

## Cancer research

# Modeling bone metastasis of cancer *in vitro* using a triple co-culture setup

## Abstract

Bone metastasis is a process by which tumor cells leave their site of initiation, invade the surrounding extracellular matrix, travel through the blood circulation, and ultimately metastasize to the bone [1–4]. While studies have modeled different aspects of this process with respect to physical interactions and signaling between cells, a model system that faithfully mimics the process of bone metastasis *in vitro* has not yet been demonstrated. Here, we have modeled bone metastasis *in vitro* by establishing a triple co-culture setup utilizing Thermo Scientific™ Nunc™ cell culture inserts in a carrier plate system. We have incorporated

the different constituents involved in the process, including cancer cells, extracellular matrix proteins, endothelial cells, and bone cells, in a single system (Figure 1) [5–7]. Using prostate cancer cells as an example, we have successfully demonstrated trans-endothelial migration to bone cells using this method [7–10]. This protocol will aid in a better understanding of the complexity of bone metastasis at both the cellular and molecular level and can be applied to the development of other experimental models to study intracellular and intercellular interactions across multiple cell types, within a single model system [11].

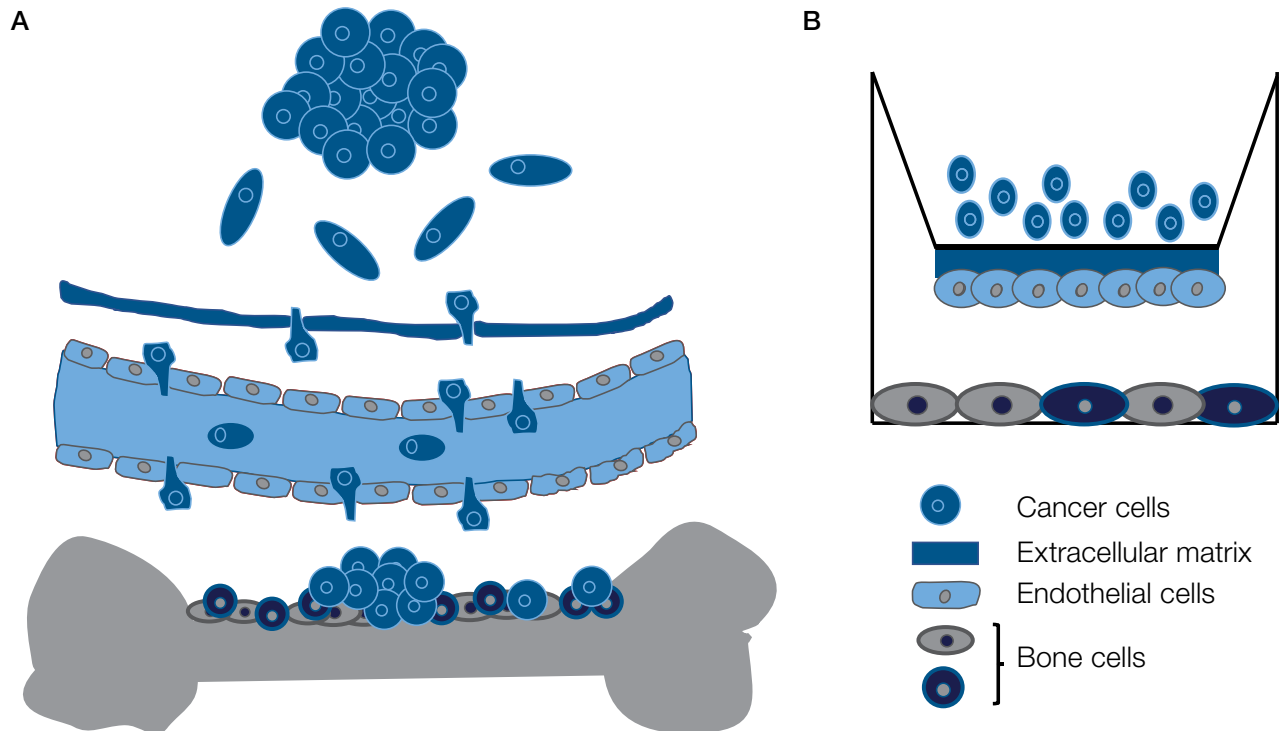


Figure 1. Overview of (A) bone metastasis and (B) its associated model utilizing Nunc cell culture inserts.

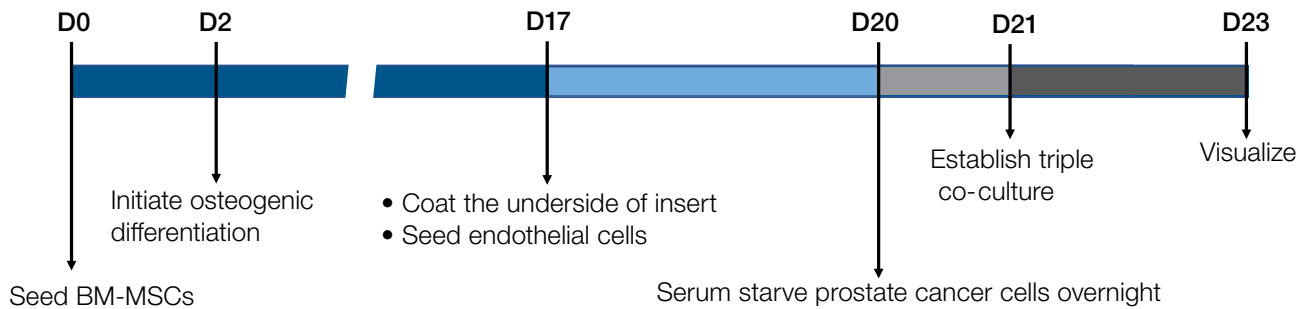


Figure 2. Timeline for establishing the bone metastasis model from day 0 (D0) to day 23 (D23).

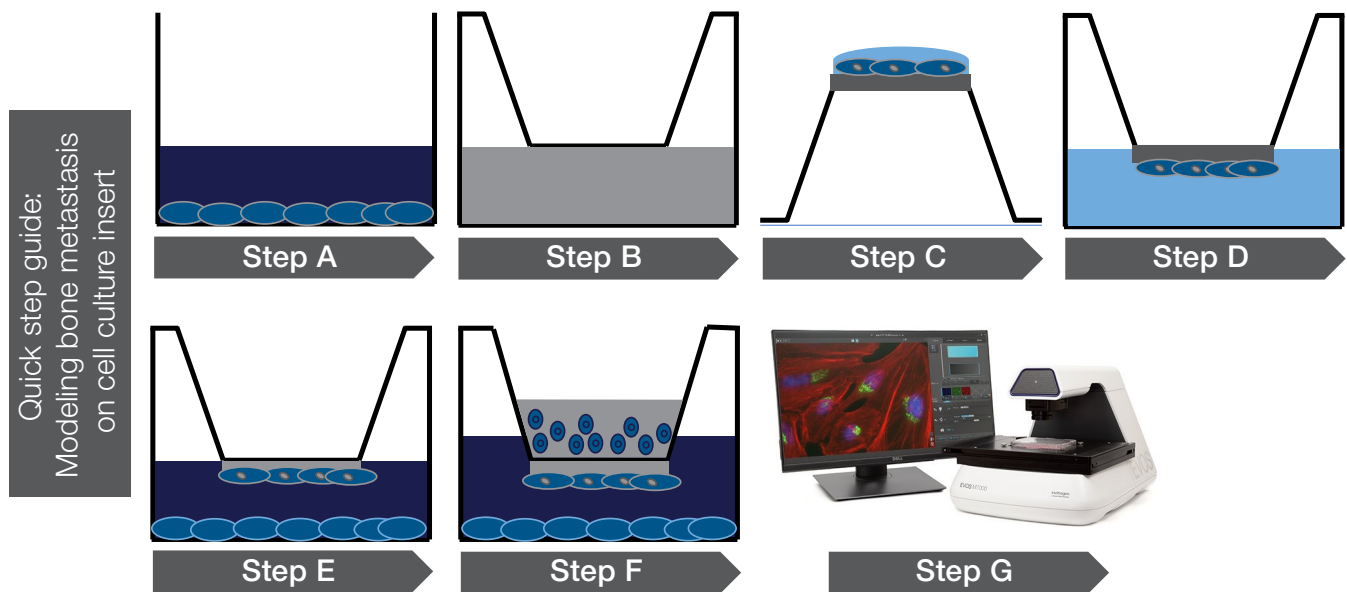


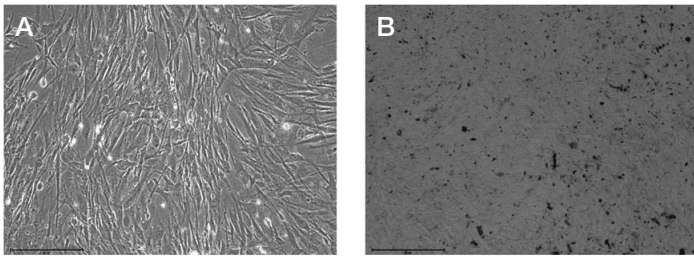
Figure 3. Illustration of the experimental setup for establishing the bone metastasis model.

## Procedure

Follow the timeline outlined in Figure 2. Also refer to Figure 3 for an illustration of the experimental setup.

### Step A: Differentiating bone marrow-derived mesenchymal stromal cells (BM-MSCs) to the osteogenic lineage

1. Dilute Gibco™ CELLstart™ Substrate 1:100 in DPBS with calcium and magnesium (+/+) and coat plasticware for 1 hour at 37°C before seeding BM-MSCs.
2. Grow BM-MSCs in Gibco™ StemPro™ MSC SFM XenoFree complete medium on plasticware coated with CELLstart Substrate for at least 1 passage post-revival before differentiation to the osteogenic lineage.
3. When cells reach ~80% confluency (Figure 4A), aspirate medium from cells and rinse once with DPBS without calcium or magnesium (-/-).
4. Dissociate cells using Gibco™ CTS™ TrypLE™ Select Enzyme and make a single-cell suspension in complete medium. Perform cell count and viability measurements using the Invitrogen™ Countess™ 3 Automated Cell Counter.
5. Seed 5,000 cells/cm<sup>2</sup> onto 24-well CELLstart Substrate-coated plates in StemPro MSC SFM XenoFree complete medium; allow the cells to reach 50% confluency (takes 2–4 days).
6. After cells have reached 50% confluency, aspirate the complete medium and add 1 mL of supplemented Gibco™ StemPro™ Osteogenesis Differentiation Medium to each well.
7. Continue to change medium every other day. Allow the cells to differentiate for 21 days (Figure 4B).



**Figure 4. Morphology of BM-MSCs and osteocytes.** (A) 80% confluent BM-MSCs. (B) BM-MSCs differentiated to the osteogenic lineage for 21 days. At this point, individual cells cannot be distinguished. Scale bar: 275  $\mu$ m.

### Step B: Creating the extracellular matrix on the underside of the cell culture insert

1. Thaw Gibco™ Geltrex™ matrix overnight at 4°C and then dilute to 3% in DPBS –/– on ice to make a working solution of Geltrex matrix (for example, add 30  $\mu$ L of Geltrex matrix to 970  $\mu$ L of cold DPBS).
2. Keeping the inserts at the highest position in the carrier plate, add 1.5 mL of Geltrex matrix working solution to each receiver well. This volume is recommended for a 24-well plate and should be adjusted for larger wells.
3. Incubate inserts at 37°C for 2 hours.

**Note:** Use cell culture inserts with 8  $\mu$ m pore size; smaller pore size will prevent cancer cell migration.

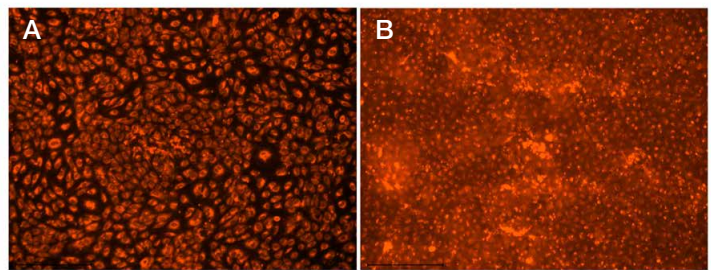
### Step C: Making the endothelial cell barrier on the underside of the insert

1. Dissociate Gibco™ Human Umbilical Vein Endothelial Cells (HUVECs) using TrypLE Express Enzyme and resuspend as singlets at a concentration of  $2 \times 10^6$ – $2.5 \times 10^6$  live cells/mL in complete medium.
2. Transfer the carrier plate containing inserts from step B to a new uncoated 24-well plate.
3. Place the lid on the carrier-containing plate, hold the plate from both sides, invert the plate, and place the lid down on the biosafety cabinet (BSC) surface.
4. Gently remove the receiver plate to avoid disturbing the inserts. At this point, the inserts are upside down.
5. Add 100  $\mu$ L of HUVEC suspension (prepared in step C.1) to the underside of each insert. Be careful to not touch the membrane with the pipette tip during this process. This seeding density is at near confluency.
6. Gently place the receiver plate back and transfer the setup to a 37°C incubator for overnight incubation. Be careful to not disturb the cell suspension on the underside of the insert.
7. The following day, remove the plate from the incubator and bring it back to the BSC. Flip the plate upright by holding it from the sides. At this point, the inserts are upright.

8. Add prewarmed complete medium for HUVECs in the receiver well (1.5 mL/well).
9. Incubate the plate at 37°C until the HUVECs form a tight monolayer (~4 days).

### Step D: Confirming endothelial cell monolayer formation on the insert's underside

1. Reconstitute Invitrogen™ CellTracker™ Red CMTPX Dye per the manufacturer's instructions.
2. Prepare 1  $\mu$ M working solution of CellTracker Red CMTPX Dye in the HUVEC basal medium.
3. Aspirate medium from the receiver wells of the plate prepared in step C. Add 1.5 mL working solution of CellTracker Red CMTPX Dye to each well and incubate the plate for 30–45 minutes at 37°C.
4. Using forceps, place one representative insert in the bottom position in the height-adjustable carrier in order to confirm monolayer formation. Visualize the cells using the RFP filter on the Invitrogen™ EVOS™ M7000 Imaging System; once a confluent monolayer is confirmed, proceed to step E. Refer to Figure 5 for representative images of cell confluency.



**Figure 5. Visualization of HUVEC monolayer using CellTracker Red CMTPX Dye.** Representative images of (A) semi-confluent HUVECs and (B) confluent monolayer of HUVECs seeded on the underside of the insert. Cells were stained with CellTracker Red CMTPX Dye.

### Step E: Establishing a co-culture of endothelial cells and BM-MSC–derived osteocytes

1. Keeping the inserts in the top position of the carrier plate, transfer the carrier containing inserts with HUVEC cells on the underside to the 24-well plate containing BM-MSC–derived osteogenic cells.
2. Add 200  $\mu$ L of serum-free DMEM on the insert and equilibrate the cultures at 37°C while the prostate cancer cells are being processed for establishing the triple co-culture setup.

### Step F: Establishing a triple co-culture setup

1. Grow PC-3 cells in DMEM supplemented with 10% FBS for at least 2 passages post-revival.
2. The day before establishing triple co-culture, make sure the prostate cancer cells have reached ~70% confluency. Serum starve the cells overnight.

3. Prepare a 1  $\mu\text{M}$  working solution of Invitrogen™ CellTracker™ Blue CMAC Dye in serum-free DMEM.
4. Aspirate medium from the prostate cancer cells and incubate the cells in the working solution of CellTracker Blue CMAC Dye in serum-free DMEM for 30–45 minutes at 37°C.
5. Dissociate the cells using TrypLE Express Enzyme and make a single-cell suspension in DMEM basal medium containing 0.2% FBS.
6. Take cell count and add  $1 \times 10^5$  cells on the insert from step E.
7. Incubate the triple co-culture setup for 48 hours at 37°C.
8. Prepare a negative control (prostate cancer cells in basal medium containing 0.2% FBS on the insert and the same medium in the receiver well) and a positive control (prostate cancer cells in basal medium containing 0.2% FBS on the insert and complete medium, i.e., DMEM supplemented with 10% FBS, in the receiver well) for the assay.

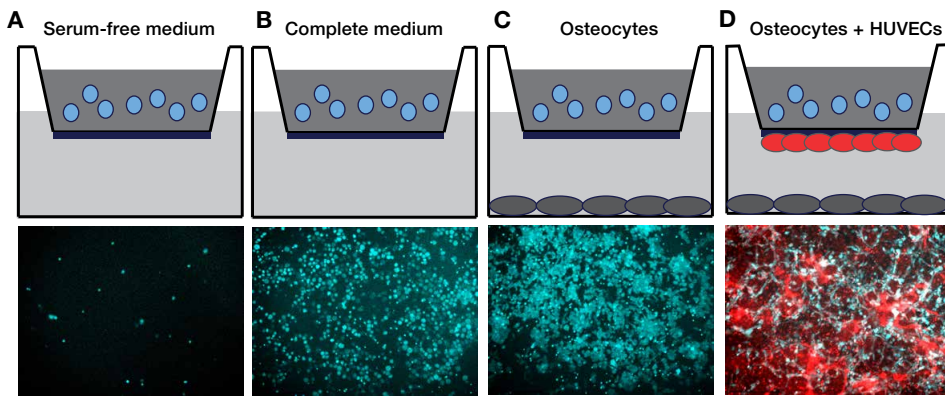
### Step G: Visualizing trans-endothelial migration of cancer cells using live-cell imaging

1. At the end of the assay, aspirate medium from the insert.
2. Using a cotton swab pre-wetted in DPBS –/–, scrape the cells off from the top of the insert.
3. Transfer the carrier containing inserts to an unused 24-well plate and place the inserts in the bottommost position.
4. Using the DAPI and RFP filters, visualize the invading prostate cancer cells as well as endothelial cells using the EVOS M7000 Imaging System for automated image capture.

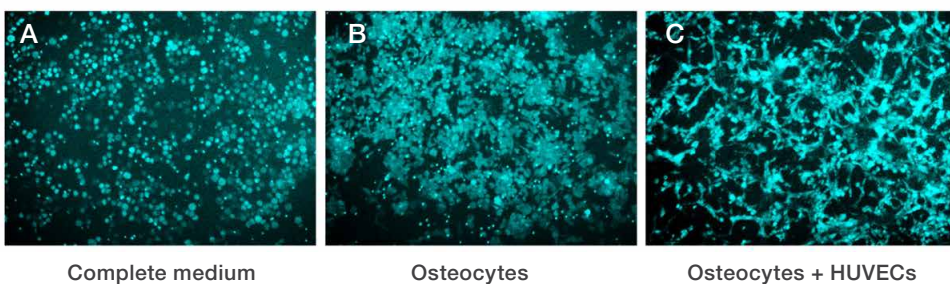
#### Tip: Use CellTracker dyes in this setup

- The use of CellTracker dyes in different colors helps visualize and differentiate between multiple cell types in the same field. In this study, use of CellTracker Red and Blue dyes helped distinguish between endothelial cells and the invading prostate cancer cells in the same field.

- The traditional method of visualizing invading cells requires fixing of the cells and staining with crystal violet stain. The use of CellTracker dyes allows for visualization of cell invasion in real time, thus providing a more convenient acquisition and quantitative analysis with live-cell imaging.



**Figure 6. Visualization of trans-endothelial migration of prostate cancer cells in the bone metastasis model.** Migration of PC-3 cells in response to (A) serum-free medium, (B) complete medium containing 10% fetal bovine serum, and (C) osteocytes. (D) Trans-endothelial migration of PC-3 cells in response to osteocytes. Bottom panels are images captured using the EVOS M7000 Imaging System using the 10x objective and DAPI and RFP filters. Top panels are illustrations of the experimental design. The components are color coded as follows: PC-3 cells: light blue, ECM: dark blue, osteocytes: gray, HUVECs: red. PC-3 cells and HUVECs were labeled with CellTracker Blue and Red dyes, respectively, before initiating the experiment.



**Figure 7. CellTracker dye enables visualization of cell morphology upon migration.** Morphology of migrated PC-3 cells in response to (A) complete medium, (B) osteocytes only, and (C) in the bone metastasis setup. Images were captured using the EVOS M7000 Imaging System using the 10x objective.

#### Data visualization

The use of CellTracker dyes allows for visualization of the migrating live cancer cells (blue) and the endothelial cells (red) in the same field of view in real time. For example, in Figure 6, PC-3 cells, which are highly metastatic prostate cancer cells, are labeled with CellTracker Blue dye, while HUVECs are labeled with CellTracker Red dye. In presence of serum-free medium in the receiver well, very few cells migrated to the underside of the insert (Figure 6A). As expected, the presence of complete medium containing 10% FBS in the receiver well caused a significant number of PC-3 cells to migrate towards the chemoattractant (Figure 6B). The presence of osteocytes in the receiver well also caused a substantial number of PC-3 cells to migrate (Figure 6C). Moreover, the presence of both osteocytes and HUVECs in the co-culture system resulted in a distinctive migration pattern of PC-3 cells (Figure 6D).



The use of CellTracker dye also helped visualize the morphology of the migrated PC-3 cells in the triple co-culture setup. As seen in Figure 7, compared to the rounded morphology of the migrated cells in response to complete medium, PC-3 cells migrating in response to osteocytes show a mix of rounded and elongated cells. Interestingly, cells undergoing trans-endothelial migration in response to osteocytes were mostly elongated.

#### Notes:

- BM-MSCs should be used within 5–6 doublings from revival for the best osteogenic differentiation results.
- Seeding more than 5,000 cells/cm<sup>2</sup> of BM-MSCs for differentiation might result in lifting of the cells from the plastic by the end of 21 days.
- HUVECs should be healthy (>90% viable) before seeding on the underside of the inserts.

#### Ordering information

Product	Cat. No.
<b>Plastics</b>	
Nunc Cell Culture Inserts in Carrier Plate System	<a href="#">141006</a>
Nunc Cell-Culture Treated Multidishes	<a href="#">142475</a>
<b>Media, supplements, and reagents</b>	
DMEM, high glucose, GlutaMAX Supplement, pyruvate	<a href="#">10569010</a>
Human Large Vessel Endothelial Cell Basal Medium	<a href="#">M200500</a>
Large Vessel Endothelial Supplement	<a href="#">A1460801</a>
CTS GlutaMAX-I Supplement	<a href="#">A1286001</a>
CTS DPBS with calcium, magnesium	<a href="#">A1285801</a>
CTS DPBS without calcium, magnesium	<a href="#">A1285601</a>
Penicillin-Streptomycin	<a href="#">15140122</a>
Fetal Bovine Serum	<a href="#">26140079</a>
StemPro MSC SFM XenoFree	<a href="#">A1067501</a>
StemPro Osteogenesis Differentiation Kit	<a href="#">A1007201</a>
TrypLE Express Enzyme (1X), phenol red	<a href="#">12605010</a>
CTS TrypLE Select Enzyme	<a href="#">A1285901</a>
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	<a href="#">A1413201</a>
CELLstart Substrate	<a href="#">A1014201</a>
CellTracker Red CMTPX Dye	<a href="#">C34552</a>
CellTracker Blue CMAC Dye	<a href="#">C2110</a>
<b>Equipment</b>	
Countess 3 Automated Cell Counter	<a href="#">AMQAX2000</a>
EVOS M7000 Imaging System	<a href="#">AMF7000</a>
<b>Cells</b>	
PC-3 prostate cancer cell line	<a href="#">CRL-1435 (ATCC)</a>
Human Umbilical Vein Endothelial Cells	<a href="#">C0035C</a>
StemPro BM Mesenchymal Stem Cells	<a href="#">A15652</a>
<b>Miscellaneous</b>	
Cotton swabs and forceps	Multiple vendors

#### Conclusion

Nunc cell culture inserts in a carrier plate system enables growth of more than one cell type in a single setup, thus aiding in making complex disease models. Using this system, we have shown a step-by-step method for establishing a physiologically relevant model for bone metastasis, using prostate cancer cells, osteocytes, and endothelial cells. The prostate cancer cells migrated differently towards the bone cells in the presence and absence of the endothelial cell barrier [9,12]. This process can also be visualized via live cell imaging, whereby CellTracker dyes help differentiate the various cell types. Overall, this protocol will help gain a holistic understanding of the events and molecules involved in the process of cancer cell migration towards bone [13–15].

## Authors

Isha Dey, Matthew Dallas, Suchitra Sajja, Kanika Singh, and Rayees B. Ahamed, Thermo Fisher Scientific

## References

- Berish RB, Ali AN, Telmer PG et al. (2018) Translational models of prostate cancer bone metastasis. *Nat Rev Urol* 15(7):403–421.
- Coleman RE (2006) Clinical features of metastatic bone disease and risk of skeletal morbidity. *Clin Cancer Res* 12(20):6243s–6249s.
- Iuliani M, Simonetti S, Ribelli G et al. (2020) Current and emerging biomarkers predicting bone metastasis development. *Front Oncol* 10:789.
- Mishra A, Shiozawa Y, Pienta KJ et al. (2011) Homing of cancer cells to the bone. *Cancer Microenviron* 4(3):221–235.
- Khodarev NN, Yu J, Labay E et al. (2003) Tumour-endothelium interactions in co-culture: coordinated changes of gene expression profiles and phenotypic properties of endothelial cells. *J Cell Sci* 116(6):1013–1022.
- Mierke CT (2008) Role of the endothelium during tumor cell metastasis: is the endothelium a barrier or a promoter for cell invasion and metastasis? *J Biophys* 2008:183516.
- Orr FW, Wang HH, Lafrenie RM et al. (2020) Interactions between cancer cells and the endothelium in metastasis. *J Pathol* 190(3):310–329.
- Molla MS, Katti DR, Katti KS (2019) An *in vitro* model of prostate cancer bone metastasis for highly metastatic and non-metastatic prostate cancer using nanoclay bone-mimetic scaffolds. *MRS Advances* 4(21):1–7.
- Wang W, Yang X, Dai J et al. (2019) Prostate cancer promotes a vicious cycle of bone metastasis progression through inducing osteocytes to secrete GDF15 that stimulates prostate cancer growth and invasion. *Oncogene* 38(23):4540–4559.
- Zhang X (2019) Interactions between cancer cells and bone microenvironment promote bone metastasis in prostate cancer. *Cancer Commun* 39(1):76.
- Wang M, Xia F, Wei Y et al. (2020) Molecular mechanisms and clinical management of cancer bone metastasis. *Bone Res* 8(1):30.
- Sznurkowska MK, Aceto N (2021) The gate to metastasis: key players in cancer cell intravasation. *FEBS J*.
- Thomas RJ, Guise TA, Yin JJ et al. (1999) Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology* 140(10):4451–4458.
- Yang C, Xia B-R, Jin W-L et al. (2019) Circulating tumor cells in precision oncology: clinical applications in liquid biopsy and 3D organoid model. *Cancer Cell Int* 19:341.
- Yap Y-S, Leong MC, Chua YW et al. (2019) Detection and prognostic relevance of circulating tumour cells (CTCs) in Asian breast cancers using a label-free microfluidic platform. *PLoS One* 14(9):e0221305.