

In Vitro Modeling of the Interaction between Bone Marrow and Disseminated Prostate Cancer Cells



Dissertation

zur Erlangung des Doktorgrades
der Biomedizinischen Wissenschaften
Dr. rer. physiol.

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Susanna Maria Lissek
aus
Frankfurt am Main

Regensburg, den 26. Februar 2025

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Dekan: Prof. Dr. Dirk Hellwig

Betreuer: Prof. Dr. med. Christoph A. Klein

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***Erst wenn wir den unermesslichen Wert jedes Lebewesens vollständig
anerkennen, eröffnen sich uns neue Wege – denn wahrer Fortschritt beginnt mit
Empathie.***

Persönliches Statement

“Ask the experimenters why they experiment on animals, and the answer is: ‘Because the animals are like us.’ Ask the experimenters why it is morally okay to experiment on animals and the answer is: ‘Because the animals are not like us.’ Animal experimentation rests on a logical contradiction”

Zitat von Charles R. Magel Philosophy Professor

Abbreviations

ACK Ammoniumchlorid-Kalium

APC Allophycocyanin

AR androgen receptor

bFGF fibroblast growth factor-basic

BFP blue fluorescent protein

BM bone marrow

BOECs blood outgrowth endothelial cells

BSA bovine serum albumin

CB-EPCs cord blood-derived endothelial progenitor cells

CK Cytokeratin

CPC calcium phosphate cement

CRPC castration-resistant prostate cancer

CTCs circulating tumor cells

DCCs disseminated cancer cells

DCCD disseminated cancer cells density

ECFCs endothelial colony forming cells

ECM extracellular matrix

EGF epidermal growth factor

EGM-2 endothelial growth medium

ELISA enzyme-linked immunosorbent assay

EpCAM epithelial adhesion molecule

EPCs endothelial progenitor cells

Etdh1 ethidium homodimer 1

FACS fluorescently activated cell sorting

FCS fetal bovine/calf serum

FHL2 four and a half LIM domains 2

GFP green fluorescent protein

hbECM human bone extracellular matrix

HBSS Hanks' balanced salt solution

hPL human platelet lysate

HPLM human plasma-like medium

HSCs hematopoietic stem cells

HUVECs human umbilical vein endothelial cells

IGF-1 insulin-like growth factor

iMSCs immortalized MSCs

iPSCs induced pluripotent stem cells

LSM laser scanning microscopy

MACS magnetic cell sorting

Matrigel basement membrane matrix derived from the Engelbreth-Holm-Swarm mouse sarcoma

max-z maximum intensity projection

MM master mix

MNCs mononuclear cells

MSCs mesenchymal stromal cells

O-MSCs osteogenic mesenchymal stromal cells

PBMCs peripheral blood mononuclear cells

pB-EPCs peripheral blood-derived endothelial progenitor cells

PBS phosphate buffered solution

PDGF- $\beta\beta$ platelet-derived growth factor BB

PDX patient-derived xenograft

PFA paraformaldehyde

P/S penicillin-streptomycin

RT roomtemperature

TGF- β transforming growth factor beta

WTA whole transcriptomic amplification

wBM whole bone marrow

YFP yellow fluorescent protein

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1 Introduction

1.1 Cancer and the metastatic colonization

Cancer remains a major global health concern, ranking among the top two causes of mortality worldwide alongside cardiovascular diseases [1]. In men, prostate cancer remains one of the most commonly diagnosed cancers and a significant contributor to cancer-related mortality [2]. Although cancer is mainly characterized by its uncontrolled cellular proliferation driven by transformed cells that evolve through natural selection [3], it is the dissemination and colonization of malignant cells at distant anatomical sites that primarily drive cancer-related fatalities. Metastasis is a dynamic and heterogeneous process in which cancer cells acquire traits for dissemination, dormancy escape, and immune evasion, ultimately leading to fatal outcomes, since unlike primary tumors, which can often be cured with localized therapies, metastatic cancer is a systemic disease that disrupts multiple organs by colonizing them directly or altering their metabolism through secreted factors [4]. Rooted in Stephen Paget's "seed and soil" theory, the concept of site-specific metastasis emphasizes that metastasizing cancer cells (the "seeds") can only flourish in supportive organ microenvironments (the "soil") [5]. Different cancers exhibit distinct metastatic behaviors in terms of latency, target organ preference, and lesion characteristics. Prostate cancer, for example, follows a slow-progressing metastatic pattern, often relapsing many years or even decades after the initial diagnosis, with a strong tendency for bone as the primary metastatic site. Breast cancer also metastasizes to bone; however, while prostate cancer metastases predominantly induce osteoblastic lesions, breast cancer metastases are more commonly associated with osteolytic lesions. In contrast, lung cancer metastasizes rapidly, often within months, and primarily spreads to the brain [6].

Decades of research have established the pivotal role of the tumor microenvironment,

particularly the bone marrow (BM) niche in metastatic colonization, where bidirectional crosstalk between disseminated cancer cells (DCCs) and the bone stroma influences key processes such as homing, seeding, dormancy, and metastatic outgrowth [7]. Within this specialized microenvironment, stromal cells, extracellular matrix components, and signaling molecules create a supportive niche that provides essential nutrients and growth factors, enabling DCC survival and proliferation [7]. The presence of DCCs in the BM or lymph nodes can be detected as early as the time of primary tumor removal, and is clinically associated with shorter overall survival and poorer outcomes in prostate cancer patients [8, 9, 10]. Given that prostate cancer often progresses slowly over more than a decade before distant metastases become clinically apparent [11], understanding these microenvironmental interactions in more detail is especially critical for developing better targeted adjuvant therapies to inhibit metastatic outbreaks. However, detecting these early events remains challenging because DCCs are exceptionally rare - approximately one in a million BM cells [12] - which complicates both their detection and functional manipulation. While advances in single cell analysis have provided more detailed profiles of the genetic attributes of DCCs, many questions persist. For instance, the unexpected expression of hematopoietic markers by DCCs suggests a highly plastic phenotype that is not yet fully understood [9, 10]. Furthermore, physiologically relevant models that can maintain and expand these early colonizing cells are still lacking. Therefore, developing functional systems for experiments with patient derived DCCs is essential, as it would not only deepen our basic understanding of metastatic colonization but also inform the development of novel therapeutic strategies aimed at preventing metastatic outgrowth.

1.2 Models in metastasis research

Given the distinct metastatic behaviors of different cancer types, understanding the mechanisms governing metastasis requires robust experimental models. Metastasis is a complex multistep process that involves early dissemination, immune evasion, and outgrowth at distant sites [13]. No single experimental model can fully recapitulate all aspects of human metastatic progression [14]. Therefore, combining *in vitro* and *in vivo* approaches is essential for obtaining comprehensive insights into metastatic dynamics and identifying potential therapeutic vulnerabilities.

To achieve this, researchers utilize a diverse range of models, each designed to address specific aspects of metastasis while navigating inherent limitations. Traditional two-dimensional cell culture systems remain a cornerstone of cancer research due to their reproducibility, scalability, and cost-effectiveness. However, they fail to capture the complexity of the *in vivo* environment, particularly the three-dimensional architecture and microenvironmental cues that regulate tumor progression. This limitation is especially evident in their inability to fully replicate critical cell–cell and cell–matrix interactions, which are key drivers of metastasis [15, 16, 17]. In contrast, 3D cell culture systems incorporate fundamental features that influence cell structure, adhesion, mechanotransduction, and signaling in response to soluble factors, thereby regulating overall cellular function in ways that differ dramatically from traditional 2D formats [18]. Advancements in bioengineering have led to the development of various 3D models that incorporate elements of the natural cellular environment. These models range from multicellular spheroids and organoids to scaffold-based co-culture systems, and multiorgan chips that integrate microfluidics, thereby offering a diverse array of platforms capable of more faithfully recapitulating physical relevant conditions [17, 19]. Despite these advancements, many researchers still rely on murine models for studying complex diseases like cancer, particularly the metastatic cascade. Such models include cell line injection models, bone implant models, and patient-derived xenograft (PDX) models [20]. Although animal models overcome many limitations of simple culture systems by providing a whole-organism physiological context that includes complex microenvironments and, in some cases, partially humanized immune systems [21, 22], they suffer from species-specific differences that can limit the direct translation because in many times cancer cell lines are used instead of patient derived cancer cells. In contrast to the widespread reliance on mouse models, *in vitro* platforms have successfully generated patient-derived circulating tumor cell models, including organoid-based prostate cancer models [23, 24] and lung cancer microfluidic systems [25]. However, because circulating tumor cells (CTCs) are typically rare in nonmetastatic stages, their expansion is generally only feasible in patients with advanced disease, regardless of whether *in vitro* or *in vivo* methods are used. Expanding DCCs from nonmetastatic patients would be highly valuable for gaining deeper insights into the reactivation mechanisms of early metastasis founder cells. Since, attempts to culture and expand BM derived DCCs from nonmetastatic patients have largely been unsuccessful [12], with only anecdotal reports

[26, 27] highlighting their distinct biology. Recent studies suggest that pre-malignant or dormant DCCs in the BM may require additional genomic or phenotypic alterations to transition into overt metastases [12]. Understanding their interactions with the BM niche and the factors driving their reactivation is crucial, as this clinically dormant phase can turn into late relapses years after treatment. Therefore, the development of dedicated *in vitro* models that accurately mimic key features of the BM microenvironment is essential to advance functional studies on patient-derived M0-stage DCCs.

1.3 *In vitro* models of bone marrow

The bone microenvironment is complex and consists of two main tissue types, cortical bone and trabecular bone, along with a dynamic extracellular matrix composed of organic components such as proteoglycans, collagen, osteonectin, and osteocalcin, and inorganic minerals like hydroxyapatite. The bone microenvironment is continuously remodeled and comprises a wide range of resident cells such as osteoblasts, osteoclasts, osteocytes, immune cells, and adipocytes that collaborate with a complex vascular network. This intricate system generates specialized microenvironments that support the production of blood cells, maintain stem cell populations, and foster the survival of disseminated cancer cells [28]. Within the BM, two critical niches have been identified: the endosteal niche and the perivascular niche [7]. The endosteal niche is predominantly formed by bone-forming cells, and the perivascular niche, defined by endothelial and stromal cells enriched with CXCL12 and stem cell factors. Both niches are essential for regulating cell fate and function and conventional two-dimensional cell culture systems fail to adequately replicate the complex three-dimensional architecture of bone or the intricate cell–matrix and cell–cell interactions essential for its microenvironment. This limitation has driven the development of more advanced *in vitro* models [29].

Several approaches have been explored to mimic the BM microenvironment for studying prostate cancer metastasis. One scaffold-based study utilized 3D-printed polycaprolactone (PCL) frameworks with human osteoprogenitor and prostate cancer cell line cells, demonstrating that cancer–bone stroma interactions and responses to antiandrogens such as bicalutamide and enzalutamide, influence tumor progression in a mineralized setting [30]. Another approach used PCL/hydroxyapatite-nanoclay

based 3D scaffolds seeded with human mesenchymal stromal cells (MSCs) to recreate a bone-like microenvironment, revealing that less aggressive prostate cancer cell line derived cells formed multicellular tumoroids, whereas highly metastatic PC-3 cells proliferated extensively without tumoroid formation. These findings highlight the potential of scaffold-based models to capture key aspects of tumor–bone interactions and therapeutic responses [31].

Such methods effectively replicate the mineralized endosteal niche; however, they often lack a vascular component, which is essential for nutrient delivery, waste removal, and cell trafficking in the BM. To overcome this limitation, researchers have developed more sophisticated models that integrate both endosteal and perivascular elements. Braham et al. [32] employed bioprinted calcium phosphate cement (CPC) scaffolds seeded with osteoblasts and incorporated endothelial progenitor cells (EPCs), MSCs, and hematopoietic stem cells (HSCs) within a Matrigel matrix to create a balanced mineralized and vascularized microenvironment for multiple myeloma research. Similarly, Baldassarri et al. [33] utilized decellularized bovine bone scaffolds layered with a fibrin hydrogel containing MSCs, endothelial cells, and a breast cancer cell line to reconstruct both the mineralized and vascular components of the BM niche. Their study demonstrated that breast cancer cells exhibited higher proliferation rates in osteoblast-enriched conditions compared to the combined niche containing both osteoblasts and perivascular-like elements. These integrative approaches significantly enhance the physiological relevance of *in vitro* models, providing a more accurate platform for studying metastatic colonization and tumor–microenvironment interactions.

Another interesting strategy for generating BM *in vitro* involves organoid models derived from induced pluripotent stem cells (iPSCs). These self-organizing three-dimensional structures develop vascular, hematopoietic, and mesenchymal compartments that emulate key features of the human BM niche, including the endothelial-to-hematopoietic transition and multilineage hematopoiesis [34]. However, this approach lacks the inorganic bone component, which is essential for fully recapitulating the biomechanical and structural properties of the native BM microenvironment.

BM microenvironment, several limitations remain that underscore the need for further refinement and innovation. One notable issue is the extensive reliance on cancer cell lines. While cell lines are often favored for their perceived reproducibility, Ben-David et al. have demonstrated significant clonal variation among clones derived from

the same cell line across different laboratories, revealing extensive clonal diversity and challenging the assumption of consistency [35]. Although these cell lines remain valuable for development and optimization, their extensive use may be insufficient for uncovering the deeper mechanistic aspects of cancer biology, particularly when it comes to understanding the behavior of DCCs. In fact, all of the cancer models described above have relied on cancer cell lines to study various aspects of tumor progression and metastasis. In animal models, investigating metastatic processes, particularly in the context of DCCs, also commonly involves the injection of cancer cell lines. This is typically performed either as xenografts, where human cancer cell lines are implanted into immunodeficient mice, or as syngeneic models, in which murine cancer cell lines are introduced into immunocompetent mice [36]. While widely used, these approaches may not accurately replicate metastatic processes in patients, raising concerns about their translational relevance, particularly in capturing the complex biology of human DCCs.

Another concern of *in vitro* BM models is the underrepresentation of patient- or tissue-specific MSCs and most importantly endothelial cells. While some studies incorporate MSCs derived from BM, these cells are typically obtained from healthy donors rather than from patients. Moreover, the endothelial compartment is almost exclusively modeled using human umbilical vein endothelial cells (HUVECs), which do not accurately reflect the specialized endothelial environment of the BM. The suitability of HUVECs as a universal model should be questioned, as endothelial cells from different vascular sources exhibit distinct gene expression profiles and functional responses [37].

And the final point to mention as limitation in *in vitro* models is the widespread use of animal-derived materials, such as fetal calf serum (fetal bovine/calf serum (FCS)), which raises further concerns regarding the physiological relevance of these models. The undefined composition of FCS introduces variability, increases the risk of contamination, and may significantly influence experimental outcomes [38, 39]. A study comparing transcriptomic profiles of MSCs cultured with FCS or human platelet lysate (hPL) identified 69 significantly differentially expressed genes, with notable enrichment especially in extracellular matrix-related pathways. While their findings suggested that culture media do not select for distinct MSCs subpopulations, the differential expression of ECM-associated genes[40], along with the broader concerns regarding *in vitro* modeling, raises a critical question: to what extent do artificial conditions, such as the use of

animal-derived serum and non-patient-specific cell lines, compromise the ability of *in vitro* models to accurately replicate the human physiological microenvironment?

While current *in vitro* models provide valuable insights, their reliance on artificial culture conditions, including animal-derived materials and non-patient-specific cell lines, raise serious concerns about their physiological relevance. In the long run, transitioning to patient-derived models represents the safest approach to achieve a more accurate representation of both human and patient-specific biology in order to advance our understanding of DCC biology and developing novel adjuvant therapies.

1.4 Goal of this thesis

As detailed in the introduction, existing models for investigating DCCs within their native microenvironment are limited. Traditional two-dimensional culture systems and animal models often fall short in replicating the intricacies of the human bone marrow environment, which creates substantial challenges when studying DCCs originating from prostate cancer patients. Therefore, the overall goal of this thesis was to establish a physiologically relevant *in vitro* BM model to support the ex vivo culture and functional studies of these rare DCC populations. By investigating how DCCs interact with the BM niche during early stages of bone metastasis, this work aimed to clarify processes underlying tumor dormancy, reactivation, and progression.

In pursuit of this goal, emphasis was placed on using human-derived materials to closely mimic the BM environment. Patient-derived BM aspirates were collected (with informed consent) from prostate cancer patients undergoing primary tumor resection. However, due to the limited availability of patient samples and the very low abundance of DCCs in each specimen, prostate cancer cell lines were employed in certain optimization steps. Ultimately, the objective was to isolate epithelial adhesion molecule (EpCAM)-positive DCCs from patient-derived BM aspirates and incorporate them into the *in vitro* BM model for expansion and functional assays (**Figure 1.1, A**).

As a starting point, a three-dimensional BM niche model originally developed by Gelinsky and Alblas et al. [32] was replicated. This model employs a CPC scaffold co-seeded with osteoblasts, MSCs, and EPCs in a two-niche setup, providing a robust foundation for customization through timed or spatial incorporation of additional cell

populations. However, significant optimization was required to adapt the model to the unique requirements of prostate cancer DCCs. In particular, challenges such as extremely low cancer cell numbers, the limited lifespan of patient-derived primary cells, the aim to replace animal-derived components with human-derived alternatives, and the implementation of appropriate analytical techniques had to be overcome. Accordingly, three specific aims were addressed (**Figure 1.1, B**):

- **SA1: Developing BM model conditions:** A two-niche BM model was established to integrate prostate cancer DCCs by optimizing technical parameters, refining quantification methods, and replacing animal-derived components with exclusively human-derived materials to more accurately replicate the human BM microenvironment.
- **SA2: Patient-specific applications in BM models:** Integration of patient-derived cells into the BM model by initially using cells from M1-stage patients' BM or bone metastasis biopsies to refine culture conditions and address the significant challenges of cultivating M0-stage DCCs
- **SA3: Microenvironmental studies:** Conducting functional studies to evaluate how distinct BM niche components and EpCAM expression influence cancer cells, starting with experiments on prostate cancer cell lines to identify key microenvironmental factors that support the expansion of patient-derived tumor cells.

By systematically optimizing the 3D BM niche model and integrating patient-specific materials, this thesis sought to overcome the limitations of conventional systems and advance the study of prostate cancer DCC.

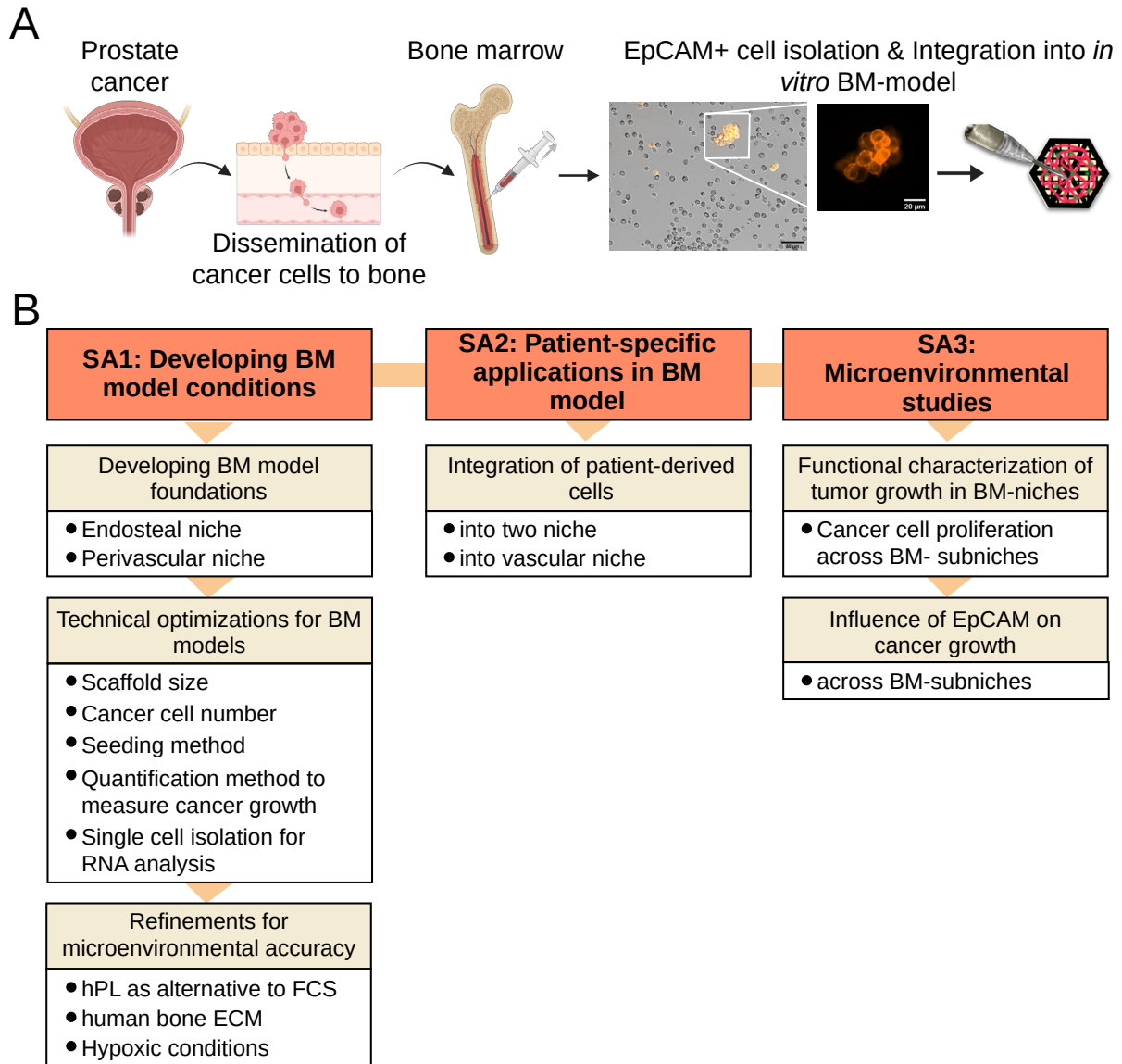


Figure 1.1: **Overview of the goal of the study.** Patient-derived bone marrow aspirates are used to isolate EpCAM-positive DCCs, which are subsequently integrated into the *in vitro* BM model. Figure created with BioRender, with additional images of DCCs and the scaffold provided by the author

2 Materials

2.1 Material

2.1.1 Media composition

2.1.1.1 MSC Expansion Medium

Table 2.1: MSC Expansion Medium Composition

Component	Final Concentration
α -MEM (22561 Gibco, with 2 mM L-glut)	-
FCS or hPL	10%
L-ascorbic-acid-2-phosphate	0.2 mM
penicillin-streptomycin (P/S)	1%
fibroblast growth factor-basic (bFGF) (add fresh)	1 ng/mL

2.1.1.2 Osteogenic Medium

Table 2.2: MSC Osteogenic Medium Composition

Component	Final Concentration
α -MEM (22561 Gibco, with 2 mM L-glutamine)	-
FCS or hPL	10%
L-ascorbic-acid-2-phosphate	0.2 mM
P/S	1%
β -glycerophosphate	10 mM
Dexamethasone (D8893, Sigma)	10 nM (10^{-8} M)

2.1.1.3 Adipogenic Medium

Table 2.3: MSC Adipogenic Medium Composition

Component	Final Concentration
α -MEM (22561 Gibco, with 2 mM L-glut)	-
FCS or hPL	10%
P/S	1%
Dexamethasone (D8893, Sigma)	1 μ M (10^{-6} M)
IBMX (I7378, Sigma)	0.5 mM
Indomethacin (I5879, Sigma)	0.2 mM
Insulin (I0516, Sigma)	1.72 μ M

2.1.1.4 Endothelial Growth and Two-Niche Medium

Table 2.4: Endothelial Growth and Two-Niche Medium Composition

Component	Final Concentration
EBM™ Basal Medium (CC-3156)	-
EBM™ SingleQuots™ Supplements (Lonza CC-4176) - leaving out Gentamicin sulfate-Amphotericin (GA-1000)	-
FCS or hPL	10%
P/S	1%
Heparin	only for hPL: 10 U/ml

2.1.1.5 Media for PC3 & LNCaP cell line

Table 2.5: PC3 & LNCaP cell media Composition

Component	Final Concentration
RPMI 1640	-
FCS	10%
L-Glutamine	2 mM
P/S	1%

2.1.1.6 Media for VCaP cell line

Table 2.6: VCaP cell media Composition

Component	Final Concentration
DMEM (PAN/P04-03596 w:4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate)	-
FCS	10%
P/S	1%

2.1.1.7 Media for tumor digestion

Table 2.7: Tumor digestion media Composition

Component	Final Concentration
DMEM/Hams's F12	-
bovine serum albumin (BSA)	1%
Hepes	10 nM
P/S	1%

2.1.2 Chemicals and commercial solutions

Table 2.8: Reagents

Name	Company	Catalogue
α -MEM	Gibco	22561
ACK lysis buffer	Gibco	A1049201
Alexa Fluor Plus 647 Phalloidin	Invitrogen	A30107
Alizarin Red S	Sigma	A5533
Amphotericin B	Gibco	15290-018
BD Cell Recovery Solution	Corning	354253
bFGF	Peptotech	AF-100-18B
BSA fraction V	VWR	441555J
Cetylpyridium chloride monohydrate	Sigma	C9002
Chloroform		
Collagen I	Corning	354236
Collagen I	Roche	11179179001

Continued on next page

Table 2.8 – continued from previous page

Name	Company	Catalogue
Collagenase	Sigma	C9407
Cryo-SFM	PromoCell GmbH	C-29912
DAPI	Sigma	MBD0015
Dexamethasone	Sigma	D8893
Dihydrotestosteron (5a-Androstan-17 ¹² -ol-3-one)	Sigma	A8380
Dispase 5 U/ml (in Hank'Balanced Salt Solution)	STEMCELL Technologies	7913
DMEM/Hams F12 Medium	PAN Biotech	P04-41500
DMEM w: 4 mM L-glutamine 4500 mg/L glucose 1 mM sodium pyruvate and 1500 mg/L sodium bicarbonate	PAN Biotech	P04-03596
eBioscience Fixable Viability Dye eFluor 450	Invitrogen	65-0863-14
EGM®-2 Endothelial Cell Growth Medium-2 BulletKit®	Lonza	CC-3162
Fetal bovine/calf serum - FBS Good	PAN Biotech	P40-37500
Fetal bovine/calf serum - FBS SeraPlus	PAN Biotech	P30-3702
FicollPaque Plus		
Freezing medium CryoSFM	Promocell	C29910
Geltrex LDEV-freie Basalmembran-Matrix mit reduziertem Wachstumsfaktor	Thermo Fisher Scientific	A1413202
Gentamicin	Gibco	15750060
Glycerin-2-phosphat Dinatriumsalz Hydrat (β-Glycerophosphate)	Sigma	G9422-50G
Heparin	Sigma	H3393
Hepes	Gibco	15630106
human AB serum	Bio Rad	805135

Continued on next page

Table 2.8 – continued from previous page

Name	Company	Catalogue
human Platelet Lysate (hPL)	selfmade	
Hyaluronidase	Sigma Aldrich	H4272
IBMX	Sigma	I5879
Indomethacine	Sigma	I7378
Insulin human	Sigma	I9278
L-ascorbic-acid-2-phosphate	Sigma	A8960-5G
Liberase TL	Roche	5401020001
Live/Dead Viability/Cytotoxicity Kit for mammalian cells	Invitrogen	L3224
Matrigel	Corning	356231
Matrigel	Corning	354263
Methanol		
Oil Red O	Sigma	O0625
Paraformaldehyde	Sigma	P6148-500G
Penicillin (10.000U/ml) / Streptomycin (10mg/ml)	PAN Biotech	P06-07100
Percoll	Thermo Fisher Scientific	17089101
Prostaglandin E2	Peptotech	3632464
RPMI	PAN Biotech	P04-17500
Thrombin from human plasma	Sigma	T6884-100UN
Trehalose	Carl Roth	8897.1
Trypan blue	Sigma Aldrich	T8154-20ml
TrypLE	Gibco	12604-013
Trypsin/ EDTA (10x) (0.05% Trypsin/ 0.02% EDTA)	PAN Biotech	P10-024100

Continued on next page

Table 2.8 – continued from previous page

Name	Company	Catalogue
Vybrant Multicolor Cell-Labeling Kit	Invitrogen	V22889

Table 2.9: Extracellular matrices used in 3D cell culture

Name	Company	catalogue	comments
Corning® Matrigel® Matrix High Concentration (HC), Growth Factor Reduced (GFR) *LDEV-free, 10 mL	Corning	354263	In the Braham et al. publications [32, 41, 42], this matrigel formulation Matrigel was used at a 50% dilution. However, due to handling difficulties, an alternative Matrigel formulation was used following the failure of initial tests
Corning® Matrigel® Growth Factor Reduced (GFR) Phenol-Red free 10ml	Corning	356231	used without dilutions, protein concentrations varied from 8.6 - 9.7 µg/ml
Geltrex LDEV-freie Basalmembran-Matrix mit reduziertem Wachstumsfaktor	Thermo Fisher Scientific	A1413202	used only during supply shortage of Corning Matrigel

2.1.3 Antibodies & microbeads

Table 2.10: Antibodies & microbeads

Name	Company	Catalogue
Brilliant Violet 421 anti-human CD146 Antibody	Biolegend	361003
Brilliant Violet 421 anti-human CD73 (Ecto-5'-nucleotidase) Antibody	Biolegend	344007
CD31 Microbead Kit Human	Miltenyi Biotec	130-091-935
FITC anti-human CD90 (Thy1) Antibody	Biolegend	328107
PE anti-human CD34 Antibody	Biolegend	343505
PE/Cyanine7 anti-human CD105 Antibody	Biolegend	323217
PE/Dazzle 594 anti-human CD309 (VEGFR2) Antibody	Biolegend	359917
PerCP/Cyanine5.5 anti-human CD45 Antibody	Biolegend	304027
Purified anti-human CD309 (VEGFR2) Antibody	Biolegend	359902
Purified Mouse IgG1 Isotype Ctrl Antibody	Biolegend	400101
recombinant CD31	Invitrogen	MA5-29475
human CD11b-APC (Clone: M1/70.15.11.5)	Miltenyi Biotec	130-091-241
human CD235a (Glycophorin A) MicroBeads	Miltenyi Biotec	130-050-501
human (Clone: HEA-125) CD326 (EpCAM)-PE	Miltenyi Biotec	130-098-118
human CD33-APC (Clone: AC104.3E3)	Miltenyi Biotec	130-091-731
Anti-APC-MicroBeads	Miltenyi Biotec	130-090-855
human CD45-APC	Invitrogen	MHCD4505-4
Anti-FHL2	Sigma (Prestige Antibodies)	HPA006028
CD34 Microbead Kit Human	Miltenyi Biotec	130-046-702

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Table 2.10 – continued from previous page

Name	Company	Catalogue
TotalSeq™-B0251 anti-human Hashtag 1 Antibody	Biolegend	394631
TotalSeq™-B0252 anti-human Hashtag 2 Antibody	Biolegend	394633
TotalSeq™-B0253 anti-human Hashtag 3 Antibody	Biolegend	394635
TotalSeq™-B0254 anti-human Hashtag 4 Antibody	Biolegend	394637
TotalSeq™-B0255 anti-human Hashtag 5 Antibody	Biolegend	394639
TotalSeq™-B0256 anti-human Hashtag 6 Antibody	Biolegend	394641
TotalSeq™-B0257 anti-human Hashtag 7 Antibody	Biolegend	394643

2.1.4 Consumables

Table 2.11: Consumables

Name	Company	Catalogue
Rotilabo® syringe filter PVDF sterile 22 µm	ROTH	P666.1
Rotilabo® syringe filter PVDF sterile 45 µm	P667.1	
Injekt® with luer lock 10 ml	Roth	58.1
Cell Strainer 40µm	Becton Dickinson	352340
Cellstar® serological pipette 2 ml	Greiner Bio-One	710180
Cellstar® serological pipette 5 ml	Greiner Bio-One	606180
Cellstar® serological pipette 10 ml	Greiner Bio-One	607180
Cellstar® serological pipette 25 ml	Greiner Bio-One	760180
Centrifuge tube 15 ml	Greiner Bio-One	188271
Centrifuge tube 50 ml	Greiner Bio-One	227261
MACS Separation Columns LS	Miltenyi Biotec	130-042-401

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Table 2.11 – continued from previous page

Name	Company	Catalogue
Cryogenic Tubes	Thermo Fisher Scientific	377267
Suspension culture plates 24 well	Sigma CellStar Greiner	M9312
6 Well Cell Culture Plate	Greiner Bio-One	657160
24 Well Cell Culture Plate	Greiner Bio-One	662160
Pre-Separation Filters (30 µm)	Miltenyi Biotec	130-041-407
MACS Separation Columns MS	Miltenyi Biotec	130-042-201
VWR Zellkulturplatten 96-Well-F steril	VWR	734-2327

2.1.5 Devices

Table 2.12: Devices

Device	Company	Model
Laser scanning microscope	Zeiss	LSM980
Automatic cell counter	ThermoFisher	Countess III
Inverted fluorescence microscope	Olympus	IX81
Micromanipulator	Eppendorf	PatchMan NP 2
Flow Cytometer	Becton Dickinson	FACSCelesta
Flow Cytometer	Beckman Coulter	Gallios
Corning® CoolCell™ LX Zelleinfrierbehälter	Corning	432002

3 Methods

3.1 Cell culture of cell lines

3.1.1 PC3 cells

PC3 cells, along with the transduced clones PC3-GFP and PC3-EpIC-YFP, have been maintained in the laboratory for an extended period. To ensure their authenticity, the cells are routinely subjected to short tandem repeat (STR) marker analysis for authentication. All PC3 cell variants were cultured in expansion medium, with RPMI serving as the basal medium supplemented with FCS as the serum component (see Section 2.1.1.5). Cells were passaged upon reaching near-confluency, with the splitting ratio varying depending on experimental requirements, up to a maximum of 1:12.

3.1.2 Immortalized MSCs and EPCs

Immortalized and stably transduced MSCs, referred to as iMSCs (iMSC_HY-GSH17_BFP⁺), and bone marrow-derived EPCs, referred to as iEC (iEC_HY-GSH32_tdTomato-Luc⁺), were kindly provided by Dr. Hind Medyouf from Georg Speyer Haus, Frankfurt. Immortalized MSCs are labeled with blue fluorescent protein (BFP), and the iEC cells are labeled with tdTomato, enabling easier tracking and visualization.

Upon arrival, iMSCs were initially cultured in StemMACS™ MSC expansion medium (Miltenyi, Cat# 130-091-680) until a sufficient number of cells were cryopreserved. Subsequently, the culture medium was transitioned to the standard MSC expansion medium composition (**Table 2.1**). The iECs were cultured in endothelial growth medium (EGM-2) medium (Lonza) supplemented with 20% FCS until an adequate number of cryovials were preserved. Once an adequate stock was established, the serum concentration was reduced to the standard level of 10% FCS for subsequent experiments.

3.2 Cell culture of primary cells

3.2.1 Isolation protocols

3.2.1.1 Mesenchymal stromal cells: Isolation from BM aspirates

Human MSCs were isolated from bone marrow aspirates obtained with informed consent and approved by the University of Regensburg Ethics Committee (ethics vote number 18-948-101). Two isolation approaches were initially evaluated: direct isolation from whole bone marrow (wBM) and isolation from mononuclear cells (MNCs) following density gradient centrifugation.

1. *wBM*: For the wBM plating approach, only half of the fresh BM aspirate was allocated to the research project. The received sample had been pre-washed twice with Hanks' buffer. Approximately 1 mL of the sample was treated with ACK lysis buffer to remove erythrocytes. After lysis, the cells were counted, and 500,000 cells per cm² were seeded into cell culture flasks for expansion.

2. *MNCs*: The remaining BM aspirate was processed using density gradient centrifugation with a 65% Percoll solution ($\rho = 1.083 \text{ g/cm}^3$) to isolate MNCs (for details on the preparation of MNCs, see Section 3.2.1.3). The isolated MNCs were seeded at a density of 2 million cells per T75 cell culture flask, corresponding to 26,666 MNCs/cm². Within a few days, cell colonies began to form, and cells were expanded by replating after approximately 11 days in culture

3.2.1.2 Endothelial progenitor cells: Isolation from BM or peripheral blood

Attempts to isolate EPCs from BM:

1. *Differential adhesion strategy [43]*: MNCs isolated from BM aspirates were seeded into a culture flask and incubated for 24 hours to allow MSCs to adhere. The supernatant, containing EPCs and HSCs, was transferred to a Falcon tube and gently centrifuged at 500 ×g for 5 minutes. The pellet was resuspended in endothelial growth medium (EGM-2; Lonza) supplemented with 10% FCS and seeded at a density of 260,000 cells per well (136,842 cells/cm²) onto collagen I-coated wells of a 24-well plate to promote EPCs adherence.

2. *CD34 cell isolation using magnetic cell sorting (MACS)*: CD34² cells were isolated from

the MNCs fraction using magnetic cell sorting (MACS), following the manufacturer's instructions and 50 000 cells/ 24 well (26 315 cells/cm² were plated onto collagen I-coated plates and cultured in endothelial growth medium (EGM-2; Lonza).

3. *CD31 cell isolation using MACS*: CD31² cells were isolated from the MNCs fraction via magnetic cell sorting (MACS), following the manufacturer's instructions, with an initial input of approximately 70 million MNCs, yielding an output of 4.48 million CD31⁺ cells. The isolated cells were plated across all 24 wells of a 24-well plate at a density of approximately 98,245 cells/cm² onto collagen I-coated plates and cultured in endothelial growth medium (EGM-2; Lonza).

Isolation of EPCs from adult peripheral blood: For the vascular part of the model, EPCs are isolated from the peripheral blood of an adult male donor and characterized as blood outgrowth endothelial cells (BOECs). First, the MNCs fraction from 100 ml of peripheral blood was isolated by pre-diluting the blood 1:1 with pre-warmed phosphate-buffered saline solution (PBS) and carefully layering it onto Ficoll-Paque. The tubes were then centrifuged at $740 \times g$ for 30 minutes. Then, the interphase was carefully collected and washed twice with DMEM (low glucose). After the final washing, half of the MNCs were resuspended in EGM-2 medium supplemented with 20% fetal calf serum (FCS), 1% P/S, and the other half in EGM-2 medium supplemented with human platelet lysate (hPL), 1% P/S, and 10 U/ml heparin (only in the hPL-supplemented medium). Before seeding, the 24-well plates were coated with 5 $\mu\text{g}/\text{cm}^2$ Collagen I (Corning) for 1 hour at roomtemperature (RT). The seeding density was 6.25×10^6 cells per well (3.3×10^6 cells/cm²). For the first five days, the medium was changed daily. Subsequently, the medium was changed every 2-3 days until colonies appeared (approximately around day 10). As soon as a colony was large enough, the cells were transferred into a 12-well plate and cultured until confluent. By passage 3, the cells were sufficiently expanded to be cryopreserved.

3.2.1.3 Disseminated cancer cells: Isolation from BM or metastasis samples

BM aspirates: Bone marrow aspirates were obtained with informed consent and in compliance with the approval of the University of Regensburg Ethics Committee (ethics vote number 18-948-101). A maximum of 10 mL of bone marrow was transferred into a 50 mL tube and diluted with Hanks' balanced salt solution (HBSS). The sample was then

centrifuged at $200 \times g$ for 10 minutes at 4°C (acceleration: 9, brake: 3). After a second wash with HBSS, the supernatant was removed as thoroughly as possible. Next, the bone marrow was overlaid onto 65% Percoll solution ($\rho = 1.083 \text{ g/cm}^3$). To determine the number of Percoll tubes needed, the total volume of the bone marrow was divided by 6. In 15 ml tubes, 6 ml of 65% Percoll solution was added and carefully overlaid with 9 ml of bone marrow diluted with HBSS. The samples were centrifuged at $1000 g$ for 20 minutes at 4°C (acceleration 9, brake 3). The interphase containing MNCs was carefully collected and transferred into a 50 ml tube, which was then filled up to 50 ml with phosphate buffered solution (PBS). This was followed by centrifugation at $500 g$ for 10 minutes at 4°C (acceleration 9, brake 3). Then the supernatant was discarded and resuspended in PBS. For counting $10 \mu\text{l}$ cell suspension was mixed with $10 \mu\text{l}$ of Trypan blue and counted with a hemocytometer, excluding dead MNCs from the count. To enrich the fraction containing DCCs, most of the hematopoietic cells were excluded using MACS. The following Allophycocyanin (APC)-conjugated antibodies were employed to exclude dendritic cells, monocytes, granulocytes, macrophages, and natural killer cells (CD11b), as well as myeloid cells (CD33). Additionally, the common leukocyte marker (CD45) was added. Glycophorin A-conjugated microbeads were used to capture erythrocytes in the MACS column. The unlabeled cell suspension was then stained with EpCAM-PE and subsequently screened under a fluorescence microscope (Olympus XI81). Single cells with high EpCAM expression were isolated using a micromanipulator and transferred into a tube containing lysis buffer for whole transcriptomic amplification (WTA). If a sufficient cell number was available, cryopreservation of cells was intended after density-gradient centrifugation (before immune cell depletion) and also after depletion if a significant number of DCCs were found during screening.

Bone metastasis biopsies: Biopsy punches of bone metastasis were minced to get small pieces in a petri dish using two scalpels, with a small amount of digestion medium added to keep the tissue moist. Subsequently, depending on the size of the tumor, 2-10 ml of digestion medium is added and the sample is digested for 20-40 minutes at 37°C . After 10 min of the digest, the sample was pipetted up and down using a 10 ml serological pipette to mechanically enhance dissociation. After digestion, the entire sample is transferred to a 50 ml Falcon tube, and the petri dish is rinsed with PBS. The sample is then centrifuged at $300 \times g$ for 10 minutes, the supernatant is discarded, and the pellet is resuspended in 1 ml of pre-warmed Trypsin/EDTA by gently swirling

the Falcon tube. The suspension is incubated at RT for 5 minutes. To inactivate the enzyme activity, basal medium containing 10% FCS is added. Afterwards, the cell suspension is filtered through a 40 µm filter to remove larger clumps. The filter is thoroughly rinsed, and the cell suspension is centrifuged at 300 x g for 5 minutes. Finally, 10 µl of cell suspension is mixed 1:1 with Trypan blue, and the viable cells are counted. Subsequently, some cells were stained for EpCAM and at least ten cancer cells were isolated for whole transcriptome analysis. The remaining cell suspension was either collected by Fraunhofer ITEM for attempts to establish organoid cultures, cryopreserved for future use, or incorporated into BM- model cultures.

3.3 Generation of the BM-Model

3.3.1 Model overview

3.3.1.1 Schematic of the two-niche BM model

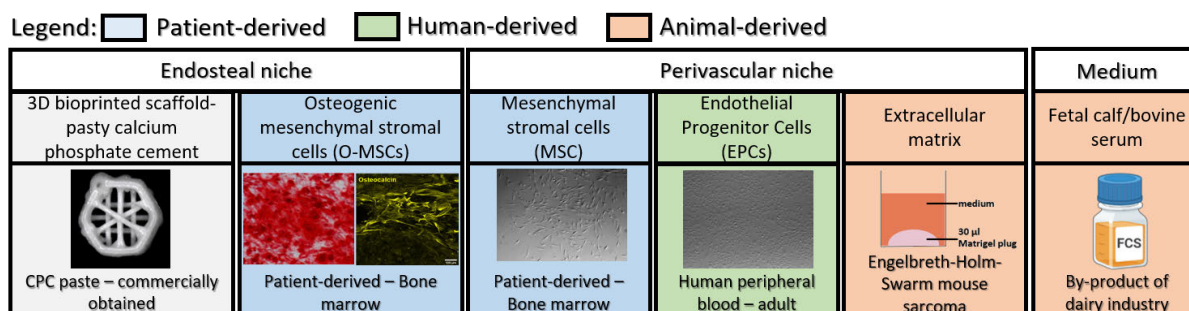


Figure 3.1: **Overview of all components of the bone marrow model setup.** Schematic representation of the BM model without modifications for replacing animal-derived components.

3.3.1.2 Endosteal niche

To build the endosteal niche, CPC scaffolds were pre-soaked in MSCs expansion medium for a few days prior to cell seeding. For cell seeding, 100,000 MSCs were applied to 5 mm scaffolds in 30 µL of medium, or 50,000 MSCs were seeded onto 3 mm scaffolds in 15 µL of medium. The cell suspension was carefully dispensed onto

the scaffold, ensuring complete coverage to facilitate cell attachment. Subsequently, the plates were placed on a shaker at 70 rpm for 2 hours to enhance cell distribution. Following this, 500 μ L of expansion medium **Table 2.1** was gently added, and the plates were returned to the shaker for an additional hour. The constructs were then incubated under static conditions. The following day, the medium was replaced with osteogenic differentiation medium (**Table 2.2**). If seeding was performed on a Friday, osteogenic medium was applied immediately during plating. The cells were differentiated for a minimum of 14 days and up to 28 days, until calcium deposits were observed in MSCs cultured on a 2D well plate as a reference.

3.3.1.3 Perivascular niche

To generate the perivascular niche of the BM model, MSCs and EPCs were co-cultured in basement membrane matrix derived from the Engelbreth-Holm-Swarm mouse sarcoma (Matrigel) (Corning, Cat# 356231 or ThermoFisher, Cat# A1413202). Various cell densities and ratios were tested during the optimization process. For functional experiments, the optimal conditions were determined to be 2,000 MSCs/ μ L and 2,000 EPCs/ μ L, maintaining a 1:1 ratio. The cell mixture was carefully layered onto the pre-prepared scaffold containing functional osteoblasts. To minimize the risk of cell suspension leakage, a volume of 30 μ L was applied to the 5 mm scaffolds, while 10 μ L was used for the 3 mm scaffolds. The Matrigel was allowed to polymerize for 20–25 minutes, after which EGM-2 medium was added to the two-niche setup. When the vascular niche was established without the endosteal niche, the cell mixture was deposited onto a pre-warmed 24-well plate, allowed to polymerize, and subsequently supplemented with EGM-2 medium.

3.3.1.4 BM model setup details for the Investigation of PC3 cells interacting with BM microenvironment

The following experimental setup was used for the functional studies of PC3 cells in BM niches (Section 4.6):

- **CPC scaffold:** 3 mm
- **MSC on scaffold:** 50,000 cells

- **Osteoblast differentiation time:** 18-28 days
- **Cell numbers for vascular niche:** 20,000 MSCs and 20,000 EPCs (1:1 ratio)
- **Volume of Matrigel:** 10 μ l
- **Seeding method:** Simultaneous
- **Cancer cells:** 20–40 PC3 cells (either GFP or EplC-YFP labeled)
- **Culture time:** 10 days
- **Serum:** 10% FCS

3.3.2 Seeding strategies for cancer cells to BM model

Cancer cells were incorporated into the BM model using two distinct strategies: (1) seeding or injection onto the established model (**Figure 3.2, A**), and (2) co-seeding simultaneously with vascular niche cells (**Figure 3.2, B**). For the 'on-top seeding' approach, cancer cells were manually selected using micromanipulation under a microscope. The selected cells were suspended in a small volume (maximum 5 μ L) containing a 1:1 mixture of Matrigel and PBS, and carefully deposited onto the fully developed vascular niche to ensure optimal cell integration. For the simultaneous seeding approach, which was employed for experiments requiring a larger number of samples, a master mix of all vascular niche cell types was first prepared in Matrigel. Cancer cells were prepared separately at a concentration of 500,000 cells/mL. From this suspension, the required number of cancer cells, typically in a volume of 1–2 μ L, was added to the master mix to ensure uniform distribution within the vascular niche. In both approaches, cancer cells were cultured in EGM-2 medium under standard conditions for 10 days. (37°C, 5% CO₂)

3.4 Humanization of the BM-model

3.4.1 hPL preparation and usage

3.4.1.1 Protocols for hPL preparation and its use as an FCS alternative

hPL was prepared in collaboration with the Department of Transfusion Medicine at UKR using expired platelet apheresis donations. The protocol was adapted from Hemeda et

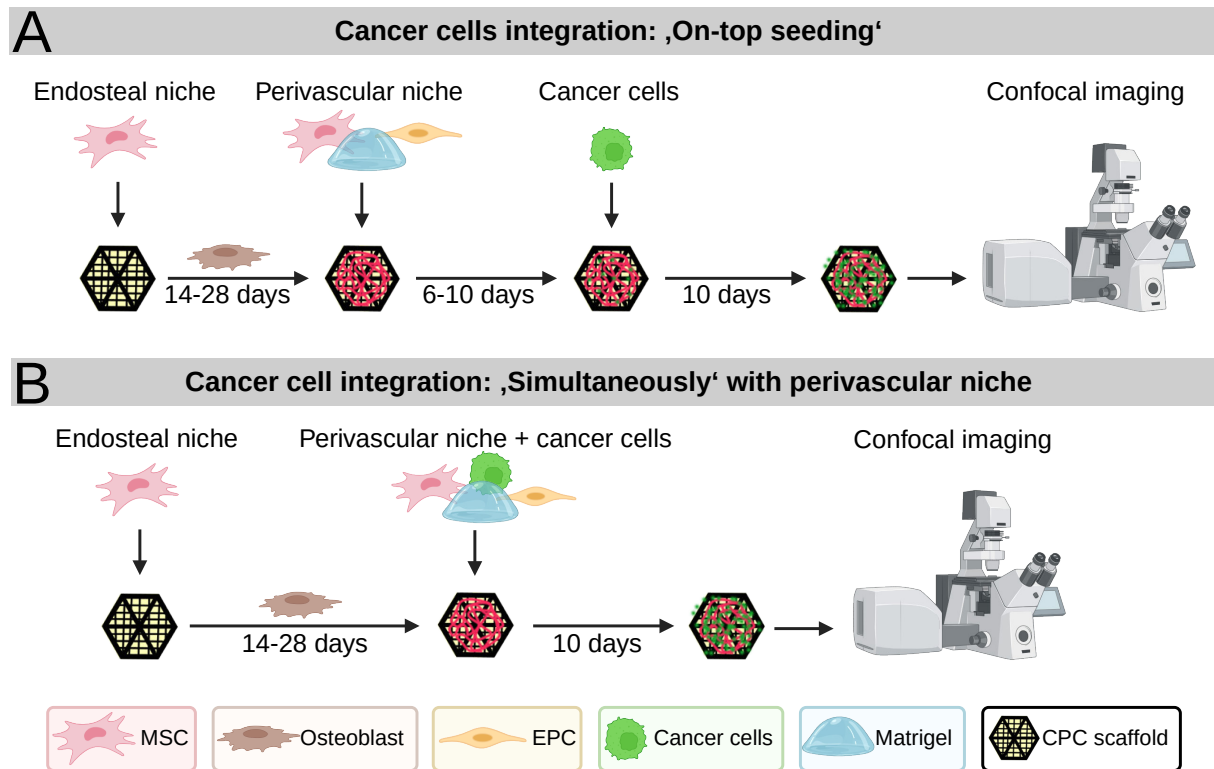


Figure 3.2: **Schematic of two different cancer cell integration methods into two niche BM model. A:** Introduction of cancer cells by seeding onto the fully developed two-niche BM model. **B:** Incorporation of cancer cells into the vascular niche during perivascular niche seeding by co-mixing MSCs, EPCs, and cancer cells in Matrigel. Figure created with BioRender, with additional scaffold images provided by the author

al. with slight modifications [44]. Over the course of the PhD project, three independent batches of hPL were generated. For each batch, two to four expired platelet apheresis donations were pooled and subjected to three to four freeze-thaw cycles, alternating between freezing at -20°C and thawing at 37°C to achieve platelet lysis. Following lysis, the samples were centrifuged at 3,000 × g for 30 minutes to remove cellular debris. The resulting supernatant was filtered through a 0.45 µm filter to ensure sterility and clarity. The filtered hPL was then aliquoted and stored at -20°C until further use.

3.4.1.2 Preparation of fibrinogen-depleted hPL medium for MSC Expansion

The preparation of fibrinogen-depleted hPL medium was performed based on an adapted protocol from Hemeda et al.[44]. To formulate the expansion medium, αMEM was supplemented with 1% P/S, 0.2 mM L-ascorbic acid-2-phosphate, and 10% hPL (**Table 2.1**). Fibrin clotting was induced by incubating the medium at different temperatures, including RT, 4°C, and 37°C. Clot formation was facilitated by vigorous shaking, followed by centrifugation at 2,000 × g for 15 minutes to separate the fibrin clot. The supernatant was then subjected to sterile filtration using a 0.22 µm filter to remove any residual particulates. The resulting fibrinogen-depleted medium was then utilized for MSCs expansion.

3.4.2 Generation of human bone ECM hydrogel

The protocol for hydrogel preparation was adapted with modifications from Sawkins et al. [45], originally developed for producing hydrogel from bovine bone. The starting material consisted of a spine segment from a male donor, the rapid autopsy program, approved by the Ethics Committee of the University of Regensburg (ethics vote number 21-2440-101). Pathologists sectioned the bone using a diamond saw to separate the cancellous and compact bone regions. The cancellous bone portion was then stored at -20°C until further processing. The first step in hydrogel preparation involved pulverizing the bone fragments (initial weight: 5.5 g). Pulverization was performed using a coffee grinder with the addition of a small amount of sterile water to aid the process. Prior to grinding, the bone pieces were immersed in liquid nitrogen to maintain a sufficiently low temperature, ensuring efficient pulverization. After pulverization, the bone material was subjected to

centrifugation at $3200 \times g$ to remove the water added during grinding. The next step involved demineralization using a 0.5 N HCl solution (25 ml per 1 g bone), with continuous shaking at 300 rpm for 24 hours at RT. The removal of HCl was achieved through slow centrifugation, followed by filtration of the supernatant to prevent the loss of smaller particles. This step was followed by two additional washes with sterile distilled water to ensure thorough removal of residual acid. Subsequently, a 1:1 methanol-chloroform mixture was used to remove lipids. The sample was incubated for 1 hour on a shaker, then allowed to settle for a few minutes before the mixture was carefully removed using a 40 μ m filter. The first wash was performed with pure methanol, followed by a second wash with sterile distilled water to eliminate any remaining solvents. The resulting bone particles were then subjected to a decellularization step by incubating them overnight at 37°C in a solution containing 0.05% trypsin and 0.02% EDTA to effectively remove cellular components from the bone matrix. The trypsin was subsequently removed, and the bone samples were washed in PBS containing 2% P/S for 24 hours at RT. Following the removal of PBS, the bone particles were snap-frozen in liquid nitrogen and stored at -80°C until further processing. To generate the hydrogel, the bone matrix was subjected to pepsin digestion. As freeze-drying was not feasible, the bone matrix was dried as much as possible under sterile conditions. Subsequently, it was resuspended in a solution of pepsin at a concentration of 2 mg/mL in 0.01 M HCl and incubated on a shaker at 250 rpm at RT for 42 hours, until most of the particles were dissolved. The resulting viscous solution was then transferred to a fresh tube and neutralized by adding 10× PBS and NaOH until a pH of 7 was achieved. The protein concentration of the resulting solution was measured using a Nanodrop device, with Matrigel of known concentrations serving as a reference.

3.5 Qualitative and quantitative analysis methods

3.5.1 Comparative analysis of hPL and FCS in supporting isolation of MSCs

To evaluate the effectiveness of hPL as an alternative to FCS in MSCs culture, 2 million BM MNCs were seeded into T75 cell culture flasks containing MSCs expansion medium

supplemented with either 10% hPL or 10% FCS. The first medium exchange was performed after 48 hours, followed by subsequent changes every 2–3 days. On day 11, cell colonies were assessed microscopically, and cells were enzymatically detached, counted, and compared across the different serum conditions to evaluate cell proliferation efficiency. The comparative analysis was performed using samples obtained from 13 different patients.

3.5.2 Bone differentiation marker: Osteocalcin

3.5.2.1 Staining protocol for detecting osteocalcin

Endosteal niches were prepared by seeding MSCs onto CPC scaffolds, with a density of 5×10^4 cells on 3 mm scaffolds and 1×10^5 cells on 5 mm scaffolds and differentiated for 14–21 days in osteogenic medium (**Table 2.2**). Following fixation with 4% paraformaldehyde (PFA) for 1 hour at RT, the samples were permeabilized for 15 minutes with 0.02% Triton in PBS. Subsequently, the samples were blocked with 5% BSA in PBS for 1 hour at RT. The primary antibody incubation was then performed using mouse anti-human osteocalcin at a concentration of 10 $\mu\text{g/ml}$ in blocking buffer, incubating overnight at 4°C. The samples were washed four times with PBS-T (0.05% Tween-20; one quick wash and three 30-minute washes). Following the washes, the secondary antibody (anti-mouse Alexa Fluor 555 Plus, 2 $\mu\text{g/ml}$), DAPI (1 $\mu\text{g/ml}$), and phalloidin (Alexa Fluor 647, 1:400) were added in blocking buffer and incubated again overnight at 4°C.

3.5.2.2 Quantitative measurements of osteocalcin signal

The osteocalcin signal was quantified using ImageJ by measuring the mean gray value in the AlexaFluor 555 channel, which represents osteocalcin expression. An isotype control was used as a background reference. Mean gray value intensities were measured across multiple regions within each sample and normalized to the isotype control. Each data point represents the osteocalcin intensity of an individual region within the entire sample, with samples obtained from two different MSCs donors.

3.5.3 Endothelial marker: CD31

3.5.3.1 Staining protocol for visualizing endothelial structures

Vascular niches were prepared using MSCs and EPCs in the respective ratio (4:1, 2:1 or 1:1). During the ratio testing, 30 μ l Matrigel drops were used, while 10 μ l Matrigel drops were used for the cell number testing. Therefore, cell concentrations are reported in cells per μ l. The tested cell concentrations were 500 MSC/ μ l, 1000 MSC/ μ l, and 2000 MSC/ μ l. To visualize the entire niche, DAPI (nuclei) and phalloidin (F-actin) staining were utilized. After ten days of culture, the Matrigel drops were fixed with 4% PFA for 1 hour at RT. After washing once with PBS, the vascular niches were permeabilized for 15 min with 0.2% Triton X100 in PBS. Subsequently, the samples were blocked with 5% BSA for 1 hour at RT. After blocking, the primary antibody (mouse anti-human CD31, clone: WM59) was added at a concentration of 10 μ g/ml in blocking solution and incubated overnight at 4°C. The following day, the samples were washed four times with PBS-T (0.05% Tween-20, one quick wash, and three 30-minute washes). After washing, the secondary antibody (anti-mouse Alexa Fluor 555 Plus, 2 μ g/ml), DAPI (1 μ g/ml), and phalloidin (Alexa Fluor 647, 1:400) were added in blocking buffer and incubated again overnight at 4°C.

3.5.3.2 Quantitative measurements of tube length

For each individual sample, four randomly selected regions were imaged using a Zeiss LSM980 confocal microscope equipped using a 10 $\times$ objective. All z-planes of the CD31 channel (Alexa Fluor 555 Plus) were acquired to capture the full three-dimensional structure of the vascular network.

To quantify the total tube length, the acquired images were first processed using ImageJ by generating a maximum intensity projection. Subsequently, a graphics tablet was used to manually label the tube structures, which were then measured in ImageJ. The total tube length for each image was determined by summing the measurements of all identified tube structures, and the results were plotted for further analysis. Each data point represents a single region from an individual sample. The experiment was conducted with 3–4 replicates per condition, using cells from a single MSCs and EPCs donor.

3.5.4 Live/Dead assay: Viability assessment

Scaffolds containing osteogenically differentiated MSCs from two donors were stained after 18–20 days of culture using Calcein AM (4 μ M) and Ethidium Homodimer-1 (2 μ M) to assess cell viability, along with Hoechst 33342 for nuclear staining. The samples were incubated with the staining solution for 30 minutes, followed by a single washing step with PBS. Imaging was performed immediately using a Zeiss LSM980 confocal microscope.

3.5.5 Flow cytometry: Analysis of surface markers for cell characterization

Flow cytometry was performed to characterize the surface markers of MSCs and EPCs based on established criteria. Surface markers for MSCs characterization were selected according to Dominici et al. [46], while markers for EPCs characterization were selected based on endothelial lineage-specific expression profiles associated with endothelial colony forming cells (ECFCs), as described by Poldervaart et al. [47]. For both cell types, cells were first stained with the eBioscience fixable viability dye eFluor 780 in serum-free PBS for 15 minutes at RT in the dark, followed by a washing step with FACS buffer (PBS + 2% FCS, 2 mM EDTA, and 0.01% sodium azide). Cells were then blocked with 10% AB serum in PBS for 10 minutes before antibody incubation. MSCs were stained with APC anti-human CD31, Brilliant Violet 421 anti-human CD73, FITC anti-human CD90, PE anti-human CD34, PE/Cyanine7 anti-human CD105, and PerCP/Cyanine5.5 anti-human CD45. EPCs were stained with APC anti-human CD31, Brilliant Violet 421 anti-human CD146, PE/Cyanine7 anti-human CD105, PerCP/Cyanine5.5 anti-human CD45, and PE anti-human CD34, with appropriate isotype controls included. After incubation for 20 minutes at RT in the dark, cells were washed, fixed with FluoroFix™ Buffer (BioLegend), and stored at 4°C until acquisition. Data acquisition was performed using a Gallios flow cytometer (Beckman Coulter) for MSCs and a FACSCelesta (BD) for EPCs, with subsequent analysis conducted using FlowJo version 10.

3.5.6 Multipotency Assays of MSCs for osteogenic and adipogenic lineages

MSCs were seeded at 15,000 cells/cm² in 6-well plates (144,000 cells per well) in duplicate for osteogenic and adipogenic differentiation, with an additional undifferentiated control. Osteogenic and adipogenic differentiation were induced using the respective media (**Table 2.2**, **Table 2.3**), while controls were maintained in expansion medium (**Table 2.1**). After 14–30 days, depending on the donor and mineralization rate, cells were fixed with 4% PFA for 20 minutes at RT. Osteogenic cultures were stained with freshly filtered 2% Alizarin Red S solution, washed with distilled water, and imaged. Adipogenic cultures were stained with 0.3% Oil Red O solution for 10 minutes and then imaged. For quantification, Alizarin Red S was extracted with 10% cetylpyridinium chloride in 10 mM Na₂PO₄ (pH 7.2) and absorbance measured at 595 nm, with correction at 655 nm. Oil Red O was extracted using 4% Igepal (Sigma) in isopropanol for 15 minutes, and absorbance was measured at 540 nm.

3.5.7 Volume approximation of PC3 cell growth

3.5.7.1 Sample acquisition with confocal microscopy

BM niche samples were fixed with 2% PFA for 1 hour at RT and stored in PBS containing 1% P/S until imaging. Depending on the analysis, samples were stained with additional antibodies, such as CD31 with AlexaFluor 555 Plus, and counterstained with DAPI (1 µg/mL) and Phalloidin AlexaFluor 647 to visualize the F-actin cytoskeleton. Imaging was performed using a Zeiss LSM980 confocal system. Samples were placed upside down on a thin coverslip and covered with PBS to maintain hydration during imaging. For growth analysis, the PC3 cell signal (either GFP or YFP) was acquired to capture the entire cellular distribution using a step size of 6 µm and a resolution of 512 px, with the entire sample imaged via tile scanning. High-resolution imaging was performed with a step size of 1–3 µm and a resolution of 1024 px, allowing for detailed visualization of cellular structures.

3.5.7.2 Measurement pipeline

An image analysis pipeline was developed using ImageJ macros to quantify cancer cell growth and distribution in the BM model. Complete tile images were processed by extracting the GFP/YFP channel, converting it to 8-bit grayscale, and applying a threshold of 18. The threshold value was manually verified, with adjustments made only when necessary to account for variations in background intensity or signal quality, ensuring accurate segmentation. Segmented images were analyzed by measuring the area, and the results were saved as TIFF files for visualization and CSV files for quantitative analysis. To estimate the 3D volume of cancer cell clusters, the measured area in pixels was converted to square micrometers (μm^2) using the known pixel size ($2.75 \mu\text{m}^2$). The cluster volume was then calculated by multiplying the measured area by the step size ($6 \mu\text{m}$). This standardized pipeline ensured reproducible and comparable results across different experimental conditions.

3.5.8 High-resolution imaging of patient-derived samples

Adhesive slides containing cells derived from patient samples of breast, prostate, or bladder cancer were used to optimize a staining protocol for high-resolution imaging. The use of these samples was approved by the University of Regensburg Ethics Committee (ethics vote number 18-948-101). The slides were prepared by plating the cells, allowing them to settle, and then discarding the supernatant by flipping the slides. The slides were then air-dried and stored at -20°C until further processing. For immunofluorescence staining of four and a half LIM domains 2 (FHL2) and Cytokeratin (CK) 8/18/19, the slides were thawed to RT and moistened with PBS. The cells were fixed with 2% PFA for 10 minutes, washed twice with PBS, and permeabilized with 0.05% Triton X-100 for 5 minutes. After two additional washing steps, the cells were blocked with PBS containing 10% AB serum for 1.5 hours at RT. Primary antibody staining was performed overnight at 4°C in PBS supplemented with 10% AB serum, using monoclonal mouse anti-human CK 8/18/19 and polyclonal rabbit anti-human FHL2. After washing with PBS, the slides were incubated with secondary antibodies (anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor Plus 647), along with DAPI in PBS containing 5% AB serum and 2.5% goat serum for 1.5 hours. After secondary antibody incubation, slides were

washed three times for 10 minutes with PBS, mounted using SlowFade Glass Soft-Set Antifade Mountant (Invitrogen, S36917), and imaged using a Zeiss LSM980 confocal microscope. Image processing and analysis were performed using ImageJ (v.1.53q) to generate maximum intensity projections or sum projection images of the z-stacks.

3.5.9 Statistical analysis

All graphs report the statistical significances using parametric tests. For comparisons involving only two groups, the Student's t-test was employed, while comparisons across multiple groups were evaluated using analysis of variance (ANOVA) followed by post-hoc multiple comparison tests (Tukey or Bonferroni) to identify pairwise differences. Given the relatively low sample sizes and the challenges in reliably confirming normality assumptions, non-parametric tests were sometimes applied additionally (as noted in the text, with graphs always displaying parametric statistical significance). For non-parametric retesting, the Mann-Whitney U test was used for two-group comparisons, and the Kruskal-Wallis test (or multiple Mann-Whitney tests) was applied for comparisons involving more than two groups. Significance levels are indicated as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

3.5.10 Software

Zotero was used for reference management and citation generation. GraphPad Prism (versions 9 and 10) was used for statistical analyses and graph generation. Fiji/ImageJ was employed for processing and quantifying microscopic data. Adobe Illustrator (version 2020) was used for figure design, while BioRender.com was used to create schematic illustrations, with references provided in the respective figures. LaTeX (TeXstudio) was used for writing and formatting this document.

3.5.11 Large language models

During the refinement of this document, I used ChatGPT, a Large Language Model (LLM), to enhance readability and improve the scientific tone of specific text passages.

Importantly, while the phrasing was optimized for clarity and precision, the factual content and original meaning of the text remained unchanged.

3.6 Single-cell sequencing

3.6.1 Single-cell preparation for 10x Genomics: Enzymatic digestion, staining

To prepare a single-cell suspension from the BM model after the full culture period, a mixture of enzymes was used. The digestion process involved Dispase (4 U/mL in HBSS) for 30 minutes at 37°C, followed by Collagenase (0.3 mg/mL) in HBSS containing BSA (final concentration 0.5%) to protect cells during enzymatic digestion for an additional 30 minutes. The final step involved TrypLE, which was added directly to the sample and incubated for another 30 minutes, with repeated resuspension of the cell suspension during the incubation. This stepwise protocol was optimized as part of the bachelor thesis by Mira-Viola Mayr, titled "Optimization of the 3D In Vitro Bone Marrow Model for Single-Cell Sequencing." Following enzymatic digestion, all subsequent steps were performed on ice using precooled centrifuges to preserve cell viability and prevent degradation. After digestion, 4–6 individual samples were pooled and washed once with HBSS to remove residual enzymes. Next, each sample was incubated with 50 µL of blocking solution (Human TruStain FcX, Fc Receptor Blocking Solution; BioLegend, Cat# 422301) prepared in HBSS with 2% FCS, and incubated for 10 minutes to prevent non-specific binding. Seven different hashtag antibodies were pre-diluted 1:10 in HBSS, and each sample condition was incubated with a specific hashtag antibody at a final concentration of 0.25 µg/mL in a total volume of 100 µL. Additionally, 1 µL of pre-diluted (1:10) viability dye was added to each sample to achieve the recommended final dilution of 1:1,000, as specified in the manufacturer's protocol. After 30 minutes of incubation on ice in the dark, each sample was filled with 3 mL of MACS buffer (0.5% BSA, 2 mM EDTA in 1× PBS) with an additional 2% FCS, followed by centrifugation at 500 × g for 5 minutes at 4°C. The supernatant was carefully removed via pipetting to avoid disturbing the pellet. The pellet was then resuspended in 2 mL of MACS buffer for an additional wash to remove excess antibodies, followed by another centrifugation step at 500 × g

for 5 minutes at 4°C. After the second wash, the supernatant was carefully removed, and the cell pellet was resuspended in 100 µL of MACS buffer (+2% FCS) for sorting. To remove any clumps, cells were filtered through a 40 µm sterile filter, and the filter was rinsed with buffer up to a final volume of 500 µL.

3.6.1.1 Fluorescence-activated cell sorting and cell pooling for 10x genomics

Fluorescence-activated cell sorting (FACS) was performed at the Flow Cytometric Cell Analytics and Cell Sorting Core Facility of the Leibniz Institute for Immunotherapy (LIT), which provides advanced flow cytometry and cell sorting services under the leadership of Assoc. Prof. Hoffmann. Sorting was conducted by Rüdiger Eder. The gating strategy was designed to select live, single cells while minimizing doublet contamination. PC3 cells were identified and sorted based on GFP labeling, whereas niche cells were selected as unlabeled cells, using only a viability stain to exclude dead cells. The number of sorted cells for each condition is detailed in Table 3.1. Following sorting, all collected cells were pooled and directly loaded onto two lanes of a 10x Genomics Chromium Next GEM Chip for single-cell RNA sequencing.

Table 3.1: Fluorescence-activated cell sorting for viable cells and targeted cell number of each cell type

Condition	Niche cells	Cancer cells
Two niche	15,000	5,000
Endosteal only	5,000	5,000
Vascular only	10,000	5,000
Cancer only	-	5,000
Two niche control	15,000	-
Endosteal control	5,000	-
Vascular control	10,000	-
Total	80,000	

3.6.2 Library preparation and sequencing

Library preparation and sequencing were performed at the Next Generation Sequencing (NGS) Core Facility of the Leibniz Institute for Immunotherapy (LIT), which provides

high-throughput sequencing services under the leadership of Prof. Dr. Michael Rehli. Single-cell RNA sequencing was performed using the Chromium GEM-X Single Cell 3' Kit v4 (10X Genomics), following the 3' gene expression protocol. Single-cell feature barcoding was incorporated using the Chromium GEM-X Single Cell 3 Feature Barcode Kit v4, in conjunction with the Chromium GEM-X Single Cell 3' Chip Kit v4. Libraries were pooled and quantified before sequencing. Sequencing was carried out on the NovaSeq 6000 platform using the S2 Reagent Kit v1.5 (100 cycles). Dual indexing was achieved with the Dual Index Kit TT Set A and NT Set A, each supporting 96 reactions. Data processing, including FASTQ file generation and secure data transfer, was managed by the service provider.

3.6.3 Bioinformatic analysis

3.6.3.1 Single-cell RNA-seq transcriptomic analysis

The transcriptomic data analysis was performed by Dr. Renato Liguori at the Institute of Bioinformatics and Computational Pathology, Universitätsklinikum Erlangen. Raw sequencing data were initially processed using Cell Ranger (10X Genomics) to demultiplex samples and generate gene expression count matrices. Subsequent analyses were conducted using the Seurat package (v.5.2.0) in R (v.4.4.1). To ensure data quality, quality control (QC) measures were applied to filter out low-quality cells and genes. Specifically, genes with fewer than 300 counts, cells with fewer than 500 or more than 100,000 detected genes, and cells with a mitochondrial gene ratio exceeding 20% were removed. After QC, the data were normalized, and highly variable features were identified for downstream analysis. For dimensionality reduction, principal component analysis (PCA) was performed, and the first 50 principal components (PCs) were selected for further exploration. To account for potential batch effects, data integration was performed using the Harmony algorithm (v.1.2.3), ensuring a more accurate clustering of cell populations. Clustering analysis was then carried out using an optimized resolution of 1.0, allowing the identification of distinct cellular populations.

3.6.3.2 Differential expression analysis

To identify differentially expressed genes (DEGs) across conditions, differential expression (DE) analysis was performed using the MAST method within the Seurat framework. Genes with an adjusted p-value < 0.05 were considered statistically significant.

3.6.3.3 Pathway enrichment analysis

Pathway enrichment analysis was conducted using the clusterProfiler package (v.4.12.6). Gene Set Enrichment Analysis (GSEA) was performed using the Gene Ontology (GO) gene set file (c5.go.v2024.1.Hs.symbols.gmt) downloaded from the Molecular SignaturesDatabase (MSigDB). Pathways were considered significantly enriched if they met the threshold of $p.adjust < 0.05$ and a normalized enrichment score (NES) < -1 or > 2 . To visualize the results, heatmaps were generated, highlighting both shared and condition-specific pathway alterations.

4 Results

4.1 Sample acquisition for modeling prostate cancer in vitro

To model the human-specific BM microenvironment *in vitro*, a diverse collection of patient-derived samples was obtained, ensuring a broad representation of metastatic (M1) and non-metastatic (M0) conditions. A total of 37 patient samples were collected: one healthy donor, 20 M0-stage prostate cancer patients, 12 M1-stage prostate cancer patients, and four donors with bone metastases from various primary cancers (**Table 4.1**). For most patient samples, the focus was first on isolating DCCs for further characterization as potential metastasis-initiating cells, while from a subset of samples additional cellular components (MNCs, MSCs) and non-cellular components (BM-plasma) were also isolated.

The overall workflow for collecting various components from a BM aspirate is illustrated in **Figure 4.1**. After receiving the BM aspirate, first BM plasma was recovered from six M0 and eight M1 samples for future analyses of secreted factors. The mononuclear cell fraction was then isolated by density gradient centrifugation, and multiple vials were cryopreserved before partially depleting immune cells (CD34+, CD11b+, and CD33+) to enhance DCC detection. From the pre-depletion MNC fraction, MSCs were expanded for 11 M0 patients, 8 M1 patients, one bone metastasis donor, and one healthy donor. Among the M0-stage patients, 12 samples yielded a total of 46 isolated DCCs, while 9 M1-stage samples contributed 66 DCCs. Additionally, 3 bone metastasis samples provided 36 isolated cancer cells.

These outcomes illustrate the versatility of this biobank for a wide range of downstream applications, including transcriptomic and functional assays. The collection of vials with MNCs, immune cell depleted BM, expanded MSCs, BM plasma, and isolated DCCs

enables investigation of the human BM microenvironment from multiple perspectives while accounting for patient heterogeneity. In the following section, we present the results of the isolation process for patient-derived MSCs from multiple donors and describe the development of the endosteal niche within the BM model.

Table 4.1: Biobank samples

Cancer Status	Total pa- tients	Patients with vials of MNC fraction	Patients with BM after de- pletion	Patients with ex- panded MSCs	Patients with BM plasma	Patients with isolated DCCs	Total isolated DCCs
Healthy	1	-	-	1	-	-	-
M0 Patient	20	2	1	11	6	12	46
M1 Patient	12	7	7	8	8	9	66
Bone Metastasis	4	-	-	1	-	1	36

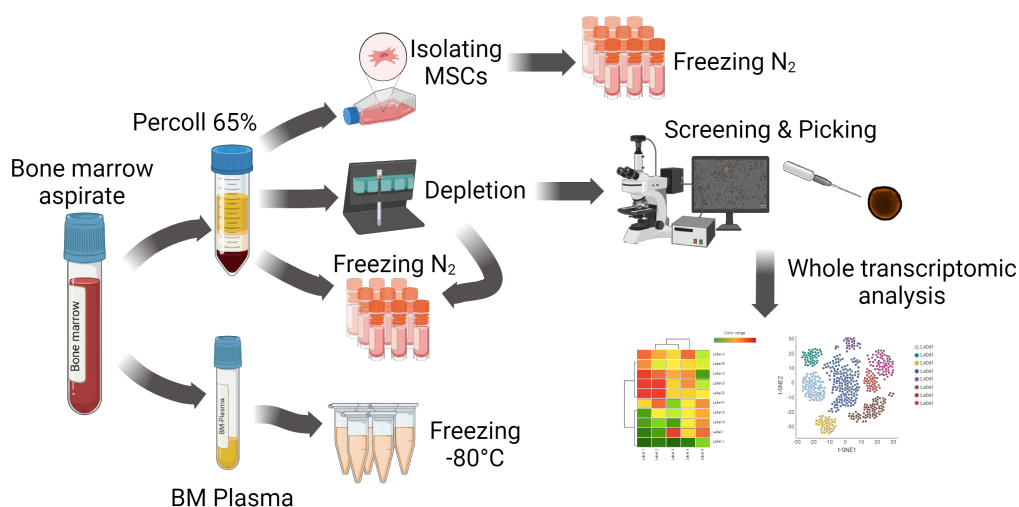


Figure 4.1: **Workflow to establish biobank from prostate cancer patients.** MNCs before and after depletion, MSCs, BM-plasma, EpCAM⁺ cells from BM-aspirates were preserved to establish a biobank from prostate cancer patients with different cancer status. Figure created with BioRender.com.

4.2 Establishing the foundations of BM niches in vitro

4.2.1 Endosteal niche: primary MSC isolation to functional validations

4.2.1.1 Primary MSC isolation and viability in extended culture

The development of a functional endosteal niche requires a reliable source of MSCs, which were isolated from prostate cancer patient BM samples. To ensure efficient isolation of MSCs, two protocols were compared for their effectiveness: First, the direct plating of wBM aspirates [32] and the more commonly used method to plate MNCs after density centrifugation [48]. Cell characterization was performed according to the minimal criteria for defining multipotent mesenchymal stromal cells [46] using flow cytometry for phenotypic analysis and differentiation to osteoblasts and adipocytes for functional analysis.

MSCs from two BM samples, D1 and D2, were isolated via direct plating of wBM or plating MNCs. Additionally, MSCs previously isolated in the laboratory were incorporated into the analysis as a control. Results from flow cytometry to determine phenotypic characterization showed similar expression levels for all positive markers, including CD90, CD105, and CD73. CD45 and CD31 were used as negative markers. CD34 was employed also as a negative marker based on established minimal criteria for BM-derived MSCs [46], although recent studies indicate that tissue-resident MSCs from bone marrow and adipose tissue do express CD34, with this expression diminishing upon cell culturing [49]. The only significant variation was observed between the two plating methods for D1. Cells cultured from wBM exhibited a higher proportion of CD34- and CD45-positive cells, along with a detectable CD31-positive subpopulation, compared to those grown from MNCs (Figure 4.2, A). This suggests that wBM-derived cultures comprise a more heterogeneous cell population.

In addition to analyzing marker expression profiles, the differentiation potential of the cells was assessed. Cells from D1 demonstrated the ability to differentiate into both adipocytes and osteoblasts, irrespective of the plating method. However, cells from D2 directly plated from whole bone marrow did not differentiate into osteoblasts, whereas cells isolated by density centrifugation to obtain the mononuclear cell fraction successfully differentiated into both osteoblasts and adipocytes (Figure 4.2, B).

Although the sample size was small, the observed discrepancies in differentiation

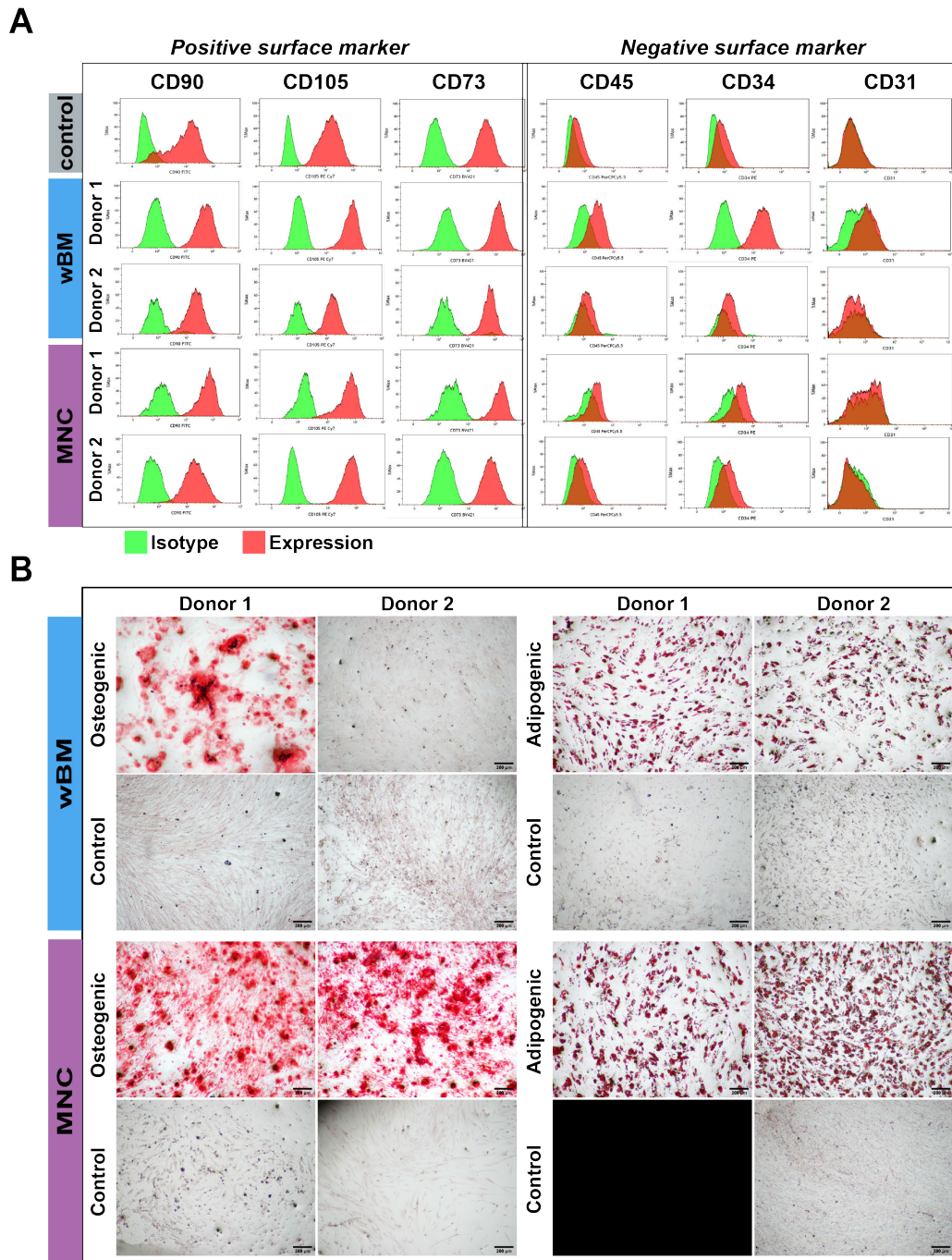


Figure 4.2: **Phenotypic (A) and functional (B) characterization of MSCs isolated from two donors using different plating methods (wBM or MNC).** **A:** Surface marker expression for MSCs was analyzed by flow cytometry. The isotype control is shown in green, with the expression of the respective antibody displayed in red. The control cells represent the marker profile of MSCs isolated from BM by a colleague. **B:** MSCs were induced to differentiate into osteoblasts (left) or adipocytes (right). Calcium deposits produced by osteoblasts were stained with Alizarin Red S, and lipid droplets accumulated in the cytoplasm of adipocytes were stained with Oil Red O. Controls show MSCs cultured in normal expansion medium. Scale bar: 200 μ m

variability and surface marker expression when plating the wBM, combined with the fact that density gradient centrifugation is a standard step in processing BM aspirates and renders MNC plating easily compatible with downstream procedures for isolating DCCs, led us to select the MNC plating method for all subsequent MSC isolations.

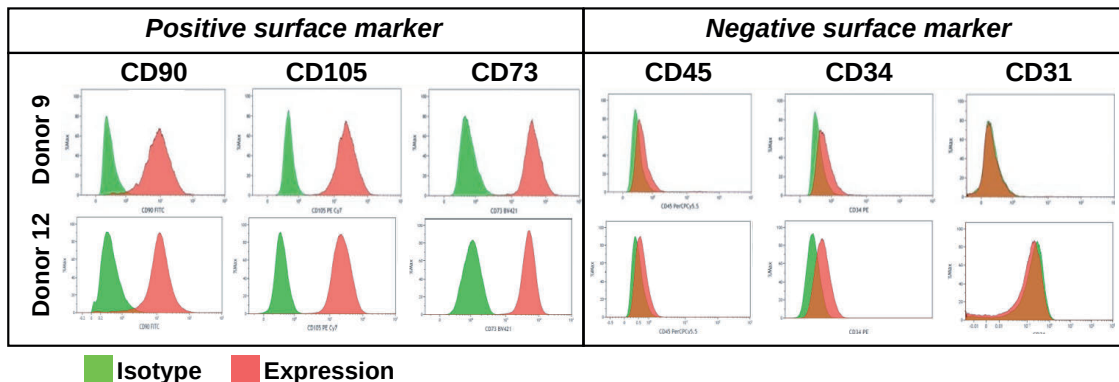
To further validate the robustness of the MSC isolation process and ensure the consistency of the resulting cultures across different donors, additional samples were evaluated for their differentiation potential and surface marker expression profiles. Flow cytometry analysis was performed on MSCs for two additional donors, using the same markers as previously assessed (**Figure 4.3, A**). Osteogenic differentiation potential was tested in six additional donors, including one immortalized MSC line (iMSC), while adipogenic differentiation was assessed in three other donors (**Figure 4.3, B**). Variations were noted in the extent of calcium deposition or lipid droplet formation (qualitative observation) and the time required for differentiation. Despite these differences, cells isolated from all donors successfully underwent differentiation within a period of 14 to 31 days. During the course of this project, MSCs were isolated from a total of 21 patient samples, including one healthy donor, eleven M0 patients, eight M1 patients, and one bone metastasis biopsy.

With successful MSC isolation and confirmation of their osteogenic differentiation potential, we proceeded to establish the endosteal niche of the BM model. This was achieved by implementing the CPC scaffold using an approach based on the protocol outlined by Braham et al. [32]. Upon initial establishment of the endosteal niche, cell viability was qualitatively assessed following the differentiation period. Given that osteoblast differentiation typically occurs within 14–21 days, viability was evaluated using live/dead staining on day 18 for Donor 12 and day 20 for Donor 19. Calcein AM was used to label viable cells, while ethidium homodimer-1 identified dead cells by penetrating compromised cell membranes and staining their DNA (**Figure 4.4**). Qualitative analysis of the staining indicated that most scaffold surfaces were covered with viable (green) cells. As anticipated in long-term cultures, a few regions in D19 exhibited accumulations of dead (red) cells, although the majority of cells remained viable. Due to technical issues, only half of D12 was imaged, which revealed only minimal dead cells throughout the observed areas.

Following confirmation that most cells remained viable during the differentiation period, we evaluated their osteoblastic activity using osteocalcin as a marker. After 21 days of

A

Flow cytometry



B

Osteogenic & adipogenic differentiation

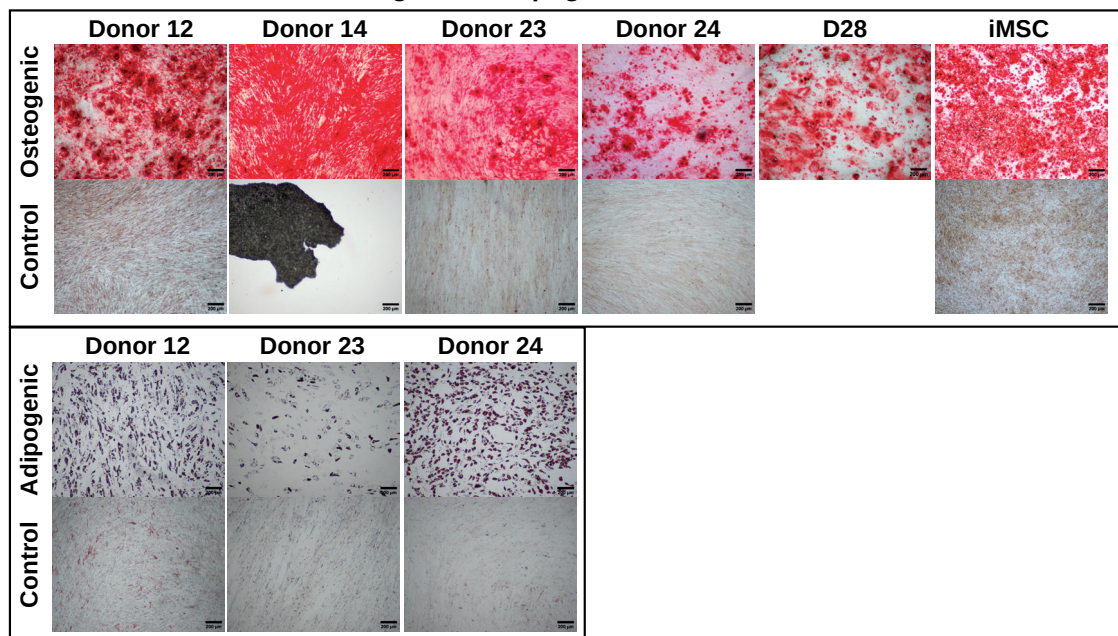


Figure 4.3: **Phenotypic (A) and functional (B) characterization of MSCs isolated from additional donors using MNC plating method.** **A:** Surface marker expression for MSCs was analyzed by flow cytometry. The isotype control is shown in green, with the expression of the respective antibody displayed in red. **B:** Cells were induced to differentiate either in osteoblasts (top) or adipocytes (bottom). Calcium deposits produced by osteoblasts were stained with Alizarin Red S and lipid drops accumulated in the cytoplasm of adipocytes were stained with Oil Red O. Control showed MSCs cultured in normal expansion medium. Scale bar: 200 μ m

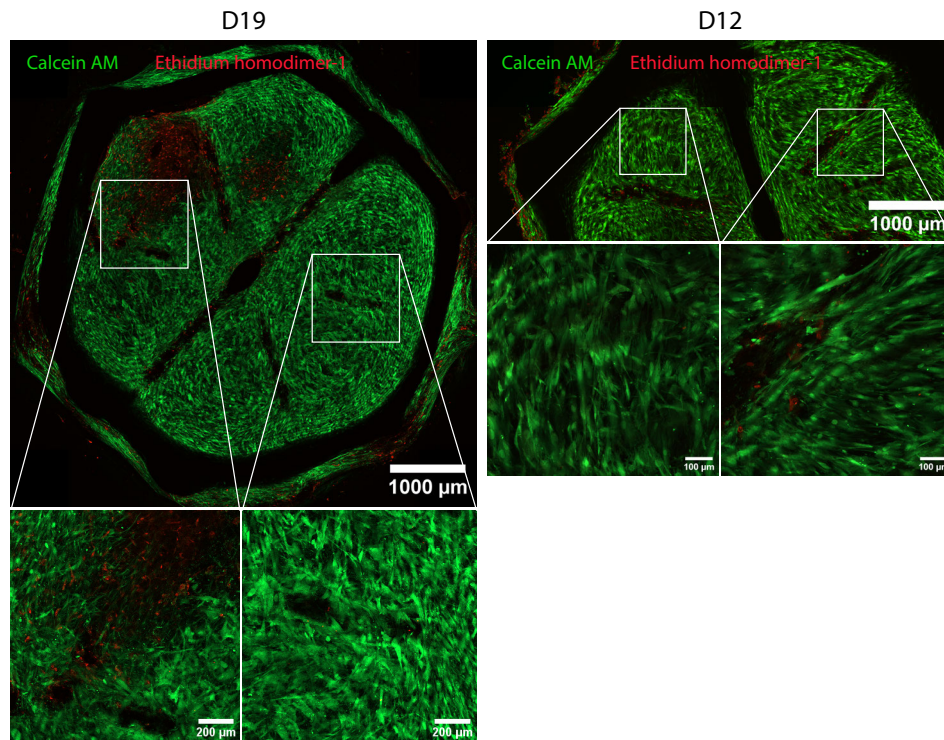


Figure 4.4: **Assessment of MSC viability following osteoblast differentiation on CPC scaffold.** MSCs seeded on CPC scaffolds were cultured in osteogenic differentiation medium for 18 days (D12) or 20 days (D19) and imaged using an LSM980 confocal microscope. The images display the max-Z projection of the tile scans (top) and magnified regions (bottom). Viable cells are indicated in green (Calcein AM), while dead cells are marked in red (ethidium homodimer-1). Scale bars for the tile scans are 1000 µm, while for the magnified regions, they are 200 µm for D19 and 100 µm for D12.

culture, fluorescent staining was performed on MSCs cultured in osteogenic medium (to induce differentiation) and on undifferentiated MSCs maintained in expansion medium. Initial observations revealed positive osteocalcin staining in both groups (**Figure 4.5**). To further clarify these differences, we examined samples from two additional donors (D23 and D24) and quantified the staining intensity by measuring the mean gray value of fluorescence intensity, normalized to an isotype control (**Figure 4.6**, A and B). In differentiated samples, normalized intensity values reached up to 21.3 (mean: 8.6), whereas undifferentiated samples showed values up to 6.8 (mean: 1.8). Undifferentiated samples consistently exhibited some osteocalcin staining, suggesting a degree of spontaneous differentiation, possibly due to prolonged culture, high cell density, or indicating that the CPC scaffold may possess osteoinductive properties. However, in this particular experiment, the staining intensity was markedly lower than in the osteogenically induced

samples.

Statistical analysis using one-way ANOVA with post hoc Tukey's test confirmed significant differences between differentiated samples and both the undifferentiated samples and the isotype control. Although additional non-parametric testing (accounting for the limited sample size) yielded a p-value of 0.2 for the comparison between undifferentiated and differentiated samples, the overall trends support enhanced osteoblastic activity in the osteogenically induced group. Furthermore, imaging an entire scaffold revealed that osteocalcin staining was particularly pronounced along the scaffold's sides in the differentiated sample, in contrast to the undifferentiated sample, further confirming enhanced osteoblastic activity after 21 days of culture. (**Figure 4.6, C**).

Overall, these findings confirm osteocalcin expression and support the conclusion that MSCs cultured in osteogenic medium undergo osteogenic differentiation

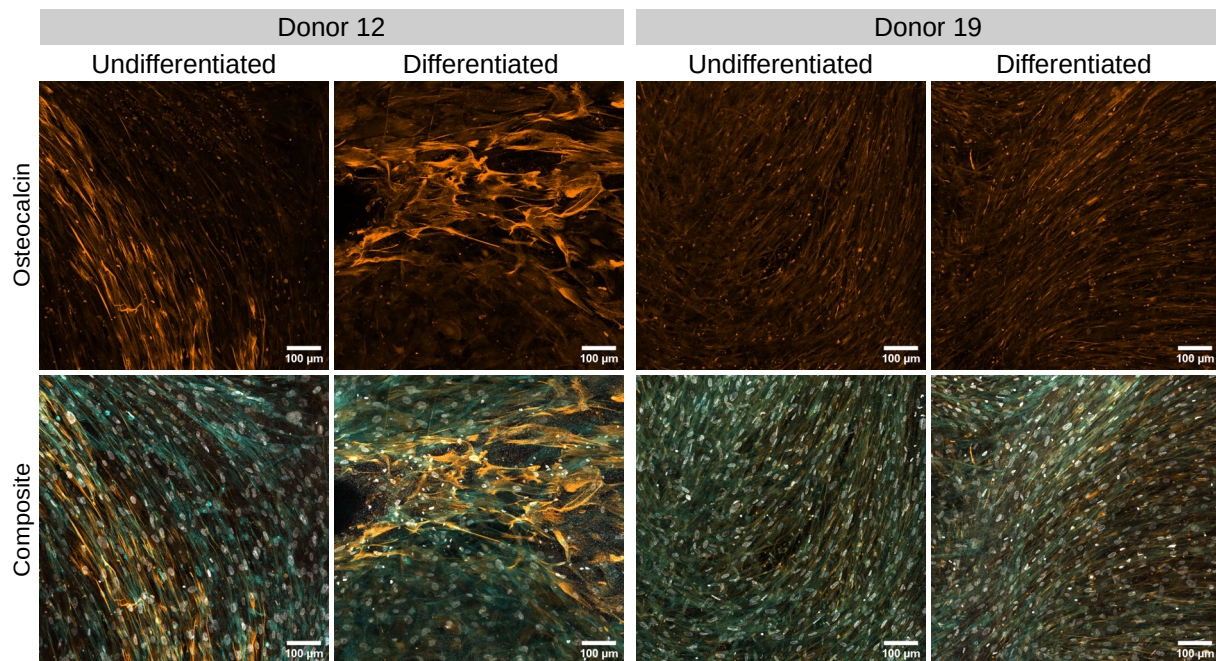


Figure 4.5: **Osteocalcin expression in differentiated and undifferentiated MSCs.** Representative images of two different MSC donors cultured either in osteogenic medium (differentiated) or expansion medium (undifferentiated) for 19 days. The top row displays the osteocalcin channel (orange), while the bottom row shows the composite image of osteocalcin (orange), nuclei (gray), and F-actin (cyan). Scale bar: 100 µm.

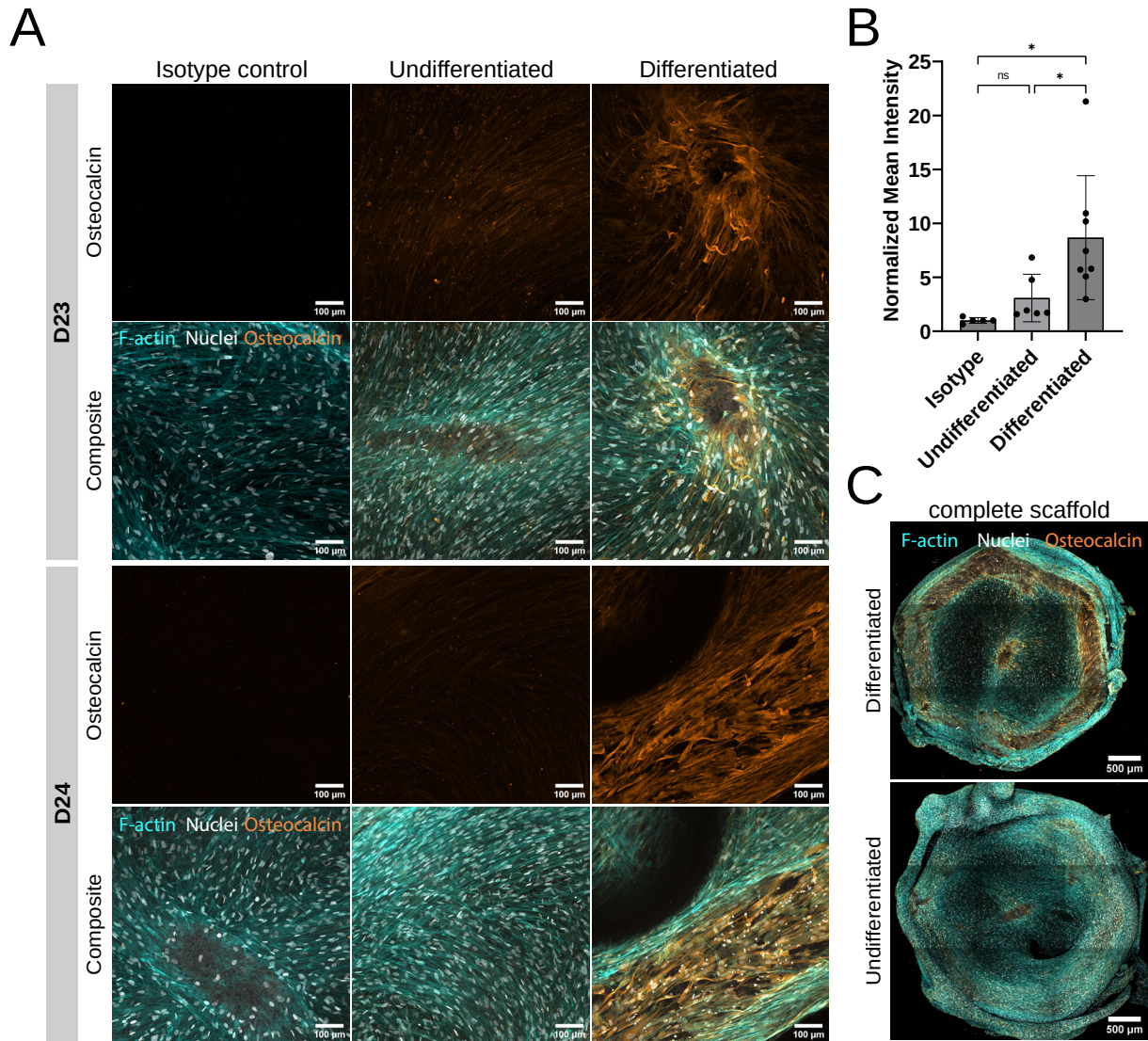


Figure 4.6: Osteocalcin expression indicates osteoblastic activity of MSCs after 21 days of differentiation. **A:** Representative images from two donors showing osteocalcin staining, including undifferentiated samples and isotype controls. The top row for each donor displays the osteocalcin channel (orange), while the bottom row shows the composite image of osteocalcin (orange), nuclei (gray), and F-actin (cyan). Scale bar: 100 μm . **B:** Quantitative analysis of osteocalcin expression based on the mean gray value intensity of multiple regions, normalized to the isotype control. Statistical significance was determined using one-way ANOVA with post hoc Tukey's multiple comparison test. **C:** Whole-scaffold images showing one sample from each donor, cultured in either differentiation medium (differentiated) or expansion medium (undifferentiated). Osteocalcin is displayed in orange, nuclei in gray, and F-actin in cyan. Scale bar: 500 μm .

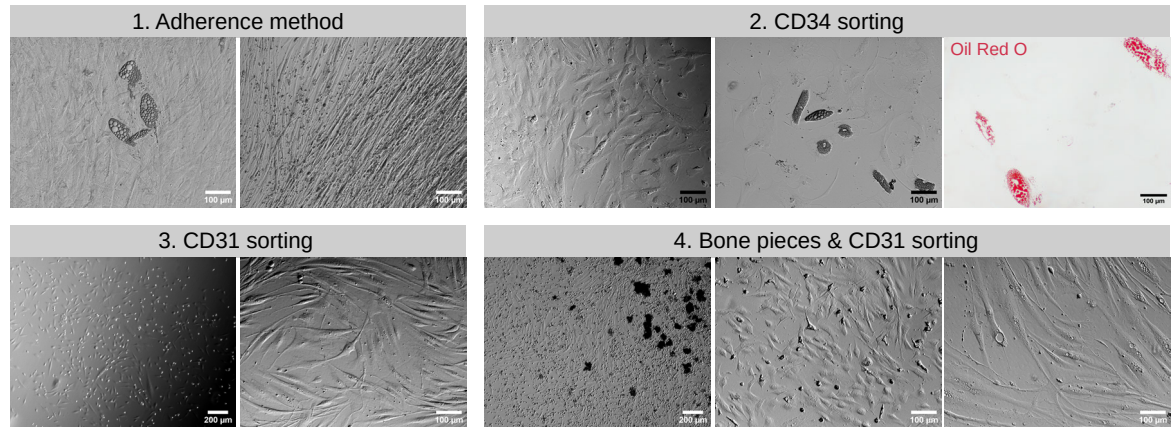
4.2.2 Perivascular niche: EPC isolation, comparison, and validation

4.2.2.1 Overcoming challenges in isolation and characterization of primary EPCs

The second niche we aimed to recapitulate from the BM microenvironment is the perivascular niche. Co-culturing MSCs and EPCs in 3D scaffolds supports early luminal structure formation and accelerates vascular differentiation, enhancing prevascularization [50, 51, 32]. While the previous section detailed the isolation of human MSCs from BM, this section first describes the procedure for isolating EPCs, which are essential for vascular formation, and then explains the setup of the co-culture to generate a perivascular niche. To achieve the goal of a fully patient-derived in vitro model, we attempted to isolate EPCs directly from BM. Therefore, various approaches were tested, with the first attempt exploiting the differential adhesion properties of specific cell types to selectively isolate EPCs. According to Fang et al. [43], BM-derived MSCs is the first cell type to adhere to standard cell culture plasticware. Following this approach, after 24 hours of incubation, the supernatant, presumably containing EPCs and HSCs, was transferred to a second culture flask coated with collagen I to facilitate the adherence of EPCs. Collagen I, along with fibronectin, is commonly used to support the expansion of endothelial cells [52, 53]. The culture medium EGM-2 from Lonza, supplemented with 10% FCS, was chosen for its specific formulation designed to promote endothelial cell proliferation and functionality. However, a limitation of using this proprietary formulation is the inability to directly compare the concentrations of the growth factors. Cultured cells eventually exhibited a mesenchymal phenotype, characterized by elongated morphology and the presence of lipid droplets, indicative of differentiation toward an adipogenic lineage (**Figure 4.7**, A1). The second approach for isolating BM-derived EPC involved first enriching CD34⁺ cells via MACS, followed by plating them on collagen I-coated plates and culturing in EGM-2. This method was chosen due to the role of CD34 in enriching progenitor cell populations [54], including endothelial progenitors from BM [50]. However, this approach also yielded cells displaying mesenchymal characteristics, including the presence of lipid droplets indicative of an adipogenic lineage, as confirmed by Oil Red O staining (**Figure 4.7**, A2). Furthermore, an attempt was made to directly isolate BM cells expressing CD31 via magnetic-activated cell sorting (MACS) from the mononuclear cell fraction of BM. Although it is known that other cell populations in the BM also express CD31, the rationale in all attempts was that the endothelial-promoting

medium might selectively support the expansion and enrichment of endothelial cells. In some cases, cells initially exhibited a cobblestone morphology characteristic of endothelial cells; however, with extended culture time, they transitioned to a mesenchymal phenotype. (**Figure 4.7**, A3). Another approach, which involved using small bone fragments as a cell source, allowing cells to migrate out of the fragments, and then rapidly sorting for CD31-positive cells, also proved challenging. The rapid outgrowth of presumably MSCs dominated the culture, making it impossible to establish a population exhibiting endothelial characteristics (**Figure 4.7**, A4). In addition to displaying the elongated morphology typical of mesenchymal cells, the isolated BM cells were stained for surface markers used to identify endothelial cells (i.e., CD31, CD144, and CD309), but the staining results were overall negative. In contrast, the immortalized EPCs line used as a control demonstrated clear CD31 staining, confirming the specificity of the staining protocol. Conversely, staining for CD90, a marker typically associated with mesenchymal cells and absent in endothelial cells, showed positive signals in the cells isolated and cultured for four passages (**Figure 4.7**, B). These results indicate that the isolated cells are of mesenchymal origin.

A



B

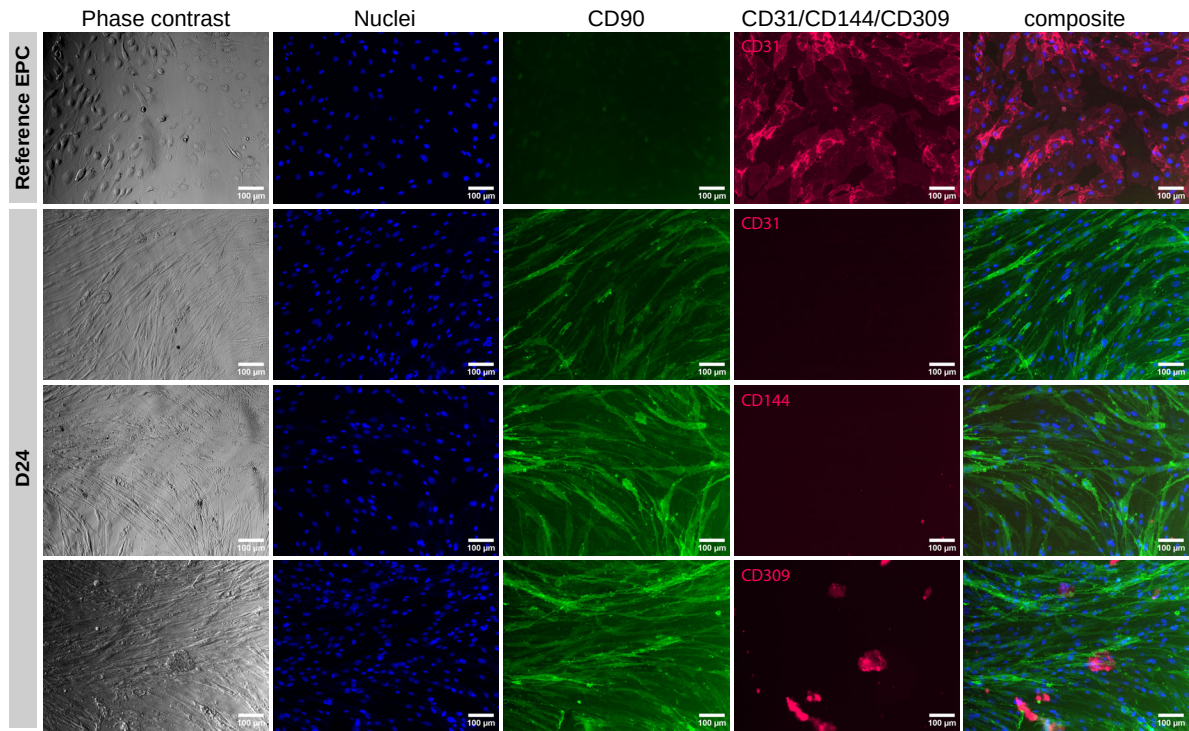
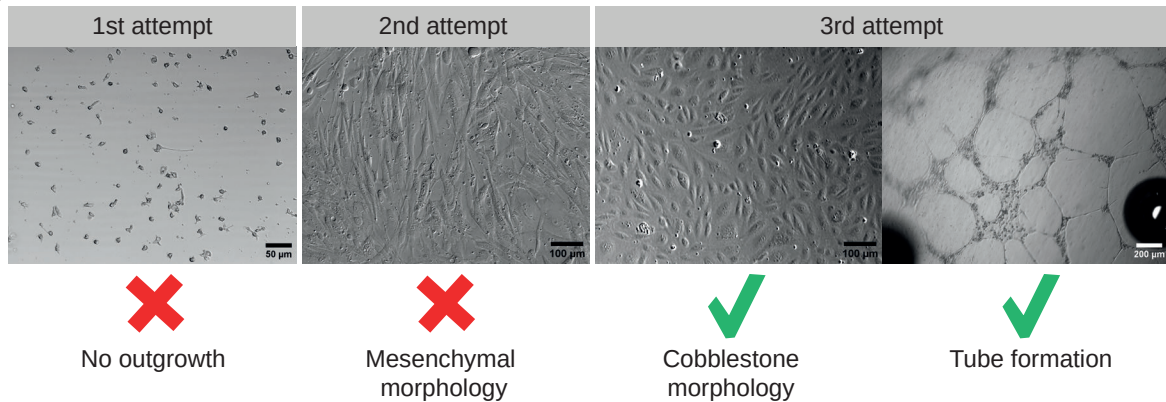


Figure 4.7: **EPCs isolation from BM** **A:** Representative images of the tested cell isolation methods. 1. Adherence-based method yielding cells with a mesenchymal phenotype. 2. Plating of CD34+ cells resulting in adipogenic features, confirmed by Oil Red O staining of lipid droplets after extended culture. 3. Plating of CD31+ cells demonstrating elongated mesenchymal characteristics. 4. Plating of small bone fragments followed by CD31 sorting, resulting in a mesenchymal phenotype similar to direct CD31 sorting from BM aspirates. **B:** Fluorescence staining for mesenchymal marker CD90 (FITC, green) and endothelial markers CD31, CD144, and CD309 (APC, red). Scale bar: 100 μ m

Since the isolation of EPCs from BM was unsuccessful, we turned to peripheral blood. We focused on BOECs, also known as late outgrowth endothelial cells or ECFCs, which display genuine endothelial properties and robust angiogenic capacity [55, 56]. However, circulating EPCs are exceedingly rare, representing only between 0.01% and 0.0001% of the total mononuclear cell fraction in normal peripheral blood samples, necessitating either very large volumes of blood or the pooling of multiple samples [57]. The first two attempts using approximately 20 mL of blood resulted in either no cell expansion or the isolation of a mesenchymal cell population. Subsequent efforts with a larger volume (100 mL) of donated blood, combined with a significantly higher cell seeding density of 3.3 million cells/cm² and an increased serum concentration (20%, compared to the initial 12%), successfully generated a homogeneous EPCs culture. These cells exhibited the characteristic cobblestone morphology typical of endothelial cells [56] and demonstrated the ability to form tubular structures when cultured on basement membrane extract (Matrigel) [56] (**Figure 4.8**, A). Further characterization revealed the expression of surface markers CD31, CD105, and CD146, with no detectable expression of the leukocyte marker CD45 or the mesenchymal-associated marker CD90. The primary distinction between adult peripheral EPCs and cord blood-derived endothelial progenitor cells (CB-EPCs) was the expression of CD34. Approximately 50% of CD31+ cells from adult peripheral EPCs co-expressed CD34, whereas only about 2% of CB-EPCs exhibited CD34 expression.

In the original protocol of the BM model we are adapting, neonatal EPCs were utilized [32]; however, limited access to cord blood and concerns regarding the relevance of neonatal cells to the characteristics of EPCs in older individuals led to the selection of adult peripheral blood-derived endothelial progenitor cells (pB-EPCs) as the next best option following the unsuccessful isolation of EPCs from BM. CB-EPCs provided by the Alblas lab in Utrecht were used exclusively for comparison with the pB-EPCs isolated in-house. Due to time constraints, an in-depth comparison between neonatal and adult-derived EPCs was not performed. Instead, efforts focused on validating the ability of adult pB-EPCs to form angiogenic structures, aiming to mimic the perivascular niche of the bone marrow. As demonstrated, the isolated pB-EPCs successfully formed angiogenic-like structures and were subsequently utilized for the establishment of the perivascular niche within the BM model.

A



B

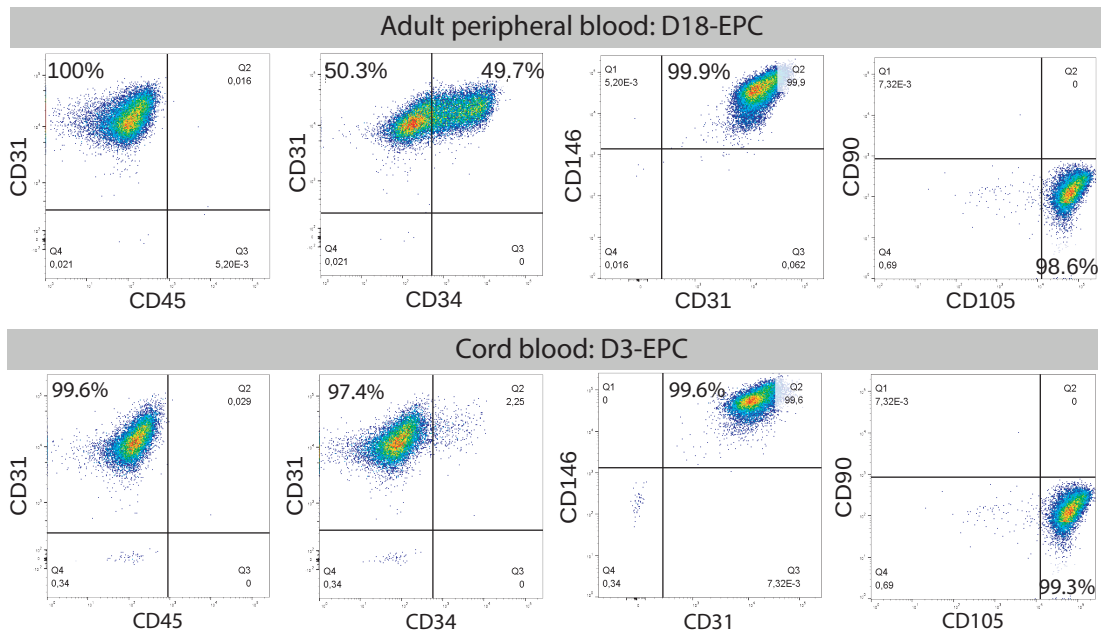


Figure 4.8: **EPC isolation from peripheral blood** **A**: Representative images from three independent attempts to isolate EPCs from peripheral blood. Minor adjustments were made between attempts, ultimately resulting in successful isolation in the third attempt, which yielded cells exhibiting cobblestone morphology and the capacity to form tubular structures *in vitro*. **B**: Surface marker expression profile of pB-EPCs compared with CB-EPCs.

4.2.2.2 Assessment of in vitro perivascular niche formation

With all the necessary primary cells available, the second BM niche, the perivascular niche, was established by co-culturing EPCs and MSCs in Matrigel. CD31-positive tubular structures were evaluated using fluorescence staining, and total tube length was measured with ImageJ software (**Figure 4.9, A**). Initial experiments followed the conditions described in the original publication, employing an MSC to EPC ratio of 4:1 [42]. However, differences in cell sources and culture environments can influence cell behavior, prompting a re-evaluation of cell densities and, subsequently, cell ratios within our specific experimental setup.

Three cell densities were tested using the proposed 4:1 ratio. Condition 1: (0.5×10^6 MSCs/ml with 0.125×10^5 EPCs/ml); condition 2: (1×10^6 MSCs/ml with 0.25×10^5 EPCs/ml); condition 3: (2×10^6 MSCs/ml and 0.5×10^5 EPCs/ml). Over the 14-day incubation period (with measurements on days 2, 5, 8, 11, and 14), condition 3 yielded the most extensive prevascular structures at every time point, whereas condition 2 consistently showed moderate tube formation and condition 1 produced the lowest total tube lengths with almost no increase over time. Notably, by day 14, condition 2 surpassed condition 1, but both remained well below the performance of condition 3 (**Figure 4.9, B**).

Normalization to the first measured time point proved unsuitable, as substantial network formation had already taken place, particularly in condition 3, emphasizing the need for a day 0 baseline measurement. Moreover, analyzing the entire sample rather than selected regions might have reduced variability, although this was not feasible with the current method. Statistical analysis using a mixed-effects model with Tukey's multiple comparison test confirmed that the highest cell density resulted in a significantly greater tubular length compared to both the medium and lowest densities.

Given the limited replicates in our biological setting, where assuming normality is challenging, we also performed non-parametric Mann–Whitney tests with Bonferroni–Dunn correction. These additional tests further supported our findings: for example, when comparing condition 3 to condition 1, significant differences were observed on days 5 ($p=0.045$), 8 ($p=0.000198$), 11 ($p=0.021978$), and 14 ($p=0.007770$); and comparing condition 3 to condition 2 revealed significant differences at all time points (with p values ranging from 0.023310 to 0.000007). Notably, condition 1 and condition 2 differed

significantly only on day 14 (**Table A1**). Consequently, the data clearly indicate that condition 3 produced the most robust networks and was therefore selected for future experiments.

Because the 4:1 ratio showed inconsistent performance across multiple experiments (data not shown), additional tests were conducted in both vascular-only and two-niche setups to evaluate alternative ratios alongside 4:1. Due to the extensive experimental design and the time-intensive nature of confocal microscopy, these comparisons were restricted to the highest cell density. The evaluation revealed that both the 1:1 and 2:1 ratios significantly outperformed the 4:1 ratio in supporting network formation, particularly within the two-niche system (**Figure 4.10**, A & C). In the two-niche setup, the 1:1 ratio generated notably more CD31-positive structures than the 4:1 ratio.

Because assuming normality in a very small sample size is difficult, we present results from both one-way ANOVA (assuming normality) and the nonparametric Kruskal–Wallis test. Both methods indicate that the 1:1 ratio significantly outperforms the 4:1 ratio (ANOVA: $p < 0.0001$, Kruskal–Wallis; adjusted $p = 0.0231$), reinforcing the conclusion that the 1:1 ratio promotes more robust prevascular structures. The ANOVA results depicted in the graph include comparisons of network formation both across different ratios within the same niche configuration and between the two-niche and vascular-only setups for the same ratio; however, cross comparisons involving different niche configurations at varying ratios were excluded to maintain a focus on biologically relevant contrasts. To validate the specificity of the CD31 antibody, an isotype control was used, which confirmed that the observed staining corresponds to endothelial structures (**Figure 4.10**, B).

It should be acknowledged that donor-specific variability in the MSC-mediated support of network formation may influence the results. The quantitative analyses presented here were conducted using MSC donor D24 and EPCs donor D18, with 3–4 biological replicates per condition. Images from an additional MSC donor (D23) exist, but network structures were not quantified because only 1:1 ratio data were available. Nonetheless, qualitative observations using donor D23 showed robust network formation at the 1:1 ratio with the highest tested cell density in both vascular-only and two-niche setups (**Figure 4.11**, top images).

Based on these findings, in which the 1:1 ratio produced a more robust prevascular network, we selected the 1:1 ratio for subsequent functional studies investigating cancer

cell growth in the BM model to ensure optimal niche formation. With the performance of both niches now validated, the groundwork is established for further optimization and integration of cancer cells into the BM model.

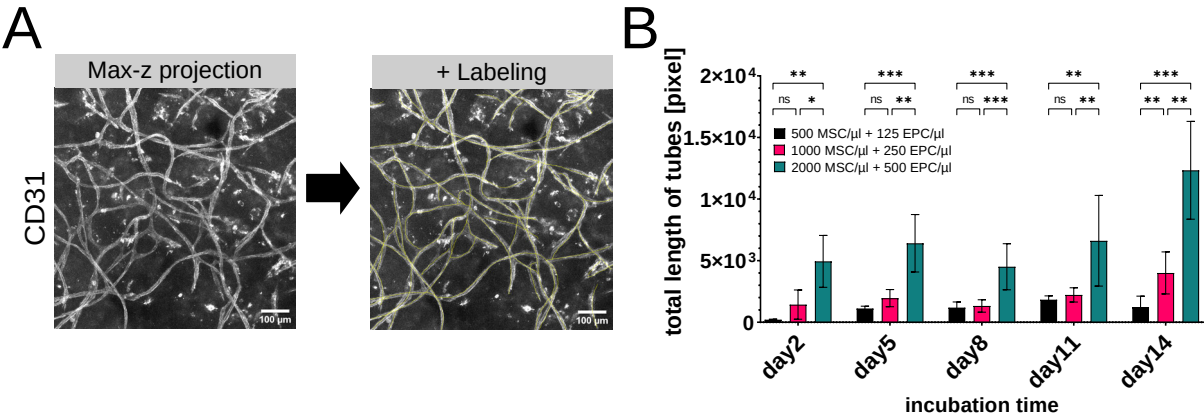


Figure 4.9: Cell density of MSCs and EPCs influences CD31+ prevascular network formation A: CD31–positive tubular structures visualized in a maximum intensity projection (max-z) through fluorescence staining. The tubular structures were manually labeled, and their total length was measured using ImageJ software to assess vascular network formation. B: Quantification of the total length of CD31–positive tubular structures formed under three different cell density conditions, tracked over 14 days. Statistical analysis was performed using a mixed–effects model with Tukey’s multiple comparison test.

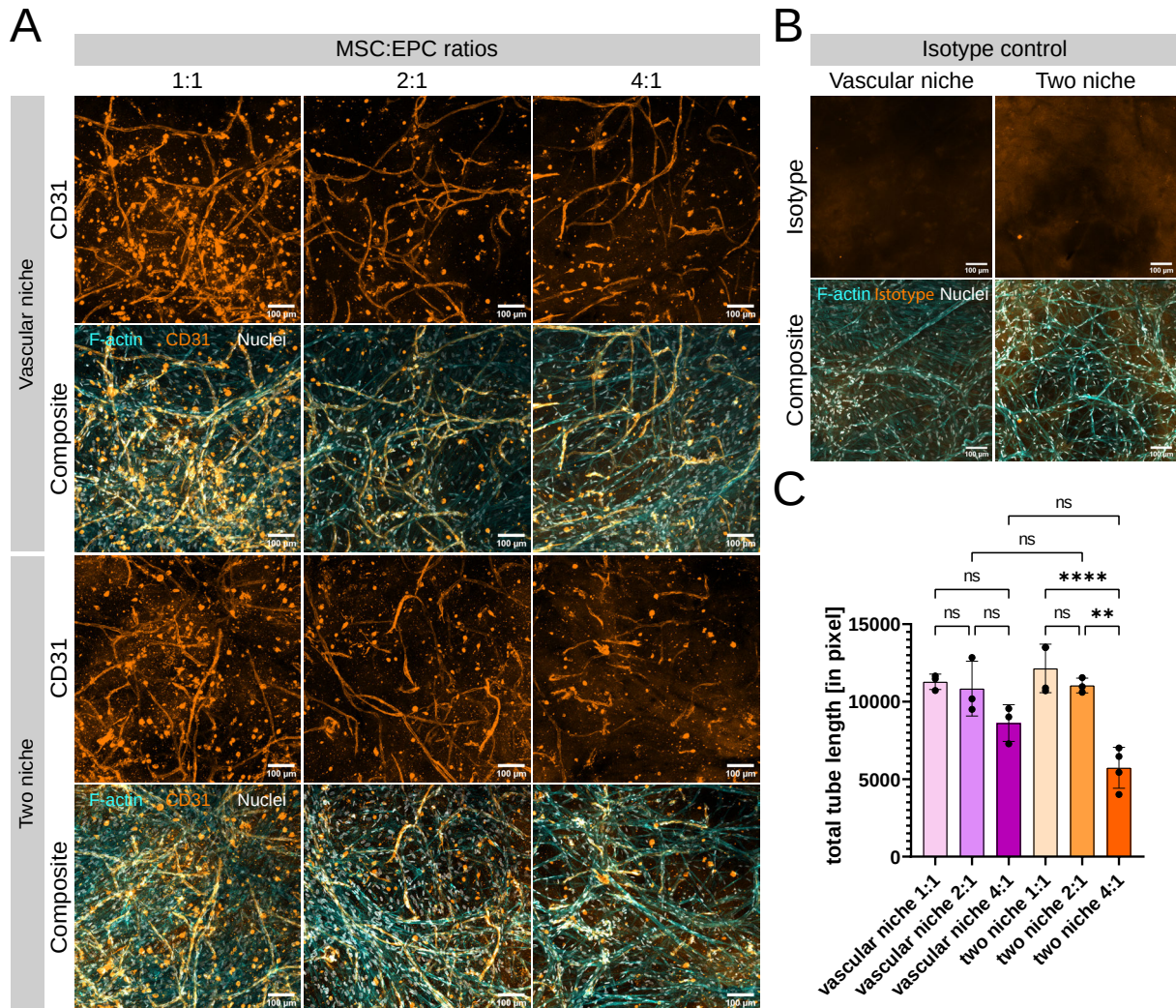


Figure 4.10: The ratio of MSCs to EPCs influences the functional characteristics of the prevascular niche **A:** Representative images of the three tested MSC to EPC ratios (1:1, 2:1, and 4:1) used to establish a functional compartment. The upper panel displays the vascular niche alone, while the bottom panel illustrates the combined two-niche system. Fluorescent signals are shown as orange (CD31), cyan (F-actin), and gray (nuclei), with a scale bar of 100 μ m. These images correspond to the highest cell density tested. **B:** Representative images of the isotype control, used to confirm the specificity of the antibody binding to CD31. **C:** Quantification of the total length of CD31-positive tubular structures formed at the highest cell density (2×10^6 MSCs/ml and 0.5×10^5 EPCs/ml), comparing three different MSCs to EPCs ratios (1:1, 2:1, and 4:1). Statistical significance was determined using one-way ANOVA with post hoc Bonferroni correction. Each sample was derived from one biological experiment with 3–4 replicates, and four randomly selected regions per replicate were measured.

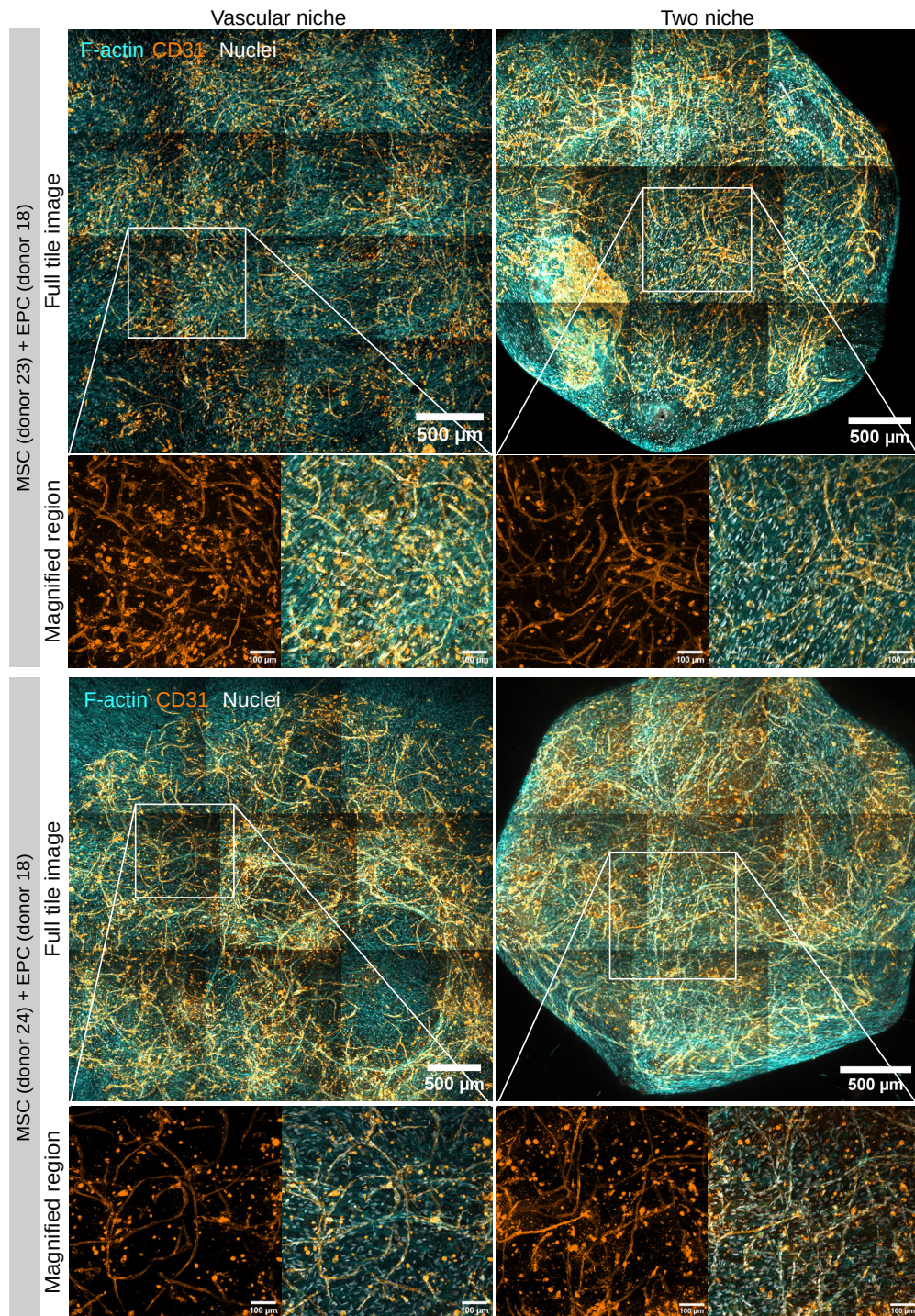


Figure 4.11: **Visualization of full tile images of the vascular niche and the combined two-niche model.** Representative complete tile images of the vascular niche (left) and the two-niche model (right) generated using MSCs from two different donors (Donor 23 and Donor 24). Fluorescent signals are shown as orange (CD31), cyan (F-actin), and gray (nuclei). Scale bars: 500 μm for the full tile images and 100 μm for the magnified regions.

4.3 Optimization of the technical aspects of the BM-model

Following the establishment and validation of the two niche BM models, this chapter provides a detailed account of the integration of cancer cells into the model and outlines the technical optimization steps necessary to investigate the interaction of each BM niche on cancer cell growth.

4.3.1 Optimization of seeding methods and cell quantity

An essential component of the optimization process involved refining the seeding method used to introduce cancer cells into the BM-model. In the original protocol, cancer cells were seeded simultaneously with the vascular niche cells. The proposed modification was designed to allow both niches to fully develop before introducing the cancer cells, thereby more closely replicating the physiological scenario in human patients, where DCCs) enter a fully functional bone marrow environment. To evaluate this approach, we manually selected 5-10 cancer cells under a microscope and suspended them in 3 μ l of Matrigel in low-binding tubes. This mixture ensured that the drop adhered to the scaffold and did not float away when medium was added. This mixture was carefully distributed onto the complete model setup and is referred to as the "seeding on top" method (**Figure 4.12, A**). Initially, this approach appeared successful. However, after conducting multiple experiments to investigate the localization of the cancer cells, we observed that, in most cases, cancer cells failed to migrate deeper into the BM-model. Instead, they remained and proliferated primarily on top of the vascular niche. In some instances, cancer cells localized near vascular cells (**Figure 4.12, B1**); however, in other cases, they predominantly grew on the layer above the entire model (**Figure 4.12, B2**). Notably, no interactions or localization with osteoblasts were observed, suggesting limited spatial integration within the BM-model.

To overcome this limitation, we tested an alternative strategy by directly injecting cancer cells into the perivascular niche. In our initial evaluation, we delivered as few as five cancer cells in 1 μ L of PBS using a fine pipette tip to confirm their incorporation into the model. However, the method proved challenging to execute and unreliable in consistently delivering cells into the niche. After several attempts to refine the technique,

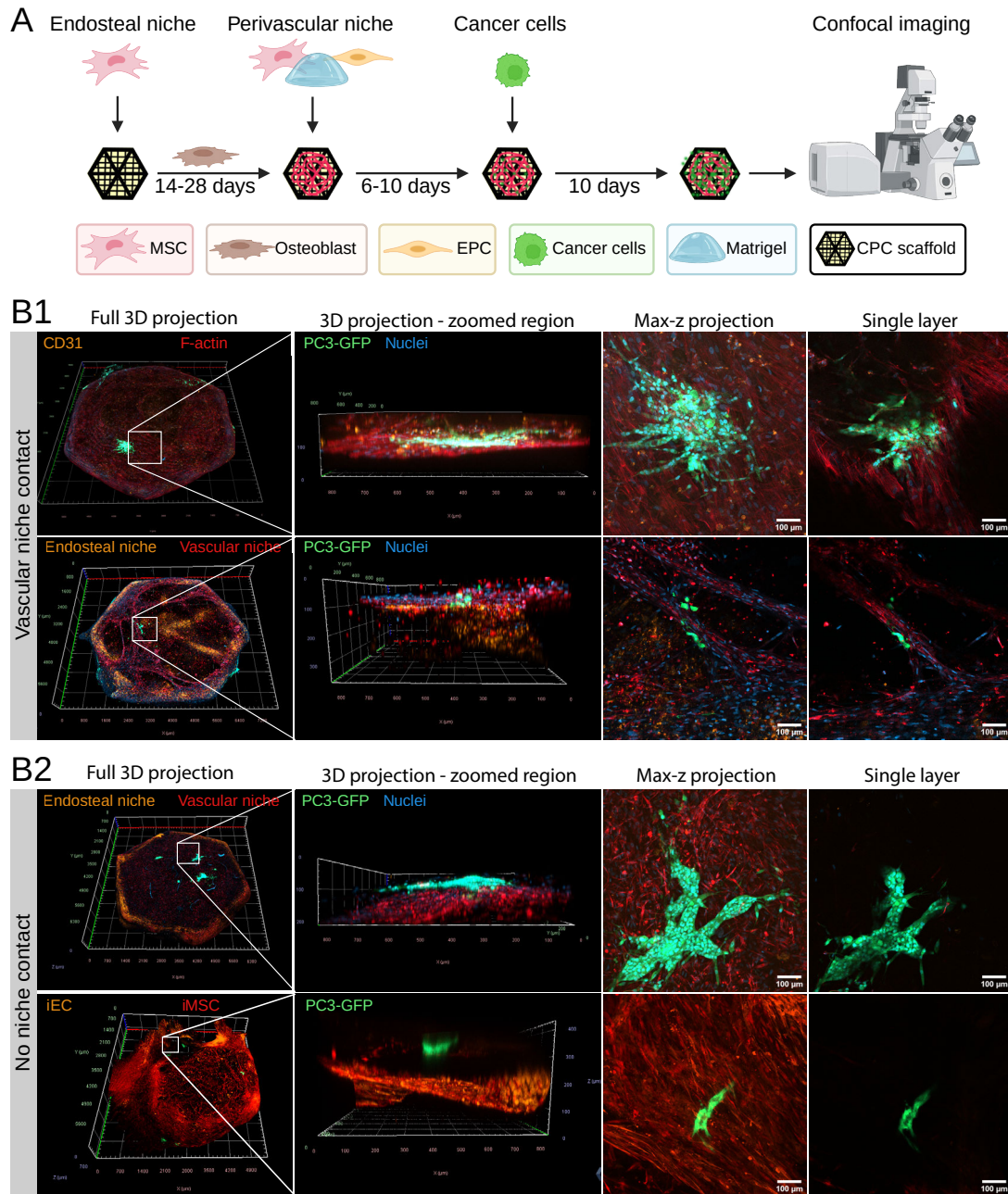


Figure 4.12: Impact of seeding strategies on spatial integration and interaction with BM niche cells.
A: Schematic workflow for establishing the complete BM model incorporating cancer cells. Cancer cells are introduced following the full development of the endosteal and perivascular niches, either by on-top seeding or through direct injection into the perivascular niche. Figure created with BioRender.com, with additional images of the scaffolds provided by the author **B:** Cancer cells (PC3-GFP) introduced via on-top seeding were observed either in close proximity to the vascular niche cells (B1) or predominantly within the layer above the vascular niche (B2). Images show various stainings or labels (indicated in each row). The left two columns display the 3D projection (including a zoomed region), the third column shows the max-z projection, and the rightmost column presents a single plane

we compared colony development using confocal imaging between injections of five cells and seeding five cells on the surface. Although colony numbers varied between both methods, likely due to the low cell count, the injection method resulted in no colony formation in two cases. **Figure 4.13**, part B, illustrates the average number of colonies counted after ten days of culture, highlighting the increased risk of significant cell loss associated with the injection method. In addition to the high risk of cell loss, this method also carried the potential for uncontrollable risk of damaging the existing niche structure. This limitation was attributed to the use of a standard pipette tip, rather than a specialized microsyringe device designed for precise microinjections under microscopic control. In conclusion, despite extensive attempts to optimize the model for greater human relevance by first establishing the vascular niche, this approach proved challenging in terms of reproducibility and ensuring that cancer cells could effectively interact with both niches. Consequently, we went back to the simultaneous seeding strategy as described in the original publication[32].

Having decided on a seeding method for implementing cancer cells into the model, the next critical step was to optimize the number of cancer cells used. This required balancing the need to closely mimic the low DCC counts typically observed in patient-derived BM samples while ensuring that the cell quantity was sufficient to enable reliable and quantifiable experimental analyses. To address this, we initiated our investigation using PC3 cells, a prostate cancer cell line derived from a bone metastasis of a grade IV prostatic adenocarcinoma in a 62-year-old white male. Following the protocol described by Braham et al. [32], 100 green fluorescent protein (GFP)-expressing PC3 cells were initially introduced into the model. However, this cell number resulted in an excessively high cancer cell burden within the model after just seven days of culture. (**Figure 4.14**, A). To better simulate patient conditions, the starting cell number was incrementally reduced to 20, 10, or as few as 5 cells. Results showed that introducing 10 or 20 cells yielded a robust number of cancer cell colonies after ten days of culture. In contrast, seeding just 5 cells proved insufficient, as only a very limited number of cells were detectable after ten days (**Figure 4.14**, B, C, D). Representative images of cancer cell numbers in the BM-model are not directly comparable due to experimental variability and the time-intensive nature of the model, which required iterative adjustments across experiments. In summary, the initial goal was to introduce a minimum of 5–10 cancer cells per sample to closely replicate the patient-derived DCC situation. However, for

functional studies using cancer cell lines, such as investigating the interactions between specific niche cells and cancer cells, this approach was refined. The optimized range of 20–30 cells per sample was adopted to balance the need for experimental practicality with maintaining model fidelity.

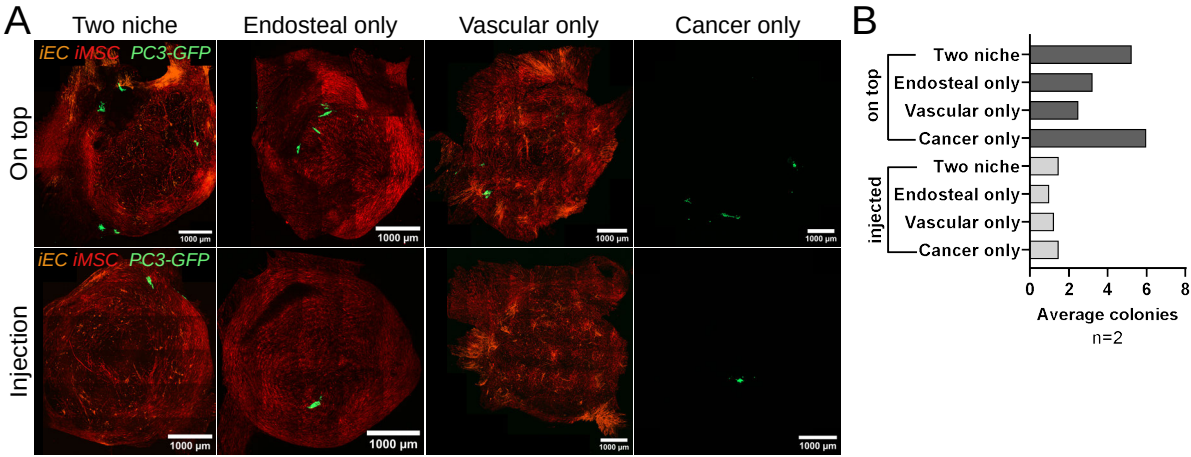


Figure 4.13: **Comparison of "on top" vs. "injection" seeding methods: Evaluation of colony formation.** **A:** Representative fluorescent microscopy images showing max-z projections of PC3-GFP (green) colony outgrowth across various niche configurations. Cells are labeled as follows: iEC (orange), iMSC (red), and PC3-GFP (green). Scale bar: 1000 μ m. **B:** Quantitative analysis of PC3-GFP colony outgrowth, showing the average colony size from two tested samples.

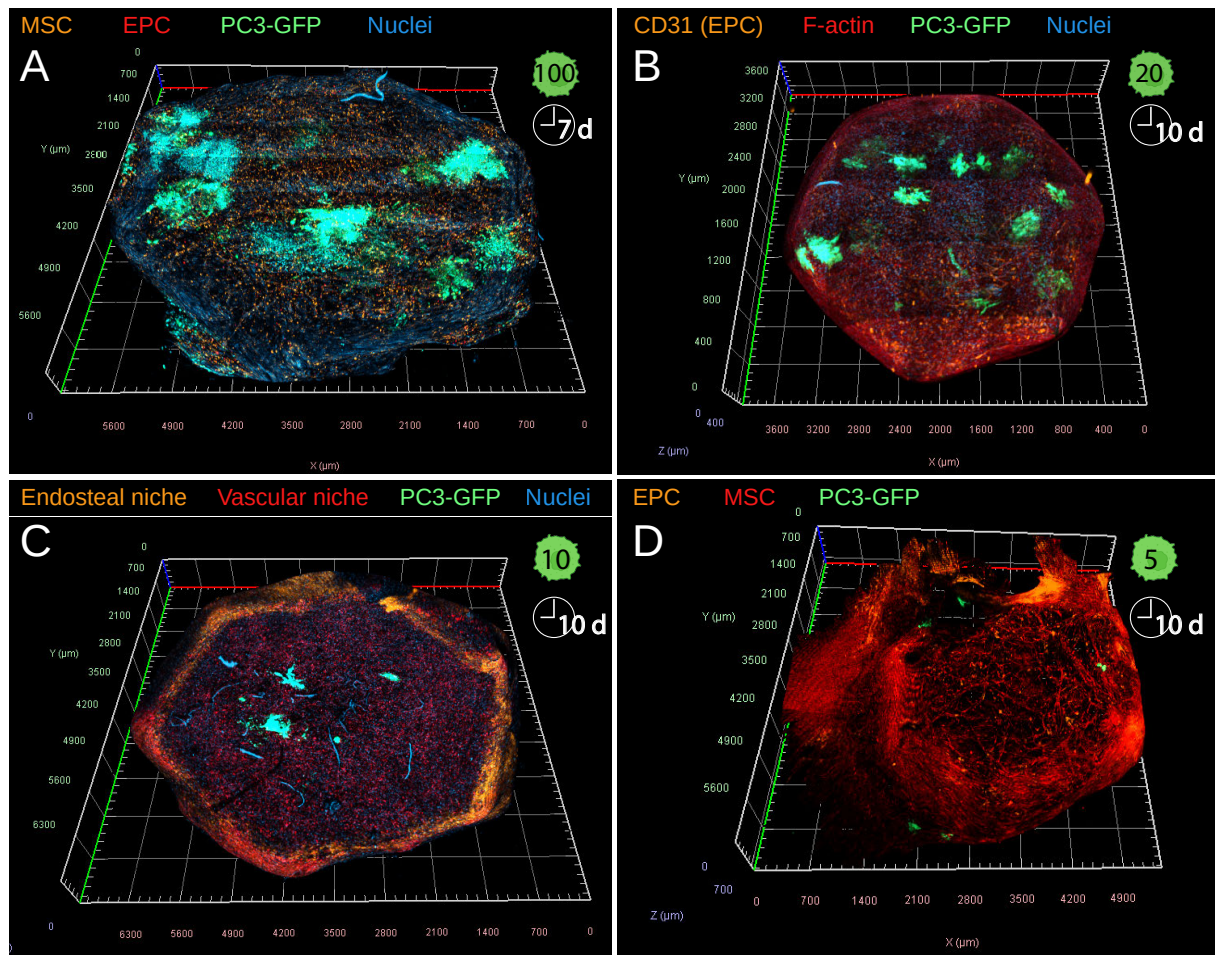


Figure 4.14: Fluorescent 3D projection images of two niche models depicting PC3-GFP cell colony outgrowth at varying initial cell numbers **A:** 100 initial PC3-GFP cells seeded simultaneously with niche-forming cells on a 5 mm scaffold and cultured for 7 days. Labels: MSCs (orange), EPCs (red), nuclei (blue), and PC3-GFP cells (green). **B:** 20 initial PC3-GFP cells seeded simultaneously with niche-forming cells on a 3 mm scaffold and cultured for 10 days. Labels: CD31 (orange, visualizing the EPC-derived network), f-actin (red), nuclei (blue), and PC3-GFP cells (green). **C:** 10 initial PC3-GFP cells seeded using the on-top method on a 5 mm scaffold and cultured for 10 days. Labels: Endosteal niche (orange), vascular niche (MSCs and EPCs, red), nuclei (blue), and PC3-GFP cells (green). **D:** 5 initial PC3-GFP cells seeded using the on-top method on a 3 mm scaffold and cultured for 10 days. Labels: MSCs (orange), EPCs (red), and PC3-GFP cells (green).

4.3.2 Overcoming challenges in confocal imaging of 3D in-vitro samples

The use of 3D tissue cultures necessitates advanced imaging techniques, such as laser scanning microscopy (LSM) or light-sheet microscopy, to achieve high-resolution analysis. At our university, imaging was conducted using a Zeiss LSM 980 confocal microscope. Consequently, the experimental model was specifically configured to ensure compatibility and optimal visualization with this imaging system. Working with a CPC-based scaffold presents significant challenges due to its opacity, which prevents light penetration. The first critical step in imaging the two-niche system was determining an appropriate sample-mounting strategy compatible with the microscope. Sectioning the model for internal imaging was not feasible, as the entire construct needed to remain intact to facilitate the relocation of all cancer cells. To enable imaging, the entire model was carefully handled with tweezers and positioned upside down on a thin cover slip. A second cover slip was placed on top, after adding a small amount of PBS to ensure the sample remained immersed in liquid. Imaging was conducted exclusively with 10x or 20x objectives, which eliminated the requirement for specific mounting media. This configuration allowed for repeated imaging of fixed samples, as they could be conveniently stored in PBS between sessions without the need for specialized mounting techniques. A notable challenge when imaging a 3D sample placed upside down was the dome-like structure formed by the Matrigel, preventing the formation of a flat imaging surface. This structural characteristic had to be carefully considered during three-dimensional image analysis to ensure accurate data interpretation. The next challenge was the slow acquisition speed of laser scanning microscopy. With the model measuring approximately 4.6 - 5 mm in diameter and up to 1 mm in thickness, its relatively large size resulted in imaging acquisition times of several hours per sample for full tile imaging depending on the amount of fluorescent channels. This lengthy process proved impractical for experiments involving multiple conditions and replicates. To address this, we aimed to downscale the model by utilizing smaller CPC scaffolds. These scaffolds were produced by our collaborators, Dr. Tilmann Ahlfeld and Richard Frank, from the group of Prof. Michael Gelinsky at TU Dresden. Due to limitations in the printing process, the smallest scaffold that could retain all essential features of the larger version had a diameter of 2.6–3 mm (**Figure 4.15, A**). The smaller scaffold successfully

preserved the grid-like architecture designed to mimic the trabecular bone region of the BM. To accommodate the reduced scaffold size, the seeding density of MSCs was adjusted from 100,000 to 50,000 cells. Viability was re-assessed using Calcein AM and ethidium homodimer 1 (Etdh1) staining (**Figure 4.15**, B), which demonstrated good cell coverage and predominantly viable cells after 21 days on the smaller scaffolds. Additionally, osteoblastic activity was evaluated through osteocalcin staining, using an immortalized MSC cell line expressing the fluorescent protein BFP. The results confirmed osteogenic activity throughout the scaffold (**Figure 4.15**, C). Additionally, the Matrigel volume for generating the vascular niche was reduced from 30 μ l to 10 μ L per scaffold to prevent spillover. These adjustments successfully downscaled the model size, enabling faster image acquisition and improved practicality for experiments involving multiple conditions and replicates

4.3.3 Development of a quantitative cell growth measurement pipeline using confocal microscopy

After optimizing the model size, seeding method, and appropriate cancer cell number, the next crucial step was to establish a reliable approach for quantifying cancer cell growth dynamics within the distinct niches of the BM-model (**Figure 4.16**, A). The objective is to evaluate the effect of each BM niche on cancer cell growth, aiming to identify which components of the microenvironment support or inhibit cancer cell proliferation. This requires the ability to perform precise growth measurements within the individual niche compartments of the BM-model. Given the complexity of 3D cultures, fluorescence intensity alone was considered an unreliable metric, primarily due to the technical limitations of laser scanning confocal microscopy. Although the Zeiss LSM980 system enabled imaging at depths of up to 700–800 μ m, the tradeoff was an uneven fluorescence intensity across focal planes due to laser power attenuation, light scattering, and absorption. Additionally, the variable spatial distribution of cells within the BM-model further complicated consistent signal detection. In order to address these challenges, the cancer cell cluster volume was used as an alternative quantitative metric. The prostate cancer cell lines used in growth experiments constitutively expressed GFP or yellow fluorescent protein (YFP) throughout their cytoplasm, enabling 3D volumetric reconstruction from confocal image stacks. Complete samples were imaged using tile

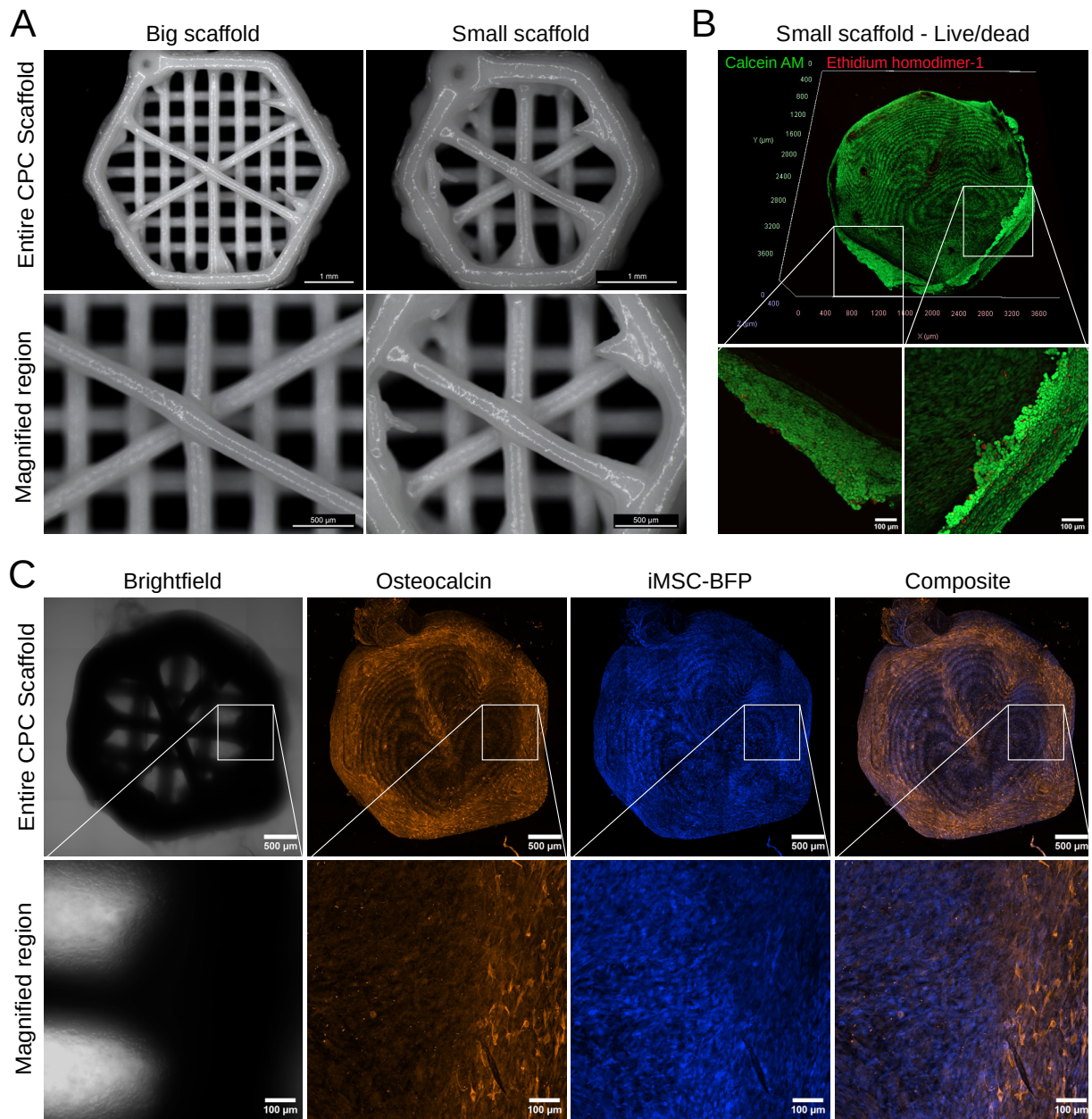


Figure 4.15: Downscaling of the CPC scaffold size for an optimized model design. **A:** Overview of CPC scaffold designs in two sizes: the larger scaffold (4.5–5 mm in diameter) and the smaller scaffold (2.6–3 mm in diameter). Scale bars: 1 mm for the entire scaffold and 500 µm for the magnified regions. The images were kindly provided by Dr. Tilmann Ahlfeld. **B:** Viability of the reduced cell number on the smaller scaffold, visualized using Calcein AM (green) and Etdh1 (red) staining. A 3D projection of the full scaffold is shown alongside two magnified regions. The magnified regions display a scale bar of 100 µm. Dimensions of the full scaffold are represented along the X and Y axes in micrometers (µm). **C:** Osteogenic activity demonstrated by Osteocalcin staining (orange) after 22 days of differentiation in immortalized MSCs (iMSCs) expressing the fluorescent protein BFP (blue). Scale bars: 500 µm for the entire scaffold and 100 µm for the magnified region. The iMSCs were generously provided by Dr. Hind Medyouf from Georg Speyer Haus, Frankfurt.

stitching, covering as many z-planes as necessary to capture the entire GFP or YFP signal for an approximation of the volume of cancer cells. Variables to consider for a volume approximation was for example the step size and the tile image resolution to balance spatial resolution and imaging time required. Initially, a step size of 18-20 μm was used, acknowledging that some cellular details might be missed. However, this compromise was necessary due to the large 5 mm scaffolds, which required extensive imaging time. With the transition to smaller scaffolds, the step size was reduced to 6 μm , enabling more precise volume quantification. To accommodate the increased number of z-planes while ensuring efficient data acquisition, the individual tile size was reduced to 512 px $\times$ 512 px. Furthermore, by using a step size smaller than the average cell diameter of PC3 cells, we ensured sufficient overlap between image slices, thereby enhancing the accuracy of the volume approximations. To establish a reproducible measurement pipeline, area measurement was performed using ImageJ, supported by a custom-written macro to enable batch processing of large image datasets. A consistent threshold was applied across all samples to ensure comparability and reduce measurement bias. Manual adjustments were only made when variations in background intensity or signal quality required threshold refinement for accurate segmentation. After measuring the area in pixels, it was converted to square micrometers (μm^2) by multiplying by the known pixel size (2.75 μm^2). Finally, the estimated cell cluster volume was calculated by multiplying the measured area in μm^2 by the step size in μm , providing an approximate 3D representation of the cell clusters within the engineered BM-model (**Figure 4.16**, B). This imaging and measurement pipeline was applied in the functional studies described in Chapter 3.6 to evaluate the growth behavior of PC3 cells within the distinct BM niches.

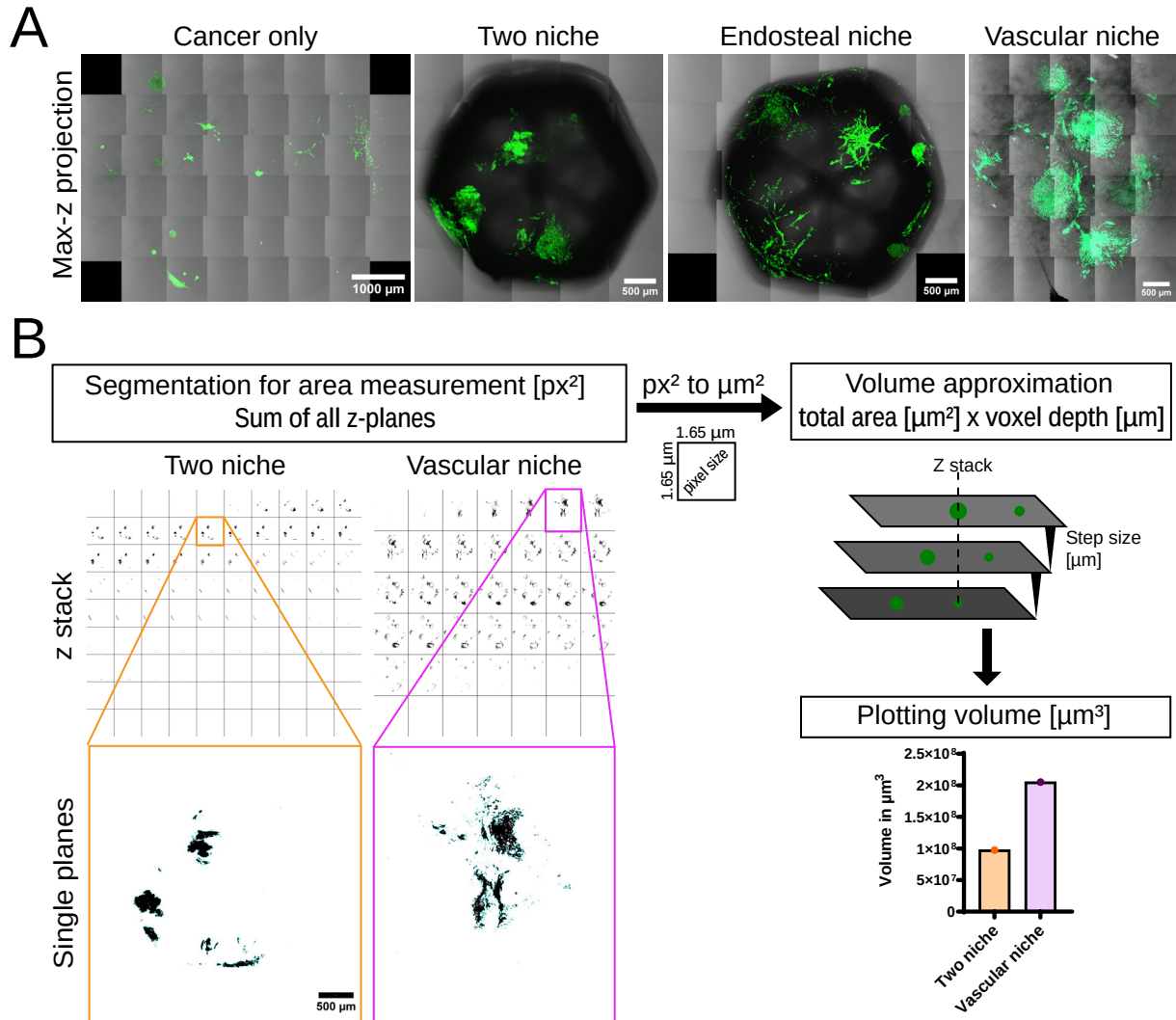


Figure 4.16: **Overview of quantification pipeline to evaluate cancer cell growth in BM niches.** Representative images of all distinct niche conditions showing the maximum z-projection of PC3-GFP (green) cell growth after 10 days. B: Overview of PC3 cell cluster volume measurement using segmentation to determine the fluorescent signal area of PC3-GFP cells in each z-plane using ImageJ. Subsequent calculations transformed pixel area (px^2) into μm^2 , followed by volume approximation through multiplication by the voxel depth.

4.3.4 Optimization of cancer cell quantity and standardization

The described pipeline for determining the volume of cancer cell clusters within the BM-model requires a consistent initial cell number or appropriate normalization to enable comparison of approximated cell cluster volumes between samples. Thus, controlling the initial seeding cell number was essential, but achieving consistent seeding at such low densities proved technically challenging. Initially, we considered seeding additional samples fixed on day 1 or 2 alongside those cultured for 10 days. These early-fixed samples were intended to serve as reference points for generating a standard curve that correlates seeding cell numbers with initial cell volumes, thereby normalizing for cell seeding differences. However, this approach was abandoned after the first attempt, as it significantly increased imaging time and became impractical for large-scale experiments. Moreover, even with a well-established standard curve, accurately determining the initial seeding cell number for all conditions, including those involving the CPC scaffold, remains nearly impossible. Manual picking of individual cells was also not feasible for experiments with high sample numbers. Therefore to address these challenges, the protocol was adjusted by adopting a master mix (MM) approach, reaching for a seeding density of 20-30 cancer cells per sample. However, due to the inclusion of multiple niche conditions in a single experiment, two separate master mixes were required for the four conditions tested (MM1: two-niche and vascular niche; MM2: cancer-only and endosteal niche). To ensure comparable seeding densities between these master mixes, cell numbers were counted in numerous samples without scaffolds immediately after seeding. The seeded cell numbers presented here are derived from the experiment evaluating PC3 cell growth dynamics (Section 4.6), which yielded cell counts ranging from 20 to 44 (**Figure 4.17**). Although the cell counts ranged from 20 to 44, these differences were uniformly distributed across the two master mixes (MM1 and MM2) in all three independent experiments. Statistical analysis confirmed no significant differences between the master mixes, with one-way ANOVA (assuming normality) yielding p-values ranging from 0.1343 to >0.9999 and the nonparametric Kruskal–Wallis test yielding p-values all >0.9999. When data from all three experiments were combined as biological replicates, comparable cell distributions between MM1 and MM2 were confirmed (**Figure 4.17**, B). This was further supported by statistical testing, which revealed no significant difference between the groups using both an unpaired

t-test ($p=0.0958$) and a Mann–Whitney test ($p=0.2246$). This approach represents a compromise between accuracy and feasible experimental handling in setups involving multiple conditions and replicates. Nevertheless, careful monitoring is essential in every new experimental setup, as low initial cell numbers may introduce significant variability, potentially affecting cell growth quantification.

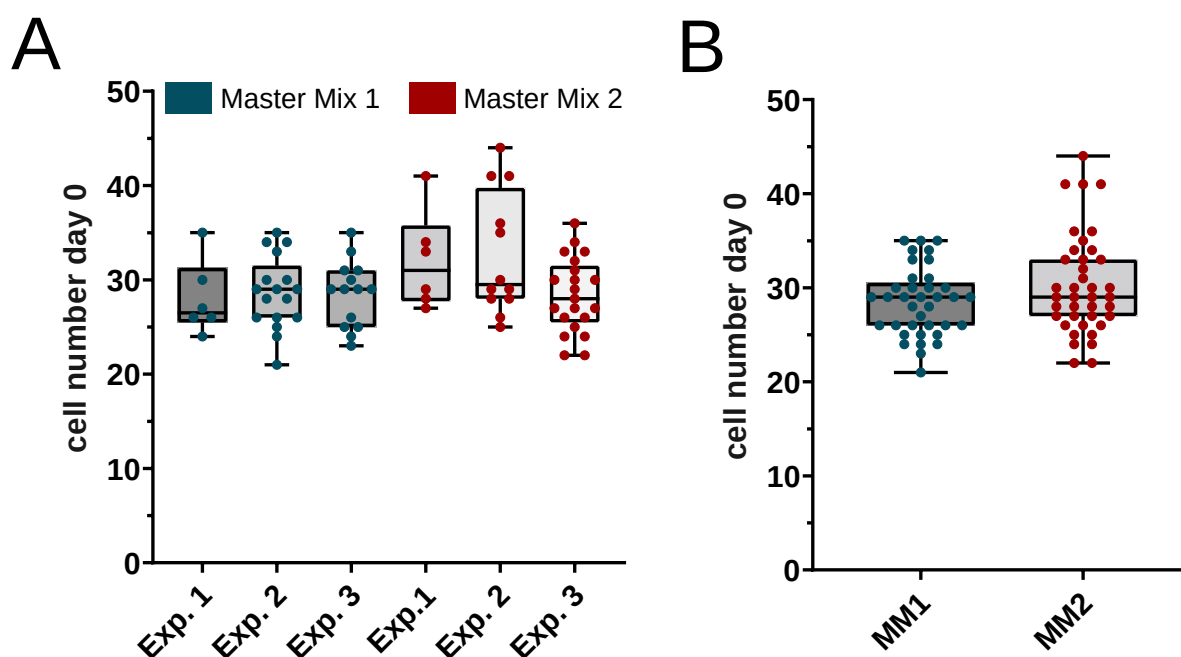


Figure 4.17: **Cell counts on day 0 using the master mix (MM) approach** A: Scatter plot showing the starting cell numbers from all prepared MMs across three independent experiments. Statistical analysis using the either ANOVA and Kruskal-Wallis test revealed no significant differences in cell numbers across experimental conditions. B: Combined analysis of all biological replicates comparing cell distributions between MM1 and MM2 using either t-test or Mann-Whitney U test showed no statistically significant differences. Exp: Experiment, MM: Master Mix

4.3.5 Protocol development for single-cell transcriptomics in BM-model applications

After establishing a robust pipeline for quantitative microscopic evaluation, the next objective was to extend our analyses beyond imaging to include transcriptomic and genomic data. To this end, we developed a protocol for dissociating the model components into a single-cell suspension, thereby enabling downstream molecular analyses. The

optimization of this dissociation protocol was carried out as part of Mira-Viola Mayr's bachelor thesis, titled "Optimization of the 3D In Vitro Bone Marrow Model for Single-Cell Sequencing," under my supervision. The optimized protocol is shown in **Figure 4.18** and begins with a Dispase treatment (4 U/ml) for 30 minutes at 37°C (1), during which the Matrigel is loosened by repeated pipetting up and down. Without washing, Collagenase is then added at a concentration of 0.3 mg/ml (2), followed by another 30-minute incubation, again with pipetting to ensure proper dissociation. The final enzyme treatment involves TrypLE (3), which is added directly to the sample and incubated for an additional 30 minutes, with the cell suspension resuspended multiple times during the incubation. After the enzyme treatments, the samples are pooled together and washed once (4) to remove residual enzymes (**Figure 4.18**, A). This method consistently produced a viable single-cell suspension from the BM-model, with an average cell viability of 80-90%. The only exception was osteoblasts from the endosteal niche when cultured alone, which showed slightly lower viability, averaging just below 80% (**Figure 4.18**, C1). Another important factor to consider was the total cell count after dissociation, which determined how many wells needed to be pooled to obtain a sufficient number of cells for sequencing. As expected, the cancer-only condition was the most limiting, yielding between 100 and 1,200 cells per well. Among the BM niches, the two-niche model produced approximately 60,000 cells per sample. The endosteal niche yielded the lowest recovery, averaging 20,000 cells per sample, while the vascular niche had the highest recovery, averaging 80,000 cells per sample (**Figure 4.18**, D). Building on the results obtained during the bachelor thesis, as summarized above, minor adjustments were implemented to optimize the protocol before employing the 10x Genomics platform for transcriptomic analysis of various cell types across all niches. Since the droplet formation process of the 10x platform requires exclusively viable cells, and despite achieving high viability rates, cell sorting was performed using a fluorescently activated cell sorting (FACS) machine to eliminate dead cells and ensure a purely viable suspension. Additionally, FACS enabled precise control over the selection and quantity of niche and cancer cells included in the analysis. This workflow allowed for the recovery of all cell types as single cells from the complex 3D BM model, facilitating detailed downstream analyses.

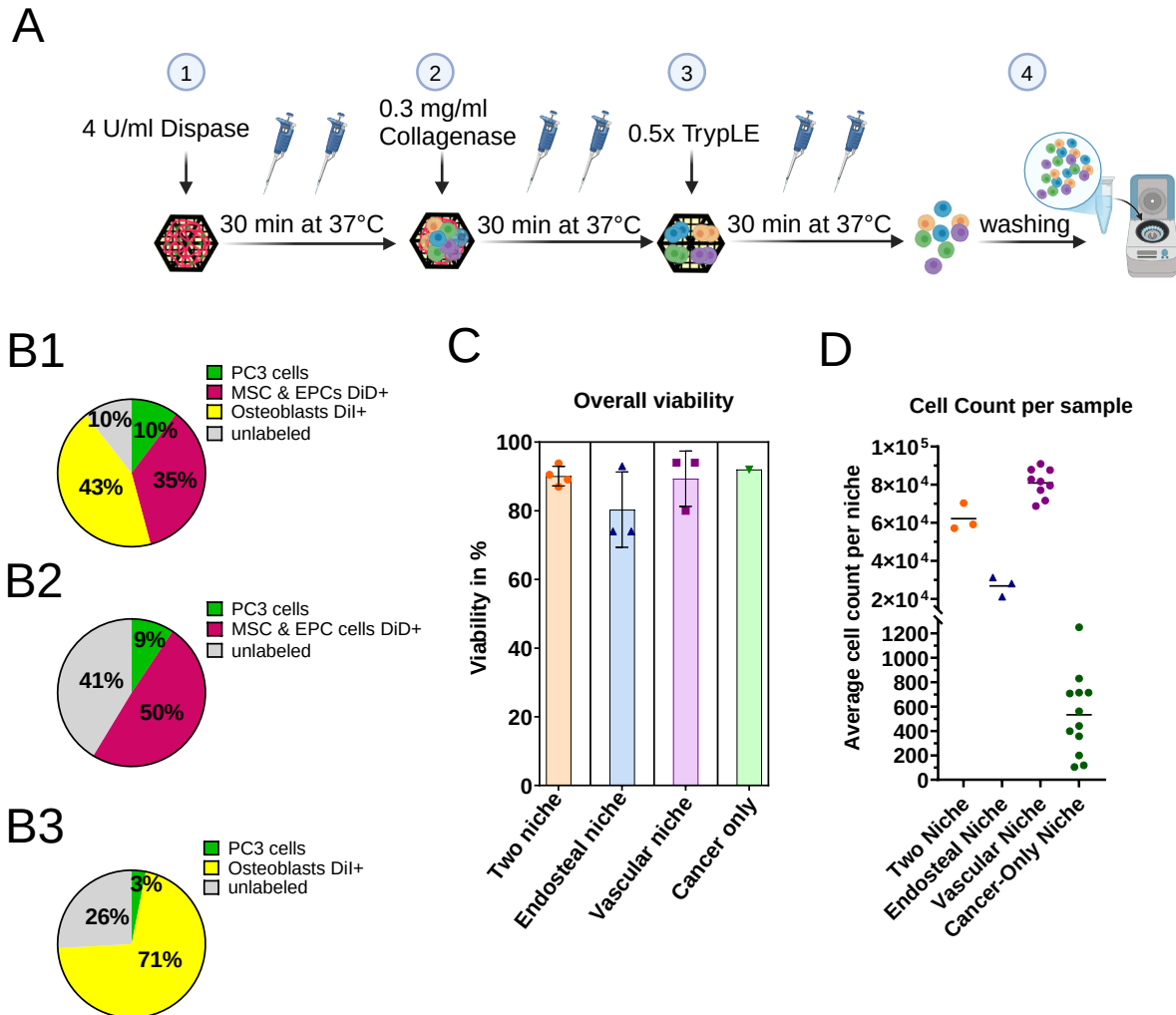


Figure 4.18: Workflow of dissociation protocol of the BM-model to generate a viable single-cell suspension for transcriptomic analysis. A: Protocol summary for generating a viable single-cell suspension, as suggested by Mira-Viola Mayr. Figure created with BioRender.com, with additional images of the scaffold provided by the author. B: Cell type distribution of specific niches after dissociation: B1: Two-niche, B2: Vascular niche, and B3: Endosteal niche. Cells were pre-labeled at the beginning of the experiment with DiD (MSCs and EPCs) or DiI (Osteoblasts), while unlabeled cells represent those that lost the label over time. C: Overall cell viability from specific niches after dissociation. D: Total cell count of all cell types per sample from specific niches following dissociation. The data presented in this figure was generated as part of the bachelor thesis work by Mira-Viola Mayr.

4.4 Refinement of the BM-model

Building on the technical optimizations detailed in the previous chapter, this chapter focuses on refining the BM-model to more accurately replicate the physiological conditions of human bone marrow. The goal was not merely to replicate the BM-model established by Braham et al. [32], but to advance it by incorporating exclusively human-derived materials and, wherever feasible, isolating cells directly from prostate cancer patients.

Specifically, this chapter explores the integration of human-derived components, such as hPL as an alternative to FCS, and the development of organ-specific extracellular matrix (ECM) hydrogels to replace Matrigel. These refinements are intended to enhance the model's microenvironmental fidelity, providing a more physiologically relevant platform for studying prostate cancer DCCs.

4.4.1 Human platelet lysate as alternative to FCS

4.4.1.1 Preparation of human platelet lysate from apheresis donations for the use in cell culture

To reduce reliance on animal-derived components and advance toward a more physiologically relevant and humanized alternative to FCS for cell culture, we investigated the use of hPL in all steps of the BM-model setup. Three hPL batches were prepared over the course of the PhD in cooperation with the Department of Transfusion Medicine at UKR (**Table 4.2**). Balancing availability, an increased number of donors would be desirable to minimize batch variability. At least two expired apheresis donations from two different donors were pooled together, and the platelets were lysed by three freeze (-20°C) and thaw (37°C) cycles. The supernatant was collected after centrifugation at 3000 x g for 30 min and filtered through a 0.45 µm filter and stored at -20°C until use (**Figure 4.19**, A). Protocol was adapted from Hamed et al. [44].

Further for the preparation of hPL-supplemented medium, it is essential to inhibit clotting, which can be achieved either by adding heparin or through mechanical fibrinogen depletion [44]. For example, during MSC expansion, the protocol involved first preparing the expansion medium by combining all reagents (aMEM + 1% P/S + 0.2 mM L-ascorbic-acid-2-phosphate + 10% hPL). To induce fibrin clotting, the medium was incubated at different temperatures (RT, 4°C, and 37°C). Subsequently, the fibrin clots were removed

by centrifugation, and the supernatant was sterile-filtered through a 0.22 μ m PES filter (**Figure 4.19**, B). All cell cultures supplemented with hPL were handled in the same way as those cultured with FCS.

For MSC expansion medium prepared with hPL-1 or hPL-2, mechanical fibrinogen depletion was fully effective. However, with hPL-3 and EGM-2 medium, fibrinogen removal was insufficient to prevent gelling at 37°C. In these cases, 10 U/mL of heparin was added to the medium to prevent clotting during culture.

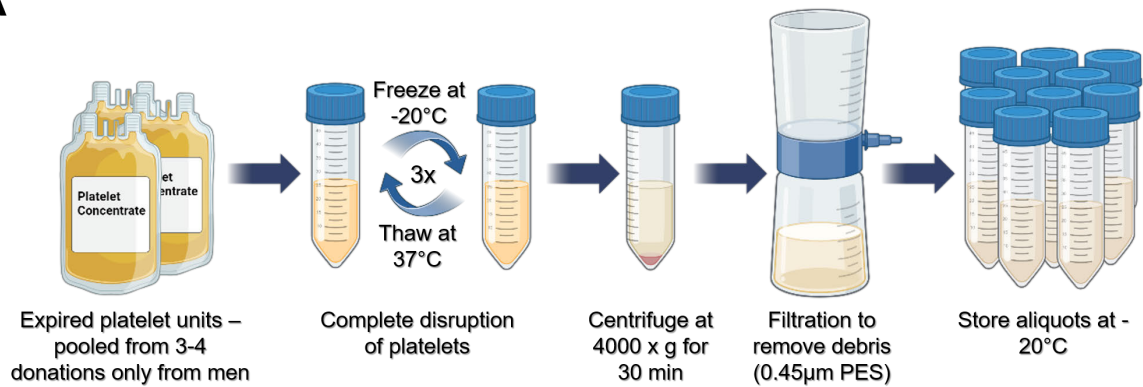
To evaluate the suitability of the prepared hPL for cell culture applications, its growth factor composition was analyzed. An enzyme-linked immunosorbent assay (ELISA) was performed for each batch of hPL to quantify key growth factors, including bFGF, epidermal growth factor (EGF), insulin-like growth factor (IGF-1), transforming growth factor beta (TGF- β), and platelet-derived growth factor BB (PDGF- $\beta\beta$), which are critical for supporting cell proliferation and maintaining functionality in the BM-model. The concentrations of growth factors in the prepared hPL batches were as follows: bFGF ranged from 31.03 ± 7.67 pg/ml, EGF from 150.33 ± 45.61 pg/ml, IGF-1 from 175 ± 99.87 pg/ml, TGF- β from 5.41 ± 3.57 ng/ml, and PDGF- $\beta\beta$ from 1.44 ± 0.57 ng/ml (**Table 4.3**). Although the results revealed variations among batches, all batches exhibited comparable efficacy in supporting cell culture. Given the focus on prostate cancer research, female donations were excluded to avoid introducing gender-specific hormonal differences into the study. As a result, the first batch (hPL-1), which included a female donor, was used exclusively for initial pilot experiments and was replaced by batch 2 and 3 for all subsequent experiments.

Table 4.2: hPL-batches

hPL batch	Received date	Volume received	Pooled	Donors	Women excluded	Lysate processed
1	2019-11-04	$\approx$ 600 ml	yes	3	no	2019-11-04
2	2019-11-30	$\approx$ 2 L	yes	4	yes	2019-12-16
3	2021-08-27	675 mL	yes	2	yes	NULL

As previously mentioned, the mechanical depletion protocol was ineffective for endothelial medium (EGM-2). Consequently, 10 U/ml of heparin was consistently added to all hPL-based EGM-2 media to prevent clot formation. Additionally, in some instances,

A



B

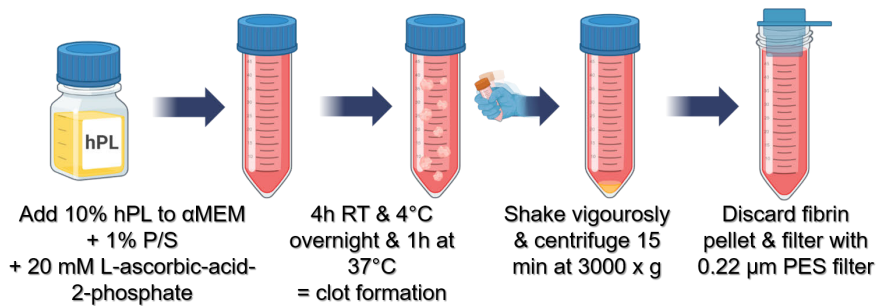


Figure 4.19: **Preparation protocol for hPL using expired platelet donations.** This protocol was adapted from Hemeda et al.[44]. **A:** Expired platelet units from male donors were pooled, and the platelets were lysed through repeated freeze-thaw cycles. **B:** Workflow for the mechanical removal of fibrinogen from the MSC expansion medium prior to its use in cell culture. Figure created with BioRender.com.

Table 4.3: Concentration of growth factors in hPL

Growth factor	hPL-1	hPL-2	hPL-3	Averaged + SD
bFGF [pg/ml]	39.25	24.02	29.83	31.03 ± 7.69
EGF [pg/ml]	124	203	124	150.33 ± 45.61
IGF-1 [pg/ml]	260	200	65	175.0 ± 99.87
TGF-β [ng/ml]	5.75	1.68	8.8	5.41 ± 3.57
PDGF-ββ [ng/ml]	1.21	2.08	1.02	1.44 ± 0.57

heparin was incorporated into the MSC expansion medium prepared with the third hPL batch (hPL-3) due to occasional failures of the mechanical fibrinogen depletion protocol for this batch. Since heparin was not included in the FCS-based medium, it was important to assess its potential effect on cell viability under the concentration used in the hPL-supplemented conditions. Therefore, MSCs and EPCs were cultured in FCS-based medium with or without 10 U/ml heparin, and WST-1 assays were performed on days 1–3 to measure metabolic activity (**Figure 4.20**). While a small increase in EPC activity was observed on day 2 under the heparin condition, this effect was not seen on days 1 and 3. Non-parametric analyses (Mann–Whitney tests with Bonferroni–Dunn correction) showed no significant differences, and although a two-way ANOVA suggested significance for EPCs on day 2, the lack of any lasting effect on day 3 implies that the observed variation may be random. Overall, these data so far indicate that heparin alone exerts minimal effects on the viability of MSCs or EPCs at the tested concentration.

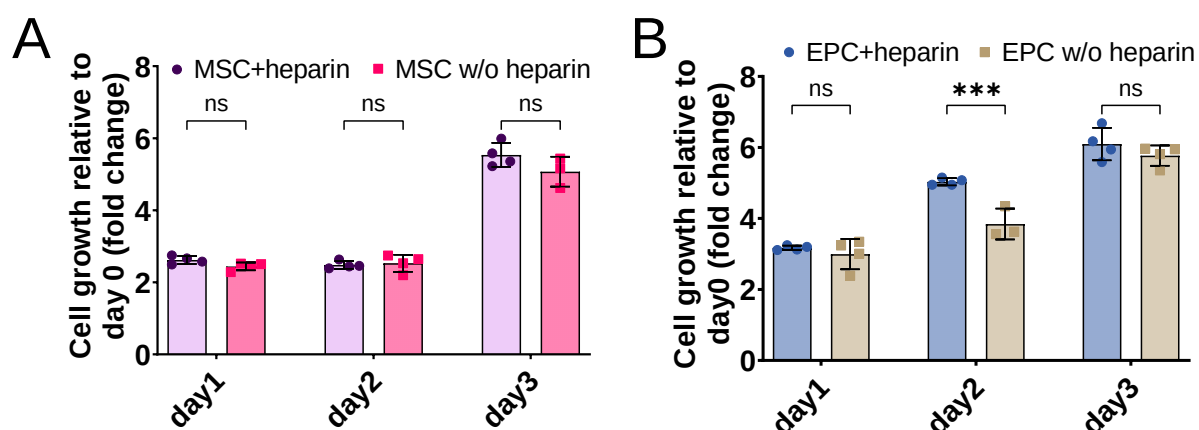


Figure 4.20: Heparin does not affect MSC and EPC proliferation. Proliferation of MSCs and EPCs was assessed using the WST-1 viability assay in the presence or absence of 10 U/ml heparin. Absorbance was measured at 450 nm with a reference wavelength of 650 nm. The assay was performed with one biological replicate and four technical replicates per condition. Mean absorbance on day 0 served as the baseline, and values for days 1, 2, 3, and 4 are presented as fold changes relative to this baseline. Statistical significance was evaluated using a two-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

4.4.1.2 Evaluation of hPL on MSC culture

After confirming that heparin does not significantly affect the performance of the full expansion medium, the suitability of hPL for culturing MSCs was investigated as a crucial first step toward replacing FCS with hPL in the BM-model. This evaluation exclusively utilized the hPL-2 batch, which did not require the addition of heparin. For the assessment, two million MNCs isolated from BM were cultured in media supplemented with either 10% FCS or 10% hPL. Within the first few days of culture, it was apparent that the hPL-supplemented medium facilitated improved cell adherence and enhanced colony formation compared to the FCS-supplemented medium. While the initial colonies were not quantified, cell counts conducted on day 11 consistently demonstrated a markedly higher cell yield in the hPL supplemented cultures. This trend was observed across all thirteen BM samples obtained from prostate cancer patients, from which MSCs were isolated (see **Figure 4.21**, A). To further validate the observation that hPL supports MSC proliferation more effectively than FCS, a WST-1 assay was performed over three days to assess cell viability and differences in proliferation rates between FCS and hPL supplemented media. Specifically, at day 2, hPL cultures exhibited up to a 9 fold increase in metabolic activity relative to day 0, whereas FCS cultures showed up to a 6 fold increase, resulting in a 3 fold difference between conditions. By day 3, hPL supplemented cultures reached up to a 15 fold increase, compared to a 9.4 fold increase in FCS supplemented cultures, corresponding to a 5.6 fold difference. All values are reported as fold changes relative to day 0. Statistical significance was primarily determined using a two way ANOVA, which confirmed these differences. Recognizing that the assumption of normality may be questionable with low sample numbers, we also performed the Mann Whitney test with Bonferroni correction. Although the adjusted p values from these nonparametric tests did not reach conventional significance thresholds, likely due to the conservative nature of the correction, this viability assay confirmed the observed trend that hPL supplemented medium produced a considerably higher number of MSCs when comparing the same donors under FCS and hPL conditions.

Having observed a greater supportive effect of hPL in MSC isolation and expansion, the next step involved testing the characterization criteria for MSC identification, as defined by Dominici et al. [46], to evaluate any differences when using hPL compared to FCS. As previously described in Section 4.2.1.1, flow cytometry was employed to

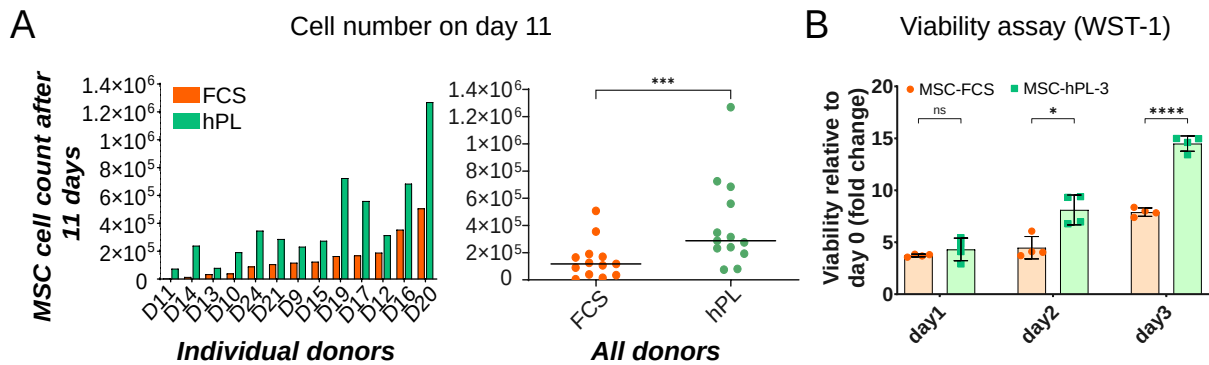


Figure 4.21: hPL enhances MSC isolation and viability compared to FCS. **A:** The total cell number was counted after 11 days in culture, following the initial seeding of two million MNCs. MSCs were isolated from the bone marrow of individual prostate cancer patients ($n=13$). Results demonstrated a higher initial cell count after 11 days in culture. Data was analysed using Wilcoxon-signed Rank test | * = $p < 0.05$ | ** = $p < 0.01$ | *** = $p < 0.001$ **B:** Proliferation of MSCs and EPCs was assessed using the WST-1 viability assay. Absorbance was measured at 450 nm with a reference wavelength of 650 nm. Technical replicates ($n=4$ per condition) were used. Day 0 mean absorbance served as the baseline, with values on days 1, 2, 3, and 4 shown as fold changes relative to this baseline. Statistical significance was determined Statistical using a two-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

analyze the surface marker profiles of MSCs from two donors, comparing cells from the same donors that were isolated and expanded in either FCS- or hPL-supplemented medium. No significant differences could be detected. No significant differences were observed between the conditions. The expression of the positive surface markers CD90, CD105, and CD73 was comparable across both conditions. For the negative surface markers, a slight increase in CD34-positive cells was noted in the hPL-cultured samples. Additionally, results from Donor 9 indicated a marginal increase in CD45 expression in the hPL-cultured cells; however, as this increase was not observed in the second donor and remained within a low expression range, it is likely a donor-specific variation rather than a consistent effect of hPL (**Figure 4.22, A**).

In addition to assessing the surface marker profile, the differentiation potential of MSCs was evaluated to confirm whether hPL supports the cells' multipotent lineage capacity. MSCs from three different donors were induced to differentiate into osteoblasts and adipocytes either with FCS or hPL supplemented medium. Successful differentiation was confirmed by staining with Alizarin Red S, which detects calcium deposits indicative of osteogenesis, and Oil Red O, which stains lipid droplets characteristic of adipogenesis. Both osteogenic and adipogenic lineages were effectively induced

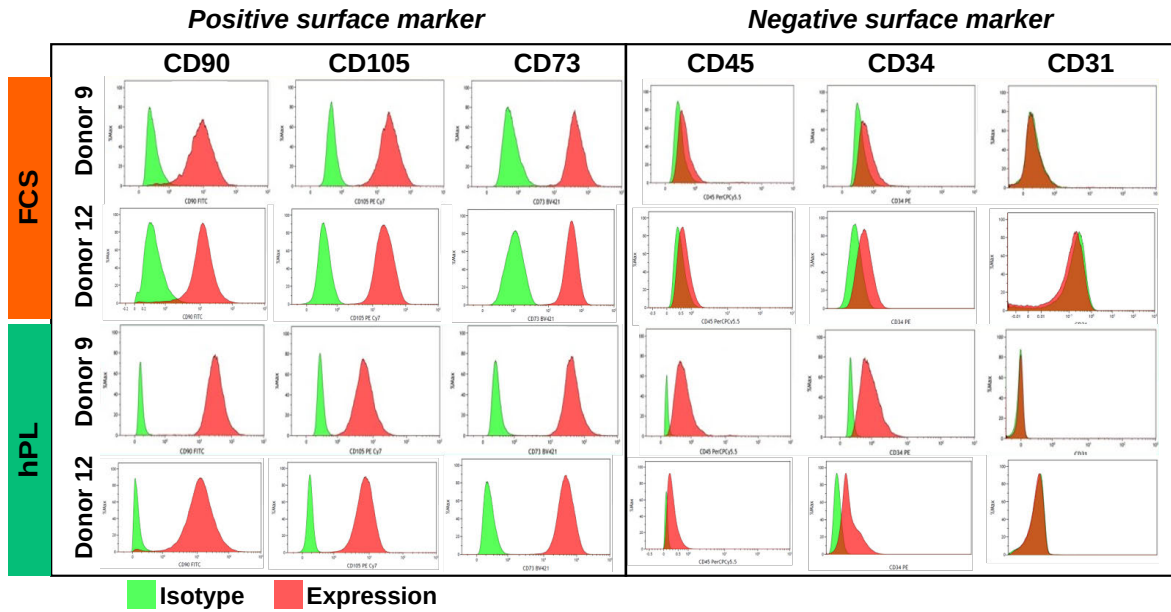
under both conditions. While donor-specific variability was observed among different samples, no visible differences were evident between the hPL- and FCS-supplemented conditions. It is important to note that this analysis was qualitative, aiming to confirm whether differentiation could be induced with hPL, rather than quantitatively assessing whether hPL-cultured cells exhibited a greater differentiation potential. (**Figure 4.22, B**). In summary, hPL demonstrated strong potential for supporting the isolation, expansion, and maintenance of characteristic surface markers and differentiation potential of MSCs derived from human BM. Building on these findings, the next step focused on evaluating the effect of hPL on the culture of EPCs, the second critical cell type in the BM-model.

4.4.1.3 Evaluation of hPL on EPC culture

To evaluate the potential of hPL as a supplement for EPCs, we first examined their functional capability to form tubular structures on Matrigel. A qualitative tube formation assay was performed, as shown in (**Figure 4.23, A**). Three endothelial primary cell types were included: CB-EPCs (D3), HUVECs obtained from a commercial source and maintained in our lab, and in-house isolated adult pB-EPCs (D18). Unfortunately, the images were taken at a late time point, 22 hours after seeding, by which time some of the tubular structures had already begun to degrade. Despite this limitation, tube formation was observed in all cases, regardless of whether FCS or hPL was used in the medium. The observed differences are difficult to attribute conclusively to the choice of serum, as tubes were formed in all conditions. It is possible that for pB-EPCs, tube formation occurred more rapidly with hPL than with FCS, leading to earlier degradation of the structures by the time of imaging. In alignment with the perivascular niche setup of the BM-model, which utilizes a serum concentration of 10–12%, an additional experiment was conducted using 12% serum in the medium, with either FCS or hPL to evaluate tube formation on Matrigel. This experiment exclusively utilized pB-EPCs, as these cells serve as the primary endothelial cell type in all BM-model experiments. To capture an earlier stage of tube formation, images were taken 15 hours after seeding. The results demonstrated minimal differences in network formation between pB-EPCs cultured with FCS and those cultured with hPL (**Figure 4.23, A**).

Similar to the characterization performed for MSCs, the surface marker profile of pB-EPCs from donor D18 was analyzed following isolation and expansion in media

A



B

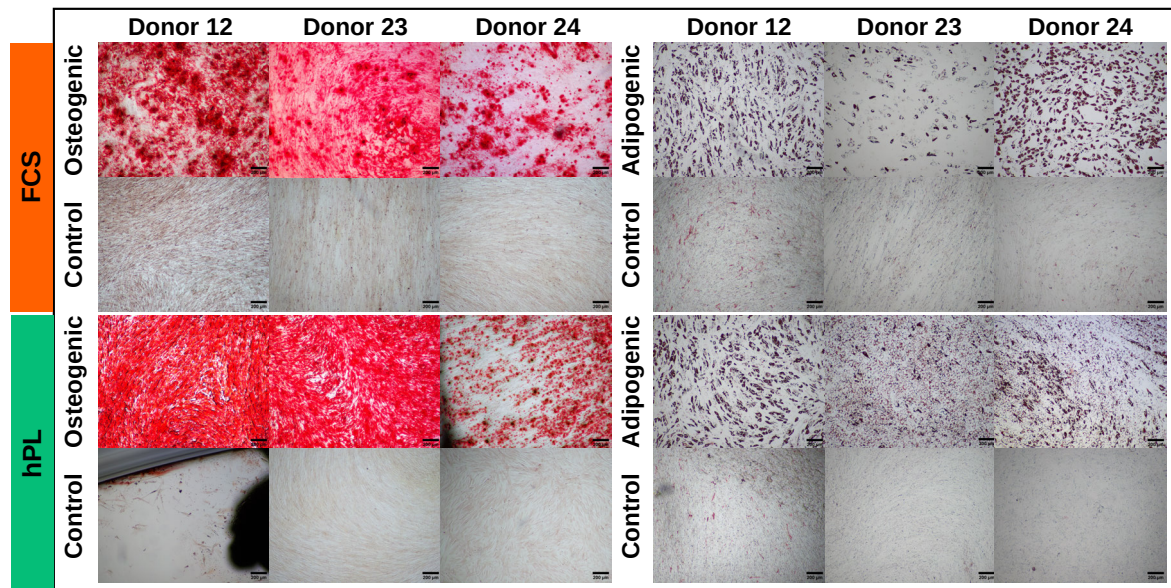
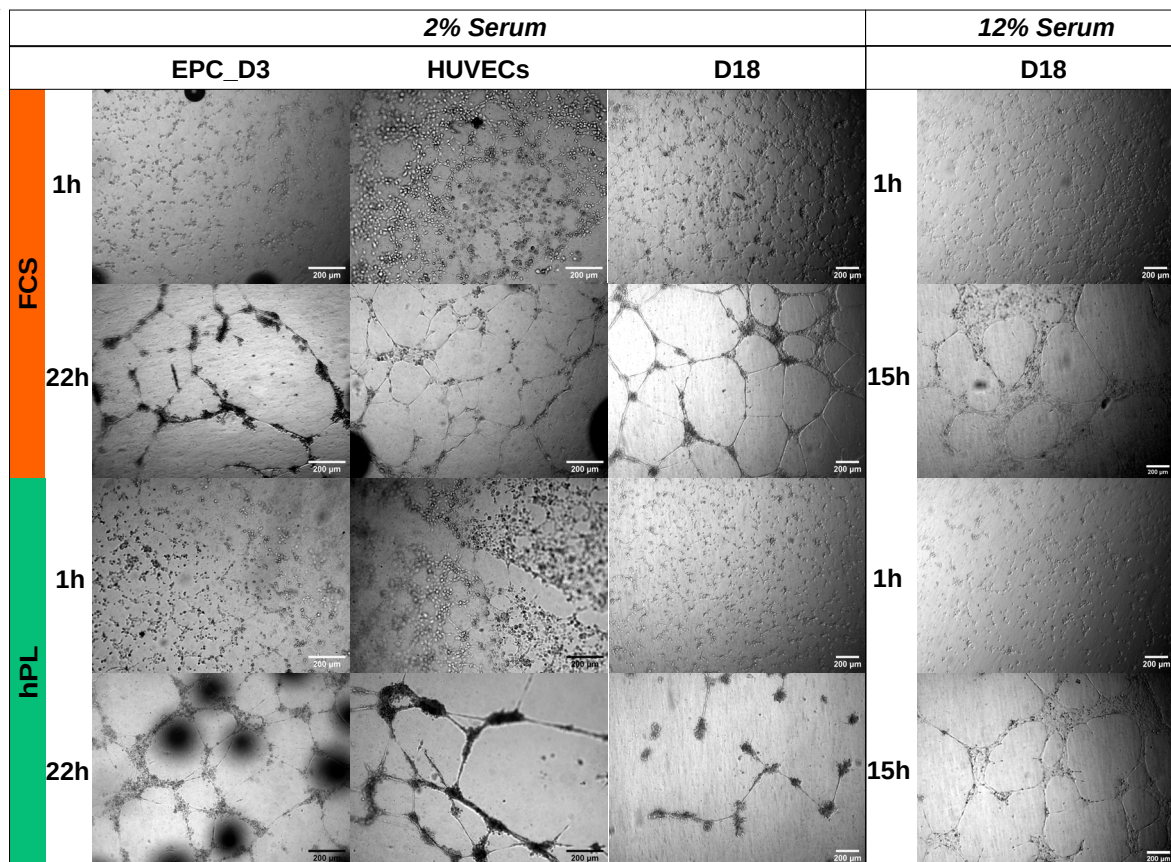


Figure 4.22: **Comparison of phenotypic and functional characterization of MSCs cultured with hPL or FCS. A:** Surface marker profiles of MSCs from two donors cultured in either FCS or hPL. Isotype controls are shown in green. **B:** Multipotency of MSCs from three donors was assessed by inducing osteoblast (left) and adipocyte (right) differentiation in hPL- or FCS-supplemented media. Calcium deposits were stained with Alizarin Red S, and lipid droplets with Oil Red O.

A



B

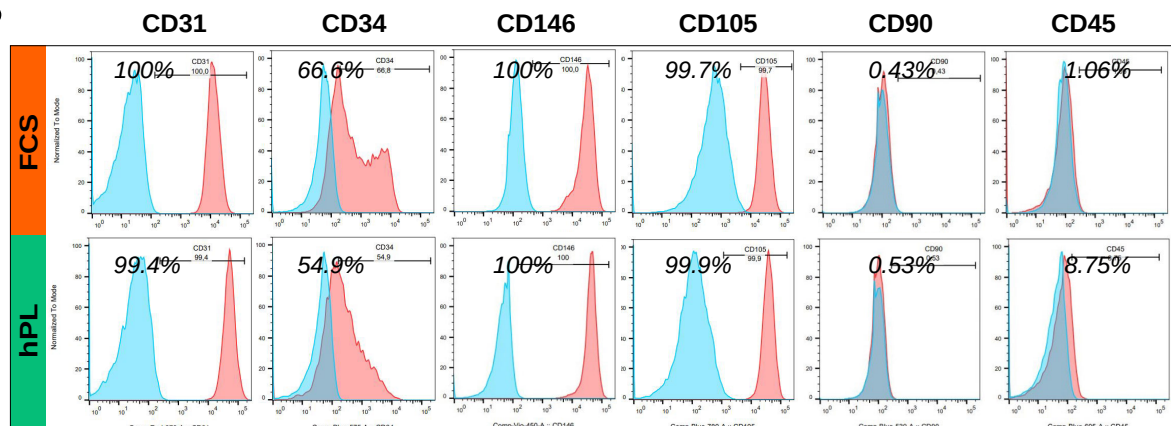


Figure 4.23: Comparison of FCS vs hPL in the functional and phenotypic characterization of EPCs
A: Validation of in vitro tube formation capability using hPL versus FCS at two serum concentrations (2% and 12%) with three different EPCs types **B:** Comparison of the surface marker profiles of pB-EPCs cultured in either FCS or hPL. Light blue represents the isotype control.

supplemented with either FCS or hPL. The results demonstrated the successful isolation of a homogeneous endothelial cell culture, with 100% of the cells expressing the endothelial markers CD31, CD146, and CD105. Additionally, CD34 was expressed in more than half of the cells (FCS: 66.6%, hPL: 54.9%), while only a small fraction of cells expressed markers from other lineages, such as CD45 and CD90 (**Figure 4.23, B**). These findings confirm the successful isolation of a pure population of EPCs, regardless of the serum supplement used during expansion.

An additional indication of the superiority of hPL as a medium supplement over FCS was observed in the proliferative capacity of EPCs. In cultures supplemented with FCS, EPCs typically exhibited growth arrest around passage 11. Out of curiosity, the serum of cells that had stopped proliferative activity (no growth observed six days post-splitting) was switched to hPL-supplemented medium. Remarkably, these cells resumed proliferation and achieved full confluence following the serum change (**Figure 4.24, A**).

Furthermore, a viability assay (WST-1) was performed on EPCs, following the same protocol previously utilized for MSCs, to evaluate differences in proliferation rates between FCS- and hPL-supplemented conditions. The results demonstrated an even higher difference than for the tested MSCs showing a metabolic activity significantly higher for EPCs cultured with hPL. Specifically, EPCs cultured in hPL-supplemented medium exhibited a 17-fold increase in metabolic activity by day 3 relative to day 0, whereas those cultured in FCS-supplemented medium demonstrated only a five-fold increase (**Figure 4.24, B**). In summary, hPL exhibited superior performance in supporting the expansion and maintenance of primary adult pB-EPC cultures compared to FCS.

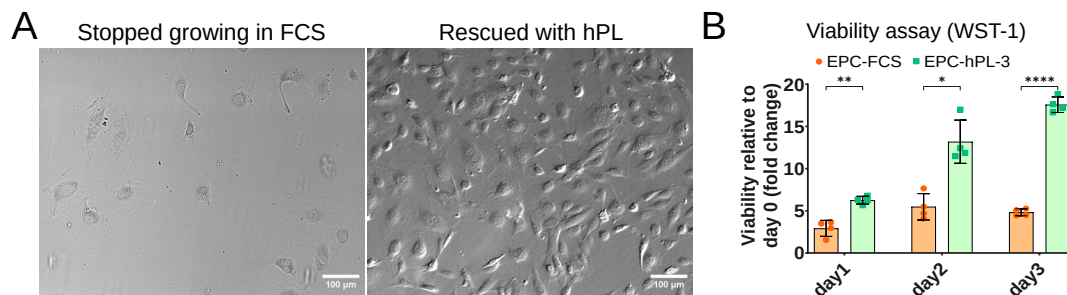


Figure 4.24: **hPL demonstrates superior efficacy in supporting EPC growth in vitro** **A:** The rescue experiment showed that EPCs, which had stopped proliferation in FCS-supplemented medium, resumed growth in hPL-supplemented medium. **B:** EPC proliferation was assessed using the WST-1 viability assay. Absorbance at 450 nm (reference 650 nm) was measured with four technical replicates per condition. Mean absorbance on day 0 served as the baseline, and fold changes were calculated for days 1–3. Statistical significance was evaluated using a two-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

4.4.1.4 Use of hPL in the 3D BM-model set-up

The final and most critical evaluation of hPL as an alternative to FCS involved its application in the 3D BM-model setup. Validation experiments were performed to determine whether both the endosteal and perivascular niches could be successfully established using hPL-supplemented medium, ensuring its compatibility with the model's requirements.

Initially, MSCs were cultured under osteogenic conditions for up to three weeks in medium supplemented with either hPL or FCS, followed by fixation and osteocalcin staining to evaluate osteoblastic activity. Validation of the staining protocol was performed in a 2D culture system using an isotype antibody as a negative control. The results demonstrated positive osteocalcin staining in differentiated MSCs, while no staining was observed in the isotype control (**Figure 4.25**), confirming the specificity of the staining method.

Qualitative analysis of osteocalcin expression on CPC scaffolds, performed using MSCs from two different donors, revealed comparable levels of expression under both hPL- and FCS-supplemented conditions (**Figure 4.26, A**). Subsequent analysis of osteocalcin expression in the complete two-niche setup, which incorporated osteogenic differentiation and an additional ten days of vascular niche implementation, demonstrated osteogenic activity in both FCS- and hPL-supplemented conditions. However,

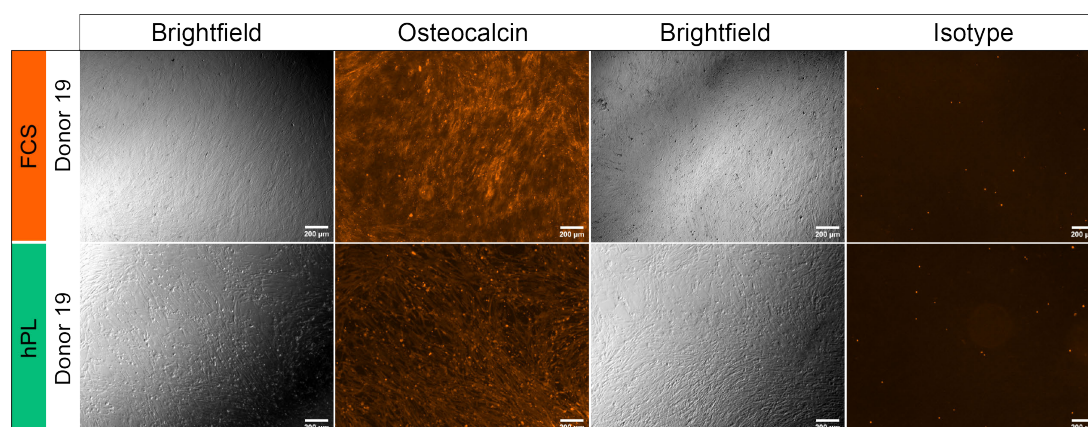


Figure 4.25: **Validation of osteocalcin immunofluorescence staining in 2D.** MSCs were cultured in a 2D setup and differentiated for 19 days in osteogenic medium. Cells were stained with an osteocalcin-specific or isotype control antibody and imaged using an Olympus IX81 microscope. Scale bar: 200 µm.

due to a technical issue with the microscope, tile images for the hPL condition cover only half of the scaffold (**Figure 4.26, B**). Having demonstrated that hPL supports the functionality of the endosteal niche with no observable differences compared to FCS, CD31 staining revealed comparable prevascular structure development in both media conditions. This observation was based on qualitative analysis and was tested using two different MSC donors in combination with pB-EPC donor 18 (**Figure 4.27**).

Finally, as the last step to evaluate hPL, the complete two-niche BM-model was established under both FCS and hPL conditions, incorporating ten PC3 cells. After ten days of culture, both conditions resulted in visible PC3 colony outgrowth with similar amounts. This indicated no substantial differences in cancer cell growth or niche development when substituting FCS with hPL (**Figure 4.28**).

In conclusion, this study demonstrated that hPL represents a viable and effective human-derived alternative to the animal-derived FCS. Notably, in standard 2D cultures of primary cells, hPL consistently outperformed FCS, supporting superior cell isolation, expansion, and viability. In 3D co-culture setups, where analyses were predominantly qualitative, hPL exhibited performance comparable to FCS, successfully supporting the development and functionality of both the endosteal and perivascular niches, as well as cancer cell growth within the BM-model. These findings establish hPL as a robust substitute for FCS, advancing the model toward a more humanized and physiologically relevant culture system.

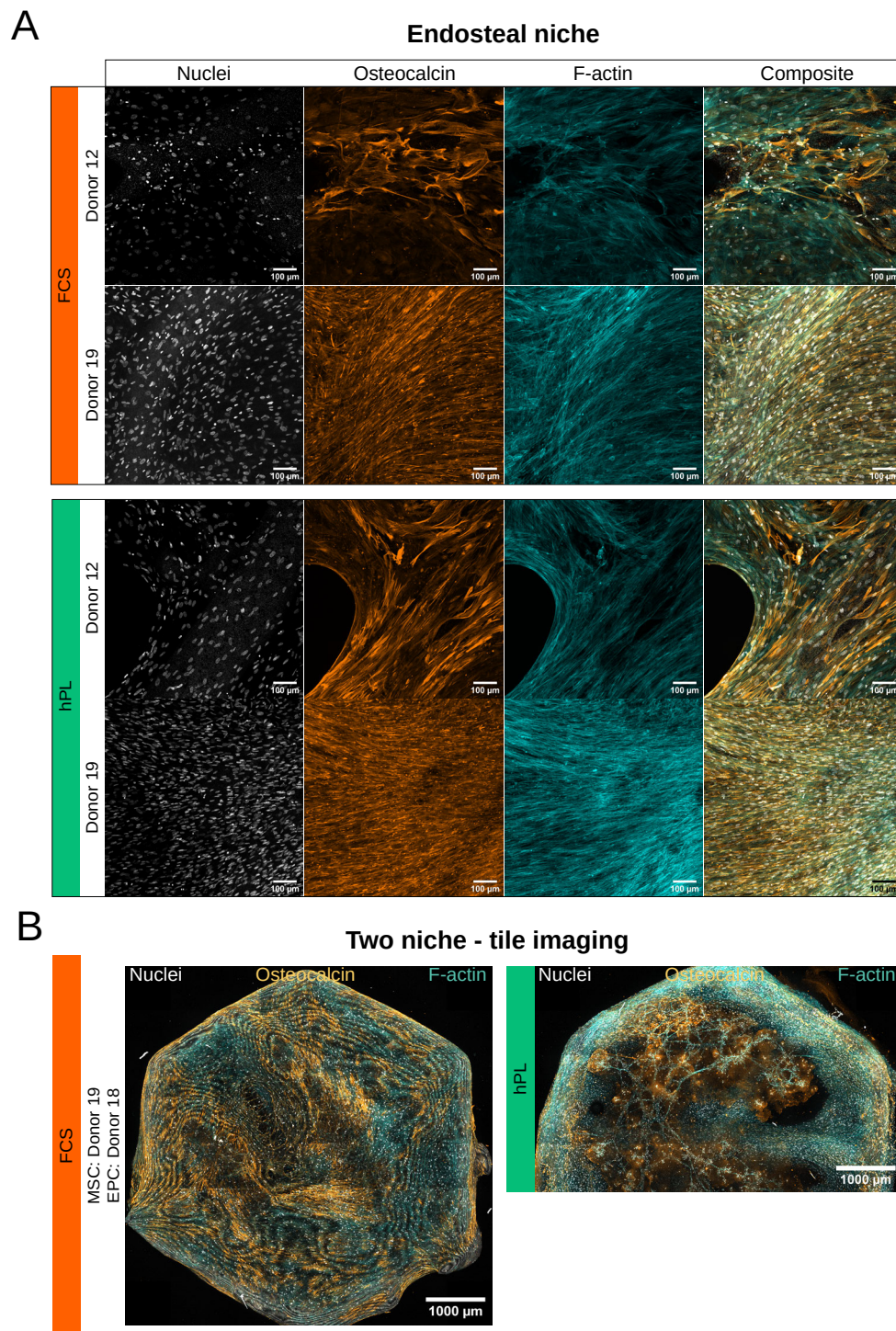


Figure 4.26: **Validation of the endosteal niche using hPL-supplemented medium** **A**: Representative images from two donors (Donor 12 and Donor 19) showing osteocalcin staining after 19 days of differentiation on CPC scaffolds in either FCS or hPL-supplemented medium, confirming osteoblastic activity in the endosteal niche. Nuclei are depicted in gray, osteocalcin in orange, and F-actin in cyan. **B**: Whole-scaffold images illustrating one sample per medium condition. Nuclei are shown in gray, CD31 in orange, and F-actin in cyan.

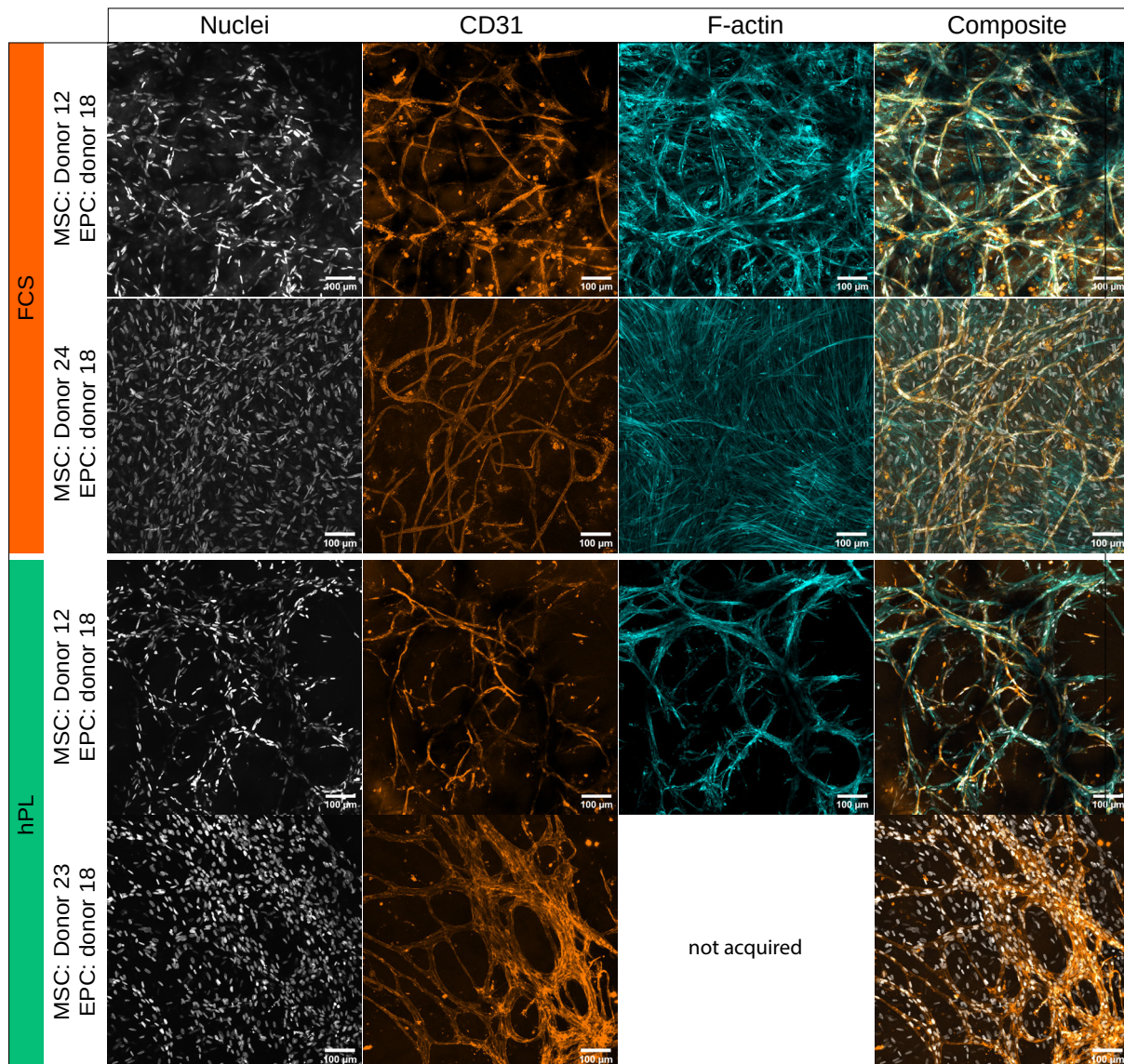


Figure 4.27: **Validation of the vascular niche using hPL-supplemented medium.** Representative images from two donors illustrating CD31-positive prevascular structures formed in the vascular niche using FCS- and hPL-supplemented medium. Nuclei are depicted in gray, CD31 in orange, and F-actin in cyan.

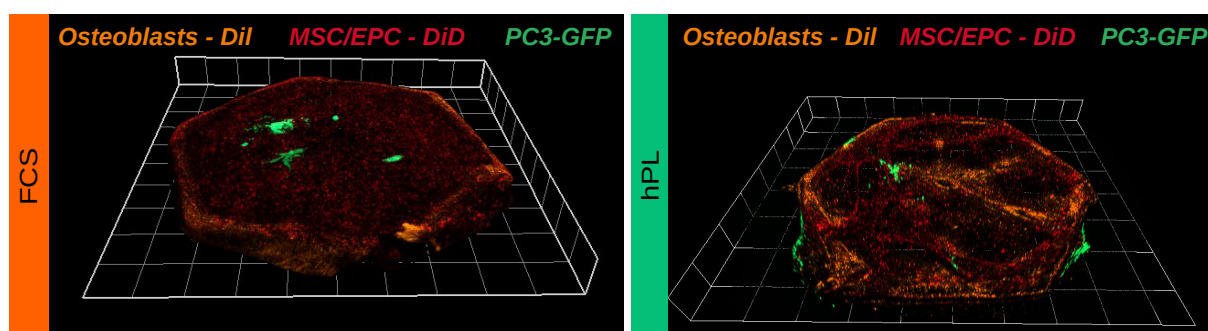


Figure 4.28: **Complete two-niche BM-model including PC3 cells cultured Under FCS or hPL serum conditions** Representative images depicting the fully established two-niche BM-model, incorporating PC3 cells under FCS- and hPL-supplemented medium conditions. The endosteal niche shows osteoblasts stained with DiI (orange) on a CPC scaffold, while the vascular niche comprises MSCs/EPCs stained with DiD (red) in Matrigel and PC3-GFP cells (green).

4.4.2 Human bone ECM hydrogel as alternative to Matrigel

Building upon the successful integration of hPL as a human-derived serum alternative, the next step focused on replacing Matrigel with an organ-specific human bone extracellular matrix (ECM) hydrogel to further enhance the physiological relevance of the BM-model. While many complex 3D models rely on Matrigel, collagen, or synthetic hydrogels as substitutes for ECM, these materials lack the organ specificity. Each organ possesses a distinct ECM composition; for bone, this includes a predominant presence of type I collagen, alongside other collagens and non-collagenous proteins. The two-niche BM-model developed by Braham et al. [32] for studying multiple myeloma incorporated Matrigel, a mouse-derived ECM hydrogel predominantly composed of laminin, collagen IV, and heparan sulfate proteoglycans. Matrigel is sourced from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor known for its abundance of extracellular matrix proteins. It is widely used as a reliable ECM hydrogel for various 3D culture models. However, its ECM composition differs significantly from that of human bone marrow, limiting its capacity to accurately replicate the physiological microenvironment of the BM. To address this limitation, we aimed to enhance our BM-model by developing a human-derived, organ-specific ECM hydrogel that more accurately mimics the unique characteristics of the BM microenvironment. This refinement aimed to potentially improve the support for the challenging-to-culture DCCs. Access to human bone tissue was facilitated through a rapid autopsy program dedicated to studying

metastases in deceased cancer patients. This invaluable resource allowed for the preparation of a human-derived bone extracellular matrix (ECM), tailored to replicate the bone microenvironment. We adapted the protocol established by Sawkins et al. [45] to generate an ECM hydrogel derived from bone tissue. Pulverizing the relatively large human bone pieces presented significant challenges during the facilitation of the protocol and required the use of a coffee grinder in combination with liquid nitrogen to maintain a sufficiently low temperature for effective pulverization. Additionally, the unavailability of a freeze dryer for human samples necessitated the omission of the lyophilization step in subsequent preparations, introducing slight procedural deviations from the original protocol. Despite these challenges and modifications, the protocol yielded an adequate amount of hydrogel after pepsin digestion and neutralization, achieving a protein concentration of approximately 3 mg/mL. While the target concentration was 10 mg/mL, this could not be achieved because the hydrogel became excessively viscous and partially polymerized at higher concentrations, making it unsuitable for pipetting. The hydrogel, with a protein concentration of approximately 3 mg/mL, successfully polymerized at 37°C. To assess its potential as a Matrigel replacement, the hydrogel's biocompatibility was evaluated by culturing cells (MSCs and EPCs) to establish the perivascular niche, employing both microscopic observation and a viability assay (WST-1).

Microscopic observation revealed that cells cultured in the human bone extracellular matrix (hbECM) hydrogel formed numerous partially developed network-like structures. However, these networks exhibited a less coordinated 3D architecture compared to those formed in Matrigel. In Matrigel, MSCs and EPCs demonstrated a tendency to form large network-like clusters over a ten-day period. Distinct network connections began to emerge within the first three days, and by day ten, extensive tubular structures had developed, becoming too dense for effective visualization with brightfield imaging. Cells cultured in hbECM also exhibited numerous cellular connections and elongated structures after three days. However, the supportive capacity of hbECM appeared less robust compared to Matrigel. Cells cultured in hbECM showed reduced dispersion throughout the gel height, remaining closer together, which may indicate a limited ability to support the formation of extensive three-dimensional networks similar to those observed in Matrigel (**Figure 4.29**, A). To facilitate more detailed visual analysis of the cellular network, hbECM droplets were cultured on glass coverslips for confocal imaging. However, the hydrogel failed to adhere to the glass surface, resulting in

floating droplets within the well. This led to altered cellular development, with cells forming dense, ball-like structures within the gel (**Figure 4.29, B**), ultimately preventing successful imaging. While hbECM may more closely replicate the composition of the bone marrow microenvironment, its hydrogel properties for in vitro culture require further refinement to optimize its capacity to support three-dimensional cellular architecture and functionality.

Cell viability assessments indicated that cells thrived within hbECM, fulfilling a key requirement for its prospective use as a hydrogel in the BM model. Notably, metabolic activity in hbECM differed from that in Matrigel, with significantly higher activity observed on days three and seven. Specifically, multiple Mann–Whitney tests confirmed significance on day 3 ($p = 0.0381$) and day seven ($p = 0.0173$), while a two-way ANOVA demonstrated a highly significant difference on day 7 ($p < 0.0001$). By day ten, however, metabolic levels in hbECM had aligned with those observed in Matrigel (**Figure 4.29, C**), suggesting that cells proliferated more actively during the first week in hbECM. It is possible that the initially enhanced proliferation in hbECM was attributable to the hydrogel's reduced capacity to support a fully three-dimensional cellular architecture. Without sufficient structural support, cells may quickly reach spatial limitations and be unable to reorganize effectively, resulting in an early plateau in proliferation. In conclusion, our pilot experiments demonstrated that hbECM maintained cell viability at levels comparable to Matrigel, fulfilling the minimal requirements for its use in the BM-model. However, the observed insufficient structural support indicates that further evaluation is necessary to fully characterize and optimize the hydrogel for our application. While these initial findings are promising regarding the use of an organ-specific hydrogel derived from human bone, extensive additional work is required to validate its properties for its intended applications.

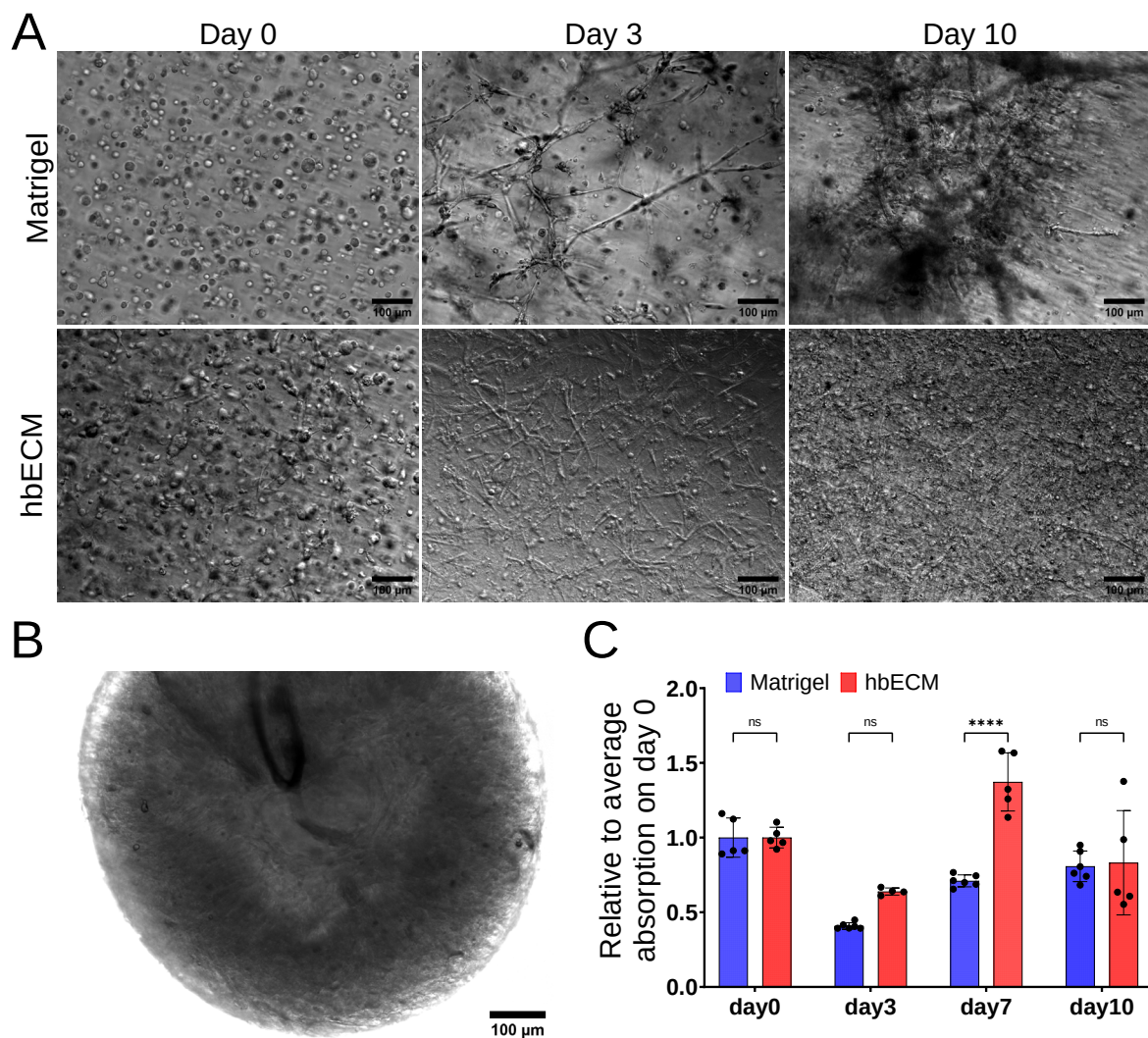


Figure 4.29: **Microscopic and viability assessment of hbECM as a potential alternative to Matrigel in BM model applications** **A:** Representative phase-contrast images showing the development of MSCs and EPCs cultured in the respective ECM conditions (hbECM or Matrigel) at days 0, 3, and 10. Scale bar: 100 µm. **B:** Phase-contrast image illustrating the development of hbECM on a glass surface, where the hydrogel failed to adhere, forming a circular structure that floated in the medium. Scale bar: 100 µm. **C:** Metabolic activity of cells cultured in either hbECM or Matrigel, as assessed by the WST-1 viability assay on days 0, 3, 7, and 10. Results are presented as values relative to the mean absorbance on day 0. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc tests.

4.4.3 MSC isolation in low oxygen condition

To build upon the refinements initiated thus far, the next step in optimizing the BM-model involved addressing another critical physiological parameter of the BM microenvironment: its low oxygen tension. The bone marrow environment exhibits significantly lower oxygen levels compared to atmospheric conditions, which typically contain approximately 21% oxygen. Oxygen concentrations within the BM can range from around 4% in capillaries to as low as 0.1% in the subendosteal regions [58]. This hypoxic environment plays a pivotal role in regulating the behavior and functionality of MSCs, which are central to the BM-model's development. The following section explores the effects of isolating MSCs under low oxygen conditions, aiming to enhance their physiological functionality within the context of the BM-model. Given the technical and financial challenges associated with maintaining oxygen levels below 5%—largely due to the significant nitrogen required to sustain such hypoxic conditions—we conducted our experiments under oxygen tensions of 5–5.6% O₂. This level, while not fully replicating the hypoxic environment of the BM, represents a practical compromise for studying the influence of reduced oxygen on MSC behavior.

This proof-of-concept experiment aimed to isolate and differentiate MSCs under low oxygen conditions and test if MSCs retain their multilineage potential under varying oxygen and serum conditions. The findings demonstrated that MSCs could indeed be isolated under hypoxic conditions. In the differentiation assays, the only significant difference was observed with the hPL-1 condition, where cells in normoxia (21% O₂) exhibited fewer calcium deposits compared to those cultured under low oxygen (5.6% O₂), which produced abundant calcium deposits. No significant differences were observed for FCS or hPL-2 conditions. Similarly, no deviations were noted in adipogenic differentiation; MSCs in all conditions produced substantial lipid droplets, as stained by Oil Red O (**Figure 4.30, A**). Calcium production was further quantified by measuring Alizarin Red S staining using spectrophotometry, confirming the visual observation that only the hPL-1 condition under 21% O₂ showed a significant difference (**Figure 4.30, B**). Quantification of Oil Red O staining revealed minor variations but no significant changes compared to the osteogenic conditions. Additionally, it should be noted that this experiment was conducted with a single MSC donor and was designed solely to demonstrate that MSCs retain their multilineage potential under varying oxygen and

serum conditions. This pilot study investigated whether MSCs could be successfully isolated and differentiated under hypoxic conditions (5.6% O₂) while maintaining their multilineage potential across different oxygen and serum conditions. The results confirmed that MSCs could indeed be isolated and expanded in a hypoxic environment. In osteogenic differentiation assays, a notable difference was observed with medium supplemented with hPL-1: MSCs cultured under normoxic conditions (21% O₂) exhibited fewer calcium deposits compared to those cultured under hypoxia (5.6% O₂), which displayed abundant calcium deposits. No differences were observed for MSCs cultured with FCS or hPL-2 under either oxygen condition. Adipogenic differentiation assays showed consistent outcomes across all conditions, with substantial lipid droplet formation visualized by Oil Red O staining (**Figure 4.30, A**). Quantitative analysis of Alizarin Red S staining, used to measure calcium deposits spectrophotometrically, confirmed that the hPL-1 condition under normoxia exhibited lower calcium production compared to its hypoxic counterpart. However, because only two wells were measured, no statistical test was performed. Differences were observed between hPL-1 and both FCS or hPL-2 under normoxic conditions, whereas under hypoxic conditions, the two measurements of Alizarin Red S were very similar (**Figure 4.30, B**). Quantification of Oil Red O staining revealed only minor variations in adipogenic differentiation, with no clear differences across the tested conditions. Although limited by the use of a single MSC donor and two replicates, our data support that MSCs retain their multilineage potential under low oxygen conditions. These results indicate that hypoxic environments do not compromise the inherent differentiation capabilities of MSCs.

This chapter summarized key refinements aimed at replicating the physiological conditions of human BM. hPL was validated as a human-derived alternative to bovine FCS, effectively supporting the isolation, expansion, and functionality of MSCs and EPCs in the complete BM-model. A pilot study on a human bone-derived hydrogel to replace Matrigel showed partial success, supporting cell viability but raising questions about its structural integrity at 3 mg/mL protein concentration. Finally, MSCs cultured under low oxygen retained their multilineage potential, though full BM-model experiments comparing hypoxic and normoxic conditions remain pending. While these promising results represent significant progress, they are only an initial step toward refining the BM-model. Due to the scope of this thesis, subsequent experiments were performed using FCS as the serum supplement, Matrigel as the ECM, and normoxic conditions.

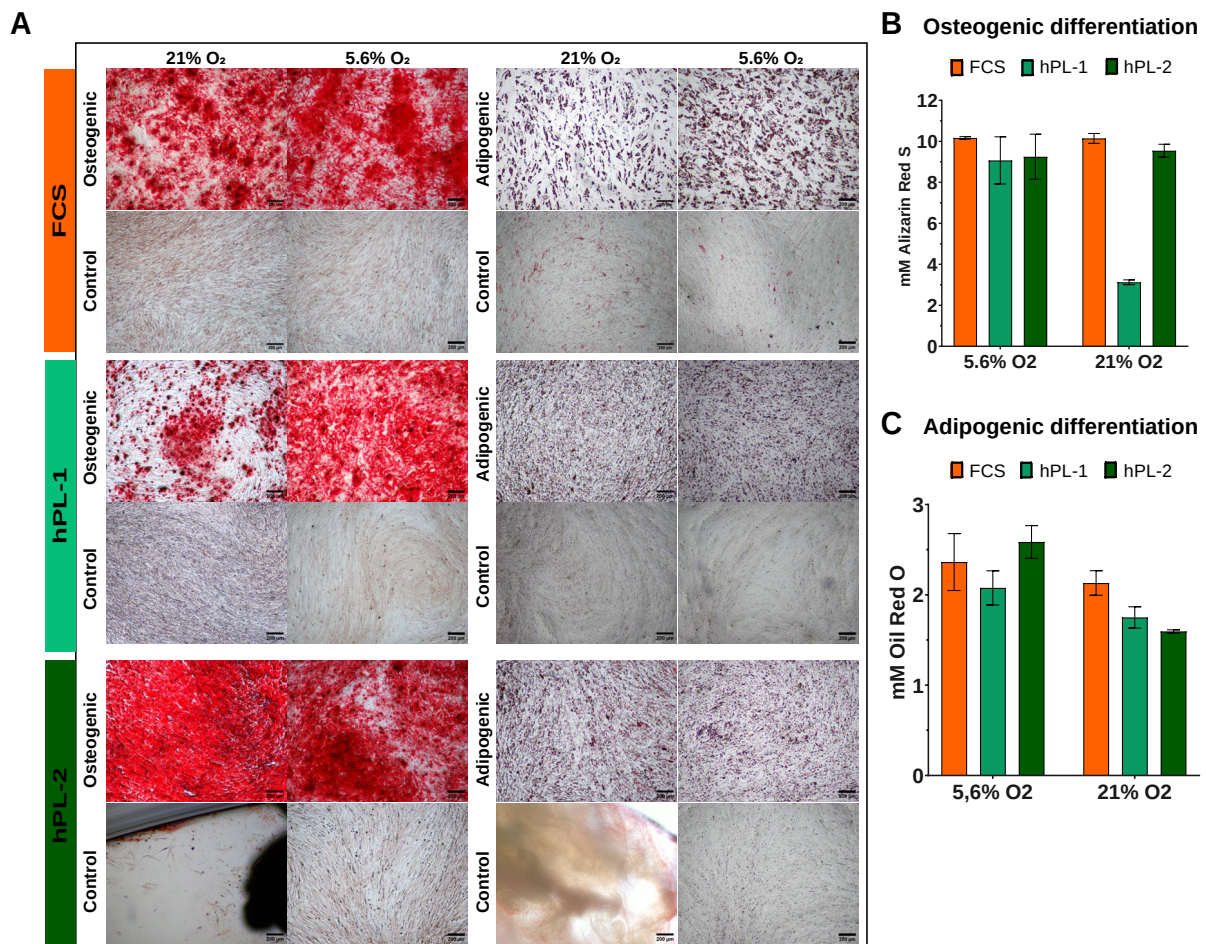


Figure 4.30: MSCs retain their multilineage potential under varying oxygen and serum conditions A: Multipotency of MSCs was evaluated by inducing differentiation into osteoblasts (left) and adipocytes (right) using two different hPL batches (hPL-1 and hPL-2) or FCS. Osteogenic differentiation was visualized through calcium deposits stained with Alizarin Red S, and adipogenic differentiation was assessed by lipid droplet formation stained with Oil Red O. **B:** Quantitative analysis of osteogenic differentiation was performed by measuring Alizarin Red S absorbance at 595 nm. **C:** Quantification of adipogenic differentiation was performed by measuring Oil Red O stain absorbance at 540 nm. Data for graphs B and C were obtained using a single MSC donor (Donor 12) with two biological replicates, each measured in duplicate. No statistical analysis because n=2.

4.5 Growing patient-derived cancer cells in BM-model

Building upon the technical optimizations and refinements discussed in the previous sections, the next step involved introducing patient-derived cancer cells into the BM-model for the first time. With the model's capacity to replicate key BM niches validated using human-derived components and its design further optimized to mimic aspects of the physiological microenvironment, this section examines its ability to support the survival and growth of patient-derived DCCs. The following section outlines pilot experiments aimed at culturing patient-derived BM samples within the BM-niches.

4.5.1 Pilot experiments to culture patient-derived BM in BM-niches

BM samples from prostate cancer patients were routinely screened for DCCs using EpCAM staining after immune cell depletion from the mononuclear cell (MNCs) fraction. Samples with a high abundance of EpCAM-positive cells and clusters were selected for implementation in the BM-model (**Figure 4.31, A**). The first depicted patient sample was obtained from an 84-year-old prostate cancer patient with metastases. This sample exhibited a high enrichment of EpCAM-positive cells and clusters. Notably, the patient had not undergone hormone deprivation therapy prior to surgery. To introduce patient-derived cells into the BM-model, two experimental approaches were tested in this pilot study: (1) seeding the entire MNCs fraction and (2) isolating and seeding individual EpCAM-positive cancer cells and clusters.

In the first approach, the entire mononuclear cell (MNCs) fraction was used after depleting CD45-, CD11b-, and CD33-positive immune cells. The remaining cells were stained with the lipid dye DiO and co-seeded with vascular niche cells onto the endosteal niche. After 14 days in culture, a few single DiO-labeled cells were still detectable (**Figure 4.31, B**). However, this approach lacked the specificity needed to differentiate between cancer cells and BM cells within the culture. Despite this limitation, the detection of DiO-labeled cells after two weeks of culture represents an initial step toward maintaining patient-derived cells within the BM model over extended periods.

In the second approach, single cells and cell clusters enriched for EpCAM-positive cancer cells were manually isolated using a micromanipulator. These isolated cells and clusters were subsequently co-cultured with vascular niche cells on the CPC scaffold.

fold and maintained in culture for 14 days. While this method enabled the precise isolation of cancer cells, the number of cells obtained was limited by the practical constraints of the handling process. The low cell number posed significant challenges in locating and identifying the cells within the BM model after the culture period. This limitation highlights the need for further optimization to improve the detectability and tracking of DCCs in the model. In this particular setup, the EpCAM-positive cells were not pre-stained prior to seeding. Instead, after 14 days of culture, the samples were fixed and subsequently stained for EpCAM to identify and evaluate the presence of cancer cells. Despite the challenges of identifying DCCs within the BM model using confocal microscopy, two EpCAM-positive cells were successfully detected. Both cells exhibited detectable EpCAM staining, though it was challenging to ascertain whether it conformed to the typical membrane-specific pattern. While one image suggested a possible membrane-specific localization, the overall staining was difficult to evaluate with confidence. Additionally, the evaluation of the DAPI nuclear staining was less distinct compared to that of other niche cells. Without further analysis using markers for apoptosis or other viability indicators, it is not possible to draw definitive conclusions regarding the survival of these cells after the 14-day culture period (**Figure 4.31**, C). This initial attempt to culture patient-derived DCCs within the BM model highlights both the potential for detecting and maintaining these cells over an extended culture period and the challenges associated with their identification and viability assessment. Furthermore, with the availability of a second patient sample, an additional attempt was made to culture patient-derived cancer cells. In this instance, cells isolated from a bone metastasis biopsy were co-seeded exclusively with vascular niche cells, as the endosteal niche was not prepared at the time. The protocol for preparing a cell suspension from the bone biopsy is detailed in the methods section. The resulting cell suspension was highly enriched with EpCAM-positive cells, as shown in **Figure 4.32**, A. Cancer cells were seeded either in Matrigel alone to assess their survival capability in the absence of supporting niche cells or co-cultured within the vascular niche of the BM-model (**Figure 4.32**, B & C).

The results of cells cultured in Matrigel alone demonstrated that some cancer cell clusters remained detectable for up to 35 days. However, assessing their growth over time was challenging, as consistent tracking of individual spheres throughout the culture period was not possible without live-cell imaging. **Figure 4.32** B shows four distinct

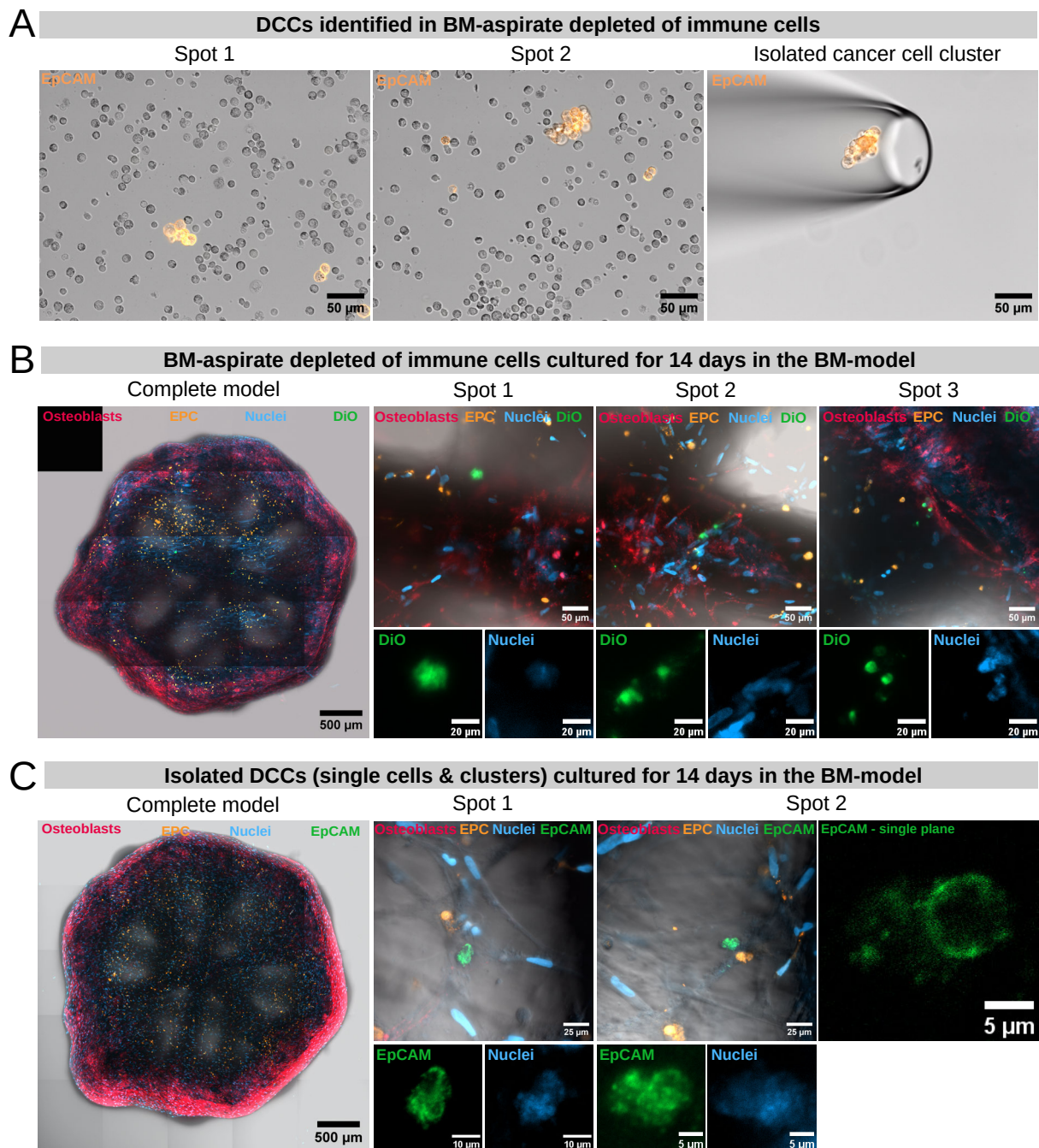


Figure 4.31: **Patient-derived DCCs isolated and cultured in the two-niche BM model.** **A:** Representative images of BM aspirate following immune cell depletion, with DCCs stained for EpCAM (orange) and isolated via micromanipulation. **B:** Depleted MNCs fraction from BM aspirate, labeled with DiO (green), was cultured for 14 days in the BM model. Osteoblasts are red, EPCs orange, nuclei blue, and F-actin red. Images show summed z-projections and also **C:** Single DCCs/clusters were isolated and cultured for 14 days in the BM model. After fixation, samples were stained for EpCAM (green). Osteoblasts (red), EPCs (orange), nuclei (blue), and F-actin (red) are shown. Images display summed z-projections, with a zoomed region (right) highlighting a single plane to focus on the EpCAM pattern.

spheres observed in the cultures, including the largest sphere, approximately 148 μm in diameter (1), identified on day 20 of culture. Smaller spheres, around 45 μm in diameter (4), showed no discernible size change by day 35. While the absence of live-cell imaging limited definitive conclusions about growth dynamics, the observation that some cancer cell clusters could be maintained in culture for over a month is encouraging, as it may provide a potential interim solution for preserving cancer cells until the BM-model is fully prepared.

Other cells from the biopsy-derived suspension were co-seeded with vascular niche cells. Before co-culture, the cancer cells were labeled with CMFDA dye for tracking and cultured in hPL-supplemented medium for up to 12 days. Fixation was performed on days 1, 5, and 12, followed by additional staining for EpCAM. CMFDA-positive (green) cells were detected at all time points, with representative images shown for days 5 and 12. Observations indicated a comparable number of CMFDA-positive cells on days 5 and 12, suggesting that most cells remained stable throughout the culture period (**Figure 4.32**, C1). However, further examination revealed that not all CMFDA-positive cells exhibited an EpCAM signal, in contrast to the strong EpCAM expression observed in cells shown in panel A prior to seeding. Representative magnified regions from samples collected on days 1, 5, and 12 are shown in panel C2, highlighting cells that are EpCAM-positive, CMFDA-positive, or double-positive. The sample from day 1 demonstrates a well-stained EpCAM/CMFDA double-positive cell cluster, clearly indicating the presence of cancer cells and the successful implementation and staining of EpCAM. The magnified area shown for day 5 reveals one EpCAM/CMFDA double-positive cell alongside three CMFDA-positive but EpCAM-negative cells. This observation could be attributed to factors such as a reduction in EpCAM expression over time, or insufficient staining sensitivity to detect lower levels of EpCAM. The magnified region from the day 12 sample reveals an EpCAM-positive cell cluster with nearly undetectable CMFDA labeling, indicating that the cluster may have been present initially with suboptimal staining or that cell proliferation during the 12-day culture period led to dye dilution (**Figure 4.32**, C2). Further investigation is required to clarify the survival and potential proliferation of these cells during the culture period. Nonetheless, these two experiments represent promising initial attempts to integrate patient-derived cancer cells into the BM model.

4.6 Investigation of PC3 cells interacting with BM microenvironment

Building on initial efforts to culture patient-derived cancer cells in the BM model, the influence of distinct BM niche components on prostate cancer cells remains unclear. While these studies demonstrated the feasibility of integrating patient-derived cells, they did not address how specific niche cell types affect tumor cell behavior. This chapter focuses on functional experiments to examine the differential impact of BM subniches on prostate cancer cells, aiming to elucidate the microenvironment's role in cancer cell dynamics and identify factors critical for supporting the **in vitro!** (**in vitro!**) expansion of patient-derived DCCs.

4.6.1 Growth dynamics reveal the complex interplay between PC3 cells and bone marrow subniches

Given the challenges of directly studying patient-derived DCCs, these experiments used the prostate cancer cell line PC3 to explore how distinct BM subniches influence tumor cell proliferation through interactions with niche cells. Therefore, PC3 cell line was introduced into the individual niches of the BM model to investigate the specific effects of the endosteal and vascular niches on prostate cancer cell growth behavior. PC3 cells were seeded in either a single niche (endosteal or vascular), in ECM alone (Matrigel), or in the combined two-niche model. This approach allowed for a comparative assessment of niche-specific influences on cancer cell growth and interactions within the engineered BM microenvironment. Colony volumes were measured as described in section 4.3.3. A low initial seeding density of 20–40 PC3 cells was chosen to mimic the DCC scenario in patients while maintaining sufficient cell numbers to enable a quantitative evaluation of the experiment, allowing for an assessment of the supportive or inhibitory roles of BM niches. Furthermore, two clones of the PC3 cell line were utilized. The PC3-EpIC-YFP clone was transduced with a vector to overexpress the intracellular domain of EpCAM and to express the fluorescent protein YFP in the cytoplasm. The PC3-GFP clone, serving as a vector control, was transduced to express GFP in the cytoplasm. Results obtained from three independent biological replicates revealed a consistent trend:

the vascular niche supported PC3 colony expansion most effectively. The endosteal niche alone also promoted an increase in colony volumes, though to a lesser extent than the vascular niche. Interestingly, the combination of the two niches exhibited a more inhibitory effect on colony expansion compared to either niche alone. However, colony volumes were still greater than in the control, where PC3 cells were cultured alone in Matrigel without any other cell types. To be more precise, when examining each PC3 clone GFP or EpiC-YFP individually, significant differences in colonies were observed compared to the cancer-only control. For PC3-GFP cells, colony volumes increased by up to 6.6-fold in the endosteal niche ($p = 0.0022$) and up to 8.5-fold in the vascular niche ($p < 0.0001$). However, in the combined two-niche model, growth was limited to a maximum 4.5-fold increase ($p = 0.3224$), which was not significant (**Figure 4.33**, A). Similarly, for PC3-EpiC-YFP cells, colony volumes increased up to 5-fold in the endosteal niche ($p = 0.0182$) and up to 11-fold in the vascular niche ($p < 0.0001$), but only up to 3.5-fold in the combined two-niche model (0.6598) compared to the mean of the respective cancer-only control (**Figure 4.33**, B). Notably, the difference in colony expansion between the combined two-niche model and the vascular niche alone was significant for both PC3-GFP ($p = 0.0073$) and PC3-EpiC-YFP ($p < 0.0001$) clones. As both clones exhibited the same trend, the graph in **Figure 4.33**, C combines the data from both, revealing an even more pronounced significant difference between the various niche conditions. These findings underscore the distinct impact of BM niche environments on PC3 colony expansion, highlighting the vascular niche's robust stimulatory effect, while the integration of both vascular and endosteal niches attenuates this support.

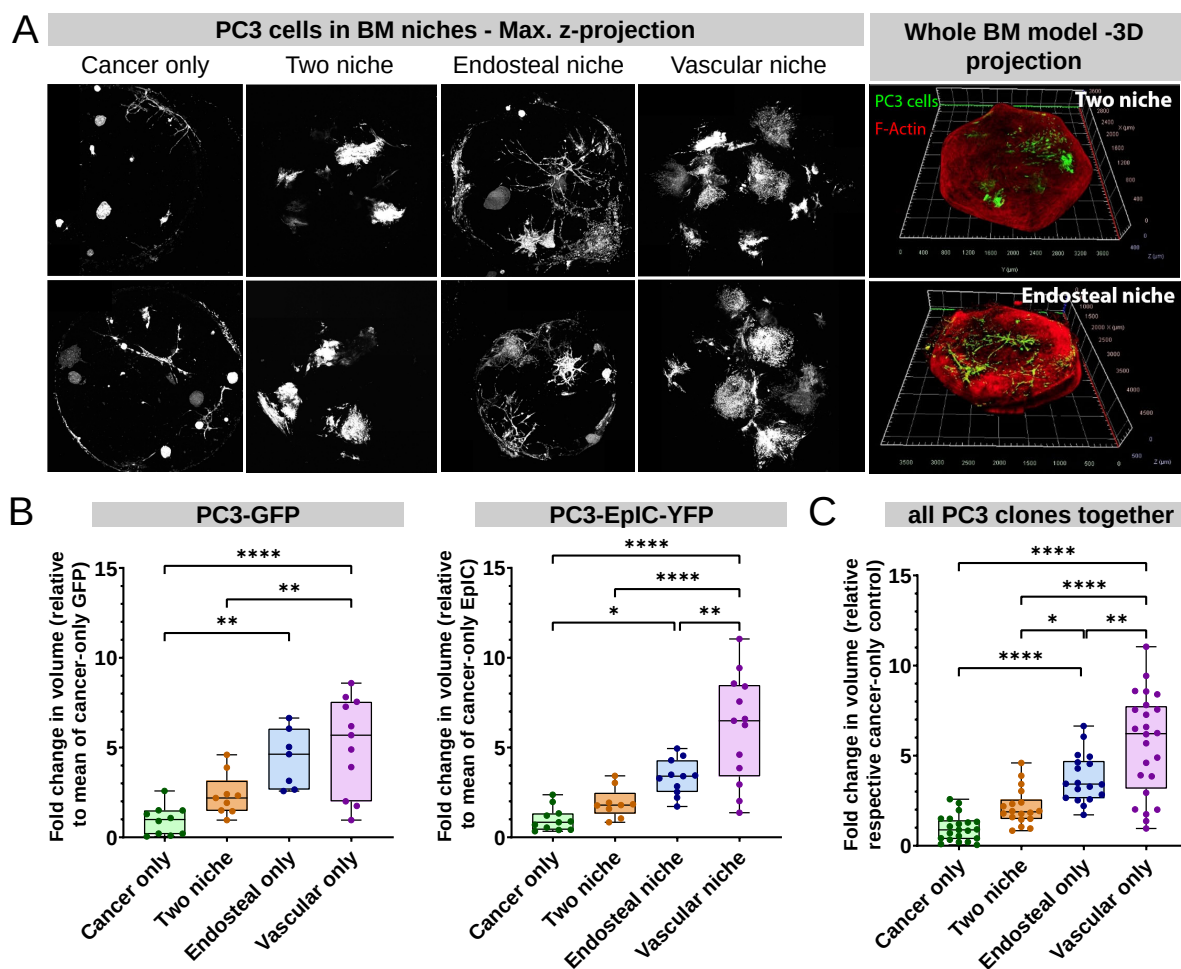


Figure 4.33: **Comparison of colony expansion of PC3 cells across BM niches** **A:** Representative images of the PC3 colony patterns in the specific niches after 10 days of culture. **B:** Quantitative analysis of PC3-GFP colony volume measurements across single and combined BM niches, normalized to the mean of the cancer-only GFP control. **C:** Quantitative analysis of PC3-EpiC-YFP colony volume measurements across single and combined BM niches, normalized to the mean of the cancer-only EpiC-YFP control. **D:** Graph showing data of both PC3 clones together, normalized to the mean of the respective cancer-only control. Each data point represents a sample from three independent biological replicates. Statistical test used: One-way ANOVA with Tukey's multiple comparison test.

To further investigate the specific contributions of different cell types within the in vitro BM niches, additional experimental conditions were introduced. For conditions involving the endosteal niche, the setup included scaffolds seeded with either undifferentiated MSCs or differentiated MSCs, as well as a two-niche configuration without the scaffold. For the vascular niche, additional conditions included PC3 cells co-cultured with either MSCs or EPCs alone. As before, the cancer-only condition, in which cancer cells were

seeded alone in Matrigel, served as the control. The results showed that the use of differentiated or undifferentiated MSCs on a CPC scaffold did only reveal a notable difference for PC3-GFP, but not for PC3-EpIC-YFP samples (**Figure 4.34**, Left A & B).

When examining the contribution of individual cell types within the vascular niche, the colony volume outcomes of PC3-GFP and PC3-EpIC-YFP cells showed very comparable trends. EPCs alone provided minimal support for colony expansion (PC3-GFP: 2.4-fold; PC3-EpIC-YFP: 2.5-fold), while MSCs alone supported expansion up to 3.6-fold for PC3-GFP and 3.5-fold for PC3-EpIC-YFP relative to their respective cancer-only controls. The fully functional vascular niche, incorporating both cell types, promoted colony expansion more effectively than either cell type alone, with increases of up to 4.8-fold for PC3-GFP and 6.4-fold for PC3-EpIC-YFP (**Figure 4.34**, Right A & B). These findings highlight that the formation of a prevascular network offers greater support for cancer cell colony expansion compared to the contribution of individual cell types alone. However, further experiments are necessary to elucidate the specific roles of MSCs differentiation status and the CPC scaffold in this process.

In summary, the findings reveal that the bone marrow microenvironment profoundly shapes the colony expansion behavior of prostate cancer cells.

4.6.2 Impact of EpCAM intracellular domain on PC3 colony growth in the BM model

In the previous chapter, colony growth experiments were performed using two PC3 clones (PC3-GFP and PC3-EpIC-YFP), although their differences were not examined in detail. In this section, colony volume measurements were normalized separately for each niche by referencing the mean colony volume of the respective PC3-GFP control group (e.g., in the cancer-only condition, both PC3-GFP and PC3-EpIC-YFP data were normalized to the mean of the cancer-only PC3-GFP group). This normalization allowed us to assess whether overexpression of the intracellular domain of EpCAM (EpICD) influences the colony behavior of PC3 cells within the BM niches. EpICD can translocate into the nucleus and, in combination with transcription factors and adaptor molecules such as FHL2, β -catenin, and Lef1, binds to promoter regions of genes that regulate cell division (e.g., cyclin D1), pluripotency, and EMT-associated processes including tight junction formation, cell adherence, and migration [59]. Under

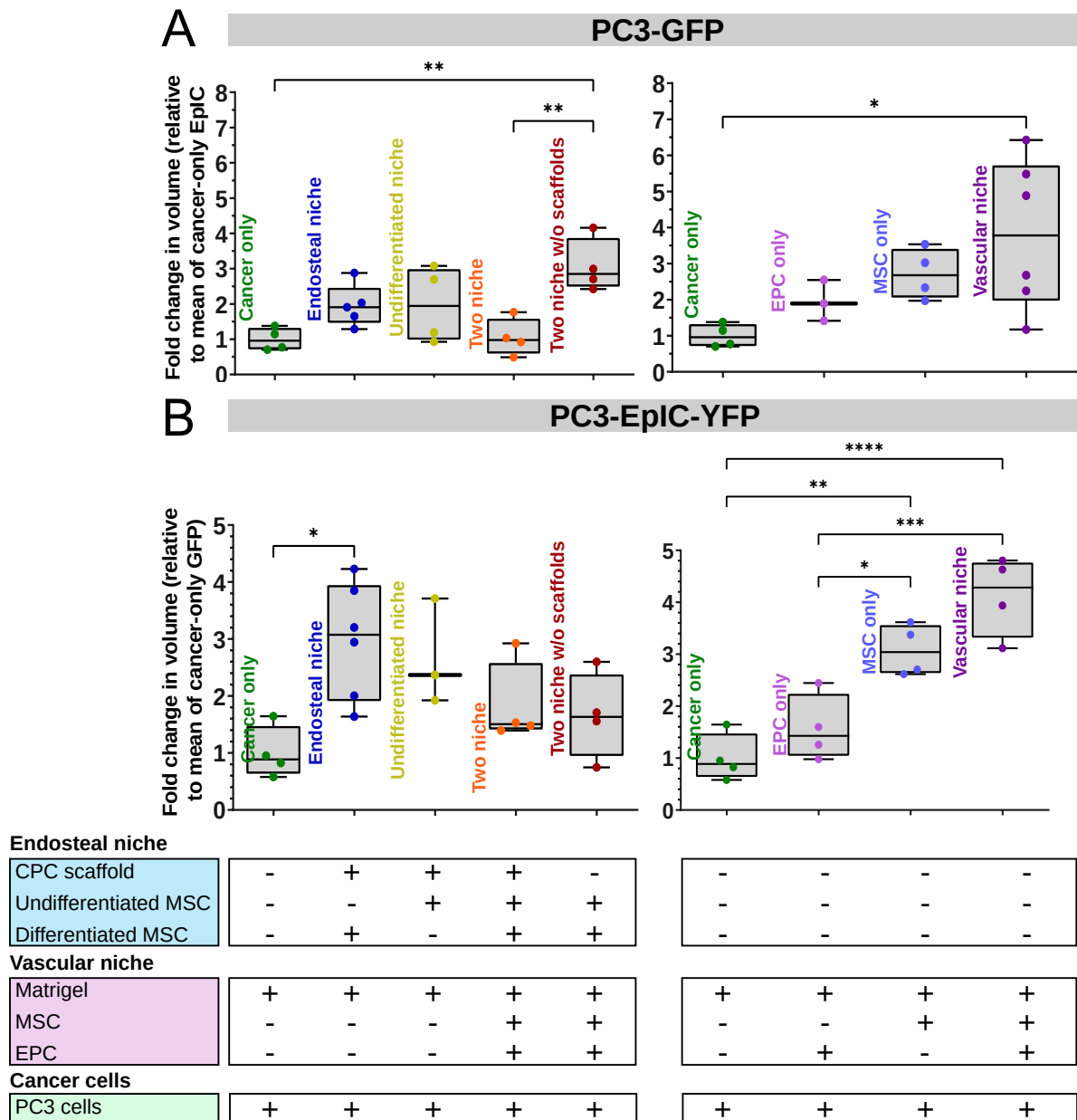


Figure 4.34: **Investigation of the individual components contributing to the niche effect.** **A:** Quantitative analysis of PC3-GFP volume measurements across additional conditions to further explore the effect of the endosteal niche. **B:** Quantitative analysis of PC3-EpIC-YFP volume measurements across additional conditions to further investigate the effect of the vascular niche. Each data point represents a sample from a single biological experiment. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons. Data without asterisks are not statistically significant.

standard 2D culture conditions, however, the PC3-EpIC-YFP cell line did not exhibit noticeable differences in proliferation compared to the the vector control clone GFP (data not shown). Moreover, across various BM niches and in three independent experiments, no statistically significant differences in colony volume were observed between the PC3-EpIC-YFP and PC3-GFP cell lines, as determined by both unpaired t-tests (parametric) and Mann–Whitney tests (nonparametric) (**Figure 4.35, A–D**). Both cell lines exhibited the strongest colony expansion within the vascular niche, although the PC3-EpIC-YFP cell line showed greater variability in colony volume outcomes (**Figure 4.35 D**). Microscopic evaluation confirmed that overexpression of EpIC did not alter colony volume patterns. A pending transcriptomic analysis is essential to fully elucidate the impact of EpIC on cellular responses within the BM microenvironment.

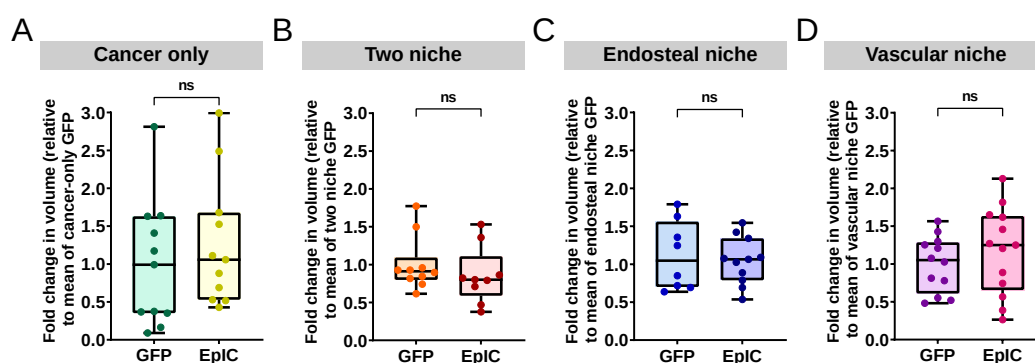


Figure 4.35: Comparison of EpIC-overexpressing and GFP control PC3 cells across BM niche. PC3 cells were cultured under various conditions (Matrigel alone, two-niche, endosteal niche, and vascular niche) for 10 days and volume of cell clusters was measured. Statistical analysis was performed using the t-test. Each data point represents a sample from three independent biological replicates. Data are normalized to the mean volume of the corresponding GFP control condition: **A**: Cancer only condition, **B**: two-niche condition, **C**: endosteal niche condition, and **D**: vascular niche condition.

4.6.3 Transcriptomic insights reveal the complex interplay between PC3 cells and bone marrow niche components

To complement the microscopic findings, PC3 cells and niche cells were analyzed in parallel using single-cell transcriptomics (10x Genomics). This analysis revealed differential regulation of over 200 pathways in PC3 cells and nearly 60 pathways in the niche cells (**Figure 4.36**). For a comprehensive list of the enriched pathways, please refer to **Table A2** for PC3 cells and **Table A3** for niche cells in the appendix. When

evaluating the differential pathway analysis of PC3 cells cultured in either niche, their gene expression profiles were compared to those of PC3 cells cultured alone in Matrigel, which served as the baseline. Notably, the differentially regulated pathways observed in the two-niche environment were not merely the additive effects of individual niche conditions. PC3 cells grown in the niches were compared to PC3 cells grown in Matrigel as baseline (**Figure 4.36**, A). Niche cells exposed to PC3 cells also showed marked alterations, especially in the two-niche condition, whereas the vascular niche alone did not yield significant pathway changes (**Figure 4.36**, B).

As an example which attracts interest, immune-related pathways were markedly modulated. In the two-niche (TN) condition, PC3 cells significantly upregulated pathways associated with chemokine receptor binding (NES = 1.89), antimicrobial humoral immune response (NES = 1.89), and chemokine activity (NES = 1.85), while downregulating leukocyte tethering/rolling (NES = -2.19) and positive regulation of macrophage activation (NES = -2.25). In the endosteal niche (endo), enriched pathways included cytokine activity (NES = 1.84), neutrophil chemotaxis (NES = 1.81), and complement activation (NES = 1.88). In the vascular niche (vasc), significant increases were observed for chemokine receptor binding (NES = 1.88), chemokine activity (NES = 1.91), neutrophil chemotaxis (NES = 1.68), and granulocyte chemotaxis (NES = 1.61) (**Figure 4.37**, A). Niche cells also showed pronounced immune-related enrichment, particularly in the endosteal compartment and under two-niche conditions. In the latter, niche cells displayed increased immune receptor activity, regulation of granulocyte differentiation, phagocytosis, and platelet activation. Under endosteal-only conditions, exposure to cancer cells significantly enhanced pathways related to neutrophil chemotaxis, MHC protein complex binding, MHC class II protein complex formation, granulocyte chemotaxis, and leukocyte chemotaxis (**Figure 4.37**, B). These results underscore the bidirectional interactions between tumor cells and their bone marrow microenvironment, warranting further evaluation. Additional heatmaps in the appendix further illustrate selected differentially expressed Gene Ontology (GO) pathways. Pathways were randomly selected from the filtered list using criteria of p-value < 0.05 and NES less than -1 or greater than 2 to highlight the observation that the differentially regulated pathways in the two-niche environment are not simply the additive effects of the individual niches. This selection emphasizes both common and unique pathway alterations across the different niche configurations (see **Figure A1** and **Figure A2**).

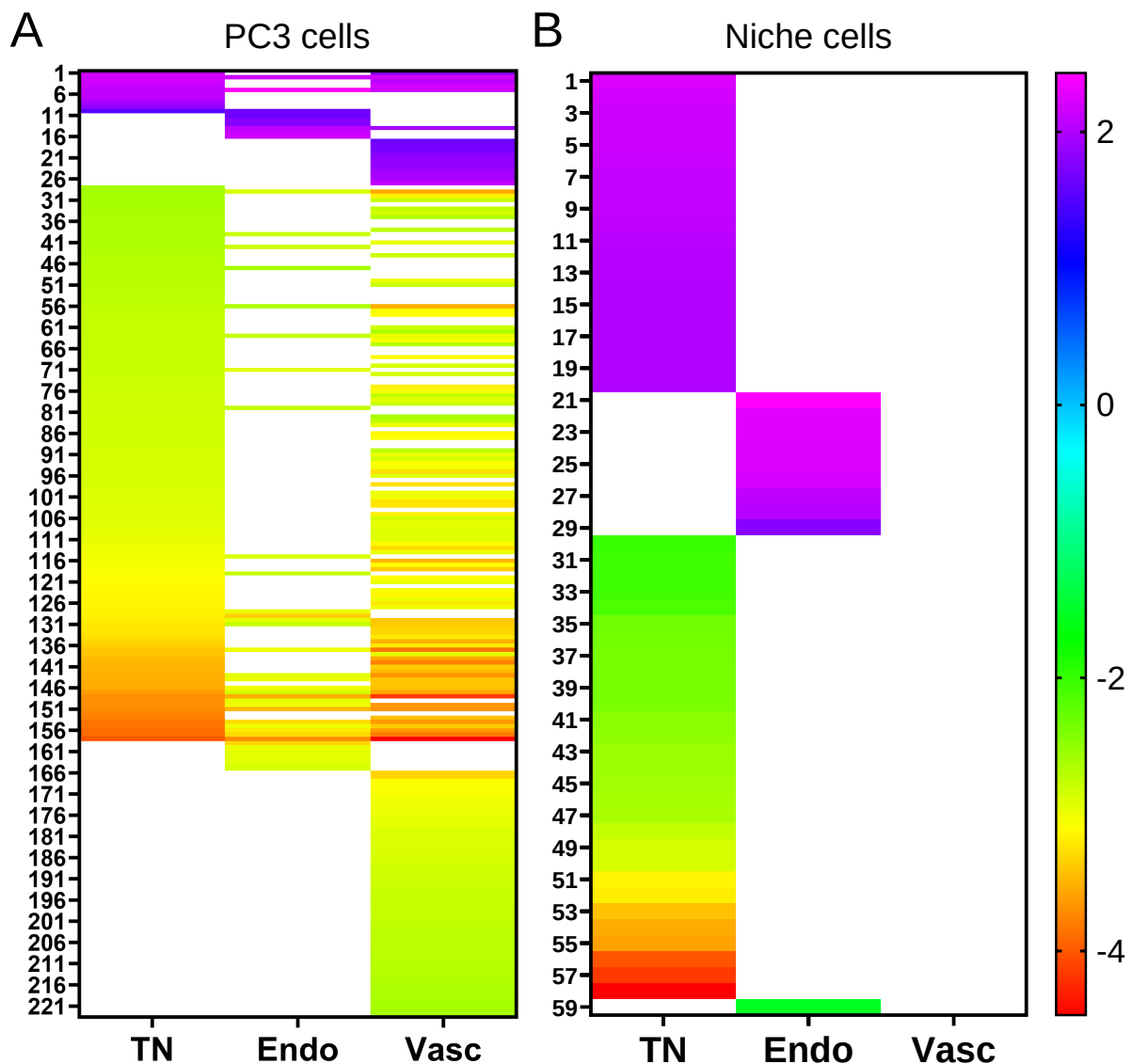
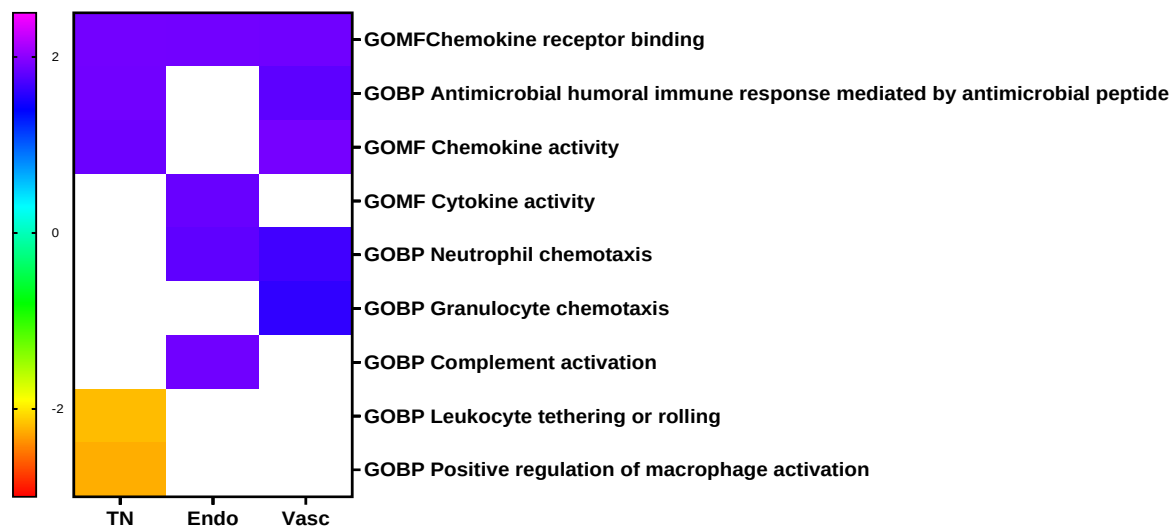


Figure 4.36: Differential expression of gene pathways in the two-niche and single-niche setups. Transcriptional differences in PC3 cells and niche cells under distinct niche conditions. Heatmaps depict differentially expressed pathways in (A) PC3 cells and (B) niche cells co-cultured under different niche configurations: two-niche (TN: endosteal + vascular), endosteal-only (endo), and vascular-only (vasc) setups. Gene Set Enrichment Analysis (GSEA) was performed using the Gene Ontology (GO) database, categorizing gene functions into Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). Pathways with significant differential expression (p -value < 0.05 , NES < -2 or > 1) are shown. **A:** Differentially expressed pathways in PC3 cells co-cultured under two-niche or single-niche conditions, compared to PC3 cells cultured independently. **B:** Differentially expressed pathways in niche cells co-cultured with PC3 cells, compared to niche cells cultured alone. The full list of enriched pathways is provided in the appendix: PC3 cells (**Table A2**) and niche cells (**Table A3**).

A PC3 cells - immune related pathways



B Niche cells - immune related pathways

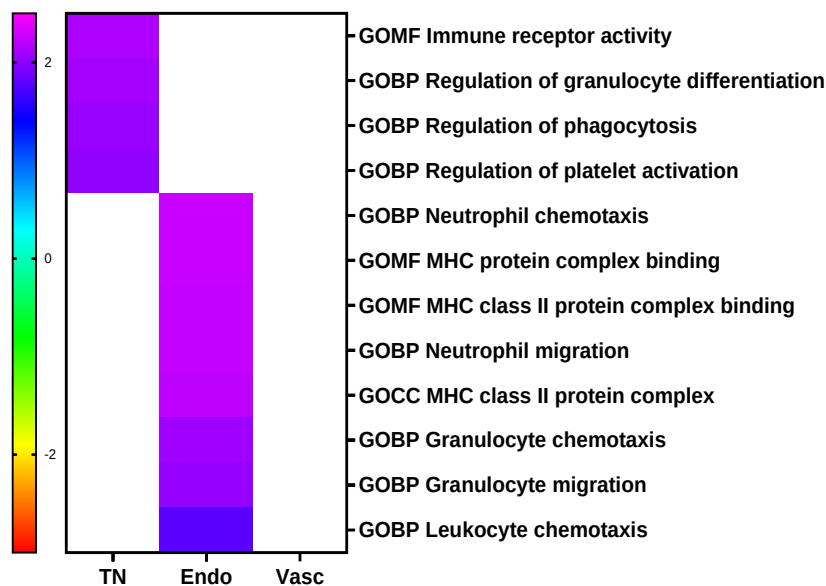


Figure 4.37: **Differentially expressed immune and inflammatory pathways in two-niche and single-niche setups.** Heatmaps show transcriptional differences in PC3 cells (A) and niche cells (B) under two-niche (endosteal + vascular), endosteal-only, and vascular-only conditions. Gene Set Enrichment Analysis (GSEA) using the Gene Ontology (GO) database identified significantly upregulated or downregulated immune and inflammatory pathways (p -value < 0.05 , $NES < -2$ or > 1) TN: two-niche, Endo: endosteal only, Vasc: vascular only. **A:** Differentially expressed pathways in PC3 cells co-cultured under two-niche or single-niche conditions, compared to PC3 cells cultured independently. **B:** Differentially expressed pathways in niche cells co-cultured with PC3 cells, compared to niche cells cultured alone.

4.7 Imaging Optimization for patient-derived samples

4.7.1 Optimizing confocal imaging for high-resolution analysis of patient-derived samples

As part of a complementary study investigating how a dormancy-inducing 3D-engineered matrix reveals a mechanosensitive and drug-protective FHL2-p21 signaling axis [60], I optimized a staining protocol for the FHL2 protein on adhesive slides containing patient-derived cells from breast, prostate, or bladder cancer. Adhesive slides are commonly used to preserve cells from patient samples for DCC detection, enabling long-term storage for extended periods, even decades. This method offers advantages in handling and storage duration but presents challenges for specific protein stainings due to suboptimal preservation of cellular structures. In this approach, cells are air-dried on adhesive slides and stored at -20°C without chemical fixatives such as aldehydes. As a result, cellular structures may not be as well-preserved as with aldehyde-based fixation, which stabilizes cell morphology and tissue architecture through protein cross-linking. The detection of DCCs in the BM fraction of MNCs is challenging due to their rarity and the need for epithelial markers such as CK for identification. Therefore, the first challenge was achieving clear and high-resolution CK staining using the LSM980 confocal microscope. Initial attempts at CK staining resulted in high background signals, likely caused by mismatched refractive indices during imaging. To address this, SlowFade Glass mounting medium (ThermoFisher Scientific), with a refractive index of 1.51 optimized for oil immersion objectives, was applied. This adjustment significantly reduced background staining and enabled clear detection of CK. In parallel, optimizing the staining protocol for FHL2 required several rounds of testing. After evaluating different concentrations of the primary and secondary antibodies, the best results were obtained using the primary anti-FHL2 antibody at a working concentration of 2 µg/ml and the secondary anti-rabbit AlexaFluor 647 antibody at 5 µg/ml. The combination of the optimized mounting medium for CK staining and the refined antibody protocol for FHL2 resulted in the first successful detection of both markers, as shown in **Figure 4.38**, A. The goal of the staining was to enable precise localization of the FHL2 protein within the nuclear or cytoplasmic regions of the cell. To test this, the MDA-MB-231 cell line was used, revealing a diverse distribution pattern of FHL2 across these compartments. In some cells, FHL2 was

predominantly localized in the nucleus (**Figure 4.38**, A (1)), while in others, it was more evenly distributed throughout the cytoplasm and nucleus (**Figure 4.38**, A (2)). Following this, patient samples with known high disseminated cancer cells density (DCCD) from prostate and bladder cancer were imaged to identify Cytokeratin-positive (CK) cancer cells in BM or lymph nodes. The diverse distribution pattern of FHL2 observed in MDA-MB-231 cells was also detected in patient-derived cells. The first image, derived from a prostate cancer patient with known metastasis (M1)), shows a CK-positive cancer cell in contact with a CK-negative non-cancer cell. Both cells express FHL2, with the cancer cell displaying predominantly cytoplasmic FHL2 expression, while the non-cancer cell shows an even distribution of FHL2 throughout the cell (**Figure 4.38**, B (1)). The second image, from the same BM sample, shows another CK-positive cancer cell, this time exhibiting an even distribution of FHL2 throughout the entire cell (**Figure 4.38**, B (2)). The third image is derived from a lymph node sample of a bladder cancer patient without known metastases (M0). Among these, two cells exhibit high FHL2 expression throughout the entire cell, while the third cell displays no detectable FHL2 expression (**Figure 4.38**, B (3)). More examples from a breast cancer lymph node sample are presented in the publication by Bakhshandeh et al. [60] and illustrate further the diverse FHL2 expression patterns across individual cancer cells and multicellular clusters. The analysis of FHL2 localization in several patient-derived cancer samples revealed significant heterogeneity in its expression, potentially reflecting the context-dependent regulation of FHL2 driven by microenvironmental cues and intrinsic cellular properties. However, conclusions regarding the dormant or proliferative states of these cells cannot be drawn without the inclusion of additional markers to assess their cell cycle status.

In conclusion, the development of an optimized staining and imaging protocol enables high-resolution visualization of protein expression patterns in patient-derived cells stored on adhesive slides. This advancement offers a robust platform for future studies on cellular localization. Furthermore, it holds significant potential for advancing research on rare DCCs, facilitating a closer examination of critical biomarkers in patient samples and paving the way for deeper insights into tumor biology and disease mechanisms.

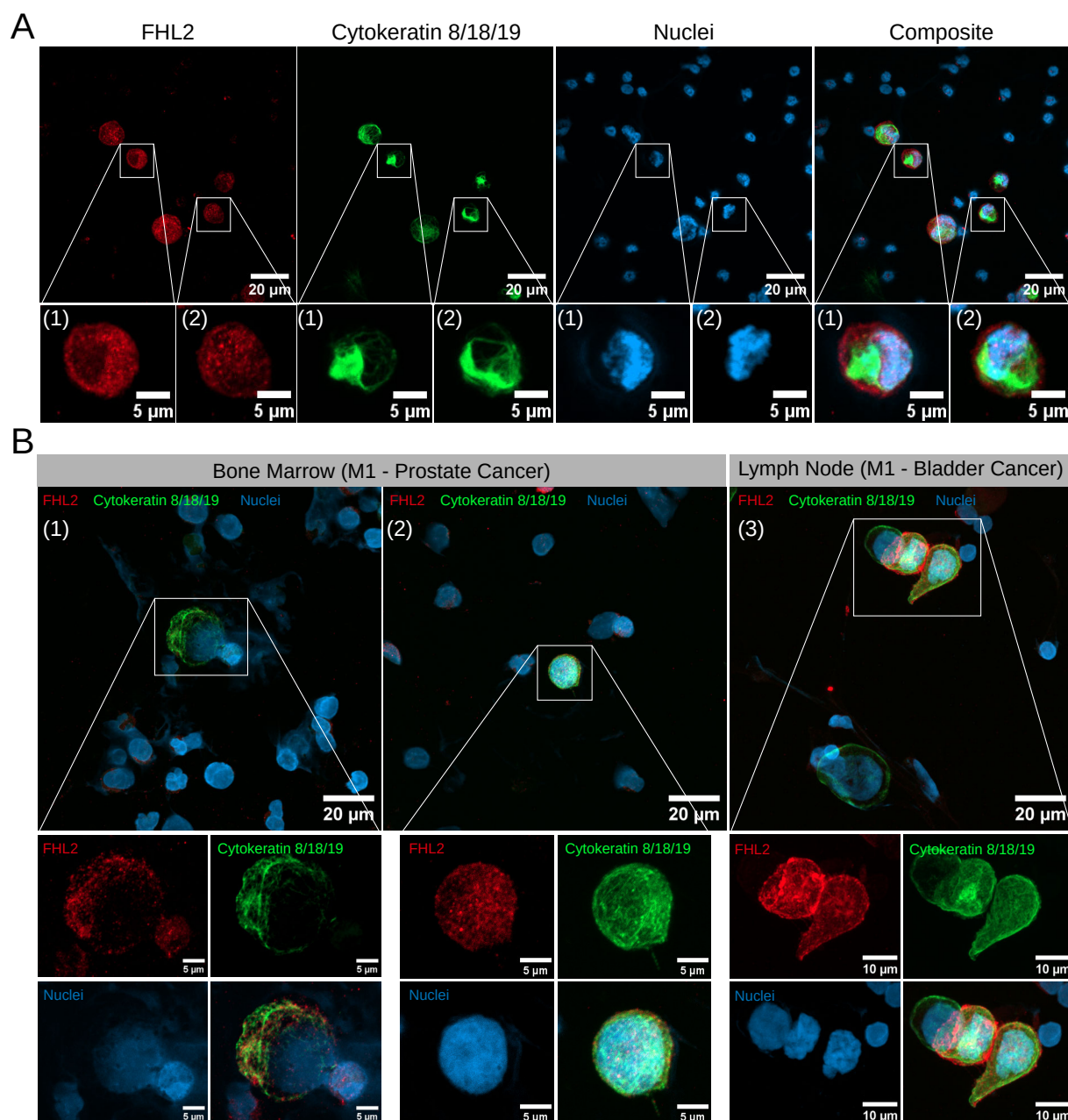


Figure 4.38: High-resolution imaging for analyzing FHL2 expression and localization in patient-derived cancer cells. **A:** Representative images of MDA-MB-231 cells spiked into peripheral blood mononuclear cells (PBMCs), demonstrating successful staining of CK (green) and FHL2 (red), with nuclei labeled in blue. Scale bars: 20 μm for the full region and 5 μm for the zoomed single-cell region. **B:** Representative images of patient-derived cancer cells from the BM of a metastatic prostate cancer patient ((1) and (2)) and from a lymph node sample of a bladder cancer patient without macrometastases ((3)). FHL2 is shown in red, CK in green, and nuclei in blue. Images represent maximum intensity Z-projections. Scale bars: 20 μm for the full image, 5 μm for single-cell zooms of (1) and (2), and 10 μm for (3).

5 Discussion

Despite extensive efforts, patient-derived M0 DCCs have consistently failed to expand in both *in vivo* and *in vitro* models. For instance, xenotransplantation into immunodeficient mice using various injection routes (subcutaneous, orthotopic, intrafemoral, and intravenous) has not yielded successful engraftment [12]. In contrast, only a subset of DCCs from advanced M1-stage prostate cancer patients have demonstrated engraftment [12], indicating that existing models lack the necessary microenvironmental cues to support the survival and outgrowth of early DCCs. Recent advances in the *in vitro* field, including state-of-the-art bioprinting and perfusion systems, aim to enhance the physiological relevance of bone metastasis models [61]. However, most of these approaches rely on widely used allogeneic cell lines, which, while highly reproducible and comparable across studies, fail to capture the inherent heterogeneity of patient-specific tumor biology.

Therefore, the primary objective of this project was to establish a culture system capable of supporting the expansion of early-stage M0 DCCs. Our approach focused on developing a personalized and humanized two-niche BM model, which allows for the controlled introduction of specific cytokines, cell types, and extracellular components, thereby enabling systematic optimization of experimental conditions to better mimic critical aspects of the tumor microenvironment. Although our model has not yet fully recapitulated the complete human BM niche and the ultimate goal of expanding M0 DCCs remains unachieved, it has provided valuable insights into the effect of microenvironmental factors, but require further refinement to support DCC survival and proliferation.

In this section, I discuss the methodological and biological aspects, as well as its limitations and perspectives of my experiments for future research.

5.1 Methodological aspects

Impact of the CPC scaffold in the BM model

To generate the endosteal niche *in vitro*, it is essential to have functionally active osteoblasts. We assessed osteogenic activity by staining for osteocalcin, also known as bone gamma-carboxyglutamic acid protein, a marker of mature osteoblast differentiation [62].

In contrast to Braham et al. [32], who reported no detectable osteocalcin expression in undifferentiated MSCs cultured on CPC scaffolds for 28 days in non inducing medium, our experiments consistently demonstrated osteocalcin expression in MSCs on the CPC scaffold for MSCs cultured in normal expansion medium. Quantitative analysis confirmed detectable osteocalcin expression in all samples, regardless of their differentiation status. Although MSCs cultured in expansion medium exhibited lower osteocalcin levels than those maintained in osteogenic medium, these findings suggest that MSCs on the CPC scaffold undergo spontaneous differentiation. This raises the question of whether the scaffold inherently possesses osteoinductive properties, priming MSCs toward the osteogenic lineage and promoting osteocalcin expression even in the absence of supplemental osteogenic factors.

The CPC material employed in this study is formulated from α -tricalcium phosphate powder and an oil-based liquid carrier that, upon contact with an aqueous environment, converts into nanocrystalline hydroxyapatite [63]. This transformation not only creates a supportive microenvironment for cell proliferation, in which cells readily adhere, grow, and function, but also lays the foundation for subsequent tissue remodeling[63]. To further enhance osteoinductivity and support additional cellular functions, such as angiogenesis growth factors, bioactive proteins, or peptides (for example, collagen [64] or VEGF [65]) can be incorporated into the composition. Although calcium phosphate materials are generally classified as non-osteoinductive, certain formulations have been shown to exhibit inherent osteoinductive properties, enabling bone formation in non-osseous sites without the need for exogenous osteogenic stimuli [66]. It remains unclear whether the CPC scaffold directly stimulates osteoblastic differentiation without exogenous induction factors or if potential staining artifacts account for the observed osteocalcin expression in cells maintained in expansion medium, though isotype control testing ruled out unspecific

antibody binding. Clarifying these discrepancies with the findings of Braham et al., who used the same CPC constructs, requires further functional investigations into the properties of CPC scaffolds. Thus far, the only experiment that omitted the scaffold was designed solely to compare PC3 cell growth in its presence or absence, and it did not specifically assess the scaffold's osteoinductive potential. Consequently, no definitive conclusions can be drawn regarding the isolated effects of the CPC material.

In conclusion, while the incorporation of the inorganic bone component enhances the model's physiological relevance, further investigation is required to elucidate the specific contributions of the CPC scaffold. A comprehensive evaluation of the osteogenic activity within the endosteal niche should include additional markers, such as type I collagen, osteopontin, bone sialoprotein, osteonectin, fibronectin, and bone morphogenetic proteins [66], as well as regular monitoring of calcium levels in the culture medium to maintain optimal niche conditions.

The Role of human platelet lysate and human bone ECM hydrogel in enhancing BM microenvironment authenticity

While the osteoinductive properties of the CPC scaffold contribute to mimicking the endosteal niche, further optimization of culture conditions was necessary to better resemble the human niche. Therefore, we tested replacing traditional animal-derived serum with human-derived alternatives.

hPL for replacement of fetal calf serum as source of growth factors

Traditionally, animal-derived components such as FCS have been used to supply essential growth factors for cell survival and proliferation [67]. However, species-specific differences and ethical concerns may compromise experimental relevance [67, 68]. In clinical settings, FCS poses a risk of pathogen transmission and can trigger immune responses due to xenogeneic antigens. Studies have shown that MSCs cultured in FCS internalize significant amounts of bovine proteins, potentially provoking humoral responses [69]. In basic research, this internalization may alter intracellular signaling and gene expression. For example, Fernandez-Rebollo et al. demonstrated that replacing FCS with human platelet lysate leads to significant changes in gene expression profiles [40], underscoring the critical impact of serum choice on cellular behavior.

hPL and serum-free, chemically defined media offer promising alternatives. Although chemically defined media provide greater experimental control, they often require extensive optimization, rely on proprietary formulations with limited transparency, and are significantly more expensive than FCS [67]. While commercially available hPL is generally more costly than FCS, it can often be obtained at a lower cost from transfusion centers, using expired platelet apheresis donations [67]. Therefore, hPL presents a cost-effective, broadly applicable human alternative, particularly for primary cells. Our experimental findings underscore the advantages of using hPL, especially in the culture of BM derived MSCs and peripheral blood-derived EPCs. MSCs cultured with hPL exhibited shorter doubling times, sustained proliferative capacity over more passages, higher initial cell yields, and maintained differentiation potential comparable to FCS-based cultures. Similarly, for EPCs not only showed enhanced proliferation and extended lifespan in hPL-based media, but hPL also rescued EPCs that had ceased proliferating in FCS, enabling them to resume growth.

While hPL has consistently delivered promising results across our applications, standardization remains an area for further optimization. A systematic review by Guiotto et al. [70] highlighted considerable variability in growth factor levels among platelet-derived products (e.g. bFGF, 0–5.5 ng/ml (mean 0.23 ng/ml); EGF, 0–20 ng/ml (mean 2.77 ng/ml); IGF-1, 0–501.62 ng/ml (mean 78.81 ng/ml); TGF- β , 0–250 ng/ml (mean 57.24 ng/ml); and PDGF-BB, 0.1–22.14 ng/ml (mean 7.5 ng/ml) [70]. Our hPL batches were within the ranges of these values, however exhibited lower mean concentrations (e.g. bFGF 0.03 ng/ml, EGF 0.15 ng/ml, IGF-1 0.17 ng/ml, TGF- β 5.41 ng/ml, and PDGF-BB 1.44 ng/ml). Compared to FCS, hPL was shown to contain higher levels of key growth factors [71], and consequently many studies opt for 5% hPL rather than 10% to support MSC growth [72].

Since established cell lines have been cultured for decades in FCS they may require a gradual transition to hPL. However this was noted in our experiments only in 2D but not in 3D culture PC3 cells.

Remaining challenges, including batch-to-batch variability of hPL [73], the technical demands of fibrinogen depletion (adapted from Hemeda et al. [44]), and donor-specific factors such as gender and blood group, were considered. Our study revealed that fibrinogen depletion's success depended on both the hPL batch and basal medium: while hPL-1 and hPL-2 performed well in MSC expansion medium, hPL-3 often induced

polymerization in the incubator, and the process was entirely ineffective in EGM-2, highlighting the influence of media-specific factors on fibrin polymerization. To minimize potential hormonal influences, we exclusively utilized male donor apheresis products. Beyond gender-related differences, blood group variability was considered. In our study, hPL batches derived from mixed (batch 1&2), exclusively group O (batch 3), or exclusively group AB (batch 4) donations showed no noticeable differences in cell culture outcomes, suggesting that blood group variability may not significantly affect hPL-based cultures in contrast to some previous studies who suggested pooling donations from groups O and AB to reduce MSC exposure to antigens and isoagglutinins [74].

Overall, hPL proved to be an excellent replacement for FCS, effectively enhancing cell proliferation in primary cell culture. Our findings, together with previous studies [75, 72, 44, 68, 74, 76, 77, 70], confirm that hPL is a highly effective human alternative for expanding MSCs and EPCs. Further studies are recommended, particularly to evaluate whether lower concentrations of hPL (below 10%) might enhance vascular niche formation. In conclusion, while standardization and optimized fibrinogen depletion remain areas for improvement, the advantages of using human-derived growth factors outweigh these challenges by eliminating the risks associated with xenogeneic components. As mentioned before, the internalization of bovine proteins from FCS can alter intracellular signaling and gene expression, potentially leading to unpredictable cellular behavior in human cell culture systems.

In conclusion, while standardization and optimized fibrinogen depletion remain areas for improvement, the advantages of using human-derived growth factors outweigh these challenges, particularly by eliminating the risks associated with human cells internalizing xenogeneic proteins that can alter signaling and gene expression.

Human bone-derived ECM to replace animal-derived ECM

Since the ECM plays a pivotal role in directing cellular behavior, morphology, and function, we initiated a pilot experiment to develop a polymerizing hydrogel derived from human bone as an alternative to traditional mouse-derived hydrogels.

The ECM is a complex and diverse network of secreted and cell-bound components that play vital roles in maintaining tissue function, regulating cell signaling, and governing cellular behavior. Key ECM constituents, including structural proteins, glycoproteins, proteoglycans, and cell surface receptors, orchestrate a functional framework that

mediates cell adhesion and signal transduction, thereby contributing to both health and disease processes [78]. The bone marrow extracellular matrix functions not only as a critical mechanical framework upholding tissue integrity, but also as a dynamic repository of growth factors and cytokines that regulate hematopoietic stem cell activities and impact leukemia progression [79].

Natural ECM hydrogels for cell culture, such as collagen, fibrin, hyaluronic acid, Matrigel, chitosan, alginate, and silk fibrils, are inherently biocompatible and bioactive and support diverse cellular functions [80]. However, individual materials like collagen, fibrin, and hyaluronic acid represent only a single protein class and therefore capture only one facet of the ECM's complexity. Although Matrigel provides a more complex protein environment, it is derived from Engelbreth-Holm-Swarm mouse sarcoma and more closely mimics a tumor microenvironment rather than the BM niche.

Although, we also used Matrigel throughout this project, we addressed these limitations by starting a pilot experiment to transition from non-organ-specific, animal-derived Matrigel to a human-derived, organ-specific ECM by isolating a hydrogel from human bone. Cells cultured in the bone ECM hydrogel exhibited a more restricted three-dimensional distribution compared to those in Matrigel. Specifically, they tended to accumulate at the bottom of the hbECM scaffold, with limited vertical dispersion, leading to fewer fully developed three-dimensional structures, although initial structures were evident. In contrast, cells in Matrigel were uniformly distributed throughout the entire gel height. Notably, our hbECM formulation had a total protein concentration of approximately 3 mg/mL, which may be insufficient to provide the necessary structural support. By comparison, Matrigel is typically used at 8–12 mg/mL [81] (with our Matrigel batches measuring 8–9.6 mg/mL). These observations underscore the need to optimize the protein concentration of the hbECM. Others showed that a diverse composition of ECM proteins can be achieved [82] and therefore our vision to develop a more physiologically relevant *in vitro* model that more accurately replicates the native BM microenvironment than Matrigel or single protein gels, may not be out of reach.

Isolation of autologous EPCs from BM

While the incorporation of human-derived non-cellular components, such as hPL and hbECM, improves the similarity of the model to the physiological niche, we addressed

the essential cell types from a single donor to generate a personalized representation of the BM cellular composition. While obtaining MSCs from BM was straightforward, the isolation of autologous EPCs posed a significant challenge. Endothelial cells are essential for the vascular niche, as the BM microenvironment relies on a specialized vascular network composed of macrovascular and microvascular compartments, including arterioles, venules, and capillaries [83]. This network plays a crucial role in regulating hematopoiesis and osteogenesis by supplying nutrients and facilitating key cell signaling interactions [84].

To develop an accurate *in vitro* BM model, establishing a functional vascular niche is important. Most *in vitro* models of endothelial function utilize HUVECs, which have become a widely accepted standard. However, concerns remain regarding their suitability as a universal model, given the significant differences in gene expression profiles and functional responses between endothelial cells of distinct vascular origins [37]. Although HUVECs are commonly used to study general endothelial biology, their limited tissue specificity may not accurately reflect the unique characteristics of BM endothelial cells. To establish a more physiologically relevant model, we aimed to isolate EPCs from BM, using differential adherence, sorting for CD34-positive or CD31-positive cells, and promoting endothelial outgrowth from bone fragments.

Since the adherence method described by Fang et al. [43] resulted in a highly heterogeneous population with no clear enrichment of endothelial progenitors, we next applied CD34-based sorting, aiming to selectively enrich endothelial progenitors while excluding non-adherent hematopoietic stem cells. However, the expected enrichment was not observed, likely due to the dynamic expression of CD34 among endothelial progenitors.

Recognizing that CD34 is not an exclusive marker of endothelial progenitors, we also employed CD31, which is expressed on both endothelial and hematopoietic cells [85]. While previous studies have shown that culturing CD31-positive BM cells under optimized conditions can promote endothelial outgrowth and vasculogenic potential [86], our attempts did not yield a homogeneous endothelial culture. This may be due to early EPCs lacking CD31 expression, only acquiring it during differentiation as they lose CD133 expression [87].

Given these limitations with direct CD34 or CD31-based selection, we explored an alternative strategy by culturing small bone fragments under endothelial growth

conditions to promote spontaneous endothelial outgrowth, followed by CD31-positive cell enrichment once cobblestone-like colonies appeared to remove contaminating MSCs. This approach was inspired by reports of successfully isolating endothelial cells from BM biopsies [88]. However, this strategy also failed to yield a homogeneous endothelial population, likely because small endothelial colonies were not detected early enough before mesenchymal cells overgrew the culture. Rafii et al. [89] utilized collagenase digestion and *Ulex europaeus* lectin-based purification to expand EPCs from human BM aspirates, which may be tested in future attempts.

Due to the difficulties in isolating EPCs from BM, we explored peripheral blood as an alternative source. Peripheral blood is a proficient source of colony-forming EPCs[90, 56], allowing us to generate patient-matched endothelial cells despite the challenges associated with bone marrow isolation. In our third attempt using peripheral blood, we processed a relatively large volume (100 mL) under high-serum conditions (20%) and seeded cells at a high density of 3.3×10^6 cells/cm². These conditions resulted in the successful isolation and expansion of ECFCs (referred to as EPCs) from a healthy male donor. However, for patient-derived samples, only a limited blood volume is typically available, requiring the development of a robust protocol that can reliably isolate EPCs from smaller volumes. Notably, Martin-Ramirez et al. [56] reported successful EPC outgrowth in 80% of donors using only 27mL of blood, a clinically acceptable and practical volume. However, as donor age may impact onto the results, we propose initially testing this protocol on peripheral blood samples from older, healthy donors to evaluate its reproducibility in our setting. Once optimized, this approach can be extended to prostate cancer patients, allowing the derivation of both EPCs and MSCs from a single donor while accommodating clinical constraints on blood collection.

5.2 Biological aspects

The BM microenvironment shapes PC3 cancer cell behavior: Microscopic and transcriptomic insights

Due to the complexity of the cellular and non-cellular components of the BM niche, it quickly became clear that expanding DCCs in a humanized *in vitro* model could not be achieved within the scope of a thesis. However, as I had isolated DCCs from prostate

cancer patients with and without metastasis in parallel to my other experiments, thereby paved the way for an in-depth transcriptomic analysis of single DCCs freshly isolated from their niche. This will provide a “ground-truth” of a niche-associated DCC phenotype to compare with model DCCs. To this end, PC3 bone metastatic prostate cancer cells were exposed to niches of varying complexity and profiled using 10x Genomics single-cell transcriptomics. The goal of this experiment will be to test whether and which of the cellular components of the niche shift the PC3 phenotype towards a DCC-like phenotype. Since the DCC data are not available yet, we first asked the question whether or not cellular interactions are able to change the phenotype of PC3 and bone marrow niche cells.

With microscopic analysis, colony volume approximations revealed that PC3 cells formed the largest colonies in the vascular niche, followed by those in the endosteal niche, which exhibited a more dispersed, migratory growth pattern. In the combined vascular and endosteal niches, colony growth resembled that in the vascular niche but with significantly reduced volume. In all co-culture conditions, colony expansion was more pronounced than in PC3 cells cultured alone in Matrigel, which showed a slower overall increase in colony size. The method used to measure cytoplasmic volume does not directly correlate with cell number, meaning that changes in cell shape rather than proliferation may have influenced the observed differences. Nuclear staining, while potentially more accurate for cell quantification, was impractical due to the depth of the model requiring continuous laser adjustments across the z-stack. Similarly, flow cytometry using counting beads proved unsuitable due to inconsistent and unrealistic results arising from uncertainties in sample volume loss that compromised accurate quantification of the low cell numbers. Although our measurement method limits direct conclusions about proliferation, the observed effects are consistent with Baldassarri et al.[33], who found that breast cancer cells were more proliferative in osteoblast-enriched conditions compared to a combined niche with osteoblasts and perivascular-like elements. Notably, their study did not analyze the vascular niche in a separate condition.

Transcriptomic analysis confirmed that cancer cells are profoundly influenced by their microenvironment, interacting distinctly within each niche. Our pathway enrichment analysis from single-cell data reveals that the differentially regulated pathways in the two-niche environment are not simply the additive effects of individual niches, emphasizing

the importance of microenvironmental complexity in shaping cancer cell behavior.

This differential enrichment is exemplified by the pro-inflammatory profile of PC3 cells within the niches. When both niches are present, PC3 cells consistently upregulate chemokine receptor binding, which is the only pathway enriched across all conditions, along with chemokine activity and an antimicrobial humoral immune response mediated by antimicrobial peptides. Whereas under single niche conditions (endosteal or vascular), they further enhance additional inflammatory responses such as cytokine activity, complement activation, neutrophil chemotaxis, and granulocyte chemotaxis, with some pathways uniquely enriched in one niche and not observed when both niches are combined. Due to time constraints, our analysis is ongoing. More in-depth single-cell investigations are underway to provide a nuanced understanding, including a comparative evaluation of PC3 cell phenotypes from the BM model with isolated DCCs from the BM of patients.

In summary, our findings underscore the importance of physiologically relevant models in cancer research. Conventional 2D cultures and animal models fail to capture key cellular interactions in the human microenvironment, where the complexity of the BM niche plays a crucial role in regulating tumor cell fate and balancing dormancy and proliferation [91]. This study lays the foundation for future investigations into the mechanisms governing early DCC colonization and their interactions within the niche.

5.3 Limitations

Challenges in interpreting quantitative data

One limitation of this study is the low number of experimental repetitions and biological replicates, particularly in terms of individual donors. Although we had a sufficient number of MSC donors, the extensive workload required to generate the BM model, taking approximately 2–3 months for each complete experiment (including primary cell preparation, niche differentiation, cancer cell culture, and subsequent microscopic analysis), limited the number of repetitions and technical replicates. The imaging process was especially labor-intensive, with each sample requiring at least 2 hours for lower-resolution tile scanning, which quickly resulted in a substantial time commitment and logistical challenges, ultimately restricting the number of repetitions. Among the

various experiments, the investigation of PC3 cell growth within the individual niches was performed three times independently using the same cell donors, providing the most robust dataset. However, additional experiments using different MSC donors would be valuable to confirm that the observed growth-supporting or limiting trends are consistent across donor variability.

Another limitation involves the interpretation of statistical significance given the relatively small number of replicates. In most experiments, I applied parametric tests, including ANOVA and t-tests under the assumption of normality. This assumption could be supported by the Central Limit Theorem when measurements are derived from randomly selected fields of view in consistently seeded cultures or from random samples of uniformly treated batches. However, because in most cases normality was difficult to assess, I additionally reported results from non-parametric tests, such as the Mann-Whitney U test or Kruskal-Wallis test, to provide a more comprehensive statistical evaluation. While this approach helps account for potential deviations from normality, we acknowledge the overall limitations in statistical robustness due to the relatively small sample size. Increasing the number of experimental repetitions and technical replicates would significantly enhance statistical power, thereby improving the reliability, reproducibility, and generalizability of our findings. However, given the extensive workload required to execute such complex 3D models, future studies must carefully balance feasibility with scientific rigor. This necessitates either focusing on highly specific research questions that can be robustly addressed within the model or implementing comprehensive readout strategies that yield deeper insights into the biology of metastatic colonization.

Humanization as part of improving physiological relevance

Beyond the challenges associated with experimental repetition and statistical robustness, it is essential to critically interpret our claim that transitioning to a fully humanized model setup enhances biological relevance. One major limitation of our study is that we lack direct experimental validation proving that humanization leads to a more physiologically accurate representation of the human condition. Our work demonstrates that hPL is a robust alternative to FCS in cell culture, effectively promoting proliferation, survival, and the maintenance of cellular functions in primary cells. Although we did not directly confirm

that FCS changes cellular responses due to its xenogeneic molecular composition, Fernandez-Rebello et al. provide compelling evidence that serum choice significantly influences cellular transcriptomes. They observed that replacing FCS with hPL induced significant gene expression changes, particularly with an enrichment in ECM-related categories even after only two days in culture[40]. While these effects were interpreted as moderate in their study, they underscore the potential impact of serum selection on cellular biology. Moreover, our 10x sequencing experiment revealed that alterations in the niche setup lead to substantial transcriptomic changes in PC3 cells, suggesting that humoral factors such as those present in serum may similarly drive transcriptional changes that influence cellular responses and function.

In summary, direct evidence is still limited, our results support the premise that prioritizing human-based culture conditions is advisable to minimize interspecies variability. However, to further substantiate this claim, additional experiments are required to precisely assess the impact of humanizing the culture system within the context of the bone marrow microenvironment.

5.3.1 Perspectives

Personalization of the BM model and uncovering niche drivers of the DCC phenotype

Investigating cancer cell response within the BM niche requires models that recapitulate human physiological conditions more accurately than conventional 2D cultures or animal models. A patient-specific, human-derived model is particularly valuable for studying DCCs because it may capture individual heterogeneity and offers deeper insights into dormancy, reactivation, and metastatic progression. However, given the complexity of this task, as observed in this study, we propose two complementary strategies: (1) refining the BM model with human-derived components and further enhancing physiological authenticity, and (2) employing a systematic “shortcut” using established cancer cell line to pinpoint which niche factors most strongly contribute to the generation of a DCC-like phenotype.

1) Refining the BM model for greater physiological relevance

To create an even more physiologically robust and personalized BM model, we propose

to follow the line of thought initiated in this study by using hPL, adopting a human plasma-like medium, and standardizing hypoxic conditions and considering androgen supplementation. Further adaptations to the in-vivo situation could include the incorporation of human bone ECM hydrogel (hbECM) instead of Matrigel (**Table 5.1**).

These refinements address key limitations in replicating *in vivo* conditions, such as the high glucose content of synthetic media, variable oxygen levels, and the reliance on animal-derived components in a human microenvironment. Building on these physiological improvements, one may further consider (see **Figure 5.1**) to integrate patient-derived DCCs by incorporating the whole BM cellular fraction or just the EpCAM positive cell fraction. When using the entire BM mononuclear fraction, which includes immune cells, the model must be autologous to prevent unwanted immune reactions. An initial strategy could comprise the following steps: First, BM MNCs are isolated, from which MSCs are expanded in hPL-supplemented media and the remaining MNC suspension cryopreserved for later co-culture. In parallel, EPCs are expanded from peripheral blood in EGM-2 with hPL. Once an osteoblast-enriched endosteal niche is established on a CPC scaffold, the cryopreserved MNCs are thawed and combined with MSCs and EPCs within a 3D hydrogel, maintained in a human plasma-like medium for 10–14 days. The resulting multicellular niche is then evaluated using confocal microscopy to assess cellular interactions and single-cell transcriptomics for in-depth analysis, which may include novel spatial transcriptomics technologies and offer novel ways to study patient-derived DCCs in their niche.

Table 5.1: Potential Next Steps for Refinement of the Personalized BM Model

Refinement area	Objective	Current challenges	Proposed next steps
Integration of hPL	Fully incorporate hPL as human derived supplement	Limited pilot experiments with patient-derived DCCs	Replace FCS entirely with hPL across the model

Continued on next page

Table 5.1 – Continued from previous page

Refinement Area	Objective	Current Challenges	Proposed Next Steps
Transition to human plasma-like medium (HPLM)	Accurately mimic the <i>in vivo</i> plasma composition	Synthetic media lack the physiological relevance of <i>in vivo</i> plasma composition and often contain high glucose levels; no tests were run so far	Test HPLM as basal media in the BM model setup using either hPL or patient-derived BM plasma
Integration of hbECM	Use tissue-specific ECM for better recapitulation of the native BM microenvironment	Low protein concentration and missing composition details	Optimize protein concentration of prepared hbECM and characterize its structural and protein composition
Hypoxic culture conditions	Simulate the low-oxygen BM environment	Low oxygen condition applied only in selected experiments	Standardize hypoxic conditions throughout all experiments
Androgen supplementation	Investigate androgen receptor dynamics in tumor progression	Could be used to compare androgen-sensitive and insensitive	Evaluate androgen supplementation in patient-derived cultures

2) Identification of niche-factors responsible for the BM-DCC phenotype

Although this personalized approach is comprehensive, it demands significant resources, careful patient recruitment, and likely numerous iterations. Consequently, we propose a parallel "shortcut" strategy that dissects niche-specific effects onto PC3 prostate cancer cells in controlled single- and multi-niche cultures. By comparing PC3 behavior in vascular, endosteal, and combined niches, one can rapidly identify which components, such as osteoblast-derived cues or perivascular signals drive a DCC-like phenotype. Insights from these simplified experiments may further guide the refinement of the fully personalized model, allowing us to focus on the most influential cellular, matrix, or signaling elements before introducing rare patient-derived DCCs.

Ultimately, these complementary approaches are expected to converge to create a robust, translationally useful BM model. Insights from PC3-based niche dissection will help identify the key cellular components, growth factors, and culture conditions necessary to recapitulate early DCC behavior, thereby guiding the physiological refine-

ments that enhance the model's ability to elucidate cancer dormancy and progression through the integration of patient-derived cells. As the model becomes increasingly authentic and personalized, it opens new avenues for investigating DCC biology and testing therapeutic strategies to prevent metastatic outgrowth in prostate cancer and beyond.

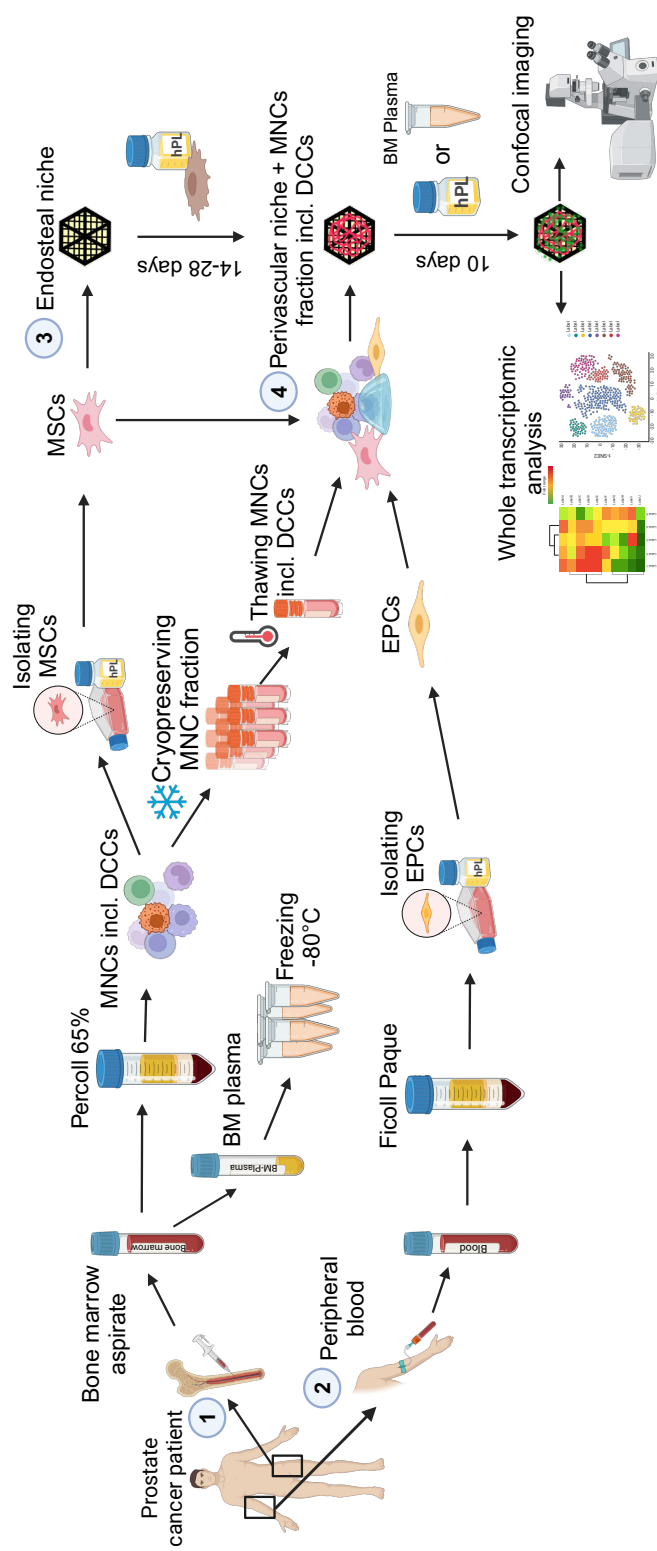


Figure 5.1: Pathway to generate a complete personalized BM model for studying patient-specific DCCs. Created with BioRender.com

5.4 Conclusion

Our work demonstrated that prostate cancer cells engage in dynamic crosstalk with the BM microenvironment, with each niche distinctly influencing colony growth and inducing specific pathway alterations. Recognizing the complexity of the native metastatic niche in prostate cancer, we emphasize the need for human-relevant three-dimensional (BM) models that faithfully recapitulate both the cellular and molecular landscapes observed in patients. This can be achieved by systematically evaluating cell lines (e.g. PC3 cells) across various BM niches to identify the cellular and non-cellular components that shape disseminated cancer cell (DCC) phenotypes.

A diverse biobank comprising M0 and M1 stage DCCs, BM-MNCs, MSCs, and BM plasma was established, providing a robust foundation for future investigations. This resource enables comparative analyses of niche-driven phenotypes with those observed in freshly isolated, patient-derived DCCs, facilitating deeper insights into tumor-microenvironment interactions. Our work also focused on optimizing the model for scenarios with low cancer cell numbers, developing refined microscopic analysis protocols, and establishing a robust dissociation protocol for single-cell sequencing. By integrating complementary analytical techniques, such as advanced microscopy and single-cell transcriptomics, we will continue to refine both cellular and non-cellular components to more accurately recapitulate the patient-derived DCC phenotype *in vitro*. Future refinements include adopting BM-typical hypoxic conditions, fully humanizing the model by replacing xenogeneic supplements (e.g., FCS) with hPL, incorporating human and organ-specific extracellular matrix materials (hbECM), and personalizing all cell types within the model to enhance its physiological relevance. Although the *in vitro* expansion of M0 DCCs was beyond the scope of this thesis, this work established a robust framework for a humanized and personalized 3D BM model that recapitulates the metastatic niche in a physiologically relevant context. By addressing the limitations of traditional 2D models, which are overly simplistic, and murine models, which fail to fully represent human biology. This approach provides a more predictive platform for studying prostate cancer dissemination. Ultimately, it lays the foundation for the development of targeted therapies aimed at intercepting metastasis at its earliest and most vulnerable stages.

6 English summary

This thesis establishes a physiologically relevant *ex vivo* bone marrow model that incorporates both endosteal and perivascular niches to investigate the early stages of metastatic colonization in prostate cancer. By utilizing human-derived materials, the model was designed to more accurately recapitulate the bone marrow microenvironment and aimed to capture the rarity and heterogeneity of disseminated cancer cells through advanced imaging and single-cell sequencing techniques. Our findings reveal that prostate cancer cells engage in dynamic crosstalk with distinct microenvironmental niches, influencing colony growth and modulating cellular pathways. Furthermore, we demonstrate that FCS can be successfully replaced by hPL, to avoid animal derived supplements in the culture. Additionally, we optimized protocols for low-cell-number scenarios and refined both microscopic analysis and cell dissociation to enable single-cell transcriptomic applications within the model. Although achieving the patient-derived DCC phenotype and successfully expanding patient-derived DCCs remain future goals, this work establishes a robust foundation. Proposed refinements include the introduction of hypoxic conditions, the adoption of fully humanized supplements, the integration of human-derived and organ-specific extracellular matrices, and the personalization of all cell types. These advancements will further enhance the model's predictive power for studying cancer dissemination in a clinically relevant setting and support the development of targeted therapies.

7 Deutsche Zusammenfassung

Diese Dissertation etabliert ein physiologisch relevantes ex vivo Knochenmarkmodell, das sowohl die endostiale als auch die perivaskuläre Nische integriert, um die frühen Stadien der metastasierenden Kolonisation bei Prostatakrebs zu untersuchen. Bei der Optimierung des Knochenmarkmodells konnten wir zeigen, dass das fötale Kälberserum (FCS) erfolgreich durch humanes Thrombozytenlysate (hPL) ersetzt werden kann, um die Kultivierung primärer Zellen zu unterstützen und tierische Komponenten zu vermeiden. Durch den vermehrten Einsatz von Zellen direkt isoliert vom Patienten sollte das Modell so konzipiert werden, dass es die Komplexität und Heterogenität einzelner Patienten und des Knochenmarkmilieus genauer abbildet. Zur Erfassung der Seltenheit und Diversität disseminierter Krebszellen wurden zudem Protokolle angepasst, um diese Zellen mittels fortschrittlicher Bildgebung und Einzelzellsequenzierung effektiv zu untersuchen. Bei der Transkriptomanalyse zeigen unsere Ergebnisse, dass Prostatakrebszellen in den verschiedenen Nischen des Knochenmarks unterschiedliche Interaktionen mit den Nischenzellen eingehen, welche das Koloniewachstum beeinflussen und spezifische zelluläre Signalwege modulieren.

Obwohl das Erreichen des in Patienten beobachteten DCC-Phänotyps und die erfolgreiche Expansion von DCCs aus Patienten noch zukünftige Ziele sind, bildet diese Arbeit ein solides Fundament. Vorgeschlagene Optimierungen umfassen das Kultivieren unter hypoxischen Bedingungen, den ausschließlichen Einsatz humaner Komponenten, die Integration humaner und organspezifischer extrazellulärer Matrix-Hydrogele (hbECM) sowie die personalisierte Isolierung aller Zelltypen. Zusammengefasst könnten diese Schritte die Aussagekraft des Modells zur Untersuchung der Krebszelldissemination in einem klinisch relevanten Kontext weiter verbessern und die Entwicklung gezielter Therapien unterstützen.

A Appendix

Table A1: Pairwise Mann–Whitney test p-values for comparisons between conditions comparing cell densities in vascular niche

Day	Condition 1 vs Condition 2	Condition 1 vs Condition 3	Condition 2 vs Condition 3
Day 2	0.357 (ns)	0.278 (ns)	0.023 (*)
Day 5	0.424 (ns)	0.045 (*)	0.002 (**)
Day 8	> 0.999 (ns)	0.0002 (***)	0.00003 (****)
Day 11	0.833 (ns)	0.022 (*)	0.0005 (***)
Day 14	0.023 (*)	0.008 (**)	0.0002 (***)

Table A2: List of differentially expressed GO pathways in PC3 cells cultured in either of the niches compared to those cultured in Matrigel. NES = Normalized Enrichment Score; only pathways with an adjusted p-value < 0.05 and NES scores < -2 or > 1 are shown

Pathway Number	GO Term	NES: Two Niche	NES: En-dosteal only	NES: Vascular only
1	GOCC_COLLAGEN_TRIMER	1.90110688		1.604739003
2	GOMF_CHEMOKINE_RECEPTOR_BINDING	1.894049038	1.888100313	1.882519319
3	GOBP_ANTIMICROBIAL_HUMORAL_IMMUNE_RESPONSE_MEDIATED_BY_ANTIMICROBIAL_PEPTIDE	1.885240867		1.796963035
4	GOMF_CHEMOKINE_ACTIVITY	1.854079602		1.905458249
5	GOBP_DISRUPTION_OF_ANATOMICAL_STRUCTURE_IN_ANOTHER_ORGANISM	1.830777119	2.101569438	1.873657087
6	GOCC_A_BAND	1.811812802		
7	GOMF_SERINE_TYPE_ENDOPEPTIDASE_INHIBITOR_ACTIVITY	1.800151742		
8	GOMF_PROTEASE_BINDING	1.698390632		
9	GOMF_ENDOPEPTIDASE_REGULATOR_ACTIVITY	1.608959643		
10	GOCC_EXTERNAL_ENCAPSULATING_STRUCTURE	1.360705738	1.456572082	
11	GOBP_RESPONSE_TO_BACTERIUM		1.457693079	
12	GOCC_COLLAGEN_CONTAINING_EXTRACELLULAR_MATRIX		1.542156319	
13	GOCC_EXTERNAL_SIDE_OF_PLASMA_MEMBRANE		1.558403285	
14	GOBP_NEUTROPHIL_CHEMOTAXIS		1.809519608	1.678455277
15	GOMF_CYTOKINE_ACTIVITY		1.844389442	
16	GOBP_COMPLEMENT_ACTIVATION		1.880217714	
17	GOCC_MONOATOMIC_ION_CHANNEL_COMPLEX			1.432877595
18	GOMF_SERINE_HYDROLASE_ACTIVITY			1.498008185
19	GOCC_CATION_CHANNEL_COMPLEX			1.499930624
20	GOMF_MONOATOMIC_ANION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY			1.56321516
21	GOBP GRANULOCYTE_CHEMOTAXIS			1.606854811
22	GOMF_CHLORIDE_CHANNEL_ACTIVITY			1.624536111
23	GOMF_CHLORIDE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY			1.631276803
24	GOMF_ABC_TYPE_TRANSPORTER_ACTIVITY			1.674704035
25	GOMF_CALCIUM_CHANNEL_REGULATOR_ACTIVITY			1.71298271

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Table A2 – continued from previous page

Pathway Number	GO Term	NES: Two Niche	NES: Endosteal only	NES: Vascular only
26	GOBP_OLIGOPEPTIDE_TRANSPORT			1.727664047
27	GOBP_RESPONSE_TO_ACIDIC_PH			1.823227148
28	GOBP_PROTEIN_METHYLATION	-2.001047921		
29	GOBP_RNA_METHYLATION	-2.001112606	-2.235662165	-2.766945861
30	GOBP_MITOCHONDRIAL_RNA_METABOLIC_PROCESS	-2.006559605		-2.458663789
31	GOBP_POSITIVE_REGULATION_OF_MRNA_PROCESSING	-2.009968093		-2.157382316
32	GOMF_PRE_MRNA_BINDING	-2.01430743		
33	GOBP_REGULATION_OF_TRANSCRIPTION_BY_RNA_POLYMERASE_I	-2.018011739		-2.121510112
34	GOMF_INTRAMOLECULAR_TRANSFERASE_ACTIVITY	-2.021039891		-2.21923866
35	GOMF_SNORNA_BINDING	-2.024581919		-2.075413302
36	GOMF_CATALYTIC_ACTIVITY_ACTING_ON_A_TRNA	-2.028431228		
37	GOBP_NEGATIVE_REGULATION_OF_ERBB_SIGNALING_PATHWAY	-2.028602096		
38	GOMF_RIBOSOME_BINDING	-2.030084435		-2.114005808
39	GOMF_STRUCTURAL_CONSTITUENT_OF_RIBOSOME	-2.033121699	-2.154635651	
40	GOBP_POSITIVE_REGULATION_OF_MYOBlast_DIFFERENTIATION	-2.038581485		
41	GOBP_METALLO_SULFUR_CLUSTER_ASSEMBLY	-2.04407236		-2.306287477
42	GOBP_MACROMOLECULE_METHYLATION	-2.045018197	-2.176370697	
43	GOBP_RNA_EXPORT_FROM_NUCLEUS	-2.050596299		
44	GOBP_TRANSCRIPTION_BY_RNA_POLYMERASE_I	-2.05359171		-2.160847078
45	GOBP_REGULATION_OF_EXTRINSIC_APOPTOTIC_SIGNALING_PATHWAY_IN_ABSENCE_OF_LIGAND	-2.067244723		
46	GOBP_PROTEIN_INSERTION_INTO_ER_MEMBRANE	-2.074318681		
47	GOBP_METANEPHROS_DEVELOPMENT	-2.076665323	-2.004566374	
48	GOBP_POSITIVE_REGULATION_OF_CELL_CYCLE_G1_S_PHASE_TRANSITION	-2.077625869		
49	GOMF_RIBONUCLEOPROTEIN_COMPLEX_BINDING	-2.082060582		
50	GOBP_MITOCHONDRIAL_RNA_PROCESSING	-2.090907837		-2.307007815
51	GOBP_PROTEIN_INSERTION_INTO_MEMBRANE	-2.092309299		-2.148768756
52	GOBP_POSITIVE_REGULATION_OF_RNA_SPLICING	-2.098294513		
53	GOBP_CYTOPLASMIC_TRANSLATIONAL_INITIATION	-2.100821449		
54	GOCC_CLATHRIN_COATED_ENDOCYTIC_VESICLE_MEMBRANE	-2.102988226		
55	GOMF_RRNA_BINDING	-2.114247295		
56	GOBP_TRNA_MODIFICATION	-2.125743706	-2.04011199	-2.75968723
57	GOCC_TELOMERASE_HOLOENZYME_COMPLEX	-2.128865857		-2.369706303
58	GOBP_TELOMERASE_RNA_LOCALIZATION	-2.132380633		-2.404851163
59	GOBP_POSITIVE_REGULATION_OF_NEURON_DIFFERENTIATION	-2.132560795		
60	GOBP_REGULATION_OF_MRNA_SPLICING_VIA_SPLICEOSOME	-2.133762228		
61	GOBP_REGULATION_OF_GASTRULATION	-2.138835413		-2.187468321
62	GOCC_NUCLEAR_REPLICATION_FORK	-2.139564737		-2.013081119
63	GOBP_RRNA_MODIFICATION	-2.141319119	-2.132777947	-2.306655551
64	GOBP_POSITIVE_REGULATION_OF_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION_TO_TELOMERE	-2.142164834		-2.376011733
65	GOBP_DNA_REPLICATION_CHECKPOINT_SIGNALING	-2.143914474		-2.083603813
66	GOBP_METANEPHROS_MORPHOGENESIS	-2.14428478		
67	GOBP_REGULATION_OF_DNA_REPLICATION	-2.147309296		
68	GOBP_PROTEIN_LOCALIZATION_TO_NUCLEAR_BODY	-2.147951966		-2.410742716
69	GOCC_TRANSCRIPTION_ELONGATION_FACTOR_COMPLEX	-2.152353346		
70	GOBP_MITOCHONDRIAL_RIBOSOME_ASSEMBLY	-2.153738699		-2.207769871
71	GOCC_RIBOSOME	-2.154446174	-2.266641586	
72	GOCC_PROTEIN_FOLDING_CHAPERONE_COMPLEX	-2.156277396		-2.226522854
73	GOBP_POSITIVE_REGULATION_OF_NEURAL_PRECURSOR_CELL_PROLIFERATION	-2.159577418		
74	GOBP_POSITIVE_REGULATION_OF_MAP_KINASE_ACTIVITY	-2.166460509		
75	GOMF_ATP_DEPENDENT_PROTEIN_FOLDING_CHAPERONE	-2.177605136		-2.464542328
76	GOBP_DNA_TEMPLATED_DNA_REPLICATION_MAINTENANCE_OF_FIDELITY	-2.179656072		-2.358780279
77	GOBP_MITOTIC_METAPHASE_CHROMOSOME_ALIGNMENT	-2.179779151		-2.115640887
78	GOBP_PROTEIN_LOCALIZATION_TO_NUCLEOLUS	-2.186643401		-2.273009617
79	GOCC_OUTER_KINETOCHORE	-2.188661288		-2.170014492
80	GOBP_ADHERENS_JUNCTION_ORGANIZATION	-2.189062387	-2.120049065	
81	GOBP_LEUKOCYTE_TETHERING_OR_ROLLING	-2.189752939		
82	GOMF_ENDONUCLEASE_ACTIVITY_ACTIVE_WITH_EITHER_RIBO_OR_DEOXYRIBONUCLEIC_ACIDS_AND_PRODUCING_5_PHOSPHOMONOESTERS	-2.193254331		-2.005747975
83	GOMF_3_5_DNA_HELICASE_ACTIVITY	-2.195180953		-2.100104751

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Table A2 – continued from previous page

Pathway Number	GO Term	NES: Two Niche	NES: Endosteal only	NES: Vascular only
84	GOCC_PRP19_COMPLEX	-2.196377569		-2.327008115
85	GOBP_ERBB4_SIGNALING_PATHWAY	-2.197785441		
86	GOBP_SPLICEOSOMAL_SNRNP_ASSEMBLY	-2.199516206		-2.423630038
87	GOMF_RNA_POLYMERASE_ACTIVITY	-2.19985319		-2.379190837
88	GOMF_RIBONUCLEASE_P_RNA_BINDING	-2.200712096		
89	GOCC_MULTIMERIC_RIBONUCLEASE_P_COMPLEX	-2.200712096		
90	GOMF_RIBONUCLEASE_P_ACTIVITY	-2.203449581		-2.105198571
91	GOCC_NUCLEAR_EXOSOME_RNASE_COMPLEX	-2.203580672		-2.319124836
92	GOMF_TRANSLATION_INITIATION_FACTOR_ACTIVITY	-2.204981481		-2.200777457
93	GOMF_DNA_POLYMERASE_BINDING	-2.210207043		-2.390291602
94	GOCC_U5_SNRNP	-2.212565479		-2.339025542
95	GOCC_SM_LIKE_PROTEIN_FAMILY_COMPLEX	-2.214756657		-2.538619267
96	GOCC_RIBONUCLEASE_P_COMPLEX	-2.220565372		-2.252489202
97	GOBP_POSITIVE_REGULATION_OF_EPITHELIAL_CELL_DIFFERENTIATION	-2.225524667		
98	GOBP_POSITIVE_REGULATION_OF_CELL_MIGRATION_INVOLVED_IN_SPROUTING_ANGIOGENESIS	-2.227072691		-2.535161224
99	GOBP_REGULATION_OF_CELL_MIGRATION_INVOLVED_IN_SPROUTING_ANGIOGENESIS	-2.231748658		
100	GOCC_U2_SNRNP	-2.234519494		-2.318421764
101	GOBP_U2_TYPE_PRESPICEOSOME_ASSEMBLY	-2.234519494		-2.341603155
102	GOBP_REGULATION_OF_PROTEIN_LOCALIZATION_TO_CHROMOSOME_TELOMERIC_REGION	-2.245766575		-2.5214099
103	GOMF_DNA_HELICASE_ACTIVITY	-2.247711046		-2.503162176
104	GOBP_POSITIVE_REGULATION_OF_MACROPHAGE_ACTIVATION	-2.249592042		
105	GOBP_PSEUDOURIDINE_SYNTHESIS	-2.25953828		-2.462090462
106	GOBP_TRNA_5_LEADER_REMOVAL	-2.262395301		-2.203061103
107	GOMF_TRNA_SPECIFIC_RIBONUCLEASE_ACTIVITY	-2.266457836		-2.287350193
108	GOMF_RNA_ENDONUCLEASE_ACTIVITY_PRODUCING_5_PHOSPHOMONOESTERS	-2.268976648		-2.288546498
109	GOCC_REPLICATION_FORK	-2.278726753		-2.255692073
110	GOBP_POSITIVE_REGULATION_OF_TELOMERASE_RNA_LOCALIZATION_TO_CAJAL_BODY	-2.285978229		-2.257456525
111	GOBP_MATURATION_OF_LSU_RRNA_FROM_TRICISTRONIC_RRNA_TRANSCRIPT_SSU_RRNA_5_8S_RRNA_LSU_RRNA	-2.291940373		-2.2593464
112	GOBP_TRNA_5_END_PROCESSING	-2.316850453		-2.423700389
113	GOBP_DNA_CONFORMATION_CHANGE	-2.323601684		-2.563461198
114	GOCC_PROTEASOME_REGULATORY_PARTICLE_BASE_SUBCOMPLEX	-2.328028328		-2.320429253
115	GOBP_RNA_MODIFICATION	-2.342682616	-2.23715229	
116	GOMF_DNA_REPLICATION_ORIGIN_BINDING	-2.348221048		-2.723101418
117	GOCC_SNO_S_RNA_CONTAINING_RIBONUCLEOPROTEIN_COMPLEX	-2.366586101		-2.392742526
118	GOCC_SMALL_NUCLEAR_RIBONUCLEOPROTEIN_COMPLEX	-2.3688294		-2.62851212
119	GOBP_TRNA_PROCESSING	-2.390644092	-2.131400757	
120	GOCC_DNA_REPLICATION_PREINITIATION_COMPLEX	-2.403206844		-2.405938496
121	GOBP_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION_TO_MITOCHONDRIAL_MEMBRANE	-2.411628383		-2.323805168
122	GOBP_FAT_SOLUBLE_VITAMIN_METABOLIC_PROCESS	-2.414113546		
123	GOBP_REGULATION_OF_DNA_TEMPLATED_DNA_REPLICATION_INITIATION	-2.425416655		-2.378628482
124	GOCC_PRERIBOSOME_SMALL_SUBUNIT_PRECURSOR	-2.427936128		-2.440816023
125	GOBP_MITOTIC_DNA_REPLICATION	-2.435945024		-2.328689591
126	GOCC_MITOCHONDRIAL_SMALL_RIBOSOMAL_SUBUNIT	-2.441506796		-2.496281548
127	GOCC_CMG_COMPLEX	-2.444548872		-2.329159503
128	GOCC_LARGE_RIBOSOMAL_SUBUNIT	-2.451776959	-2.316593688	
129	GOCC_RIBOSOMAL_SUBUNIT	-2.462132531	-2.606516272	
130	GOBP_ENDONUCLEOLYTIC_CLEAVAGE_OF_TRICISTRONIC_RRNA_TRANSCRIPT_SSU_RRNA_5_8S_RRNA_LSU_RRNA	-2.468560878	-2.266931913	-2.64744119
131	GOBP_MATURATION_OF_LSU_RRNA	-2.496555414	-2.150091019	-2.631269223
132	GOCC_SPLICEOSOMAL_TRI_SNRNP_COMPLEX	-2.506047718		-2.591892581
133	GOBP_MATURATION_OF_5_8S_RRNA_FROM_TRICISTRONIC_RRNA_TRANSCRIPT_SSU_RRNA_5_8S_RRNA_LSU_RRNA	-2.520463612		-2.569912832
134	GOCC_U2_TYPE_CATALYTIC_STEP_2_SPLICEOSOME	-2.538801827		-2.495620764
135	GOBP_DNA_UNWINDING_INVOLVED_IN_DNA_REPLICATION	-2.576058269		-2.739950635
136	GOCC_CATALYTIC_STEP_2_SPLICEOSOME	-2.601626427		-2.492507381

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Table A2 – continued from previous page

Pathway Number	GO Term	NES: Two Niche	NES: Endosteal only	NES: Vascular only
137	GOCC_PRERIBOSOME_LARGE_SUBUNIT_PRECURSOR	-2.648264784	-2.335244444	-3.00044392
138	GOBP_CELL_CYCLE_DNA_REPLICATION	-2.651277168		-2.292208658
139	GOCC_PROTEASOME_COMPLEX	-2.698038758		-2.789109086
140	GOCC_U2_TYPE_SPLICEOSOMAL_COMPLEX	-2.711633789		-2.95480154
141	GOCC_PRECATALYTIC_SPLICEOSOME	-2.722583735		-2.623671419
142	GOCC_PROTEASOME_ACCESSORY_COMPLEX	-2.736416194		-2.696337561
143	GOCC_90S_PRERIBOSOME	-2.750055186	-2.292450716	-2.875806273
144	GOBP_MATURATION_OF_SSU_RRNA_FROM_TRICISTRONIC_RRNA_TRANSCRIPT_SSU_RRNA_5_8S_RRNA_LSU_RRNA	-2.752733622	-2.32285209	-2.665398246
145	GOBP_REGULATION_OF_DNA_TEMPLATED_DNA_REPLICATION	-2.763457156		-2.651666886
146	GOBP_RIBOSOME_ASSEMBLY	-2.76865144	-2.328518035	-2.673928664
147	GOBP_MATURATION_OF_5_8S_RRNA	-2.837821434	-2.21499277	-2.785085585
148	GOBP_RIBOSOMAL_SMALL_SUBUNIT_BIOGENESIS	-2.888352799	-2.752003583	-3.279609001
149	GOBP_MITOCHONDRIAL_TRANSLATION	-2.893257664	-2.292333274	
150	GOBP_RNA_5_END_PROCESSING	-2.893783267	-2.330126775	-2.862756391
151	GOCC_SMALL_SUBUNIT_PROCESSOME	-2.907934187	-2.711953887	-2.868109497
152	GOBP_DNA_TEMPLATED_DNA_REPLICATION	-2.936317896		
153	GOBP_DNA_REPLICATION_INITIATION	-2.963756845		-2.686626249
154	GOBP_RIBOSOMAL_LARGE_SUBUNIT_BIOGENESIS	-2.998031431	-2.547782274	-2.848104434
155	GOCC_MITOCHONDRIAL_LARGE_RIBOSOMAL_SUBUNIT	-3.01342931	-2.327130355	-2.622600443
156	GOBP_MATURATION_OF_SSU_RRNA	-3.049286836	-2.495229175	-2.850065897
157	GOCC_ORGANELLAR_RIBOSOME	-3.054796494	-2.575113199	-2.991358106
158	GOCC_PRERIBOSOME	-3.152052718	-2.895620213	-3.521698775
159	GOBP_RRNA_PROCESSING		-2.577259887	
160	GOCC_KERATIN_FILAMENT		-2.312186248	
161	GOBP_RRNA_METABOLIC_PROCESS		-2.288768357	
162	GOCC_SMALL_RIBOSOMAL_SUBUNIT		-2.281050059	
163	GOBP_MITOCHONDRIAL_GENE_EXPRESSION		-2.26346613	
164	GOCC_AXON_INITIAL_SEGMENT		-2.263284894	
165	GOBP_TRNA_METABOLIC_PROCESS		-2.213224562	
166	GOMF_RNA_METHYLTRANSFERASE_ACTIVITY			-2.581427879
167	GOMF_TRNA_BINDING			-2.57634516
168	GOBP_FOREBRAIN_REGIONALIZATION			-2.389202508
169	GOBP_HEPARAN_SULFATE_PROTEOGLYCAN_METABOLIC_PROCESS			-2.387944736
170	GOBP_TRNA_DECAY			-2.38699098
171	GOBP_REGULATION_OF_MITOCHONDRIAL_TRANSLATION			-2.371923721
172	GOBP_REGULATION_OF_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION_TO_CHROMOSOME			-2.340981316
173	GOBP_TRNA_METHYLATION			-2.331935811
174	GOCC_PROTEASOME_CORE_COMPLEX			-2.3193497
175	GOMF_ATP_DEPENDENT_ACTIVITY_ACTING_ON_RNA			-2.317674249
176	GOMF_PSEUDOURIDINE_SYNTHASE_ACTIVITY			-2.2983834
177	GOBP_PROTEIN_LOCALIZATION_TO_NUCLEOPLASM			-2.296443818
178	GOMF_LIGASE_ACTIVITY_FORMING_CARBON_OXYGEN_BONDS			-2.285884845
179	GOMF_TRNA_METHYLTRANSFERASE_ACTIVITY			-2.267056592
180	GOBP_CHAPERONE_MEDIATED_PROTEIN_FOLDING			-2.24409621
181	GOBP_AMINO_ACID_ACTIVATION			-2.241051495
182	GOBP_DE_NOVO_PROTEIN_FOLDING			-2.23879919
183	GOMF_SINGLE_STRANDED_DNA_HELICASE_ACTIVITY			-2.235368307
184	GOMF_NUCLEOSOME_BINDING			-2.235220665
185	GOBP_RIBOSOMAL_SUBUNIT_EXPORT_FROM_NUCLEUS			-2.228587004
186	GOCC_DNA_REPAIR_COMPLEX			-2.223631267
187	GOMF_CATALYTIC_ACTIVITY_ACTING_ON_A_RRNA			-2.205988177
188	GOBP_NUCLEOLAR_LARGE_RRNA_TRANSCRIPTION_BY_RNA_POLYMERASE_I			-2.198443705
189	GOBP_POSITIVE_REGULATION_OF_TRANSCRIPTION_BY_RNA_POLYMERASE_I			-2.192881034
190	GOBP_TRNA_THREONYLCARBAMOYLADENOSINE_METABOLIC_PROCESS			-2.187184673
191	GOBP_SPLICEOSOMAL_TRISNRNP_COMPLEX_ASSEMBLY			-2.183448982
192	GOBP_MITOCHONDRIAL_TRNA_PROCESSING			-2.171131581
193	GOBP_CHAPERONE_MEDIATED_PROTEIN_COMPLEX_ASSEMBLY			-2.167355543
194	GOCC_EUKARYOTIC_48S_PREINITIATION_COMPLEX			-2.156505174
195	GOBP_GLOMERULAR_MESANGIUM_DEVELOPMENT			-2.151027259

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Table A2 – continued from previous page

Pathway Number	GO Term	NES: Two Niche	NES: Endosteal only	NES: Vascular only
196	GOBP_REGULATION_OF_ATTACHMENT_OF_SPINDLE_MICROTUBULES_TO_KINETOCHORE			-2.147713081
197	GOCC_EUKARYOTIC_TRANSLATION_INITIATION_FACTOR_3_COMPLEX			-2.145451927
198	GOBP_REGULATION_OF_TRANSLATIONAL_FIDELITY			-2.141531757
199	GOBP_TRNA_WOBBLE_BASE_MODIFICATION			-2.140494598
200	GOBP_RNA_CAPPING			-2.134779113
201	GOCC_U12_TYPE_SPLICEOSOMAL_COMPLEX			-2.124743594
202	GOBP_MITOTIC_SPINDLE_ASSEMBLY			-2.119657409
203	GOCC_ENDOPEPTIDASE_COMPLEX			-2.112188729
204	GOBP_PROTEASOME_ASSEMBLY			-2.107524092
205	GOBP_POSITIVE_REGULATION_OF_EMBRYONIC_DEVELOPMENT			-2.104782781
206	GOBP_POSITIVE_REGULATION_OF_CELL_CYCLE_G2_M_PHASE_TRANSITION			-2.100192592
207	GOBP_PROSTAGLANDIN_TRANSPORT			-2.099019735
208	GOBP_FORMATION_OF_CYTOPLASMIC_TRANSLATION_INITIATION_COMPLEX			-2.097285168
209	GOBP_POSITIVE_REGULATION_OF_MITOCHONDRIAL_TRANSLATION			-2.091290307
210	GOMF_NUCLEOSOMAL_DNA_BINDING			-2.085896821
211	GOCC_PRESPLICEOSOME			-2.084358959
212	GOBP_DNA_STRAND_RESECTION_INVOLVED_IN_REPLICATION_FORK_PROCESSING			-2.078987419
213	GOBP_REGULATION_OF_MITOCHONDRIAL_GENE_EXPRESSION			-2.076188385
214	GOBP_DNA_STRAND_ELONGATION_INVOLVED_IN_DNA_REPLICATION			-2.070118685
215	GOBP_RIBOSOMAL_LARGE_SUBUNIT_ASSEMBLY			-2.049411157
216	GOBP_REGULATION_OF_SPINDLE_ORGANIZATION			-2.042779836
217	GOBP_PROTEIN_INSERTION_INTO_MITOCHONDRIAL_OUTER_MEMBRANE			-2.035335593
218	GOBP_TELOMERE_MAINTENANCE_VIA_TELOMERE_LENGTHENING			-2.031632393
219	GOMF_TRANSLATION_FACTOR_ACTIVITY_RNA_BINDING			-2.031429982
220	GOCC_PRC1_COMPLEX			-2.018199109
221	GOBP_REGULATION_OF_PROTEIN_EXPORT_FROM_NUCLEUS			-2.00755394
222	GOBP_RRNA_METHYLATION			-2.004125204
223	GOBP_REGULATION_OF_ENDOTHELIAL_CELL_DIFFERENTIATION			-2.003086557

Table A3: List of differentially expressed Gene Ontology (GO) pathways in niche cells co-cultured with PC3 cells under different niche conditions. The table presents differentially expressed pathways in niche cells co-cultured with PC3 cells under three niche conditions: two-niche (TN), endosteal-only (Endo), and vascular-only (Vasc), compared to the respective niche cells cultured without PC3 cells. Only pathways with an adjusted p-value < 0.05 and a Normalized Enrichment Score (NES) < -2 or > 1 are included.

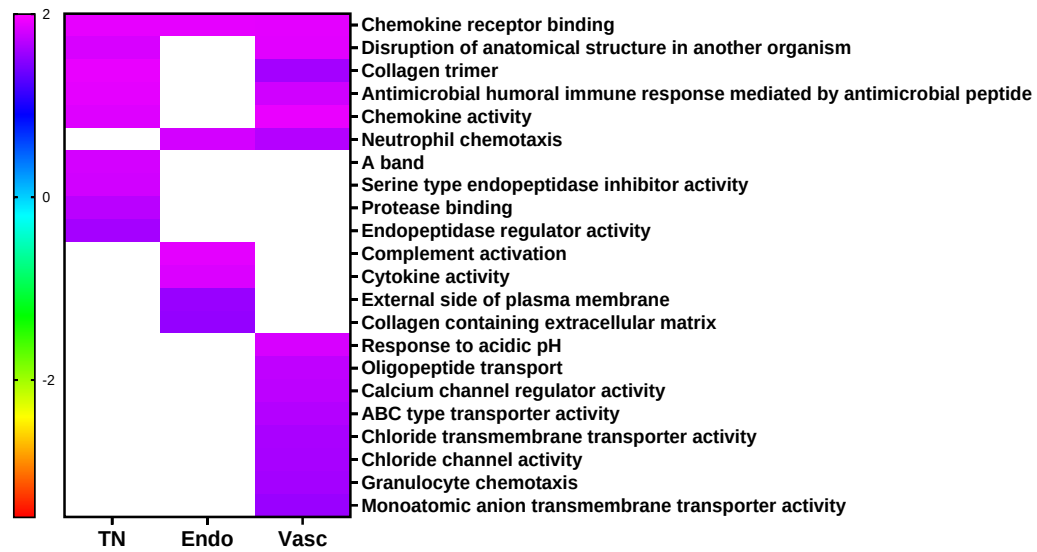
Pathway Number	GO Term	NES: Two Niche	NES: Endosteal Only	NES: Vascular Only
1	GOBP_ENDOCRINE_PROCESS	2.23732544		
2	GOBP_REGULATION_OF_SYSTEMIC_ARTERIAL_BLOOD_PRESSURE_MEDIATED_BY_A_CHEMICAL_SIGNAL	2.18540399		
3	GOBP_NEGATIVE_REGULATION_OF_ENDOTHELIAL_CELL_PROLIFERATION	2.15150826		
4	GOBP_REGULATION_OF_SYSTEMIC_ARTERIAL_BLOOD_PRESSURE_BY_HORMONE	2.15138218		
5	GOMF_IMMUNE_RECEPTOR_ACTIVITY	2.14512613		
6	GOBP_NEGATIVE_REGULATION_OF_BLOOD_VESSEL_ENDOTHELIAL_CELL_MIGRATION	2.12996754		
7	GOBP_CELL_FATE_DETERMINATION	2.10010623		
8	GOBP_REGULATION_OF GRANULOCYTE DIFFERENTIATION	2.09968373		
9	GOBP_GLOMERULUS_DEVELOPMENT	2.09356998		
10	GOBP_NEGATIVE_REGULATION_OF EPITHELIAL CELL MIGRATION	2.0843049		
11	GOBP_REGULATION_OF PHAGOCYTOSIS	2.05555718		
12	GOBP_LYMPH_VESSEL_DEVELOPMENT	2.03356616		
13	GOBP_POSITIVE_REGULATION_OF_MONOATOMIC_ION_TRANSPORT	2.02437982		
14	GOBP_REGULATION_OF PLATELET ACTIVATION	2.02203482		
15	GOBP_POSITIVE_REGULATION_OF CYTOSOLIC CALCIUM_ION CONCENTRATION	2.02075669		
16	GOMF MOLECULAR TRANSDUCER ACTIVITY	2.00802184		
17	GOBP_CGMP_MEDIATED_SIGNALING	2.00767421		

Continued on next page

Table A3 – continued from previous page

Pathway Number	GO Term	NES: Two Niche	NES: Endosteal Only	NES: Vascular Only
18	GOBP_ARTERY_MORPHOGENESIS	2.00225463		
19	GOMF_HORMONE_BINDING	2.00196271		
20	GOBP_ARTERY_DEVELOPMENT	2.0004193		
21	GOCC_CYTOSOLIC_LARGE_RIBOSOMAL_SUBUNIT		2.42964118	
22	GOBP_NEUTROPHIL_CHEMOTAXIS		2.26058058	
23	GOMF_MHC_PROTEIN_COMPLEX_BINDING		2.25870972	
24	GOMF_MHC_CLASS_II_PROTEIN_COMPLEX_BINDING		2.24253106	
25	GOBP_NEUTROPHIL_MIGRATION		2.2416664	
26	GOCC_MHC_CLASS_II_PROTEIN_COMPLEX		2.2117172	
27	GOBP_GRANULOCYTE_CHEMOTAXIS		2.0883062	
28	GOBP_GRANULOCYTE_MIGRATION		2.04407411	
29	GOBP_LEUKOCYTE_CHEMOTAXIS		1.78643593	
30	GOBP_ELECTRON_TRANSPORT_CHAIN	-2.01006908		
31	GOMF_PROTON_TRANSPORTING_ATP_SYNTHASE_ACTIVITY_ROTATIONAL_MECHANISM	-2.05112934		
32	GOBP_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION_TO_MITOCHONDRIAL_MEMBRANE	-2.05739552		
33	GOBP_MITOCHONDRIAL_ELECTRON_TRANSPORT_CYTOCHROME_C_TO_OXYGEN	-2.06035725		
34	GOBP_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY	-2.11804163		
35	GOMF_ELECTRON_TRANSFER_ACTIVITY	-2.31131422		
36	GOCC_PROTON_TRANSPORTING_ATP_SYNTHASE_COMPLEX	-2.32726745		
37	GOCC_CYTOCHROME_COMPLEX	-2.34870928		
38	GOBP_NADH_DEHYDROGENASE_COMPLEX_ASSEMBLY	-2.35608328		
39	GOBP_NUCLEOSIDE_TRIPHOSPHATE_BIOSYNTHETIC_PROCESS	-2.36002456		
40	GOCC_MITOCHONDRIAL_PROTEIN_CONTAINING_COMPLEX	-2.36977208		
41	GOBP_OXIDATIVE_PHOSPHORYLATION	-2.45271238		
42	GOCC_INNER_MITOCHONDRIAL_MEMBRANE_PROTEIN_COMPLEX	-2.47318792		
43	GOMF_OXIDOREDUCTION_DRIVEN_ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	-2.55456381		
44	GOMF_NADH_DEHYDROGENASE_UBIQUINONE_ACTIVITY	-2.56941237		
45	GOBP_ATP_BIOSYNTHETIC_PROCESS	-2.58042077		
46	GOBP_CYTOPLASMIC_TRANSLATION	-2.62204133		
47	GOBP_ATP_SYNTHESIS_COUPLED_ELECTRON_TRANSPORT	-2.62649665		
48	GOCC_NADH_DEHYDROGENASE_COMPLEX	-2.76239307		
49	GOCC_RESPIRASOME	-2.85995455		
50	GOMF_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_NAD_P_H_QUINONE_OR_SIMILAR_COMPOUND_AS_ACCEPTOR	-2.86171516		
51	GOBP_PROTON_MOTIVE_FORCE_DRIVEN_ATP_SYNTHESIS	-3.14556188		
52	GOCC_SMALL_RIBOSOMAL_SUBUNIT	-3.18656728		
53	GOCC_CYTOSOLIC_RIBOSOME	-3.4278352		
54	GOCC_CYTOSOLIC_SMALL_RIBOSOMAL_SUBUNIT	-3.54292761		
55	GOCC_LARGE_RIBOSOMAL_SUBUNIT	-3.5957738		
56	GOCC_CYTOSOLIC_LARGE_RIBOSOMAL_SUBUNIT	-4.01388812		
57	GOCC_RIBOSOMAL_SUBUNIT	-4.16246897		
58	GOMF_STRUCTURAL_CONSTITUENT_OF_RIBOSOME	-4.46976095		
59	GOMF_CIS_REGULATORY_REGION_SEQUENCE_SPECIFIC_DNA_BINDING		-1.53009962	

A PC3 cells - upregulated



B PC3 cells - downregulated

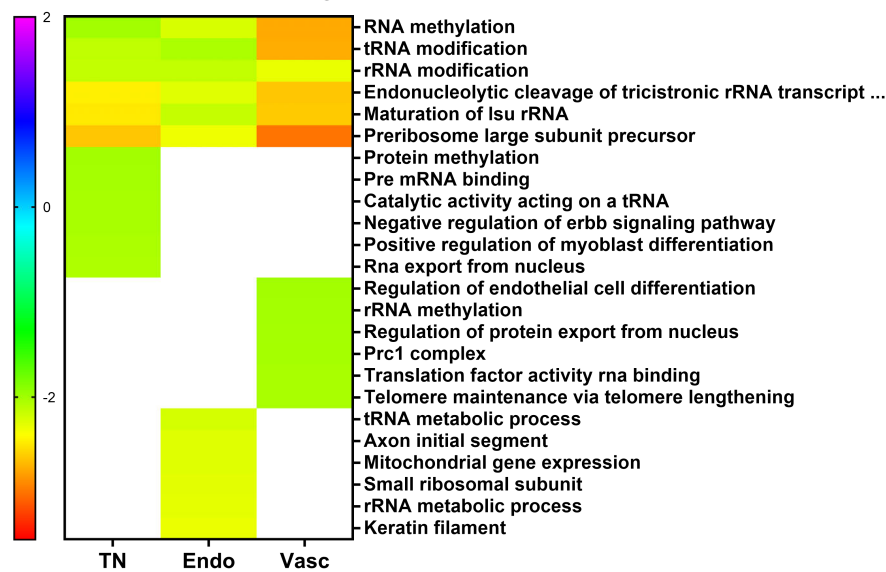
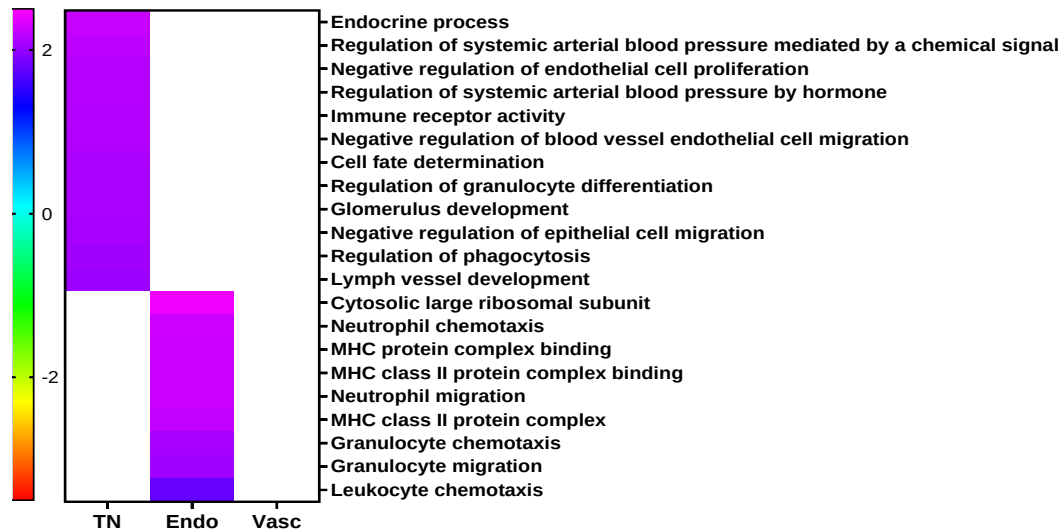


Figure A1: Selected differentially expressed gene pathways in PC3 cells cultured under two-niche and single-niche conditions. Transcriptional differences in PC3 cells and niche cells across distinct niche configurations. Heatmaps illustrate differentially expressed Gene Ontology (GO) pathways in PC3 cells cultured under three niche conditions: two-niche (TN; endosteal + vascular), endosteal-only (Endo), and vascular-only (Vasc), compared to PC3 cells grown independently in Matrigel. Pathways were randomly selected from the filtered list based on p -value < 0.05 and $NES < -2$ or > 1 , including 5–10 differentially expressed pathways shared across at least two or all three niche conditions, as well as 5–10 uniquely enriched pathways in each niche condition (TN, Endo, or Vasc). Only pathways with p -value < 0.05 are shown. (A) Upregulated pathways. (B) Downregulated pathways.

A Niche cells - upregulated



B Niche cells - downregulated

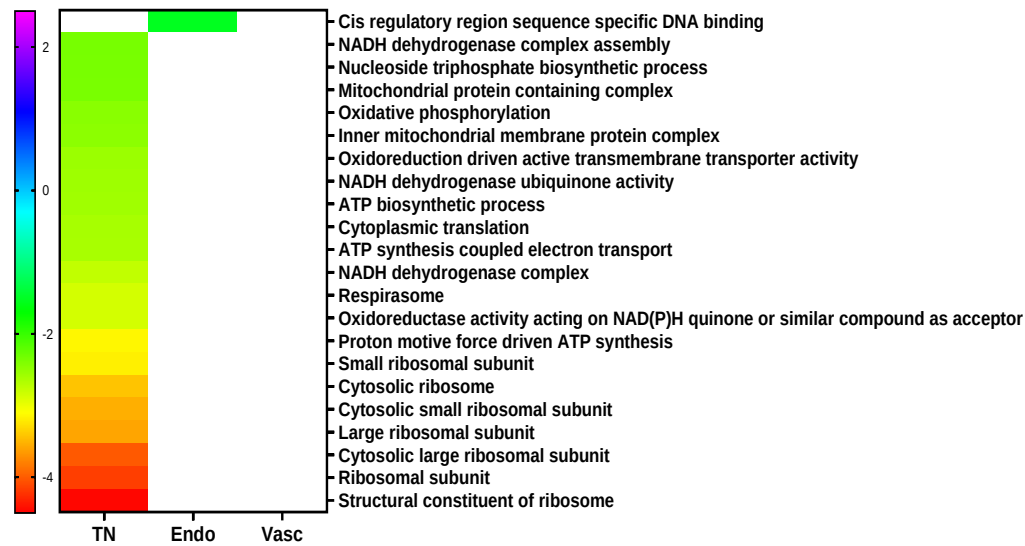


Figure A2: **Selected differentially expressed gene pathways in niche cells cultured with or without PC3 cells.** Heatmaps illustrate differentially expressed Gene Ontology (GO) pathways in niche cells cultured under three conditions: two-niche (TN; endosteal + vascular), endosteal only (Endo), and vascular only (Vasc), in the presence of PC3 cells compared to their respective conditions without PC3 cells. Pathways were randomly selected from the filtered list based on $p\text{-value} < 0.05$ and $NES < -2$ or > 1 , including 5–10 differentially expressed pathways shared across at least two or all three niche conditions, as well as 5–10 uniquely enriched pathways in each niche condition (TN, Endo, or Vasc). Only pathways with $p\text{-value} < 0.05$ are shown. (A) Upregulated pathways. (B) Downregulated pathways.

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