

Video Article

In Vivo Model for Testing Effect of Hypoxia on Tumor Metastasis

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Abstract

Hypoxia has been implicated in the metastasis of Ewing sarcoma (ES) by clinical observations and *in vitro* data, yet direct evidence for its pro-metastatic effect is lacking and the exact mechanisms of its action are unclear. Here, we report an animal model that allows for direct testing of the effects of tumor hypoxia on ES dissemination and investigation into the underlying pathways involved. This approach combines two well-established experimental strategies, orthotopic xenografting of ES cells and femoral artery ligation (FAL), which induces hindlimb ischemia. Human ES cells were injected into the gastrocnemius muscles of SCID/beige mice and the primary tumors were allowed to grow to a size of 250 mm³. At this stage either the tumors were excised (control group) or the animals were subjected to FAL to create tumor hypoxia, followed by tumor excision 3 days later. The efficiency of FAL was confirmed by a significant increase in binding of hypoxyprom-1 in the tumor tissue, severe tumor necrosis and complete inhibition of primary tumor growth. Importantly, despite these direct effects of ischemia, an enhanced dissemination of tumor cells from the hypoxic tumors was observed. This experimental strategy enables comparative analysis of the metastatic properties of primary tumors of the same size, yet significantly different levels of hypoxia. It also provides a new platform to further assess the mechanistic basis for the hypoxia-induced alterations that occur during metastatic tumor progression *in vivo*. In addition, while this model was established using ES cells, we anticipate that this experimental strategy can be used to test the effect of hypoxia in other sarcomas, as well as tumors orthotopically implanted in sites with a well-defined blood supply route.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54532/>

Introduction

Ewing sarcoma (ES) is an aggressive malignancy affecting children and adolescents.¹ The tumors develop in soft tissues and bones, commonly in limbs. While the presence of metastases is the single most powerful adverse prognostic factor for ES patients, the mechanisms underlying their development remain unclear.² Tumor hypoxia is one of the few factors implicated in ES progression. In ES patients, the presence of non-perfused areas within the tumor tissue is associated with poor prognosis.³ *In vitro*, hypoxia increases invasiveness of ES cells and triggers expression of pro-metastatic genes.⁴⁻⁶ However, despite these lines of evidence, no direct proof for hypoxia-induced ES progression and spread exists. Moreover, the mechanisms by which hypoxia exerts such effects are, at present, unknown. Hence, we have created an *in vivo* model to fill the gap between existing *in vitro* data and clinical observations. This model system enables direct testing of the effects of hypoxia on tumors occurring in their natural environment, using magnetic resonance imaging (MRI) to follow tumor progression and metastasis *in vivo* in combination with *ex vivo* pathological and molecular analyses (Figure 1).

Since no established transgenic model of ES is currently available, the *in vivo* studies on metastatic properties of these tumors rely on injections of human cells into immunocompromised mice. While the use of immunologically impaired animals may underestimate the impact of the immune system on the disease progression, the ability to use human cells increases translatability of such studies. Among different xenograft models, systemic injections into tail vein are the easiest to perform, yet they omit the initial steps of tumor cell intravasation and escape from the primary site of growth.⁷⁻¹² On the other hand, orthotopic xenografting, which involves injections of tumor cells into bones (femur, rib) or muscles, is more technically challenging, but also more biologically relevant to human cancer.¹³⁻¹⁶ However, in the past, the high morbidity associated with rapid

growth of primary tumors has often necessitated animal euthanasia before metastasis development. In this study, we employed a previously established model of cell injections into the gastrocnemius muscle followed by excision of the resulting primary tumor combined with longitudinal monitoring of metastatic progression by MRI.^{17,18} Such injections into gastrocnemius muscle in close proximity to the tibia allow for tumor growth in two natural ES environments — muscles and bones — and result in distant metastases to locations typically affected in humans.¹⁸ Thereby, this model accurately recapitulates the metastatic processes occurring in ES patients during disease progression.

The localization of primary tumors in the lower hindlimb also facilitates the precise control of the blood supply to the tumor tissue. Femoral artery ligation (FAL) is a well-established technique utilized in angiogenesis research to block blood flow to distal regions of the leg and investigate tissue vascularization in response to ischemia.^{19,20} Importantly, the initial drop in blood flow is followed by collateral vessel opening and tissue reperfusion observed approximately 3 days after FAL.²⁰ Thus, when performed in a tumor-bearing limb, this model recreates hypoxia/reperfusion events that occur naturally in rapidly growing tumors and enables the escape of metastatic tumor cells due to restoration of perfusion to the lower hindlimb via newly opened collateral vessels.²¹ Importantly, this procedure must be performed when the tumor size is small enough to prevent excessive hypoxia in control tumors (typically at the tumor-bearing calf volume of 150 - 250 mm³), ensuring significant differences in levels of tumor hypoxia between control and FAL-treated groups.

In addition to longitudinal monitoring of the effect of hypoxia on ES latency and the frequency of metastases, this model also allows for the collection of tissues and the development of new cell lines from both primary tumors and metastases. Importantly, previous work established that metastases-derived cell lines exhibit enhanced metastatic potential upon reintroduction to animals, indicating that tumor dissemination is associated with permanent changes in the tumor cell phenotype, and thereby validating the use of these cell lines to decipher the metastatic processes.¹⁸ Collectively, these models can now be used for the genetic and molecular analyses required for identifying hypoxia-induced metastatic pathways.

As hypoxia is a pro-metastatic factor enhancing the malignancy of various tumors, our model can be used as a platform to investigate the role of hypoxia in other tumor types that naturally develop in limbs, such as osteosarcoma and rhabdomyosarcoma.²¹⁻²³ Moreover, a similar approach can be applied to malignancies growing in other anatomical locations with a well-defined route of blood supply. Ultimately, the model can be modified and its utility further extended, depending on individual research needs.

Protocol

All procedures were approved by the Georgetown University Institutional Animal Care and Use Committee.

1. Cell Preparation for Orthotopic Injections

1. Culture human ES cells under standard conditions. Use approximately one 15-cm cell culture plate not exceeding 70% of confluency for injection of 5 mice.
NOTE: For this study, SK-ES1 cells were cultured in McCoy's 5A medium with 15% fetal bovine serum (FBS) on collagen-coated plates and TC71 cells were cultured in RPMI with 10% FBS and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Both media were supplemented with antibiotics — penicillin (100 units/ml), streptomycin (100 µg/ml) and fungizone (1 µg/ml).
2. Wash the ES cells with phosphate buffered saline (PBS) and trypsinize at 70% confluence with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) for 5 min.
3. Remove the ES cells from the plate with cell culture media, then centrifuge for 5 min at 200 x g at room temperature. Re-suspend the ES cells in 10 ml of cold PBS and then count the number of cells.
4. Centrifuge ES cells for 5 min at 200 x g at room temperature, and then re-suspend at 2×10^7 (SK-ES1) or 10^7 (TC71) cells per ml in cold PBS. Keep the final cell suspension on ice while performing injections.

2. Orthotopic Injection of ES cells into Gastrocnemius Muscle

1. Use 4-6 week old female SCID/beige mice.
2. To inject ES cells, gently hold the mouse and stabilize its left leg between the fourth and fifth fingers, exposing the medial side of the lower hindlimb.
3. Using a 28 G $\frac{1}{2}$ needle, inject 0.1 ml of the previously prepared cell suspension that contains either 2×10^6 (SK-ES1) or 10^6 (TC71) ES cells into the gastrocnemius muscle (**Figure 2A**).
NOTE: Although maximum volume for intramuscular injections is typically 0.05 ml, for this particular procedure the volume increase to 0.1 ml is necessary due to the high cell number injected. This procedure has been approved by the Georgetown University Institutional Animal Care and Use Committee.
 1. Insert the needle into the gastrocnemius muscle anteriorly at approximately a 30 - 45 degree angle in the direction of the tibial crest/tuberosity.
 2. Slightly withdraw the needle once it touches the tibial crest/tuberosity. Slowly inject the cell suspension solution, gradually withdrawing the needle to release pressure.
4. Monitor the injected mice over the next 24 hr for signs of distress.
NOTE: Investigators with less experience in animal handling should consider anesthetizing mice for tumor cell injections. Some institutions may require anesthesia for safety reasons.

3. Monitoring Primary Tumor Growth

1. Monitor the growth of primary tumors daily until the tumor size reaches the desired volume.

NOTE: In the current study, a calf volume of 250 mm³ was used as a starting point of the experiment (**Figure 2B**). Typically, it takes approximately 1 - 2 weeks for the tumors to reach this size.

1. Measure the calf size daily with digital calipers via its medial-lateral and anterior-posterior lengths.
2. Determine the calf volume by the formula $(D \times d^2/6) \times 3.14$, where D is the longer diameter and d is the shorter diameter of the tumor-bearing lower hindlimb.

NOTE: The size of the normal adult mouse calf is of approximately 40 - 50 mm³. Its volume will increase due to tumor growth and at the later stages the calf will be mainly comprised of tumor tissue.

4. Femoral Artery Ligation (FAL) for Inducing Hypoxia in the Tumor-bearing Hindlimb

1. Prepare the surgical tools needed for this operation: curved or pointed fine forceps, pointed forceps, surgical scissors and a needle holder. Sterilize these tools prior to surgery using an autoclave or a hot-bead sterilizer. Additionally, have fine cotton swabs ready for this surgery. NOTE: It is recommended that the tools be re-sterilized at the tips as needed during the procedure.
2. Inject analgesic agent (Carprofen 5 mg/kg) subcutaneously (SQ). To detect and confirm hypoxia, inject hypoxyprobe-1 (pimonidazole, 60 mg/kg). NOTE: This dose equates to 1.5 mg per mouse and is achieved by injecting 0.1 ml of 15-mg/ml hypoxyprobe solution in PBS intraperitoneally (IP). The hypoxyprobe is then detectable postmortem in animal tissues by immunohistochemistry.
3. Place the mouse in an anesthesia induction chamber containing 3 - 5% isoflurane in 100% oxygen at a flow rate of 1 L/min.
4. Leave the mouse in the induction chamber until it is unresponsive to external stimuli. Then remove the animal from the induction chamber. Place the animal in the supine position on a sterile drape placed atop a warming pad on the operating surface. Use a nose cone to connect it to a continuous flow of 1 - 3% isoflurane in 100% oxygen at a flow rate of 0.8 L/min.
 1. Apply sterile non-medicated ophthalmic ointment to each eye to prevent corneal drying. To thoroughly depilate the surgical area, apply hair removal cream, leaving it on the skin for no more than 10 sec. Then wipe off the hair removal cream using an ethanol prep pad.
5. Extend and secure the hindlimb with a piece of tape approximately 45 degrees from the midline of the mouse. Once the hindlimb is secure, wipe the exposed skin with 10% povidone/iodine swab/solution, followed by ethanol, repeating two more times each. For the remainder of the surgical procedure, use a stereo microscope to obtain an enlarged view of the hindlimb region.
6. Using pointed forceps and surgical scissors, make an incision of the skin, approximately 1 cm long, from mid-thigh towards the inguinal region. Using saline-moistened fine cotton swabs, gently brush away subcutaneous fat tissue surrounding the thigh muscle.
7. Carefully reveal the underlying femoral artery via blunt dissection through the subcutaneous fat tissue. Stabilize the wound and surgical field to expose the vasculature of the mid-upper adductor muscle.
8. Using fine forceps, gently pierce through the membranous femoral sheath to expose the neurovascular bundle. Using a clean set of fine forceps, dissect and separate the femoral artery from the femoral vein and nerve at the proximal location near the groin, distal to the inguinal ligament. Use caution to avoid piercing the femoral vein wall.
9. Following dissection, pass a strand of 6-0 silk suture underneath the femoral artery and distal to the branch of the lateral circumflex femoral artery (LCFA). Occlude the femoral artery using double knots (**Figure 3**).
10. Close the incision using 6-0 polypropylene sutures. After closing the incision, inject SQ 0.5 ml of warm saline for fluid balance therapy. Place the animal on top of a draped warm pad in the recovery cage and monitor continuously until awake.
11. Monitor the animals during first 6 hr after surgery and inject the analgesic agent (Carprofen 5 mg/kg, SQ) every day for 3 days. Remove sutures 10 days post-surgery using sterile scissors.

5. Primary Tumor Excision by Leg Amputation

NOTE: Amputate the tumor-bearing lower hindlimb when the calf size reaches 250 mm³ for the control group or 3 days after FAL for the hypoxic group.

1. Shave hair from the tumor-bearing limb from the distal tibia to the pelvic region with hair clippers while gently holding the animal. Inject the analgesic agent (Carprofen 5 mg/kg, SQ) before the procedure.
2. Place the mouse into an anesthesia induction chamber containing 3 - 5% isoflurane in 100% oxygen at a flow rate of 1 L/min. Leave the mouse in the induction chamber until it is unresponsive to external stimuli. Then remove the animal from the induction chamber.
3. Place the animal in the right lateral recumbent position on a sterile drape placed on a warming pad on the operating surface. Use a nose cone to connect it to a continuous flow of isoflurane 1 - 3% in 100% oxygen at a flow rate of 0.8 L/min. Apply sterile non-medicated ophthalmic ointment to each eye to prevent corneal drying.
4. Prepare the surgical site using 10% povidone/iodine swab/solution, followed by ethanol, repeating 3 times. Apply a sterile gauze (e.g., surgical drape) over the mouse to obtain a sterile surgical field.
5. Make a middle femoral circumferential skin incision, followed by blunt dissection and retraction of the skin proximally. Expose the medial femoral neurovascular pedicle on the median side of the leg, and then ligate near the inguinal ligament using 4-0 coated (polyglactin 910) absorbable suture material.
6. Perform a mid-femoral transection of muscle groups with scissors, followed by blunt dissection of soft tissue to the coxofemoral joint. Using a bone cutter, perform a mid-femoral osteotomy. Using a sterile fine cotton swab or an absorbable gelatin sponge, gently press the osteotomy site to minimize and prevent bleeding.
7. Close the overlying skin using surgical wound clips and inject 0.5 ml of warm saline SQ for fluid balance therapy. Place the animal on top of a draped warm pad in the recovery cage and monitor continuously until awake.
8. Upon surgery, collect tissue samples from primary tumors for RNA, DNA or protein isolation, snap freeze in liquid nitrogen and store at -80 °C. For primary cell culture, collect tissue samples at this step, as described in section 9 below. Fix the remaining limb tissue in 10% neutral-buffered formalin for histology and immunochemistry, including hypoxyprobe-1 detection.

9. Monitor the animals for locomotion, pain and food consumption during the first 6 hours after surgery, then every day for 3 days. Inject the analgesic agent (Carprofen 5 mg/kg, SQ) daily for 3 days. Remove the wound clips 10 days after amputation using a wound clip remover.

6. Monitoring Mice for the Presence of Metastases

1. Observe the mice daily and evaluate them for clinical signs of metastasis at least twice a week.
 1. Observe the animals for the presence of macrometastases presenting as masses that develop in various locations, typically shoulders, contralateral legs and jaws. To this end, carefully palpate head, neck and axillary regions, and contralateral hindlimb. Check for internal organ metastases via abdominal distension along with MRI scanning.
 2. Check for the presence of lung metastases by pressing the xyphoid process (the lower end of the sternum) with index finger.²⁴
NOTE: This pressure diminishes the diaphragmatic respiration capacity. Mice with advanced lung metastases show signs of respiratory distress manifested by laborious breathing.
 3. Observe the animals for neurological symptoms, such as leg paralysis and ataxia suggesting metastases to the central nervous system.
 4. Monitor the mice at least once per week for weight loss, as an indication of potential disease progression. Body weight loss exceeding 15% of the pre-procedural weight is considered a humane endpoint.

7. Magnetic Resonance Imaging (MRI) for Detecting Metastases

1. Perform MRI to detect metastases at desired time points.¹⁸
NOTE: In the current study, a 7-Tesla horizontal spectrometer was used. MRI was performed at days 15 and 35 post-amputation for SK-ES1 cells and at day 15 for TC71 cells.
2. Place the mouse into an anesthesia induction chamber containing 1 - 3% isoflurane in a gas mixture of 30% oxygen and 70% nitrous oxide.
3. Leave the mouse in the induction chamber until it is unresponsive to external stimuli. Then remove the animal from the induction chamber.
4. Transfer the anesthetized mouse onto a stereotaxic holder with respiration and temperature monitorization with continuous administration of 1.5% isoflurane and 30% nitrous oxide. Apply sterile non-medicated ophthalmic ointment to each eye to prevent corneal dryness. Image the animal either in a 40 or 23 mm Bruker mouse volume coil for whole body or brain imaging, respectively.
5. Use a two-dimensional, T2-weighted RARE sequence: TR = 3,000 msec, TE = 24 msec, matrix = 256, FOV = 4.35 x 3.0 cm, slice thickness = 0.5 mm, RARE factor = 4 and averages = 4.¹⁸
6. Place the animal in a warm recovery cage and monitor continuously until awake.
NOTE: The mice will recover rapidly since a shallow plane of anesthesia is used.
7. Monitor the animals during the first 6 hr after imaging to make sure that there are no adverse effects of the anesthesia.

8. Euthanasia and Necropsy

1. Euthanize the mice once the animals present with metastases detectable by MRI and/or clinical symptoms of disease progression.
NOTE: In the current study, the mice were sacrificed at days 50 and 25 post-amputation for animals bearing SK-ES1 and TC71 xenografts, respectively. In some cases, earlier euthanasia was necessary due to a high metastasis burden.
2. Euthanize the mice by exposure to CO₂ at 1.5 L/min (CO₂ at 10 - 30% of the euthanasia chamber vol/min). To ensure animal death, perform cervical dislocation after CO₂ exposure.
3. Spray the entire mouse with 70% ethanol and place it into the laminar flow hood. Collect the blood by heart puncture using a 25 G ½ needle with a 1 ml syringe and transfer to a blood collection tube containing 2 mg of EDTA.
4. Collect the following tissues: spleen, adrenal glands, ovaries, kidneys, liver, lungs, brain, right leg, bone marrow from both humeri and spine, as well as macroscopic metastases present in other locations.^{25,26}
5. Fix half of each tissue in 10% neutral-buffered formalin for histology and immunochemistry, including hypoxyprobe-1 detection. Snap freeze the other half in liquid nitrogen, then store at -80 °C for RNA, DNA or protein isolation.^{25,26} For primary cell culture, collect tissue samples as described in section 9 below.

9. Primary Cell Culture

1. To perform primary cell culture, dissect tissues from the amputated limb (section 5 above) or during the necropsy (section 8 above) under sterile conditions in a laminar flow hood.
2. Prepare cell culture media appropriate for the cell line used for orthotopic injections, supplemented with penicillin (200 units/ml), streptomycin (200 µg/ml), fungizone (1 µg/ml), and 0.2% mycoplasma prophylactic antibiotic. Place 2.5 ml of the primary culture medium into a 6-cm cell culture plate.
3. Select viable tumor tissue areas from primary tumors or metastases, and then isolate two to three segments at 2-3 mm each using sterile scissors.
NOTE: Viable tumor tissue is usually found on the edges of the tumor and can be discriminated from necrosis by its pinkish or reddish color, significant luster and overall wet appearance. In contrast, necrotic tissue is commonly seen in the center of the tumor and presents as a whitish/cream color mass with a dull, cheesy appearance.²⁷
4. Transfer the isolated segments to a 6-cm cell culture plate containing primary culture medium described in step 9.2.
5. Culture cells under standard conditions, as described in section 1 (NOTE) and 9.2.^{18,28} Check the culture for cellular outgrowth arising from the tissue pieces, which should be observed within a few days. Once cells reach confluence, trypsinize and propagate them according to standard cell culture techniques.
NOTE: The primary cell culture can be subsequently used to evaluate growth and metastatic properties of the cells derived from control and hypoxic tumors, as well as their molecular features, as previously described.¹⁸

Representative Results

Following injection of ES cells into gastrocnemius muscle, the primary tumors are allowed to grow to a calf size of 250 mm³ (**Figure 1, 2**). The time necessary for the tumors to reach this volume typically ranges from 10 - 15 days for TC71 to 20-25 days for SK-ES1 xenografts, respectively. Tumors at a calf volume of 250 mm³ exhibit a relatively low level of endogenous hypoxia (approximately 3% of tumor tissue), based on hypoxyprobe-1 (pimonidazole) staining (**Figure 4A, C**). Importantly, in these control tumors, the positive staining for hypoxyprobe-1 was observed solely in the areas of physiological hypoxia, *i.e.*, in tumor cells that are distant from the vasculature and start to undergo cell death (**Figure 4A**). Such staining exhibits a characteristic "air-brush" pattern, while the bulk of healthy tumor tissue remains negative for hypoxyprobe-1.⁶ In contrast, FAL performed at this stage creates profound hypoxia, as evidenced by the positive staining for hypoxyprobe-1 observed in the vast majority of the tumor cells at 4 hr post-FAL (**Figure 4B**). Notably, the hypoxyprobe-1-positive tumor cells are also observed in close proximity to blood vessels and the characteristic pattern of physiological hypoxia is lost. In these FAL-treated tumors, 73% of tissue consists of hypoxic tumor cells (**Figure 4C**). The tumor tissue also exhibits the first signs of hypoxia-induced damage, including cell shrinkage and loose structure (**Figure 4B**).

The effectiveness of FAL was further supported by the complete inhibition of primary tumor growth. During the 3-day period between FAL and amputation, the size of primary tumors did not increase (**Figure 5A**). In contrast, primary tumors from mice subjected to sham surgery continued to grow rapidly, reaching a volume of approximately 650 mm³, thus mimicking the growth rate of tumors in control mice not subjected to surgery (**Figure 5A**).¹⁸ In line with these data, histopathological analysis revealed extensive areas of necrosis in FAL-treated primary tumors harvested 3 days post-surgery (**Figure 5B**). Nevertheless, there were groups of viable tumor cells present within the tumor tissue, usually located at the edges of the tumor, close to the vasculature (**Figure 5B**). To investigate the oxygenation status of these cells we used hypoxypromes, which are activated in live hypoxic cells, subsequently gaining the ability to covalently bind to proteins within these cells.²⁹ As such, these probes serve as a permanent marker of the cells that experienced hypoxia at any point in time. Thus, to detect changes in the oxygen levels of the tumor cells throughout the duration of the experiment, we used two different hypoxypromes that can be detected independently by immunohistochemistry. The probes were administered at two time points — immediately before FAL (hypoxyprobe-1) and prior to amputation (hypoxyprobe-2, CCI-103F) — and their localization was detected in tumors harvested 3 days post-FAL (**Figure 1**). Hypoxyprobe-1, which was present in the system at the time of FAL, marked a majority of the tumor cells, as the tissue became severely hypoxic (**Figure 5C**). This labelling is also evident in areas of necrosis/apoptosis, since these cells were still alive at the time of FAL and therefore capable of permanently binding hypoxyprobe-1, as shown in **Figure 4B**. In contrast, hypoxyprobe-2 administered 2 days post-FAL only stained viable cells, as the cells in necrotic areas were already dead and unable to activate the probe at the time of its administration (**Figure 5D**). Interestingly, we also observed viable tumor tissue adjacent to the vasculature that was initially hypoxic (hypoxyprobe-1-positive), but adequately oxygenated at the later time point (hypoxyprobe-2-negative) (**Figure 5C, D**). This finding is in agreement with previous reports indicating that collateral vessel-dependent tissue reperfusion restores blood flow in the hindlimb 3 days post-FAL.²⁰ The viable tumor cells remaining in the tumor tissue 3 days post-FAL were highly invasive, as evidenced by their massive intravasation on vascularized edges of the tumor (**Figure 5E**). Some of the cells within the vessel lumen were positive for hypoxyprobe-1, indicating that they had become hypoxic upon FAL, yet remained viable. Collectively, these data suggest that tissue reperfusion following severe hypoxia may facilitate the escape of cells from the primary tumors.

Based on the pilot experiment, widespread metastases were detectable by MRI approximately 35 days post-amputation for SK-ES1 xenografts, while TC71 cells typically metastasized within 15 days. Consequently, for comparative analysis of metastasis latency and frequency, MRI was performed at day 15 and 35 post-amputation for the animals bearing SK-ES1 tumors, while one imaging at day 15 was sufficient for mice with TC71 tumors. Euthanasia was performed at day 50 and day 25 for the animals with SK-ES1 and TC71 xenografts, respectively. As previously reported, the most common types of metastases included soft tissue masses in the shoulder area, as well as bone metastases to contralateral legs, maxillary area and spine for SK-ES1 cells, and lung and pelvic tumors for TC71.¹⁸ In addition, frequent metastases to internal organs, including adrenal glands, lungs and liver, as well as bone marrow infiltration, were observed in mice bearing FAL-treated SK-ES1 xenografts (**Figure 6**). In general, hypoxia significantly increased the overall (SK-ES1) or site-specific (TC71) frequency and multiplicity of metastases in both cell lines. However, TC71 xenografts also developed frequent recurrent masses at the site of amputation (25% for control and 40% for FAL-treated tumors), as previously described for the large TC71 primary tumors.¹⁸

The tissues from control and FAL-treated primary tumors and metastases can also be a source of material for comprehensive molecular analyses aimed at the identification of hypoxia-activated pathways involved in ES dissemination. For example, we observed significant differences in metabolomic profiles of control and hypoxic primary tumors (data not shown). We were also able to successfully isolate multiple cell lines from all of the above tissues. In many cases, the cells derived from FAL-treated tumors exhibited noticeable changes in morphology, as compared to the original cells and those derived from control tumors (**Figure 7A**). The purity of these cell cultures was confirmed by positive immunostaining for the Ewing sarcoma marker, CD99 (**Figure 7B**). Moreover, 100% of cells in the established cell lines had a positive signal in fluorescence *in situ* hybridization (FISH) with human centromeric probes, often presenting with increased chromosome numbers (**Figure 8A**). The human origin of these cells was further confirmed by analysis of the metaphase chromosomes (**Figure 8B**) that exhibited cytogenetic rearrangements previously described for SK-ES1 cells in both the original cell line and cells derived from FAL-treated tumors (**Figure 8B**).³⁰

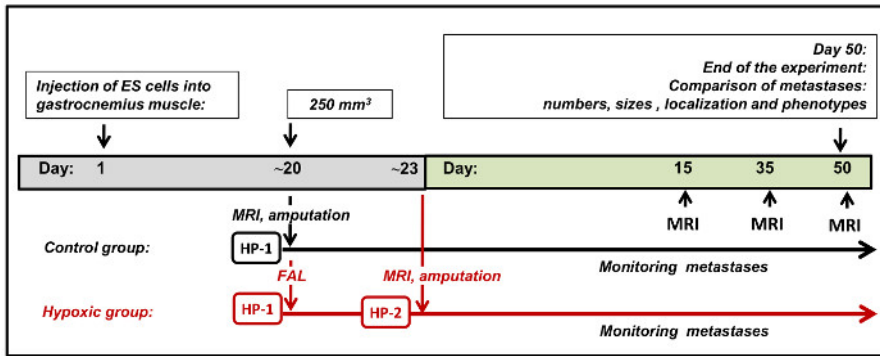


Figure 1. Outline of the ES Hypoxia Model. ES cells ($1 - 2 \times 10^6$) were injected into gastrocnemius muscles of SCID/beige mice. Once the resulting primary tumors reached a calf volume of 250 mm³, the mice were divided into two experimental groups. Mice from the control group were injected with hypoxyprobe-1 (HP-1), and the primary tumors were excised by limb amputation. Mice from the hypoxic group were injected with hypoxyprobe-1 and then subjected to femoral artery ligation (FAL). Two days later the mice were injected with hypoxyprobe-2 (HP-2), and then the tumor-bearing limbs were amputated three days post-FAL. Animals from both groups were monitored by periodic magnetic resonance imaging (MRI). Once metastases became apparent based on imaging and clinical signs, the mice were euthanized and the presence of metastases was confirmed by necropsy and histopathological analyses. The time of tumor growth, imaging and euthanasia is based on the SK-ES1 cell rate of growth and metastasis. Note that while hypoxyprobe-2 was necessary to establish the method and visualize changes in hypoxia levels during the period between FAL and limb amputation, its use is not essential for the actual experiments. Therefore, this step is not included in the protocol. [Please click here to view a larger version of this figure.](#)

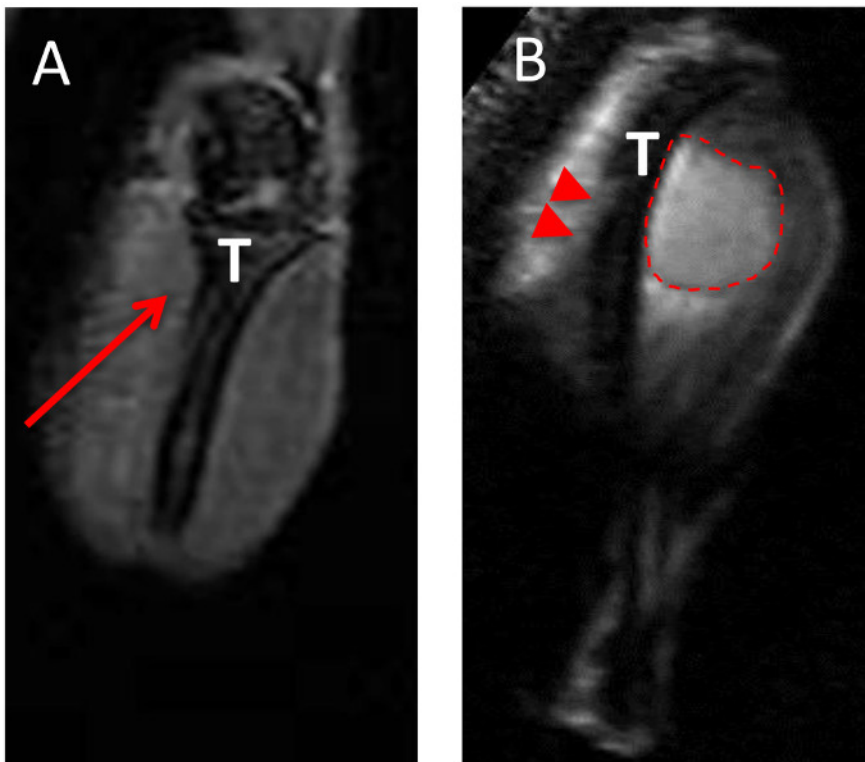


Figure 2. Primary Tumor Growth. **A.** Magnetic resonance image (MRI) of an intact hindlimb. The red arrow indicates the site and direction of the tumor cell orthotopic injection. **B.** Primary tumor at a calf volume of approximately 250 mm³ growing in the gastrocnemius muscle (red outline). Red arrowheads indicate sites of bone destruction. T - tibia. [Please click here to view a larger version of this figure.](#)

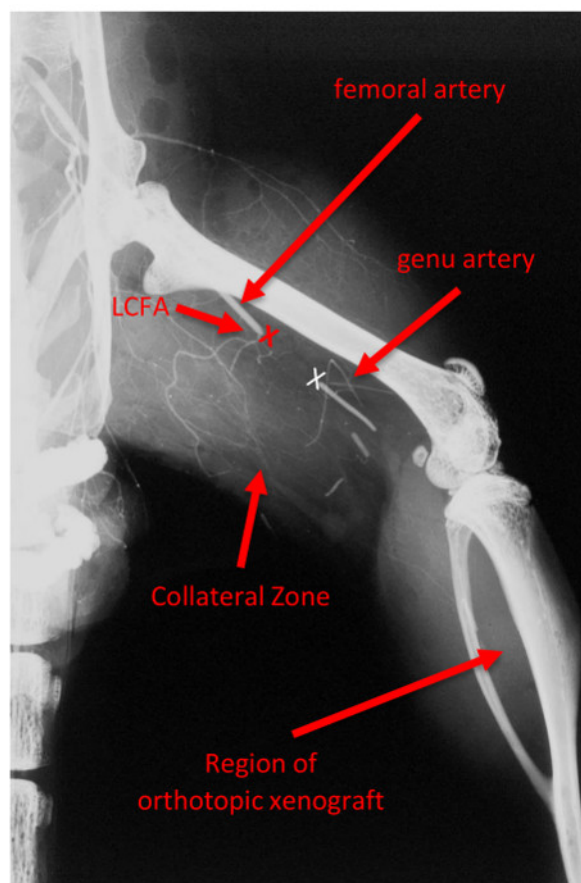


Figure 3. The Site of Femoral Artery Ligation. A representative postmortem x-ray arteriogram of a 129S1/SvJ mouse perfused with barium sulfate immediately after ligation is shown to highlight the presence of pre-existing collateral vessels. Note that while the image depicts two ligations (Xs), one distal to the lateral circumflex femoral artery (LCFA) (red X) and another proximal to the genu artery (white X), the tumor hypoxia model only used the proximal ligation, near the inguinal ligament and distal to the LCFA (red X). The region of collateral vessel development is shown as described (faint vessels), and they are identified by their tortuous, cork-screw shape. [Please click here to view a larger version of this figure.](#)

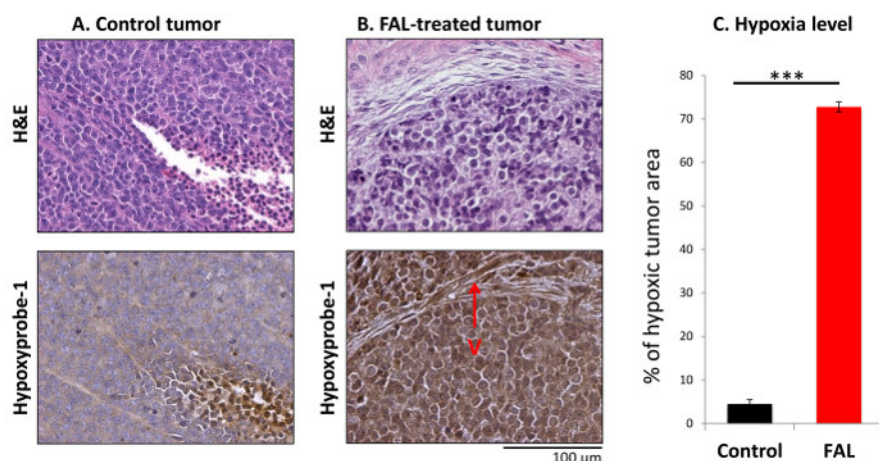


Figure 4. Tumor Hypoxia Induced by Femoral Artery Ligation (FAL). **A.** A control tumor at a calf volume of 250 mm³ stained by hematoxylin & eosin (H&E) and immunostained for hypoxyprobe-1 (brown). Hypoxyprobe-1 immunostaining is solely observed in the areas of physiological tumor hypoxia, where the tumor cells distant from vasculature are deprived of oxygen and start to undergo cell death. **B.** FAL-treated primary tumor at a similar size harvested 4 hr post-ligation and stained with H&E and immunostained for hypoxyprobe-1. The majority of tumor cells are highly positive for hypoxyprobe-1, including those in close proximity to the vasculature. V - blood vessel. **C.** The area of positive staining for hypoxyprobe-1 was quantified in control and FAL-treated tumors using ImageJ software. Error bars represent SEM. *** - p < 0.001 [Please click here to view a larger version of this figure.](#)

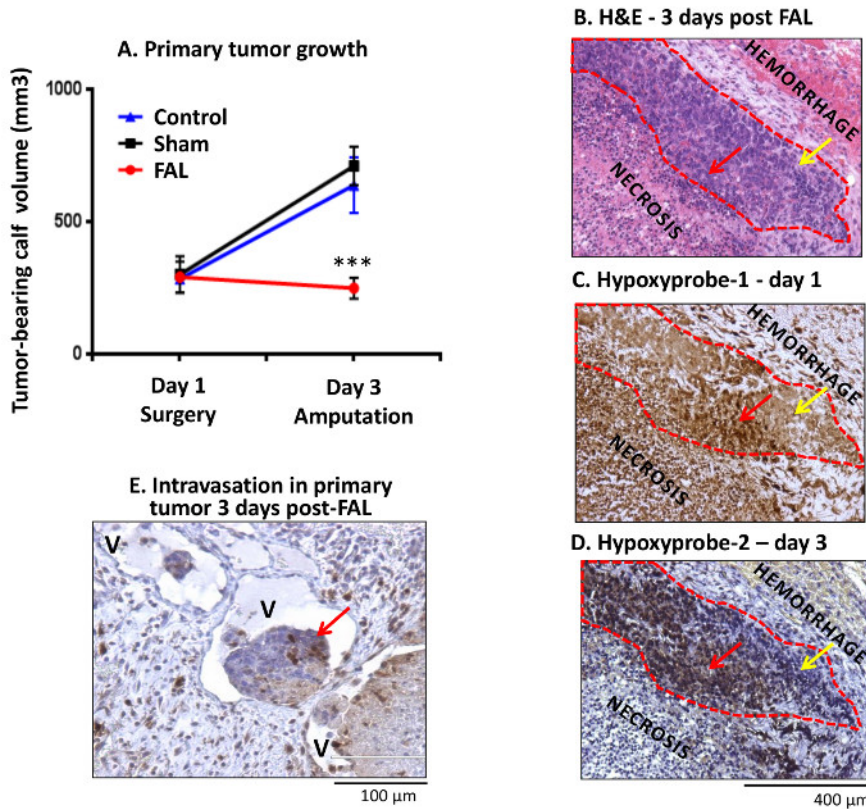


Figure 5. Effect of Femoral Artery Ligation (FAL) on Primary Tumor Growth. **A.** Primary tumor growth during the 3-day period between surgery and amputation in FAL- ($n = 4$) and sham surgery-treated ($n = 4$) animals, as compared to intact control tumors ($n = 6$). Error bars represent SD. *** - $p < 0.001$, as compared to both control and sham surgery-treated tumors. **B.** Hematoxylin and eosin (H&E) staining of a FAL-treated tumor harvested 3 days post-ligation reveals widespread necrosis within tumor. The red outline indicates an area of viable tumor cells in close proximity to a highly vascularized region perfused 3 days post-FAL. **C.** FAL-treated tumor tissue harvested 3 days post-surgery immunostained for hypoxypore-1. Hypoxypore-1 was injected before FAL. Therefore, the positive immunostaining indicates the existence of extensive tissue hypoxia immediately post-surgery. **D.** Hypoxypore-2 was injected 2 days post-FAL, and the tissue collected 24h later, at amputation. Positive hypoxypore-2 staining identifies tissue hypoxia at the time of amputation. The red arrow indicates tissue that is hypoxic at both time points while the yellow arrow indicates an area adjacent to the vasculature that was hypoxic immediately after FAL (hypoxypore-1-positive), but negative for hypoxypore-2 at the time of excision, which is indicative of tissue reperfusion. The lack of hypoxypore-2 staining in necrotic areas suggests that the cells in this region were not viable at the time of its administration and therefore unable to actively bind the probe. The staining presented in panels B-D was performed on serial tissue sections. **E.** Intravasation in FAL-treated tumor 3 days post-FAL. The immunostaining identifies viable cells positive for hypoxypore-1 within the vessel lumen (red arrow). [Please click here to view a larger version of this figure.](#)

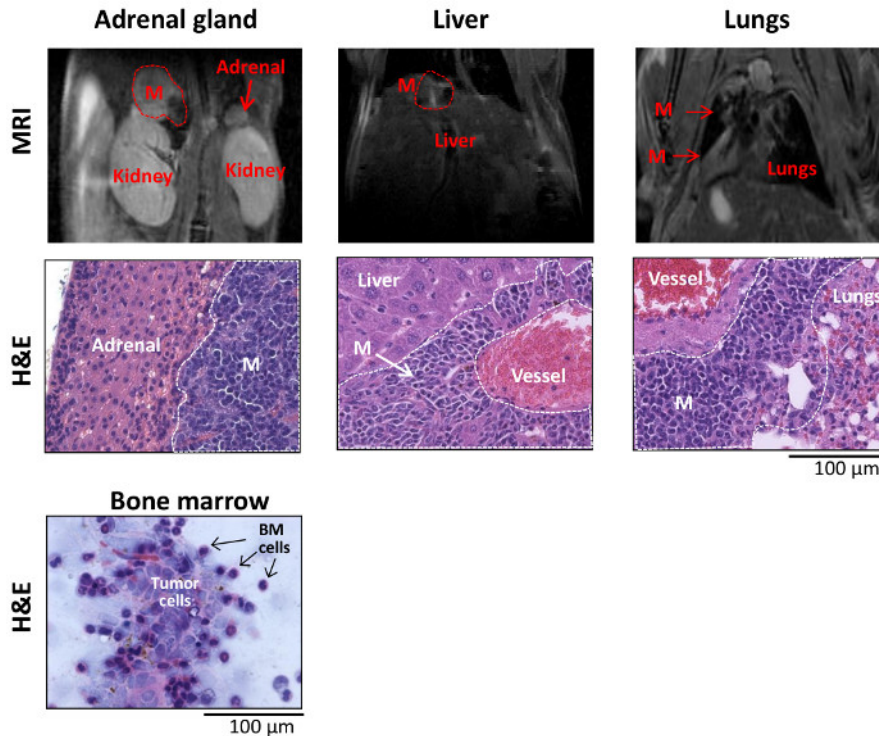
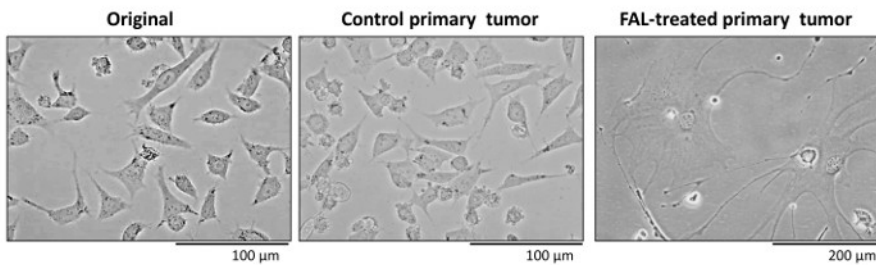


Figure 6. Examples of Metastases in Mice Bearing FAL-treated SK-ES1 Tumors. The metastases were detected by magnetic resonance imaging (MRI) and confirmed by necropsy and histopathological analyses (hematoxylin & eosin staining, H&E). M - metastasis. [Please click here to view a larger version of this figure.](#)

A. Changes in morphology of SK-ES1 cells derived from FAL-treated primary tumors



B. CD99 immunostaining in cells derived from FAL-treated tumors

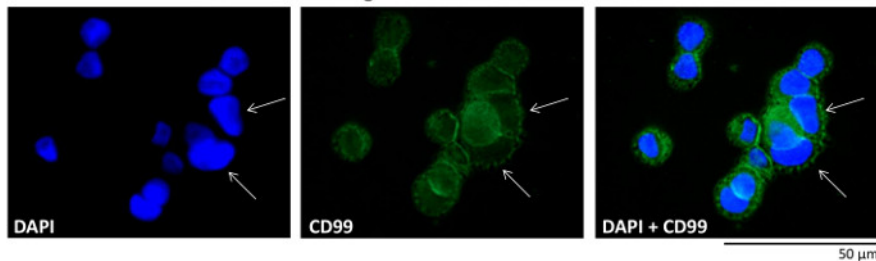
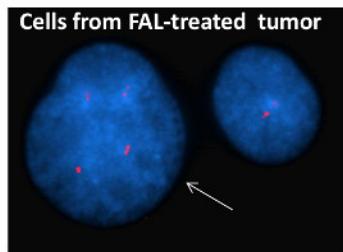


Figure 7. Characteristics of Cells Derived from FAL-treated Primary Tumors. **A.** Viable cell cultures were established from tissues harvested from control and hypoxic primary tumors and their morphology was compared to the original cell lines used for initial orthotopic injections. The cells derived from FAL-treated primary tumors exhibit dramatic changes in their sizes and morphology. Representative images of the original and primary tumor-derived SK-ES1 cells are shown. **B.** Representative images of cells derived from FAL-treated tumors immunostained for the ES marker, CD99, and counterstained with the DNA-binding dye, DAPI. All cells are CD99-positive, including hypertrophic cells containing enlarged nuclei (white arrow). [Please click here to view a larger version of this figure.](#)

A. FISH – human centromeric probe



B. FISH in metaphase chromosomes

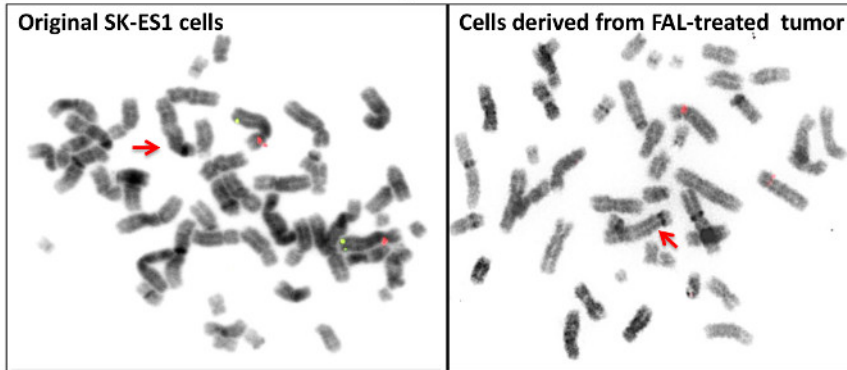


Figure 8. Chromosomal Analysis of the Cells Derived from FAL-treated Tumors. **A.** Fluorescence *in situ* hybridization (FISH) analysis in interphase cells showing two nuclei with signals for human centromeric 4 probe. The large nucleus with four signals indicates a tetraploid (4n) cell (white arrow). **B.** FISH analysis in representative metaphases of the SK-ES1 original cell line and of the cell derived from FAL-treated primary tumor. Red arrows indicate *inv(1)(p13.1q21)*, a non-random cytogenetic rearrangement characteristic of the SK-ES1 cells. Red signals: human centromeric 4 probe; Green signals in the original SK-ES1 cells: *NPY5R* probe on chromosome 4q32.2. [Please click here to view a larger version of this figure.](#)

Discussion

Our model involves the comparison of metastasis in two experimental groups — a control group, where tumors are allowed to develop in the hindlimb followed by amputation upon reaching a calf volume of 250 mm³, and a hypoxia-exposed group, in which the tumor-bearing hindlimb is subjected to FAL at the same volume, followed by amputation 3 days later. Even though in these experiments the FAL-treated tumors are amputated with a slight delay, as compared to the control tumors, their size does not increase during the 3-day period between ligation and amputation. Therefore, this approach enables the comparison of metastatic potential between tumors of comparable sizes but with dramatically different levels of hypoxia. In contrast, using sham surgery, a commonly utilized control for surgical interventions, followed by 3 days of tumor growth would result in significant differences in tumor size between the experimental groups, with the sham surgery-treated primary tumors possessing significant levels of endogenous hypoxia. Based on the pilot experiments, sham surgery does not significantly affect tumor growth or its hypoxia levels and can thus be eliminated from the experimental design. Instead, the strategy of comparing tumors of similar sizes and different hypoxia levels is a highly relevant model for elucidating the effects of hypoxia on dissemination of the disease. Importantly, unlike previous *in vitro* studies, this approach allows for comprehensive evaluation of the pro-metastatic actions of hypoxia in a natural tumor microenvironment.⁴⁻⁶

The goal of the experimental design was to achieve a low basal level of metastases that would allow for the detection of a hypoxia-induced stimulatory effect on their formation. We established that a calf size of 250 mm³ at FAL and amputation was optimal for SK-ES1 primary tumors. However, this parameter will need to be optimized for each cell line, depending on their metastatic potential and local invasiveness. For tumors with higher metastatic potential, the primary tumor size at amputation may need to be decreased. For example, TC71 tumors exhibit a high local invasiveness manifested by frequent formation of recurrent tumors at the site of amputation.¹⁸ Once such masses develop, the animals have to be excluded from analyses, as it would not be clear if subsequent distant metastases originated from the primary tumors or from recurrent tumors that often grow rapidly and exhibit high levels of endogenous hypoxia. From previous experience, independent of the cell line used, 150 mm³ is the minimal tumor-bearing calf size that can be reliably measured and normalized. If decreasing the tumor size at FAL and amputation does not eliminate recurrent tumors or decrease the basal metastasis rate sufficiently, the particular cell line in question may not be suitable for these experiments.

Likewise, it is important to establish the timeline of MRI and euthanasia for each individual cell line, depending on the latency of its metastasis. Notably, the surgical wound clips that interfere with MRI can be removed no earlier than 10 days post-surgery, and this period must sometimes be extended due to complications with healing. Thus, in this study we established day 15 as the time of the first MRI.

Another important consideration in this model is maintaining a level of hypoxia capable of inducing metastatic processes without causing complete tumor necrosis, which would prevent tumor cell dissemination. Although we have not encountered this problem in this study, if needed, the severity of hypoxia may be regulated by altering the exact site and number of ligations in relation to the branches of the femoral artery, such as the LCFA and genu artery.²⁰ Thus, a ligation proximal to the LCFA will create more severe ischemia in the hindlimb circulation, thereby

increasing the hypoxic conditions in the distal leg. In contrast, ligating distal to subsequent branches of the femoral artery will decrease the resulting hypoxia in the lower hindlimb.

Importantly, there were no severe adverse effects of combined FAL and amputation procedures and no deaths related to the surgeries, as previously reported for these techniques performed separately.¹⁸⁻²⁰ Thus, the success of the method is limited mainly by the rate of tumor take at the primary site and the frequency of recurrent tumors, both of which are cell line-dependent.

As hypoxia has been implicated as a pro-metastatic factor in many tumor types, this approach can also be suitable for directly testing its effects and defining its mechanisms of actions in other sarcomas that develop in limbs.²³ Moreover, a similar strategy can be applied to other malignancies growing in orthotopic environments with a well-defined blood supply route. Thus, with appropriate modifications, this model can be a useful tool in research beyond the field of ES biology.

Disclosures

The authors have nothing to disclose.

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