

Ewing sarcoma PDX models

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Abstract

Ewing sarcoma (EWS) is a rare malignant pediatric tumor and patient derived xenografts (PDXs) could represent a possibility to increase the number of available models to study this disease. Compared to cell derived xenografts (CDX), PDXs are reported to better recapitulate tumor microenvironment, heterogeneity, genetic and epigenetic features and are considered reliable models for their better predictive value when comparing preclinical efficacy and treatment response in patients. In this chapter, we extensively describe a method for generating Ewing sarcoma PDX models for their validation and molecular characterization.

Key words: Ewing sarcoma, Patient-derived xenografts, immunodeficient mice, preclinical models, Pediatric tumors

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1. Introduction

Historically, the discovery of athymic immunosuppressed mice (*nude* mice) in 1962 changed the paradigm of cancer research [1]. Indeed, since that time, it is possible to graft tumor cells or human tumor fragments on these T cells deficient mice and thus avoid transplant rejection. Using for the first time this approach, Rygaard and Povlsen [2] implanted under the skin of nude mice a fragment of colon cancer of a 71-year-old patient. This tumor developed as a differentiated adenocarcinoma like that of the donor. This model could be propagated over a period of seven years, representing seventy-six successive transplants of the nude mouse tumor. Subsequently, other immunodeficient murine models (SCID, NOD-SCID, NSG...) have been developed and also used for this purpose. During the 80's, evidences for a good correlation between the response to certain chemotherapies in patients and in associated patient derived xenograft (PDX) models was reported but did not draw much attention [3]. In parallel, subcutaneous cell line derived xenograft models (CDXs) in immunocompromised mice were also developed and emerged as an easier model to work with for preclinical studies. However, the predictive value of CDX models seemed, already at the time, less convincing than PDX models. Indeed, a study synthesizing the responses to numerous cytotoxic agents, in a panel of 39 CDX, had demonstrated the low correlation between the efficacy of these drugs in these models and in patients [4]. However, for reasons of accessibility to PDX models, ease of implementation of CDX models as well as the emergence of other mouse models (including transgenic models), PDX models have fallen into oblivion for almost three decades. During this period, most preclinical studies in mice, whether conducted by the academic laboratories or the pharmaceutical industry, have been based on these poorly predictive CDX models. However, in the last decade, a considerable effort has been made to rehabilitate PDX models for preclinical research in view of their faithfulness to recapitulate tumor heterogeneity, genetic and epigenetic features and for their better predictive value when comparing preclinical efficacy and treatment response in patient [5–11]. As any models, PDX present also some disadvantages that should be taken into consideration. For instance, PDX are generated into immunodeficient mice, which are, at the time of the expansion of immunotherapy-based approaches, not suitable for these preclinical studies. Humanized PDX models have been developed to circumvent to some extent this aspect [12]. Another illustration of potential caveats to consider in PDX models is for instance the cross-species signaling dysfunction. Indeed, murine hepatocyte growth factor (HGF) does not recognize and activate its human MET orthologous receptor [13], which is problematic when testing molecules targeting this pathway. However, this can be addressed using transgenic immunodeficient mice expressing human HGF [14]. as illustrated above. Alternative approaches have been developed to circumvent some of the PDX drawbacks and scientists must carefully consider, if the use of PDX is the most suitable model to conduct their research.

In this regard, Ewing sarcoma PDX, could provide a better option to reproduce the pathogenesis of this rare pediatric and adolescence bone tumor. Indeed, a recent study showed that gene expression profile analysis of the PDX and of the cell culture obtained from the same patient demonstrated a higher concordance between the PDX and the human tumor than the cell culture [11].

With the idea of sharing our experience to generate Ewing sarcoma PDX models in the past years, in this chapter, we specifically focused on methodological aspects and also describe how to validate and characterize these models.

2. Materials

2.1 Tissue collection and preparation

1. Biological safety cabinet Biosafety Level 2 (BSL2).
2. Petri Dishes 60 mm.
3. 50 ml sterile tubes.
4. Sterile Fine Scissors, scalpels and tweezers.
5. Culture medium (i.e. RPMI or IMDM) plus 10% Fetal bovine serum (FBS, optional). and Penicillin-Streptomycin.
6. Fetal bovine serum (certified or tested to comply with S(O)FP animal facility requirements).
7. Dimethyl sulfoxide (DMSO), sterile-filtered.
8. Freezing medium (90% FBS; 10% DMSO).
9. Sterile cryovials.
10. Mr. Frosty™ Freezing Container.
11. Vacutainer blood collection EDTA-treated tubes.
12. -80°C ultra-freezer and liquid nitrogen storage tank.
13. Histopaque-1077.
14. Parafilm and specimen jar labels.
15. Ice bucket with pre-chilled cold packs.
16. Dry ice.
17. Isothermic box and absorbent material.

2.2 Tumor engraftment

1. NSG (NOD.Cg-Prkdcscid IL2rgtm1Wjl/SzJ) mice (5-10 weeks old) are preferred for the establishment of PDX models but similar results can be achieved with SCID (CB17/Icr-Prkdcscid/IcrIcoCrI) or Swiss Nude (CrI:NU(Ico)-Foxn1nu) immunodeficient strains [15] (see Note 1).
2. Anesthetic solution, for example a mixture of Xylazine (20mg/ml, Rompun 2% or Xilor 2%) and Tiletamine+Zolazepam (Zoletil 50/50 mg/ml), should be prepared at the time of use by diluting the drugs in phosphate buffered saline (PBS). Xylazine should be diluted 12,6x and Tiletamine+Zolazepam 7.875x. For example, by adding to 250µl of PBS, 25µl of Xylazine and 40 µl of Tiletamine+Zolazepam for a final volume of 315µl.
3. 70% Ethanol (v/v): 70 ml ethanol (absolute), 30 ml sterile deionized water.
4. 0.1% chlorhexidine gluconate solution.
5. Sterile surgical gloves.
6. Sterile gauze compress.
7. Mice trimmer.

8. Polystyrene disposable sterile forceps.
9. Dissecting scissors, forceps and tweezers, sterile autoclaved.
10. Standard pattern Forceps- straight 14.5cm.
11. London Forceps-angled 16cm.
12. Adson Forceps-Serrated straight.
13. Michel Structure Clips and clip applier.
14. Sterile 9 mm wound clips, wound clip applier and wound clip remover autoclaved.
15. Sterile calipers for measurement of tumor size.

2.3 Passages and tumor collection from mice

1. Ice.
2. 96% Ethanol.
3. Sterile tweezers and scissors.
4. Sterile Phosphate-buffered saline (PBS).
5. 10% neutral buffered formalin solution (*see Note 2*).
6. 1.5 mL sterile cryovial tubes.
7. Plastic petri dishes.
8. Liquid nitrogen.
9. Ultrafreezer -80°C.

2.4 Ewing Sarcoma PDX validation

Histology (*see Note 2*)

1. Paraffin wax.
2. Microtome.
3. Hentellan jars.
4. Xilene, 100%; 96%, 70% ethanol.
5. Pre-coated slides.
6. Haemotoxylin and Eosin.
7. Acid ethanol.
8. Deionized water (dH₂O).
9. Xilene-based mounting medium.
10. Microscope.

Immunohistochemistry (see Note 2)

1. Xilene, 100%; 96%, 70% ethanol.
2. dH₂O.
3. Hematoxylin.
4. Sterile Phosphate-buffered saline (PBS).
5. Antigen Unmasking: 10 mM Sodium Citrate Buffer (see Note 3).
6. Methanol (see Note 4).
7. 37% hydrogen peroxide (see Note 4).
8. ABC Reagent: (Vectastain ABC Kit, or equivalent).
9. Diamino-Benzidine (DAB) stock solution 100x (see Note 5).
10. Xilene-based mounting medium.
11. Microscope.

PDX chimerism

1. Mortar and pestle.
2. Liquid nitrogen.
3. RNase free water.
4. DNase I (RNase free).
5. RNA extraction reagent or kit (Trizol, RNeasy plus mini kit or equivalent).
6. Reverse transcription kit (High-Capacity cDNA Reverse Transcription Kit or equivalent).
7. Quantitative PCR reagents (Power SYBRTM Green PCR Master Mix or equivalent).
8. Primers:

TBP_Hs_forward:	5'-AGAACAACAGCCTGCCACCTTAC- 3'
TBP_Hs_reverse:	5'-GGGAGTCATGGCACCTGAG- 3'
Tbp_mm_forward:	5'-CCCTTGTACCCTTCACCAATGAC - 3'
Tbp_mm_reverse:	5'-TCACGGTAGATAACAATATTTTGAAGCTG- 3'
TBP_Hs+mm_forward:	5'-TGCACAGGAGCCAAGAGTGAA- 3'
TBP_Hs+mm_reverse:	5'-CACATCACAGCTCCCCACCA- 3'

3. Methods

3.1 Tissue collection, preparation and delivery of fresh and frozen samples

Before starting tissue collection, the research project involving PDX establishment must be approved by the Institutional Ethic Committee. Patients must receive exhaustive explanation regarding the project and authorize the use of their samples by signing an informed patient consent form. Furthermore, the use of animals for experimentation is strictly framed and all experiments made on animals must be tested in the respect of the guidelines established by Veterinary ethic Committee and National Law (see Note 6).

All tissue and blood preparation should be performed in a Biological Safety Cabinet (BSC) using sterile instruments and technique. Sterility must be kept during all procedures.

When receiving the sample for the establishment of PDX models, it is important to simultaneously preserve and collect patient samples (blood and tumor) for the subsequent molecular characterization procedure of these models. Therefore, if possible, it is recommended to keep at least one frozen piece and one formalin-fixed, paraffin-embedded (FFPE) tumor piece as well as a blood sample. Since blood/bone marrow transplantation can occur in these patients, this information should be asked to the clinicians before considering using the blood sample for sequencing approaches. (see Note 7).

Ideally, engraftment of the tumor sample should occur at the same location than the surgery/biopsy. However, if not possible, the following procedure can be used to transport these sample.

1. The tumor should be maintained at 4°C in culture medium until implantation (see Note 8).
2. Transfer the tumor material into a sterile petri dish along with a small volume of the culture medium used for transportation to keep the tissue wet. Evaluate the material before cutting to make sure that viable tumor tissue is being implanted avoiding necrotic areas as well as normal tissues (bone, cartilage, connective tissue).
3. Cut the tissue into fragments using a sterile scalpel or fine scissors. If possible, the fragment dedicated to the PDX implantation should have a diameter of 3-4 mm (corresponding to 10-30 mm³ tissue fragment); engraftment of fragments of smaller dimensions may have lower probability to be successful.
4. For future characterization, patient's tumor tissue should be snap frozen as soon as possible (i.e. within a couple of minutes) in liquid nitrogen immediately following surgery and then stored at -80°C.
5. For molecular characterization, a constitutional (germ line) sample (most frequently a patient's blood sample) prior to the initiation of chemotherapy, should be used (see Note 9).
6. If the tumor implantation needs to be delayed, samples should be suspended in 1ml of freezing medium, place into the slow-rate freeze container and store at -80°C overnight. Subsequently, vials should be transferred in a liquid nitrogen storage tank until delivery to the animal facility.
7. A fragment of tumor tissue must be available for histopathology and immunohistochemistry and should be fixed in a 10% formalin solution (see Note 2), routinely processed, and embedded in paraffin.

8. Any remaining tissue can be used for *in vitro* cultures.
9. In case of delivery of a patient tumor fresh sample to a distant animal facility, specimens must be placed in pre-chilled medium vials and shipped in sterile tubes, filled with culture medium +10% FBS.
10. Vials should be sealed with parafilm and wrapped with adsorbent material to avoid liquid leaks.
11. To maintain cold temperature, samples must be transported with pre-chilled cold packs in an isothermic box for implantation into mice

In case of shipping of patient tumor frozen samples, stored in liquid nitrogen, to a distant animal facility,

12. Cryotubes should be properly labelled and placed in a sealable plastic bag.
13. The plastic bag should be placed in a screw cap plastic container.
14. The container should be placed in a polystyrene box containing enough dry ice for shipment (*see* Note 10)

3.2 Tumor engraftment over or under the inter-scapular brown fat pad

All experiments involving live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and performed following National and International law.

1. Shave mice with the mice trimmer if necessary.
2. Prepare as many tumor fragments (4mm × 4mm) as mice to be engrafted in a petri dish and keep the tissue moist with some drops of PBS (Figure 1).
3. Anesthetize immunodeficient mice with Xylazine, Tiletamine and Zolazepam solution mixture. 40µl of the final solution should be administered by intramuscular injection in an anterior leg of the mouse. (*see* Note 11).
4. Transfer the animal to the surgical platform in a sterile field and place it in a prone position.
5. When the mouse is fully anesthetized (*see* Note 12), disinfect the skin with sterile gauze compress embedded with chlorhexidine solution. Repeat this procedure twice with new embedded compresses.
6. With sterile scissors, make a small (4-6 mm) incision (parallel to the spinal column) between the shoulder blades being careful not to cut any muscle. There should be little or no bleeding with this procedure.
7. Make a subcutaneous pocket by inserting the round tip of a tweezer into the center of the transplantation site and make a pocket about 4-6 mm long using blunt dissection technique and reaching the area of the inter-scapular brown fat pad.
8. Insert the tumor fragment into the pocket with the tweezer by placing it over the area of the brown fat pad. Carefully remove the tweezers once the tissue has been placed.
9. Close the incision with a wound clip by using the wound clip applicator.

Alternatively, the tumor fragment can be implanted under the fat-pad (*see* Note 13) following the point 1 to 6 of the above procedure and continue with the alternative procedure described below (point 7 to 13):

- 10 Using your left hand (if right-handed), hold and lift the mouse skin (approximately 1 cm from the incision site) with disposable sterile forceps. 11 With your right hand, introduce London forceps into the incision and gently pull out the brown fat pad, keep the fat pad grabbed outside the mouse and avoid touching the surface of the skin.
- 12 With your left hand, release the skin and put the disposable sterile forceps on the side.
- 13 With your left hand and Adson forceps, hold the fat pad a few millimeters away from the London forceps and hold it firmly.
- 14 With your right hand, release the contention of the London forceps on the fat pad (still hold with your left hand) and with the angled tips of the London forceps, drill a small hole in the membrane between the mouse spine and the fat pad and release slightly the London forceps to generate a cavity under the fat pad.
- 15 With your right hand and London forceps, take a tumor fragment and insert it in the cavity under the scapular fat pad.
16. With Adson forceps in your left hand, place the fat pad under the mouse skin.
- 17 Close the incision with a wound clip by using the wound clip applier.

After the surgery:

- 18 Identify animals by the current method used in the laboratory (*see* Note 14)
- 19 Return the mouse to its sterile home cage and keep it in a warm place until it awakens.
- 20 Monitor the mice daily for 3-5 days post-surgery and remove wound clips within 7 – 10 days.
- 21 Check tumor growth weekly and measure its dimension with a sterile caliper.
- 22 When the tumor reaches the end-point size, mice are euthanized (*see* Note 15).

3.3 Passages and tumor collection from mice

Necropsy must be performed very carefully recording tumor features such as color, vascularization, consistency, necrotic and viable areas and trying to identify any potential sites of metastasis by inspecting lungs, brain and all of the organs in the peritoneum. Ewing sarcoma tumors typically show white color and a very soft loose consistency.

1. Euthanize the mouse using the method approved by the appropriate Ethics Committee.
2. Lay the mouse in a supine position on the surgical platform and clean the incision area and the whole mouse with ethanol. The hair must be completely wet.
3. Make an incision to expose the tumor. Carefully detach the skin from the tumor using tweezers and scissors.
4. Carefully remove the tumor.
5. Put the tumor sample in a petri dish and place it on ice.
6. Divide the tumor into representative portions for different applications, such as passage in other mice, viable fragment freezing, histology, nucleic acid and protein isolation, and generation of viable cell lines.
7. Put sufficient fresh tumor samples in PBS for passage in other mice (*see* Note 16), for viable fragment freezing and generation of viable cell lines (Figure 1)

8. Fix some tumor samples in 10% neutral buffered formalin solution for histology (*see* Note 2)
9. For nucleic acid and protein extraction, put some small (diameter 2-3 mm) tumor fragments in 1.5ml conical polypropylene tubes and immediately snap-freeze the vials by submerging them in liquid nitrogen and then transfer the vials at -80°C in an ultra-freezer.

3.4 Ewing Sarcoma PDX validation

The comparison between PDX models at different *in vivo* passages and patient's tumors should be done in all PDX obtained and include the following techniques.

1. Histology

As first step, histopathologic features of the patient Hematoxylin-Eosin-stained slides and the PDX derived samples should be compared to gain information about histologic similarity (Figure 2A).

- 1.1 The tissues from human tumor and PDX have to be fixed in 10% buffered formalin, routinely processed, and embedded in paraffin (*see* Note 2).
- 1.2 Serial, 4- μ m-thick, paraffin sections are mounted on pre-coated slides and processed as described in Immunohistochemistry section 2.1 according to standardized procedures [11].
- 1.3 After, incubate slides with Hematoxylin for 3 minutes and rinse with dH₂O and with tap water for 5 minutes;
- 1.4 Dip 8-12x(fast) in Acid ethanol to destain slides.
- 1.5 Rinse with tap water and for dH₂O for at least 5 minutes.
- 1.6 Stain the slides with Eosin (30 seconds).
- 1.7 Dehydrate slides as described in Immunohistochemistry section 2.6.
- 1.8 Coverslip slides using a xylene-based mounting medium (*see* Note 17).

The comparative morphological analysis between patient's tumor and corresponding PDX should include the evaluation of the following characteristics:

- a) Tumor cellularity;
- b) Pattern of growth;
- c) Mitotic activity;
- d) Cell morphology, including degree of pleomorphism and differentiation.

Histologically, conventional Ewing sarcoma is uniformly made of sheets of small round cells that are closely packed and without matrix [16]. The chromatin is finely stippled, and nucleoli are usually not evident. Usually, extensive deposits of glycogen are observed in the cytoplasm, that is generally scarce. A 'large cell', or 'atypical', variant of Ewing sarcoma has been reported [17]; the main difference of these cells from conventional Ewing sarcoma are larger-size nuclei with irregular contours. In the case of Peripheral Neuro Ectodermal Tumors (PNET), the presence of rosette pattern is common (*see* Note 18). The analysis and comparison of these morphological

characteristics between the tumor of origin and the PDXs allows to classify the PDX models in three main categories:

Complete identity: No difference between patient and xenograft-derived samples;

Similar pattern: Changes in growth pattern and overall cellularity allowed, but no differences in mitotic activity or/and pleomorphism;

Morphologic Shift: significant morphological differences concerning all the characteristics indicated above [7, 11].

The comparison should be done in all the generations of PDX obtained.

2. Immunohistochemistry

Patient samples and the matched xenograft tissues should be assessed for markers of proliferation, resistance to apoptosis or neural differentiation like Ki-67, Caspase 3 (total); Caspase 3 (cleaved), S-100 or Neuron-Specific-Enolase (NSE). In addition, a number of diagnostically delineating markers should be performed based on the histopathologic diagnosis like CD99 antigen (Figure 2B), FLI1, CAV1, BCL11B or GLG1, NKX2-2 [7, 11, 18, 19]. Every analysis should be accompanied by appropriate positive and negative controls. Moreover, all stained sections should include non-tumor mouse cells, such as endothelial cell, myopericytes and fibroblasts which must be constantly negative.

The procedure here presented is an ABC avidin/biotin method, routinely used for IHC staining. However, in alternative, standard operative procedures of each laboratory could be followed.

Don't allow slides to get dry during all steps of this procedure.

2.1. Deparaffinization/Rehydration

2.1.1. Incubate slides in two washes of xylene for 30 minutes each.

2.1.2. Incubate slides in three washes of 100% ethanol for 5 minutes each.

2.1.3. Incubate slides in one wash of 95% ethanol for 5 minutes.

2.1.4. Incubate slides in one wash of 70% ethanol for 5 minutes.

2.1.5 Rinse slides with dH₂O for 5 minutes each.

2.2. Antigen Unmasking (if necessary)

2.2.1. Put slides in Hellendal jars (10 slides/jar) with citrate buffer.

2.2.2. Bring slides to boil in sodium citrate buffer for 3 cycles of 5 min each at 750 W (replace buffer after every cycle).

2.2.3. Cool at room temperature for 20 min without changing the buffer of the last cycle.

2.2.4. Put slides in PBS for 3-5 min (until starting immunostaining).

2.3 Inhibition of endogenous peroxidase

2.3.1 Incubate slides with inhibition solution for 30 min at room temperature.

2.3.2. Wash twice (5 min each) with PBS and once with distilled water (5 min).

2.4 Immunostaining

2.4.1. Incubate slides for 15 min at room temperature with Horse normal serum (kit Mouse) or Goat normal serum (kit Rabbit) diluted in PBS.

2.4.2. Discart normal serum without allowing slides to dry.

2.4.3. Incubate slides overnight at +4°C in a humidified chamber with the primary antibodies diluted in PBS (*see Note 19*).

2.4.4. Wash slides twice (5 min each) with PBS and once with dH₂O water (5 min).

2.4.5. Incubate slides for 30 min at room temperature with the biotinylated secondary Ab diluted in PBS (*see Note 20*).

2.4.6. Wash slides twice (5 min each) with PBS and once with dH₂O water (5 min).

2.4.7 Incubate slides for 30 min at room temperature with ABC.

2.4.8. Wash slides twice (5 min each) with PBS and once with dH₂O water (5 min).

2.5 Development of immunoreaction

2.5.1 Defreeze Diamino-Benzidine (DAB) stock solution 100x (5 gr/100 ml PBS) and prepare DAB working solution (*see Note 5*).

2.5.2 Incubate slides for 5 min at room temperature with DAB 1x and add 5-100 µl of H₂O₂ and incubate for additional 5-10 min (*see Note 21*).

2.5.3. Wash slides twice (5 min each) with PBS and once with dH₂O water (5 min).

2.5.4. Counterstain nuclei with pre-filtered hematoxylin (30 sec - 3 min).

2.5.5. wash slides with distilled water for 3-5 min.

2.6 Dehydration

2.6.1. Incubate slides in one wash of 70% ethanol for 2 minutes.

2.6.2. Incubate slides in one wash of 95% ethanol for 2 minutes.

2.6.3. Incubate slides in three washes of 100% ethanol for 5 minutes each.

2.6.4. Incubate slides in two washes of xylene for at least 2 minutes each.

2.6.5. Mount coverslips with xylene-based mounting medium.

3. Ewing Sarcoma PDX: evaluating human and murine chimerism

In PDX, the human component arises from the tumor fraction whereas the microenvironment derives from immunodeficient murine stromal cells (human stroma that is present at the time of engraftment is rapidly replaced by murine cells within the first passages) [10, 20]. Evaluating the proportion human and murine derived tissue in a PDX is a simple method that can be routinely used as a first step of model validation. This approach is also useful to promptly detect spontaneous murine tumors that occasionally develop in these immunodeficient strains [21, 22]. For this, RT-QPCR with a set of 3 primers recognizing respectively the murine, the human or both orthologous transcripts of the Tbp, TBP genes can be used [23]. In Ewing sarcoma PDX, the murine stroma typically represents 5-15% of the tumor (Figure 2C). In parallel, the presence of the EWSR1-ETS fusion transcript (Figure 2D) can be used to confirm the proper derivation of an Ewing sarcoma model [24]. It is recommended to perform these RT-QPCR validation methods with all tumors growing on mice from P0 to P2 and later on, every third passage with one or two tumors per passage.

3.1 Isolation of RNA

3.1.1 Homogenize a snap frozen PDX tumor sample (10-30mg) with a mortar and pestle, transfer sample powder into a 2ml Eppendorf tube and add 500 µl Trizol reagent without thawing samples.

3.1.2 Incubate the homogenized samples for 5 minutes at room temperature.

3.1.3 Add 0.1ml of pure chloroform, shake firmly for 15 seconds.

3.1.4 Incubate samples for 2-3 minutes at room temperature.

3.1.5 Centrifuge sample at 14000rpm for 15 minutes at 4°C.

3.1.6 Transfer aqueous (clear upper) phase to a fresh tube.

3.1.7 Add 1µl glycogen as carrier to explant samples.

3.1.8 Precipitate RNA by adding 250 µl isopropanol and incubate at -20°C overnight.

3.1.9 Spin samples at 4000rpm for 30 minutes at 4°C.

3.1.10 Remove isopropanol and wash RNA pellet once with 500µl 75% Ethanol.

- 3.1.11 Air dry pellet for 5 minutes.
- 3.1.12 Resuspend RNA in 20 µl RNase free water H₂O and incubate 10 minutes at 55-60°C.
- 3.1.13 Add 2 µl DNase I (RNase free) to 20 µl mRNA and incubate for 30 minutes at 37°C.
- 3.1.13 Samples can now be stored at -80°C or used immediately for reverse transcription.
- 3.1.14 Alternatively a RNA extraction Kit can be use (RNeasy plus mini kit or equivalent).

3.2 Reverse Transcription

- 3.2.1 use 1µg of RNA for each reaction (extracted with a method described above).
- 3.2.2 add reverse transcription buffer, dNTP, random primers and reverse transcriptase and complete with water according to manufacturer instructions.
- 3.2.3 Incubate reverse transcription sample according to manufacturer instructions.

3.3 Quantitative PCR

- 3.3.1 Mix forward and reverse primers for each primer set (TBP_Hs, Tbp_mm and TBP_Hs+mm) to prepare a 10µM stock solution (for example 20 µl of 100µM TBP_Hs_forward, 20 µl of 100µM TBP_Hs_reverse and 160µl of water).
- 3.3.2 Add to the cDNA reaction 200µl of molecular grade water.
- 3.3.3 Perform duplicate QPCR reactions for all 3 primer sets as following:
 - 9µl of diluted cDNA
 - 1µl of 10µM primer stock solution
 - 10µl of 2X Power SYBR™ Green PCR Master Mix
- 3.3.4 Run RT-QPCR program and determine Ct and melting temperature for each reaction.
- 3.3.5 Determine the fraction of murine and human tissue with the following formula:

$$\text{Human fraction} = \frac{2^{-(\overline{CT}(\text{TBP_Hs}) - \overline{CT}(\text{TBP_Hs+mm}))}}{2^{-(\overline{CT}(\text{TBP_Hs}) - \overline{CT}(\text{TBP_Hs+mm}))} + 2^{-(\overline{CT}(\text{TBP_mm}) - \overline{CT}(\text{TBP_Hs+mm}))}}$$

$$\text{Murine fraction} = 1 - \text{Human fraction}$$

4. Ewing Sarcoma PDX: STR profiling

When generating or using several PDX simultaneously, the risk of inadvertent model exchange has to be considered and authentication through short-tandem repeat (STR) profiling is highly recommended to confirm their identity (for instance with PowerPlex® 16 HS System, ref. DC2101 from Promega and following step by step

manufacturer recommendation). It is recommended to perform STR validation with at least one tumor per passage and confirm its identity with the profile of the matched original patient tumor (Figure 2E).

5. Ewing Sarcoma comprehensive molecular characterization

Concurrently with morphological and immunohistochemical evaluation of markers of EWS, validation of EWS-PDX models should include assessment of type of fusion transcripts as well as the occurrence of the few recurrent secondary alteration present in Ewing Sarcoma (mutation in *STAG2*, *TP53* and deletion of *CDKN1A*) that is expected to mirror the profile of the original tumor samples [11, 25]. In addition, to gain a better insight into the similarity of the PDX with the original tumor, it would be a good practice to perform a global gene expression correlation analysis between gene expression profiles of EWS samples and the corresponding PDX, including the comparison of PDX of different *in vivo* passages. Moreover, due to the small number of EWS patients, an effort at national and European level should be done, such as that it is currently set up in the consortia dedicated to the generation of a large cohort of pediatric PDX solid tumor entities, (ITCC-P4 project <https://www.itccp4.eu/>) and to generate an exhaustive molecular characterization of these models and their matched patient tumors. These comprehensive analyses will include low coverage whole genome sequencing (lcWGS), whole exome sequencing (WES), DNA methylation profiling and RNA sequencing for all PDX and matched tumor as well as lcWGS and WES for patient germline DNA. These extensively characterized PDX models shall provide to the scientific community and the pharmaceutical industry, state of the art pediatric PDX models (including for Ewing sarcoma) to transform preclinical investigation into successful clinical trial and innovative therapies against these aggressive pediatric cancers.

4. Notes

1. Human tumor fragments can be engrafted in mice with different degree of immunodeficiency. NOD-SCID-Il2rg^{-/-} (NSG) mice are strongly recommended, at least for the first engraftment, thanks to their high level of immunodeficiency and the long lifespan. Nude and SCID mice can be used as well, but lower immunodeficient mice or mice with a shorter survival should be employed only in later passages when PDX *in vivo* growth is stabilized and usually faster than initial passage. Nude mice can be useful when testing drug efficacy against PDX because they are less sensitive to cytotoxic and radiation therapies compared to NSG mice.
2. Chemicals must be handled under a fume hood accordingly to MSDS.
3. Citrate buffer: for 1 L add 2.94 g sodium citrate trisodium salt dihydrate and 2.10 g citric acid anhydrous to 1 L dH₂O. Adjust pH to 6.0.
4. Endogenous peroxidase inhibition solution (to be dissolved immediately before use): for 100 ml add 1 ml of H₂O₂ to 99 ml methanol.
5. To prepare 100x DAB working solution dilute 5 gr of DAB in 100 ml of PBS and store 1 ml aliquots at -20°C. . Diamino-Benzidine is highly dangerous and may cause cancer. Handle with care strictly following the MSDS.
6. In the planning of the experiment, the 3R guidelines should be applied: reducing the number of animals by protocol; refining, that is, improving animal welfare

experimentation by limiting suffering and stress; replace the use of animals by alternative methods as possible. The availability of alternative methods (*in vitro* systems, mathematics modeling) allowed to reduce number of animals used for biomedical research. However, there is no current model for the moment completely substitute PDX models.

7. All fresh human tissue, including whole blood and its components, must be handled under Biosafety Level 2 (BSL2) conditions. All work is conducted in a biological safety cabinet (BSC) using personal protective equipment and avoiding the use of sharp tools where possible. All tools potentially exposed to the human material must be treated with a 10% sodium hypochlorite solution for a minimum of 10 minutes, double bagging for autoclaving and UV disinfection system. Follow all waste disposal regulation when disposing waste materials.
8. Tumor tissue should be implanted as soon as possible, an ideal time is within 2 hours from surgery, but implantation in the 24 hours post-surgery can yield successful PDX.
9. Blood could be collected and stored as whole blood sample in vials with EDTA (minimum 2 ml, preferentially 5 – 10 ml), at -80°C; or in alternative, it is possible separate plasma from blood cells. In this case, whole blood needs to be centrifuged within 2 hours of collection at 300 for 15 minutes at room temperature to separate blood cells from the plasma. After, blood cells should be diluted 1:2 in PBS, separated in the different subfractions with Histopaque-1077, according to manufacturer instructions. Plasma (for cell free DNA extraction) and peripheral mononuclear cells PBMC need to be stored at -80 °C.
10. In case of shipping by air transport, packaging must be done in compliance with IATA packaging instruction and properly labeled for biological substance category B (UN3373) and dry ice (class 9 hazard label, UN 1845, indicating weight of dry-ice content).
11. The volume of 40µl of the final anesthetic solution is the dose recommended for 5-10 weeks old mice. Patient-derived tumor samples at the first implant in mice can have highly variable latency and growth rates. In the fastest growing PDX, tumor graft can become palpable around two weeks after implantation, but it is common for Ewing sarcoma tumor grafts to take several months or up to one year to become palpable and additional 2-3 months to be ready to be collected for the first time. For this reason, mice not older than 5-10 weeks should be used. If the tumor fragment is frozen prior to implantation, when re-transplanting the tumor fragment, some additional months could be required. In our experience, in Ewing sarcoma often the re-transplanted tissue will show reduced latency compared to the original patient derived sample. When cohorts of mice are transplanted with similar size fragments from the same stabilized PDX sample, tumors will usually grow with similar latency.
12. Make sure that the mouse is in plane of anesthesia before starting surgery and that the tongue is in the proper position. If some twitching is present, allow more time for the anesthesia to take effect.
13. The expected rate of successful engraftment with the implant over the interscapular fat pad for EWs should be around 24% [11], implant into the interscapular fat pad can give an uptake of 45-50%.
14. It is strongly advisable to keep in separate cages PDXs deriving from different patients. On each cage put a label indicating strain, gender and the unique identification number of the mouse, followed by the PDX unique identification

- code, number of passages in vivo, for example P1 for the first implant, P2 for the second implant and so on, and the date of the implant.
15. If the mice show any sign of discomfort or illness prior to tumor growth, or the tumor starts to ulcerate, the mice should be euthanized.
 16. The procedure for a passage is identical to the one described under point 3.2. In this case, PDX tumor fragments (typically 4x4 mm) are grafted in 2-4 immunodeficient mice (one tumor fragment per mouse).
 17. Place a drop of mounting medium on the slide taking care to leave no bubbles and allow the mounting medium to cover all the tissue under the coverslip. Dry overnight in the hood.
 18. Rosette is defined as a group of cells characterized by nuclei at the periphery and cytoplasm projections toward the center of this structure.
 19. Refer to product data sheet for recommended antibody diluent. Optimal dilution should be standardized using proper controls.
 20. During the incubation with the biotinylated secondary antibody, prepare the Avidin-Biotin peroxidase complex (ABC) according to manufacturer's instruction
 21. Check microscopically the development of the reaction every 2-3 min. The reaction will be stopped when positive control included in the jar developed

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Figure legends:

Figure 1 Generation of EWS-PDX model. Tumor samples obtained from surgical or biopsy specimens are divided in small fragments and are used as follow: subcutaneously implanted in mice, snap-frozen and stored in biobank and dissociated to establish primary cell cultures. When the tumor reaches the ethical size, mice will be euthanized. At every passage, tumor is divided into representative portions for different applications, such as model establishment or propagation (so-called “passage”), viable fragment freezing, histology, nucleic acid and protein isolation, and generation of viable cell lines. From the third passage, this model is defined as established and can be used for preclinical studies for instance.

Figure 2 A-B) Histologic and immunohistochemical features of patient’s tumors and corresponding PDX at different *in vivo* passages. EW PDXs consist of small round cell sheets, closely packed and without matrix, as patient’s tumors. CD99 antigen expression of PDX reflected that of patient’s tumor. **C)** Absolute proportion of human derived tumor and murine stroma tissues in two EWS PDX models across several passages, **D)** Evaluation of the EWSR1-ETS fusion transcript in EW PDXs and in patient’s tumor samples. Positive controls are included. **E).** STR profiling between patient’s tumor and PDX allows for the authentication of models along passages. PDX derived from tumor B displays a different STR profile than the one derived from tumor A



