Video Article

Establishment of a Primary Culture of Patient-derived Soft Tissue Sarcoma

Alessandro De Vita[1], Laura Mercatali[1], Giacomo Miserocchi[1], Chiara Liverani[1], Chiara Spadazzi[1], Federica Recine[1], Alberto Bongiovanni[1], Federica Pieri[2], Davide Cavaliere[3], Valentina Fausti[1], Dino Amadori[1], Toni Ibrahim[1]

[1]Osteoncology and Rare Tumors Center, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS

[2]Pathology Unit, Morgagni-Pierantoni Hospital

[3]Unit of Surgery and Advanced Oncologic Therapies, Morgagni-Pierantoni Hospital

Correspondence to: Alessandro De Vita at alessandro.devita@irst.emr.it

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Abstract

Soft tissue sarcomas (STS) represent a spectrum of heterogeneous malignancies with a difficult diagnosis, classification, and management. To date, more than 50 histological subtypes of these rare solid tumors have been identified. Thus, due to their extraordinary diversity and low incidence, our understanding of the biology of these tumors is still limited. Patient-derived cultures represent the ideal platform to study STS pathophysiology and pharmacology. We thus developed a human preclinical model of STS starting from tumor specimens harvested from patients undergoing surgical resection. Patient-derived STS cell cultures were obtained from the surgical specimens by collagenase digestion and isolated by filtration. Cells were counted, seeded, and left for 14 days in standard monolayer cultures and then processed by downstream analysis. Before performing molecular or pharmaceutical analyses, the establishment of STS primary cultures was confirmed through the evaluation of cytomorphologic features and, when available, immunohistochemical markers. This method represents a useful tool 1) to study the natural history of these poorly explored malignancies and 2) to test the effects of different drugs in an effort to learn more about their mechanisms of action.

Video Link

The video component of this article can be found at https://www.jove.com/video/56767/

Introduction

Soft tissue sarcomas (STS) include a spectrum of heterogeneous lesions of mesenchymal origin accounting for 1% of all solid tumors[1,2], and the new World Health Organization classification recognizes the presence of more than 50 different subtypes[3]. Among these, the most common histotypes are adipocyte sarcoma or liposarcoma and leiomyosarcoma, accounting for 15% and 11% of all adult STS, respectively[4,5]. Although STS can develop in different parts of the body, the extremities and retroperitoneum are the most common sites, occurring in 60% and 20% of cases, respectively[6].

The cornerstone of therapy for localized disease is wide surgical resection, whereas the benefits of adjuvant and neoadjuvant chemotherapy are still unclear and are only considered in selected cases[7]. In the metastatic setting, chemotherapy is the standard of care but shows limited results[8]. A better understanding of the molecular biology of STS would help to improve the current differential diagnosis, optimize the available treatments, and identify new targets for therapy.

Within this context, research into cancer has been performed for many years using immortalized cell lines[9]. These experimental models are normally cultured in standard monolayer supports or implanted into immunodeficient rodents to establish *in vivo* xenograft models[10]. Immortalized cell lines represent a manageable and easy-to-store material with which an ample number of research experiments can be performed. They are, in fact, the least expensive and most widely used tool in preclinical studies[11].

However, cell-line based cancer models also have some limitations, *e.g.* prolonged culture in a monolayer system together with an increasing number of passages induces phenotypic and genetic drift, making cells less likely to recapitulate key tumor features. Furthermore, cell line cultures fail to reproduce the cell-to-cell interaction and signaling molecule crosstalk that mimic the biological behavior of tumors and characterize the tumor microenvironment. These issues form the basis of the gap existing between preclinical and clinical results[12].

Given the above, the interest in patient-derived primary cultures has increased[13]. Indeed, the excision of malignant and normal tissue reduces the risk of losing cancer cell phenotype and heterogeneity, thus offering starting material that is more representative of the tumor microenvironment. Moreover, the use of fresh tumor specimens harvested from patients undergoing surgical resection enables us to investigate single tumors and to compare different lesions from the same part of a patient's body[14]. For the above reasons, tissue-derived cell cultures provide a valuable material to study tumor pathophysiology and pharmacology[15,16,17], especially in STS in which commercially available sarcoma

cell lines are limited[18]. We thus optimized a method to establish a patient-derived STS primary cell culture starting from surgically excised tumor tissue[13,19,20]. Our method consists of disaggregating the tumor specimen into small tissue fragments followed by overnight enzymatic tissue dissociation to obtain a single cell suspension. The following day, the enzymatic digestion is stopped by adding fresh culture media and the obtained suspension is filtered to remove tissue fragments, cell-aggregates, and excess matrix material or debris. Finally, a fraction of isolated tumor cells is cytospinned onto glass slides, fixed and stored for downstream analysis aimed at confirming the establishment of STS primary culture by cytomorphological, immunohistochemical and molecular cytogenetic evaluation (*e.g.* FISH analysis of MDM2 amplification represents the standard differential diagnosis for a well-differentiated liposarcoma and the dedifferentiated liposarcoma)[4]. The remaining cells are seeded into a standard monolayer culture for gene expression profiling and pharmacological studies. All the experiments are performed using low passage primary cultures to avoid the selection of specific cancer subclones, and analyzed by an experienced sarcoma pathologist. We have thus created a fully human STS *ex-vivo* model[13,19,20].This highly versatile, easy to handle model could represent a useful tool for different types of preclinical research, *e.g.* to identify new molecular markers for diagnosis and prognosis or to gain a deeper understanding of the activity and mechanisms of action of standard and new drugs used in the management of STS.

Protocol

STS cells are isolated from a tumor mass surgically excised from patients with STS. The protocol has been approved by the Local Ethics Committee and is performed according to Good Clinical Practice guidelines and the Declaration of Helsinki. All patients gave written informed consent to take part in the study. The surgical specimens are analyzed by an experienced sarcoma pathologist and processed within 3 hours of surgery.

1. Tumor Specimen Collection and Processing:

1. Collect tumor specimens with the help of a sarcoma pathologist in a 100-mL sterile urine container with 50 mL of DMEM low glucose medium sealed with a paraffin film.
2. Store the samples at 4 °C for a maximum of 3 h after tumor resection.
3. Perform all the following steps under a sterile tissue culture hood.
    1. Sterilize the hood with 70% ethanol and wear sterile gloves.
    2. Sterilize steel inox tweezers with 70% ethanol (use a sterile gauze).
    3. Remove the paraffin film from the urine container and discard.
    4. Wash exterior of the urine container with 70% ethanol.
    5. Open a square petri dish.
    6. Open the urine container and transfer the tumor specimens into the square petri dish using the sterilized steel inox tweezers.
4. Wash the tumor specimens with PBS twice.
5. Open a sterile surgical scalpel.
    1. Finely shred the tumor specimens into 1 - 2 mm^3 pieces.
    2. Collect one quarter of the total sample in a 2-mL tube.
    3. Add 1 mL of trizol reagent (purchased) to the tube and immediately freeze at -80 °C (the sample will be used in the downstream analysis for the STS gene expression evaluation).
6. Collect the rest of the finely shredded tumor material in a 15-mL tube.
    1. Add 4 mL of collagenase type I solution (collagenase type I is dissolved in PBS, at a concentration of 4 µg/mL).
    2. Dilute the collagenase type I solution with 4 mL of DMEM high culture media (final concentration of collagenase type I is 2 µg/mL).
    3. Close the 15-mL tube and seal with paraffin film.
    4. Put the 15-mL tube in a rolling shaker in an incubator at 37 °C in stirring conditions for 15 min to activate the digestion.
    5. After 15 min store the tube in the rolling shaker under stirring conditions at room temperature overnight.
7. The following day put the 15-mL tube under the sterile hood.
8. Gently filter the cell suspension through a 100 µm sterile filter attached to the top of a 50-mL tube.
9. Wash the filter with DMEM high culture media supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin.
10. Discard the 100 µm sterile filter, close the 50-mL tube and centrifuge at 225 x g for 5 minutes at room temperature.
11. Discard the supernatant by inverting the tube.
12. Re-suspend the cells with 1 - 2 mL DMEM high culture media supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin.
    Note: The volume depends on the amount of tumor sample processed.
13. Count the cells with a Neubauer chamber.
    1. Dilute the sample 1:2 in trypan blue and use 10 µL of the dilution to count cells.
    2. Count at least twice and calculate the mean of the replicates to know the total mean number of cells collected.
14. Plate cells in a standard monolayer culture at a density of 80,000 cells/cm^2.
    Note: Always include controls in experiments. Perform at least 3 technical replicates for each condition. Perform at least 2 biological replicates. For preclinical pharmacology, gene expression and protein expression analyses, experiments should be carried out with a low number of culture passages within 30 - 45 days of cell seeding to avoid losing cancer cell phenotypes.
15. Cytological sample preparation.
    1. Collect 100,000 cells in 200 µL of DMEM high culture media supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin.
    2. Pipette the solution (200 µL pipette) into a disposable funnel equipped with a filter and mounted with a glass slide.
    3. Open the cytocentrifuge and insert the samples.
    4. Run the cytocentrifuge at 18.6 x g for 20 min.

5. Discard the funnel equipped with the filter.
6. Fix the slides in acetone for 10 min followed by chloroform for 5 min.
7. Store the slides at -20 °C.
   Note: Thaw and hydrate the samples before staining and perform the staining following the manufacturer's instructions. Prepare at least 3 technical replicates.

2. STS Cell Cultures:

1. Change media once a week (DMEM high culture media supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin).
   1. Discard the culture medium using a 200 - 1,000 µL pipette.
   2. Add new fresh medium using a 200 - 1,000 µL pipette.
   3. Cell passage:
      1. Detach cells when the confluence is around 90%:
         Note: STS cell culture amplification should be performed once a week on the basis of the culture conditions.
      2. Discard medium, wash with PBS and add 2-3 mL of trypsin 1x in PBS
         Note: The volume of trypsin used depends on the amount of cultured cells obtained.
      3. Incubate cells for 3 - 5 min at 37 °C.
      4. Collect detached cells from the plate using a 200 - 1,000 µL pipette.
      5. Pipette the detached cells into a 15-mL tube and add 4 mL of DMEM high culture media supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin to stop the enzymatic reaction.
      6. Centrifuge at 225 x g for 5 min at room temperature.
      7. Discard the supernatant.
      8. Re-suspend the cell pellet by adding 1 mL of DMEM high culture media supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin.
      9. Seed the cells in a new culture plate or a flask with a final volume according to the manufacturer's instructions.
         Note: Do not split cells more than 1:3.

3. Downstream Analyses:

Note: A variety of different experiments can be carried out using this model (see below). It is, therefore, crucial to decide during the design phase of the study which analyses will be performed so that an adequate number of samples and replicates can be prepared.

1. Proliferation assays such as MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
2. Apoptotic assays such as terminal deoxynucleotidyl transferase (TdT) nick end labeling (TUNEL) assay
3. Immunohistochemical analysis
4. Gene expression analysis
5. Western blot analysis
6. ELISA assay
7. Fluorescence-activated cell sorting (FACS) analysis
8. *In vivo* xenograft model

Representative Results

We designed a simple method to obtain the establishment of a primary culture of patient-derived soft tissue sarcoma and report here an example of results obtained on one specific STS histotype[13]. The protocol was used for the establishment of primary cultures of different STS histotypes including well-differentiated liposarcoma, dedifferentiated liposarcoma, myxoid liposarcoma, pleomorphic liposarcoma, myxofibrosarcoma, undifferentiated pleomorphic sarcoma, GIST, and desmoid fibromatosis.

The success rate for the establishment of primary cultures was 54% (13 primary cultures from 24 STS surgical samples) for surgical specimens derived from patients treated with chemotherapy, radiotherapy or not treated. Conversely, it was 72% (13 primary cultures from 18 STS surgical samples) for surgical samples derived only from untreated patients.

Tumor cells were isolated from a well-differentiated/dedifferentiated liposarcoma (ALT/DDLPS) surgically removed from a 54-year-old patient. MDM2 amplification analysis, currently performed for the standard differential diagnosis of ALT/DDLPS and reviewed by an experienced sarcoma pathologist, was positive, confirming the presence of an ALT/DDLPS lesion (**Figure 1A**). Cytological analysis of MDM2 amplification in isolated tumor cells confirmed the establishment of a patient-derived ALT/DDLPS primary culture (94.6% of cultured cells were positive for MDM2 amplification) (**Figure 1B**). The primary culture continued to proliferate after passages in standard monolayer cultures and cell viability was monitored by MTT and Tunel assay.

This model enabled us to test the sensitivity of the ALT/DDLPS primary culture to different drugs currently used or under clinical evaluation for the treatment of ALT/DDLPS such as ifosfamide (IFO), epirubicin (EPI), the combination IFO+EPI, trabectedin (TRABE), and eribulin (ERI), performed using the MTT assay. The results confirmed the sensitivity of the cultures to chemotherapy (**Figure 2A**). The most active treatment was IFO+EPI, a first-line therapy used in advanced or metastatic ALT/DDLPS. Furthermore, cell morphology was examined after drug exposure, results in ighlighting morphological changes in the primary culture with respect to control and the findings of the cytotoxic assay (**Figure 2B**). As mentioned in the protocol, several downstream analyses can be performed with this model. **Figure 3** provides a schematic representation of the method and includes a timeline within which the experiments should be performed to obtain translational results.

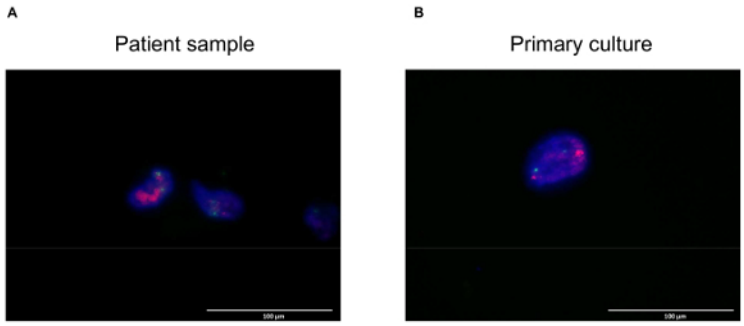

Figure 1. Establishment of patient-derived primary culture of ALT/DDLPS. A) *In situ* hybridization (FISH) performed to detect MDM2 amplification in a patient tissue specimen (100X image). **B)** *In situ* hybridization (FISH) performed to detect MDM2 amplification in a primary culture (100X magnification). Please click here to view a larger version of this figure.

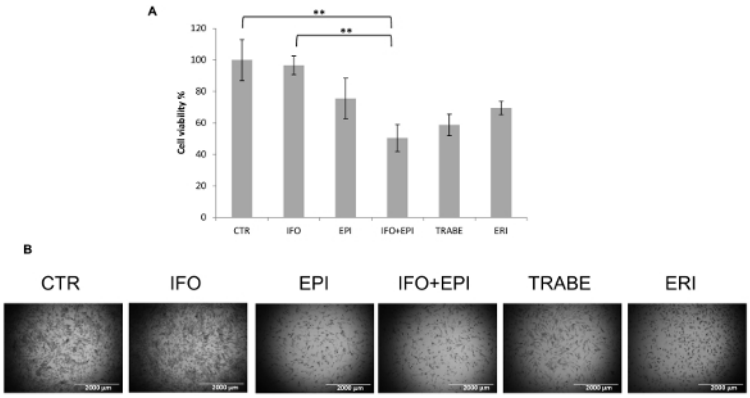

Figure 2. Chemotherapy in an ALT/DDLPS primary culture. A) The cells were treated with: ifosfamide (IFO), epirubicin (EPI), IFO+EPI, trabectedin (TRABE), and eribulin (ERI). Three independent replicates were performed for each experiment. Data are presented as mean ± standard error (SE), as stated, with n indicating the number of replicates. Differences between groups were assessed by a two-tailed Student's *t*-test, *p <0.05. **B)** Images of ALT/DDLPS primary culture after drug exposure (2X magnification). Please click here to view a larger version of this figure.

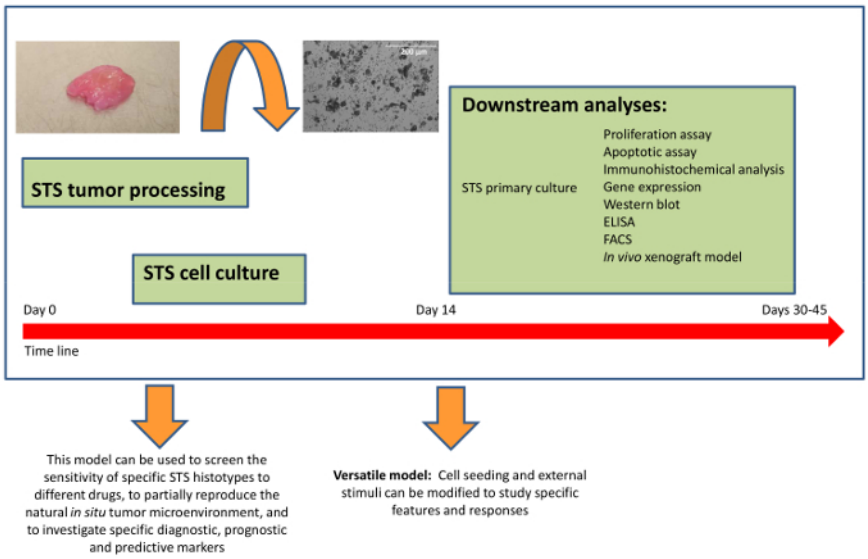

Figure 3: STS primary culture model. Schematic representation of the preclinical model of STS primary culture including the timeline and the possible downstream analyses. Please click here to view a larger version of this figure.

Discussion

Well defined preclinical models are needed to elucidate the molecular background of tumors, predict poor prognosis, and develop new therapeutic strategies for cancer patients. This is especially important for rare tumors such as STS whose high heterogeneity in terms of morphology, aggressive potential, and clinical behavior challenges our understanding of STS biology and patient management[21]. Moreover, the few commercial sarcoma cell lines available limits preclinical investigations into this group of mesenchymal tumors[18]. *Ex vivo* models thus represent a valuable research resource for STS. We developed a fully human *in vitro* model of STS starting from surgically resected tumor tissue (**Figure 3**). During the optimization of the method, several critical issues were addressed and resolved. The first problem concerned the time interval between surgical treatment and sample processing. Initially, when tumor specimens arrived at our biosciences laboratory after a certain time, they were stored overnight at 4 °C and processed the next day. The STS cells isolated from these samples exhibited low proliferation activity and poor viability in standard monolayer culture. This can, in part, be attributed to the fact that whole tumor specimen is kept for a prolonged period of time in the urine container before being processed. We thus modified the starting time of the sample processing to a maximum of 3 h after surgery to ensure that the primary culture continued to proliferate in standard monolayer culture and exhibit good viability. We also optimized the cell disaggregation process: small homogeneous tissue fragments are needed to facilitate the enzymatic digestion, and this was achieved using a scalpel. Moreover, although the protocol worked with specimens of different sizes, the establishment of a primary culture starting from specimens of limited size (<2 cm) is more difficult to achieve. The collagenase concentration was another important issue because too high anenzyme concentration can cause cellular stress, leading to phenotypic changes in the STS primary culture or a decrease in tumor cell survival. Good enzymatic digestion is required to separate cells from matrix material and debris. We finally optimized the enzyme concentration to use in the digestion phase. Moreover, to limit cellular stress and provide nutrients for STS cells during the isolation phase we re-suspended the enzyme in culture media instead of other reagents such as PBS.Finally, several experiments performed on different STS primary cultures such as liposarcoma and myxofibrosarcoma revealed changes in gene expression over time and increased sensitivity to chemotherapy[13,20]. These findings are attributable to the prolonged culture of patient-derived STS cells in a monolayer system together with an increasing number of passages, both of which induce phenotypic and genetic drift. The decreasing reliability of this culture system over time to recapitulate tumor tissue, as previously shown, represents one of the most important limitations of *ex vivo* models[22,23]. We thus optimized the protocol by reducing the time in which to perform experiments (within 30 - 45 days of tumor cell seeding) and by using low passage cultures to obtain reproducible and translational results. Moreover, the primary cultures can be frozen, but their subsequent viability is dependent on several factors including tumor location, STS histotype, amount of surgical specimen and number of culture passages performed.

Our system has a number of advantages, *i.e.* it is a fully human preclinical model, in contrast to other murine-based sarcoma cell line models[24,25,26,27]. Furthermore, whilst the confirmation of the establishment of STS primary cultures, achieved by cytomorphological, immunohistochemical and molecular cytogenetic evaluation, with the support of an experienced sarcoma pathologist, is mandatory in our protocol, this is not always the case for other STS primary culture models. In some papers, downstream analysis of cells recovered from surgical specimens was performed without confirming the diagnosis the STS cell percentage in the primary cultures[28]. Our system is also versatile, reproducible and can be used to perform a wide variety of experiments. Moreover, cell seeding, and external stimuli can be modified to study specific features and responses. The development of this model enabled us to investigate the sensitivity of patient-derived STS cells to different drugs currently used or under clinical evaluation for the treatment of STS. This model, therefore, represents an important preclinical tool for predicting response to therapy and, if further optimized, could potentially be used to improve personalized therapy. It could also be used to identify diagnostic or prognostic molecular markers, especially important in STS where few specific biomarkers are available. One of the limitations of the protocol is the relatively short time available to perform downstream analysis after the establishment of the primary culture. The protocol could be improved by performing gene expression profiling and clustering analysis for both tumor and primary cultures derived from the same patient over time to assess the capacity of the primary culture to mimic the heterogeneity of the tumor tissue, especially for those STS

histotypes for which a diagnostic marker is not available. Such analyses would also help to further characterize how the cells in the obtained culture differ from those derived directly from tumor samples.

In conclusion, the establishment of robust preclinical models is essential to advance our understanding of tumor pathology. We believe that our model will help to uncover the molecular mechanisms that are responsible for the poor prognosis of STS and will contribute to the identification of new therapeutic strategies.

Disclosures

The authors have no conflicts of interest to disclose.

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References

1. Siegel, R.L., Miller, K.D., Jemal, A. Cancer statistics. *CA Cancer J Clin.* **66** (1), 7-30 (2016).
2. Linch, M., Miah, A.B., Thway, K., Judson, IR., Benson, C. Systemic treatment of soft-tissue sarcoma gold standard and novel therapies. *Nat Rev Clin Oncol.* **11** (4), 187-202 (2014).
3. Fletcher, C.D. The evolving classification of soft tissue tumours - an update based on the new 2013 WHO classification. *Histopathology.* **64** (1), 2-11 (2014).
4. De Vita, A., et al. Current classification, treatment options, and new perspectives in the management of adipocytic sarcomas. *Onco Targets Ther.* **9** (12), 6233-6246 (2016).
5. Recine, F., et al. Update on the role of trabectedin in the treatment of intractable soft tissue sarcomas. *Onco Targets Ther.* **10**, 1155-1164 (2017).
6. Ducimetière, F., et al. Incidence of sarcoma histotypes and molecular subtypes in a prospective epidemiological study with central pathology review and molecular testing. *PLoS One.* **6** (8), e20294 (2011).
7. Nussbaum, D.P., et al. Preoperative or postoperative radiotherapy versus surgery alone for retroperitoneal sarcoma: a case-control, propensity score-matched analysis of a nationwide clinical oncology database. *Lancet Oncol.* **17** (7), 966-975 (2016).
8. The ESMO/European Sarcoma Network Working Group. Soft tissue and visceral sarcomas: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* **25** (Suppl 3), 102-112 (2014).
9. Paul, J. The Cancer Cell *in Vitro.*: A Review. *Cancer Research.* **22**, 431-440 (1962).
10. Morton, C.L., Houghton, P.J. Establishment of human tumor xenografts in immunodeficient mice.*Nat Protoc.* **2** (2), 247-50 (2007).
11. Centenera, M.M., Raj, G.V., Knudsen, K.E., Tilley, W.D., Butler, L.M. Ex vivo culture of human prostate tissue and drug development. *Nat Rev Urol.* **10** (8), 483-487 (2013).
12. Vannucci, L. Stroma as an Active Player in the Development of the Tumor Microenvironment. *Cancer Microenviron.* **8** (3), 159-166 (2015).
13. De Vita, A., *et al*. Activity of Eribulin in a Primary Culture of Well-Differentiated/Dedifferentiated Adipocytic Sarcoma. *Molecules*. **3**, 21 (12), E1662 (2016).
14. Failli, A., et al. The challenge of culturing human colorectal tumor cells: establishment of a cell culture model by the comparison of different methodological approaches. *Tumori.* **95** (3):343-347 (2009).
15. Steinstraesser, L., et al. Establishment of a primary human sarcoma model in athymic nude mice. *Hum. Cell.* **23**, 50-57 (2010).
16. Raouf, A., Sun, Y.J. In vitro methods to culture primary human breast epithelial cells. *Methods Mol. Biol.* **946**, 363-381 (2013).
17. Daigeler, A. Heterogeneous in vitro effects of doxorubicin on gene expression in primary human liposarcoma cultures. *BMC Cancer.* **29**, 313 (2008).
18. Pan, X., Yoshida, A., Kawai, A., Kondo, T. Current status of publicly available sarcoma cell lines for use in proteomic studies.*Expert Rev Proteomics.* **13** (2), 227-40 (2016).
19. Liverani, C., et al. Innovative approaches to establish and characterize primary cultures: an *ex vivo.* 3D system and the zebrafish model. *Biol Open.* **15**, 6(2), 309 (2017).
20. De Vita, A., et al. Myxofibrosarcoma primary cultures: molecular and pharmacological profile. *Ther Adv Med Oncol.* **9**, (12) 755-767 (2017).
21. Blay, J.Y., Ray-Coquard, I. Sarcoma in 2016: Evolving biological understanding and treatment of sarcomas. *Nat Rev Clin Oncol.* **14** (2), 78-80 (2017).
22. Dairkee, S.H., Deng, G., Stampfer, M.R., Waldman, F.M., Smith, H.S. Selective cell culture of primary breast carcinoma. *Cancer Res.* **55** (12), 2516-9 (1995).
23. Kar, R., Sharma, C., Sen, S., Jain, S.K., Gupta, S.D., Singh, N. Response of primary culture of human ovarian cancer cells to chemotherapy: In vitro individualized therapy. *J Cancer Res Ther.* **12** (2):1050-5 (2016).
24. Ravi, A., et al. Proliferation and Tumorigenesis of a Murine Sarcoma Cell Line in the Absence of DICER1. *Cancer Cell.* **21** (6), 848-855 (2012).
25. Zhong, X.Y., et al. Effect of vegf gene knockdown on growth of the murine sarcoma cell line MS-K. *Genes Cells.* **16** (6), 625-38 (2011).
26. Kidani, T., Yasuda, R., Miyawaki, J., Oshima, Y., Miura, H., Masuno, H. Bisphenol A Inhibits Cell Proliferation and Reduces the Motile Potential of Murine LM8 Osteosarcoma Cells. *Anticancer Res.* **37** (4), 1711-1722 (2017).
27. Sugiyasu, K., et al. Radio-sensitization of the murine osteosarcoma cell line LM8 with parthenolide, a naturalinhibitor of NF-κB. *Oncol Lett.* **2** (3):407-412 (2011).
28. Daigeler, A., et al. Heterogeneous in vitro effects of doxorubicin on gene expression in primary human liposarcoma cultures. *BMC Cancer.* **29** (8), 313 (2008).

