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The effect of Imp-2 on Ewing sarcoma cancer stem cells: when epigenetics orchestrate tumor growth

Presented by

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Contents

Introduction

Ewing's sarcoma family tumors

Ewing sarcoma is part of the Ewing's sarcoma family tumors (ESFTs) that includes peripheral primitive neuroectodermal tumor (PNET), extraosseous ewing sarcoma, Askin's tumor and atypical Ewing sarcoma. These tumors share histological and immunohistochemical similarities and display a single chromosomal translocation. ESFT is the second most frequent malignant bone tumor, after osteosarcoma, of adolescents and young adults. ESFTs are very aggressive and often relapse after treatment.

Ewing sarcoma, on which this work will focus, occurs predominantly in the femur, the pelvis and less commonly in the upper extremities, axial skeleton, ribs and face with a peak incidence between 10 and 15 years of age. The histology of these tumors reflects poor differentiation and presents as small round cells with a halo of cytoplasm around the nucleus. They are therefore often referred to as small round blue cell tumors. Immunohistochemical analysis of ESFT reveals expression of the lymphoid cell adhesion receptor CD99, neural markers including NSE, S-100, synaptophysin and CD56 and mesenchymal markers including vimentin. Macroscopically, Ewing sarcoma is grey-white with zones of necrosis and fibrosis. It is associated with a survival rate of 65% at 5 years if treated with current multimodal therapy, which includes surgery, chemotherapy and radiation. However, Ewing sarcoma often displays multidrug resistance, which explains the frequent relapse.

The underlying event in Ewing sarcoma pathogenesis is the non-random balanced chromosomal translocation between chromosome 11 and chromosome 22, t(11q23;22q12). It causes fusion between EWS gene on chromosome 22q12 and an ETS family gene on chromosome 11q24 (FLI gene in 85% of cases or ERG, ETV1, ETV2, FEV) (1). This translocation plays an essential role in the development and the maintenance of ESFT (2) and it is also used for diagnosis by FISH and RT-PCR.

The fusion gene formed encodes an aberrant transcription factor, which drives the expression or repression of genes implicated in regulation of cell proliferation and transformation by binding to their promoters and thus playing a crucial role in Ewing sarcoma pathogenesis.

The cancer stem cell model

Tumors are heterogeneous, which allows rapid adaptation and favors survival in a changing environment. One major source of tumor heterogeneity is believed to reside in cancer stem cells (CSCs) in addition to genetic mutations, epigenetic changes and influences of the tumor microenvironment (3).

Ewing sarcoma appears to follow the CSC model (4). This model assumes that a tumor is organised in a cellular hierarchy with populations of cells (CSCs) that show self-renewal capacity and that can generate populations of more differentiated cells with reduced proliferative and tumor initiating ability (tumor bulk) (5). In tumors following this model the CSCs are responsible for tumor initiation and resistance to therapy because of their reduced sensitivity to current treatment and their capacity to develop resistance mechanisms (5). It has been observed that CSCs of various types of cancer could survive chemotherapy and create *de novo* growth *in vitro* and *in vivo*. *In vivo*, an enrichment in CD133+ cells (associated with CSC functions in diverse cancer types) has been observed after treatment of xenotransplanted colorectal cancer cells with chemotherapy (6). CSCs have shown to use various mechanisms of resistance including overexpression of anti-apoptotic proteins and drug efflux pumps. They also display a low proliferation rate compared to the rest of the tumor bulk that preserves them from chemotherapy which targets the most rapidly proliferating cells (6).
Considering these observations, the cancer stem cell model predicts that cure of a cancer should only be possible if all the CSCs in the tumor are killed or induced to lose their pluripotency.

CSCs have the capacity to form spheres in non-adherent serum-free conditions *in vitro* and these cells can regenerate a tumor which is a phenocopy of the primary tumor after xenotransplantation into immunocompromised mice (5). A possible way to isolate these

cells is through expression of cell surface markers that are associated with CSCs in some cancers (e.g. $CD34^{+}$ $CD38^{-}$ cells in AML or $CD44^{+}$ $CD24^{-low}$ in breast cancer) (5) (3). However none of these markers is truly specific and new approaches are required to isolate these cells reliably in different cancer types (7).

The CSC model has been contrasted with the stochastic model in which all tumor cells have the capacity to initiate tumor growth under appropriate conditions (8) but both models have limitations. Currently the tendency is to unify these two models in a single dynamic model in which the population of CSCs is continuously adapting due to the accumulation of genetic mutations and epigenetic modifications. At late stages of tumor progression cell hierarchy tends to disappear leading to a mass of cells most of which may display CSC characteristics (7).

The cancer stem cell model in Ewing sarcoma

Isolation of Ewing sarcoma CSC remains difficult because of the lack of specific markers. Nevertheless, it has been shown that tumor cells which express the surface marker CD133 can induce tumor growth *in vivo* and form spheres in low attachment culture conditions in contrast to CD133- cells (4). Thus, these cells seem to have CSC characteristics and maintain the capacity to differentiate into adipocytes, chondrocytes and osteocytes *in vitro* just like the mesenchymal stem cells (MSCs), which are their most likely cells of origin (9). Indeed it has been shown that MSCs undergo transformation after induction of EWS-FLI protein expression (10).

Epigenetics and Ewing sarcoma

A major mechanism responsible for tumor heterogeneity and CSC specification is epigenetic regulation of gene expression, which can promote cancer cell survival and thus provide them a growth advantage over other cells. It also constitutes one of the mechanisms that underlie cancer cell plasticity (11).

The term “epigenetics” was used to describe heritable changes in the cellular phenotype that were independent of alterations in the DNA sequence. Currently this term refers to DNA methylation, non-coding RNA expression and chromatin modifications that

regulate DNA accessibility to transcription factors and repair complexes. Thus, epigenetics affects different fundamental DNA processes including transcription, repair and replication.

Chromatin conformation is determined by multiple mechanisms that include the constellation of histone modifications resulting in chromatin condensation (heterochromatin) or relaxation (euchromatin). Chromatin is a complex structure in which the DNA surrounds multiple histone octamers known as the nucleosomes. Pairs of histones H2A, H2B, H3 and H4 compose this structure which is encircled 1,5 times by DNA thus forming the primary chromatin structure. In the nucleus of a cell this structure is compacted and precisely organised allowing appropriate DNA processing (12). The different histones mentioned above can be modified by addition or removal of methyl and acetyl groups on their N-terminus. The resulting marks play a role in the chromatin accessibility and the binding of transcription factors which directly influence DNA transcription and the corresponding gene expression. The histone marks can be categorized according to their function (12) :

- Promoter activation marks (H34Kme2, H3K4me3, acetylation);
  Promoter repressive marks (H3K9me3, H3K27me3).
- Enhancer activation marks (H3K4me1, 2, H3K27ac, H2AZ);
  Enhancer repressive marks (H3K9me2, H3K9me3, DNAme).
- Gene body marks such as H3K36me3 which correlate with gene expression levels.

Epigenetic regulation participates in numerous key biological processes including cell differentiation during embryonal development and plays an important role in the pathogenesis of numerous diseases when it becomes disrupted.

Modifications of the epigenome have been observed in multiple cancer types. These epigenetic changes consist in differences of DNA methylation, histone marks and the non-coding RNA landscape (13). They arise early in transformation and participate in the regulation of different mechanisms on which tumor cells relies to promote growth and survival.

One key function of epigenetic modifications in cancer is to drive the specification and maintenance of CSCs. Thus, genes which play a role in differentiation, are silenced by specific histone repressive marks such as H3K27me^3 or by hypermethylation of their promoters leading to the conservation of the stem cell state (14) whereas others that determine pluripotency are activated by loss of histone methylation marks and acquisition of acetylation marks.

Paediatric cancers, which often have an explosive evolution, are typically associated with few mutations and a relatively stable genome. Thus, their development seems to arise in the context of disruption of differentiation programs that are dependent on epigenetic regulation (15). An interesting observation in paediatric cancers is the presence of mutations on genes that affect epigenetic regulation such as methyltransferase, acetylase and histone demethylase coding genes (11).

Regarding Ewing Sarcoma, it has been shown that the EWS-FLI1 protein binds to promoters of genes that play a role in the epigenetic regulation such as the methyltransferase EZH2 of the polycomb group complex (16). The resulting epigenetic changes enhance the expression of several genes important for tumorigenesis.

Recent development of new technologies has allowed detailed analysis of epigenetic regulation of gene expression. The ChIP-seq technique (chromatin immunoprecipitation followed by sequencing) allows genome wide visualisation of the chromatin landscape of any cell type using antibodies that recognize specific chromatin marks. Thus, ChIP-seq allows detailed assessment of all the relevant chromatin modifications and thereby prediction of specific gene activity or repression. Promoters and enhancers bearing dual marks indicate genes that are poised for induction.

In this work, we first compared the epigenome of a CSCs population to that of the tumor bulk containing more differentiated cells using ChIP-seq.
Then, referring to the chromatin pattern showing active gene transcription, a set of genes specifically targeted by EWS-FLI1 in CSCs was selected from these data. We focused on one of these genes, *IMP2*, because it was shown to be important for CSC metabolism in glioblastoma (17).

Next, we confirmed Imp-2 overexpression in CSCs by qPCR and Western blot and finally we started investigations of the functional role of Imp-2 in the metabolism of Ewing sarcoma CSCs *in vitro* and *in vivo*.

Material and methods

Cell culture

Cells were obtained from 4 Ewing sarcoma family tumor (ESFT) samples removed by surgery as previously described (19): ESFT2, ESFT3, ESFT4 and ESFT5. Dead and immune cells were removed (CD45+, Milteny beads and columns) and remaining cells were sorted (Milteny beads and columns) for CD133 expression. Spheres were cultured in IMDM medium (Gibco) completed with 20% KO serum (Gibco), 10ng/mL LIF (Millipore), 10ng/mL recombinant human EGF (Invitrogen), 10ng/mL recombinant human bFGF (Invitrogen) and penicillin streptomycin 1% (PS) in ultra-low attachment flasks (Corning). Adherent cells were obtained from spheres by cultivating them in IMDM medium (Gibco) supplemented with 10% FCS (Gibco) and 1% PS.

Treatments of cells

ESFT4 cells were treated for 72h with 1µM rotenone to inhibit OXPHOS or with 25mM oxamic acid to inhibit glycolysis in adherent and sphere conditions.

Imp-2 knockdown and retroviral infection

Short hairpin RNA sequence targeting Imp-2 was antisense strand (5' TTTCTATGGATATCTACCC3'). This sequence was inserted into PGIPZ vector according to the manufacturer's recommendations.

Imp-2 sh RNA or control plasmids were transfected into 293T cells to produce the virus used to infect ESFT5 spheres: Imp2-sh ESFT5 and Ctrl-ESFT5 respectively. Viral supernatant was concentred by ultracentrifugation using Optima XL-80K Ultracentrifuge (Beckman coulter) at 19'500 rpm during 90min at 4°. Concentrated virus was added to dissociated ESFT5 spheres. Cells were selected 72h later with puromycin (1/500) resistance during 5 days. The efficiency of Imp-2 depletion was verified by Western blot analysis.

RNA acquisition, real-time PCR and Western blot

Total RNA was obtained using miRCURY miRNA extraction kit (Exiqon) as recommended. Real time PCR was performed as previously described(20). TaqMan probe included 18S. Primer sequences for *IMP2* SYBR Green gene expression quantification were: forward 5'-AGCTAAGCGGGCATCAGTTTG-3'; reverse 5'-CCGCAGCGGGAAATCAATCT-3'.

Western Blot was performed according to standard procedures. Anti-Imp2 (1mg/mL; MBL, RN008P) antibody was used. Secondary antibody was HRP-conjugated goat anti-mouse (GE Healthcare). Imp2 was detected as a doublet.

ChIP-seq

ChIP-seq data for ESFT3 samples were obtained from previous studies of our group. Samples were fixed as follows: 10 mio cells were incubated for 10 minutes at 37° in medium containing 1%PFA, reaction was stopped by adding glycine, samples were then washed and snap frozen. Chromatin immunoprecipiation was performed by our collaborators (N. Riggi, G. Boulay and Miguel N Rivera) as follows: chromatin was fragmented to a size range of 200–700 bases with a Branson 250 sonicator and was immunoprecipitated with antibodies against H3K4me3 (Millipore), H3K27me3 (Millipore), H3K27ac (Abcam, Active Motif), H3K4me1 (Abcam), H3K36m3 (Abcam), H3K9ac (Abcam) and FLI1 (Santa Cruz, catalog no. sc-356). Antibody-chromatin complexes were then pulled down with protein G-Dynabeads (Life Technologies), washed, and then eluted. Crosslink reversal, RNase A, and proteinase K treatments were performed. Immunoprecipitated DNA was extracted with the Min-Elute PCR purification kit (QIAGEN). ChIP DNA was quantified with Qubit, and used to prepare sequencing libraries. ChIP DNA and input controls were then sequenced with the Hi-Seq Illumina genome analyzer. Obtained sequences were aligned to a reference genome HG19 using BVA. To visualise the ChIP-seq tracks we used IGV software.

Proliferation assay

Proliferation of Ctrl-ESFT5 and sh-Imp2 ESFT5 cells was assessed with Cell Proliferation ELISA BrdU colorimetric kit (Roche) according to the manufacturer's protocol. Proliferation was measured every 24 hours up to 72 hours. Quadriplicates were established to assess internal variability.

Mouse treatment and tumor measurement

Experimental protocols involving mice were approved by the Veterinary Service of the Canton of Vaud (Etat de Vaud, Service Vétérinaire), under authorization number VD2488. Two groups of NOD SCID gamma KO mice, four mice per group, were anesthetized and injected with 10000 sphere-derived Ctrl-ESFT5 or sh-Imp2 ESFT5 cells beneath the renal capsule and allowed to engraft for 49J. When control tumors reached 1 cm^3, all mice were sacrificed. Injected kidneys were weighed and their size (short and long axis length) was measured.

Statistical analysis

All statistical analysis was performed using graphpad prism 6. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

Results

Chromatin associated with the Imp-2 gene shows a differential permissive transcription pattern with EWS-FLI1 binding sites and activating histone marks in ESFT CSCs that are absent in differentiated bulk tumor cells

We analysed ChIP-seq data from a fresh primary Ewing sarcoma sample (ESFT3), cells grown as tumorigenic spheres in serum-free conditions or cultured, in the presence of serum, as adherent differentiated non-tumorigenic cells. We compared the histone modification patterns between the three populations of cells and found significant differences in numerous gene coding and non-coding regions.

Following these observations, we focused on the *IMP2* gene (or *IGFBP2,* Insulin-like Growth Factor Binding Protein 2) because it displayed important differences in histone modifications between spheres and adherent cells and has been related to cancer stem cell metabolism in glioblastoma (17).

Figure 1 shows differential binding of the EWS-FLI1 transcription factor to the promoter of *IMP2*. The binding of the fusion protein is stronger in ESFT3 spheres than in the adherent counterpart cells. Interestingly, the activating H3K27ac mark is lower at the same locations on *IMP2* in adherent cells and present in spheres. Similar differences in H3K27ac are observed at a more distal location on the *IMP2* gene suggesting other active enhancers in spheres. As this mark is related to active enhancers, these are likely to be active in CSCs relative to bulk tumor cells.

Conversely, the polycomb repressive mark H3K27me3 seems to be more abundant in ESFT3 adherent cells than in the corresponding spheres. Finally, the H3K36me3 mark, which is directly correlated to the gene expression level, is more highly expressed in spheres than in adherent cells.

Taken together these results suggest differential regulation of *IMP2* by EWS-FLI1 between sphere CSCs, adherent and bulk tumor cells. *IMP2* displays all features of an actively transcribed gene in CSCs whereas it seems repressed in bulk tumor cells.

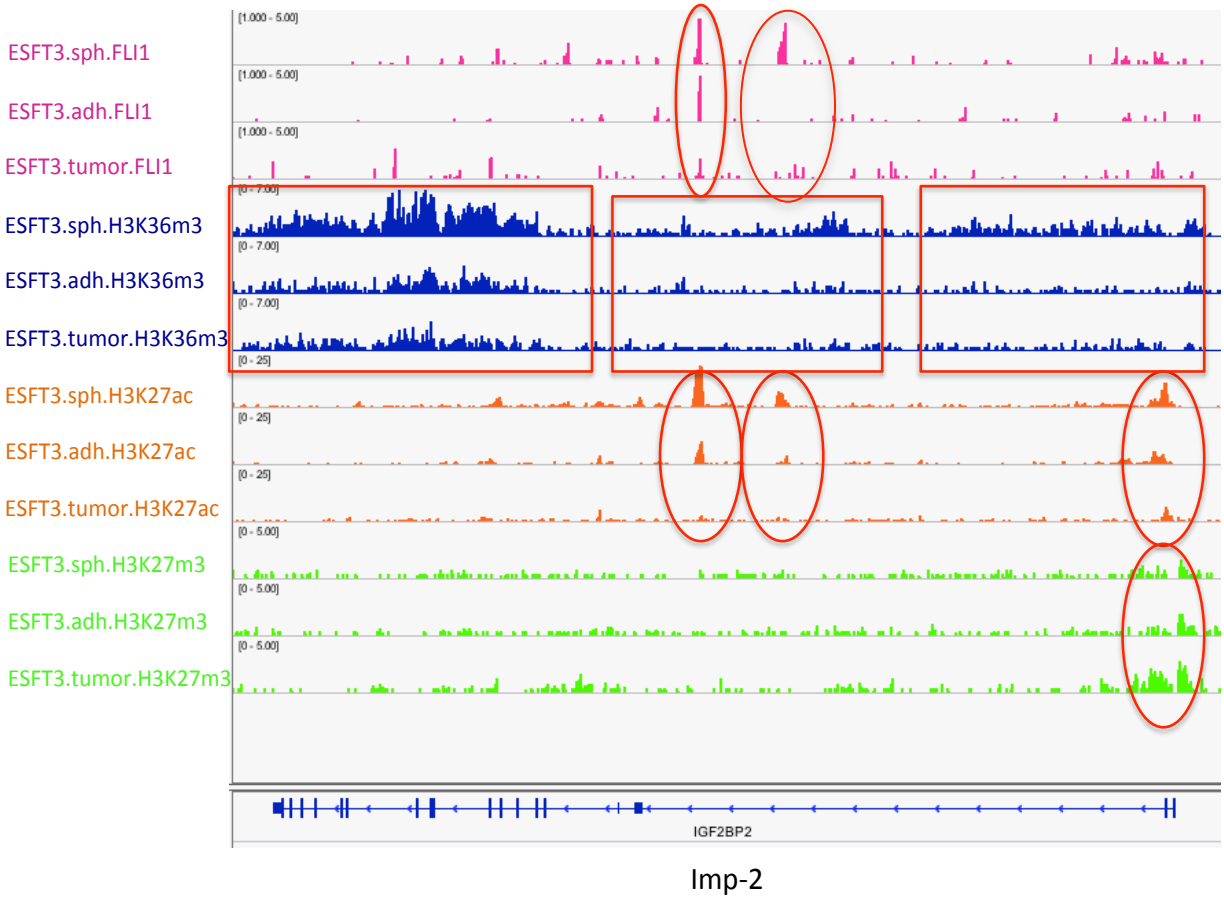


Figure 1: Differential binding of EWS-FLI1 in spheres, adherent and primary tumor on the *IMP2* gene (pink) and its consequences on chromatin modification (H3K36m3 blue, H3K27ac orange, H3K27me3 green).

Imp-2 is highly expressed in Ewing sarcoma CSCs

To confirm differential expression of Imp-2 in Ewing sarcoma spheres (CD133+) and adherent cells (CD133-), we performed real time PCR (RT-qPCR) on four different primary samples (ESFT2, ESFT3, ESFT4 and ESFT5). The results showed a strong upregulation of *IMP2* transcripts in CSCs compared to adherent cells in all samples except ESFT4 (Fig2-A).

We then performed a Western blot analysis on these samples in spheres and adherent cells to assess whether the Imp-2 protein expression followed the same trend. As expected, the levels of Imp-2 protein were more elevated in spheres than in adherent cells consistent with the results obtained by RT-qPCR (Fig2-B).

These observations confirm ChIP-seq data predictions regarding Imp-2 expression in ESFT3, and show the differential expression of Imp-2 in different primary ESFT samples.

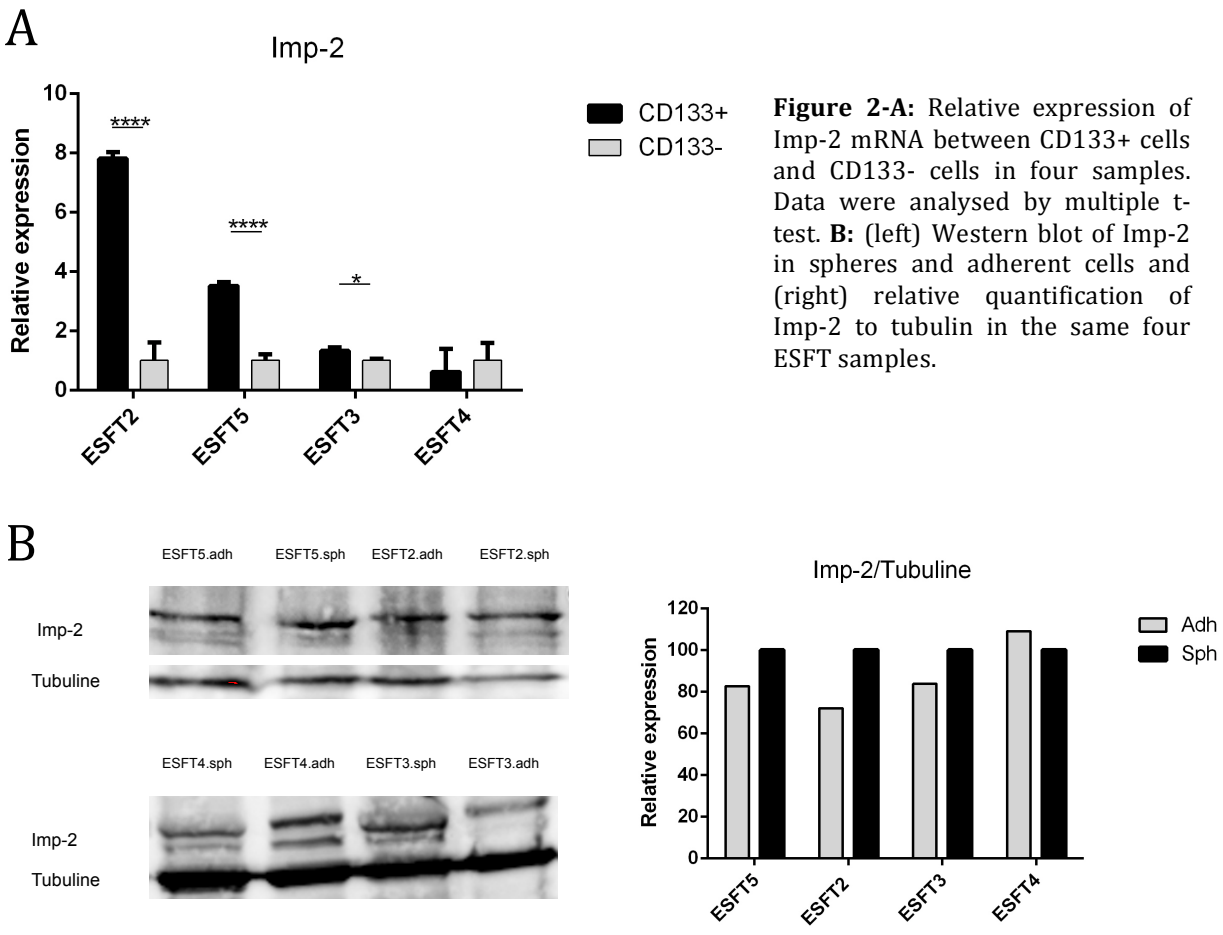


Figure 2-A: Relative expression of Imp-2 mRNA between CD133+ cells and CD133- cells in four samples. Data were analysed by multiple t-test. **B:** (left) Western blot of Imp-2 in spheres and adherent cells and (right) relative quantification of Imp-2 to tubulin in the same four ESFT samples.

Imp-2 plays a role in cell proliferation in Ewing sarcoma

In order to assess the impact on tumor growth of Imp-2 overexpression in Ewing sarcoma CSCs we performed knock down experiments using an shRNA for Imp-2 on ESFT3 and ESFT5 and monitored cell growth.

ESFT3 and ESFT5 sphere cells were infected with a lentivirus containing an Imp-2 or scrambled shRNA. Imp-2 knock down efficiency was assessed by Western blot analysis (Fig-3B). Unfortunately only ESFT5 cells were successfully infected (data not shown for other ESFTs).

We then used a BrdU proliferation assay and checked the 24h, 48h and 72 hours proliferation rate of both control and Imp-2-depleted cells.

After 48h the results showed a higher proliferation rate in control cells compared to sh-Imp-2 cells (Fig.3), consistent with the implication of Imp-2 in cell proliferation. Although the difference is still present at 72h it was not significant due to a high intra sample variability and cell confluence.

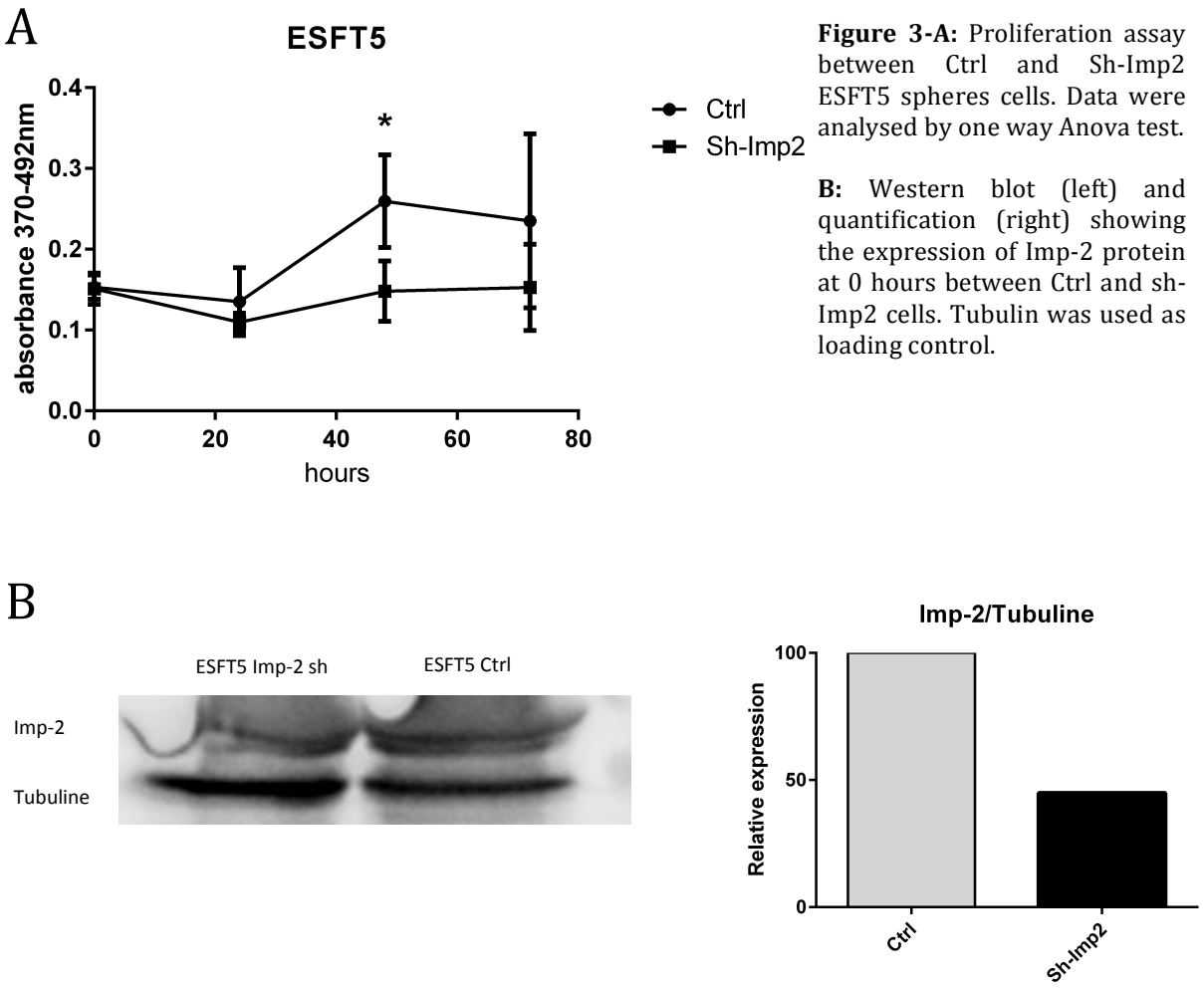


Figure 3-A: Proliferation assay between Ctrl and Sh-Imp2 ESFT5 spheres cells. Data were analysed by one way Anova test.

B: Western blot (left) and quantification (right) showing the expression of Imp-2 protein at 0 hours between Ctrl and sh-Imp2 cells. Tubulin was used as loading control.

Depletion of Imp-2 impairs tumor growth *in vivo*

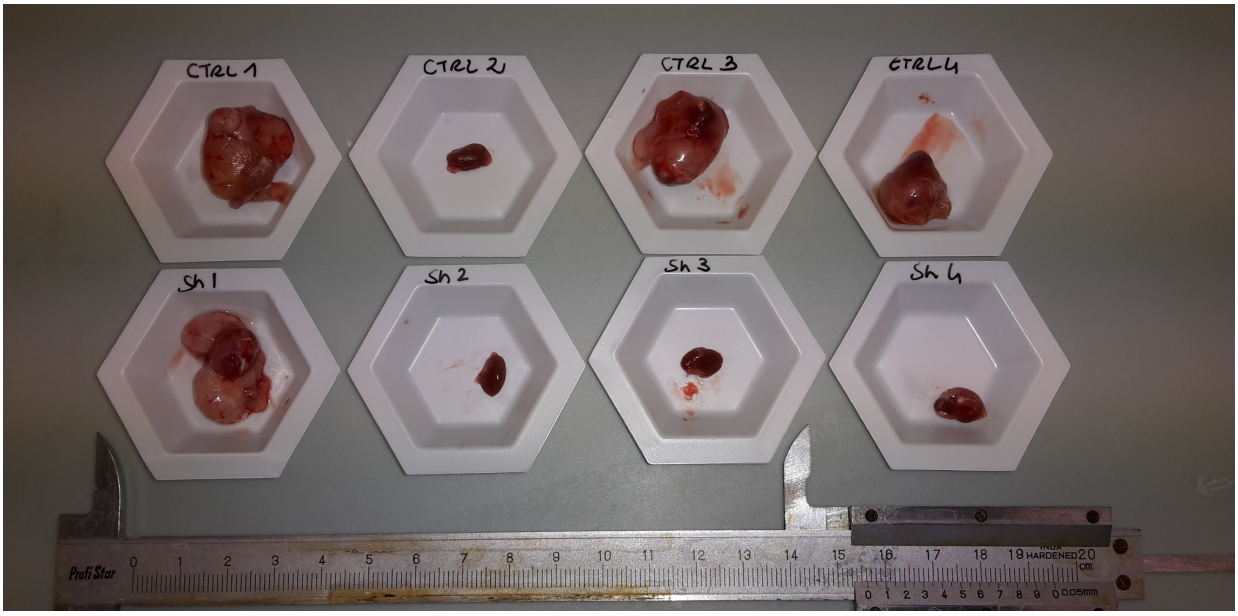
In order to assess a possible effect of Imp-2 depletion on tumor growth *in vivo* we performed a tumorigenicity assay using two groups of four mice each.

We injected sh-Imp2 or control ESFT5 cells in the subrenal capsule of immunocompromised (NOD-SCID cgKO) mice. After eight weeks, mice were sacrificed and kidneys with or without tumors were weighed.

Figure 4 shows that three mice had tumors in the control group whereas only one mouse had a macroscopically visible tumor in the Imp-2 depleted group. Unfortunately, due to the small number of mice the results were not statistically significant but a clear trend can be observed.

Taken together, these results show that at least in one primary ESFT sample Imp-2 depletion impaired *in vitro* and *in vivo* CSC growth and may impair *in vivo* tumor forming capacity of CSCs.

A



B

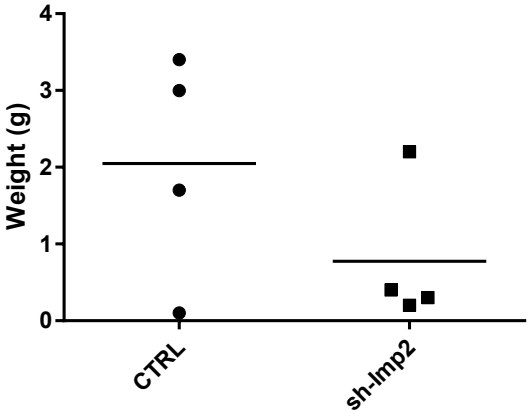


Figure 4-A: Macroscopic view of the four mouse kidneys injected with the ESFT5 sh-Imp2 and control cells.
B: Comparison between tumor and kidney weight of ESFT5 sh-Imp2 and control cells. Results were analysed by a student-t test.

Ewing sarcoma CSCs and bulk tumor cells might follow different metabolic pathways

There are many ways to influence cellular proliferation, one of them being the regulation of its glucose and protein metabolism.

It is now well established that cancer cells tend to use aerobic glycolysis (or Warburg effect) to metabolise glucose even in the presence of oxygen. Aerobic glycolysis produces only 2 molecules of ATP for 1 molecule of glucose, in contrast to the mitochondrial oxydative phosphorylation OXPHOS, which produces 36 molecules of ATP per molecule of glucose. One of the current explanations for this phenomenon is that aerobic glycolysis allows formation of more biomass for the proliferating cells than OXPHOS (18).

In glioblastoma, overexpression of *IMP2* was shown to enhance OXPHOS in CSCs. In contrast, differentiated adherent bulk tumor cells which show *IMP2* downregulation relied preferentially on glycolysis for energy production (17).

We therefore tried to analyse the metabolism of ESFT4 (as other cells were unavailable at that time) CSCs *versus* adherent bulk tumor cells. Cells were either plated as adherent monolayers in four plates or suspended as spheres in four flasks. We then added oxamic acid (25mM), which is a molecule that inhibits glycolysis by interfering with the lactate dehydrogenase, or rotenone (1µM), which inhibits OXPHOS by binding to the respiratory chain, or DMSO as a control. After five days, we observed differential morphological changes between these two populations of cells. Administration of oxamic acid to CSCs seems to only mildly affect sphere growth as, even if smaller, they are still present and proliferating, whereas with rotenone sphere formation is completely disrupted. Adherent cells, on the contrary, show a strong growth inhibition by oxamic acid whereas rotenone affects only the morphology of the cells but proliferation seems to be conserved (Fig.5).

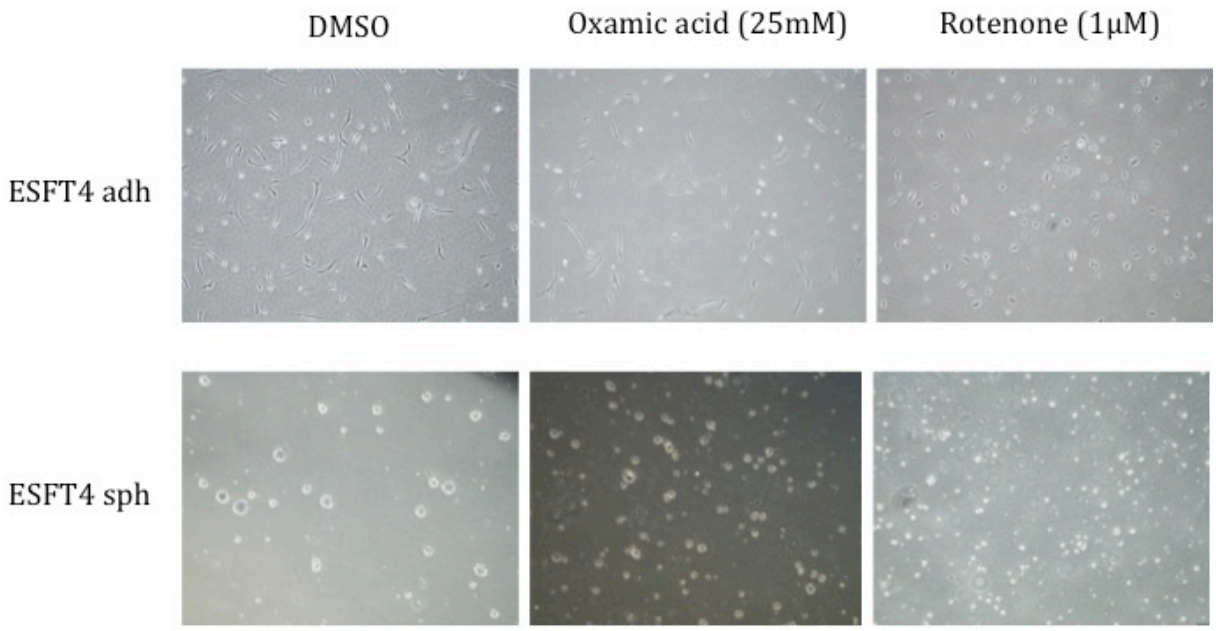


Figure 5: representative pictures of ESFT4 cell cultures in adherent and sphere conditions after 72 hours treatment with oxamic acid or rotenone. DMSO was used as control.

These data suggest that Imp-2 could play the same role on the metabolic switch between OXPHOS and glycolysis in ESFT CSCs as in glioblastoma (17). Furthermore, Imp-2 could be implicated in the determination of the metabolic pathway used by cells. However, these data are very preliminary and warrants further investigations.

Discussion

A major challenge in cancer treatment resides in the targeting of specific mechanisms that underlie tumor initiation and progression. The discovery that development of multiple cancers depends on the survival of a specific sub-population of cells within the tumor provides new hope in the field of cancer research (21). Tumor heterogeneity and mechanisms of emergence of CSCs remain poorly understood and need to be investigated in order to develop new therapeutic strategies that selectively target defined cell subpopulations. Regarding Ewing sarcoma, studies revealed the existence of a subpopulation of cells that displays CSC properties (4) but the pathways that promote its emergence and survival remain to be fully understood. In our study, we used the ability of primary Ewing sarcoma CSCs to proliferate in low attachment serum free culture conditions and their expression of the surface marker CD133 to isolate them from the rest of the tumor bulk (4). Even if these two methods are not totally specific for CSC isolation, they represent a reliable way to obtain CSC enriched populations (22) (4).

Studying the epigenetic mechanisms that promote tumorigenesis has shown promising results in several cancers (13). The ChIP-seq technique allowed identification of a specific set of genes, which is essential for the propagation and treatment of glioblastoma (23). Understanding the epigenetic mechanisms underlying tumor formation is particularly crucial in paediatric cancers as these tumors show important dysregulation of gene expression programs with only few mutations (15). 60% of ESFT bear a single non-random translocation as the only mutation, highlighting the importance of epigenetic modifications in the genesis of ESFT, specifically in the generation of intratumor heterogeneity. Analysis of the epigenetic landscape of primary ESFT CSCs and differentiated bulk tumor cells using ChIP-seq techniques revealed significant differences in histone methylation and acetylation pointing towards differential gene expression profiles between spheres and adherent cells. Therefore correlation between histone code assessed by ChIP-seq and genes expression represents a powerful tool for the comprehension of carcinogenesis (14).

As ESFT CSCs and differentiated cells display very different phenotypes, despite the same genetic background, it is particularly interesting to assess how EWS-FLI1 binds and modulates gene expression in the two cell populations. The mechanism by which this is achieved, as well as the study of the genes specifically induced or repressed in one or the other cell population, may provide clues as the mechanism underlying CSCs generation and may lead to the discovery of new therapeutic targets. In this study, we chose to focus on a specific gene, *IMP2*, in order to first confirm that chromatin immunoprecipitation of histone modifications effectively correlates with and is able to efficiently predict gene expression. *IMP2* has been shown to play a crucial role in glioblastoma CSC metabolism and CSC generation, we therefore wanted to assess whether those findings might be applied to ESFT CSCs. Analysis of the histone code in conjunction with EWS-FLI1 binding in ESFT3 allowed us to identify a gene, which is differentially expressed between non-tumorigenic differentiated cells and CSC. Interestingly, this differential expression at the mRNA and protein levels is also observed in two other ESFT samples. However, ChIP-seq analysis remains to be done in ESFT2 and ESFT5 and other ESFT samples in order to ensure the reproducibility of our results.

As Imp-2 was shown to be crucial for CSC proliferation in glioblastoma we wanted to assess the effect of Imp-2 on the proliferation of our ESFT CSCs enriched cell populations.

IMP2 is expressed during development in different organs including gonads, small intestine and bone marrow. Its related protein is believed to play role in organogenesis and it participates to RNA regulation. Interestingly, Imp-2 has been related to multiple malignancies like breast cancer (24) and hepatocellular carcinoma (25). In glioblastoma, Imp-2 showed to promote CSC proliferation by supporting the main mechanism of energy production in gliomaspheres: OXPHOS (17).

Proliferation assays on Imp-2 depleted ESFT5 cells revealed that Imp-2 promotes CSC proliferation. *In vitro,* the difference of cell proliferation between Ctrl-ESFT5 cells and sh-Imp2 ESFT5 cells was significant at 48h. However at 72 hours, high proliferation rate and high cell numbers in both cell populations (confluent cells) reduced the specificity and account for the large variability of these results.

As *in vitro* proliferation results are not always reflective of *in vivo* cellular behaviour, we injected these cells into immunocompromised mice. Interestingly, *in vivo*, three mice developed grossly visible tumors after xenotransplantation of Ctrl-ESFT5 cells, whereas only one mouse injected with sh-Imp2 ESFT5 showed a visible tumor. These promising findings have to be confirmed in other ESFT samples and other *in vivo* experiments including more mice. Nevertheless, these results seem to highlight the importance of Imp-2 in tumor formation capacity of ESFT CSCs.

Numerous studies focusing on cancer metabolism showed that malignant cells preferentially use glycolysis as main energy source in order to produce enough metabolites for biomass formation. This mechanism is called the Warburg effect (18). However recent studies revealed that certain CSCs such as epithelial ovarian CSCs (26) or glioblastoma CSCs (17), preferentially use the OXPHOS pathway to metabolise glucose. To get a hint of a possible mechanism explaining the proliferative advantage of cells with high expression of Imp-2, we assessed whether Ewing sarcoma CSCs showed the same trend. Therefore, we added an inhibitor of the complex 1of the mitochondrial respiratory chain in our ESFT4 spheres and adherent cultures. The same was done with oxamic acid, an inhibitor of lactate dehydrogenase, in order to impair glycolysis. Interestingly, microscopic analysis revealed a diminution of cell numbers in sphere cultures with rotenone but no effect in the presence of oxamic acid. The opposite effect was observed in adherent culture conditions suggesting a differential glucose metabolism in these two cell populations with a preference of spheres for the OXPHOS pathway. Even if other experiments are needed in order to give more details on oxygen consumption or lactate production to confirm our results, this observation revealed an interesting feature of ESFT CSCs metabolism that appears to recapitulate observations in glioblastoma cells (17). Considering the positive impact of Imp-2 on OXPHOS in gliomaspheres and its impact on *in vitro* and *in vivo* ESFT tumor growth, its effect on ESFT CSCs metabolism should be investigated.

Although this study remains to be completed with other mechanistic experiments, it proposes a new way to investigate Ewing sarcoma CSCs gene expression in order to understand crucial mechanisms of CSCs generation and survival. Analysing epigenetic changes between CSCs and bulk tumor cells revealed differential EWS-FLI1 mediated

regulation mechanism of a specific gene, *IMP2*. Differential expression between CSCs and bulk tumor cells was then confirmed in several other primary ESFT samples and seems to have a functional *in vivo* impact as downregulation eradicated the tumor forming capacity of these cells. The elucidation of the CSC regulating pathways might provide new approaches to specifically target tumor-initiating cells.

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