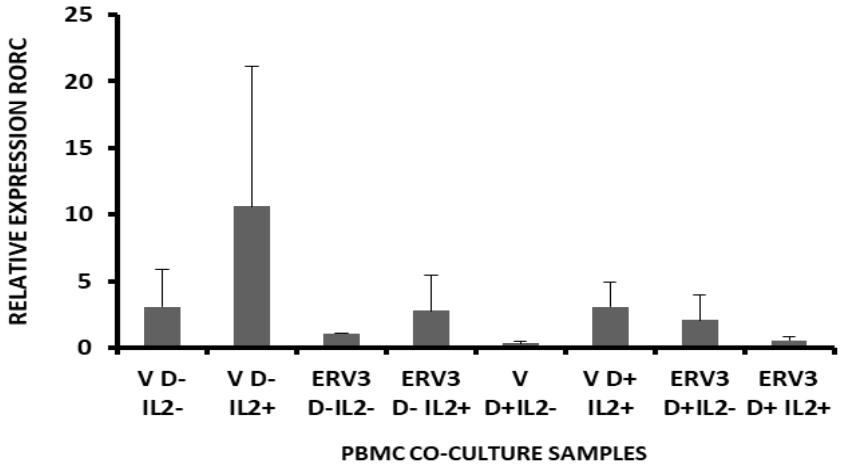


Figure 45. Relative expression of RORC in the co-culture samples (PBMC cells in contact with A673 cells). The abbreviations are: V (empty vector), ERV3 (vector with insert), D- (without doxycycline), D+ (with doxycycline), IL2- (without IL2), and IL2+ (with IL2). The $2^{-\Delta\Delta Ct}$method was performed for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

A control experiment was done to measure the impact of doxycycline in the expression of transcription factors in PBMC. These PBMCs were cultured without A673 cells. There were two groups. The first one was done under the same conditions as the co-culture. The second one was left without changes during the week. The doxycycline was added the first day and the analysis was done on day 7. The results of the two qRT-PCRs are shown from Figure 46 to 49.

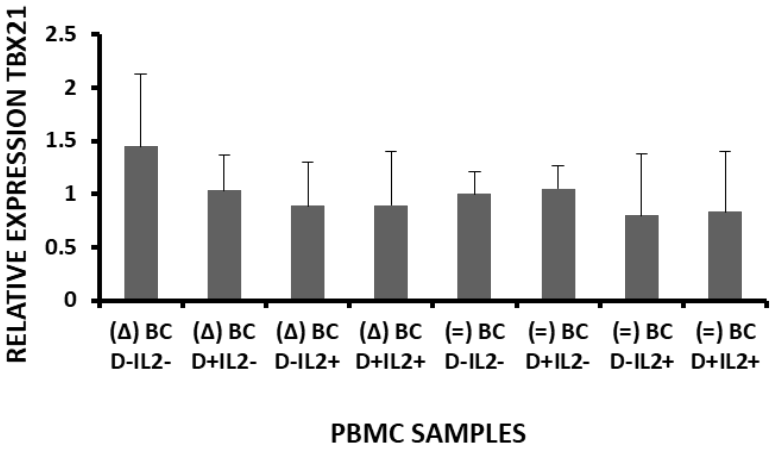


Figure 46. Relative expression of TBX21 in PBMC samples. The (Δ) is used for the samples that were divided imitating the experiment from the co-culture. The (=) sign is used for the samples that remained without change for 7 days. BC is used for PBMC. D- means without doxycycline and D+ means with doxycycline. IL2- means without IL2 and IL2+ means with IL2. The $2^{-\Delta\Delta Ct}$ method was used for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

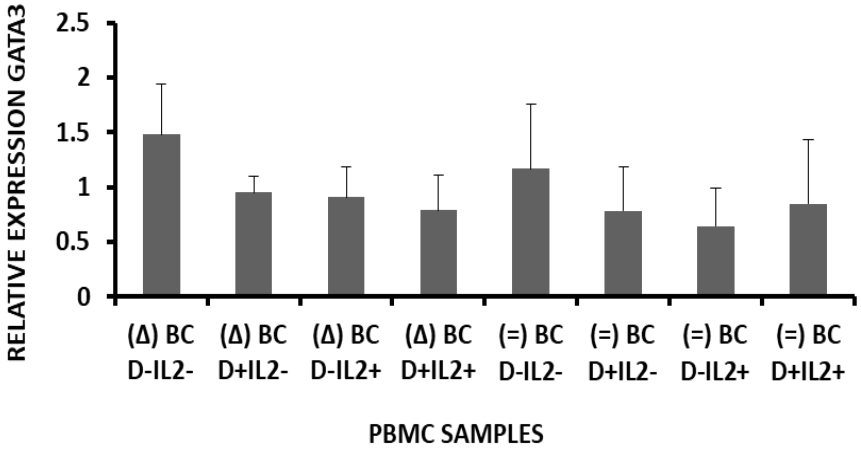


Figure 47. Relative expression of GATA3 in PBMC samples. The (Δ) is used for the samples that were divided imitating the experiment from the co-culture. The (=) sign is used for the samples that remained without change for 7 days. BC is used for PBMC. D- means without doxycycline and D+ means with doxycycline. IL2- means without IL2 and IL2+ means with IL2. The $2^{-\Delta\Delta Ct}$ method was used for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

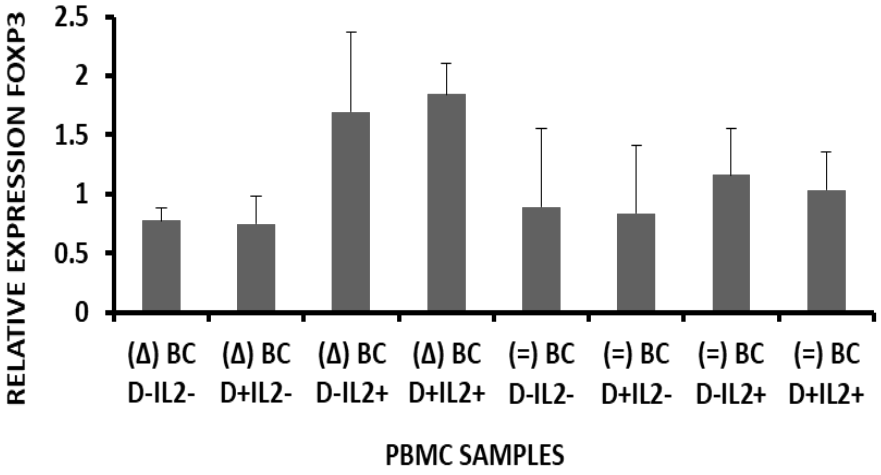


Figure 48. Relative expression of FOXP3 in PBMC samples. The (Δ) is used for the samples that were divided imitating the experiment from the co-culture. The (=) sign is used for the samples that remained without change for 7 days. BC is used for PBMC. D- means without doxycycline and D+ means with doxycycline. IL2- means without IL2 and IL2+ means with IL2. The $2^{-\Delta\Delta Ct}$ method was used for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

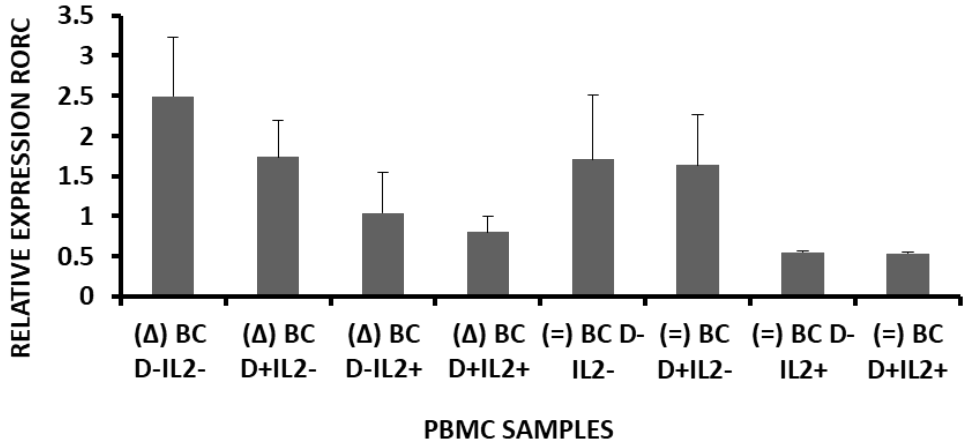


Figure 49. Relative expression of RORC in PBMC samples. The (Δ) is used for the samples that were divided imitating the experiment from the co-culture. The (=) sign is used for the samples that remained without change for 7 days. BC is used for PBMC. D- means without doxycycline and D+ means with doxycycline. IL2- means without IL2 and IL2+ means with IL2. The $2^{-\Delta\Delta Ct}$ method was used for the determination of the gene expression. For comparative analysis, HPRT1 was used as housekeeping control and the median of all samples was set as 1. The experiment was performed two times.

5. Discussion

Several experiments were run to gather information about the differences between cells transfected with empty control vector and a vector with ERV3 or ZNF117. Likewise, it was important to establish the differences of gene expression in presence and absence of doxycycline. The discussion follows the order from the results.

5.1 B cell lines

Three different cell lines were cultivated, KM-H2, L-428 and P493-6. This experiment was run to compare the different expression of genes in presence of tetracycline. The new gene measured in this experiment was myc. The proto-oncogene c-myc (myc) activates the regulation of cell proliferation, differentiation and apoptosis potently. Genetic alterations in myc contribute to various tumors, mainly B-cell lymphomas (Pajic et al., 2000; Nguyen et al., 2017). The cell line P493-6 is a B-cell line. The special condition of this cell line is that carries a tetracycline-regulated myc (Pajic et al., 2000). In the qRT-PCR, the samples having tetracycline were downregulated in presence of tetracycline. This was the expected effect. The expression of myc was unchanged in the other two cell lines.

When measuring the expression of ERV3, KM-H2 and L-428 showed no significant difference with or without antibiotic. Interestingly, P493-6 showed upregulation in the presence of tetracycline. This was the exact effect showed in a study of expression and regulation of ERV3 in HL cells (Kewitz and Staege, 2013). There, the authors compared ERV3 expression in cells growing in medium (cycling cells) and cells growing in medium with tetracycline (arrested cells). They also proved that ERV3 was increased in cells under inhibited proliferation conditions. It is upregulated in cell cycle arrested leukemia cells (Abrink et al., 1998). In choriocarcinoma cells, the transgenic expression of ERV3 inhibits cell proliferation (Lin et al., 2000). The high expression of ERV3 in cells under anti-proliferative conditions might indicate a tumor suppressing activity (Kewitz and Staege, 2013). The results from the qRT-PCR with ZNF117 were the same as with ERV3. The presence of tetracycline seems to have an effect in the expression of both genes in the P493-6 cells.

5.2 Cell screening

Many studies have revealed the presence of ERV3 in tissues. A screening was made with 42 samples of tumor and normal tissues. They showed similar results to the literature. A negative control was made with one sample of mouse tissue. The relative expression of ERV3 was undetectable. ERV3 is absent from the mouse genome (Lee et al., 2006). It has been detected

only in humans and primates (Yuan et al., 2001). Several studies have used it as a marker for the presence of human DNA (Kim et al., 2006; Gage et al., 2011; Barletta et al., 2014).

ERV3 is extensively expressed in human tissues and cells (Andersson et al., 1996). Eighteen normal tissues were analyzed in the screening. Placenta showed the highest relative expression in this experiment. This result has been until now the same one shown in the literature (Larsson et al., 1994; Andersson et al., 1996; Muir et al., 2004). It is also expressed in other human reproductive tissues (Larsson et al., 1994). Ovary showed a high relative expression after placenta, breast and white blood cells. Placenta has the highest value of ERV3 expression and the lowest one of ZNF117. The meaning of the low expression of ZNF117 in placenta is interesting to follow as ERV3 seems to play a main role in it.

Among the analyzed tumor tissues, THP1 cells showed the highest expression with ERV3 and with ZNF117 screening. Studies have shown that several tumors displayed a significant positivity but there are no specific patterns seen in this study. A673 cell line, mainly used during the experiments, had a relative low expression of ERV3 and ZNF117. This fact is important because it makes the cells appropriated for the study. Published data from ERV3 presence in tumors is found in journals but there are still no studies regarding ZNF117. Therefore, more studies should be done with more samples to clarify the meaning of these values and their variation between ERV3 and ZNF117. Especially, studies with THP1 should be done because this cell line showed high expression of ERV3, ZNF117 and fusion protein ERV3 ZNF117.

The fusion transcript ERV3-ZNF117 was also measured. Placenta has a low expression just as for ZNF117. THP1 shows a high expression but in comparison to the screening of ERV3 and ZNF117, this expression is just one third of theirs. The meaning of these values can be interpreted as the amount of fusion transcript inside each tissue. The difference between the single measure of each one and the measure of both together (ERV3-ZNF117) still needs to be clarified.

Using the data from the cell screening, the mole weight of DNA was calculated in every cell line. This is a more accurate method to note the differences between cell lines. The results were exactly the same as the ones obtained with the relative expression in the qRT-PCR.

5.3 Transgenic A673 cells

5.3.1 Expression of ERV3, ZNF117 and ERV3-ZNF117

The transfection of ERV3 was realized in A673 cells, Ewing sarcoma cells, because of the easy management of the transfection. Besides, these cells have a rapid growth allowing enough number of cells for the experiments. The A673 cell line was derived from an Ewing sarcoma and it has a complex karyotype with presence of a t(11; 22) translocation (Roberts et al., 1999). Cell lines are useful tools in experiments. The main experiments were made with this cell line and with the vector pRTS1. This vector is an EBV-derived plasmid which has various important characteristics. For example, it has a low background activity, it is highly inducible in the presence of doxycycline and it has a graded response to increasing concentrations of the inducer (Bornkamm et al., 2005). The included luciferase gene is circumscribed by SfiI sites, making it useful as for cloning genes of interest (Bornkamm et al., 2005). Due to this site, the cloning in the described experiment was always made using the restriction enzyme SfiI.

In order to study the expression of ERV3, different experiments were done using doxycycline to induce the cells. The relative expression of ERV3 was higher when doxycycline was added. This effect was only seen in the cells having pRTS1xERV3. Despite the fact that the relative expression increased four times with the induction, the over-expression seems to be relatively weak. It is not known if the low relative expression at the end depends on the specific insert. Further studies should be done to analyze this effect.

The transfection of ZNF117 was made following the same conditions as the ERV3 transfection, which was done months earlier. In this transfection, the relative expression of ZNF117 was also upregulated in presence of doxycycline. The expression was double than without the induction, but the values were low. As expressed before for ERV3, further studies should be done to analyze if the low values obtained are due to the insert.

To characterize the gene expression in more detail, the relative expression was measured through a period of seven days to notice the differences of ERV3 expression. The highest expression was seen on day three with pRTS1xERV3 with doxycycline. The expression was five times higher than the control without induction. After this highest point, the relative expression descended gradually until it reached the same level from the other samples. This effect shows a variation of inducibility through time. Here, doxycycline was added in the first hours of the experiment and the conditions of the cells were not changed during the week. In this experiment, the relative expression of ERV3 on day three is twice as high as the value

gotten from independent experiments in one measure point. This is actually the highest value of overexpression obtained during the experiments. As written before, further studies should be done to prove if that is the usual effect from ERV3 insert in this vector.

ZNF117 was measured in the transfected cells with pRTS1xERV3. When induction was done with doxycycline, the signal was higher in samples having antibiotic. However, the results were low relative expression in all samples, being the highest point also on day three. This shows that when ERV3 is upregulated it does not necessarily comes with the upregulation of ZNF117. Probably high expression of ERV3/ZNF117 is not compatible with cell growth. In previous studies (Kewitz and Staege, 2013) it was shown that expression of ERV3 increases in cells that have stopped proliferation. If cells with high expression cannot proliferate, this might reduce the overall low expression seen in the transfected cells.

The third experiment done with the kinetics from ERV3 was to measure the relative expression of the fusion transcript ERV3-ZNF117. The values obtained were higher than in the analysis of ZNF117 alone but lower than the ones from ERV3. The samples with doxycycline had higher relative expression than the ones without it. The results of day three showed also the highest values as seen in the experiments of kinetics before. On day four and five the signal from vector with ERV3 without doxycycline went higher than the other values. ERV3 seems not to induce the fusion transcript ERV3-ZNF117.

The experiment made with the cells transfected with pRTS1xZNF117 showed an upregulation of ZNF117 when induced with doxycycline. The value was two-fold higher. This increase is lower than the one seen with the ERV3 transfection (five times higher). When ERV3 and the fusion protein ERV3-ZNF117 were measured in the transfected cells with pRTS1xZNF117, they were not upregulated in presence of doxycycline. ZNF117 forms a cluster on human chromosome 7 with highly homologue other zink finger proteins (Kewitz and Staege, 2013). Until now, the physiological function of ZNF117 has not been clarified but there is a high possibility that it contributes to the effects of ERV3. Further studies are needed to know more about this gene.

5.3.2 Expression of EGFP

In the experiments done by Bornkamm et al., 2005, they induced EGFP by adding doxycycline to pRTS1 transfected clones. They proved that EGFP expression decreased considerably after removing doxycycline. However, the decrease was slowly over time (Bornkamm et al., 2005). This was proved by measuring the EGFP gene in the transfected cells in different time periods.

The data shown in Table 13 were from experiments done with transgenic cells after 7 months of the transfection. The expression of EGFP was extremely different among experiments. However, the tendency remained in all the measurements. This is supported by the fact that the inducibility of the vector varies over time. Experiments done in another research showed that after 9 months the inducibility decreased significantly (Bornkamm et al., 2005).

As expected, the relative expression of EGFP was only high in the samples having doxycycline. However, the highest value came from the empty vector and not from the vector having ERV3 insert. The difference between both was almost three-fold. To prove if that was the tendency, two other experiments were run, a FACS analysis and one qRT-PCR with hygromycin phosphotransferase. The FACS analysis showed a similar effect. The empty vector showed a higher expression than the vector with insert when GFP was measured. The difference here between both was of two-fold. The experiment with hygromycin phosphotransferase supports the results from the previous experiments. The samples having doxycycline were showing higher relative expression than the ones without it. This is not expected but suggests that the treatment of the cells with doxycycline not only affects the bidirectional GFP/Insert promoter but also the constitutive promoter used for hygromycin phosphotransferase expression. Besides, the empty vector showed a higher relative expression than the vector with ERV3 insert, two-fold also. Further studies are to be made to prove if this lower expression of the vector having the insert is an effect from the insert itself.

5.4 Expression of various genes in the transgenic cells

Several genes were studied in the transgene cells such as ADRB1 (Adrenoreceptor B1), LIPI, EWS-FLI1, CCND1 (Cyclin D1), KCNAB3 (potassium voltage-gated channel subfamily A regulatory beta subunit 3) and XIST (X Inactive Specific Transcript). LIPI and EWS-FLI1 are specific genes in Ewing sarcoma (Pfeifle et al., 2009). LIPI showed no clear effect with none of the transgene cells regardless if they were with insert or not, or if they were induced with doxycycline or not. XIST was also analyzed. This gene triggers X chromosome inactivation. If XIST is lost, X is reactivated and other genome changes happen, leading to cancer (Yildirim et al., 2013). As mentioned with LIPI, no clear effect was seen.

EWS-FLI1 expression was similar to the one observed with KCNAB3 and CCND1. The three of them showed a clonal effect. Each gene was highly expressed in the empty vector and had a lower expression in the vector with ERV3. No important difference is seen in the presence of doxycycline. KCNAB3 is a gene expressed in ES but just in a few number of normal tissues.

Besides, CCND1 is a gene expressed in ES and in a lower level in all other tissues (Staege et al., 2004).

Finally, ADRB1 is like KCNAB3, a gene expressed in ES but just in a few number of normal tissues (Staege et al., 2004). This gene is upregulated in the empty vector in presence of doxycycline, but downregulated in the vector with ERV3 induced with doxycycline. Overall, the gene expression profile suggests clonal variation. It is important to emphasize that the experiment was repeated only two times to be considered with high statistical significance.

5.5 CCND1 and doxycycline in A673 cells

Due to the results obtained with CCND1, an additional experiment was done with this gene. The relative expression of CCND1 in A673 cells with different doses of doxycycline showed no regular tendency. There was a lot of variation among experiments having the same condition. The only repeated effect was with the cells having the highest dose of doxycycline. The relative expression of CCND1 in these cells decreased after day 9 suggesting that high doses of doxycycline may play a role in the cyclins. However, the experiment should be repeated because of the various differences among the rest of the results.

5.6 Co-culture of A673 and PBMC

On day 7, both cell compartments (A673 and PBMC) from this experiment were analyzed. Altogether, no clear effects of ERV3 on the immunostimulatory activity of A673-cells could be observed. The percentages of CD3 positive cells from the bottom were lower than the corresponding percentages of the cells from the supernatant. All these cells were taken from data of the lymphocyte gate. This is likely a consequence of the presence of tumor cells in this compartment. The size of the tumor cells is variable and at least some of the cells might be included in the lymphocyte gate. Additional antibody combination that can better separate tumor cells and PBMCs should be used in future experiments.

Besides counting the cells and analyzing them in FACS, a qRT-PCR was done. The values were different in the analysis of cells from the bottom of the wells and from the supernatant. As a marker for the Th1 response, the T-box transcription factor (TBX21) was measured. Among the cells from the bottom there was an upregulation in the presence of IL-2, being especially high in pRTS1xERV3 samples without doxycycline suggesting a strong stimulus from this interleukin on the Th1 response interacting with ES tumor cells. In contrast, among PBMCs from the supernatant in both experiments TBX21 was downregulated in presence of IL-2, especially

with the samples having ERV3 insert. Probably, the stronger interaction with tumor cells supported a response in the cells from the bottom. In the Th2 response, the zinc protein transcription factor GATA3 was measured. The gene was upregulated in the cells from the bottom in the presence of IL-2, especially with ERV3 insert. On the contrary, it was downregulated among PBMCs in the presence of IL-2. It showed a similar expression as with TBX21.

In the Treg response, Forkhead box P3 (FOXP3) was measured. FOXP3 was upregulated in all samples from the bottom and from the supernatant in the presence of IL-2. This supports the fact that the presence of IL-2 stimulates the Treg response. Among the Th17 response, the retinoic acid receptor-related orphan receptor gamma (RORC) was measured. In this measurement no common tendency was seen. RORC was downregulated in presence of IL-2 in the experiment where PBMCs were measured alone. Further studies should be done to see if this tendency can be reproduced. There are various differences among the experiments. The reason can be due to the different origin of the PBMCs (different donors). The different origin from cells might give different immunological responses.

6. Summary

6.1 Summary

The present investigation analyzed expression of the endogenous retrovirus 3 (ERV3) in tumor cells.

Upregulation of ERV3 in cell-cycle arrested P493-6 cells was proven to be a consequence of cell cycle arrest and not of the addition of tetracycline to B cells. Multiple articles have shown the expression of ERV3 in normal tissues and tumor cells. The cell screening performed in this study revealed similar results from the ones published until now. Placenta had the highest expression of ERV3 followed by ovary and breast. In contrast, the screening showed that placenta had only low ZNF117 expression. A common high expression was seen in from ERV3 and ZNF117 in THP-1 cells. The fusion transcript ERV3-ZNF117 was also measured in some tissues and tumor cells and it showed a similar expression pattern as ZNF117 with high expression only in THP-1 cells.

Among the experiments with cells transfected with pRTS1xERV3, ERV3 was upregulated in presence of doxycycline. However, when ZNF117 was measured in these transgene cells the expression remained unchanged. In the experiments with cells transfected with pRTS1xZNF117, ZNF117 was upregulated in presence of doxycycline. In these cells, ERV3 was measured in presence of doxycycline and no upregulation was evidenced. These results suggest that expression of ERV3 is not regulated by ZNF117 and *vice versa*.

The expression of some typical Ewing Sarcoma associated antigens was measured in presence and absence of ERV3 with and without doxycycline. Overall, the gene expression profile suggested a clonal variation with no impact of ERV3 on the expression of these genes. In particular, cyclin D1 showed reduced expression in the presence of doxycycline. The additional experiment with different concentrations of doxycycline in A673 cell cultures showed an impact of doxycycline on expression of CCND1 and cell proliferation. Especially after day 9, the cell numbers in the presence of highest concentrations of doxycycline were decreased. Further studies should be done to investigate the effects of doxycycline on Ewing sarcoma cells. Co-culture experiments of A673 cells and PBMC showed no clear effect of ERV3 expression on the immunological response.

Further experiments are needed in order to gain insight into the function of ERV3/ZNF117 in tumor cells. The transgenic cell lines described in the present study might be useful for further studies.

6.2 Zusammenfassung

In der vorliegenden Untersuchung wurde die Expression des Endogenen Retrovirus 3 (ERV3) in Tumorzellen untersucht. Die Hochregulation von ERV3 in Zellzyklus-arretierten P493-6-Zellen konnte als eine Folge des Zellzyklus-Arrestes bestätigt werden und nicht als Effekt von Tetracyclin auf B-Zellen. Mehrere Publikationen haben die Expression von ERV3 in normalen Geweben und Tumorzellen gezeigt. Das in dieser Studie durchgeführte Zellscreening ergab ähnliche Ergebnisse wie die bisher veröffentlichten. Plazenta hatte die höchste Expression von ERV3, gefolgt von Eierstock und Brustdrüse. Im Gegensatz dazu zeigte das Screening, dass die Plazenta nur eine geringe Expression von ZNF117 aufweist. Eine gemeinsame hohe Expression wurde für ERV3 und ZNF117 in THP-1-Zellen beobachtet. Das Fusionstranskript ERV3-ZNF117 wurde auch in einigen Geweben und Tumorzellen gemessen und zeigte ein ähnliches Expressionsmuster wie ZNF117 mit hoher Expression nur in THP-1-Zellen.In den Experimenten mit Zellen, die mit pRTS1xERV3 transfiziert wurden, wurde ERV3 in Gegenwart von Doxycyclin hochreguliert. Die Expression von ZNF117 änderte sich jedoch nicht. In den Experimenten mit Zellen, die mit pRTS1xZNF117 transfiziert wurden, wurde ZNF117 in Gegenwart von Doxycyclin hochreguliert. In diesen Zellen wurde ERV3 in Gegenwart von Doxycyclin unverändert niedrig exprimiert. Diese Ergebnisse legen nahe, dass die Expression von ERV3 nicht durch ZNF117 reguliert wird und *vice versa*.

Die Expression einiger typischer Ewing-Sarkom-assoziierter Antigene wurde in Gegenwart und Abwesenheit von ERV3 mit und ohne Doxycyclin gemessen. Insgesamt zeigte das Genexpressionsprofil eher eine klonale Variation ohne Einfluss von ERV3 auf die Expression dieser Gene. Insbesondere Cyclin D1 zeigte reduzierte Expression in Anwesenheit von Doxicyclin. Zusätzliche Experimente mit verschiedenen Konzentrationen von Doxycyclin in A673-Zellkulturen zeigten einen Einfluss von Doxycyclin auf die Expression von CCND1 und die Zellproliferation. Besonders nach dem 9. Tag waren die Zellzahlen in Gegenwart der höchsten Doxycyclin-Konzentrationen gesunken. Weitere Studien sollten durchgeführt werden, um die Auswirkungen von Doxycyclin auf Ewing-Sarkomzellen zu untersuchen. Co-Kultur-Experimente von A673-Zellen und PBMC zeigten keinen klaren Effekt der ERV3-Expression auf die immunologische Reaktion.

Um weitere Einblicke in die Funktion von ERV3/ZNF117 in Tumorzellen zu erhalten, sind weitere Experimente notwendig. Die in der vorliegenden Studie beschriebenen transgenen Zelllinien könnten für derartige weitere Studien nützlich sein.

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Thesen

1. ERV3 is upregulated in cell-cycle arrested P493-6 cells.
2. While ERV3 is highly expressed in placenta, ZNF117 and ERV3-ZNF117 are almost undetectable.
3. ERV3 and ZNF117 are highly expressed in THP1 cells.
4. The expression of ERV3 in transfected cells with vector pRTS1xERV3 could be switched on by addition of doxycycline.
5. The expression of ZNF117 in transfected cells with vector pRTS1xZNF117 could be switched on by addition of doxycycline.
6. ERV3 upregulation has no effect on ZNF117 or ERV3-ZNF117 expression and *vice versa*.
7. The treatment of pRTS1-transfected cells with doxycycline might not only affect the bidirectional promoter driving expression of GFP and the gene of interest but also the constitutive promoter used for hygromycin phosphotransferase expression.

Selbständigkeitserklärung/Declarations

1) I declare that I have not completed or initiated a doctorate procedure at any other university.

2) I declare that all information given is accurate and complete. The thesis has not been used previously at this or any other university in order to achieve an academic degree.

3) I declare under oath that this thesis is my own work entirely and has been written without any help from other people. I complied with all regulations of good scientific practice and I used the sources mentioned and included all the citations correctly both in word or content.

Halle(Saale), 08.11.2018

Yomara Yardén Bustamante Rivera

Curriculum Vitae

Name:	Yomara Yardén Bustamante Rivera
Date of birth:	18.07.1984
Place of birth:	Guatemala City, Guatemala

1990-2000	Elementary School. English American School, Guatemala City
2001-2007	Medicine Study in San Carlos de Guatemala University. Graduation on January 31st 2007.
01.12.-31.01.2006	Practice in the Laboratory of the Human Genome Sequencing Center at Baylor University, Houston, Texas. Medicine Thesis: Biotechnological Knowledge in Fellows and Medicine Students in Guatemala.
2008-2011	Fellow in Pediatrics. Roosevelt Hospital Guatemala City. Hospital Nacional, Guastatoya, El Progreso, Guatemala. San Carlos de Guatemala University.
11.02.2012	Master in Pediatrics. Master Degree from San Carlos de Guatemala University Thesis: Mutations in Connexin26 in children with Deafness in Guatemala.
11.2015-12.2015	Forschungs-Praktikum in der Forschungslabor Kinderklinik I Universität Halle-Wittenberg.
06.2012-02.2017	Pediatrician in the Oncological Hospital, Unidad Nacional de Oncología Pediátrica (UNOP). Guatemala City.
03.2017-05.2018	Investigation Project for the MD Degree (Dr.med.) Forschungslabor, Kinderklinik I Universität Martin-Luther, Halle-Wittenberg.
25.10.2018	Defense of the thesis: Expression of Endogenous Retrovirus 3 (ERV3) in tumor cells.

Halle (Saale), 8.11.2018

Yomara Yardén Bustamante Rivera

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