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The role of EZH2 Polycomb group gene in primary cell permissiveness for EWS-FLI1 oncogene expression

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Abstract

Ewing's sarcoma (ES) is a malignant tumor that can be defined by the characteristic chromosomic translocation t(11;22)(q24;q12), which then leads to the formation of the fusion protein EWS-FLI1, responsible for malignant transformation. The expression of many genes can be modified by EWS-FLI1 in ES, including EZH2 (Enhancer of Zeste, Drosophila, Homolog 2). This methyltransferase is a member of the Polycomb group genes, which are capable of inducing epigenetic modifications that silence or induce target gene expression. EZH2 is part of the Polycomb Repressive Complex 2 (PRC2), which inhibits DNA transcription by adding a trimethyl group to histone H3. This provides a mechanism by which EZH2 can participate in generating conditions that help maintain the cell at a stem-like state.

While expression of EWS-FLI1 in primary cells has not been successful, primarily due to an oncogenic stress response, mesenchymal stem cells (MSCs) were able to survive and successfully express EWS-FLI1. Therefore, it is likely that plasticity is important for cells to sustain overexpression of EWS-FLI1. Thus, inducing EZH2 expression could possibly increase cell permissiveness to EWS-FLI1 expression.

This work shows that, following transduction of fibroblasts with EZH2, primary cells become more permissive and are capable of expressing EWS-FLI1. Thus, EZH2 provides a permissive environment for oncogenic transformation.

Keywords: Cancer - epigenetics - Ewing's sarcoma - transformation - permissiveness.

Introduction

1. Pediatric tumors: Ewing's sarcoma family of tumors

Cancer is the second leading cause of death among children in the U.S., just after accidents. Developing cancer during childhood remains rather rare, with an incidence of just above 16 per 100'000 people per year under the age of 20, and prognosis has significantly improved in the last 40 years. The causes leading to cancer in children remain unclear, although research is pointing toward a multicausal etiology, as complex interactions between environmental and genetic factors are believed to be linked to tumorigenesis (1). Therefore, prevention of pediatric tumors is a challenge yet to be addressed. Nevertheless, research has led to a better understanding of the mechanisms behind cancer and has helped improve treatments and outcome. For instance, leukemia and lymphoma are the most frequent cancers in children, accounting for about 60% of all cancer between the ages 0 and 18 years, and outcome has drastically improved with an actual average of 70% 10-year survival rate for the most frequent blood cell malignancies (2). In contrast, primary bone tumors, which are less common and account for 5% of all pediatric malignancies, bear a much poorer prognosis, with 5-year survival rates as low as 20% for metastatic osteosarcomas (3).

Among childhood malignancies, Ewing's sarcoma (ES) counts as one of the most aggressive ones with a 5-year survival rate of less than 30% in some severe cases (4). This malignancy was first described by James Ewing in 1921 as a radiosensitive undifferentiated tumor of long bones. Further analysis of various tumors with histological and immunochemical features similar to those of ES, such as peripheral Primitive Neuroectodermal Tumor (PNET) or Askin tumor, helped define a group of neoplastic diseases under the Ewing Sarcoma Family Tumor (ESFT) denomination, which are all small round blue cell tumors (5).

ES is the second most common primary bone malignancy of children after osteosarcoma (6). Incidence of ES is the highest among children between 10 to 15 years of age, with a slight male predominance. Long bones of the extremities and pelvis are most commonly involved, but ES can also occur in soft tissues. Even though under one fifth of patients are clinically metastatic at time of diagnosis, mortality is high, primarily due to relapse that occurs in up to 90% of cases. Metastasis is suspected to occur early in disease progression likely due to single tumor cells that have left the bulk and disseminated in the body before the time of diagnosis. Lungs and bone marrow are the two main sites of metastases.

Since the introduction of systemic chemotherapy as standard treatment, survival rate has dramatically risen from 10% to up to 75% for localized tumors (7). The current standard treatment involves a multidisciplinary approach: surgery is combined to multidrug chemotherapy and radiation therapy (6). Hopefully, further

and deeper understanding of ES biology will ultimately lead to new selective, targeted and more efficient therapies using less toxic agents.

2. Cellular and molecular biology of Ewing's sarcoma

There is much debate around the cellular origin of ES. The first hypothesis suggested a neural origin, in part because common markers can be found on ES cells and neural crest cells and because ES cells could undergo neural differentiation (8). However, as ES can involve bones or soft tissue, it is conceivable that ES could derive from more multipotent undifferentiated cells. Recent data provide evidence that ES probably derives from mesenchymal stem cells (MSCs), which can differentiate into adipocytes, chondrocytes and osteoblasts, express neural markers, and are known for being capable of migrating from bone marrow to extraskeletal sites (9).

Several non-random chromosomal changes have been described in ESFT and all involve EWSR1 (Ewing sarcoma region 1), a gene found on chromosome 22. In more than 85% of all ES cases, EWSR1 is fused to FLI1 on chromosome 11 after translocation t(11;22)(q12;q24) to encode the 68kDa aberrant chimeric protein EWS-FLI1 (figure 1), located in the cell nucleus (10). This fusion is a signature of ESFT (11).

EWSR1 encodes a ubiquitously expressed protein, member of the TET gene family, containing a RGG (arginine - glycine - glycine) repeat region, that binds RNA. It regulates transcription by interacting with RNA Polymerase II (RNA PolII) and various transcription activators or repressors. EWS is also believed to be involved in gene splicing (12). Knock-down of EWSR1 in mice leads to an arrest in hematopoietic stem cell (HSC) growth and renewal (13).

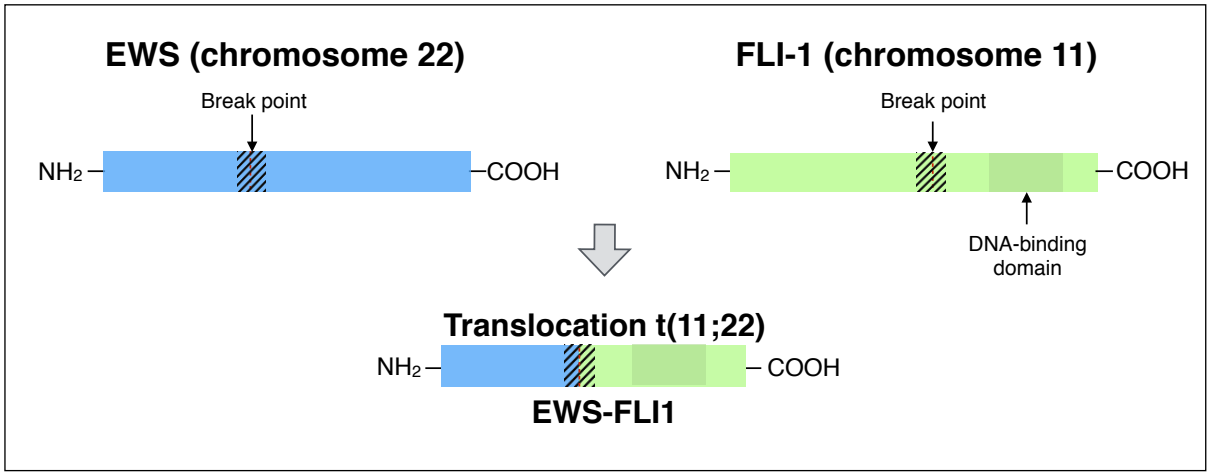

Figure 1. Schematic representation of the EWS-FLI1 chimeric protein as a result from the t(11;22) translocation. The C-terminal portion of transcription factor EWS is fused to the N-terminus of FLI-1, which contains the DNA-binding domain. The diagonal lines show the possible locations of breakpoint regions and various sequences juxtaposing the fusion points found in ESFT. Based on (11).

FLI1 (Friend's leukemia virus induced erythroleukemia 1) is a member of the ETS transcription factor family, in which all members share a common C-terminal DNA binding domain. The malignant potential of FLI1 has been shown through induced overexpression of FLI1, which causes increased growth and self-renewal of erythroid progenitors (14). Also, the capacity of FLI1 to inhibit retinoblastoma (Rb) expression helps cells enter the S phase of the cell cycle. Knock-down of FLI1 leads to disrupted T and B cell development as well as impaired vascular endothelial differentiation (15).

When translocation occurs, DNA-binding properties of FLI1 joined to transcription activation capacities of EWS lead to either induction or repression of an altered gene repertoire. Candidate targets relevant for malignant transformation and tumor progression have been identified and include, among others, induction of MYC and PDGFC, which promote cell proliferation and survival, as well as repression of p21 and IGFBP3, helping the cell escape from apoptosis and growth inhibition (16). The mechanisms by which EWS-FLI1 ultimately influences expression of targets are not fully elucidated yet, but a study has shown that EWS-FLI1 induces RNA helicase A (RHA) expression, which in turn binds to EWS-FLI1 target promoters, thus enhancing EWS-FLI1 function (17). This shows EWS-FLI1 can act directly or indirectly in order to modify gene expression.

The EWS-FLI1 fusion protein acts as an aberrant transcriptional regulator, leading to cell differentiation arrest and oncogenesis (9). A study in which EWS-FLI1 expression in ESFT cell lines was inhibited showed a decrease in cell growth *in vitro*, as well as in tumor forming capacity *in vivo* (18), pinning the tumorigenic function of EWS-FLI1. Expression of EWS-FLI1 can probably be modulated based on changes in DNA conformation such as histone methylation, an important epigenetic process.

3. Epigenetics: key to understanding cancer

Although the genetic events that underlie carcinogenesis and subsequent tumor progression are beginning to be well understood, it is becoming increasingly apparent that epigenetic modifications play an equally important and sometimes even dominant role. Activation of oncogenes and corresponding oncoproteins, or loss of tumor suppressor genes, for instance, are commonly referred to as major actors in cellular transformation. In most cases these events help deregulate the cell cycle, render cells insensitive to pro-apoptotic signals and provide them with autonomy with respect to growth factor signaling. In addition to this, oncogenic events can affect differentiation by altering epigenetic regulatory circuits that involve DNA and histone methylation, other forms of histone modification and microRNA (miRNA) biogenesis.

The epigenetic state of primary cells is believed to determine whether they may or may not be permissive for oncogene-induced transformation. Thus, in depth understanding of epigenetically regulated cell permissiveness for oncogene expression and the mechanisms that regulate epigenetic changes upon

transformation are key steps toward developing rational mechanism-based targeted anti-cancer therapies.

Polycomb group (PcG) genes encode proteins that create epigenetic alterations in the DNA structure. These are modifications in the genome expression without changes in the primary DNA sequence. PcG proteins can act through various mechanisms, primarily histone methylation or acetylation. Histones are instrumental in packaging DNA to chromosomes and can influence DNA transcription by modifying chromatin conformation. Thus, PcG proteins can influence cell division, differentiation, survival and senescence (19–22). The PcG gene EZH2 (Enhancer of Zeste, Drosophila, Homolog 2) is over-expressed in many tumors including ES and has been correlated with the most aggressive forms of prostate and breast cancer (23,24). The Polycomb Repressive Complex 2 (PRC2), which contains EZH2 as its catalytic core protein, can inhibit gene expression. EZH2 is a methyltransferase responsible for adding a trimethyl group to lysine 27 of histone H3, causing a reversible repression of transcription (25). Its target genes are mostly regulators of cell cycle and differentiation (22,24,26,27), so that an increased expression of EZH2 as seen in ES will help keep the cell in a state of plasticity with abnormal cell growth and proliferation (10,23). We know that subpopulations of cancer cells are often quite similar to stem cells in regard to gene expression and chromatin signature (24). Therefore, we can hypothesize that plasticity induced by PcG EZH2 may be an important feature for transformation. Indeed, it has been questioned whether the increase in EZH2 expression in tumors is found because EZH2 is a marker of proliferation or an active contributor to transformation (16).

4. Polycomb group genes and Ewing's sarcoma

Attempts to induce ES formation through overexpression of EWS-FLI1 in human cells have failed (28). Normal primary cells do not tolerate expression of EWS-FLI1 and therefore die or inhibit its expression. The oncogenic stress caused by EWS-FLI1 leads to expression of genes inhibiting the cell cycle, thus inducing cell apoptosis. However, expression of EWS-FLI1 is well tolerated by certain cell types such as mesenchymal stem cells (MSCs) (28), which supports the hypothesis that MSCs are the ES initiating cells.

Therefore, it can be expected that a stem state is critical to help the cell tolerate overexpression of EWS-FLI1. Furthermore, EZH2 is found to be increased in MSCs (28). Thus, inducing expression of EZH2 could promote cell plasticity and increase its permissiveness for expression of EWS-FLI1, leading to transformation. This action is probably indirect and is likely to be mediated by other proteins important for EWS-FLI1 expression.

Specific aim of the study

The aim of this work was to investigate whether increased EZH2 expression, leading to newly acquired plasticity of the cell, can induce EWS-FLI1 protein expression in differentiated cells such as fibroblasts. Here, we induced expression of both genes on a human fibroblast lineage, MRC5, by cell infection with viruses containing the genes. RNA and protein expression levels for EWS-FLI1 were analyzed and compared depending on presence or absence of induced EZH2 expression.

Materials and methods

Cell culture

293T cells were used as packaging cells for transfection. MRC5 immortalized fibroblast-like cells, originally developed from human lung tissue of a male 14-week-old fetus after abortion, were used as target cells for infection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin and streptomycin (PS), 1% non essential amino acids (NeAA) and 10% fetal bovine serum (FBS) at 37°C under a humidified 5% CO_2 atmosphere.

Maxiprep

To obtain greater quantities of DNA, Maxiprep was performed according to the manufacturer's instructions (JetStar 2.0 Plasmid Purification Kit). Plasmid quantification was done using NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, USA).

Plasmid transfection and viral infection

MRC5 cells were infected with two different plasmids to obtain conditional overexpression of EZH2 and stable expression of EWS-FLI1 genes. EZH2 was overexpressed using the pINDUCER20 lentiviral plasmid (28). Transduced cells were selected for Neomycin resistance and the transcription of EZH2 gene was induced by the addition of doxycyclin (DOX) at the working concentration of 1:500 to the culture medium for 2 days. Untreated DOX samples were used as controls. Another lentiviral plasmid containing a Puromycin resistance gene was used to induce the stable expression of EWS-FLI1 gene (figure 2).

Viruses were produced by 293T cell transfection when cells were at 30% confluence, using FuGENE® 6 Transfection Reagent (Promega) according to the manufacturer's protocol. pMD2G and pCMVΔR8.74 vectors were used respectively as envelop and packaging plasmids. After 48h of incubation, the supernatant was collected and filtered (0.45μm) in order to get rid of any residual cells and collect the isolated virus.

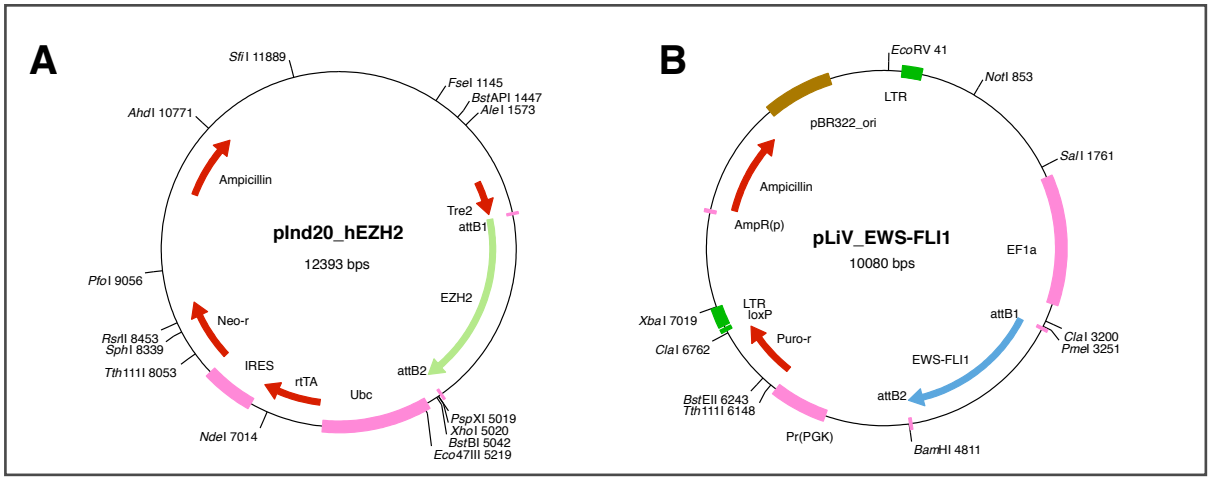

Figure 2. Plasmid maps. A. pINDUCER20 containing EZH2 and neomycin resistance genes. **B.** pLIV plasmid containing EWS-FLI1 and puromycin resistance genes.

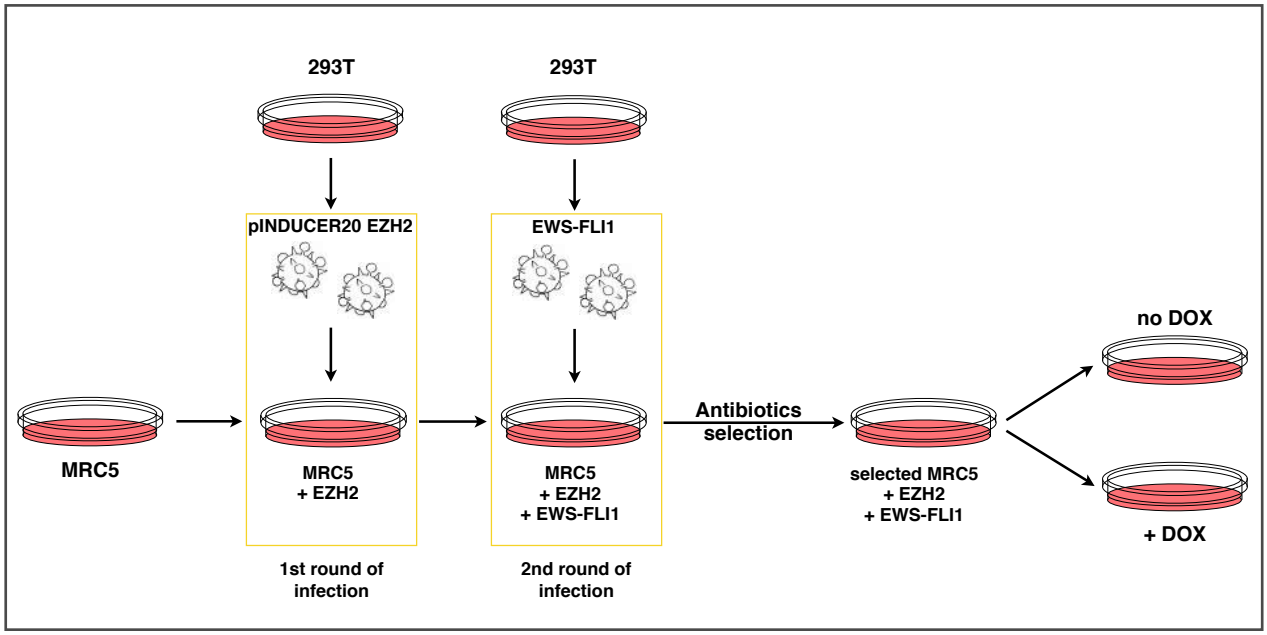

Figure 3. Transfection and infection steps. The viruses produced by 293T cells were collected and added to MRC5 culture medium. Transduced MRC5 were selected with antibiotics (Neomycin and Puromycin) before induction. Here, only simultaneous selection is depicted. We started induction with DOX (100ng/µL). Each final sample ±DOX was analyzed (PCR and Western blot).

Culture medium containing the virus and Polybreene 1000x was added to the target cells, MRC5, at 30-40% confluence.

We proceeded to two rounds of infection (figure 3). Firstly, MRC5 were infected with inducible EZH2 containing lentiviruses. Secondly, $MRC5^{EZH2}$ were infected with lentiviruses containing EWS-FLI1. Infected MRC5 underwent antibiotic selection for 48h with puromycin and/or neomycin (G418) at concentrations of 2.5μg/mL and 800μg/mL respectively either simultaneously or sequentially. Antibiotic concentrations were previously determined by testing MRC5 sensitivity to different doses of each antibiotic.

After MRC5 EZH2+EWS-FLI1 double infection, in presence or not of EZH2 induction by DOX, samples were snap frozen for RNA extraction or prepared for protein extraction.

RNA extraction and quantitative real-time polymerase chain reaction (RQ-PCR) assays for gene expression

Real-time PCR detects and measures the fluorescence generated by the amplified and accumulated specific cDNA strands throughout the course of the PCR, so that it is possible to quantify the relative expression of amplified transcripts during the log phase of the reaction.

Total RNA was extracted from MRC5 fibroblasts using TRIzol® reagent (Life Technologies) according to manufacturer's instructions. We performed retrotranscription on 500ng of isolated RNA, which served as template for cDNA synthesis using random primers (Promega, C1181).

EZH2 (Taqman probe Hs00172783_m1, Applied Biosystems) level of expression was analyzed using TaqMan® transcription kit (Applied Biosystems).

EWS-FLI1 expression was assessed by gene-specific Sybr green primers from Microsynth (see probe sequences in Table 1). RQ-PCR was performed with ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA). Gene transcription was compared to that of housekeeping reference genes PP1A (Sybr green probe, Microsynth, see probe sequences in Table 1) or 18s (Taqman, probe Hs99999901_s1, Applied Biosystems). Samples were run in triplicates, and fold change of transcription was calculated using the ΔC_t comparative method. Equal amounts of cDNA were used in all experiments.

Table 1. Primers used for the RQ-PCR	
PP1A	
Forward	AACGTGTTCTTC
Reverse	TTATGGCGTGTGAAGTCA
EWS-FLI1	
Forward	GCTAGGCGACTGCTGGTC
Reverse	GTCAACCTCAATCTAGCACAGGG

Western blot analysis

Whole cell lysates were prepared from MRC5 cultures using nuclear lysis buffer (50mM Tris pH 7.5, 0.5M NaCl, 1% NP40, 1% DOC, 0.5% SDS, 2mM EDTA) containing a protease inhibitor cocktail. Lysates were sonicated for 10 seconds. Total protein underwent electrophoresis through 8% polyacrylamide gel and transferred to a Whatman® Protran® BA83 nitrocellulose membrane for 2 hours at 250mA. Membranes were blocked in 5% milk in phosphate-buffer saline (PBS) containing 0.5% tween 20 (PBST) for 1 hour, then hybridized with rabbit anti-EZH2 (Active Motif) and goat anti-EWS (N-18, Santa Cruz Biotechnology) antibodies at dilutions of 1:2000 and 1:200 respectively for 1h in 2.5% milk at room temperature. Membranes were then washed in PBST and hybridized with horseraddish peroxidase-conjugated secondary goat anti-rabbit and rabbit anti-goat antibodies (Dako) in 2.5% milk for 90 minutes at room temperature. Control of protein amount was performed with mouse anti-α-tubulin antibody (Calbiochem) (1:5'000) for 1 hour in 2.5% milk at room temperature. Bands were visualized using SuperSignal West Femto kit (Thermo Scientific) detection reagents. Quantification of band intensity was performed by calculating the ratio between band intensity of the studied protein and that of α-tubulin in the same samples.

Results

Transcription of the EWS-FLI1 gene is strongly increased in the presence of EZH2.

For EZH2 overexpression, we used the pINDUCER20 lentiviral plasmid (29) containing EZH2. In order to avoid toxicity after infection, we tried selecting infected cells with puromycin and neomycin in two different ways. In some samples, both antibiotics were added simultaneously in the culture medium after the two rounds of infection (sim). In other samples, we proceeded to a two-step selection. As a first step, we added neomycin to the culture medium after the first round of infection. Then, as a second step, a sequential selection with puromycin was performed following the second round of infection (seq).

We first validated EZH2 induction in DOX treated MRC5$^{EZH2+EWS-FLI1}$ infected cells compared to untreated controls. RNA analysis with RQ-PCR showed successful induction of EZH2 in both (sim) and (seq) selection conditions following DOX treatment, with a more important induction in (sim) selected cells (figure 4).

In spite of the basal expression of EZH2 being low in wild-type cells, a slight increase of its expression in MRC5^{+DOX} was enough to cause a significant induction of EWS-FLI1 RNA expression (figure 4). The most important induction of EWS-FLI1 was seen in (sim)$^{+DOX}$.

Protein expression of EWS-FLI1 rises in the presence of EZH2.

To evaluate whether EZH2 overexpression allows the expression of EWS-FLI1 protein, we proceeded to western blot analysis.

Protein expression of EZH2 was induced in both (seq)$^{+DOX}$ and (sim)$^{+DOX}$ cells, although increase in the former was more important than in the latter, with difference of α-tubulin to EZH2 band intensity ratios of 0.4 and 0.05 respectively (figure 5). This is contradictory with previous RQ-PCR results, in which EZH2 induction was more important in simultaneously selected samples (figure 4).

The expression of EWS-FLI1 protein was successfully induced in DOX treated MRC5$^{EZH2+EWS-FLI1}$ simultaneously selected cells compared to control (figure 5A). No expression of EWS-FLI1 was observed in (seq)$^{+DOX}$ samples, despite EZH2 overexpression (figure 5B).

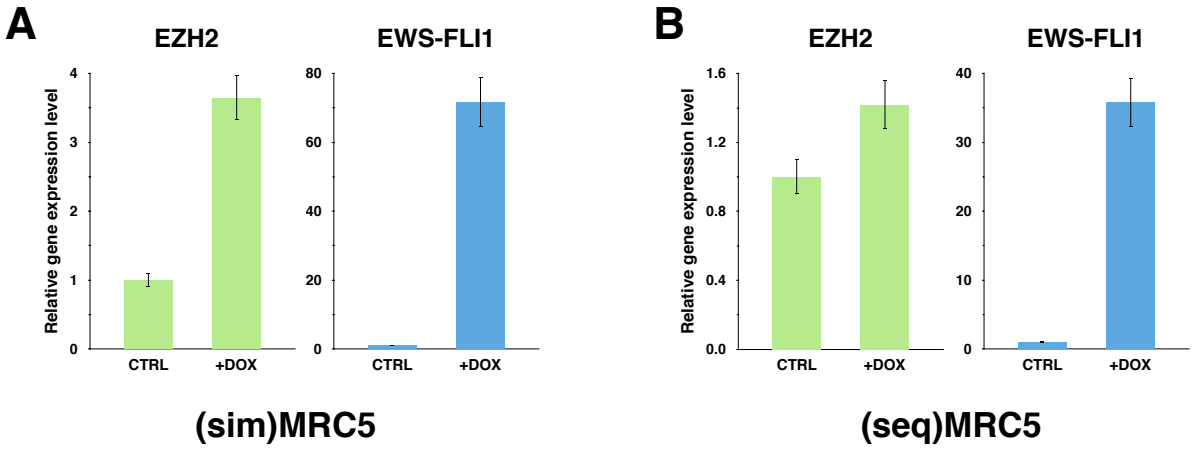

Figure 4. Relative expression of RNA in MRC5$^{EZH2+EWS-FLI1}$. A. Simultaneously selected (sim)MRC5. A 3.65-fold increase of EZH2 expression was seen in presence of DOX, showing induction was successful. EWS-FLI1 was highly expressed in samples containing DOX. The housekeeping gene used was 18s. **B.** Sequentially selected (seq)MRC5. Induction of EZH2 was successful, though less than in (sim) samples. EWS-FLI1 was induced after EZH2 overexpression. The housekeeping gene used was PP1A. Abbreviations: CTRL, control cells; +DOX, doxycyclin induced cells.

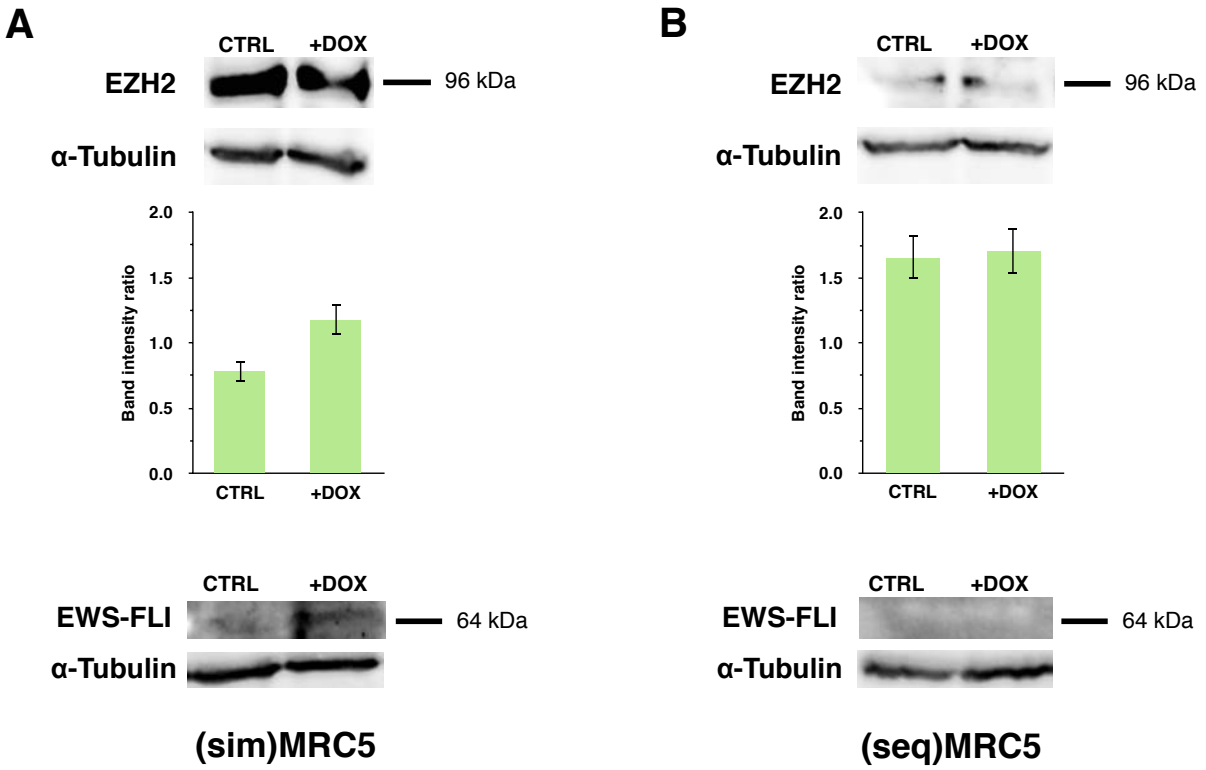

Figure 5. Western blot results in MRC5$^{EZH2+EWS-FLI1}$. The amounts of protein loaded on the gel were not the same for (sim) and (seq) samples. **A.** Simultaneously selected (sim)MRC5. EZH2 was induced after addition of DOX in culture medium, as seen with increased band intensity in +DOX samples. EWS-FLI1 protein expression was induced *de novo* after EZH2 overexpression. **B.** Sequentially selected (seq)MRC5. EZH2 protein expression was slightly increased in the presence of DOX. EWS-FLI1 was not detectable in either induced or not induced conditions. Abbreviations: CTRL, control cells; +DOX, doxycyclin induced cells.

Discussion and Perspectives

We have found that EWS-FLI1 expression is induced *de novo* in simultaneously selected MRC5 cells that overexpress EZH2, therefore confirming our hypothesis that PcG may increase cell permissiveness for oncogene expression. However, the experiment should be repeated, as we found induced EWS-FLI1 protein expression in simultaneously infected cells, but not in sequentially selected samples. For more precise results, we should better calibrate the DOX induction conditions on cells containing the inducible plasmid alone.

There are many hypotheses as to how EZH2 and EWS-FLI1 work together. Recent work from our laboratory using ChIP-seq (chromatin immunoprecipitation sequencing) assessed epigenetic changes, focusing on the EZH2 gene portion, in cells isolated from ES. EWS-FLI1 was observed to bind to the EZH2 DNA sequence more readily in spheres than in adherent cells (figure 6).

In order to form spheres, cells have to be grown in non-adherent conditions. Cells usually require adherence to survive, which means that the properties of cells

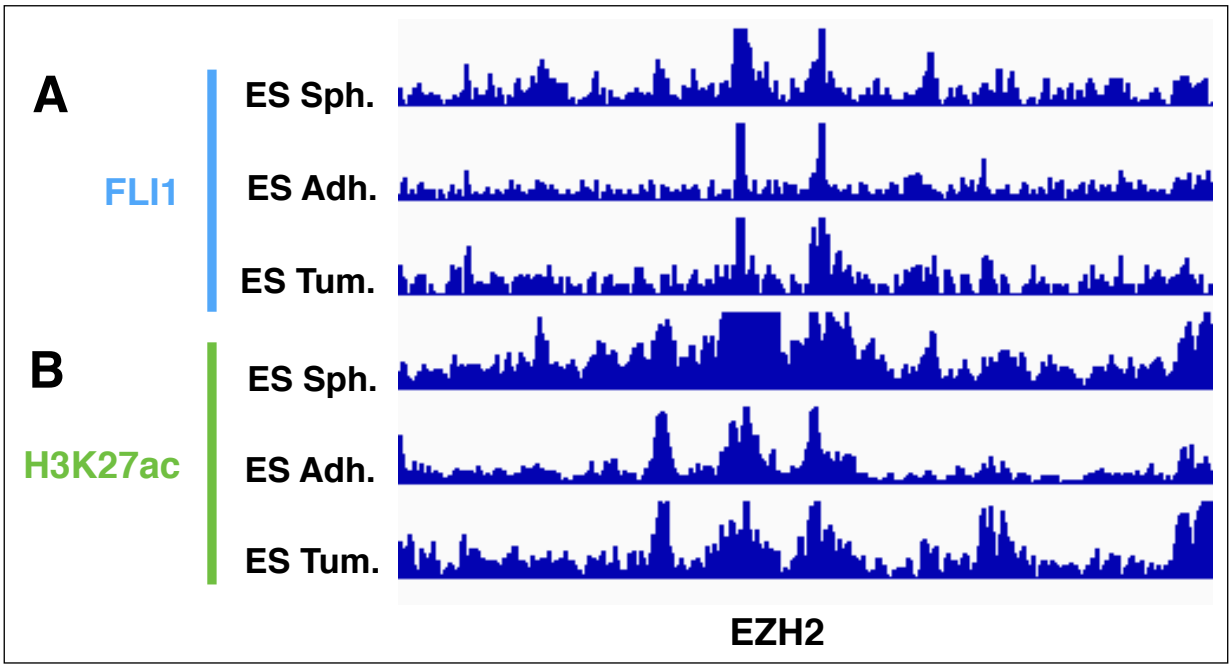

Figure 6. EWS-FLI1 binds EZH2 acetylated sequences in tumorigenic ES cells. Higher inputs shown by blue peaks translate increased binding of antibodies to the DNA strand. **A.** FLI1 binding to EZH2 gene. The maximum input was found on sphere and tumor derived cells obtained from isolated ES cells. Less input was seen in adherent cells, which are less tumorigenic. **B.** H3K27 acetylation markers on EZH2 gene. They were identified by specific anti-acetylation markers antibodies. Input was highest in sphere derived cells. Abbreviations: ES: Ewing's sarcoma; Sph.: sphere-derived ES cells; Adh.: adherent ES cells; Tum.: tumor-derived cells. H3K27ac: H3K27 acetylation marker.

found in spheres are distinct from those of differentiated cells. Indeed, spheres are mostly comprised of tumor-initiating cells, which possess a survival advantage. We assume that these tumor-initiating cells are the cancer stem cells (CSCs) from which the tumor bulk arises. This bulk contains both the self-renewing CSCs and their more differentiated progeny (adherent cells in culture).

Anti-FLI1 antibodies bind DNA more readily in sphere-forming cells, suggesting that interaction between EWS-FLI1 and EZH2 is associated with an aggressive cell phenotype (figure 6A). Histone acetylation is a marker of active transcription of the associated DNA sequence. We see from figure 6B that the EZH2 sequence is more strongly acetylated in sphere-derived cells, meaning that transcription of this DNA portion is highly active in these cells. Such results support the likely critical role of EZH2, as it may be differentially expressed in aggressive tumor-initiating cells and associated with CSCs properties.

Fibroblasts are an unlikely ES cell of origin as in order to stably express EWS-FLI1 they require loss of either p53 (30) or p16 (31) pathways, which are not typically found in ES. It would be interesting though, to further evaluate the consequence of EWS-FLI1 expression in more differentiated cells such as MRC5 fibroblasts by analysis of CSCs cell surface markers, such as CD133. The next step would be to compare the phenotype of $MRC5^{EZH2+EWS\text{-}FLI1}$ to that of MSCs expressing EWS-FLI1, in order to see whether EZH2 is active in the early phase of transformation of differentiated cells. EWS-FLI1 expression levels obtained in fibroblasts should be compared to that in ES tumor cells and $MSC^{EWS\text{-}FLI1}$. Finally, these experiments should be repeated on primary fibroblasts, which would represent a more physiological model to understand how differentiated cells might acquire pluripotency and tumorigenicity by expressing EZH2 and thus creating a permissive environment for EWS-FLI1 expression. We could also compare life span of fibroblasts stably expressing EWS-FLI1, depending on EZH2 expression levels. It is conceivable that EZH2 facilitates survival of fibroblasts, possibly through induction of a stem cell state.

Conclusion

The aim of this work was to address the role of PcG in rendering cells permissive for EWS-FLI1 expression. We found that EZH2 may indeed help cells become more permissive for expression of an oncogene such as EWS-FLI1. EZH2 induction may be critical to allow differentiated cells such as fibroblasts to circumvent oncogenic stress from EWS-FLI1 expression and thus promote transformation. Other experiments using a large number of samples will be performed to confirm these results.

Observations from this work put together with other data from the laboratory suggest that EZH2 plays a major part in tumor cells with phenotypes similar to CSCs in ES. Furthermore, our data allow us to suggest that ES could arise from differentiated cells under the appropriate circumstances, suggesting that we cannot exclude the possibility that ES may originate from more differentiated cells.

Further research to achieve detailed understanding of the events linking EZH2 to EWS-FLI1 are underway and will provide valuable information toward developing new possible strategies for targeting ES. Ultimately, we hope similar investigations can be performed on other tumor types.

Troubleshooting

At the beginning of this work, we planned to study the role of EZH2 through a similar method but involving non-inducible plasmids. We produced retroviruses containing either EZH2 plasmid or EWS-FLI1 plasmid. The former contained a hygromycin resistance gene, and the latter a puromycin resistance gene. We used an empty hygromycin-resistant vector and then a puromycin-resistant green fluorescent protein (GFP) plasmid for negative control (figure 7). Using GFP as the final negative control allowed us to make sure infection had been successful, as cells appear green under UV light in the microscope.

We followed a pattern similar to the protocol described previously, comprising two rounds of infection. We proceeded to antibiotic selection (hygromycin), but hardly any cell infected with EZH2 survived. We first thought the rate of infection was too low, so that only a minority of cells were able to resist the selection. We also hypothesized that, if the infection had been successful, EZH2 overexpression might be toxic for the cells. Indeed, when not selected, cells infected with EZH2 seemed to proliferate more slowly than those infected with the empty vector.

After the 2nd round of infection, we observed GFP-infected samples under the microscope. As they did emit green fluorescence, we rejected possible manipulation

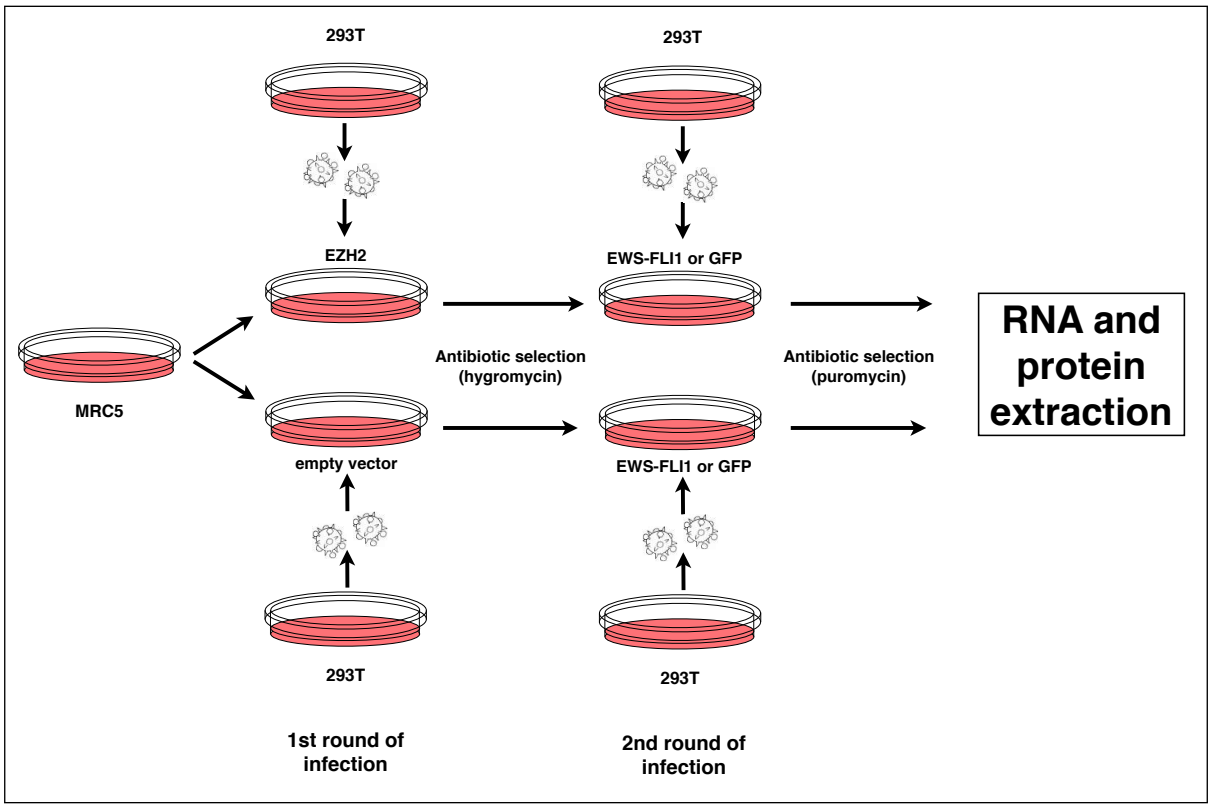

Figure 7. 1st attempt of transfection and infection. MRC5 were infected with EZH2 or an empty vector during the first round of infection. In the 2nd round of infection, each sample was infected either with EWS-FLI1 or GFP.

errors, because the same protocol had been followed for all transfections and infections.

RQ-PCR on hygromycin-selected doubly infected cells showed paradoxical results. Indeed, EZH2 expression was higher in control samples, as opposed to what should be expected. However, EWS-FLI1 expression is found only in cells infected with the gene (figure 8). This was another proof that lack of EZH2 overexpression in the samples was not due to error in manipulations but rather to the inadequacy of the plasmid itself.

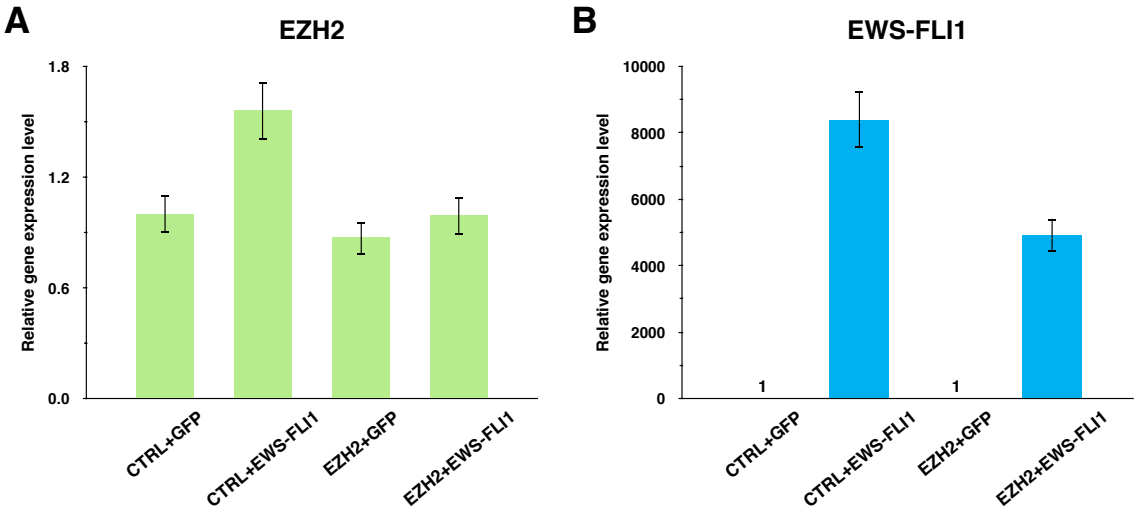

Figure 8. Relative EWS-FLI1 and EZH2 RNA expression levels on hygromycin-selected MRC5. The housekeeping gene used here was PP1A. **A.** EZH2 expression levels were increased the most in cells infected with an empty vector. **B.** EWS-FLI1 was expressed only in cells infected with the EWS-FLI1, showing successful infection.

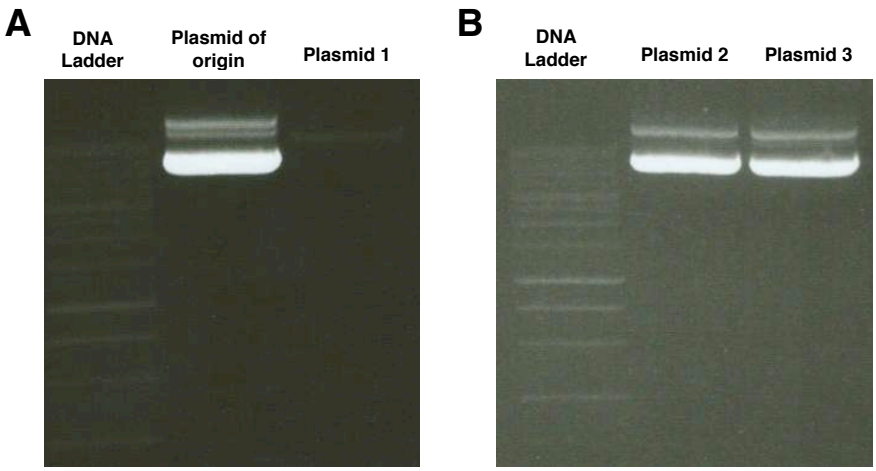

Figure 9. Electrophoresis of EZH2 plasmids on 1% agarose gel. A. Plasmid 1 was the 1st plasmid used to perform the experiments described above. Plasmid of origin is the plasmid from which the 1st plasmid was produced. The EZH2 band seen in the plasmid of origin is not found on Plasmid 1. **B.** Plasmids 2 and 3 were produced later from the same plasmid of origin, and show no sign of degradation.

Therefore, we decided to check the integrity of the EZH2 plasmid by running the plasmids on 1% agarose gel. We compared the plasmid extracted at the beginning of the work (Plasmid 1) to the plasmid of origin from which it had been electroporated into bacteria. Electrophoresis revealed that the EZH2 plasmid used in the experiments was degraded (Plasmid 1, figure 9A).

We electroporated another batch of bacteria using the plasmid of origin in order to extract new DNA. Control electrophoresis showed plasmid was conserved (Plasmids 2 and 3, figure 9B). To assess quality of transfection and infection with the newly produced plasmid, we infected both 293T and MRC5 cell lines with EZH2 from Plasmid 2 or an empty vector. RQ-PCR was performed on transfected and infected samples. EZH2 was clearly increased in transfected 293T cells, but none of the infected cells showed successful EZH2 infection (figure 10). Despite not being degraded, the EZH2 plasmid was not functional for infection.

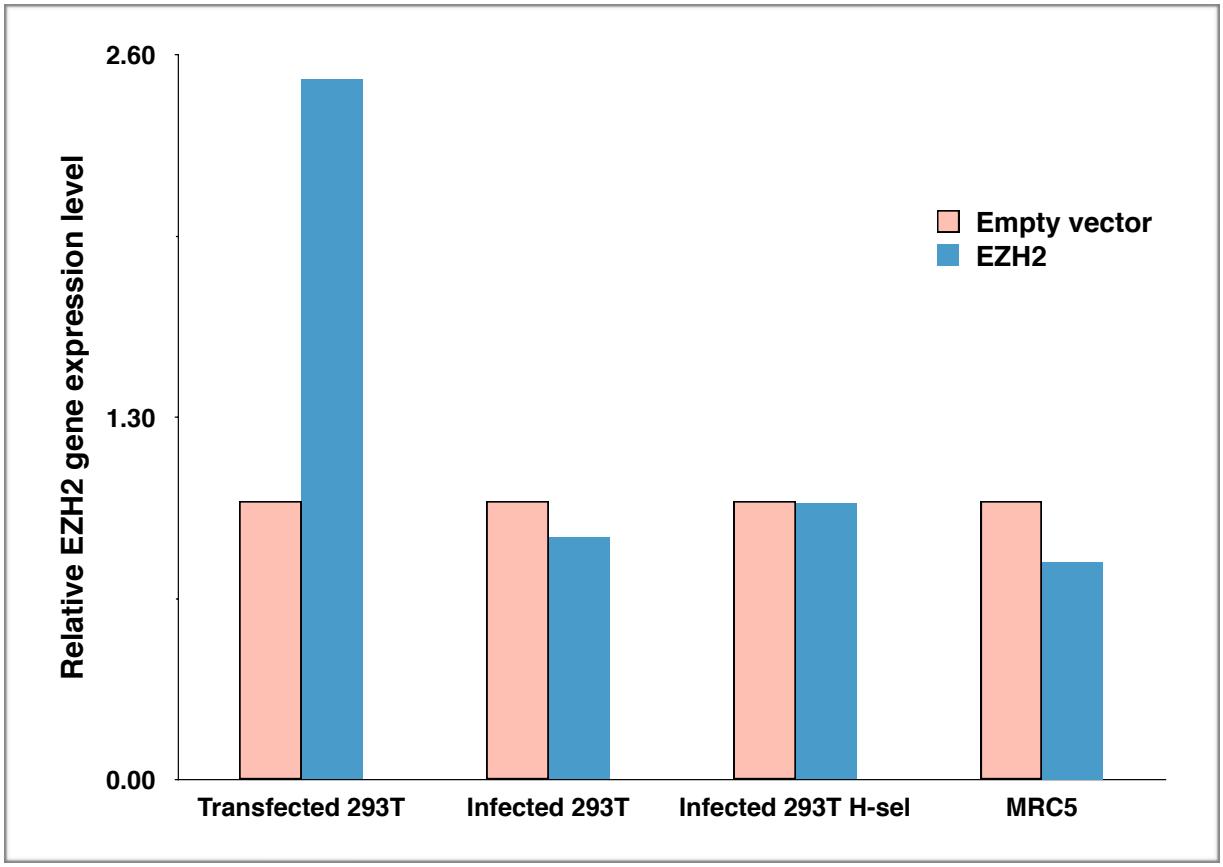

Figure 10. RQ-PCR to assess quality of transfection and infection with EZH2 plasmid. 293T cells were transfected with an empty vector (light pink, stroke) or EZH2 (blue, no stroke). Viruses were collected and used to infect two dishes of 293T cells and one of MRC5 cells. One of the 293T cell plates of each condition went through hygromycin selection (H-sel) to evaluate rate of infection. Results showed successful cell transfection with EZH2 but no induction of EZH2 was found in infected samples, as expression levels were the same as in control cells. The housekeeping gene used here was PP1A.

Consequently, a new plasmid containing EZH2 had to be synthesized for further experiments. In order to reduce toxicity to the cells, we resorted to using an inducible plasmid pINDUCER20 containing EZH2. The work that followed is presented above.

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