iScience

CellPress
OPEN ACCESS

Review

Feasibility and barriers to rapid establishment of patient-derived primary osteosarcoma cell lines in clinical management

Thomas Chow,[1,2] William Humble,[2,4] Enrico Lucarelli,[3] Carmine Onofrillo,[2,4] Peter F. Choong,[2,4] Claudia Di Bella,[2,4,5] and Serena Duchi[2,4,*]

SUMMARY

Osteosarcoma is a highly aggressive primary bone tumor that has seen little improvement in survival rates in the past three decades. Preclinical studies are conducted on a small pool of commercial cell lines which may not fully reflect the genetic heterogeneity of this complex cancer, potentially hindering translatability of *in vitro* results. Developing a single-site laboratory protocol to rapidly establish patient-derived primary cancer cell lines (PCCL) within a clinically actionable time frame of a few weeks will have significant scientific and clinical ramifications. These PCCL can widen the pool of available cell lines for study while patient-specific data could derive therapeutic correlation. This endeavor is exceedingly challenging considering the proposed time constraints. By proposing key definitions and a clear theoretical framework, this evaluation of osteosarcoma cell line establishment methodology over the past three decades assesses feasibility by identifying barriers and suggesting solutions, thereby facilitating systematic experimentation and optimization.

INTRODUCTION

Osteosarcoma (OS) is the most common primary bone cancer, where the mainstay of therapy is neoadjuvant chemotherapy (NAC), definitive surgery, and adjuvant chemotherapy. It is a highly aggressive tumor with a low 5-year survival rate (<20%) in metastatic disease.[1] The lack of meaningful improvement of 5-year survival rates in the past 30 years is largely due to the rapid onset of drug resistance[2] and clearance[3,4] during chemotherapy, with 34–68% of OS patients remaining poor responders.[2] Identifying mechanisms to overcome drug resistance is laborious and is hampered by the lack of representative cell culture models that accurately recapitulate OS biology.[5]

Commercial OS cell lines have served as readily accessible preclinical tools for studying fundamental biological mechanisms (Table 1).[26] Extensive efforts to phenotypically characterize all publicly available OS cell lines have greatly standardized *in vitro* experiments and data reliability,[27–30] although caution must be taken when using commercial cell lines. OS has a highly heterogeneous presentation with multiple histological subtypes (osteoblastic, chondroblastic, fibroblastic, telangiectatic, etc.)[1] and lack recurrent genetic markers.[31] However, the small pool of commercial OS cell lines (isolated largely pre-1980s and from predominantly Caucasian populations) results in the routine use of only three to four cell lines that may not fully reflect the diverse tumor landscape (Table 1). Genetic alterations may also occur over the cell line's long-term *in vitro* culture, causing a genetic discrepancy from the original cells and ultimately a divergence of characteristics from the original tumor, limiting their *in vitro* to *in vivo* translatability.[33,34]

Patient-derived primary cancer cell lines (PCCL) are derived directly from patient tumor tissue and undergo minimal *in vitro* passaging (subculture), thereby retaining a genetically and clonally heterogeneous cell population better representative of the source tumor and their *in vivo* characteristics compared to commercial cell lines.[35,36] Using PCCL could streamline bench-to-bedside translation of OS research and even facilitate precision oncology applications.[34,37–39] Indeed, there are emerging studies investigating the potential of PCCL to produce patient-specific data that could predict drug sensitivity using next-generation genomic analysis[5,40,41] and *in vitro* drug testing.[39,42] Although tumor organoids and tissue explants have shown promising results in predicting treatment response in lung and colorectal cancer,[43–46] rare tumors like OS with limited tissue samples may constrain the scalability of such organoid-based studies. On the other hand, PCCL can be used in conjunction with novel 3D *in vitro* models that also recapitulate the crucial tumor microenvironment (TME), while retaining greater control over experimental parameters.[47–51] A tunable patient-specific *in vitro* tumor model could strengthen the study of OS pathogenesis

[1]Melbourne Medical School, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville, VIC, Australia
[2]BioFab3D-ACMD, St Vincent's Hospital Melbourne, Fitzroy, VIC, Australia
[3]Osteoncology, Bone and Soft Tissue Sarcomas and Innovative Therapies Unit, IRCCS Istituto Ortopedico Rizzoli, Via di Barbiano 1/10, 40136 Bologna, Italy
[4]Department of Surgery, The University of Melbourne, St Vincent's Hospital Melbourne, Fitzroy, VIC, Australia
[5]Department of Orthopaedics, St Vincent's Hospital Melbourne, Fitzroy, VIC, Australia
*Correspondence: sduchi@unimelb.edu.au
https://doi.org/10.1016/j.isci.2024.110251

Table 1. Summary of publicly available human OS cell lines

	Cell line (commercial code)	Company/cell bank	PD (hrs)	Subtype	Age	Sex	Ethnicity
1	MG-63 (CRL-1427)	ATCC	48	Fibroblastic	14	M	Caucasian
2	COS-1SA-1 (formerly OsA-CL) (CRL-2098)	ATCC	24	Fibroblastic	19	M	Black
3	Saos-2 (HTB-85)	ATCC	48	Epithelial	11	F	Caucasian
4	U-2 OS (HTB-96)	ATCC	36	Epithelial	15	F	Caucasian
5	G-292 (clone A141B1) (CRL-1423)	ATCC	NA	Fibroblastic	9	F	Caucasian
6	HOS (CRL-1543)	ATCC	36	Mixed fibroblastic and epithelial	13	F	Caucasian
7	HOS-MNNG (CRL-1547, derived from HOS)	ATCC	24	Mixed fibroblastic and epithelial	13	F	Caucasian
8	HOS-143B (CRL-8303, derived from HOS)	ATCC	36	Mixed fibroblastic and epithelial	13	F	Caucasian
9	KHOS/NP (R-970-5, derived from HOS) (CRL-1544)	ATCC	NA	Fibroblastic	13	F	Caucasian
10	HAL	EuroBoNet Consortium (Norwegian Radium Hospital, Norway)	48	NA	16	M	NA
11	KPD	EuroBoNet Consortium (Norwegian Radium Hospital, Norway)	36	Osteoblastic	7	F	NA
12	MHM	EuroBoNet Consortium (Norwegian Radium Hospital, Norway)	60	Fibroblastic	41	F	NA
13	OHS	EuroBoNet Consortium (Norwegian Radium Hospital, Norway)	36	Osteoblastic	14	M	NA
14	IOR/MOS	EuroBoNet Consortium (Instituto Ortopedico Rizzoli, Italy)	72	Osteoblastic	13	F	Caucasian
15	IOR/OS9	EuroBoNet Consortium (Instituto Ortopedico Rizzoli, Italy)	72	Osteoblastic	15	M	Caucasian
16	IOR/OS10	EuroBoNet Consortium (Instituto Ortopedico Rizzoli, Italy)	72	Fibroblastic	10	F	Caucasian
17	IOR/OS14	EuroBoNet Consortium (Instituto Ortopedico Rizzoli, Italy)	48	Osteoblastic	13	M	Caucasian
18	IOR/OS15	EuroBoNet Consortium (Instituto Ortopedico Rizzoli, Italy)	48	Osteoblastic	12	F	Caucasian
19	IOR/OS18	EuroBoNet Consortium (Instituto Ortopedico Rizzoli, Italy)	60	Osteoblastic	33	M	Caucasian
20	IOR/SARG	EuroBoNet Consortium (Instituto Ortopedico Rizzoli, Italy)	72	NA	25	M	Caucasian
21	ZK-58	EuroBoNet Consortium (Heinrich-Heine University, Germany)	72	Osteoblastic	21	M	NA
22	CAL-72	EuroBoNet Consortium (University College London)	66	Osteoblastic	15	M	NA
23	11T254	EuroBoNet Consortium (Nice University Hospital, France)	NA	NA	NA	NA	NA
24	NOS-1 (RCB1032)	RIKEN-BRC	49.6	Osteoblastic	16	M	Japanese
25	NOS-2 (RCB1033)	RIKEN-BRC	NA	Osteoblastic	11	M	Japanese
26	NOS-10 (RCB2348)	RIKEN-BRC	NA	Epithelial	15	M	Japanese
27	HuO9 (JCRB0427)	JCRB	57	Fibroblastic	13	F	Japanese

(*Continued on next page*)

Table 1. *Continued*

	Cell line (commercial code)	Company/cell bank	PD (hrs)	Subtype	Age	Sex	Ethnicity
28	HuO9N2 (JCRB0428)	JCRB	120	NA	13	F	Japanese
29	HuO-31N (JCRB0413)	JCRB	NA	Osteoblastic	15	F	Japanese
30	NY (JCRB0614)	JCRB	30	Osteoblastic	15	M	Japanese

The table shows the nine cell lines available from commercial company ATCC, and the 21 cell lines available on request from cell banks or universities. Population Doubling, subtype, Age, Sex and Ethnicity are reported. Some cell lines were incompletely characterized. Adapted from these studies[27,28,30,32] with additional information from the respective cell bank websites. Abbreviations: American Type Culture Collection (ATCC), RIKEN Bioresource Center (RIKEN-BRC), Japanese Cancer Research Resources Bank (JCRB), population doubling time (PD), not specified (NA).

and facilitate the high-throughput screening of novel therapeutics to assess individual drug sensitivities.[52–55] Such models are enhanced by the timely availability of patient-specific cell lines.

Although protocols to isolate PCCL from solid tumors are available,[56,57] they are not devised with the constraints of clinical management in mind and remain a traditionally lengthy process requiring up to a year.[35] If PCCL are to be used with the intention of informing therapeutic decisions, they must be established within a clinically actionable time frame that is far shorter than the time frame traditionally allocated for research study. Streamlining and substantially contracting the time needed to establish new PCCL simultaneously addresses the paucity of available OS data by providing a source of tumor cells for subsequent studies beside clinical management. A single-site (hospital) laboratory-based protocol to rapidly establish new OS PCCL with clinical constraints in mind is a worthwhile avenue of investigation for its clinical and scientific utility. As such an aim has not been proposed before, so we need to define important parameters.

First, to define a "clinically actionable time frame", we need to briefly look at the OS management timeline. As shown in Figure 1, all patients undergo a bone biopsy for definitive diagnosis and histological subtyping. Two cycles of NAC (total of 10 weeks) are commenced (typically within two weeks of biopsy) and completed prior to surgical resection, followed by four cycles of adjuvant chemotherapy.[58] As every NAC cycle can select for resistant clones, obtaining PCCL and generating data before starting chemotherapy would be ideal. However, this would necessitate establishing and testing PCCL in the two weeks between bone biopsy (where tumor samples are obtained for processing) and the *start* of cycle 1; an extremely short time frame. Factoring the limitations of current experimental technique, a more realistic "clinically actionable time frame" would be between bone biopsy and the *end* of NAC cycle 1. This gives a more feasible period of six weeks for PCCL to be established and subsequently generate patient-specific data before the commencement of NAC cycle 2. This time frame is not unrealistic as Chew et al.[57] has established pediatric cancer cell lines from rare childhood tumors between 1 and 8 weeks from obtaining surgical specimens.

Second, the PCCL is "established" when it has been authenticated by verifying that its identity corresponds with the original tumor cell, maintains its morphology and therefore ready to be reliably used for experiments.

Third, the "established" PCCL must continue being cultured until a "sufficient number" of cells is generated for statistically significant testing. For example, chemosensitivity assays are commonly conducted with 10^4 cells/cm^2 in 24 well plates, and a triplicate assay of six concentrations of five different treatments would require roughly 10^6 cells. However, culturing PCCL within 3D *in vitro* models generally require a higher cell seeding density of 10^4–10^7 cells/cm^3.[59] Performing the same chemosensitivity assay with a concentration of 10^7 cells/cm^3 in an average 3D model volume of 30 mm^3[60,61] (a size that allows cultivation of the 3D model in a 96-well plate with 200ul of cell culture media) would roughly equate to 3x10^5 cells per single unit. If we were to encompass all 96 wells, multiply two time points per week for 4–5 weeks using six different concentrations would require a total of 10^9 cells.

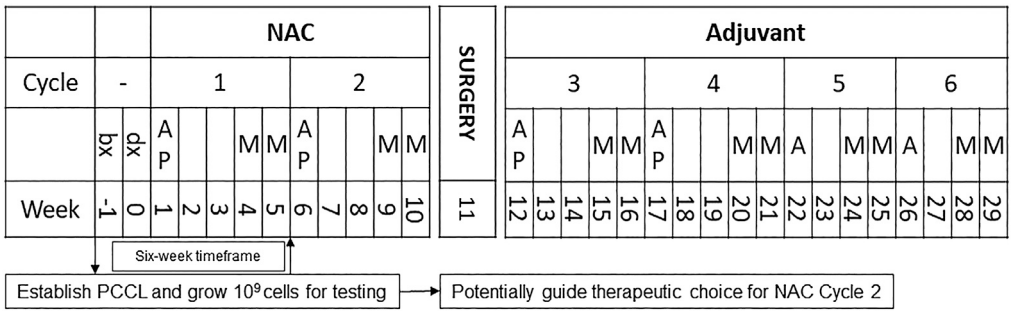

Figure 1. Timeline of OS management with potential six-week time frame for PCCL establishment

Standard multi-agent MAP chemotherapy regimen at our institution. All patients undergo a bone biopsy for histological subtyping as part of the diagnostic workup. Two cycles of NAC are given for a total of 10 weeks. Surgery is performed to remove the primary tumor. Four cycles of adjuvant chemotherapy commence postoperatively. There is a potential window of six-weeks between bone biopsy and the end of cycle 1, for PCCL to be established and tested. Adapted from Choong (2021).[58] Abbreviations: Osteosarcoma (OS), primary cancer cell line (PCCL), neoadjuvant chemotherapy (NAC), high-dose MAP (methotrexate, doxorubicin, cisplatin), bx (biopsy), dx (diagnosis).

Our aim becomes more precise with these definitions: devise a clinically based laboratory protocol that can rapidly establish and grow at least 10^9 PCCL within 6 weeks since obtaining the bone biopsy. This systematic review assesses the feasibility of our aim. We first compile all contemporary papers establishing OS cell lines in the past three decades (Figure 2, Table 2), then extract specific steps in their methodology across five domains under our unique clinical considerations (Table 3) to facilitate comparison (Tables 4, 5, and 6). The raw data is then condensed and synthesized (Table 7). In our discussion, we highlight barriers to feasibility in each domain, and thereafter suggest potential solutions to address them (Figure 3). Ultimately, this study analyzes the feasibility, barriers, and potential solutions for the rapid establishment of OS PCCL within clinical management, thereby providing a useful guide to accelerate experimental optimization.

Methods

Search strategy

An initial search in electronic databases MEDLINE and EMBASE were searched for English language articles until June 15, 2022. A second search was subsequently conducted limiting publication year 2022 - 21 January 2024 to capture any new articles published during this

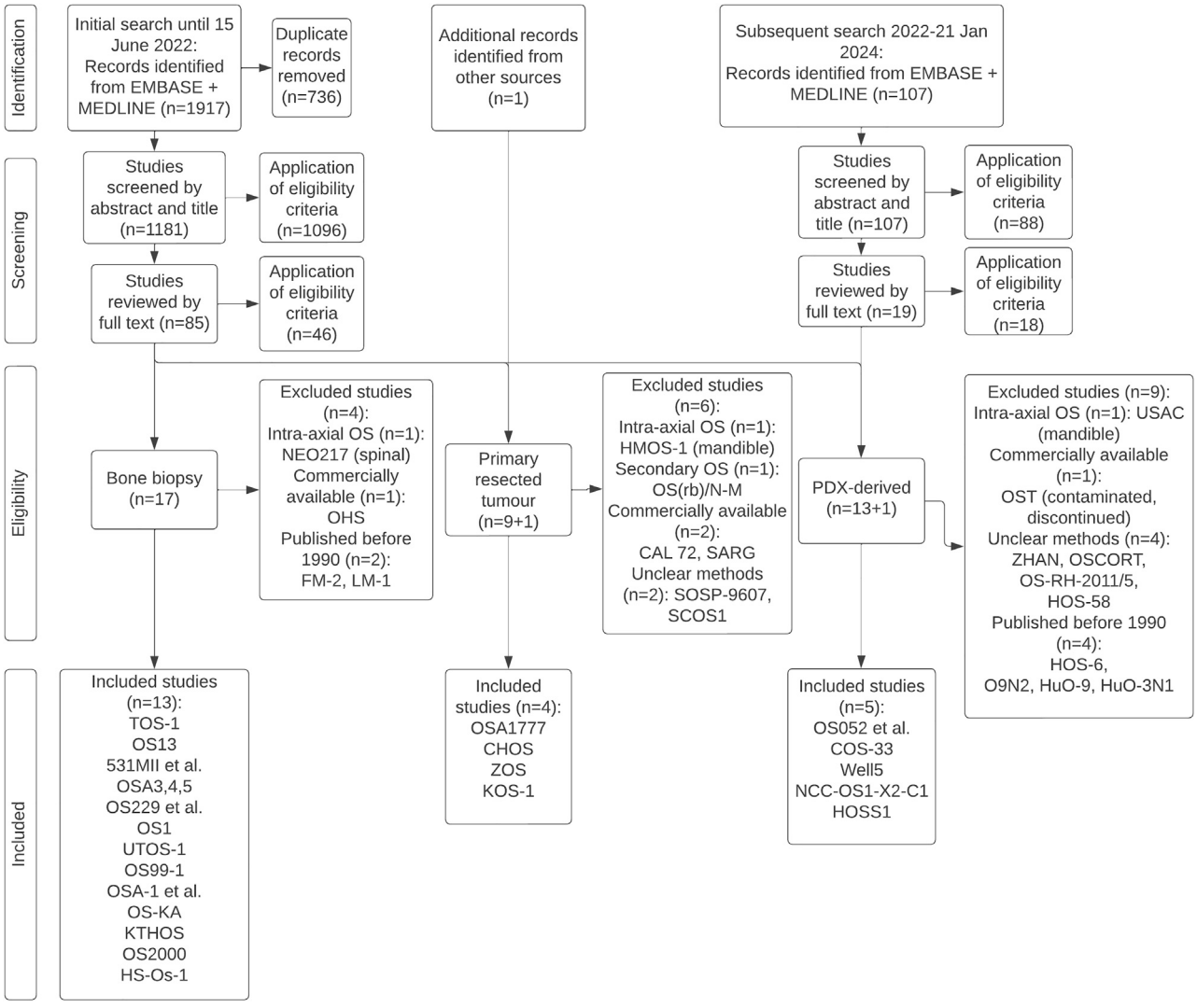

Figure 2. PRISMA Diagram outlining search strategy and eligible studies

This diagram presents the flow of information through the systematic review process, following the PRISMA guidelines. The diagram outlines the number of records identified through database searching, additional records identified through other sources, records screened, records excluded, full-text articles assessed for eligibility, and the final number of articles included in the systematic review. Arrows indicate the flow of articles at each stage, with reasons for exclusion provided where applicable. The PRISMA flow diagram provides transparency and clarity regarding the selection process for inclusion of studies in the systematic review. Abbreviations: osteosarcoma (OS), patient-derived xenograft (PDX).

Table 2. Excluded studies

Biopsy	Primary resected tumour	PDX
Excluded studies (n=4): **Intra-axial OS (n=1):** NEO217 (spinal)[6] **Commercially available (n=1):** OHS[7] **Published before 1990 (n=2):** FM-2[8] LM-1[9]	Excluded studies (n=6): **Intra-axial OS (n=1):** HMOS-1 (mandible)[10] **Secondary OS (n=1):** OS(rb)/N-M[11] **Commercially available (n=2):** CAL 72[12], SARG[13] **Unclear methods (n=2):** SOSP-9607,[14] SCOS1[15]	Excluded studies (n=9): **Intra-axial OS (n=1):** USAC (mandible)[16] **Commercially available (n=1):** OST (contaminated, discontinued)[17] **Unclear methods (n=4):** ZHAN,[18] OSCORT,[19] OS-RH-2011/5,[20] HOS-58[21] **Published before 1990 (n=4):** HOS-6,[22] O9N2,[23] HuO-9,[24] HuO-3N1[25]

		Initial search until June 15 2022	2022-21 Jan 2024
1	osteosarcoma.mp. or Osteosarcoma/	87025	95214
2	cell line.mp. or Cell Line/	1819249	1991465
3	tumor cell line.mp. or Cell Line, Tumor/	409271	435894
4	2 or 3	1819249	1991465
5	1 and 4	18182	20081
6	(establish* and "cell line").tw.	74125	79183
7	(character* and "cell line").tw.	114895	123669
8	6 or 7	168002	180428
9	5 and 8	1917	2053
10	remove duplicates from 9	1181	1271
11	limit 10 to yr = "2022 – current"	NA	107

time. The search strategy was performed with keywords such as "establish", "osteosarcoma", "human", "cell line", and conducted in Embase Classic + Embase <1947 to 2022 June 15> and Ovid MEDLINE(R) ALL <1946 to June 15, 2022> as detailed below. A general search using the same keywords in Google was also conducted to capture studies not in the database.

Article eligibility and study selection

Inclusion criteria

- English language
- Full-text articles
- Human OS only
- High-grade OS only, including all histological subtypes

Exclusion criteria

- Secondary OS [a]
- Intra-axial OS [b]
- Established from metastatic site [c]
- Obtainable from public cell banks or commercial companies [d]
- Established from existing commercial cell lines by transfecting/genetic engineering [e]
- Studies that did not outline isolation protocol/methodology in reproducible steps [f]
- Studies before year 1990 [g]

[a] Radiation-induced secondary OS was excluded to focus on primary tumor.

[b] OS commonly develops in the femur (42%), tibia (19%), humerus (10%), with the remainder in the intra-axial skeleton (pelvis, spine, mandible).[3] Intra-axial locations which do not permit oncologic resection with a curative intent was excluded as these are biased toward failure.

[c] Exclude metastasis to focus on primary tumor site.

[d] Previously 'novel' cell lines that are now commercially available from companies or cell banks were excluded

[e] Secondary cell lines were excluded to focus on primary cell lines

[f] Studies deemed to have 'unclear methods' upon data extraction could not fill at least two 'Entry' of our extraction table (see Table 3).

[g] Methods to establish cell lines have remained largely similar in the past few decades. To focus on contemporary literature, papers before 1990 were rejected. Additionally, before this time it is difficult (if not impossible) to clarify the validity of the methods; we want to identify emerging cell culture technologies might accelerate the isolation of new cell lines.

Data extraction strategy

We evaluate each study protocol in its component steps (Table 3). The Study Findings' section is organized into five 'Entry'; key steps in cell line establishment adapted from these studies.[62,63,86] The 'Principles' outline important considerations informed by our specific clinical aim and recommendations from international guidelines for cell line establishment.[87–89] The latter finally informs 'Data extraction'; the discrete information that is extracted to facilitate systematic comparison of their methodology.

RESULTS

Search results

The records identified from the database search were assessed according to the eligibility criteria, as outlined in the PRISMA diagram (Figure 2). References were included for excluded papers (Table 2). The initial search until 15 June 2022 yielded 1917 articles, with 1181 articles remaining after duplicates were removed via computational software. The title and abstract were screened, and a further 1096 articles were excluded. Of the 85 articles sought for retrieval and full text review, 20 were included in this initial search. Due to the length of the initial analysis, a subsequent search was conducted for additional articles published between 2022 and 21 Jan 2024. This subsequent search yielded 107 articles. The title and abstract were screened, with 19 articles for full text review, with one paper being eligible. In addition, a general scoping search in Google yielded one paper (we also confirmed that this paper was not in the MEDLINE or EMBASE database).

Altogether, these 22 studies were stratified according to their 'source of tissue' (Entry 2, Table 3): biopsy (n = 13), primary resected tumor (n = 4), patient-derived xenograft (PDX) (n = 5), and represented in Tables 4, 5, and 6 respectively. Discrete information was extracted from the studies as per the 'Data extraction' column in Table 3, and if not reported is stated as 'not specified'. The raw data in Tables 4, 5, and 6 was then synthesized in Table 7 to give readers an overview of the results.

Table 3. Strategy for systematic data extraction of contemporary OS cell line establishment methods

Entry	Principles	Data extraction
1. Clinical data	• What is the essential information of patient, tumor, clinical data for therapeutic correlation	• Patient characteristics (age, gender) • Bone of origin • Histological subtype
2. Source of tissue	• Obtaining tissue fits within existing clinical framework, does not delay care • Sample isolated from patient with minimal harm (potential clinical benefit outweighs risk) • Which source of tumor cells are most sterile	• Source of tumor cells • Transport conditions from operating theater to lab area
3. Disaggregation	• What is the most time-efficient method to create a single cell suspension for primary culture	• Mechanical • Enzymatic
4. Expansion	• What are specific cell culture conditions used • How many subcultures and passaging • Grow 10^9 cells within six weeks	• Culture media (type, concentration) • Incubation conditions • Frequency of media replacement • Total number of passages • Overall time taken to establish cell line
5. Authentication & Characterization	• What are the minimum tests to confirm the identity and novelty of PCCL • What *in vitro* tests are relevant for therapeutic correlation • How to ensure PCCL not contaminated with other cell types/bacteria	• Authentication assays assessing genetic uniqueness[a] and cell of origin[b] • Characterization assays studying general features of cell line[c] • Assays assessing purity[d]

'Entry' are the five key steps of cell line establishment as adapted from Richter (2021) and Langdon (2004).[62,63] 'Principles' are informed by our clinical aim and constraints. 'Data extraction' is the discrete information that will be extracted from eligible studies. Abbreviations: neoadjuvant chemotherapy (NAC), patient-derived primary cancer cell line (PCCL).

As suggested in[62,63]: These assays generally comprise of [a] DNA fingerprinting, genotype profiling, karyotype analysis; [b] PCR, cell-specific protein expression, differentiation; [c] Morphology, growth/cell cycle, invasion/mobility, tumorigenicity; [d] Mycoplasma, fibroblasts. We will categorize the assays used according to this adapted criteria.

Table 4. Studies that established PCCL from bone biopsy

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
Palmini et al.[64]	TOS-1	1. Not specified 2. Not specified 3. Telangiectatic	1. Needle aspiration 2. Sample placed in culture medium supplemented by 100IU/mL penicillin and 100 µg/mL streptomycin, pH 7.4 for transport to laboratory.	1. Mechanical dispersion 2. Enzymatic treatment in Ham's F12 Coon's modification medium with collagenase type II at 37°C.	1. Monolayer culture in GM Ham's F12 Coon's modification medium, supplemented with 10% FBS 2. 5% CO2 in air at 37°C 3. Media replaced every three days 4. Cells were harvested using Trypsin-EDTA, when 90% confluence was reached 5. One month	1. a) Not specified 1. b) RT-PCR >phenotype marker (SATB2+) >metastasis and migration markers (EZR-, AXL-) >cancer stem cell marker (PROM1+, MYC-) 2. Invasive capacity >soft agar growth assay 3. Not specified
Mizushima et al.[65]	OS13	1. 15, F 2. Distal femur 3. Not specified	1. Biopsy specimen 2. Not specified	1. Tissue was minced 2. Not specified	1. IMDM, containing 10% FBS. 2. 5% CO2 incubator 3. Not specified 4. Not specified 5. One year	1. a) Karyotype analysis >multiple numeral and structural chromosomal aberrations 1. b) RT-PCR >sarcoma-initiating gene (LIN28B+) 2. Chemosensitivity assay >Adriamycin showed anti-tumour effects Spheroid formation assay >Clonal sphere formation after limiting dilution: 100 colonies (/500cells) formed Metabolic analysis >oxygen consumption rate, extracellular acidification rate >dependent on glycolysis, not on mitochondrial oxidative phosphorylation Tumourigenicity >1x10^3 cells injection into immunodeficient mice, spontaneous tumor formation at 7 weeks 3. Not specified

(*Continued on next page*)

Table 4. *Continued*

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
Martínez-Vélez et al.[66]	531MII678R 588M 598M	1. Each obtained from a different patient. Individual characteristics not specified 2. Not specified 3. Not specified	1. Needle biopsy of 0.5mm diameter 2. Not specified	1. Sample cleaned of soft tissue under a dissecting microscope. Bone debris was separated using a 70μm nylon mesh 2. Remaining bone chips were washed with PBS and treated with 250units/mL collagenase and DNase in α-MEM for 2h. Cells were precipitated by centrifugation, washed several times to remove excess collagenase and DNase	1. α-MEM containing 10% heat-inactivated FBS, supplemented with 100units/mL penicillin and 100 μg/mL streptomycin 2. Not specified 3. Not specified 4. Passaged between 15 and 22 times, when cells were 90% confluent 5. Not specified	1. a) Genotype profile >TP53-/RB1- >variable heterozygous loss among samples 1. b) Transcriptomic profile >EBF2+, OPG+ 2. Proliferation and apoptosis assay >MTT assay 3. Not specified
Palmini et al.[67]	OSA3	1. Not specified 2. Not specified 3. Small cell	1. Needle aspiration 2. Sample placed in a culture medium supplemented by 100 IU/mL penicillin and 100 μg/mL streptomycin, pH 7.4, transported to the laboratory.	1. Mechanical dispersion, following enzymatic digestion. 2. Enzymatic treatment in Ham's F12 Coon's modification medium with collagenase type II at 37∘C.	1. Monolayer culture in Ham's F12 Coon's modification medium, supplemented with 10% FBS 2. 5% CO2 in air at 37∘C 3. Media replaced every three days 4. Cells were harvested using Trypsin-EDTA when 80–90% confluence was reached 5. Two months	1. a) Not specified 1. b) RT-PCR >phenotype (SATB2+, EWSR1+) >migration/metastasis (EZR-, AXL+) >ESC markers (Nanog+, Sox2-, KLF4+, LIN28A-, POU5F1+) >stem cell markers (PROM1-, ALDH1A1+, $CD34^{-}$) >pluripotency (MYC-) 2. Invasive capacity >Soft agar assay: clonogenic efficiency 10% 3. Not specified
Hassan et al.[68]	OS229 OS232 OS231 OS238 OS242 OS252	1. Each obtained from a different patient. Individual characteristics not specified	1. 25mg of fresh tumor was obtained 2. Not specified	1. Sample finely minced using a sterile scalpel 2. Minced tissue was incubated in 5mL of media composed of	1. Resulting cell pellet was resuspended in 20mL of cell culture media (MEM-alpha media+20% FCS+1% pen-strep) and	1. a) Not specified 1. b) Surface antigen analysis >Surface receptor expression: IGF-2R high expression

(*Continued on next page*)

Table 4. *Continued*

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
	OS290 OS293 OS308 OS311	2. Not specified 3. Not specified		MEM-alpha, 20% FCS, 0.6% collagenase Type II, and 0.002% DNAseI, for 2h. The slurry was then passed through a 70μm cell strainer. The filtered solution was centrifuged at 200×g.	subsequently plated in a Corning T75 flask. Cultured in monolayer in MEM-alpha supplemented with 10% FCS, 100U/mL penicillin, and 3 mg/mL streptomycin 2. 5% CO2 in air at 37°C 3. Not specified 4. Passaged when 80% confluence was reached 5. Not specified	>PDGFR-b, IR, IGF-1R, HER-2, c-Met, VEGFR-3 medium expression >EGFR, FGFR-2, HER-3, HER-4, VEGFR-1, VEGFR-2, FGFR-3 low expression 2. Not specified 3. Fibroblast contamination >GD2 antibody labeling to verify population consisted of OS and not fibroblasts >more than 85% were OS cells
Pereira et al.,[69] Nathan et al.[70]	OS1	1. 6, F 2. Right femur 3. Not specified	1. Bone biopsy 2. Not specified	1. Samples rinsed in PBS, minced into small fragments 2. Dispersed in PBS containing 0.25% trypsin	1. Monolayer culture in mixed culture medium containing (9:1,v/v) of RPMI 1640 and DMEM, supplemented with 15% FBS in plastic culture flasks 2. 5% CO2 in air at 37°C 3. Not specified 4. Uniform colonies were morphologically selected by removing contaminating fibroblast-like and highly proliferative cells with a TPP Cell Scraper under an Olympus IX70 inverse microscope. Total of 76 passages performed 5. 21 months	1. a) Karyotype analysis >consistent with high-grade OS >trisomy in chromosomes 5, 7, 10 >translocations and structural deletions 1. b) RT-PCR >osteogenic markers (Runx2 elevated) >p53-/RB+ Immunohistochemistry >Osteoblast-specific markers (Osteocalcin+, Collagen type I+, osteonectin+, BMP4-) Differentiation assay >Alizarin red stain: demonstrated mineralization capacity at week 2–3 Surface antigen analysis >Flow cytometry: mesenchymal stem cell surface antigens ($CD29^+$, $CD44^+$, $CD71^+$, CD105+, $CD63^-$)

(*Continued on next page*)

Table 4. *Continued*

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
						2. Morphology >phenotype maintained after long-term culture (30 passages) >cells eventually elongated to become wider and spindled-shaped. Appeared thinner as they began to migrate. Doubling time >5 days Tumorigenicity >Xenotransplantation in SCID mice, spontaneous tumor formation at 14 weeks 3. Not specified
Yasuda et al.[71]	UTOS-1	1. 18, M 2. Proximal left humerus 3. Osteoblastic	1. Open biopsy 2. Not specified	1. Not specified 2. Not specified	1. Cultured in RPMI 1640, supplemented with 100 mg/mL streptomycin, 100U/mL penicillin and 10% FBS, in a 25cm^2 plastic flask 2. 5% CO2 in air at 37°C 3. Media replaced once per week. 4. Harvested with Ca2+ and Mg2+-free PBS containing 0.1% trypsin and 0.02% EDTA, and seeded in new flasks for passaging 5. Not specified	1. a) Not specified 1. b) RT-PCR >osteoblastic differentiation markers (ALP+/OC+/OP+) Immunohistochemistry >Osteoblastic differentiation markers (OP+/OC+/ALP+) >Epithelial-mesenchymal transition markers (vimentin+) 2. Morphology >spindle-shaped with atypical nuclei >equalized after 6 passages, maintained over 50 passages >corresponds with histologic appearance of original tumor Doubling time >40h

(*Continued on next page*)

Table 4. *Continued*

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
						Tumorigenicity >1x10^8 cells injected into SCID mice, spontaneous tumor formation at 8 weeks (14000mm^3) >Corresponds histopathologically with original tumor: atypical spindle-shaped cells, formation of osteoid 3. Not specified
Gillette[72]	OS99-1	1. 11, F 2. Distal femur. 3. Osteoblastic	1. Diagnostic biopsy 2. Not specified	1. Minced under aseptic conditions 2. Tumor mince treated with 2U/mL Dispase in PBS for 1h at 37°C	1. Cells were resuspended by pipetting and plated in RPMI 1640 containing 10% FBS, nonessential amino acids, minimum essential medium (MEM) vitamins, sodium bicarbonate, sodium pyruvate, and penicillin/streptomycin. 2. 5% CO2 in air at 37°C 3. Not specified 4. Cells were subcultured at 80% confluence. >60 passages performed 5. 6 months.	1. a) Karyotype analysis >highly rearranged karyotype (hypotriploid and hypohexaploid) 1. b) qRT-PCR >Osteoblastic markers (ALP+, RUNX2+, OC+, osteonectin+) >Chondrocyte markers as negative control (aggrecan-, LINK-) >p53-, Rb- Differentiation assay >Mineralization induction after adding ascorbic acid and phosphate to growth medium >ALP and OC activity increased >consistent with osteoblastic lineage 2. Morphology >grow in clusters, numerous microvilli on cell surface Doubling time >16h Tumorigencity >3x10^5 cells inoculated on chorioallantoic membrane of 10days old chicken embryo >visible tumors at 7days incubation and could be used to establish cells in culture 3. Mycoplasma contamination >LookOut Mycoplasma PCR detection kit: absent

(*Continued on next page*)

Table 4. *Continued*

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
Veselska et al.[73]	OSA-1, OSA-2, OSA-3, OSA-5	1. 8F, 14F, 56F, 18F respectively 2. Not specified 3. OSA-1, OSA-2, OSA-3: Osteoblastic OSA-5: telangiectatic	1. Fresh tumor specimens obtained, and briefly washed in 70% ethanol, followed by two washing in PBS. 2. Not specified	1. Mechanically chopped into pieces 2mm in diameter. Samples were washed three more times in PBS, followed by centrifugation. 2. Not specified	1. Cells were seeded in 1mL of complete medium - containing DMEM supplemented with 20% FCS, 2mM glutamine, and antibiotics: 100 IU/mL of penicillin and 100 µg/mL of streptomycin, in 25cm^2 cell culture flasks 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2. 3. Volume of the medium was gradually increased to 5mL over the next 48 h. 4. Harvested using trypsin at 60% confluence, subcultured into a new flask 5. Not specified	1. a) Not specified 1. b) Immunohistochemistry >vimentin+, desmin+, S100+ >CSC marker (nestin+ except for OSA-5/CD133+) 2. Not specified 3. Not specified
Warzecha et al.[74]	OS-KA	1. 6, F. 2. Not specified 3. Not specified	1. Biopsy sample 2. Not specified	1. Not specified 2. Sample enzymatically dispersed with collagenase/dispase overnight	1. Monolayer culture in high glucose DMEM with 10% FCS and antibiotics in 75 cm^2 flasks. 2. Not specified 3. Not specified 4. Not specified 5. Not specified	1. a) Not specified 1. b) Not specified 2. Chemosensitivity assay >growth inhibition up to 90% when incubated with cyclopamine >growth inhibition up to 50% when treated with tomatidine 3. Not specified
Hitora et al.[75]	KTHOS	1. 16, F 2. Distal femur	1. Open biopsy	1. Tumor tissue minced into small fragments.	1. Monolayer culture in EMEM supplemented	1. a) Not specified 1. b) Immunohistochemistry

(*Continued on next page*)

Table 4. *Continued*

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
		3. Osteoblastic	2. Samples rinsed with PBS containing penicillin G (100U/mL) and streptomycin (100 mg/mL	2. Fragments dispersed into a single-cell suspension in PBS containing 0.1% trypsin and 0.02% EDTA	with 10% FBS, penicillin G (100U/mL), streptomycin (100 mg/mL), an L-glutamine (2 mmol/L), in a plastic culture bottle. 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2. 3. Not specified 4. Harvest using 0.1% trypsin solution when confluent. Cells were subcultured at a 1:3 or a 1:4 dilution. 106 passages performed 5. 27 months	>osteoblastic (OC+/osteonectin+) >SCF+/KIT+ 2. Morphology >spindle to pleomorphic cytoplasm >round to ovoid nuclei containing multiple prominent nucleoli >consistent with original histological features of tumor Doubling time >35.6h Tumorigenicity >5x10^6 cells subcutaneously inoculated into 6-week-old nude mice >spontaneous tumor formation at 6 weeks (3-4cm diameter) >histological features similar to original tumor 3. Not specified
Tsukahara et al.,[76] Nabeta et al.[77]	OS2000	1. 16, F 2. Left femur 3. Fibroblastic	1. Biopsy sample 2. Not specified	1. Minced into small pieces (2mm diameter) 2. Not specified	1. Monolayer culture in DMEM containing 15% FCS 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2 3. Not specified 4. Harvested using 0.25% trypsin at confluence. >50 passages performed 5. 1 year	1. a) Not specified 1. b) RT-PCR > OC-, collagen I+ 2. Tumorigenicity >1x10^7 cells injected subcutaneously into 6-week-old SCID mice >histology: large cells with nucleic dysplasia and increased chromatin condensation 3. Not specified

(*Continued on next page*)

Table 4. *Continued*

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
Sonobe et al.[9]	HS-Os-1	1. 11, F 2. Left proximal humerus 3. Osteoblastic	1. Needle biopsy 2. Not specified	1. Minced with scissors, washed with DMEM. 2. Digested with 0.25% trypsin solution at 37C for 30 min. Centrifugation at 1200rpm, for 5min. The isolated tumor cells were washed twice with DMEM	1. Approximately 1x10^6 dispersed cells were cultured in DMEM containing 10% FCS with 100 μg/mL penicillin G potassium, 100 μg/mL streptomycin sulfate, in a 25cm^2 plastic flask. 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2 3. Half volume of the fresh culture medium was exchanged twice weekly until the rapid and stable growth of the culture cells at passage 7 (6 months) 4. Cells were subcultured at confluence with a dilution of 1:3 to 1:5 every 2 to 3 weeks 5. 24 months.	1. a) Karyotype analysis >chromosome number ranged from 55 to 134 1. b) Immunohistochemistry >OC+/ALP+/vimentin+ 2. Morphology >round, polygonal shape with marked pleomorphism Doubling time >58h Tumorigenicity >5x10^6 cells inoculated into 6-week-old athymic nude mice >palpable nodules after 2–3 weeks >histological features: irregular bony trabeculae with calcification, with necrotic and haemorrhagic foci 3. Not specified

Abbreviations: growth medium (GM), fetal bovine serum (FBS), Iscove's Modified Dulbecco's Medium (IMDM), phosphate-buffered solution (PBS), fetal calf serum (FCS), Roswell Park Memorial Institute 1640 medium (RPMI 1640), Dulbecco's modified Eagle's medium (DMEM).

Table 5. Studies that established PCCL from primary resected tumor

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
Thanindratarn et al.[78]	OSA1777	1. 19, F 2. Left proximal femur 3. Not specified	1. Tumor tissues directly harvested in the operating theater. 2. Placed in sterile normal saline within a sealed microcentrifuge tube on ice for immediate transport to the laboratory	1. Specimens were washed three times with RPMI 1640 containing 1% penicillin/streptomycin (100 U/mL. Minced with a razor blade 2. Not specified	1. Cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, in tissue culture flasks. 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2 3. Media replaced every 3–4 days 4. Harvested using 0.25% trypsinization at 80–90% confluence, with subculture into new flasks. >50 passages 5. 6 months	1. a) STR profile >unique genomic identity without matching ATCC, DSMZ repositories >AMEL locus of X chromosome consistent with female patient origin >no contamination by another human cell line detected >no contamination by mouse DNA detected 1. b) Western blot >EMT markers (vimentin+/cytokeratin-) >tumor markers (p53-/MDM2+) 2. Morphology >spindle-shaped with a round to oval nuclei Doubling time >60h Chemosensitivity assay >in 2D culture: Cell viability decreased after 5days exposure. More sensitive to triple treatment (MAP) compared with single treatment >in 3D culture: decreased spheroid size after 7days exposure Spheroid formation assay >cluster formation in 3D culture by day 5 >similar growth rate to MNNG-HOS and U2OS 3. Not specified
Liu et al.[79]	CHOS	1. 58, M 2. Right scapula 3. Chondroblastic	1. Tumor samples directly harvested in the operating room 2. Placed in 0.9% sterile saline into sealed microcentrifuge tubes on ice for immediate transportation to the laboratory.	1. Minced into small pieces with a sterile scalpel and scissors. 2. Tumor mince treated with 2% collagenase II in DMEM/Ham's F-12 for 2h at 37C.	1. Cells were cultured in T75 flasks with RPMI 1640 containing 10% FBS, 100U/mL penicillin, and 100 mg/mL streptomycin 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2. 3. Not specified 4. Harvested using 0.25% trypsinization at 80–90% confluence, with serial passages every 2–3	1. a) Karyotype analysis >Cytogenetic G-banding: hypotetraploid karyotype constitute 11% of dividing cells >Loss of Y chromosome, gain of chromosome 12. 1. b) RT-PCR >chondroblast markers (ACAN+/COL II+/COL X+) >mesenchymal markers (vimentin+) >metastasis markers (ezrin+/S100A4+) Western Blot >chondrocyte (high ACAN/COL II) >osteoblastic (low ALP)

(*Continued on next page*)

Table 5. ***Continued***

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
					days >50 passages performed 5. 6 months.	2. Morphology >Spindle-shaped Doubling time >36h Chemosensitivity assay >more sensitive to cisplatin and doxorubicin, resistant to methotrexate >when compared to U2OS, MNNG/HOS Tumorigenicity >1.5x10^6 cells subcutaneously injected into nude mice >spontaneous tumor formation at 4 weeks >Histochemical analysis of cartilage matrix: purple metachromic color when stained with toluidine blue; similar to original tumor 3. Not specified
Zou et al.[80]	ZOS	1. 18, M 2. Right distal femur 3. Osteoblastic	1. Tissue specimen obtained after two cycles of NAC. 2. Rinsed with PBS containing penicillin G (100U/mL) and streptomycin (100 mg/mL)	1. Minced into 1mm^3 pieces 2. Not specified	1. DMEM (high glucose) containing 10% FBS, penicillin G (100 U/mL), streptomycin (100 mg/mL), L-glutamine (2 mmol/L) 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2. 3. Polygonal cells outgrew the explant and reached confluence after 12 days 4. Passaged every 3 days, dispersed by PBS containing 0.25% trypsin and 0.02% EDTA. Stable over >100 passages 5. Not specified	1. a) Karyotype analysis >Giemsa trypsin banding on 23rd passage >chromosome number ranged from 55 to 60 1. b) RT-PCR >osteoblastic markers (ALP+, osteopontin+, osteocalcin+) >metastatic markers ($CD44^-$, cadherin-11-) >apoptosis markers (Fas-) 2. Morphology >polymorphic appearance >nucleus is atypical and large with 2–3 prominent nucleoli. Karyokinesis is frequent and multiple nuclei can be seen. Doubling time >33.65h Invasiveness Capacity >Matrigel Invasion assay >60cells/hpf after 24h Chemosensitivity MTT assay >more resistant to MTX compared to U2-OS Tumorigenicity

(*Continued on next page*)

Table 5. ***Continued***

Reference	Cell line name	1. Clinical data 1. Patient age in year and gender (F = female, M = male) 2. Bone of origin 3. Histological subtype	2. Source of tissue 1. Source of tumor tissue 2. Transport conditions (from operating theater to lab)	3. Disaggregation 1. Mechanical 2. Enzymatic	4. Expansion 1. Culture media 2. Incubation conditions 3. Frequency of media replacement 4. Subculture, total number of passages 5. Overall time taken	5. Authentication and characterization methods 1. Authentication assays assessing: a) genetic uniqueness, b) cell of origin 2. Characterization assays studying: general features of cell line 3. Assays assessing purity (+ = present, - = absent)
						>$5x10^6$ cells injected into nude mice >average volume tumor reached 6.77cm3 within 2 months > rare osteoid was observed in histological section in keeping with original tumor 3. Not specified
Minamitani et al.[81]	KOS-1	1. 14, F 2. Left distal femur 3. Osteoblastic	1. Tissue specimen obtained at time of surgery 2. Not specified	1. Tumor tissue minced with razor blades 2. Not specified	1. Cultured in a plate with DMEM supplemented with 10% FCS, 50mg penicillin, 50mM streptomycin, and L-glutamine. 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2. 3. Not specified 4. Not specified 5. Not specified	1. a) Not specified 1. b) Immunohistochemistry >ALP+/von Kossa+/COL I+ >MMP: secrete MMP1/MMP2/MMP3 2. Morphology >polygonal shape, prominent nucleoli, marginally aggregated chromatin Doubling time >27.4h Tumorigenicity >$1x10^7$ cells subcutaneously injected into athymic nude mice >spontaneous tumor formation at 2 weeks >histologically similar to original tumor 3. Mycoplasma contamination >absent

Table 6. Studies that established PCCL from PDX

Reference	Cell line name	1. Clinical data Patient age in year and gender (F = female, M = male) Bone of origin Histological subtype	2. Source of tissue Initial PDX formation method Source of tumor tissue	3. Disaggregation Mechanical Enzymatic	4. Expansion Culture media Incubation conditions Frequency of media replacement Subculture, total number of passages Overall time taken	5. Authentication and characterization methods Authentication assays assessing: a) genetic uniqueness, b) cell of origin Characterization assays studying: general features of cell line Assays assessing purity (+ = present, - = absent)
Schott et al.[5]	OS052, OS384, OS457, OS526, OS742, OS833	1. 17F, 12F, 17M, 13M, 8F, 14M respectively 2. fibula; OS052, OS833. Tibia; OS384, OS526, OS742. Humerus; OS457 3. Not specified	1. 1mm^3 fragments of tumor tissue in Matrigel implanted under the renal capsule of NSG mice. Tumors were allowed to reach 1–2 cm^3 2. For PDX passaging, cells were implanted subcutaneously in the flank of NSG mice (5x10^5 cells) in 30mL of MEM alpha and 20mL Matrigel. Generation not specified.	1. After filtering, mouse stroma removed by depletion on a MACS column, to generate single-cell suspension 2. Digested in a collagenase buffer and filtered through a 70-mm filter.	1. standard DMEM supplemented with 10% FBS and 1% PSG. 2. Not specified 3. Cells were allowed to expand and sorted for human HLA positive to enrich for human osteosarcoma tumor cells, and this was performed twice to generate a pure population. 4. Not specified 5. Not specified	1. a) STR analysis > Genome copy-number concordance > Allele specific copy-number WGS > Single nucleotide variants (SNVs), small-scale indels, somatic structural variants 1. b) Histology > corresponds with original patient sample 2. Tumorigenicity > injecting 1x10^6 cells into the lateral tail vein of 2- to 5-month-old NSG mice. Spontaneous tumor growth >*in vivo* metastatic capacity 3. Mycoplasma testing >negative
VanCleave et al.[82]	COS-33	1. 7, F 2. Humerus 3. Osteoblastic	1. Parental PDX model (called OS-33) created by direct transplantation of untreated patient primary tumor fragment subcutaneously into an immunodeficient mouse 2. Tumor source was obtained from the third-passage tumor of the PDX line	1. Not specified 2. Tumor pieces digested using a mix of serum-free αMEM medium, Collagenase, Trypsin-EDTA, and Penicillin-streptomycin	1. Seeded into a 100-mm tissue culture dish. Initially cultured in the growth medium containing 20% FBS and 1% Penicillin-streptomycin. 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2. 3. After 48h, the cells were switched to a growth medium containing 10% FBS. 4. Harvested using Trypsin-EDTA at confluence and serially subcultured. >50 passages performed	1. a) Cytogenetic analysis >hyper-triploid clones with several complexly arranged chromosomes Genotype profile >TP53−/− 1. b) Differentiation assay >addition of BMP2 promotes differentiation to mature osteoblasts, consistent with osteoblastic origin 2. Invasive capacity >Boyden chamber-based: invasion decreased on treatment with Rapamycin

(*Continued on next page*)

Table 6. *Continued*

Reference	Cell line name	1. Clinical data Patient age in year and gender (F = female, M = male) Bone of origin Histological subtype	2. Source of tissue Initial PDX formation method Source of tumor tissue	3. Disaggregation Mechanical Enzymatic	4. Expansion Culture media Incubation conditions Frequency of media replacement Subculture, total number of passages Overall time taken	5. Authentication and characterization methods Authentication assays assessing: a) genetic uniqueness, b) cell of origin Characterization assays studying: general features of cell line Assays assessing purity (+ = present, - = absent)
					5. Not specified	>Wound healing assay: migration decreased on treatment with Rapamycin Chemosensitivity assay >mTOR and clonogenicity inhibition concentration-dependent by Rapamycin Tumorigenicity >grafted into immunocompromised mice >tumor formation histologically similar to parental PDX 3. Not specified
Yu et al.[83]	Well5	1. 16, M 2. Not specified 3. Osteoblastic	1. Fresh patient tumor tissues were dissected into small pieces (3 × 3 × 1mm^3) at 4°C, and implanted subcutaneously into the bilateral flanks of 6-to-8-week-old female NOD/SCID mice (P1). Tumor tissues from the xenograft was dissected into small pieces (2 × 2 × 1mm^3) and implanted into the bilateral flanks of another NOD/SCID mouse (P2). 2. P2 tissue at 6 weeks inoculation	1. Cut into 4mm^3 pieces 2. Type IV collagenase for 1h at 37°C. Cells were washed in PBS three times	1. Monolayer culture in DMEM supplemented with 10% FBS. 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2. 3. Flow cytometric analysis was used to distinguish between CD44$^+$ human cells and CD44$^-$ mice cells. 4. Sorted CD44$^+$ cells were passaged two times *in vitro*, and then digested and diluted into 10 divisions. Each division was digested and diluted repeatedly until the purity of the diluted osteosarcoma cells was close to 100%. 5. Not specified	1. a) Not specified 1. b) Immunohistochemistry >SSEA-4+ Differentiation assay >successful induction into osteogenic and adipogenic cells 2. Proliferation >CCK8 assay: stronger proliferative capacity than MG-63 Invasive capacity >Cell scratch assay: stronger migration capacity than MG-63 Tumorigenicity >1x10^6 cells injected into BALB/c nude mice >surface metastasis present at 6 weeks 3. Not specified

(*Continued on next page*)

Table 6. *Continued*

Reference	Cell line name	1. Clinical data Patient age in year and gender (F = female, M = male) Bone of origin Histological subtype	2. Source of tissue Initial PDX formation method Source of tumor tissue	3. Disaggregation Mechanical Enzymatic	4. Expansion Culture media Incubation conditions Frequency of media replacement Subculture, total number of passages Overall time taken	5. Authentication and characterization methods Authentication assays assessing: a) genetic uniqueness, b) cell of origin Characterization assays studying: general features of cell line Assays assessing purity (+ = present, - = absent)
Kito et al.[84]	NCC-OS1-X2-C1	1. 11, M 2. Right distal femur 3. Not specified	1. Patient resected tumor tissues were cut into 2-3mm pieces and subcutaneously implanted into the hind bilateral flanks of 6- to 12-week-old female SCID mice. Tumor was passage into recipient mice when their size reached between 500 and 1000mm^3. 2. P2 tissue used	1. Cut into small pieces. Passaged through an 18-gauge needle. Cell suspensions were collected with 40-μm nylon mesh, 2. Not specified	1. Monolayer culture in a 10-cm culture plate, in DMEM supplemented with 10% FBS, 100 U penicillin G, and 100 μg/mL streptomycin. 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2. 3. Not specified 4. >50 passages performed 5. 40 months	1. a) STR profile >comparison against cell banks (ATCC, DSMZ, JRCB): no matching reference >comparison against original tumor tissue: confirms same genetic identity Karyotype analysis >SNP array: widespread gain and loss of chromosomes 1. b) Not specified 2. Morphology >spindle-like Doubling time >29h Spheroid formation assay >readily formed spheroids 3. Mycoplasma contamination >absent
Bai et al.[85]	HOSS1	1. Not specified 2. Not specified 3. Not specified	1. Residual tumor from definitive resection was transported to laboratory in sterile RPMI medium. Tumor was minced into 2mm^3 pieces and implanted subcutaneously into flanks of 6-week-old NOD-SCID mice with equal volume cold Matrigel. Passaged when tumor size reached 1cm^3 2. Generation not specified	1. Sample mechanically minced 2. Collagenase IV (1 mg/mL) digested for 2h at 4°C	1. Primary culture in RPMI 1640 medium without FBS, containing 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor , 10 ng/mL hepatocyte growth factor overnight. 2. Standard conditions at 37°C in an atmosphere of 95% air:5% CO2 3. One week later, media changed to complete medium 10% FBS, 100U/mL penicillin, 100 μg/mL streptomycin 4. Not specified 5. Not specified	1. a) Not specified 1. b) RT-PCR >adhesion and gap junction molecules (Cx26/Cx43/Cx45 detected in spheroids, not in 2D culture) >E-cadherin higher in 3D compared to 2D >ECM-related genes upregulated in 3D compared to 2D 2. Doubling time >25h Chemosensitivity assay >apoptosis ratio higher in 2D (55–80%) compared to 3D (20–35%) at same concentration to Doxorubicin, Gemcitabine, Docetaxel Spheroid formation assay >grew stably after 8days 3. Not specified

Table 7. Comparison of tumor tissue source for establishing PCCL

Key Entry	Data extraction		Number of studies reported (n, (% rounded to nearest whole number))
1. Clinical data	Patient characteristics (age and gender)	Age • <10y/o (n=3) • 11-20y/o (n=13) • >20y/o (n=1) Gender • M (n=6) • F (n=11)	17 (77)
	Bone of origin	Scapula (n=1) Humerus (n=4) Femur (n=9)	14 (64)
	Histological subtype	Osteoblastic (n=9) Chondroblastic (n=1) Fibroblastic (n=1) Small cell (n=1) Telangiectatic (n=1)	13 (59)
2. Source of tissue	Source of tumour tissue	Biopsy (n=13) • core needle biopsy (n=11) • open biopsy (n=2) Primary resected tumour (n=4) PDX (n=5)	22 (100)
	Transport conditions to laboratory	Culture medium supplemented with penicillin and streptomycin, pH 7.4 (n=3) Sterile saline, on ice (n=2)	5 (23)
3. Disaggregation	Mechanical	Mincing with sterile scalpel/scissors (n=13) Vague methods (described as "mechanical dispersion") (n=3) Filtration (n=2)	18 (82)
	Enzymatic	Enzyme, concentration (where specified): Collagenase (n=10) • Type unspecified (n=4): 250units/mL • Type II (n=4): 0.6%, 2% • Type IV (n=2): 1mg/mL Trypsin (n=2): 0.1%, 0.25% Dispase (n=2): 2units/mL Duration: 30 minutes (n=1) 1h (n=2) 2h (n=3) >24h (n=1) Not specified (n=7)	14 (64)
4. Expansion	Culture media	DMEM (n=11), concentration not specified • High glucose type (n=2) • Unspecified glucose type (n=9) RPMI 1640 (n=5) Hams (n=2) FBS (n=1) alpha-MEM (n=1) IMDM (n=1)	21 (95)

(*Continued on next page*)

Table 7. *Continued*

Key Entry	Data extraction		Number of studies reported (n, (% rounded to nearest whole number))
	Incubation conditions	37C, 5% CO2 (n=19)	19 (86)
	Frequency of media replacement	Every 3 days (n=5) Once/week (n=1) Twice/week (n=1)	7 (32)
	Subculture, total number of passages	<25 (n=1) 50-60 (n=6) >70 (n=3)	10 (45)
	Overall time taken	>3 months (n=2) 6 months (n=3) >1 year (n=6)	11 (50)
5. Authentication and characterization methods	Authentication assays	a) Assessing genetic uniqueness	
		STR profile	3 (14)
		• Comparison of specific gene locus against cell bank depositories (ATCC, DSMZ, JRCB): confirm no matching reference, and thus a novel cell line • Comparison against original tumour tissue: confirm same genetic identity • Microsatellite loci used: DS1358, D7S820, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S17, D16S539, TH01, TPOX, CSF1PO, AMEL (X, Y), PENTA D, PENTA E, MOUSE	
		Karyotype analysis	7 (32)
		• G-banded cytogenetic – multiple, complex chromosomal aberrations (n=6) • SNP array (n=1)	
		Genotype profile	2 (9)
		• Tumour genes (TP53/RB1)	
		b) Assessing cell of origin	
		RT-PCR	8 (36)
		• Osteoblastic differentiation markers - ALP, OC, OPN, RUNX2, COL I • Chondrocyte - S100, aggrecan, LINK, COL II, COL X, ACAN • Phenotype - SATB2, EWSR1 • Migration/metastasis - CD44, cadherin-11, ezrin, AXL, S100A4 • Cancer stem cell markers - PROM1, ALDH1A1, CD34, MYC (pluripotency) • Embryonic stem cell markers - SOX2, NANOG, POU5F1, KLF4, LIN28A, SSEA-4 • Sarcoma-initiating gene - LIN28B	
		Immunohistochemistry	7 (32)
		• Osteoblast differentiation markers - OC, OPN, ON, ALP, COL I, Von Kossa (calcium deposits), BMP4 • EMT markers - vimentin, desmin, S100 • ECM degrading proteins - MMP1, MMP2, MMP3 • Cancer stem cell markers - nestin, SCF/KIT, SSEA-4	
		Western blot	2 (9)
		• Osteoblast markers – ALP • Chondrocyte – ACAN, COL II	

(*Continued on next page*)

Table 7. *Continued*

Key Entry	Data extraction		Number of studies reported (n, (% rounded to nearest whole number))
		• EMT markers - vimentin, cytokeratin • Tumour markers - p53, MDM2	
		Surface antigen analysis	2 (9)
		• Flow cytometry: mesenchymal stem cell surface antigens - CD29, CD44, CD63, CD71, CD105 (n=1) • Surface receptor profile: IGF-2R (n=1)	
		Differentiation assay	4 (18)
		• Alizarin-red assay: mineralization capacity at week 2-3 (n=1) • Mineralization induction after adding ascorbic acid and phosphate to growth medium (n=1) • Addition of BMP2 promotes differentiation into osteoblasts (n=1) • Induction into adipogenic and osteogenic cells (n=1)	
	Characterization assays	Morphology	11 (50)
		• Maintenance of phenotype across multiple passages • Imaged using phase contrast microscopy • Comparison against histological sections of original tumour for correlation	
		Doubling time (MTT assay)	11 (50)
		• <30h: 16h, 25h, 27.4h, 29h (n=4) • 30-50h: 33.65h, 35.6h, 36h, 40h (n=4) • >50h: 58h, 60h, 5 days (n=3)	
		Invasive capacity	5 (23)
		• Boyden-based chamber assay (n=2) • Cell scratch assay (n=1) • Soft agar growth assay (n=1) • Wound healing assay (n=1)	
		Spheroid formation assay	4 (18)
		• Grew stably after 5d (n=1) • Grew stably after 8d (n=1) • Duration not specified (n=2)	
		Chemosensitivity assay	7 (32)
		• Standard MAP regimes at increasing concentration levels (n=3) • Novel targets: Adriamycin (n=1), cyclopamine/tomatidine (n=1), rapamycin (n=1), Doxorubicin/Gemcitabine/Docetaxel (n=1)	
		Tumorigenicity	13 (59)
		• Growth: Assess time taken for spontaneous tumour formation and size, after injection of tumour cells into immunodeficient mice • Histopathology: Ensure xenograft tumours grown histologically identical to original	
	Assays assessing purity	Mycoplasma: LookOut PCR commercial detection kits	5 (23)
		Fibroblasts: identify via GD2 antibody labelling	2 (9)

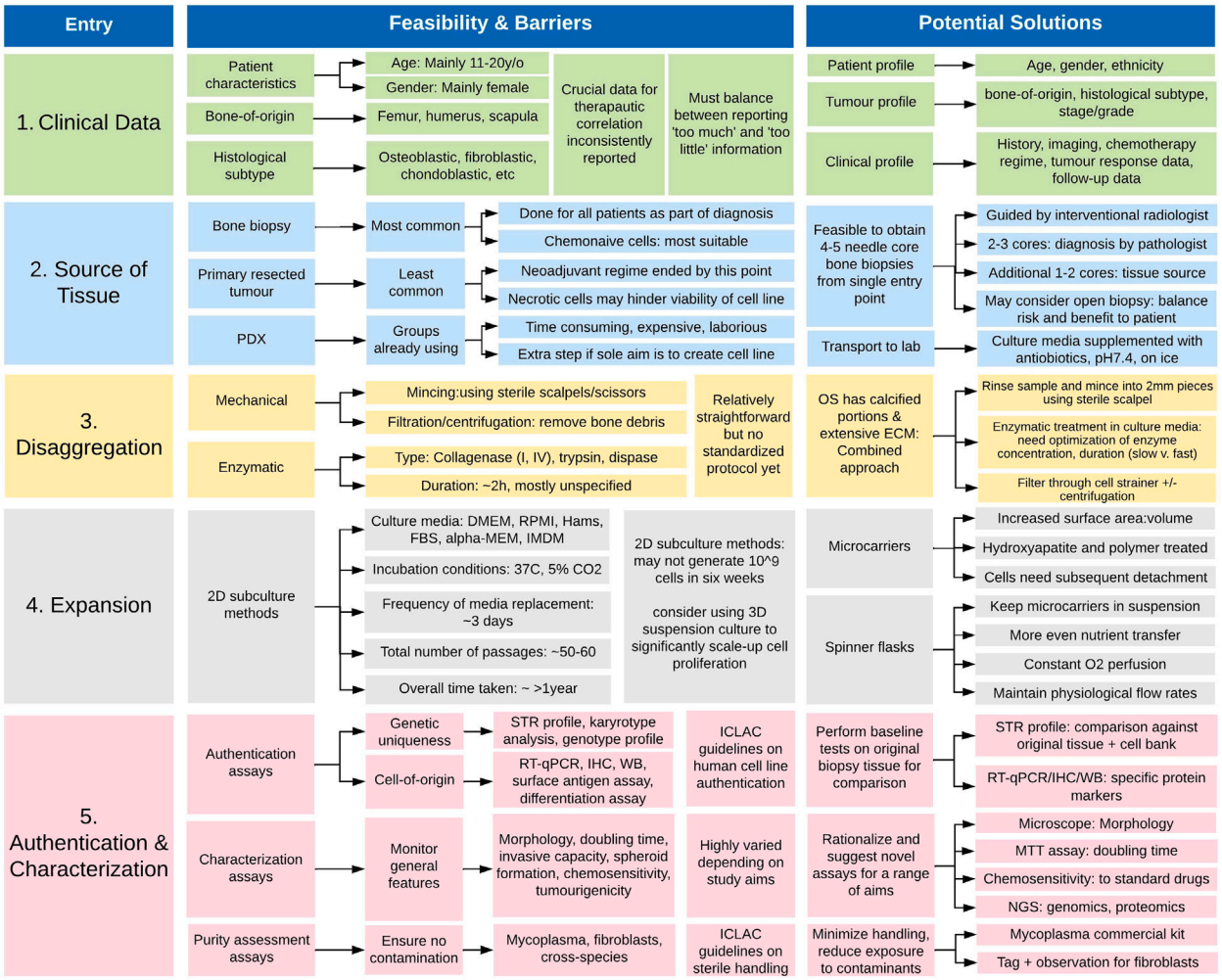

Figure 3. Workflow for establishing OS PCCL within clinical management

This workflow summarizes the extracted data, barriers to feasibility, and potential solutions for establishment of PCCL within six weeks. The overall methodology is categorized into five Entry steps. The second column summarizes the extracted data and identifies barriers in each Entry. The final column suggests potential solutions to overcome said barriers, which serves as a useful guide for future experiments. Abbreviations: Osteosarcoma (OS), primary cancer cell lines (PCCL), extracellular matrix (ECM), International Cell Line Authentication Committee (ICLAC), neoadjuvant chemotherapy (NAC), short-tandem repeat (STR), next generation sequencing (NGS), real-time PCR (RT-PCR), immunohistochemistry (IHC), western blot (WB).

Study findings

A summary of the raw data obtained from Tables 4, 5, and 6 is synthesized and presented in Table 7.

(1) Clinical Data: Patient characteristics (age, gender) were reported in n = 17 (77%) of studies. Bone of origin was reported in n = 14 (64%) of studies. Histological subtype was reported in n = 13 (59%) of studies, for which osteoblastic was the most reported.

(2) Source of tissue: The tissue source used was bone biopsy in n = 13 (59%), primary resected tumor in n = 4 (18%), and tumors in pre-existing PDX in n = 5 (23%) of studies. This makes bone biopsy the most widely utilized tumor tissue source. To maintain sterility for transport to the laboratory, samples were placed in either culture supplemented with antibiotics (n = 3, 14%), or on sterile saline on ice (n = 2, 9%). Additional methodology was reported for PDX studies, as it required the initial formation of the xenograft model.

(3) Disaggregation: Mechanical disaggregation was most performed by repeatedly mincing tumor tissue with sterile blades or scissors (n = 13, 59%) or filtration (n = 2, 9%), while n = 3 (14%) studies were unclear about their methods. Enzymatic digestion was commonly incorporated as a subsequent step following mechanical disaggregation. The most commonly reported enzymes were collagenase (Type II and Type IV) (n = 10, 45%), followed by trypsin (n = 2, 9%) and dispase (n = 2, 9%); all at varying concentrations. The duration

of enzymatic treatment where reported ranged from 30min to 24h. Following this step, the sample is often washed with media prior to culture. Only n = 3 studies performed an additional centrifugation step to obtain a cell pellet prior to culture.

(4) Expansion: The most common culture medium reported was DMEM (n = 11, 50%; high glucose type n = 2, unspecified glucose type = 9), followed by RPMI 1640 (n = 5, 23%), Hams (n = 2, 9%), alpha-MEM (n = 1, 5%), IMDM (n = 1, 5%). Culture medium was supplemented with 10% FBS in most cases. Cell lines OS1 and OS2000 were supplemented with 15% FBS and 15% FCS respectively, while OSA-1 and COS-33 were supplemented with a higher concentration (20%) of FCS and FBS respectively. Although the level of detail in reporting *in vitro* culture methods was highly variable, all studies expanded cells using 2D subculture methods and generally followed a similar procedure. Standard incubation conditions (37C, 5% CO2) were used, media was replaced every 3–4 days, and serial passaging was performed with trypsin to harvest cells when they reached 80–90% confluence and plated onto a new culture flask. The process would then be continually repeated until the cell line was established. Where reported, the most common number of passages performed was 50–60 (n = 6, 27%). Where reported, overall time taken ranged from 3 months to one year. As the papers do not have the definition of "establish" and "overall time taken" that we have provided, it is difficult to make any meaningful comparisons of time frame between studies.

(5) Authentication and Characterization Methods: All the assays used in the eligible studies were extracted and categorized. Authentication assays were divided into a) Assessing genetic uniqueness and b) Assessing cell of origin. Assays assessing genetic uniqueness include STR profile (n = 3, 14%), karyotype analysis (n = 7, 32%), genotype profile (n = 2, 9%). Assays assessing cell of origin include RT-PCR (n = 8, 36%), immunohistochemistry (n = 7, 32%), western blot (n = 2, 9%), surface antigen analysis (n = 2, 9%), differentiation assay (n = 4, 18%). The specific markers used for these assays and their function are also compiled and categorized in Table 7. The characterization assays listed in eligible studies were highly variable and depended on their aim. Characterization assays included those studying morphology (n = 11, 50%), doubling time ranging from 16h to five days (n = 11, 50%), *in vitro* invasive capacity (n = 5, 23%), spheroid formation assay (n = 4, 18%), chemosensitivity assay to standard MAP or novel drugs (n = 7, 32%), tumorigenicity by observing spontaneous tumor formation following introduction into immunodeficient mice (n = 13, 59%). Assays assessing purity included mycoplasma testing using commercial detection kits (n = 5, 23%), and fibroblast labeling (n = 2, 9%).

DISCUSSION

An experimental protocol that can rapidly establish OS PCCL within clinical management could be useful for both clinical and scientific purposes. Our study assesses the feasibility of establishing PCCL within a clinically actionable time frame of six weeks. By systematically assessing contemporary studies reporting novel OS cell line establishment in the past three decades, we aim to identify barriers to feasibility and in turn offer strategic recommendations that can accelerate protocol optimization. In general, the methodology in eligible studies were not standardized and often did not report key information; some omitted patient data, had unclear methodology, or did not authenticate their cell line. However, it is establishing a cell line within the strict time frame of six weeks which likely remains a major barrier, as studies reportedly took at least three months to be established. The tests used for authentication and characterization were also highly varied, and there is a need to rationalize and streamline these tests. In Figure 3, we present a visual summary categorized according to each of the five entries: (1) feasibility assessment and barrier identification (as per Results), followed by (2) potential solutions to each (as per Discussion). Each chapter and subchapter in the Discussion corresponds with the Data Extraction in (Column 3) Table 3. This serves as valuable theoretical groundwork that can facilitate modular experimentation and accelerate optimization of such a protocol.

Clinical data

As commercial OS cell lines are often used for scientific purposes, data sheets often publish the minimum amount of clinical data (Table 1). We followed this precedence and only extracted the age, gender, bone of origin, and histological subtype from studies; but even this small amount of data was inconsistently reported: only *n* = 17 (77%) of studies reported the age and gender of patients, *n* = 14 (64%) for bone of origin, and *n* = 13 (59%) for histological subtype. This is a concerning finding as omitting basic clinical and pathological data of donor patients makes it difficult to evaluate if the characteristic of the PCCL resembles the original tumor, making any meaningful therapeutic correlation to *in vitro* results highly challenging.[31] Beside clinical decision making, the absence of basic patient data for a newly derived PCCL prevents meaningful comparison with commercial cell lines. For example, correlative studies to identify salient risk factors in OS patients.

There needs to be greater standardization in reporting clinical data, but a fine balance will have to be struck between reporting "too much" and "too little" clinical data. Too little data (as observed in this study) may hinder effective *in vitro* to *in vivo* correlations, but too much clinical data may conversely make comparisons too cumbersome. This chapter suggests the minimum (but crucial) clinical data needed to make therapeutic correlations between PCCL and patient (Principle 1, Table 3). We suggest three clearly defined "Profiles": Patient, Tumor, Clinical – and the data in each to be recorded.

Patient profile

Osteosarcoma has a well-described bimodal distribution, with the first peak during adolescence and the other after the 6th decade of life.[79] It commonly presents from 10 to 25 years of age, and this is reflected with 76% (*n* = 13 of 17) of studies reporting patients

between 11 and 20 years old. These age differences are relevant as adult tumors manifest different clinical features and responses compared to their pediatric counterparts.[90] There is lower tolerance to aggressive chemotherapy; as a result, elderly patients have unfavorable prognosis compared to pediatric patients,[79] making it crucial to report age. Pediatric OS also has a male predominance in incidence, which is hypothesized to depend on the rapid bone growth due to the different hormonal changes in males and females during their respective pubertal windows.[90] Despite this, only 24% (n = 6 of 17) of studies reported male patients. Furthermore, only two of the eight OS cell lines sold by ATCC were derived from male patients; highlighting that current cell lines are not adequately reflecting the patient landscape. For the new PCCL to adequately reflect the patient landscape and facilitate useful comparisons, patient gender must be reported. Ethnicity was not formally included in the data extraction (as most papers omitted it). Ethnicity is reported by most commercial cell lines, largely derived from Caucasian patients (Table 1). Chemotherapy causes DNA damage at the cellular level, where genetic differences may influence responses to therapy and may be more pronounced in ethnically diverse populations.[69] Reporting ethnicity of donor patients may be useful to account for possible ethno-geographic and genetic differences when evaluating therapeutic correlation.

Tumor profile

Osteosarcomas have distinct characteristics according to their primary site,[89] however only n = 14 (64%) of papers reported tumor location. Of the reported locations, the femur (n = 9, 64%) was the most common, followed by humerus (n = 4, 29%) and scapula (n = 1, 7%), in keeping with established literature.[58,91] Reporting bone of origin (intra-axial/extra-axial, left/right, proximal/distal) can facilitate comparisons of aggressiveness and level of treatment response among different primary sites. High-grade (conventional) OS develops within medullary bone, and accounts for 75% of cases.[26] It is further classified into different histological subtypes based on the predominant extracellular matrix (ECM) and is used clinically for prognosis: osteoblastic (50%), chondroblastic (25%), and fibroblastic (25%), and rarer variants which account for <1% of cases: mixed, small cell and telangiectatic.[26] Of the n = 13 (59%) papers reporting subtypes: osteoblastic was the most common (n = 9, 69%), followed by n = 1 (7%) each of chondroblastic, fibroblastic, small cell and telangiectatic, largely in keeping with literature.[58,92] It is crucial to report histopathological subtypes as they have different characteristics which would influence their biology. Although not included in the data extraction, other important tumor data would include those influencing prognosis and aggressiveness, and include stage/grade, baseline measurements of size at diagnosis (from imaging), presence of metastasis.[35]

Clinical profile

Traditionally, cell lines were established to study OS biology and not for a clinical purpose. However, reporting key clinical characteristics will facilitate correlation of clinical observations to PCCL-generated data.[84] A brief clinical history can indicate the aggressiveness of the primary tumor: delayed diagnosis, vague presenting complaint, presence of pulmonary metastasis are poor prognostic markers which should be noted.[58]

Local imaging examines tumor morphology: (1) plain radiographs assesses for areas of calcification and bony destruction, (2) CT chest assesses for lung metastasis, (3) MRI demonstrates tumor size/location and proximity to adjacent anatomical structures, (4) PET scan reveals the most metabolically active area within the lesion and improves overall diagnostic accuracy. Reporting selected slices from diagnostic and subsequent imaging will be useful in making correlations to aggressiveness. Recording the precise chemotherapy regime (agents, doses, cycles, side effects, toxicities), and the *in vivo* treatment response data such as the tumor necrosis rate (TNR) at resection, adequacy of surgical margins will enable prognostic correlations.[93] Patients are followed-up for 8 years to assess for recurrence or metastasis, and this may be useful in assessing for 5-year survival rates.

Recording clinical data into these three Profiles helps to categorize the tremendous volume of data generated in the clinical setting. For each Profile we suggest the bare minimum data to be reported but acknowledge that it is non-exhaustive and the type/level of detail of data reported might differ based on the purpose of the PCCL. Groups interested in studying drug sensitivities might choose to highlight in further detail the chemotherapy regimens, whilst groups studying differences between histological subtypes may choose to report new bimolecular markers.

Source of tissue

There were three main solid primary tumor tissue sources to generate PCCL: bone biopsy, primary resected tumor, and PDX. In our Introduction we defined a "clinically actionable time frame" lies between bone biopsy and the start of NAC cycle 2; by the time primary resected tumor samples or PDX are created, NAC will have concluded.

Furthermore, there is no consensus for second-line therapy for poor responders, where studies that modify adjuvant treatment by dose intensification failing to demonstrate a survival benefit,[2] suggesting that any meaningful change to the chemotherapy regime should be initiated early and likely prior to NAC completion. However, we will continue to critically evaluate each source of tissue and their appropriateness under our unique clinical considerations. Namely, obtaining tissue must not delay care, must not cause additional harm to the patient, and must remain the most sterile (Principle 2, Table 3).

Table 8. Comparison of tumor tissue source for establishing PCCL

Bone needle biopsy	Primary resected tumour	PDX
Tissue readily available: existing step in management, will be performed for all cases.	Performed as definitive therapy in most cases	Initial source of tumor cell still needs to be obtained from biopsy or primary resected tumor first
Chemo-naïve tumor cells: useful for chemo-response and drug sensitivity studies	Cells exposed to NAC: useful for studying chemoresistance, and for recurrent and metastatic tumors	Validating cell lines: useful for studying effects of long-term *in vitro* culture on cells
Obtained early in management: potential 6 weeks window for isolating cell line	NAC ended by this point: may not be in time to influence any therapeutic decisions	Too time consuming: additional time needed to establish PDX, thereafter cell line
Obtaining additional samples without harm to patient is feasible: multiple cores can be obtained from a single-entry point. May not represent intratumoral heterogeneity due to small sample.	Sterility of sample hard to maintain requires analysis by pathologist first and may contain necrotic cells that impede cell line establishment	Has associated material, ethical barriers: more appropriate for groups already working with PDX

The Table summarizes the key aspects of three different sources of tumor tissue for establishing PCCL within clinical management. Abbreviations: patient-derived xenograft (PDX), neoadjuvant chemotherapy (NAC).

Bone biopsy

Bone biopsy is necessary for tissue diagnosis and histological subtyping. It is obtained for every patient, making it a ready source of untreated tumor tissue which could explain its use in majority of studies (n = 13, 59%). There were two approaches described to obtain the biopsy: Percutaneous needle core biopsy (n = 11) and open biopsy (n = 2). Percutaneous needle core biopsy is performed using a co-axial technique, where multiple cores are obtained through a single-entry site,[94] while open biopsy requires surgical incision and removal of tissue. Open biopsies are no longer routinely performed due to their higher invasiveness,[95] and are generally done only after two inconclusive needle biopsies to obtain more tissue for a definitive diagnosis.

Notably, long expansion times of at least three months was reported in studies using bone biopsy. None of the studies reported the initial number of harvested cells making it difficult to make a precise conclusion, but this could be attributed to the initial low number of cells obtained from bone biopsy.[96] Generally, the initial number of harvested cells depends on the amount and condition of surgical tissue available,[36] and one possible way of obtaining more cells is to perform open biopsies routinely for the exclusive purpose of generating PCCL. Whilst more tissue can indeed be obtained this way, the higher complication rates and seeding risk of open biopsies compared to needle biopsies will likely outweigh the benefit to the patient in the normal setting.

Instead, a potential solution is to simply obtain additional core needle biopsies. From the same (single) entry point, three to five cores can be obtained (with two to three used for histological diagnosis). It is entirely feasible to obtain one to two additional biopsies (past what is needed solely for histological diagnosis) and use them as a dedicated tissue source for PCCL isolation, without compromising timely management or sterility. Needle bone biopsy is an attractive option as the established PCCL would technically be the closest representation of the original pre-treated tumor,[97] making it a useful source of chemonaive cells. Furthermore, normal (non-cancerous) bone can also be obtained in the same procedure and serve as control samples when authenticating PCCL[98] (authentication assays).

Primary resected tumor

Primary resection is performed as definitive therapy following NAC, and the surgical specimen is evaluated for TNR: an important prognostic factor which guides subsequent management.[99,100] Only n = 4 (18%) of papers utilized this source of tissue. As the whole resected sample must be sent to the pathologist for processing and an accurate calculation of TNR, tissue availability and sterility is much harder to maintain, which could explain its lower usage compared to bone biopsy. Furthermore, cell lines established from this source are no longer chemo naive. The mixture of necrotic and live tumor cells within the sample may also hinder the establishment of a viable cell line. However, there may still be utility in isolating PCCL from primary resected tumor tissues to facilitate the study of chemo-resistant OS cells. Studies of chemo-naïve and chemo-resistant cell lines obtained from the same patient (from bone biopsy and resected tumor tissue respectively), could yield new insights into the mechanisms of drug resistance and targeted therapy.[40]

Patient-derived xenografts

Patient-derived xenografts are a widely used preclinical model that mimic *in vivo* conditions and facilitate the study of OS tumors in a more physiologically representative environment. Briefly, PDX are created by inoculating human tumor cell suspensions (initially obtained from either a biopsy or resected tissue) into immunodeficient mice to facilitate spontaneous tumor growth (which takes around 6–8 weeks). These tumors are then further inoculated into new mice to create subsequent generations. Studies using this approach (n = 5, 23%) established cell lines from a biopsy obtained from the PDX tumor (often the second or third generation); a highly time-consuming process. Furthermore, researchers using this tissue source were already working with PDX as their primary preclinical

tool, where establishing a PCCL was a secondary outcome. For the sole and primary purpose of establishing PCCL, creating a PDX would be an unnecessary step; notwithstanding the multiple barriers that arise from working with xenografts that include strict ethical guidelines, high fail graft rate, and high cost.[101] However, PDX remains useful to assess if tumorigenicity of the PCCL is retained following long-term *in vitro* cell culture (characterization assays).

By comparing the three sources of primary tumor tissue as summarized in Table 8, additional bone needle biopsies at the time of diagnosis are potentially the most feasible and appropriate for establishing chemo-naïve PCCL for therapeutic correlation. It is: (1) conducted early in OS management, (2) additional tissue samples can be obtained in the same procedure (care is not delayed as patient is not required to undergo additional steps), and (3) minimally invasive, allowing sterility to be easily maintained. It must be noted that despite these advantages, bone biopsies are obtained from a small region of tumor and are unlikely to entirely reproduce the intratumoral heterogeneity, particularly in the case of metastatic tumors. Cell lines established from primary resected tumors and PDX also have their own utility; being more relevant to the study of recurring or metastatic tumors and the long-term effect of *in vitro* propagation on PCCL, respectively.

Transport conditions to laboratory

Maintaining sterility of the tissue sample is crucial as a contaminated cell line is challenging to ameliorate afterward. Despite this, only $n = 5$ (23%) of studies reported how sample sterility was maintained on transport to the laboratory. Samples were either placed in sterile plastic containers on ice containing culture medium (RPMI 1640 or DMEM) supplemented with antibiotics (penicillin and streptomycin) at physiological pH 7.4, or in sterile saline. Immediate transport in culture media at physiological pH is best-practice and is preferable over normal saline,[62] to provide nutrients to keep tumor cells alive during transport. Keeping samples cool on ice, with antibiotic supplementation of media, will provide the crucial bacteriostatic and bacteriocidic conditions to prevent mycoplasma growth. Additionally, current best practice dictates all subsequent tissue processing and cell work should be executed aseptically in a Class II biosafety cabinet to minimize contamination.

Disaggregation

Disaggregation involves breaking down the initially solid tumor sample into smaller components to ultimately generate a cancer cell suspension for primary culture.[36] This process generally consists of mechanical dissociation and/or enzymatic digestion.[62] Mechanical dissociation physically divides the sample into smaller pieces, while enzymatic treatment digests the ECM present in the initial tissue sample. Among eligible papers, $n = 18$ (82%) of papers described mechanical methods, and $n = 14$ (64%) papers reported enzymatic methods. The overall procedure was similar among studies: samples were washed with medium or PBS to remove blood and bone debris prior to a combination of either solely or a combination of mechanical/enzymatic disaggregation, ending with a round of filtration/wash prior to primary culture. However, they differed largely in terms of the specific conditions, concentrations and duration of enzymatic disaggregation. We discuss what is likely the most time-efficient method to generate a single cell suspension for primary culture (Principle 3, Table 3).

Mechanical

This process involved physically breaking up the sample into smaller pieces and is not equipment intensive. Among the $n = 18$ papers describing mechanical disaggregation, 72% ($n = 13$ of 18) minced their samples using a sterile scalpel or scissors into 1-2mm diameter pieces, in keeping with best-practice.[62] Unfortunately, 17% ($n = 3$ of 18) of papers were vague and did not describe their mechanical methods precisely (simply stated as "mechanical dispersion"). Filtration through a nylon mesh or cell strainer to remove bone debris was employed in $n = 2$ papers, which could be due to highly heterogeneous nature of OS tumors compared to other solid tumors, with areas of necrosis but also areas of calcified growth. Biopsy samples containing more calcified areas may require additional filtration to remove bone. For solid tumor samples in general, mechanical disaggregation alone may not be sufficient to generate single cell suspensions due to substantial ECM components, compared to fluid clinical tissue such as an ascites, aspirate, or effusion, where the cancer cells are already in suspension.[62] The calcified nature of OS may require mechanical disaggregation to be followed by enzymatic digestion, a combination employed in $n = 8$ studies.

Enzymatic

Enzymatic treatment is commonly employed after mechanical disaggregation by digesting the ECM without unduly impacting tumor cell viability. Enzymatic disaggregation is carried out using specific selection of enzymes, concentrations, and exposure times to obtain the best cell yield, and there is still no standardized protocol for OS tumors.[36] Among the $n = 14$ (64%) papers reporting enzymatic methods, the most used enzymes were collagenase (Type I/IV) ($n = 10$, 71%), trypsin ($n = 2$, 14%) and dispase ($n = 2$, 14%). As bone is composed mainly of collagen I and X, collagenase is an appropriate choice and could explain its frequent use. Dispase is a protease which cleaves fibronectin, collagen IV, and to a lesser extent collagen I, whereas trypsin is often used to harvest adherent cells. Considering the extensive stromal involvement of OS, this suggests that collagenase is a suitable choice.

Unfortunately, half of papers ($n = 7$, 50%) did not specify the duration of enzymatic digestion, with a range of different concentration/enzyme unit/mL. Where mentioned, papers commonly digested tumor cells for 2h ($n = 3$, 21%), while $n = 3$ (21%) had durations of <1h, suggesting that a duration of 2h could be sufficient to achieve significant ECM digestion for primary culture. Performing enzymatic digestion overnight on ice ($n = 1$) may have a gentler effect on cells[62] but would massively prolong this step; the benefit of possible higher tumor cell yield may not outweigh the additional time required.

Following enzymatic digestion, *n* = 3 (21%) of studies performed centrifugation of the resulting cell suspension at 200g or 1200RPM for 5 min, prior to primary culture. Centrifugation can separate cell types according to their density and can remove contaminants to obtain a pure cell pellet.[62] Notably, OS has a complex tumor microenvironment (TME) with stromal and immune cell niches,[1,102] where the cellular composition of initial cell suspensions are likely be very variable. Hence, separating these components via centrifugation may be an appropriate step, but will require studies not present in current OS literature. In this chapter we suggest that Disaggregation can be performed sequentially: (1) mechanical mincing into 1-2mm^3 pieces using sterile scalpels +/− filtration of bone debris, (2) enzymatic digestion, (3) +/−centrifugation. Although we have compiled a list of enzymes and varying concentrations and duration of treatment as reported in the studies, there is no standardized protocol specific to OS yet, as evidence by the variety of approaches. Optimizing the Disaggregation step will require a period of experimentation for the optimal choice of proteolytic enzymes, working concentrations, and duration of treatment to obtain the best cell yield without excessive cell destruction for subsequent culture and Expansion.

Expansion

Expansion involves repeatedly subculturing cells until a "sufficient number" of cells are grown to facilitate statistically meaningful *in vitro* testing, which we defined to be at least 10^9 cells. Our goal is to grow 10^9 PCCL within six weeks (Principle 4, Table 3), a large number of cells that needs to be grown within a relatively short time frame. This chapter discusses the methods reported for cell expansion in current OS literature and recommends potential modes of expediting this process.

2D subculture (passaging) methods

All eligible studies utilized two-dimensional (2D) subculture strategies. Two-dimensional serial subculture involves growing cells on planar tissue culture vessels, and serially passaging cells to new vessels when confluence is reached. This process is repeated continuously until a desired amount of cells are grown. Only 50% (*n* = 11) studies reported "overall time taken", ranging from three months (*n* = 2 of 11, 18%) to one year (*n* = 6 of 11, 55%), and at first glance it may seem that none of the methodologies are fast enough. However, making any comparisons on the effectiveness of the eligible studies' methodology among each other is exceedingly difficult due to the lack of clearly defined endpoints. No paper reported the number of cells at the end of the Expansion step, making it near impossible to evaluate which method grew cells more quickly. We instead discuss the culture conditions employed by the studies and thereafter suggest avenues of exploration of novel cell culture techniques that could significantly scale-up cell proliferation.

A variety of culture media was used, and often a mixture is utilized, although the exact composition is not often reported. Roughly half of papers used DMEM (*n* = 11 of 21, 52%), with *n* = 2 using the high glucose type. High glucose DMEM is an easily accessible media which reportedly stimulates greater rates of proliferation in OS cells which rely predominantly on aerobic glycolysis,[65] making it a suitable energy source for cellular expansion. Other culture media included RPMI 1640 (*n* = 5 of 21, 24%), Hams (*n* = 2 of 21, 10%), FBS, alpha-MEM, and IMDM in turn. These are all easily accessible and appropriate culture media, with the choice seemingly dependent on availability for the specific laboratory.

Additionally, media supplements should be considered. In most of the papers, media is supplemented with 10% fetal bovine serum (FBS), a standard practice in cell culture. A higher concentration of FBS (from 15 to 20%) can be used to boost the proliferation of slow growing cells. However, the exact growth factors within FBS responsible for promoting OS cell growth are not currently known or explored. Alternatively, platelet concentrates such platelet rich plasma (PRP) may be used, where 10% PRP has been shown to double the number of mesenchymal stem cells (MSC) within the same time frame compared to 10% FBS.[103] Furthermore, as OS cells are purported to originate from MSC, PRP has also been shown to increase the proliferation of OS cells (Saos-2 and HOS) in a dose dependent manner.[104] Furthermore, PRP can be derived from the patient themselves, potentially providing a readily available source of native growth factors.

Where reported, all papers cultured cells under standard incubation conditions of 37C, 5% CO2 (*n* = 19, 86%) in a humidified incubator, with the most common media replacement frequency every 3 days. Fresh media must be replaced in a timely manner to meet the high metabolic demands of OS cells. Due to the static and planar nature of 2D culture, cells are not exposed to fresh media in the latent time between media replacement. This leads to the development of nutrient/oxygen gradients within the vessel, where heterogeneous oxygen gradients diminish the diffusion rate of gases in cells, and thereby their metabolic and proliferative capabilities[105] A system of closed-loop media replacement may be able to overcome these limitations and provide a continual source of fresh media which could likely boost cell proliferation rates (Bioreacters and Spinner flasks).

In keeping with established practice, cells were cultured in tissue culture flasks and passaged to new flasks when 80–90% confluency was reached. Only 45% (*n* = 10) of papers reported the total number of passages performed, with roughly half (*n* = 6 of 10, 45%) of these papers reporting 50 to 60 passages. Each passage requires manual handling and exposes cells to the risk of contamination, whilst the proteolytic enzyme trypsin used to detach cells from the plate invariably causes cell damage and temporarily slows the rate of proliferation. Novel culture flasks and systems capable of continual perfusion and *in-situ* visualization to assess confluency could overcome these barriers (see Bioreacters and Spinner flasks).

As mentioned, an endpoint of Expansion is not clearly defined across studies, therefore there is no means of reliably discerning the most efficient method. However, conventional adherent cell culture systems do indeed have significant limitations in terms of scalability, notwithstanding the lack of fine control over pH, gas, and metabolite concentrations, which could prolong the overall Expansion time required.

Although 2D scale-up strategies can increase the surface area to volume ratio, they largely remain susceptible to the same inefficiencies and contamination risks. Simple operations like cell seeding, media change and cell detachment/harvest are challenging when using multi-layered T-flasks due to their size and weight, while roller-flasks can enhance media and gas exchange but still require manual media

replacement. The large quantity of cells required as per the aims of our study means that even 2D scale-up approaches may be inadequate to grow 10^9 cells within six weeks. In the following section, we suggest exploring three-dimensional (3D) suspension culture as an alternative cell culture method to massively scale-up cell proliferation and significantly reduce Expansion time.[106,107]

3D suspension culture for scale-up proliferation

Three-dimensional suspension culture is a novel method to scale-up cell proliferation by four principles: (1) increased surface area to volume ratio, (2) increased availability of growth elements, (3) optimizing growth conditions by shear stress, and (4) continuous monitoring and tailoring of culture conditions.[108] Previously, suspension cultures were viewed as inappropriate for anchorage-dependent cells such as OS because these cells require attachment to a surface to proliferate.[109] However, microcarriers and bioreactors are emerging cell culture systems which may present a worthwhile avenue of exploration to bypass the limitation of scalability.

Microcarriers/microspheres. Microcarriers are small spheres that adapt anchorage dependent OS cells to suspension culture by providing a floating attachment point that vastly increase their surface area to volume.[110] They have a diameter ranging between 90 and 300μm and are available in different sizes, materials, coatings, and surface charges.[111,112] Treatment of microcarriers with biocompatible materials can improve proliferation as demonstrated with SaOS-2 cells on gelatin-treated hydroxyapatite microcarriers, which showed a much higher proliferation rate compared to static 2D controls.[113] Hydroxyapatite is a thoroughly researched bone mimic which has stiffness adequate for OS cell attachment and maintains cell viability, while gelatin improves adhesion to initiate attachment.[114,115] Combining other cell adhesion treatments can enhance cell attachment to microcarriers.[110,112] However, microcarriers are not without their challenges: it is technically difficult to dissociate cells from carriers and harvest them for use. Conventional enzymatic methods have proved inefficient in detaching cells from microcarriers and can additionally cause cell death, decreasing the pool of viable cells and potentially extending the expansion time necessary to establish a PCCL. Coatings with thermo-responsive polymers and degradable carriers are promising solutions to dissociate and harvest cells, but require further investigation in OS research. However, integrating microcarriers directly into novel 3D *in vitro* models, could nullify the need to detach cells (appropriate *in vitro* models and other testing systems).

Bioreactors and spinner flasks. Bioreactors are systems engineered for suspension culture which circulate media to continually perfuse cells (usually attached on microcarriers) within a closed-loop environment by magnetic or electronic stirring, rocking platform, pneumatic pump, or rotating culture vessel.[116] Continual perfusion makes key elements (oxygen and nutrients) more available, while preventing the accumulation of waste products and the development of nutrient, oxygen, and pH gradients inherent to 2D batch-fed cultures.[111] Many commercial iterations of bioreactors exist, most are large-scale, however spinner flasks represent a more appropriate bench-scale, ultra-high-density system.

Spinner flasks generate dynamic perfusion using a magnetic rotating spinner at the bottom of the device. This fluid flow creates shear stress at the surface of cells, which present research suggests can stimulate cell growth depending on stirring speed.[113] Studies have reported shear stresses of 3–5 cPa and superficial flow rates of 400–800 μm/s leading to increased proliferation rates and osteogenic response.[117,118] An optimal level of shear stress must be experimentally determined however, as excessive shear can lead to cell-surface detachment which would be counteractive to our aim.[119]

As outlined by Perez et al., culture media can be inoculated with cells to fill the flask with a volume of 100–200mL, after which biodegradable microcarriers are added and an initially low speed of rotation (40–60 r/min) facilitates initial cell attachment to microcarriers. Spinner flasks can be designed to contain multiple compartments to allow sequential replacement of microcarriers without the need to interrupt perfusion or compromise the sterility of the system.[120] Such systems may prolong the period of cell culture allowing researchers to obtain more cells with less material usage and from a smaller initial cell concentration. Additionally, continuous media agitation maintains microcarriers in suspension and decreases the development of nutrient gradients by continually perfusing cells with fresh media.

It is imperative that cell and viability are readily measurable and avoid contamination risks so cells can continue to grow. *In-situ* microscopy, sampling of small aliquots from cell culture, and monitoring fluorescence intensity from fluorescence-transfected cells are all possible real-time, contactless methods of measuring proliferation without interrupting cell culture. Spinner flasks can be designed with closed-loop input/output channels to facilitate media replacement and monitoring of cell culture supernatant over time, thus minimizing contamination risk and manual handling involved.

Notwithstanding the existing limitations inherent to 2D cell culture, it is ultimately the short time frame of six weeks which encourages exploring 3D suspension culture methods to massively scale up Expansion. Promisingly, microcarriers have been shown to greatly increase the surface area to volume ratio of cells suspended in culture media, enhancing proliferation rates when used in conjunction with bioreactor systems. Spinner flasks can provide an economical, bench-scale system suitable for hospital-based laboratories. This alternative cell culture system has the potential to increase cell proliferation rates, under more sterile, tightly controlled culture conditions with continuous media perfusion, that could contract the Expansion step immensely. However, this emerging technology will require an extensive period of optimization before 3D suspension scale-up systems become a routine and efficient *in vitro* cell expansion system. Avenues for exploration include.

i. Optimize microcarrier conditions: size, gelatin-coated/hydroxyapatite for initial attachment onto the growth surface, thermo-responsive coatings for cell detachment and more efficient harvest of viable cells
ii. Optimize spinner flask conditions: tighter control of critical growth parameters which include the use of a bubble-free, permeable O2 aeration system, nutrient, and media perfusion, mixing rate via magnetic spinner to provide optimal shear stress

iii. Continuous perfusion and culture: design of input/output channels and multi-chambered flasks for increased flask volume and minimized manual handling
iv. Contactless monitoring of cell density, viability using *in-situ* microscopy whilst maintaining sterility

Authentication and characterization

Authentication and characterization assays are performed to ensure that the identity and purity of the PCCL is confirmed, as the incorrect use of cell lines due to misidentification and cross-contamination damages the reliability of potential *in vitro* results.[88,89] Despite guidelines being available, the variability of approaches in cell line authentication has been a long-standing challenge among researchers. This challenge was reflected in the eligible studies, with assays performed without a clear systematic approach, at times leading to the omission of important tests. This is crucial to rectify as the authentication of tumor cells, and a detailed characterization of its features, holds immense scientific and translational significance.

A definition as to when a cell line is adequately authenticated and characterized will be helpful in determining the crucial "tipping point" of "just enough" tests to be conducted. As mentioned in the Introduction, we proposed that the PCCL is "established" when its identity corresponds with the original tumor cell, maintains its morphology, and is not contaminated by other cell types: i.e., when it is ready to be reliably used for experiments revolving around predicting the *in vivo* response of tumor cells. Such verification can often be performed on the first few cell passages.

This chapter compiles all the assays mentioned in the eligible studies and stratifies them according to those that (1) confirm identity and novelty of the newly established PCCL, (2) investigate cell behavior, and (3) ensure purity and non-contamination (Principle 5, Table 3).[34] In doing so, the assays are organized into clearly defined sections: Authentication assays assessing genetic uniqueness and cell of origin, Characterization assays studying general features of cell line, Assays assessing purity, thereby aiding systematic rationalizing for the minimum (but crucial) checkpoints for quality assurance.

Authentication assays

The International Cell Line Authentication Committee (ICLAC) defines 'Authentication' as "to confirm or verify the identity of a cell line, demonstrating that it is derived from the correct species and donor". Due to the extreme variability of OS, there is a lack of specific, recurrent molecular markers for identification.[79] Therefore, it is vital for the PCCL to be compared and validated against the original tumor tissue. A baseline reference can be created by performing selected immunohistochemistry or genetic tests on the initial bone biopsies: we have classified assays according to those assessing genetic uniqueness and those assessing cell of origin.

Assays assessing genetic uniqueness. Short tandem repeat (STR) profiling is the gold-standard for human cell line authentication and a requirement for publication,[87] despite this only $n = 3$ (14%) of papers performed this assay. This is deeply troubling as STR profiling can detect interspecies contamination and even discriminate between individuals of the same species, by testing for the presence of a relatively small number of specific allelic markers. Introns specific to OS (Entry 5a, Table 7) can be tested to ensure the genetic identity of PPCL corresponds to the original tumor and is novel by comparison against established STR profile databases (accessible here (https://iclac.org/databases/).[34] Not requiring a high level of technical expertise or equipment, STR profiling is essential for verification: it is a highly discriminatory, a requirement for publication, low-cost, and rapid.[87]

The genetic landscape of OS tumors is highly complex, demonstrating aneuploidy and inconsistent genetic aberrations, with no single targetable genetic event appearing to define this disease.[28] Chromosomal studies by performing G-banded karyotype analysis ($n = 6$, 27%) or SNP array ($n = 1$, 5%) can help shed light on the genomic drivers of OS, but with no recurring karyotype reported to date it may be difficult to confirm genetic identity of PCCL even with comparison against the baseline biopsy tissue due to its complex karyotype. Additionally, about 70% of OS cases have altered genotypes, which may include gain of chromosome 1, loss of 13 (RB1 gene), and 17 (TP53).[121] Based on the limits of current scientific understanding, a genotype profile investigating major tumor suppressor or oncogenes, such as TP53 and RB1 as in $n = 2$ (9%) of studies, may be less helpful for identification considering the high degree of genomic instability.[26]

Ensuring the genetic novelty and concordance with the original tissue is essential for verifying the new PCCL, which can easily be done with STR profiling. Although karyotyping and genotype profiling may be less helpful for the sole aim of proving identity, there remains much to be discovered on the genomic landscape of OS. Such tests can shed light on new genomic drivers and characteristics, potentially discovering new identifiable markers, particularly if used in conjunction with other genomic tests (additional assays to consider).

Assays assessing cell of origin. Assurance of identity is required to ensure that key phenotypic characteristics of the PCCL have not changed throughout its lifetime *in vitro*, by comparison against the cell type identified on initial tumor sample.[88] As evidenced by the multitude of tests used in the eligible studies, this verification can be done by a wide variety of assays. However, to avoid unnecessarily prolonging this authentication step, careful consideration must be employed to avoid doubling up on assays studying similar phenotypic characteristics.

Real-time PCR (RT-PCR) is a rapid molecular assay used to identify cell marker expression on a transcriptional level, used in $n = 8$ (36%) of studies. Specific transcriptional markers can be selected to confirm a particular OS histological subtype (osteoblastic, chondrocyte, etc.), or certain cancer characteristics (migration/metastatic markers) (Entry 5b, Table 7). With results obtainable in a few hours, RT-PCR is a powerful tool to confirm that the PCCL corresponds with the original tumor, by expression of the same selected markers.

Immunohistochemistry (IHC) identifies expression of cell markers on a translational level within the cell performed by 32% ($n = 7$) of studies. A useful tool for molecular studies, IHC studies the same markers used in RT-PCR and may be useful to ensure that the same markers are

expressed translationally. Western blot (WB) was used in $n = 2$ (9%) to quantify the expression of a specific protein in a tissue sample: cells are lysed to release their intracellular proteins which are separated by molecular weight through gel electrophoresis, before finally being transferred onto the membrane surface for quantification. Compared to RT-PCR and IHC (which are usually qualitative), WB can facilitate quantification of protein levels, which may help confirm cell of origin.

Surface antigen analysis by flow cytometry was performed in $n = 2$ (9%) of studies to analyze the presence of MSC surface antigens (CD44, CD90, and CD105) and IGF-2R. Flow cytometry can assess the expression of specific extracellular markers to confirm the cell of origin, where MSC are purported to be a cell of origin for OS although the literature is inconclusive. While tumor-associated antigens are becoming increasingly relevant for developing a serological diagnosis of OS,[78] surface antigen analysis may not yet have sufficient discriminatory power for definitive verification, considering limitations of current techniques and knowledge. One of the diagnostic properties of OS is the differentiation into osteoblasts and the ability to deposit minerals. Differentiation assays can assess mineralization capacity aided by the addition of osteogenic factor-containing media (ascorbic acid, phosphate, and BMP2) and prove osteoblastic origin as done in $n = 4$ (18%) studies. However, these assays are time consuming, with results available after two to three weeks.

Characterization assays

Considering the diversity and complexity of OS, the success of *in vitro* drug studies depends on cell lines retaining their *in vivo* characteristics,[31] characterization assays help study general features of the PCCL to enable comparative study. There is a staggering range of assays that can be done to characterize cell behavior and the non-exhaustive nature of this chapter. Instead, we aim to streamline the assays listed in the eligible studies, briefly highlight other novel assays to consider, and finally discuss 2D and 3D systems of *in vitro* testing; thereby providing readers a solid theoretical base to tackle the huge breadth of possible assays that can be used to characterize and study PCCL.

Assays listed in eligible studies. Morphological characterization was performed in 50% ($n = 11$) of studies to validate the cancer phenotype of the PCCL by correlation with histological sections of the original tumor. Visual examination with phase contrast microscopy can quickly assess if cell morphology is retained across multiple passages.

Doubling time was obtained by $n = 11$ (50%) of studies by MTT assays. In keeping with the heterogeneous nature of OS is heterogeneous, there was a wide range of reported doubling times among the newly established cell lines: 36% ($n = 4$ of 11) of studies had doubling times between 30 and 50h, in keeping with most OS commercial lines of 36h (U2OS, HOS, HOS-143B) and 48h (MG-63, SaOS-2) respectively (Table 1). Notably, $n = 4$ (36%) of studies reported doubling times <30h, suggesting that they are more aggressive than commercial cell lines. Obtaining the doubling time has great value in facilitating comparison against other primary or commercial OS cell lines as a measure of aggressiveness and baseline growth characteristics.

Several assays were used in the eligible studies ($n = 5$, 23%) to assess the invasiveness and metastatic capacity of cell lines, including Boyden-based chamber, cell scratch, soft agar growth assay, and wound healing assay. These *in-vitro* tests can provide a baseline assessment of invasive capacity without performing lengthy *in vivo* tests (which involve injecting tumor cells into PDX and subsequently assessing for lung metastasis).

Assessing the spheroid forming capacity of new cell lines generally took 5–8 days ($n = 4$, 18%) and can measure the self-renewal and multipotent nature of the cancer stem cell subpopulations within the PCCL, lending insight into the cell line's capacity for differentiation and tumorigenicity. Additionally, spheroids have shown to mimic *in vivo* conditions more reliably than standard monolayer culture, by forming an outer proliferating layer and inner necrotic core similar to solid tumors.[78] By providing a 3D environment like the native tumor, spheroids can facilitate more accurate *in vitro* studies. However, performing high throughput *in vitro* studies on spheroids may not be feasible considering the relatively protracted wait time to form spheroids, and the little control over the spheroid size which make it difficult modulate the nutrient and O2 gradients inside the aggregates and necrotic cores.[110] The rising use of 3D scaffold-based *in vitro* cell culture models (appropriate *in vitro* models and other testing systems) may address these limitations whilst still providing a tunable and relevant 3D environment for testing.

Chemosensitivity assays were performed with increasing concentrations of the standard chemotherapy drugs (doxorubicin and cisplatin) ($n = 3$ of 7, 43%) or novel drugs ($n = 4$ of 7, 57%) such as Adriamycin and cyclopamine. These newly established cell lines showed higher resistance to standard chemotherapy compared to commercial cell lines. This shows the clear preclinical utility that new PCCLs could have in generating *in vitro* data that more accurately assesses the efficacy of potential drugs before progressing onto more costly *in vivo* studies. There is also an exciting potential for these PCCL to generate patient-specific data that can inform an individual patient's chemo-response profile.

Roughly half of studies ($n = 13$, 59%) assessed tumorigenicity by inoculating human OS cells into immunodeficient mice and assessing for spontaneous tumor growth and its corresponding histology as compared to the original tissue.[122] This is important as long-term *in vitro* culture of cancer cells may result in genetic alterations that lead to loss of tumorigenicity, necessitating regular checks on tumor initiation potential, undermining the cell line's ability to sustain the original tumor characteristics and thus its predictive clinical power.[122] However, it is an overall expensive and time-consuming process taking at least six weeks for the tumor to grow and subsequently be assessed. However, should the newly established PCCL be immediately used for short-term culture, we are potentially freed from the complications secondary to long term *in vitro* culture, namely loss of heterogeneity and loss of tumorigenicity. As there are shorter alternatives such as spheroid formation assays that can also investigate tumorigenicity, it may not be strictly necessary to perform PDX formation in the short term.

Additional assays to consider. There is a focus among eligible studies on assays that interrogate cell growth, cell viability, invasiveness and chemosensitivity. Emerging technologies like next-generation sequencing (NGS) can generate a wealth of information with relatively minimal

genetic material. Genomic and transcriptomic profiles can correlate oncogenic molecular alterations with cellular drug response to build predictive models of patient drug-response.[123,124] Angiogenesis studies can also shed light on tumor invasiveness considering the hematogenous spread of OS and assess the likely efficacy of novel drugs targeting new vessel formation.

Appropriate in vitro models and other testing systems (2D or 3D). Although the above assays were conducted on cells grown in monolayer, there are growing concerns over data reliability considering the extreme differences between the *in vivo* tumor microenvironment (TME) and the artificial conditions generated *in vitro* by adhesion to 2D plastic surfaces.[125] There was $n = 2$ studies showing a chemosensitivity difference between the same PCCL cultured in 2D and 3D conditions. For reliable *in vitro to in vivo* translation of results, it is vital to optimize culture conditions and grow/test PCCL in appropriate *in vitro* models that assimilate the native TME and recapitulate more *in-vivo-like* behavior.[59] Emerging 3D *in vitro* models such as scaffold-based cell culture can recreate specific TME conditions using biocompatible materials and have shown to be more representative compared to monolayer culture.[1,126]

Using these 3D *in vitro* systems alongside traditional monolayer testing will continue to be helpful as the inter-tumor heterogeneity of OS suggests that no single model system will be effective to test the therapeutic potential of specific drugs across all OS.[63] In particular, High Content Systems can play a crucial role in advancing drug discovery and understanding cellular responses by offering the capability to analyze multiple parameters in a single experiment.

(1) Operetta CLS High-Content Analysis System: This system integrates automated microscopy and image analysis to assess various cellular features, such as morphology, fluorescence intensity, and translocation, allowing for comprehensive drug response analysis.
(2) ImageXpress Micro Confocal High-Content Imaging System: Equipped with confocal imaging capabilities, this system enables the examination of cellular responses to drugs in three dimensions, along with the assessment of parameters like cell viability, apoptosis, and protein expression levels.
(3) IN Cell Analyzer Series: The IN Cell Analyzer platform provides high-throughput, high-content imaging for drug screening applications. It allows the analysis of multiple cellular parameters, including subcellular localization, cell morphology, and dynamic cellular processes, facilitating a thorough examination of drug effects.
(4) ArrayScan XTI High-Content Analysis (HCA) Reader: This system combines high-throughput imaging with powerful bioinformatics analysis, allowing the simultaneous evaluation of cellular responses to drugs across various endpoints such as cell cycle progression, nuclear morphology, and cytoplasmic intensity.
(5) CellInsight CX7 High-Content Analysis System: This system offers a flexible and scalable platform for the analysis of cellular responses to drugs. It covers a wide range of parameters, including cell health, mitochondrial function, and intracellular signaling, providing a holistic view of drug effects on cells.

Assays assessing purity

Studies using contaminated cell lines are not reproducible, and a purity check is extremely important to exclude the possibility of working with unwanted models and producing false and unreliable data. Unfortunately, cell lines generally become cross-contaminated early due to faulty cell handling.[36] Adhering to proper handling techniques and performing purity assays will help detect cellular contamination, resulting in more reliable results. Mycoplasma is a bacterium that frequently contaminates mammalian tissue cultures, and their presence affects cell behavior. Although employed in only $n = 5$ (23%) of studies, mycoplasma screening is easily performed with commercial kits and is crucial to ensure non-contamination of the PCCL. Fibroblasts are present in the OS ECM and may be present if disaggregation of the solid tissue was not adequately performed, which may rapidly outgrowth the cancer cell population. Two studies (9%) labeled fibroblasts and scraped them off physically. Additionally, Geneticin is an antibiotic with a selective action on fibroblasts and helps their overgrowth, with little interference on cancer cell survival. In addition to these purity assessment assays, we recommend following the ICLAC guidelines on sterile handing; briefly, they include keeping the number of handlings to a minimum, reducing exposure to unsterile handling, reducing exposure to other chemicals/agents.[34]

Limitations and future perspectives

A protocol to establish PCCL prior to the second course of NAC (that could possibly guide clinical decision) has not been proposed before, and we acknowledge the immense difficulty of accomplishing this within six weeks. This study performed an extensive theoretical assessment of the past three decades of OS cell line establishment methods within a systematic framework. Although logically deduced, our proposed definitions regarding a "clinically actionable time frame", when the PCCL becomes "established", and the number of "sufficient cells" needed for adequate testing, remain largely theoretical until experimentally determined. As no existing definitions were indicated by the eligible studies, it is difficult to make any useful comparisons and critically evaluate the efficiency of one methodology over another. Furthermore, every subsequent passage or subculture will inevitably select for a dominant cancer clone. Using PCCL for reliable therapeutic correlation or scientific study must account for these inherent limitations, making it crucial to use appropriate and well-characterized *in vitro* or *in vivo* models alongside these novel cell lines. While not a step-by-step lab-based protocol at this stage, our framework serves as a useful 'map' for accelerating systematic optimization of PCCL establishment within OS management. Categorizing the cell line establishment process into five Entry points facilitates "modular" experimentation, where the potential solutions for each step can be tested in a systematic fashion. For example, refining 3D suspension cultures for scale-up proliferation can be done using readily available commercial cell lines first to optimize conditions before using patient samples in preclinical studies.

Conclusion

A protocol that can rapidly establish PCCL for OS within a clinically actionable time frame and grow sufficient cells for statistically meaningful testing would be immensely useful. New PCCL can augment the range of OS cell lines researchers can employ for preclinical study, while patient-specific data have the potential for therapeutic correlation and informing clinicians. Validating and growing 10^9 cells within six weeks from primary tumor tissue (biopsy) is unprecedented and likely exceedingly difficult with current scientific techniques. By systematically reviewing the methodology of novel OS cell lines published in the past three decades, we explore the feasibility of this aim by categorizing the methodology into five main Entry and theoretically identified barriers and potential solutions for each. This provides a "framework" for groups to compare methodology in a standardized manner and accelerate modular optimization of each step. This study has.

I. Provided definitions to enable comparison of methodology:
   a. "clinically actionable time frame" of six weeks
   b. "establish" when identity coincides with original tumor tissue
   c. "sufficient cells" for testing comprises at least 10^9 cells

II. Delineated five Entry of methodology: helps to facilitates modular and systematic experimental optimization.
III. Identified barriers and potential solutions for each Entry: groups can see state-of-the-art alongside avenues of future exploration.
IV. Compiled authentication assays according to three criteria: helps standardize validation of future cell lines.

ACKNOWLEDGMENTS

Funding for this work was gratefully received from NHMRC-MRFF Investigator Grant (Di Bella) (#1193897). We extend our sincere gratitude to Dr Alexandre Xavier Mendes, Swinburne University, for his invaluable contribution to the creation of the graphical abstract for this paper.

AUTHOR CONTRIBUTIONS

T.C.: performed the literature review and critical data analyses, prepared the Tables and Diagrams, and wrote the manuscript.

W.H.: collaborated on writing and critical analyses with a particular focus on 3D cell culture systems.

E.L. and C.O.: performed critical review of the manuscript from a cell biologist's perspective. P.F.C. and C.D.B.: performed critical review of the manuscript from an orthopedic surgeon's perspective.

S.D. conceived the study, supervised T.C. and W.H. in writing and data analysis, wrote and revised the manuscript.

DECLARATION OF INTERESTS

This work was supported by Research Endowment Fund 2022 – St. Vincent's Hospital Melbourne. The authors declare no competing interests.

REFERENCES

1. Rodrigues, J., Sarmento, B., and Pereira, C.L. (2022). Osteosarcoma tumor microenvironment: the key for the successful development of biologically relevant 3D *in vitro* models. In Vitro Models *1*, 5–27. https://doi.org/10.1007/s44164-022-00008-x.
2. Tippett, V.L., Tattersall, L., Ab Latif, N.B., Shah, K.M., Lawson, M.A., and Gartland, A. (2023). The strategy and clinical relevance of *in vitro* models of MAP resistance in osteosarcoma: a systematic review. Oncogene *42*, 259–277. https://doi.org/10.1038/s41388-022-02529-x.
3. Jafari, F., Javdansirat, S., Sanaie, S., Naseri, A., Shamekh, A., Rostamzadeh, D., and Dolati, S. (2020). Osteosarcoma: A comprehensive review of management and treatment strategies. Ann. Diagn. Pathol. *49*, 151654. https://doi.org/10.1016/j.anndiagpath.2020.151654.
4. De Luca, A., Bellavia, D., Raimondi, L., Carina, V., Costa, V., Fini, M., and Giavaresi, G. (2022). Multiple Effects of Resveratrol on Osteosarcoma Cell Lines. Pharmaceuticals *15*, 342. https://doi.org/10.3390/ph15030342.
5. Schott, C.R., Koehne, A.L., Sayles, L.C., Young, E.P., Luck, C., Yu, K., Lee, A.G., Breese, M.R., Leung, S.G., Xu, H., et al. (2024). Osteosarcoma PDX-Derived Cell Line Models for Preclinical Drug Evaluation Demonstrate Metastasis Inhibition by Dinaciclib through a Genome-Targeted Approach. Clin. Cancer Res. *30*, 849–864. https://doi.org/10.1158/1078-0432.ccr-23-0873.
6. Zhou, Z., Li, Y., Yan, X., Wang, X., Yang, C., Wei, H., Yang, X., and Xiao, J. (2017). Does rarity mean imparity? Biological characteristics of osteosarcoma cells originating from the spine. J. Cancer Res. Clin. Oncol. *143*, 1959–1969. https://doi.org/10.1007/s00432-017-2448-9.
7. Fodstad, Ø., Brøgger, A., Bruland, Ø., Solheim, O.P., Nesland, J.M., and Pihl, A. (1986). Characteristics of a cell line established from a patient with multiple osteosarcoma, appearing 13 years after treatment for bilateral retinoblastoma. Int. J. Cancer *38*, 33–40. https://doi.org/10.1002/ijc.2910380107.
8. Eilon, G., Perkins, J., and Viola, M.V. (1983). Characteristics of a calcitonin-responsive cell line derived from a human osteosarcoma. Cancer Res. *43*, 3763–3769.
9. Sonobe, H., Mizobuchi, H., Manabe, Y., Furihata, M., Iwata, J., Hikita, T., Oka, T., Ohtsuki, Y., and Goto, T. (1991). Morphological characterization of a newly established human osteosarcoma cell line, HS-Os-1, revealing its distinct osteoblastic nature. Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. *60*, 181–187. https://doi.org/10.1007/BF02899545.
10. Yoshikawa, H., Ohishi, M., Kohriki, S., Yoshiura, M., and Ohsaki, Y. (1997). Establishment and characterisation of an osteoblastic clonal cell line from human mandibular osteosarcoma (HMOS-1). Oral Oncol. *33*, 163–168. https://doi.org/10.1016/S0964-1955(96)00075-9.
11. Kawai, K., Matsuhisa, T., Sakurai, M., Taniguchi, Y., and Yatani, R. (1999). Establishment and characterization of a second primary osteosarcoma cell line (OSrb/N-M) from a patient cured of bilateral retinoblastoma. Cancer Lett. *135*, 37–46. https://doi.org/10.1016/S0304-3835(98)00272-9.

12. Rochet, N., Dubousset, J., Mazeau, C., Zanghellini, E., Farges, M.-F., Stora Novion, H.D.E., Chompret, A., Delpech, B., Cattan, N., Frenay, M., et al. (1999). Establishment, characterisation and partial cytokine expression profile of a new human osteosarcoma cell line (CAL 72) (Wiley-Liss, Inc).
13. Scotlandi, K., Serra, M., Landuzzi, L., and Baldini, N. (1992). SARG: A new human osteosarcoma cell line. Expression of bone markers and of major histocompatibility antigens. Ann. Oncol. *3*, S29–S31. https://doi.org/10.1093/annonc/3.suppl_2.S29.
14. Chen, X., Yang, T.T., Wang, W., Sun, H.H., Ma, B.A., Li, C.X., Ma, Q., Yu, Z., and Fan, Q.Y. (2009). Establishment and characterization of human osteosarcoma cell lines with different pulmonary metastatic potentials. Cytotechnology *61*, 37–44. https://doi.org/10.1007/s10616-009-9239-3.
15. Hinohara, S., Satake, N., Sekine, K., and Kaneko, Y. (1998). Abnormalities of the FHIT transcripts in osteosarcoma and Ewing sarcoma. Jpn. J. Cancer Res. *89*, 887–894. https://doi.org/10.1111/j.1349-7006.1998.tb00645.x.
16. Tachibana, H., Kakuta, S., Yagami, K., and Nagumo, M. (2000). Experimental Oral Biology: Effects of cytokines on the production of nitric oxide in a chondrogenic cell line established from human osteogenic sarcoma. Oral Dis. *6*, 303–309. https://doi.org/10.1111/j.1601-0825.2000.tb00143.x.
17. Sakayama, K., Kidani, T., Miyazaki, T., Shirakata, H., Kimura, Y., Kamogawa, J., Masuno, H., and Yamamoto, H. (2004). Effect of ketoprofen in topical formulation on vascular endothelial growth factor expression and tumor growth in nude mice with osteosarcoma. J. Orthop. Res. *22*, 1168–1174. https://doi.org/10.1016/j.orthres.2004.03.015.
18. Zhan, F.-B., Zhang, X.-W., Feng, S.-L., Cheng, J., Zhang, Y., Li, B., Xie, L.-Z., and Deng, Q.-R. (2019). MicroRNA-206 Reduces Osteosarcoma Cell Malignancy *In Vitro* by Targeting the PAX3-MET Axis. Yonsei Med. J. *60*, 163–173.
19. Harisi, R., Dudas, J., Nagy-Olah, J., Timar, F., Szendroi, M., and Jeney, A. (2007). Extracellular matrix induces doxorubicin-resistance in human osteosarcoma cells by suppression of p53 function. Cancer Biol. Ther. *6*, 1240–1246. https://doi.org/10.4161/cbt.6.8.4443.
20. Blattmann, C., Thiemann, M., Stenzinger, A., Roth, E.K., Dittmar, A., Witt, H., Lehner, B., Renker, E., Jugold, M., Eichwald, V., et al. (2015). Establishment of a patient-derived orthotopic osteosarcoma mouse model. J. Transl. Med. *13*, 136. https://doi.org/10.1186/s12967-015-0497-x.
21. Siggelkow, H., Niedhart, C., Kurre, W., Ihbe, A., Schulz, A., Atkinson, M.J., and Hüfner, M. (1998). *In vitro* differentiation potential of a new human osteosarcoma cell line (HOS 58). Differentiation. *63*, 81–91. https://doi.org/10.1046/j.1432-0436.1998.6320081.x.
22. Takaoka, K., Yoshikawa, H., Masuhara, K., Sugamoto, K., Tsuda, T., Aoki, Y., Ono, K., and Sakamoto, Y. (1989). Establishment of a cell line producing bone morphogenetic protein from a human osteosarcoma. Clin. Orthop. Relat. Res. *244*, 258–264. https://doi.org/10.1097/00003086-198907000-00028.
23. Kawai, A., Ozaki, T., Ikeda, S., Oda, T., Miyazaki, M., Sato, J., Taketa, K., and Inoue, H. (1989). Two distinct cell lines derived from a human osteosarcoma. J. Cancer Res. Clin. Oncol. *115*, 531–536.
24. Kawai, A. (1990). A newly established human osteosarcoma cell line with osteoblastic properties. Clin. Orthop. Relat. Res. *259*, 256–267. https://doi.org/10.1097/00003086-199010000-00036.
25. Yamane, T. (1985). Establishment and characterization of cell lines derived from a human osteosarcoma. Clin. Orthop. Relat. Res. *199*, 261–271. https://doi.org/10.1097/00003086-198510000-00038.
26. Gomez-Brouchet, A., Gilhodes, J., Acker, N.V., Brion, R., Bouvier, C., Assemat, P., Gaspar, N., Aubert, S., Guinebretiere, J.-M., Marie, B., et al. (2021). Characterization of Macrophages and Osteoclasts in the Osteosarcoma Tumor Microenvironment at Diagnosis: New Perspective for Osteosarcoma Treatment? Cancers *13*, 423. https://doi.org/10.3390/cancers13030423.
27. Ottaviano, L., Schaefer, K.L., Gajewski, M., Huckenbeck, W., Baldus, S., Rogel, U., Mackintosh, C., de Alava, E., Myklebost, O., Kresse, S.H., et al. (2010). Molecular characterization of commonly used cell lines for bone tumor research: A trans-European EuroBoNet effort. Genes Chromosomes Cancer *49*, 40–51. https://doi.org/10.1002/gcc.20717.
28. Mohseny, A.B., MacHado, I., Cai, Y., Schaefer, K.L., Serra, M., Hogendoorn, P.C.W., Llombart-Bosch, A., and Cleton-Jansen, A.M. (2011). Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. Lab. Invest. *91*, 1195–1205. https://doi.org/10.1038/labinvest.2011.72.
29. Kresse, S.H., Rydbeck, H., Skårn, M., Namløs, H.M., Barragan-Polania, A.H., Cleton-Jansen, A.M., Serra, M., Liestøl, K., Hogendoorn, P.C.W., Hovig, E., et al. (2012). Integrative Analysis Reveals Relationships of Genetic and Epigenetic Alterations in Osteosarcoma. PLoS One *7*, e48262. https://doi.org/10.1371/journal.pone.0048262.
30. Lauvrak, S.U., Munthe, E., Kresse, S.H., Stratford, E.W., Namløs, H.M., Meza-Zepeda, L.A., and Myklebost, O. (2013). Functional characterisation of osteosarcoma cell lines and identification of mRNAs and miRNAs associated with aggressive cancer phenotypes. Br. J. Cancer *109*, 2228–2236. https://doi.org/10.1038/bjc.2013.549.
31. Kondo, T. (2020). Current status and perspectives of patient-derived rare cancer models. Hum. Cell *33*, 919–929. https://doi.org/10.1007/s13577-020-00391-1.
32. Hattori, E., Oyama, R., and Kondo, T. (2019). Systematic review of the current status of human sarcoma cell lines. Cells *8*, 157. https://doi.org/10.3390/cells8020157.
33. Sarntivijai, S., Lin, Y., Xiang, Z., Meehan, T.F., Diehl, A.D., Vempati, U.D., Schürer, S.C., Pang, C., Malone, J., Parkinson, H., et al. (2014). CLO: The cell line ontology. J. Biomed. Semantics *5*, 37. https://doi.org/10.1186/2041-1480-5-37.
34. Mirabelli, P., Coppola, L., and Salvatore, M. (2019). Cancer cell lines are useful model systems for medical research. Cancers *11*, 1098. https://doi.org/10.3390/cancers11081098.
35. Richter, M., Piwocka, O., Musielak, M., Piotrowski, I., Suchorska, W.M., and Trzeciak, T. (2021). From Donor to the Lab: A Fascinating Journey of Primary Cell Lines. Frontiers Media S.A. *9*, 711381. https://doi.org/10.3389/fcell.2021.711381.
36. Miserocchi, G., Mercatali, L., Liverani, C., De Vita, A., Spadazzi, C., Pieri, F., Bongiovanni, A., Recine, F., Amadori, D., and Ibrahim, T. (2017). Management and potentialities of primary cancer cultures in preclinical and translational studies. J. Transl. Med. *15*, 229. https://doi.org/10.1186/s12967-017-1328-z.
37. Esparza-Lopez, J., Martinez-Aguilar, J., and Ibarra-Sanchez, M. (2019). Deriving Primary Cancer Cell Cultures for Personalized Therapy. Clin. Transl. Invest. *71*, 369–380.
38. Mitra, A., Mishra, L., and Li, S. (2013). Technologies for deriving primary tumor cells for use in personalized cancer therapy. Trends Biotechnol. *31*, 347–354. https://doi.org/10.1016/j.tibtech.2013.03.006.
39. Kodack, D.P., Farago, A.F., Dastur, A., Held, M.A., Dardaei, L., Friboulet, L., von Flotow, F., Damon, L.J., Lee, D., Parks, M., et al. (2017). Primary Patient-Derived Cancer Cells and Their Potential for Personalized Cancer Patient Care. Cell Rep. *21*, 3298–3309. https://doi.org/10.1016/j.celrep.2017.11.051.
40. Hu, Z., Wen, S., Huo, Z., Wang, Q., Zhao, J., Wang, Z., Chen, Y., Zhang, L., Zhou, F., Guo, Z., et al. (2022). Current Status and Prospects of Targeted Therapy for Osteosarcoma. Cells *11*, 3507. https://doi.org/10.3390/cells11213507.
41. Chen, X., Bahrami, A., Pappo, A., Easton, J., Dalton, J., Hedlund, E., Ellison, D., Shurtleff, S., Wu, G., Wei, L., et al. (2014). Recurrent somatic structural variations contribute to tumorigenesis in pediatric osteosarcoma. Cell Rep. *7*, 104–112. https://doi.org/10.1016/j.celrep.2014.03.003.
42. De Luca, A., Bellavia, D., Raimondi, L., Carina, V., Costa, V., Fini, M., and Giavaresi, G. (2022). Multiple Effects of Resveratrol on Osteosarcoma Cell Lines. Pharmaceuticals *15*, 342. https://doi.org/10.3390/ph15030342.
43. Wensink, G.E., Elias, S.G., Mullenders, J., Koopman, M., Boj, S.F., Kranenburg, O.W., and Roodhart, J.M.L. (2021). Patient-derived organoids as a predictive biomarker for treatment response in cancer patients. npj Precis. Oncol. *5*, 30. https://doi.org/10.1038/s41698-021-00168-1.
44. Wang, H.M., Zhang, C.Y., Peng, K.C., Chen, Z.X., Su, J.W., Li, Y.F., Li, W.F., Gao, Q.Y., Zhang, S.L., Chen, Y.Q., et al. (2023). Using patient-derived organoids to predict locally advanced or metastatic lung cancer tumor response: A real-world study. Cell Rep. Med. *4*, 100911. https://doi.org/10.1016/j.xcrm.2022.100911.
45. Su, C., Olsen, K.A., Bond, C.E., and Whitehall, V.L.J. (2023). The Efficacy of Using Patient-Derived Organoids to Predict Treatment Response in Colorectal Cancer. Cancers *15*, 805. https://doi.org/10.3390/cancers15030805.
46. Verduin, M., Hoeben, A., De Ruysscher, D., and Vooijs, M. (2021). Patient-Derived Cancer Organoids as Predictors of Treatment Response. Front. Oncol. *11*, 641980. https://doi.org/10.3389/fonc.2021.641980.
47. De Luca, A., Raimondi, L., Salamanna, F., Carina, V., Costa, V., Bellavia, D., Alessandro, R., Fini, M., and Giavaresi, G.

(2018). Relevance of 3d culture systems to study osteosarcoma environment. J. Exp. Clin. Cancer Res. *37*, 2. https://doi.org/10.1186/s13046-017-0663-5.

48. Li, Y., and Kumacheva, E. (2018). Hydrogel microenvironments for cancer spheroid growth and drug screening. Sci. Adv. *4*, eaas8998.
49. Rodrigues, J., Sarmento, B., and Pereira, C.L. (2022). Osteosarcoma tumor microenvironment: the key for the successful development of biologically relevant 3D *in vitro* models. In Vitro Models *1*, 5–27. https://doi.org/10.1007/s44164-022-00008-x.
50. Alfranca, A., Martinez-Cruzado, L., Tornin, J., Abarrategi, A., Amaral, T., de Alava, E., Menendez, P., Garcia-Castro, J., and Rodriguez, R. (2015). Bone microenvironment signals in osteosarcoma development. Cell. Mol. Life Sci. *72*, 16. https://doi.org/10.1007/s00018-015-1918-y.
51. Zhang, Y., Mai, Q., Zhang, X., Xie, C., and Zhang, Y. (2017). Microenvironment Signals and Mechanisms in the Regulation of Osteosarcoma. In Osteosarcoma - Biology, Behavior and Mechanisms, K. Honoki and K. Weiss, eds. https://www.intechopen.com/chapters/53766.
52. Gao, S., Shen, J., Hornicek, F., and Duan, Z. (2017). Three-dimensional (3D) culture in sarcoma research and the clinical significance. Biofabrication *9*, 032003. https://doi.org/10.1088/1758-5090/aa7fdb.
53. Kwakwa, K.A., Vanderburgh, J.P., Guelcher, S.A., and Sterling, J.A. (2017). Engineering 3D Models of Tumors and Bone to Understand Tumor-Induced Bone Disease and Improve Treatments. Curr. Osteoporos. Rep. *15*, 247–254. https://doi.org/10.1007/s11914-017-0385-9.
54. Sitarski, A.M., Fairfield, H., Falank, C., and Reagan, M.R. (2018). 3d Tissue Engineered *In Vitro* Models Of Cancer In Bone. ACS Biomater. Sci. Eng. *4*, 324–336. https://doi.org/10.1021/acsbiomaterials.7b00097.
55. Gaebler, M., Silvestri, A., Haybaeck, J., Reichardt, P., Lowery, C.D., Stancato, L.F., Zybarth, G., and Regenbrecht, C.R.A. (2017). Three-Dimensional Patient-Derived *In Vitro* Sarcoma Models: Promising Tools for Improving Clinical Tumor Management. Front. Oncol. *7*, 203. https://doi.org/10.3389/fonc.2017.00203.
56. Mitra, A., Mishra, L., and Li, S. (2013). Technologies for deriving primary tumor cells for use in personalized cancer therapy. Trends Biotechnol. *31*, 347–354. https://doi.org/10.1016/j.tibtech.2013.03.006.
57. Chew, N., Habarakada, D., Firestein, R., and Daniel, P. (2023). A protocol to establish cell line models from rare pediatric solid tumors. STAR Protoc. *4*, 102537. https://doi.org/10.1016/j.xpro.2023.102537.
58. Choong, P.F.M. (2021). In Sarcoma: A Practical Guide to Multidisciplinary Management, P.F.M. Choong, ed. (Springer). https://doi.org/10.1007/978-981-15-9414-4.
59. Chow, T., Wutami, I., Lucarelli, E., Choong, P.F., Duchi, S., and Di Bella, C. (2021). Creating *in Vitro* Three-Dimensional Tumor Models: A Guide for the Biofabrication of a Primary Osteosarcoma Model. Tissue Eng. Part B Rev. *27*, 514–529. https://doi.org/10.1089/ten.teb.2020.0254.
60. Boghaert, E.R., Lu, X., Hessler, P.E., McGonigal, T.P., Oleksijew, A., Mitten, M.J., Foster-Duke, K., Hickson, J.A., Santo, V.E., Brito, C., et al. (2017). The Volume of Three-Dimensional Cultures of Cancer Cells *In Vitro* Influences Transcriptional Profile Differences and Similarities with Monolayer Cultures and Xenografted Tumors. Neoplasia *19*, 695–706. https://doi.org/10.1016/j.neo.2017.06.004.
61. Monteiro, M.V., Gaspar, V.M., Ferreira, L.P., and Mano, J.F. (2020). Hydrogel 3D *in vitro* tumor models for screening cell aggregation mediated drug response. Biomater. Sci. *8*, 1855–1864. https://doi.org/10.1039/C9BM02075F.
62. Langdon, S.P. (2004). Cancer Cell Culture: Methods and Protocols*88* (Humana Press).
63. Richter, M., Piwocka, O., Musielak, M., Piotrowski, I., Suchorska, W.M., and Trzeciak, T. (2021). From Donor to the Lab: A Fascinating Journey of Primary Cell Lines. Front. Cell Dev. Biol. *9*, 711381. https://doi.org/10.3389/fcell.2021.711381.
64. Palmini, G., Romagnoli, C., Donati, S., Zonefrati, R., Galli, G., Marini, F., Iantomasi, T., Aldinucci, A., Leoncini, G., Franchi, A., et al. (2021). Analysis of a Preliminary microRNA Expression Signature in a Human Telangiectatic Osteogenic Sarcoma Cancer Cell Line. Int. J. Mol. Sci. *22*, 1163. https://doi.org/10.3390/ijms22031163.
65. Mizushima, E., Tsukahara, T., Emori, M., Murata, K., Akamatsu, A., Shibayama, Y., Hamada, S., Watanabe, Y., Kaya, M., Hirohashi, Y., et al. (2020). Osteosarcoma-initiating cells show high aerobic glycolysis and attenuation of oxidative phosphorylation mediated by LIN28B. Cancer Sci. *111*, 36–46. https://doi.org/10.1111/cas.14229.
66. Martínez-Vélez, N., Xipell, E., Vera, B., Acanda de la Rocha, A., Zalacain, M., Marrodán, L., Gonzalez-Huarriz, M., Toledo, G., Cascallo, M., Alemany, R., et al. (2016). The Oncolytic Adenovirus VCN-01 as Therapeutic Approach Against Pediatric Osteosarcoma. Clin. Cancer Res. *22*, 2217–2225. https://doi.org/10.1158/1078-0432.CCR-15-1899.
67. Palmini, G., Zonefrati, R., Romagnoli, C., Aldinucci, A., Mavilia, C., Leoncini, G., Franchi, A., Capanna, R., and Brandi, M.L. (2016). Establishment and Characterization of a Human Small Cell Osteosarcoma Cancer Stem Cell Line: A New Possible *In Vitro* Model for Discovering Small Cell Osteosarcoma Biology. Stem Cells Int. *2016*, 3042198. https://doi.org/10.1155/2016/3042198.
68. Hassan, S.E., Bekarev, M., Kim, M.Y., Lin, J., Piperdi, S., Gorlick, R., and Geller, D.S. (2012). Cell surface receptor expression patterns in osteosarcoma. Cancer *118*, 740–749. https://doi.org/10.1002/cncr.26339.
69. Pereira, B.P., Zhou, Y., Gupta, A., Leong, D.T., Aung, K.Z., Ling, L., Pho, R.W.H., Galindo, M., Salto-Tellez, M., Stein, G.S., et al. (2009). Runx2, p53, and pRB status as diagnostic parameters for deregulation of osteoblast growth and differentiation in a new pre-chemotherapeutic osteosarcoma cell line (OS1). J. Cell. Physiol. *221*, 778–788. https://doi.org/10.1002/jcp.21921.
70. Nathan, S.S., Pereira, B.P., Zhou, Y.F., Gupta, A., Dombrowski, C., Soong, R., Pho, R.W.H., Stein, G.S., Salto-Tellez, M., Cool, S.M., and van Wijnen, A.J. (2009). Elevated expression of Runx2 as a key parameter in the etiology of osteosarcoma. Mol. Biol. Rep. *36*, 153–158. https://doi.org/10.1007/s11033-008-9378-1.
71. Yasuda, T., Kanamori, M., Nogami, S., Hori, T., Oya, T., Suzuki, K., and Kimura, T. (2009). Establishment of a new human osteosarcoma cell line, UTOS-1: cytogenetic characterization by array comparative genomic hybridization. J. Exp. Clin. Cancer Res. *28*, 26. https://doi.org/10.1186/1756-9966-28-26.
72. Gillette, J.M., Gibbs, C.P., and Nielsen-Preiss, S.M. (2008). Establishment and characterization of OS 99-1, a cell line derived from a highly aggressive primary human osteosarcoma. In Vitro Cell. Dev. Biol. Anim. *44*, 87–95. https://doi.org/10.1007/s11626-007-9075-8.
73. Veselska, R., Hermanova, M., Loja, T., Chlapek, P., Zambo, I., Vesely, K., Zitterbart, K., and Sterba, J. (2008). Nestin expression in osteosarcomas and derivation of nestin/CD133 positive osteosarcoma cell lines. BMC Cancer *8*, 300. https://doi.org/10.1186/1471-2407-8-300.
74. Warzecha, J., Göttig, S., Chow, K.U., Brüning, C., Percic, D., Boehrer, S., Brude, E., and Kurth, A. (2007). Inhibition of Osteosarcoma Cell Proliferation by the Hedgehog-Inhibitor Cyclopamine. J. Chemother. *19*, 554–561. https://doi.org/10.1179/joc.2007.19.5.554.
75. Hitora, T., Yamamoto, T., Akisue, T., Marui, T., Nakatani, T., Kawamoto, T., Nagira, K., Yoshiya, S., and Kurosaka, M. (2005). Establishment and characterization of a KIT-positive and stem cell factor-producing cell line, KTHOS, derived from human osteosarcoma. Pathol. Int. *55*, 41–47. https://doi.org/10.1111/j.1440-1827.2005.01787.x.
76. Tsukahara, T., Nabeta, Y., Kawaguchi, S., Ikeda, H., Sato, Y., Shimozawa, K., Ida, K., Asanuma, H., Hirohashi, Y., Torigoe, T., et al. (2004). Identification of human autologous cytotoxic T-lymphocyte-defined osteosarcoma gene that encodes a transcriptional regulator, papillomavirus binding factor. Cancer Res. *64*, 5442–5448. https://doi.org/10.1158/0008-5472.CAN-04-0522.
77. Nabeta, Y., Kawaguchi, S., Sahara, H., Ikeda, H., Hirohashi, Y., Goroku, T., Sato, Y., Tsukahara, T., Torigoe, T., Wada, T., et al. (2003). Recognition by cellular and humoral autologous immunity in a human osteosarcoma cell line. J. Orthop. Sci. *8*, 554–559. https://doi.org/10.1007/s00776-003-0663-5.
78. Thanindratarn, P., Li, X., Dean, D.C., Nelson, S.D., Hornicek, F.J., and Duan, Z. (2020). Establishment and Characterization of a Recurrent Osteosarcoma Cell Line: OSA 1777. J. Orthop. Res. *38*, 902–910. https://doi.org/10.1002/jor.24528.
79. Liu, Y., Feng, X., Zhang, Y., Jiang, H., Cai, X., Yan, X., Huang, Z., Mo, F., Yang, W., Yang, C., et al. (2016). Establishment and characterization of a novel osteosarcoma cell line: CHOS. J. Orthop. Res. *34*, 2116–2125. https://doi.org/10.1002/jor.23245.
80. Zou, C.Y., Wang, J., Shen, J.N., Huang, G., Jin, S., Yin, J.Q., Guo, Q.C., Li, H.M., Luo, L., Zhang, M., and Zhang, L.J. (2008). Establishment and characteristics of two syngeneic human osteosarcoma cell lines from primary tumor and skip metastases. Acta Pharmacol. Sin. *29*, 325–332. https://doi.org/10.1111/j.1745-7254.2008.00756.x.

81. Minamitani, K. (2000). Establishment of an osteoblastic osteosarcoma cell line and effects of cell culture conditions on secretion of matrix metalloproteinases from the cultured osteosarcoma cells. Kurume Med. J. *47*, 115–124. https://doi.org/10.2739/kurumemedj.47.115.
82. VanCleave, A., Palmer, M., Fang, F., Torres, H., Rodezno, T., Li, Q., Fuglsby, K., Evans, C., Afeworki, Y., Ross, A., et al. (2020). Development and characterization of the novel human osteosarcoma cell line COS-33 with sustained activation of the mTOR pathway. Oncotarget *11*, 2597–2610. https://doi.org/10.18632/oncotarget.27611.
83. Yu, P., Wen, J., Wang, J., Liang, J., Shen, Y., and Zhang, W. (2019). Establishment and characterization of a novel human osteosarcoma cell line for spontaneous pulmonary metastasis research *in vivo*. Ann. Transl. Med. *7*, 573. https://doi.org/10.21037/atm.2019.09.23.
84. Kito, F., Oyama, R., Sakumoto, M., Takahashi, M., Shiozawa, K., Qiao, Z., Sakamoto, H., Hirose, T., Setsu, N., Yoshida, A., et al. (2018). Establishment and characterization of novel patient-derived osteosarcoma xenograft and cell line. In Vitro Cell. Dev. Biol. Anim. *54*, 528–536. https://doi.org/10.1007/s11626-018-0274-2.
85. Bai, C., Yang, M., Fan, Z., Li, S., Gao, T., and Fang, Z. (2015). Associations of chemo- and radio-resistant phenotypes with the gap junction, adhesion and extracellular matrix in a three-dimensional culture model of soft sarcoma. J. Exp. Clin. Cancer Res. *34*, 58. https://doi.org/10.1186/s13046-015-0175-0.
86. Miserocchi, G., Mercatali, L., Liverani, C., De Vita, A., Spadazzi, C., Pieri, F., Bongiovanni, A., Recine, F., Amadori, D., and Ibrahim, T. (2017). Management and potentialities of primary cancer cultures in preclinical and translational studies. J. Transl. Med. *15*, 229. https://doi.org/10.1186/s12967-017-1328-z.
87. Almeida, J.L., Cole, K.D., and Plant, A.L. (2016). Standards for Cell Line Authentication and Beyond. PLoS Biol. *14*, e1002476. https://doi.org/10.1371/journal.pbio.1002476.
88. Fusenig, N.E., Capes-Davis, A., Bianchini, F., Sundell, S., and Lichter, P. (2017). The need for a worldwide consensus for cell line authentication: Experience implementing a mandatory requirement at the International Journal of Cancer. PLoS Biol. *15*, e2001438. https://doi.org/10.1371/journal.pbio.2001438.
89. Kondo, T. (2020). Necessity of guidelines for publication of patient-derived cancer models. Ann. Transl. Med. *8*, 158. https://doi.org/10.21037/atm.2019.12.144.
90. Mills, L.J., Spector, L.G., Largaespada, D.A., and Williams, L.A. (2021). Sex differences in expression of immune elements emerge in children, young adults and mice with osteosarcoma. Biol. Sex Differ. *12*, 5. https://doi.org/10.1186/s13293-020-00347-y.
91. Jafari, F., Javdansirat, S., Sanaie, S., Naseri, A., Shamekh, A., Rostamzadeh, D., and Dolati, S. (2020). Osteosarcoma: A comprehensive review of management and treatment strategies, *49* (W.B. Saunders), pp. 151654. https://doi.org/10.1016/j.anndiagpath.2020.151654.
92. Broadhead, M.L., Clark, J.C.M., Myers, D.E., Dass, C.R., and Choong, P.F.M. (2011). The molecular pathogenesis of osteosarcoma: a review. Sarcoma *2011*, 959248. https://doi.org/10.1155/2011/959248.
93. Hiraga, H., and Ozaki, T. (2021). Adjuvant and Neoadjuvant Chemotherapy for Osteosarcoma: JCOG Bone and Soft Tissue Tumor Study Group (Oxford University Press), pp. 1493–1497. https://doi.org/10.1093/jjco/hyab120.
94. Crenn, V., Vezole, L., Bouhamama, A., Meurgey, A., Karanian, M., Marec-Bérard, P., Gouin, F., and Vaz, G. (2021). Percutaneous core needle biopsy can efficiently and safely diagnose most primary bone tumors. Diagnostics *11*, 1552. https://doi.org/10.3390/diagnostics11091552.
95. Pohlig, F., Kirchhoff, C., Lenze, U., Schauwecker, J., Burgkart, R., Rechl, H., and von Eisenhart-Rothe, R. (2012). Percutaneous core needle biopsy versus open biopsy in diagnostics of bone and soft tissue sarcoma: a retrospective study. Eur. J. Med. Res. *17*, 29. https://doi.org/10.1186/2047-783X-17-29.
96. Palmini, G., Zonefrati, R., Romagnoli, C., Aldinucci, A., Mavilia, C., Leoncini, G., Franchi, A., Capanna, R., and Brandi, M.L. (2016). Establishment and Characterization of a Human Small Cell Osteosarcoma Cancer Stem Cell Line: A New Possible *In Vitro* Model for Discovering Small Cell Osteosarcoma Biology. Stem Cells Int. *2016*, 3042198. https://doi.org/10.1155/2016/3042198.
97. Andrade, R.C., Boroni, M., Amazonas, M.K., and Vargas, F.R. (2021). New drug candidates for osteosarcoma: Drug repurposing based on gene expression signature. Comput. Biol. Med. *134*, 104470. https://doi.org/10.1016/j.compbiomed.2021.104470.
98. Geraghty, R.J., Capes-Davis, A., Davis, J.M., Downward, J., Freshney, R.I., Knezevic, I., Lovell-Badge, R., Masters, J.R.W., Meredith, J., Stacey, G.N., et al. (2014). Guidelines for the Use of Cell Lines in Biomedical Research (Nature Publishing Group), pp. 1021–1046. https://doi.org/10.1038/bjc.2014.166.
99. Bouhamama, A., Leporq, B., Khaled, W., Nemeth, A., Brahmi, M., Dufau, J., Marec-Bérard, P., Drapé, J.L., Gouin, F., Bertrand-Vasseur, A., et al. (2022). Prediction of Histologic Neoadjuvant Chemotherapy Response in Osteosarcoma Using Pretherapeutic MRI Radiomics. Radiol. Imaging Cancer *4*, e210107. https://doi.org/10.1148/rycan.210107.
100. He, F., Xie, L., Sun, X., Xu, J., Li, Y., Liu, R., Sun, K., Shen, D., Gu, J., Ji, T., and Guo, W. (2022). A Scoring System for Predicting Neoadjuvant Chemotherapy Response in Primary High-Grade Bone Sarcomas: A Multicenter Study. Orthop. Surg. *14*, 2499–2509. https://doi.org/10.1111/os.13469.
101. Karkare, S., Allen, K.J.H., Jiao, R., Malo, M.E., Dawicki, W., Helal, M., Godson, D.L., Dickinson, R., Macdonald-Dickinson, V., Yang, R., et al. (2019). Detection and targeting insulin growth factor receptor type 2 (IGF2R) in osteosarcoma PDX in mouse models and in canine osteosarcoma tumors. Sci. Rep. *9*, 11476. https://doi.org/10.1038/s41598-019-47808-y.
102. Smith, H.L., Beers, S.A., Gray, J.C., and Kanczler, J.M. (2020). The role of pre-clinical 3-dimensional models of osteosarcoma. Int. J. Mol. Sci. *21*, 5499. https://doi.org/10.3390/ijms21155499.
103. Lucarelli, E., Beccheroni, A., Donati, D., Sangiorgi, L., Cenacchi, A., Del Vento, A.M., Meotti, C., Bertoja, A.Z., Giardino, R., Fornasari, P.M., et al. (2003). Platelet-derived growth factors enhance proliferation of human stromal stem cells. Biomaterials *24*, 3095–3100. https://doi.org/10.1016/S0142-9612(03)00114-5.
104. Kanno, T., Takahashi, T., Tsujisawa, T., Ariyoshi, W., and Nishihara, T. (2005). Platelet-rich plasma enhances human osteoblast-like cell proliferation and differentiation. J. Oral Maxillofac. Surg. *63*, 362–369. https://doi.org/10.1016/j.joms.2004.07.016.
105. Oh, S.K.W., Chen, A.K., Mok, Y., Chen, X., Lim, U.M., Chin, A., Choo, A.B.H., and Reuveny, S. (2009). Long-term microcarrier suspension cultures of human embryonic stem cells. Stem Cell Res. *2*, 219–230. https://doi.org/10.1016/j.scr.2009.02.005.
106. Rafiq, Q.A., Coopman, K., and Hewitt, C.J. (2013). Scale-up of human mesenchymal stem cell culture: current technologies and future challenges. Curr. Opin. Chem. Eng. *2*, 8–16. https://doi.org/10.1016/j.coche.2013.01.005.
107. Sladkova, M., and De Peppo, G. (2014). Bioreactor Systems for Human Bone Tissue Engineering. Processes *2*, 494–525. https://doi.org/10.3390/pr2020494.
108. Bellani, C.F., Ajeian, J., Duffy, L., Miotto, M., Groenewegen, L., and Connon, C.J. (2020). Scale-Up Technologies for the Manufacture of Adherent Cells. Front. Nutr. *7*, 575146. https://doi.org/10.3389/fnut.2020.575146.
109. Khalili, A.A., and Ahmad, M.R. (2015). A Review of Cell Adhesion Studies for Biomedical and Biological Applications. Int. J. Mol. Sci. *16*, 18149–18184. https://doi.org/10.3390/ijms160818149.
110. Bodiou, V., Moutsatsou, P., and Post, M.J. (2020). Microcarriers for Upscaling Cultured Meat Production. Front. Nutr. *7*, 10. https://doi.org/10.3389/fnut.2020.00010.
111. Bellani, C.F., Ajeian, J., Duffy, L., Miotto, M., Groenewegen, L., and Connon, C.J. (2020). Scale-Up Technologies for the Manufacture of Adherent Cells. Front. Nutr. *7*, 575146. https://doi.org/10.3389/fnut.2020.575146.
112. Yang, F., Wang, S., Li, Y., Li, S., Liu, W., Li, Y., and Hu, H. (2022). Physical optimization of cell proliferation and differentiation using spinner flask and microcarriers. Amb. Express *12*, 63. https://doi.org/10.1186/s13568-022-01397-8.
113. Perez, R.A., Riccardi, K., Altankov, G., and Ginebra, M.P. (2014). Dynamic cell culture on calcium phosphate microcarriers for bone tissue engineering applications. J. Tissue Eng. *5*, 2041731414543965. https://doi.org/10.1177/2041731414543965.
114. Roseti, L., Parisi, V., Petretta, M., Cavallo, C., Desando, G., Bartolotti, I., and Grigolo, B. (2017). Scaffolds for Bone Tissue Engineering: State of the art and new perspectives. Mater. Sci. Eng. C Mater. Biol. Appl. *78*, 1246–1262. https://doi.org/10.1016/j.msec.2017.05.017.
115. Turnbull, G., Clarke, J., Picard, F., Riches, P., Jia, L., Han, F., Li, B., and Shu, W. (2018). 3D bioactive composite scaffolds for bone tissue engineering. Bioact. Mater. *3*, 278–314. https://doi.org/10.1016/j.bioactmat.2017.10.001.
116. Stephenson, M., and Grayson, W. (2018). Recent advances in bioreactors for cell-based therapies. F1000Res. *7*. F1000 Faculty Rev-517. https://doi.org/10.12688/f1000research.12533.1.

117. Trachtenberg, J.E., Santoro, M., Williams, C., Piard, C.M., Smith, B.T., Placone, J.K., Menegaz, B.A., Molina, E.R., Lamhamedi-Cherradi, S.-E., Ludwig, J.A., et al. (2018). Effects of Shear Stress Gradients on Ewing Sarcoma Cells Using 3D Printed Scaffolds and Flow Perfusion. ACS Biomater. Sci. Eng. *4*, 347–356. https://doi.org/10.1021/acsbiomaterials.6b00641.

118. Grayson, W.L., Marolt, D., Bhumiratana, S., Fröhlich, M., Guo, X.E., and Vunjak-Novakovic, G. (2011). Optimizing the medium perfusion rate in bone tissue engineering bioreactors. Biotechnol. Bioeng. *108*, 1159–1170. https://doi.org/10.1002/bit.23024.

119. Perez, R.A., Riccardi, K., Altankov, G., and Ginebra, M.P. (2014). Dynamic cell culture on calcium phosphate microcarriers for bone tissue engineering applications. J. Tissue Eng. *5*, 2041731414543965. https://doi.org/10.1177/2041731414543965.

120. Teixeira, G.Q., Barrias, C.C., Lourenço, A.H., and Gonçalves, R.M. (2014). A multicompartment holder for spinner flasks improves expansion and osteogenic differentiation of mesenchymal stem cells in three-dimensional scaffolds. Tissue Eng. Part C Methods *20*, 984–993. https://doi.org/10.1089/ten.tec.2014.0067.

121. Patiño-García, A., Zalacain, M., Folio, C., Zandueta, C., Sierrasesúmaga, L., San Julián, M., Toledo, G., de Las Rivas, J., and Lecanda, F. (2009). Profiling of chemonaive osteosarcoma and paired-normal cells identifies EBF2 as a mediator of osteoprotegerin inhibition to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Clin. Cancer Res. *15*, 5082–5091. https://doi.org/10.1158/1078-0432.CCR-09-0300.

122. Hattori, E., Oyama, R., and Kondo, T. (2019). Systematic review of the current status of human sarcoma cell lines. Cells *8*, 157. https://doi.org/10.3390/cells8020157.

123. Mirabelli, P., Coppola, L., and Salvatore, M. (2019). Cancer Cell Lines Are Useful Model Systems for Medical Research. Cancers *11*, 1098. https://doi.org/10.3390/cancers11081098.

124. Goodspeed, A., Heiser, L.M., Gray, J.W., and Costello, J.C. (2016). Tumor-Derived Cell Lines as Molecular Models of Cancer Pharmacogenomics. Mol. Cancer Res. *14*, 3–13. https://doi.org/10.1158/1541-7786.MCR-15-0189.

125. Smith, H.L., Beers, S.A., Gray, J.C., and Kanczler, J.M. (2020). The role of pre-clinical 3-dimensional models of osteosarcoma. Int. J. Mol. Sci. *21*, 5499. https://doi.org/10.3390/ijms21155499.

126. Molina, E.R., Chim, L.K., Barrios, S., Ludwig, J.A., and Mikos, A.G. (2020). Modeling the Tumor Microenvironment and Pathogenic Signaling in Bone Sarcoma. Tissue Eng. Part B Rev. *26*, 249–271. https://doi.org/10.1089/ten.teb.2019.0302.