Resection of the Primary Osteosarcoma Terminates Self-seeding and Facilitates Metastasis

THESIS

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By

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Abstract

**Introduction:** Pediatric osteosarcoma is the most common bone cancer in children. Despite aggressive surgical excision and chemotherapy, up to 80% of children die after developing metastasis, despite the absence of metastatic disease at diagnosis. Similar to numerous other cancers, surgical excision of the primary tumor is often followed by development of metastasis. The present study sought to characterize the effect that surgical excision of a primary osteosarcoma has on the subsequent development of lung metastasis. This work hypothesizes a more dynamic model of metastasis development, where circulating tumor cells enter and exit circulation in an equilibrium at both the site of the primary tumor (through the process of self-seeding) and at the metastatic niche (through the process of lung colonization). We specifically sought to identify the contributions of multiple tumor-derived cytokines and chemokines, including IL-6 and IL-8, oncostatin M, and SDF-1, based on our previous work implicating these cytokines in the biology of metastatic colonization of lung.

**Results:** Using a murine model of metastatic osteosarcoma, we determined that surgical removal of a primary tumor caused a significant increase in the number of tumor cells within the lungs. We determined that the presence of a primary tumor could protect lungs from developing metastatic lesions when mice were inoculated with a defined number of
osteosarcoma cells intravenously. In the absence of a primary tumor, circulating tumor
cells preferentially migrated to the lungs. We identified significant expression of IL-6, IL-
8, IL-11, Oncostatin and SDF-1 by osteosarcoma cells in culture. IL-6 and IL-8 expression
was consistently higher than that of other cytokines. All of the cytokines and chemokines
tested, including IL-6, IL-8, Oncostatin and SDF-1, induced robust directional migration
of osteosarcoma cells using \textit{in vitro} chemotaxis assays.

\textbf{Conclusion:} These results suggest that the presence of a primary tumor influences the
migration of circulating tumor cells. Circulating tumor cells preferentially migrate to the
primary tumor when present—it is the most favorable environment for growth and survival.
After resection of the primary tumor, circulating tumor cells seed to the second most
favorable location, the lungs, initiating the development of lung metastasis. Our data
suggest that IL-6 and IL-8 likely mediate this process. Both cytokines were highly
expressed in osteosarcoma cells and both were capable of inducing migration \textit{in vitro}.
While further work is needed to elucidate this biology in greater detail, this work already
suggests that an understanding of these processes could be leveraged to design therapies
that manipulate the dynamics of self-seeding and metastatic seeding to prevent and even
treat pediatric osteosarcoma.
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Chapter 1: Review of literature

Metastasis in pediatric osteosarcoma

Osteosarcoma (OS) is the most common bone tumor and deadliest cancer in children.\textsuperscript{1–3} In the United States, 1000 new cases are diagnosed every year.\textsuperscript{4} Less than 15\% of patients will have metastasis at the time of diagnosis. The standard treatment in people, is induction chemotherapy using MAP (high dose methotrexate, doxorubicin and cisplatin) followed by surgical resection of the tumor with preservation of limb function (where possible), and post-surgery chemotherapy.\textsuperscript{5} Despite advanced surgical techniques and development of adjuvant therapy, only 60-70\% of patients without clinically evident metastasis at first diagnosis will survive beyond 3 years.\textsuperscript{6} The two major challenges related to OS are resistance to chemotherapy and the development of metastatic lesions within the lungs despite successful surgical excision of the primary tumor.

Cancer progression can be divided into two different processes, the growth of the primary tumor and the development of secondary metastases. Scientists have long pondered the biologic processes that result in metastatic tumor spread. Paget’s “seed and soil” hypothesis was first published in 1889. Based on the fact that metastasis are not randomly distributed, Paget proposed that certain tumor cells (the “seed”) had specific affinity for the environment of certain organs (the “soil”) and that the cells will only seed to a compatible soil. In 1928, Ewing championed an alternative hypothesis, suggesting that the non-random
pattern of metastasis resulted from the anatomy of the vascular system, rather than from characteristics intrinsic to the metastatic tissue.\textsuperscript{7}

Both models illustrate fundamental principles that govern the probability of metastasis at a distant site, but they do not explain how many cancers will develop metastasis sometimes years after surgery, even when metastasis were undetectable at the time of surgery.

\textit{Metastatic dormancy}

Judah Folkman pioneered the field of angiogenesis. He discovered that angiogenesis is needed for tumor growth.\textsuperscript{8} Based on clinical observations that distant metastases often don’t develop until after a primary tumor is removed, he showed that avascular microscopic metastases remain dormant until an angiogenic switch allows development of neovascularization and growth of the metastatic tumor cells.\textsuperscript{8–11} This process is a primary mechanism underlying tumor dormancy—the concept that single cells or microscopic clusters of cells can remain viable but clinically undetectable for an extended period of time. The concept of tumor dormancy applies not only secondary disease but also to tumor cells within the primary tumor.\textsuperscript{12}

Several studies have demonstrated that the primary tumor actively prepares distant tissues to receive circulating tumor cells by secreting growth factors which elicit responses from those tissues that favor tumor growth.\textsuperscript{13} Under the influence of those growth factors, tumor associated cells, such as VEGFR-1 positive hematopoietic bone marrow progenitors and macrophages, gather at the “pre-metastatic niche” and create a microenvironment favorable
for metastatic colonization. The nature of the soluble factors influences the location of the metastasis, which supports the idea that the location of the metastatic site is not random and is influenced by the primary tumor itself. Once a tumor cell arrives to the metastatic niche, survival of the dormant tumor requires establishment of an equilibrium between apoptosis and cell proliferation.

In Dr. Folkman’s original work, metastatic dormancy was maintained in Lewis lung carcinoma by inhibition of angiogenesis, which resulted from secretion of anti-angiogenic factors by the primary tumor. This continued until surgical excision of the primary tumor, which halted secretion of these “metastasis suppressors,” permitting angiogenesis and rapid development of metastasis. The existence of an angiogenic switch needed to terminate dormancy is suspected in OS as well. This static model of tumor dormancy suggests that metastasis are already implanted at the time of resection of the primary tumor, but that removal of the tumor causes a switch in the physiology of that tumor from a dormant to a proliferative phenotype.

An alternative hypothesis to the classic paradigm of metastatic dormancy involves a more dynamic model that involves “self-seeding” of circulating tumor cells. The model, originally described in 2009, stipulates that many (if not most) of the circulating tumor cells (CTCs) that originate from a primary tumor and survive circulation ultimately “metastasize” back to the primary tumor (Figure 5). Those cells possess the hallmarks of metastatic cells, such as the ability to detach from the primary tumor, to lyse the proteinaceous and carbohydrate matrix, to intravasate, to adhere to the endothelium, to...
extravasate, to attach to a target location, to induce angiogenesis and finally to propagate within a new environment.

CTCs that return to the primary tumor, however, do not need to adapt to a new “soil” as they come back to a familiar microenvironment. The presence of a permeable vasculature at the primary tumor also facilitates self-seeding. This contrasts with the challenges encountered when CTCs arrest in other tissues, where they face an unfamiliar microenvironment and tight vascular capillary endothelial walls. Only the most adaptable (most aggressive) of all CTCs have the ability to seed to a secondary location. CTCs seeding back to the primary tumor contribute to the growth of the tumor and may provide an expanding population of metastatically-proficient CTCs.

Whether CTCs originate from the primary tumor or from secondary lesions, both are capable of seeding back to the primary tumor. The multidirectional circulation/seeding of CTCs has been suggested as a potential mechanism responsible for local recurrence of cancer. CTCs could potentially seed back to the primary surgical site attracted by remaining cancer cells or stroma cells.

*Circulating tumor cells and “self-seeding”*

Our work proposes an alternative explanation for this observation that metastasis often arise only after resection of the primary tumor. This alternative hypothesis suggests a more dynamic model, which invokes elements of the processes of circulating tumor cells and “self-seeding.” The “self-seeding” model was originally described Joan Massague’s group
in 2009. The concept of self-seeding stipulates that many (if not most) of the circulating tumor cells (CTCs) that originate from a primary tumor and survive circulation, ultimately “metastasize” back to the primary tumor (Figure 5). Those cells share all the hallmarks of metastatic cells, such as the ability to detach from the primary tumor, to lyse the proteinaceous and carbohydrate matrix, to intravasate, to adhere to the endothelium, to extravasate, to attach to a target location, to induce angiogenesis and finally to propagate within a new environment.

CTCs that return to the primary tumor, however, do not need to adapt to a new environment as they come back to the same, familiar, territory that they originate from. The presence of a permeable vasculature at the primary tumor site facilitates self-seeding. This contrasts with the environment encountered when CTCs arrest in other tissues, where they face challenges such as an unfamiliar microenvironment and tight vascular capillary endothelial walls. Only the most adaptable (most aggressive) of all CTCs have the ability to seed to a secondary location. CTCs seeding back to the primary tumor contribute to the growth of the tumor and may provide an expanding population of metastatically-capable CTCs.

Whether CTCs originate from the primary tumor or from secondary lesions, both are capable of seeding back to the primary tumor. The multidirectional circulation/seeding of CTCs has been suggested as a potential mechanism responsible for local recurrence of cancer. CTCs could potentially seed back to the primary surgical site attracted by remaining cancer cells or stroma cells.
The tumor microenvironment: The tumor microenvironment contributes to the progression of cancer through the interactions of cancer cells with stromal cells by way of factors, such as cytokines and chemokines, derived from the tumor itself and from the cells in the surrounding environment. Numerous tumor-derived cytokines such as IL-6, IL-11, and their receptors have been shown to be overexpressed in different cancer cells (including OS) and to act as growth factors through paracrine and autocrine mechanisms (such as in prostatic cancer). SDF-1, a chemokine naturally synthesized by osteoblasts has been shown to up-regulate production of IL-6 and to provide chemotactic and growth signals for tumor cells in its own right. The IL-11 receptor has been shown to be overexpressed in OS cells and in OS metastases. Preliminary data from our own lab showed that expression of IL-6 and IL-8 in OS cell lines (OS-17) correlates with the ability to colonize mouse lung. In samples of tumors taken from human patients, IL-6 and IL-8 were expressed at low levels in biopsies of primary tumors and at high levels in tissues from lung metastases (Figure 1).
Specifically, the IL-6 family of cytokines has been implicated as a major driver of tumor progression and metastasis. Upregulation of IL-6 has been associated with chronic inflammatory states and is strongly associated with development of cancer. The IL-6 cytokine family, including IL-6, IL-11, leukemia inhibitor factor (LIF), ciliary neutrotropic factor (CNTF) and oncostatin (OSM) share a common signaling receptor component (gp130). Importantly, overexpression of IL-6 has been implicated as an autocrine and paracrine mechanism important for the proliferation of multiple cancers, including OS. OSM has also been shown to be overexpressed and to promote invasion and angiogenesis in OS cells in human and in dogs.

IL-6 family cytokines activate the JAK/STAT3 signaling pathway, modulating gene expression promoting cell proliferation, survival, invasion and angiogenesis.
Dysregulation of this pathway enhances production of VEGF, promoting angiogenesis$^{32,40}$ and this has been shown to occur in human OS tumor cells.$^{41}$ In support of its role in OS, inhibition of IL-6 and STAT3 have been shown to significantly inhibit OS growth and metastasis in an orthotopic model.$^{24}$ IL-6 has been shown to be overexpressed not only in primary OS but also in metastatic lesions.$^{22}$

IL-8 is a chemokine of the CXC family. It is produced by mononuclear phagocytes and was initially identified as a major inflammatory cytokine responsible for chemoattraction and activation of neutrophils.$^{42,43}$ IL-8 has been more recently shown to contribute to the progression of cancer in people, through its mitogenic and angiogenic properties.$^{44}$ IL-8 is expressed in multiple cancer such as breast cancer and has been associated with metastatic potential.$^{45,44}$

Our preliminary data show that IL-6, other IL-6 family members, IL-8 and SDF-1 drive the recruitment of CTCs back to the primary tumor. These cytokines and chemokines are also expressed in the normal lung, supporting the theory that cytokines responsible for driving the recruitment of CTCs back to the primary tumor have the potential to attract CTCs to the lungs. Specifically, both IL-6 and IL-8 stimulate behaviors associated with metastasis in OS cells such as chemokinesis, proliferation, directional migration and invasive behavior (Figure 2). A better understanding of the interaction between OS cells, cytokines and lungs will provide important information that could lead to the development of therapeutic to prevent or treat metastatic disease in OS.
An orthotopic murine model of metastatic osteosarcoma

The establishment of cell lines from human and animal tumors has contributed tremendously to improve understanding of cancer. Cell lines cultured in plastic, however, do not recapitulate the complex biology of a tumor arising within a tissue, nor the critical interactions that occur between the tumor and within the primary site and at metastatic foci. This includes interactions that facilitate immune evasion or angiogenesis and those through which stromal cells provide factors that promote growth and survival of the tumor cells themselves.

The establishment of OS tumors implanted in murine models has proved a rapid and model for the study of cancer biology. For example, OS tumor cell lines injected subcutaneously or IV form solid tumors within days to weeks after implantation, shortening the timescale within which results can be obtained.
An alternative method for propagating tumors, the patient-derived xenograft (PDX), can be established by direct implantation of primary tumor fragments into mice.\textsuperscript{50,51} Advantages of this model include that the tumor piece grows initially within its native environment and never experiences the selective pressures associated with in vitro culture on plastic. These models have facilitated the screening of therapeutics through “mouse clinical trials,” studies which allow scientists to determine the response of broad panels of unique tumors with high degrees of reproducibility.\textsuperscript{52}

Orthotopic implantation involves the placement of tumor cells in the location they would normally develop, allows study of the growth of the primary tumor, and in some cases, the development of spontaneous metastasis.\textsuperscript{49,53} Establishment of an orthotopic OS model by intra-tibial injection of tumor cells has been widely used but it remains a controversial model, at least with regard to metastasis. It has been suggested that tumor cells are given direct access to blood vessels through the intraosseous injection, bypassing the first steps of establishment of CTCs.\textsuperscript{54} Direct embolization of tumor cells at the time of injection can complicate studies of metastasis.\textsuperscript{55}

The site of implantation has significant implications for the study of tumor biology. Mouse flank xenografts have been used for many years, and numerous studies have established subcutaneous tumors as a standard methodology for the study of tumor response to therapy.\textsuperscript{56,57} Because primary tumor growth, development of metastasis, and response to chemotherapy can be largely influenced by factors in the microenvironment\textsuperscript{58–60} the use of orthotopic models, where the tumor is introduced into its “natural location” (Figure 3) is often considered a more appropriate model for \textit{in vivo} study.\textsuperscript{54,61,62}
Figure 3: On the left, establishment of the xenograft by intra-tibial injection of OS-17 cells; on the right, tumor (red arrow) at the time of amputation, note the leg was clipped for surgery.

Conclusion

The mechanisms which result in the development of metastatic disease after resection of a primary tumor remain poorly understood. Some models suggest that primary tumors produce anti-angiogenic factors that suppress the growth of metastatic disease until the primary tumor is resected. Anti-angiogenic therapies, however, have failed to prevent metastasis in clinical practice. We know that CTCs circulate freely even when gross metastasis is absent. The recently-developed concept of tumor self-seeding suggests that CTCs most frequently “metastasize” back to the primary tumor, facilitating growth. This concept has shown to be operable in breast and colon cancer as well as in OS. Tumor-
derived cytokines and chemokines such as IL-6, IL-11, and SDF-1 appear to drive recruitment of these cells back to the primary tumor, which likely represents the most favorable growth environment.

If, then, the primary tumor grows by recruiting circulating tumor cells back to the primary tumor, what becomes of the circulating tumor cells when the primary tumor is removed? While removal of a primary tumor most certainly eliminates the primary source of CTCs, does it also take with it the primary means for purging these cells from circulation? Therefore, the purpose of this body of work was to test the hypothesis that excision of a primary OS tumor causes CTCs to transition from preferential colonization of the primary tumor site to colonization of lung tissue. If true, understanding the biology that drives this process will facilitate the development of therapeutics that can prevent the emergence of metastasis after resection or amputation.
Chapter 2: Material and methods

Animals:
The mice (6-week-old CB17SC scid<sup>-/-</sup> female mice) were purchased from Envigo/Harlan (Indianapolis, IN). All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) of the Research Institute at Nationwide Children's Hospital (Columbus, OH). The approved protocols were designed to minimize the numbers of mice used and to minimize any pain or distress. All manipulations were done under sterile conditions in a laminar flow hood. All surgical procedures were performed under general anesthesia and under aseptic conditions. Mice were maintained under barrier conditions.

Cell culture:
The mGRFP-labelled OS-17 cells were cultured in RPMI supplemented with 10%FBS with tetracycline at 37°C, 5% CO2. The mRFP1-labelled OS-17 cells were cultured in RPMI supplemented with 10%FBS.

Establishment of orthotopic tumors:
Anesthesia was induced with isoflurane 3.5% in 2L.min<sup>-1</sup> of O<sub>2</sub> and maintained with isoflurane 1-2% in 1 L.min<sup>-1</sup> of O<sub>2</sub>. Anesthesia was delivered with a mask (Figure 5). Mice were positioned on lateral recumbency. After the skin of the medial aspect of the proximal
left tibia was clipped with a cordless clipper (ChroMini, Wahl®) and the sterile preparation was done with the use of swab stick applicator (Chloraprep®, 2% Chlorhexidine gluconate, 70% Isopropyl alcohol, Carefusion Corp.). The left tibia was positioned vertically with the foot on the table (Figure 3). The insertion of the patellar tendon was visualized and the per-cutaneous intra-tibial injection of $10^5$ cells in 50µL was performed through the lateral aspect of the tibial plateau with a 1cc syringe on a 31G needle (Figure 3). Mice that were to receive a sham (group 1 in experiment 2) were injected RPMI with the same technique. In order to reduce post procedural pain, mice were maintained under anesthesia for 3 minutes to allow for fluid volume equilibration, which is the primary source of pain. Mice recovered in a recovery cage and were considered fully recovered when they were back to their normal level of activity and did not show any sign of pain, usually within 5-15 minutes.

**Measurement of the tumors:**

Mice were weighed daily and examined for signs of ill thrift, lameness or swelling around the surgical site. Once the tumor was palpable, at about 4-5mm in the largest diameter, the tumors were measured using an electronic caliper in orthogonal directions. Tumor growth was monitored until the tumors reach predetermined dimension (8-12mm).

**Amputation:**

At time point 1, the tumor-bearing leg of mice from group 2 were amputated by coxo-femoral disarticulation. The surgical preparation started before induction with clipping of the entire leg up to the spine and cranially up to the last rib, and ventrally up to midline.
This was done awake in order to reduce the total time under general anesthesia. Mice were anesthetized with the same protocol previously described. Pain was managed with the addition of subcutaneous buprenorphine (2 mg/kg, Buprenex® injectable 0.3mg/ml) and ketoprofen, subcutaneously (Ketofen® injectable 5mg/kg). Each mouse received 3 cc of sterile saline sub-cutaneously before the procedure to encounter for blood loss during surgery and volume loss due to amputation and evaporation. At recovery, another 2-3 cc of sterile saline was injected in case of marked blood loss. The surgical field was steriley prepped using a swab stick applicator (Chloraprep®, 2% Chlorhexidine gluconate, 70% Isopropyl alcohol, Carefusion Corp.).

The SurgiSuite Multi-functional Surgical Platform (Kent Scientific corp.) was used to perform amputations (Figure 4). It offers bright LED lighting, homeothermic control, magnetic limb positioner. The Platform has an easy-to-clean, chemical resistant surface. It includes integrated warming using a rechargeable, battery-operated far infrared warming pad located in the center of the platform. The warming pad sits below the surface to allow for a level surgical field with the rest of the platform. The surgical area is surrounded by a ferro-magnetic stainless steel plate, incorporating a window to allow for warming of the animal during a surgical procedure. LED lighting plugs directly into the USB power strip with up to 7 USB devices that can be used simultaneously. The mouse was placed in right lateral recumbency (Figure 5), with the front legs tied to the surgical table. A sterile drape was used to cover the body, except for a hole for leg placement, to maintain sterility.
The limb was amputated by coxo-femoral disarticulation. A circumferential elliptical incision around the mid-thigh was made and the skin was reflected (Figure 6). The fat on the cranial aspect of the distal femur and on the medial aspect of the thigh was dissected with the use of the cauterity pen. The femoral artery, vein and nerve bundle is directly visualized emerging from the abdominal wall (Figure 8). It was dissected using a DeBakey atraumatic tissue forceps. Once isolated, a stainless steel ligating clip (Ethicon Endosurgery, Blue Ash, OH, USA) was used to ligate the femoral artery and vein. At least 2 clips were placed, one towards the body and one towards the leg. Femoral artery, vein and nerve were then transected using Metzenbaum scissors. The gluteal muscles were incised from their insertion (third trochanter and greater trochanter) and the coxofemoral joint
capsule was transected to free the femoral head. The sciatic nerve was directly visualized caudal to the greater trochanter. It was anesthetized with lidocaine 2% diluted to 0.5% and transected with a #11 blade. The thigh muscles were transected at the level of the proximal third of the femur and reflected off of the proximal aspect of the femur.

Figure 5: Positioning of the mouse on the surgery table, note the entire leg is clipped and prepped. Anesthesia is maintained with a mask.
The muscles that originate directly on the femur (Vastus Lateralis m., Vastus Medialis m. and Vastus Intermedius m.) were not transected as they would be removed with the leg. The muscles of the hip region were sutured over the acetabulum using 6-0 polyglecaprone suture on a reverse cutting needle (Monocryl®, Ethicon).

The skin incision was closed with subcutaneous sutures using 6-0 polyglecaprone suture on a reverse cutting needle (Monocryl®, Ethicon) and reinforced with tissue adhesive. Once the surgery was done, the mice recovered in a warmed environment until they returned to normal behavior (walking, grooming) which usually took from 15 to 30 minutes. Postoperatively, mice were treated with sub-cutaneous injections of buprenorphine (2mg/kg SC, Buprenex® injectable 0.3mg/ml) every 12h for 3 days and sub-cutaneous injections of ketoprofen (Ketofen® injectable 5mg/kg) at recovery and daily
for the following 72 hours.

The tumor was harvested and dissected away from non tumoral tissues. The tumor was bisected, one half was placed in formalin and the other was placed in a 2ml cryogenic vial (Corning®) immediately placed in liquid nitrogen before storage at -73°C.

Figure 7: After skin incision and incision of the subcutaneous fat on the medial aspect of thigh, the femoral artery and vein (white arrow) are isolated.
Lung collection:

Once the blood was harvested, mice were euthanized. A stab incision was then made through the skin, on mid line, caudal to the xyphoid and the skin was opened following the midline cranially up the rostral aspect of the mandible using mini-Metzenbaum scissors. A stab incision was then made in the abdominal wall with mini-Metzenbaum scissor and continued along the last pair of ribs from ventral to dorsal on the right and left side. The diaphragm was punctured and once the negative pressure was relieved, the lungs partially deflated, the diaphragm was completely cut of its insertion on the rib cage. Then the rib cage was cut on the right and on the left sides, as dorsal as possible. Cranially, the cut was extended until the clavicles were cut, while always avoiding mid-line to preserve the trachea. All attachments of the heart and lungs to the rib cage were bluntly dissected while

Figure 8: Mouse at recovery. The surgical incision (black arrow) was closed using intradermal and skin glue.
the rib cage was elevated and pulled cranially, giving access to the entire lungs and the trachea up to the cricoid cartilage. The trachea was gently held with Debakey atraumatic tissue forceps and cut as proximal as possible. The trachea was gently pulled ventrally and separated from the esophagus by blunt dissection. The esophagus was cut just cranial and just caudal to the lungs. All attachments to the rib cage, and diaphragm were dissected away. The inferior vena cava and aorta were cut and the lungs-trachea-heart were isolated en bloc from the body. Perma-hand® Silk 4-0 (Perma-Hand® Silk Suture, 4-0, Ethicon, Johnson-Johnson, USA) was used to make a loose over-hand knot to be tied over the trachea after lung inflation. The knot was pre-placed around the trachea and an 18G catheter was placed within the tracheal lumen. Sterile 0.9% sodium chloride (BD PosiFlush™ Pre-filled Saline Syringes, 0.9% sodium chloride USP, BD Medical Corp.) was instilled in the lungs. Once they were insufflated, the catheter was quickly removed and the knot was tied. The left lung lobe was isolated and placed in OTC on dry ice before storage at -73°C. The inferior and post caval lobes were placed in a 2ml cryogenic vial (Corning®) and immediately frozen in liquid nitrogen before storage at -73°C (snap frozen). The middle and superior lobe were placed in formalin for 24hours and then switched to Dubelcco’s Phosphate Buffered Saline (DPBS) (Gibco®, Life technologies™, Thermo Fisher Scientific Inc. USA).

Genomic DNA preparation:
Genomic DNA (gDNA) was obtained from blood, bone marrow, and lungs using the Quick-gDNA™ Miniprep Kit (Quick-gDNA™ Miniprep Kit, Zymo Research). This kit
allows quick purification of high quality DNA from cells, whole blood, plasma, serum, body fluids, swans, lymphocytes, cultured cells and solid tissue as well.

Quantification of tumor cells:
CTCs were quantified in the different compartments (blood, bone marrow and lungs) by RT-PCR using primers directed at the individual fluorescent proteins. The Quick gDNA™ Miniprep Kit was purchased from Zymo Research Corp. (Irvine, CA). The PrimeTime® Gene Expression Master mix was purchased from Integrated DNA Technologies Inc (Coralville, IA).

Fluorescence Microscopy:
A Leica DMI-4000 B inverted microscope (Leica) and Leica cooled CCD camera (Leica) were used to capture images of fluorescently-labeled tumor cells with sections from OTC-embedded cryosections.

Experimental design: amputation model (Figure 9)
CB17SC scid -/- mice were given intra-tibial injections of 5.10^5 mRFP1-labelled OS-17 cells into the proximal left tibia to generate an orthotopic tumor. Once the primary tumor reached 8-10 mm diameter (Time 1), the control group (group 1) was euthanized and organs (blood, lungs, bone marrow) were harvested as described below. The treatment group (group 2) had the tumor removed by coxo-femoral disarticulation and the no treatment group (group 3) did not receive any surgical procedure. One week after treatment
(Time 2), mice from group 2 and 3 were euthanized and organs were harvested as described above. Microscopic metastatic foci were compared in the three groups by quantification using RT-PCR.

![Experimental design: metastatic model (Figure 10)](image)

**Figure 9:** At time point 0 (on the left), the xenograft was established; At time point 1, (in the middle), group 1 was euthanized and lungs were harvested, group 2 was amputated and group 3 received no treatment; At time point 2, group 2 and 3 were euthanized and lungs were harvested.

Experimental design: metastatic model (Figure 10)

CB17SC scid -/- mice were injected 5 x 10^5 eGFP-labelled OS-17 cells into the left tibial plateau.

When tumors reached criteria for amputation, mice were inoculated intravenously with 1 x 10^6 mRFP1-labelled OS-17 cells via tail vein. After one week, mice were euthanized and tumors, lungs, bone marrow and blood were collected. The distribution of the RFP-labelled
tumor cells was determined by both fluorescence microscopy and qRT-PCR of each tissue using primers directed at the individual fluorescent proteins. Using the two labels allows to differentiate CTCs originating from the primary tumor from those introduced into the bloodstream.

Figure 10: Metastatic model of OS in mice: in group 1, CTCs were injected IV in mice without primary OS. In group 2, CTCs were injected once primary OS reached greatest dimension. Mice were euthanized 1 week after injection and distribution of CTCs were assessed in lungs and primary tumor.

ELISA assays

Expression of cytokines/chemokines IL-6, IL-8, IL-11, OSM and SDF-1 by OS17 cells was quantified by ELISA. OS-17 was cultured in RPMI + 10% FBS until 80% confluent. The cells were then plated in a 6 well plate in 2 ml of RPMI and incubated at 37C for 72 h. The supernatant was harvested and centrifuged to eliminate any cellular debris. The cell-free supernatant obtained was frozen at -20C. Multiplex sandwich ELISA was performed.
on the cell-free supernatant to measure the concentration of the cytokines IL-6, IL-8, IL-11, oncostatin (OSM) and SDF-1. The Magnetic Luminex® assay was used in this experiment.

Migration Assay
To determine, the chemoattractant potential for IL-6, IL-8, OSM or SDF-1 in OS-17, transwell migration assays were performed (Figure 11) using Corning control inserts (8μM pore size). The upper compartments were seeded with $1 \times 10^5$ OS-17 cells in 200μL of RPMI, and the lower compartment was filled with 800 μL plain RPMI (negative control), RPMI supplemented with 1% FBS (positive control), or RPMI supplemented with IL-6, IL-8, OSM or SDF-1. The cells were incubated for 24 h to allow migration. The cells that migrated in the filter were fixed using Diff-Quik stain kit (Siemens). Migration was analyzed by counting the number of stained cells on the bottom of the filters.
Statistical analysis:

Statistical analyses were carried out using Prism software (Graphpad, Inc). In the metastasis quantification experiments, non-parametric Kruskal-Wallis analysis with posthoc comparisons was used to evaluate differences between groups. For cytokine expression and migration assays (which resulted with approximately normal distributions), one-way ANOVA with Tukey post-hoc comparisons was used to evaluate differences. For all experiments, p < 0.05 was considered to be statistically significant.

Figure 11: Transwell migration assay (A). The upper compartment was seeded with OS-17 in RPMI (B). The lower compartment was seeded with RPMI supplement with one of the cytokine studied (B).
Removal of the primary OS precipitate lung metastasis:

Based on numerous clinical observations, it has been postulated that primary tumors might inhibit the progression of metastasis. To assess the influence of the removal of the primary OS on lungs metastasis progression, we used a murine orthotopic model of OS. We determined that when the primary tumor was removed, a significant increase in the number of microscopic metastatic foci were found in the lungs (Figure 12). We also found that there was no significant difference between the two groups who retained primary tumors at euthanasia, whether at the early or late time points (p < 0.05).
Circulating tumor cells are attracted back to the primary OS:

We then used a metastatic model of OSA to evaluate the influence of the presence of the primary tumor when CTCs are circulating. We determined that, in the presence of a primary tumor, levels of tumor cells which remain viable within the lungs decreased significantly. Examination of the primary tumors revealed microscopic self-seeding metastatic foci within the same period of time (Figure 13). This experiment showed that CTC seeding is influenced by the presence of a primary tumor. In the presence of a primary tumor, CTCs preferentially seed back to the primary tumor. In the absence of a primary tumor, CTCs seed preferentially to the lungs, which is the most common location for metastasis in OS.

Figure 12: Percentage of tumor derived cells found in the lungs at the time of euthanasia. The group with early amputation had significantly (*) more OS-17 derived cells within the lung compared to the two groups that were euthanized bearing a primary tumor.
Figure 13: Metastatic model of OS in a murine orthotopic model of OS. On the left, minimal number of tumor cells were found in the lung when a primary OS tumor was present. On the right, where mice bore no primary tumor, metastatic foci were found within the lung.

OS-17 cells produce cytokines and chemokines capable of autocrine induction of invasive behavior:

The previous results suggest that primary OS tumors influence CTCs seeding. To better understand the mechanisms that drive CTCs to seed back to established tumors, we measured chemoattractant cytokines of the IL-6 family and others known to drive chemotaxis in other tumors assessed their capability of attracting OS-17 \textit{in vitro}.

We determined that OS-17 produce very high levels of IL-6 and IL-8. IL-11 was produced at a lower level. OSM and SDF-1 were also produced, but at very low levels compared to
IL-6 and IL-8 (Figure 14). Using a transwell migration assay, we assessed the effect of IL-6, IL-8, OSM and SDF-1 on migration and invasion of OS-17. In the absence of cytokine, minimal migration of OS-17 was noted. The positive control confirmed the potential for invasion and migration of OS-17. And IL-6, IL-8, OSM or SDF-1 all induce migration of OS-17 as well (Figure 15).

![Cytokine Expression](image)

Figure 14: Cytokine expression by OS-17
Chapter 4: Discussion

The metastatic cascade is a complex and still poorly understood process. Cancer cells interact with their microenvironment to proliferate locally and promote the growth of the tumor. Tumor cell interactions with the microenvironment are also involved in the development of metastasis.\textsuperscript{63–65} Production of anti-angiogenic factors by the primary tumor has been suggested to suppress the growth of metastatic disease until the primary tumor is resected. However, anti-angiogenic therapies have failed to prevent metastasis in clinical practice.\textsuperscript{9} The recently-developed concept of tumor self-seeding suggests that CTCs most frequently “metastasize” back to the primary tumor, which facilitates growth.\textsuperscript{16,17,20,25} CTCs return preferentially to the primary tumor due to its highly permeable vasculature.
and the lack of adaptation required for the CTCs to survive in a familiar microenvironment. This concept has been shown to contribute to the growth of the primary tumor in an orthotopic model of OS.\textsuperscript{25}

Despite complete resection of the primary tumor, 30-40\% of human patients diagnosed with OS will develop metastatic disease despite evidence of tumor spread at the time of diagnosis and surgery. The mechanisms which result in the development of metastatic disease after resection of a primary tumor remain poorly understood.\textsuperscript{66–68} We found that mice with macroscopic primary OS and absence of metastasis at the time of surgery will rapidly develop microscopic disease following amputation compared to mice that were not amputated. These results support the hypothesis that the presence of a primary tumor is protective for the development of metastasis in OS and suggest that removing the primary OS tumor may contribute to the development of metastasis within the lung.

To validate the hypothesis that the primary tumor is protecting the lungs from metastatic seeding through migration of CTCs back, we created a model in which a defined number of circulating tumor cells was introduced by tail vein injection of OS-17 in mice. When mice with primary OS were injected CTCs, the CTCs were found to seed to the primary tumor and significantly reduced numbers of metastasis were found in the lungs. When mice did not have a primary tumor, significant numbers of CTCs were found in the lungs. This second set of results supports the hypothesis that primary OS is the most favorable environment for CTCs to seed\textsuperscript{69}, and that in the absence of the primary tumor, CTCs are more likely to colonize the lung. Those results correlate with the clinical behavior of pediatric OS.
In this and other models, tumor-derived cytokines and chemokines such as IL-6, IL-11, and SDF-1 appear to drive recruitment of these cells back to the primary tumor. IL-6 and IL-8 have been shown to recruit CTCs at the primary site in OS.\textsuperscript{25} IL-6, IL-11 and OSM belong to the Il-6 family. They have been implicated in the promotion of tumor progression and tumor cells migration in multiple cancer.\textsuperscript{16,70} In a migration assay, we showed that IL-6, IL-8, SDF-1 and OSM induce invasive behavior in OS-17 cells. We can speculate that IL-6, IL-8, SDF-1 and OSM are involved in attracting OS-17 back to the primary tumor. IL-6 and IL-8 have been identified in normal lungs in a preliminary study and we can speculate that those cytokines are involved in the attraction of CTCs once the primary tumor has been removed.

If true, understanding the biology that drives this process will facilitate the development of therapeutics that can prevent the emergence of metastasis after resection or amputation. Identifying the common environmental factors within the primary tumor and the lungs responsible for attracting CTCs and allowing their development within those two compartments could lead to a potential therapeutic target for treatment or prevention of the development of metastasis in pediatric OS. We identified that OS-17 produces IL-6, IL-11, IL-8, OSM and SDF-1 in culture. These same cytokines/chemokines induced potent invasive behavior.

Future investigations should focus on cytokines of the IL-6 family. To confirm the implication of those cytokines in the progression of metastasis in OS, we could knockdown the expression of the cytokines or their receptors individually and in combinations to find the most effective combination.
Chapter 5: Limitations

Our orthotopic model was designed with intra-tibial injection of tumor cells and not tumor pieces. In this model, tumor cells may embolize at the time of injection into the tibia. That said, we did not observe any acute deaths, which might suggest pulmonary embolism as reported previously.\textsuperscript{55} To reduce the risk for tumor embolization, minimal volumes are used for injection. None of our mice developed early metastasis, which supports our assertion that intra-tibial injection is an appropriate method to initiate the development of an orthotopic model of OS in these studies.

A spontaneous model of metastasis such as our orthotopic model presents some limitations. As can be seen from the data presented, there is a high degree of variability in the timing and extent of metastasis that ultimately develops in the mice. Moreover, in the amputation model, as designed, we have not accounted for the effect of stress related to anesthesia and surgery on the recruitment of CTCs to the various tissues. Comparison with the existing groups would allow us to determine whether the stress of anesthesia might influence the flux of CTCs into the blood. Inclusion of a sham surgery group would help determine whether the level of stress experienced during a surgery may influence the flux of CTCs.

Mice were euthanized one week after amputation, so we do not know the long term effect of amputation on survival. Would mice without amputation start developing metastatic disease? Because of ethical reason, we could not let the tumor grow further without causing pain, discomfort to the mice.
Chapter 6: Conclusion

Traditional chemotherapy and surgical excision of OS in canine and pediatric patients has failed to eliminate the progression of metastasis in the patient. In this study, we identified surgical excision of the primary tumor as a potential trigger for the development of metastasis. We also confirmed that CTCs seed back to the primary tumor and that these CTCs relocate to the lungs in the absence of a primary tumors. We identified that cytokines of the IL-6 family and the chemokine IL-8 to have potential for attracting CTCs and mediating self-seeding.

A study in soft tissue sarcoma showed improved survival in patients that underwent a revision surgery after having an incomplete resection of a distal limb soft tissue sarcoma.71 There are several potential reasons for those findings, including the development of immunity after surgery. We propose that the remaining tumoral tissue at the surgical site might attract CTCs from circulation and that the second surgery facilitates elimination not only of the local disease, but also of the potent metastatic cells that seeded back.

We may be able to leverage the self-seeding mechanisms to attract CTCs to a location or a device that could be removed from the patient, reducing the amount of CTCs in the patient, which potentially could improve survival or maintain control of metastatic disease. Once we confirm and identify the cytokines capable of attracting CTCs, the concentrations of
cytokines needed, we could place an implant, such as sponge of gelatin infused with cytokines to purge the body from CTCs, subsequently removing the device to destroy them.
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