In vitro models of bone-forming tumours: from target to treatment

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Chapter 7

Osteosarcoma 3D patient derived cultures to test genome-informed personalized treatment options; a feasibility study

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Abstract

Osteosarcoma is the most common malignant bone sarcoma, characterized by a complex karyotype. Current treatment for osteosarcoma consists of (neo) adjuvant chemotherapy and surgery, which has not improved survival in the last decades. Therefore there is urgent need for novel therapeutic options for osteosarcoma patients, in particular personalized and targeted treatment options. Potential novel therapies are often tested pre-clinically in 2D *in vitro* models, however, these models do not completely recapitulate the *in vivo* situation, whereas 3D models are more representative. In this study, we have established for the first time long-term primary osteosarcoma 3D cultures, either cultured as hydrogels or microsarcs, for the purpose of pre-clinical drug testing. For seven patients, whole-exome sequencing was performed of corresponding primary tumour tissue to identify potential targets for drug treatment. Three out of seven of the established primary osteosarcoma 3D cultures harboured the same genetic alterations as the primary tumours, while for the remaining four cultures no driver mutations were found to confirm these were tumour-derived. For two patients, the primary osteosarcoma 3D cultures were suitable to evaluate personalized treatment, after which cell viability was assessed and cultures were analysed by histology and immunohistochemistry. One primary osteosarcoma 3D culture with a homozygous loss of *CDKN2A* was sensitive to CDK4/CDK6 inhibitor palbociclib, while the other carried a *MYC* amplification and was sensitive to MYC inhibitor 10058-F4, although control cultures indicated the response was not predicted by the genetic biomarker. To compare sensitivity to drugs of primary osteosarcoma 3D cultures (hydrogels and microsarcs) with 2D cultures or 2D cells propagated in a 3D environment, a panel of potential novel treatment options for osteosarcoma was used and histology and cell viability were assessed. Drug treated osteosarcoma hydrogels and microsarcs were less sensitive to drugs compared to 2D cultured cells, or 2D cells cultured as multi-cellular tumour spheroids. These results demonstrate that long-term primary osteosarcoma 3D cultures are suitable to include in a pre-clinical pipeline when testing novel therapeutic options for osteosarcoma.

Introduction

Osteosarcoma, the most common bone sarcoma is most often diagnosed in children and young adults (<30 years), but also has a second peak of incidence in the elderly (1). Osteosarcoma patient five-year survival rates are as low as 30% in cases of recurrence or relapse. Treatment strategies have not significantly improved survival rates over the last decades. Current treatment consists of highly toxic neoadjuvant methotrexate-doxorubicin-cisplatin (MAP) treatment and surgical resection as no new, improved treatment options have been identified (2). An improved multimodal treatment strategy is critical to improve outcomes for these patients.

The rarity of recurring alterations in combination with the genomic complexity and inter-tumour heterogeneity of osteosarcoma poses a challenge to overcome for future identification of novel treatment options and the development of clinical trials. By performing comprehensive analysis of clinically relevant targetable alterations and tailoring treatment strategies in patient-derived xenograft (PDX) models, Sayles and colleagues demonstrated the potential efficacy of genome informed targeted therapy (3). This approach can be applied to other representative osteosarcoma models, such as 3D cell cultures, in order to gain more information on personalized treatment strategies for the disease.

In conventional osteosarcoma 3D culture methods, cells are initially cultured in monolayer on a treated plastic surface, and subsequently transferred to low attachment or scaffold-based 3D culture. This method allows for the evaluation of treatment response that is more representative of the *in vivo* situation as compared to conventional 2D cell lines (4, 5). *In vitro* models can be further improved when derived straight from patients and by maintaining cells in extracellular matrix-based scaffolds, using methods such as organoid culture (6). In general, sarcoma cells cultured in 2D are morphologically very different from the *in vivo* situation due to a lack of cell-cell and cell-matrix attachment, and cells are prone to dedifferentiation over time (7). Continuous culturing to establish cell lines can select for specific cells and introduce additional mutations, alter gene and protein expression, and thus may alter drug response (8-10). Recapitulating the *in vivo* environment as closely as possible ensures a more representative therapeutic outcome. Organoid-like long-term culture of metastatic osteosarcoma was previously established by adaptation of epithelial organoid culture methods to osteosarcoma samples (11). However, this method was not successful for generation of long-term primary osteosarcoma tumour patient-derived cultures.

In the present study we describe culture methods tailored to the osteosarcoma microenvironment. We were able to establish long-term osteosarcoma primary 3D cultures, regardless of osteosarcoma subtype or pre-treatment. The culture was established without use of organoid culture methods commonly used for epithelial cancers, avoiding non-representative cell behaviour and increasing cost effectiveness. Instead, collagen- based

hydrogels and osteogenic growth factors were used for osteosarcoma 3D cell culture propagation. Patient cells were propagated in hydrogels immediately after collection of patient material to prevent 2D culture-based dedifferentiation. Denser cell masses, termed microsarcs, were produced from hydrogel propagated cells in order to more sufficiently mimic *in vivo* osteosarcoma upon treatment. In addition, multi-cellular tumour spheroids (MCTS) were generated from 2D primary cultures of one patient to compare 3D models created after culture in 2D to the immediate 3D propagation models. The primary osteosarcoma 3D long-term cultures were used in this study to investigate whether these models can be used for personalized targeted treatment. For this purpose, whole-exome sequencing (WES) was performed on tumour tissue of seven osteosarcoma patients to detect targetable alterations, after which the 3D primary osteosarcoma cultures of two patients were treated with drugs targeting the alterations. Furthermore, the sensitivity to drugs was compared between conventional 2D cultures, 2D cells propagated in a 3D environment, and straight-from-patient derived 3D cultures. We demonstrate use and relevance of these osteosarcoma 3D culture models in the context of precision medicine to obtain more representative pre-clinical data and improve clinical outcomes for osteosarcoma patients.

Materials and Methods

Patient samples

All tumour tissue samples used for this study were derived from the bone and soft tissue tumour biobank of the Leiden University Medical Centre (BWD005/SH/sh) and obtained from patients undergoing surgical resection. The use of tumour tissue samples for this study was approved by the LUMC ethical review board (B.16.026). Written informed consent was obtained from all participants and the study was conducted according to the code for Proper Secondary Use of Human Tissue in the Netherlands. Relevant clinical information can be found in **Table 1**.

2D primary line production

Tissue samples were obtained directly from surgery and minced with razor blades and immersed in 3mL collagenase/dispase (1mg/mL) either at 37°C for two hours or at room temperature overnight. Once digested, the samples were washed twice with DMEM:F12 (#10565018, Gibco, Waltham, USA). The remaining pieces of tissue were squeezed using a glass pipette in order to suspend the cells. After washing, the cells were transferred into 75 ml culture flasks and cultured in αMEM (#LO BE12-169F, Lonza, Basel, Switzerland) supplemented with 10% foetal bovine serum (FBS), 100U/ml penicillin and 100µg/mL streptomycin (Life Technologies Limited) and 1% NEAAs (#11140050, Gibco, Waltham, USA). Cells were grown in a humidified incubator with 1% O_2 and 5% CO_2 and cultured until stably multiplying.

Table 1. Overview of osteosarcoma patient samples used in this study. M = male; F = female; SNV = single nucleotide variant; amp = amplification; NA = not applicable

Patient ID	L6558	L6565	L6581	L6620	L6621	L6647	L6727
Subtype	Conventional, telangiectatic	Conventional	Conventional	Conventional	Conventional chondroblastic	Conventional	Conventional, telangiectatic
Location	Fibula	Humerus	Lung	Humerus	Os ilium	Abdomen	Pelvis
Age	36	57	29	51	21	25	67
Gender	M	F	M	F	M	M	F
Tumour sample	primary	primary	metastasis	primary	primary	metastasis	primary
Neo-adjuvant treatment	Yes	No	Yes, for treatment of primary tumour	No	Yes	No	Yes
Tumour specific alteration	*EPHB1* SNV exon 7 >chr3: 135162098 A>G; *PES* SNV exon12 >chr22: 30579877 C>T	*KRAS* SNV (c.35G>A; VAF: 0.64), CDKN2A loss	*F13A1* SNV exon 4, >chr6: 6266605 C>T	*MTRR* SNV intron 13, >chr5: 7897041 G>T	*MYC* amp intron 13, >chr5: 7897041 G>T	*TP53* SNV (c.775G>T; VAF: 0.74)	*TP53* SNV (c.406C>T; VAF: 0.45)
Confirmed in 3D culture (analysis method)	No (Sanger sequencing)	Yes (Cancer Hot Spot Panel)	No (Sanger sequencing)	No (Sangerse quencing)	Yes (MYC IHC staining)	Yes (P53 IHC staining) but stopped growing	No (Cancer Hot Spot Panel)
Targetable alteration	BRCAness signature	*CDKN2A* loss	None	BRCAness signature	*MYC* amp	4q12 amp (*KIT, PDGFRA, KDR*)	None
Target specific drug	PARPi	Palbociclib	NA	PARPi	JQ1 or 10058-F4	Regorafenib	NA

Osteosarcoma MCTS production from 2D culture

Multicellular tumour spheroids (MCTS) were produced from conventionally cultured (2D) primary L6565 cells (OS MCTS). The cells were suspended in αMEM (#LO BE12-169F, Lonza, Basel, Switzerland) supplemented with 10% foetal bovine serum (FBS) and 100U/ml penicillin and 100µg/mL streptomycin (Life Technologies Limited) and 1% NEAAs (#11140050, Gibco, Waltham, USA). Additional supplementation with 50 µg/ml ascorbate 2-phosphate and 100nM dexamethasone was performed directly before culture. The medium contained 0.24% (w/v) methyl cellulose in order to improve reproducibility of spheroids. The cells were then seeded onto 1% (w/v) agarose coated (ultra-low attachment) plates at a density of 20,000 cells/well. They were cultured for 7 days in order to aggregate into a large mass and were subsequently used for therapeutic testing.

Osteosarcoma 3D primary line production (hydrogels and microsarcs)

After tissue digestion the cell suspension was strained using a 70µm cell strainer (#431751, Corning, New York, USA). If blood was present in the pellet, cells were additionally washed with 2mL PBS and treated with 4mL RBC lysis buffer (#00-4333-57, Invitrogen, Waltham, USA) for 4 mins. To stop the lysis 30mL of PBS was added and the cells were washed in cell culture medium. The cells were suspended in a collagen scaffold (2.5mg/mL collagen, 1x DMEM, 8.29µM NaOH, 0,38% alginate (w/v)) and plated out either in 12-well (for propagation) or 96-well (for therapeutic compound testing) non-treated cell culture plates into 10/80µL gels respectively. The plates were incubated at 37°C for 25 mins and then left at room temperature for an additional 5 mins. In order to gelate the alginate component of the scaffold and further stiffen the gel, the samples were incubated in 0.2M $CaCL_2$ solution for 5 mins. The samples were subsequently washed twice with 0.15M NaCl solution, followed by two washes with medium and placed in culture medium containing αMEM (#LO BE12-169F, Lonza, Basel, Switzerland) supplemented with 10% foetal bovine serum (FBS) and 100U/ml penicillin and 100µg/mL streptomycin (Life Technologies Limited) and 1% NEAAs (#11140050, Gibco, Waltham, USA). Additionally, medium was supplemented with 50 µg/ml ascorbate 2-phosphate and 100nM dexamethasone (osteogenic growth factors). Cells were grown in a humidified incubator with 1% O_2 and 5% CO_2 and cultured until >80% gel confluency for either passage or treatment. Cells were regularly STR-profiled using the GenePrint 10 system kit (Promega, Madison, WI, USA) and showed the same STR profile as the original tumour. In addition, all cells used for treatment were regularly tested for mycoplasma.

To passage the osteosarcoma hydrogels, cultures were first collected and washed once with PBS. In order to digest the scaffold 0.25mg/mL LiberaseTL (#5401020001, Roche, Basel, Switzerland) solution (in PBS) was added (4,375µL/µL scaffold). The scaffolds were incubated at 37°C for digestion and checked and vortexed after the first 10mins then subsequently 5min intervals until the gel was dissolved and the cells were released into a single cell solution. The digestion was quenched using medium and washed with medium. On a bi-passage basis the cells were strained further to remove debris using autoclaved 50µm cell strainers (#04-0042-2317, Sysmex, Kobe, Japan). The cells were then resuspended in new scaffold at a ratio of 1:2

for further culture or frozen for cryostorage. All osteosarcoma lines produced could be repropagated from frozen cryostock.

For further therapeutic testing, osteosarcoma microsarcs were additionally produced from the cell lines propagated in hydrogel culture. The method used to produce microsarcs was identical to the MCTS protocol described above, but a higher seeding density (50,000 cells/well) was used. Although the aggregation method of MCTS and microsarcs was the same, the cells were propagated in different environments previous to aggregation (2D/3D culture) since the beginning of *in vitro* culture.

Next generation sequencing data analysis

For whole exome sequencing, DNA was isolated from matched pairs of primary tumour and normal frozen tissue of seven patients using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA samples were checked for degradation by gel electrophoresis. Whole exome sequencing with a minimum coverage of 100x was performed by GenomeScan BV (Leiden, the Netherlands) using the Illumina Novaseq6000 platform and the Agilent SureSelect Human All Exon V7 kit. The WES data were processed using the BioWDL somatic variant calling pipeline developed at LUMC (https://github.com/biowdl/germline-DNA/blob/v3.0.0/somatic.wdl). The quality control was first performed by FastQC (v0.11.9). Then the adapters were further clipped using Cutadapt (v2.8). The clean reads were aligned to the human reference genome GRCh38 using BWA-MEM (v0.7.17-r1188). According to the GATK best practice, duplicated mapped reads were marked using Picard (v2.20.5) and the base quality recalibration was performed using GATK4 (v 4.1.2.0) to generate the analysis-ready reads in the BAM format. Strelka (version 2.9.7) and Mutect2 (version 4.1.2.0) were used to detect small somatic variants from the matched tumour-normal samples. Only the variants meeting the criteria as determined by Strelka and Mutect2 were included in the downstream analysis. These variants were then normalized and decomposed using vt program (v2015.11.10) and subsequently annotated using VEP (v98) with SIFT and Polyphen-2 options enabled. VEP plugins and custom annotation files were also used to include annotations of CADD scores, dbNSFP3.5 (phastCons100way vertebrate & phyloP100way vertebrate), population allele frequency information (e.g., gnomAD version 3 and GoNL). Copy number variants (CNVs) were identified following GATK somatic CNV discovery best practice workflow (v4.1.4.0). Pre-selected targetable genes (**Supplementary Table 1**) were used to prioritize both small variants and CNV. Genes were labeled "targetable" when it occurred in more than one of the following targetable cancer gene panels: FoundationOne and FoundationOne Heme, MSK-IMPACT (12), Mi-oncoseq (13), and UCSF 500 Cancer Gene Panel.

For the customized Cancer Hotspot deep sequencing panel sequencing, DNA was isolated from osteosarcoma hydrogels of L6727 P5 and L6565 P15 using the Wizard Genomic DNA purification kit (Promega) according to the manufacturer's instructions. Libraries were generated using Life Technology's Ion AmpliSeq Cancer Hotspot Panel v6. Data analysis was

performed as described previously (14, 15). To confirm presence of non-driver tumour-specific variants in the 3D cultures, PCR and Sanger sequencing for the genes *EPHB1, PES, F13A1, USP43* and *MTRR* was performed.
The tumour mutational burden (TMB) was calculated per sample by dividing the number of protein-coding somatic mutations by the total size of the exome (30 Mb was used which is a commonly agreed standard). Using the fit_to_signatures_strict (cutoff = 0.010) from MutationalPatterns R-package (v3.0.1), mutational signature profiles per sample were generated based on SigProfiler exome SBS reference signatures (https://www.synapse.org/#!Synapse:syn12026190).

Immunohistochemistry
Whole slide sections of paraffin embedded tumour tissue or 4 µm paraffin embedded osteosarcoma hydrogels, osteosarcoma microsarcs or osteosarcoma MCTS were made and stained for haematoxylin and eosin or used for immunohistochemical staining after deparaffinization and rehydration. Tumour tissue sections were stained for SATB2 (clone CL0276, Sigma-Aldrich, Saint Louis, MO, USA). In addition, L6621 was stained for MYC (clone Y69, Abcam, Cambridge, UK) and L6647 was stained for p53 (clone DO-7, Dako, Agilent Technologies, CA, USA). Osteosarcoma hydrogels, osteosarcoma microsarcs, and osteosarcoma MCTS were stained for SATB2. Additional stainings were performed for L6565 hydrogels or MCTS: cleaved caspase 3 (Cell Signaling Technology, Leiden, The Netherlands), Ki67 (clone D2H10, Cell Signaling Technology), p16 (clone E6H4, CINTEC, Roche, Basel, Switzerland), Rb (clone G3245, BD Pharmingen, San Diego, CA, USA) and MYC. For SATB2, Ki67 and cleaved caspase 3 staining, antigen retrieval was performed by a 10 minute incubation in 10 mM citrate buffer (pH 6) followed by cooling down for two hours. For MYC, p53, p16 and Rb staining, antigen retrieval was performed using Tris-EDTA (pH 9). Sections were incubated with primary antibody (Ki67, 1:1600; MYC, 1:80; p53, 1:1; cleaved caspase 3, 1:800; Rb, 1:2000, SATB2, 1:10, p16, 1:1) overnight at 4 °C. The next day, sections were incubated with BrightVision one step detection system poly-HRP anti-mouse/rabbit (Immunologic, WellMed BV, Duiven, The Netherlands) for 30 minutes. Sections were washed with PBS and DAB+ Chromogen (Dako) was added to each slide for 10 minutes. Slides were counterstained with haematoxylin, dehydrated and mounted.

Drug treatment of osteosarcoma hydrogels and microsarcs
For drug treatment in osteosarcoma hydrogels, once near confluence, L6565 and L6621 was treated with palbociclib (20 or 45 µM, dissolved in PBS; Selleckchemicals, Houston, TX, USA), JQ1 (1 or 10 µM; Selleckchemicals), 10058-F4 (10 or 50 µM; Selleckchemicals) or solvent controls (PBS and DMSO) for 72 hours. Dosage for drug treatment was selected based on results from previously published studies (16-18) in which a similar concentration range was used. For drug treatment in osteosarcoma microsarcs, L6565 was seeded at 50,000 cells per well in a 96-well plate. One week after the generation of osteosarcoma microsarcs , cells were treated with palbociclib (20 or45 µM) or PBS for 72 hours.

Drug screening in L6565

For a focused drug screen in L6565 cultures, compounds were selected based on recent literature (19). These included an IGF pathway inhibitor (linisitinib) (20, 21), a tyrosine kinase inhibitor (regorafenib) (22), and an mTOR inhibitor (rapamycin) (23). In addition, standard chemotherapeutic agents used in sarcomas, i.e. doxorubicin and cisplatin were included, while palbociclib was used as positive control.

For drug screens in 2D cultured cells, L6565 cells were seeded at 4000 cells per well of a 96-well plate. For monotherapy treatment, the next day cells were treated for 72 hours with doxorubicin or cisplatin (both in-house hospital pharmacy of Leiden University Medical Center), palbociclib, rapamycin, linsitinib, or regorafenib (all Selleckchemicals) in concentrations ranging from 0.001 to 100000 nM. For combination treatments, cells were seeded in medium containing cisplatin or doxorubicin for 24 hours. The next day, cisplatin or doxorubicin was removed, and palbociclib was added for 72 hours. Pre-treatment of cisplatin and doxorubicin was based on a previously published study indicating simultaneous addition of chemotherapy with palbociclib will result in interference between the drugs (24). The combination treatment of rapamycin and palbociclib was performed by simultaneous addition of each drug 24 hours after seeding for 72 hours, which is based on the experimental set-up of a previously published study in which rapamycin and palbociclib were added simultaneously (25).

For drug screens in osteosarcoma MCTS and hydrogels, L6565 was treated with cisplatin, doxorubicin, rapamycin and palbociclib for 72 hours.

Cell viability and nuclei count assays

After drug treatment, cell viability was determined by Presto Blue Cell Viability reagent after 90 minutes (for osteosarcoma hydrogels, microsarcs or MCTS) or 60 minutes (for 2D cultured cells) incubation, after which fluorescence was measured at 550/600 nm using a microplate reader (Infinite M Plex, Tecan Group Ltd., Zürich, Switzerland) . Osteosarcoma hydrogels were formalin fixed and paraffin embedded after read-out.

2D cultured cells were fixed in formalin and Hoechst (Invitrogen Life Technologies) was added to each well after read-out. Nuclei were counted using Cellomics HCS viewer Software V9. Cell viability and nuclei count reads were determined relative to PBS or DMSO control after correcting for background reads and reads prior to the start of treatment (day 0), as described in (26)

Statistical analysis

For statistical comparisons between groups, a Kruskal-Wallis test was performed using Dunn's correction for multiple testing. All statistical analyses were performed using GraphPad Prism v.8. Comparisons were considered statistically significant using a significance level of $p \leq 0.05$.

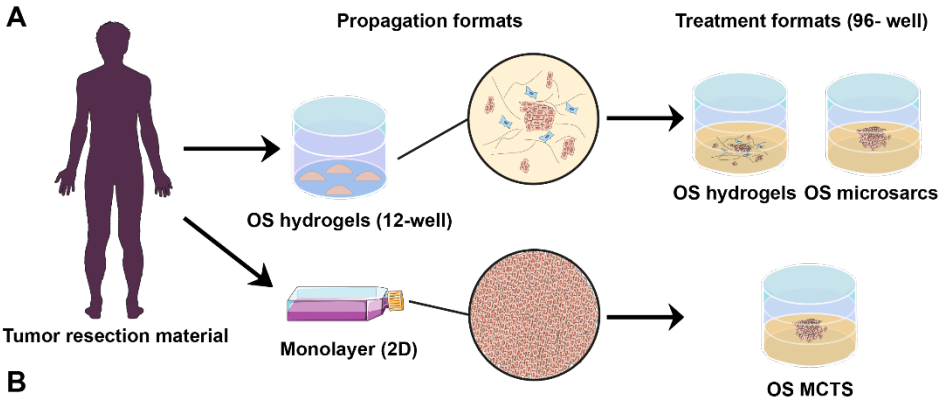
Results

Long-term genetically stable 3D osteosarcoma hydrogel cultures can be established from human osteosarcoma resection material

Osteosarcoma hydrogel lines were established from osteosarcoma resection material of seven individual patients **(Fig 1A)**. The selection consisted of a variety of osteosarcoma specimens, including pre-treated tumours and different osteosarcoma subtypes **(Table 1)**. All seven osteosarcoma hydrogel lines were cultured in collagen-based scaffolds over the course of 44-120 days to at least passage 7. This demonstrates long-term propagation of such lines fully in 3D cell culture, allowing their expansion and use for therapeutic testing and mechanistic studies whilst preserving their *in vivo* behaviour **(Fig 1B)**. However, during the COVID-19 pandemic, lab work was suspended and all cultures were frozen down, which may have affected the recovery and propagation time of cultures. Osteosarcoma hydrogels showed the lowest cell density as compared to osteosarcoma microsarcs or MCTS (**Fig 1C**). Morphology of the cells was reminiscent of the original tumour, for example, cells within osteosarcoma hydrogels of L6565 showed a spindle-shaped morphology, similar to the original tumour (**Fig 1C**). However, none of the patient-derived lines displayed evidence of osteoid deposition, and SATB2 immunohistochemistry was negative in the osteosarcoma hydrogels, microsarcs, and MCTS (**Supplementary Fig S1**). The selected patient lines (L6565 and L6621) used for therapeutic screening proliferated up to passage 30, allowing for multiple testing of therapeutic strategies.

Whole-exome sequencing of primary osteosarcoma reveals targetable alterations

For all seven patient-derived osteosarcoma hydrogels whole exome sequencing was performed on the DNA from the corresponding original tumour tissue as well as on DNA from non-neoplastic tissue from the same patient to evaluate the presence of targetable molecular alterations for each patient and to identify specific alterations that can be used to confirm tumorigenicity of each osteosarcoma hydrogel.


A
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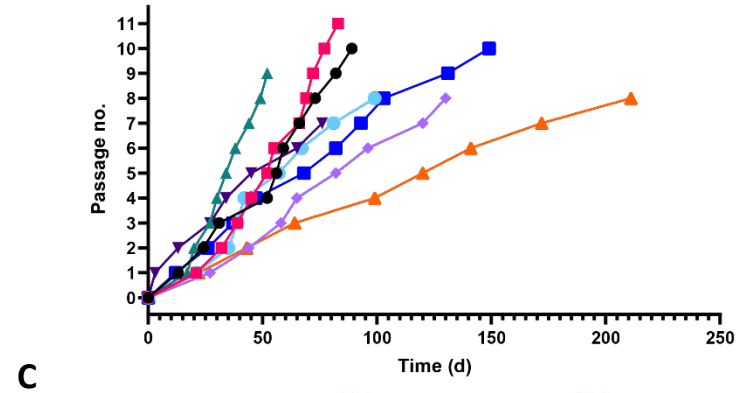
Treatment formats (96- well)
OS hydrogels (12-well)
OS hydrogels
OS microsarcs
Tumor resection material
Monolayer (2D)
OS MCTS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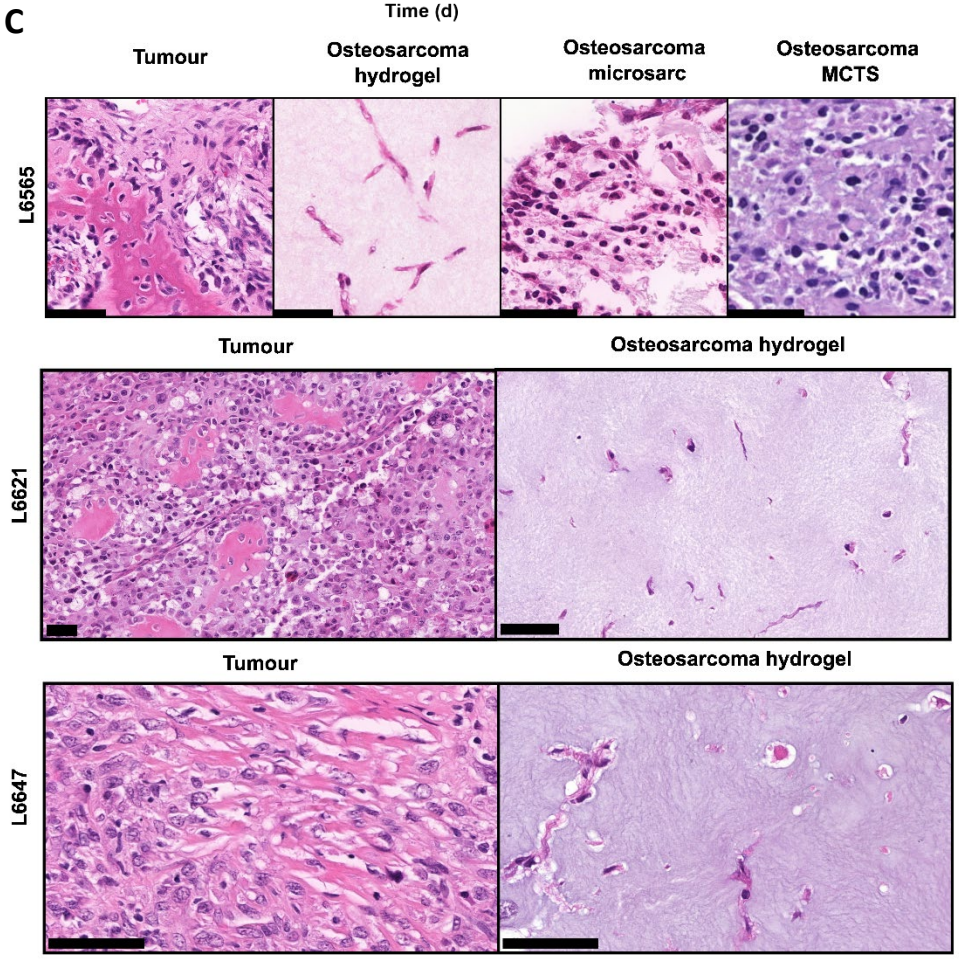
C
Tumour
Osteosarcoma hydrogel
Osteosarcoma microsarc
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L6565
Tumour
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L6621
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L6647

Figure 1. Growth rate and histology of seven established primary 3D osteosarcoma cultures. (**A**) Osteosarcoma patient resection material was digested to single-cell suspensions, which were then propagated in a collagen-based scaffold with optimized osteosarcoma medium (OS hydrogels) or cultured in monolayer (2D). For treatment of osteosarcoma hydrogels, the culture format was switched to 96-well plate hydrogels or to microsarc format. For treatment of cultures derived from monolayer, cells were seeded as multi-cellular tumour spheroids (MCTS). (**B**) Seven osteosarcoma lines were established and cultured for at least seven passages without signs of a growth plateau. (**C**) H&E staining of the original tumours and the corresponding patient-derived osteosarcoma hydrogels, microsarcs or MCTS. Scalebar represents 50 µm.

Detection of targetable alterations

WES data analysis revealed that the total number of single nucleotide variants of each tumour varied among patients (**Fig 2A**). The tumour mutational burden in all osteosarcoma patients was low to intermediate, with an average of 9.0 mutations per megabase (**Fig 2B**) (27). The copy number profiles of most of the osteosarcoma samples demonstrated a highly complex genome with many copy number alterations, although the level of complexity varied. In contrast, L6565 only showed a small number of copy number changes (L6565 and L6621 shown in **Fig 2C**, rest of samples in **Supplementary Figure S2**). Mutational signature analysis revealed that two COSMIC mutational signatures were dominant (signature SBS5 and SBS31, **Supplementary Figure S3**), although these were not associated with a specific etiology. In L6558 and L6620 the mutational signature SBS3 was identified, albeit at a low level, which is associated with a BRCAness signature, and may suggest sensitivity to PARP inhibitors (28). Both tumours did not show alterations in homologous recombination deficiency related genes (15).

All seven osteosarcoma tumour samples were analysed for the presence of targetable alterations (**Table 1**). To identify which genetic alterations are targetable for each patient, genetic alterations including single nucleotide variants and copy number alterations, were filtered using a pre-defined list of targetable genes (**Supplementary Table 1).** L6565 was revealed to have a *KRAS* mutation (c.35G>A; VAF: 0.64) and homozygous loss of *CDKN2A*, the latter is targetable by CDK4/CDK6 inhibitors such as palbociclib. L6621 showed a *MYC* amplification, which can be targeted by MYC inhibitors such as JQ1 and 10058-F4. L6647 carried an amplification in the region 4q12, which contains the genes *KIT*, *PDGFRA* and *KDR* which can be targeted by receptor tyrosine kinase inhibitors such as regorafenib. For two tumours, L6581 and L6727, no targetable alterations were identified. An overview of identified targetable alterations is shown in **Table 1**.

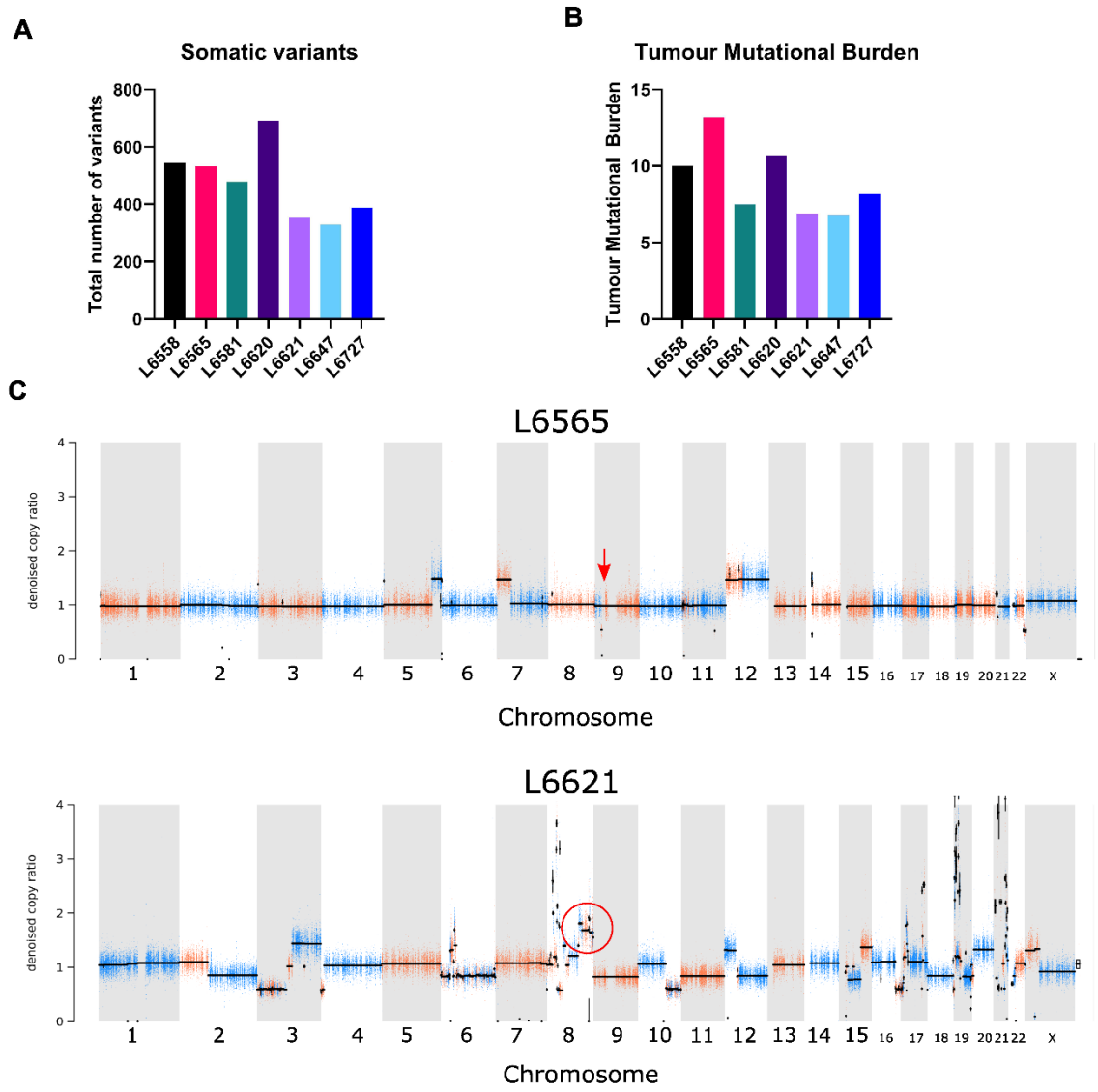


Figure 2. Whole exome sequencing analysis of osteosarcoma tumour samples. (**A**) Total number of somatic single nucleotide variants per tumour. Bars represent the number of overlapping variants identified by different variant callers (Strelka, Mutect2). (**B**) Tumour mutational burden per patient. Values are expressed as mutations per megabase. (**C**) Copy number profiles of L6565 and L6621. For L6565 the copy number loss of *CDKN2A* is indicated with a red arrow. For L6621 the MYC amplification is indicated with a red circle.

Molecular confirmation of the presence of tumour cells in the 3D cultures

The presence of tumour specific alterations was investigated in the osteosarcoma hydrogels to confirm the presence of tumour cells instead of normal fibroblasts in the 3D cultures. Using a a customized Cancer Hotspot deep sequencing panel, it was confirmed that L6565 osteosarcoma hydrogels contained the same driver mutations as the original tumour, including the identical *KRAS* mutation and homozygous loss of *CDKN2A* (**Supplementary Figure S4**). Moreover, L6565 osteosarcoma hydrogels indeed showed loss of p16 protein expression (**Figure 3**). Because for L6727, L6558, L6620, and L6581 no driver mutations were available other somatic variants were used to compare the primary tumour with the 3D cultures. Single nucleotide variants were selected on the basis of coverage (>100) and variant allele frequency (>0.3) and include a *TP53* point mutation (c.406C>T, VAF: 0.45), a *EPHB1*

point mutation, a *MTRR* point mutation, and a *F13A1* point mutation respectively. Unfortunately, these mutations were not found in the 3D cultured cells. This may imply that tumour cells were overgrown by normal fibroblasts in 3D culture. For L6621, the positive immunohistochemical staining for MYC suggested that the *MYC* amplification as identified by WES in the L6621 primary tumour tissue, was also present in the L6621 3D cultured cells (**Fig 3**. Likewise, L6647 3D cultured cells showed p53 overexpression suggestive of the presence of the *TP53* mutation (c.775G>T; VAF: 0.47) that was found using WES in the L6647 primary tumour tissue (**Figure 3**). Thus, in total three out of seven 3D osteosarcoma hydrogel cultures consisted of tumour cells and could therefore be used to test targeted therapies (**Table 1**). Unfortunately, the patient derived osteosarcoma hydrogel line L6647 did not grow sufficiently for further testing, which left two patient derived osteosarcoma hydrogel lines (L6565 and L6621) for drug testing.

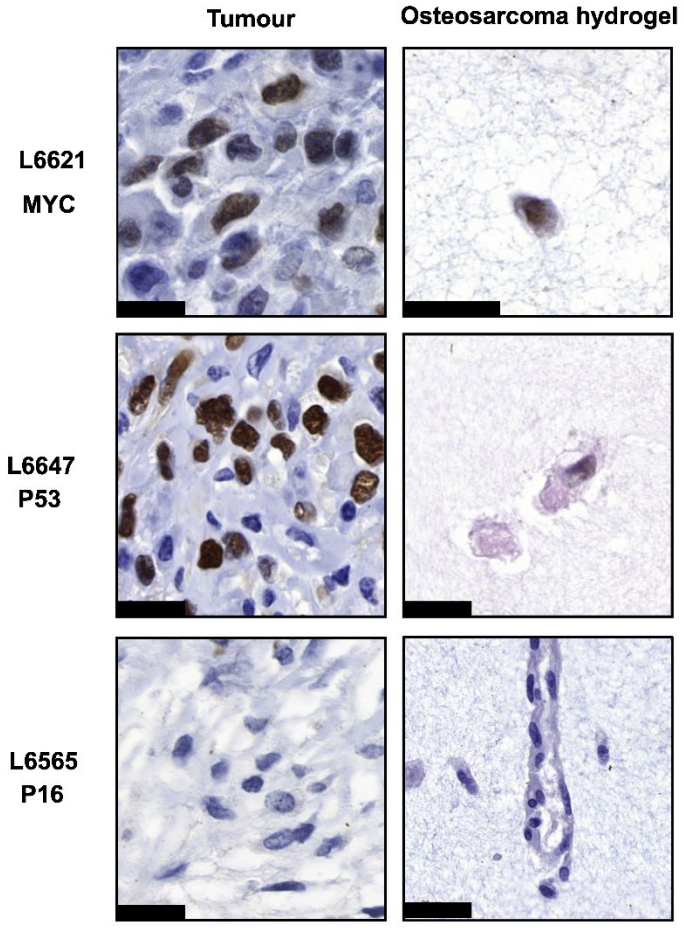


Figure 3. Osteosarcoma hydrogels carry the same alteration as the original tumour. Loss of immunohistochemical expression of P16, or overexpression of MYC and P53 were confirmed in the osteosarcoma hydrogels derived from L6565, L6621 and L6647 respectively. Scalebar represents 20 µm.

Targeted treatment of patient-derived 3D osteosarcoma cell cultures

L6621, which displayed a *MYC* amplification, was treated with BET bromodomain inhibitor JQ1, which was shown to downregulate MYC transcription (29), as well as a specific MYC inhibitor, 10058-F4 (30). Osteosarcoma hydrogels of L6621 that were treated for 72 hours with 1 or 10 µM JQ1 did not show a reduction in cell viability after treatment, whereas treatment of 10 µM or 50 µM 10058-F4 reduced cell viability to 70% ($P = 0.0046$) and 40% ($P < 0.001$) respectively (**Fig 4A**). However, the presence or absence of a MYC amplification did not predict response, since osteosarcoma hydrogels without *MYC* amplification (L6565) also showed a reduction in cell viability ($P < 0.001$)(**Fig 4B, 4C**).For L6565 the deletion of *CDKN2A* could indicate sensitivity to CDK4/CDK6 inhibition, provided Rb is functional. Rb status was determined for L6565 with immunohistochemistry and showed no loss of Rb in the osteosarcoma cells in hydrogel (**Figure 4C**). Thus, osteosarcoma hydrogels of L6565 were treated with CDK4/CDK6 inhibitor palbociclib for 72 hours which reduced cell viability ($P < 0.001$)(**Fig 4B**). However, no difference in morphology, apoptosis or proliferation between untreated and palbociclib treated osteosarcoma hydrogels was observed by Ki67 or cleaved caspase 3 staining (**Fig 4D, Supplementary Figure S5**). Since only few cells are visible per section in osteosarcoma hydrogels, L6565 was also cultured as microsarcs resulting in a similar reduction in cell viability as compared to osteosarcoma hydrogels ($P = 0.0146$) (**Fig 4E**). However, the presence or absence of *CDKN2A* did not predict the effect of palbociclib on cell viability in L6565, as L6621 osteosarcoma hydrogels (without loss of *CDKN2A*) also showed reduced cell viability upon palbociclib treatment ($P < 0.001$)(**Figure 4A**). The dosage of palbociclib used in this study was relatively high as compared to previous studies for the treatment of HER-2 negative breast cancer and breast cancer cell lines *in vitro* (31). Thus, to determine whether the dosage of palbociclib could be reduced further, different drug combinations with other promising novel osteosarcoma therapeutics were tested in 2D cultures of L6565. However, no combination therapy was able to reduce the dose of palbociclib (**Supplementary Figure S6**).

Osteosarcoma hydrogels are suitable to test novel treatment options

We further explored whether the generated osteosarcoma hydrogels could be used to test potential novel treatment options for osteosarcoma. L6565 2D cultured cells (**Fig 5A**) were used for prescreening of drugs and showed that cisplatin, doxorubicin, rapamycin (mTOR inhibitor) and palbociclib were able to induce a dose dependent response after 72 hours, whereas linsitinib (IGF pathway inhibitor) and regorafenib (broad receptor tyrosine kinase inhibitor) showed the highest IC_{50} values (2166 and 3013 nM, respectively) and induced more toxic responses at high doses. Therefore cisplatin, doxorubicin, and rapamycin were selected to validate further using multi-cellular tumour spheroids (MCTS) of L6565 cells. The same cells cultured as MCTS were less sensitive compared to 2D cultured cells (**Fig 5B**). Finally, the L6565 osteosarcoma hydrogels were less sensitive to drugs as compared to L6565 osteosarcoma MCTS or cells in monolayer (**Fig 5C**).

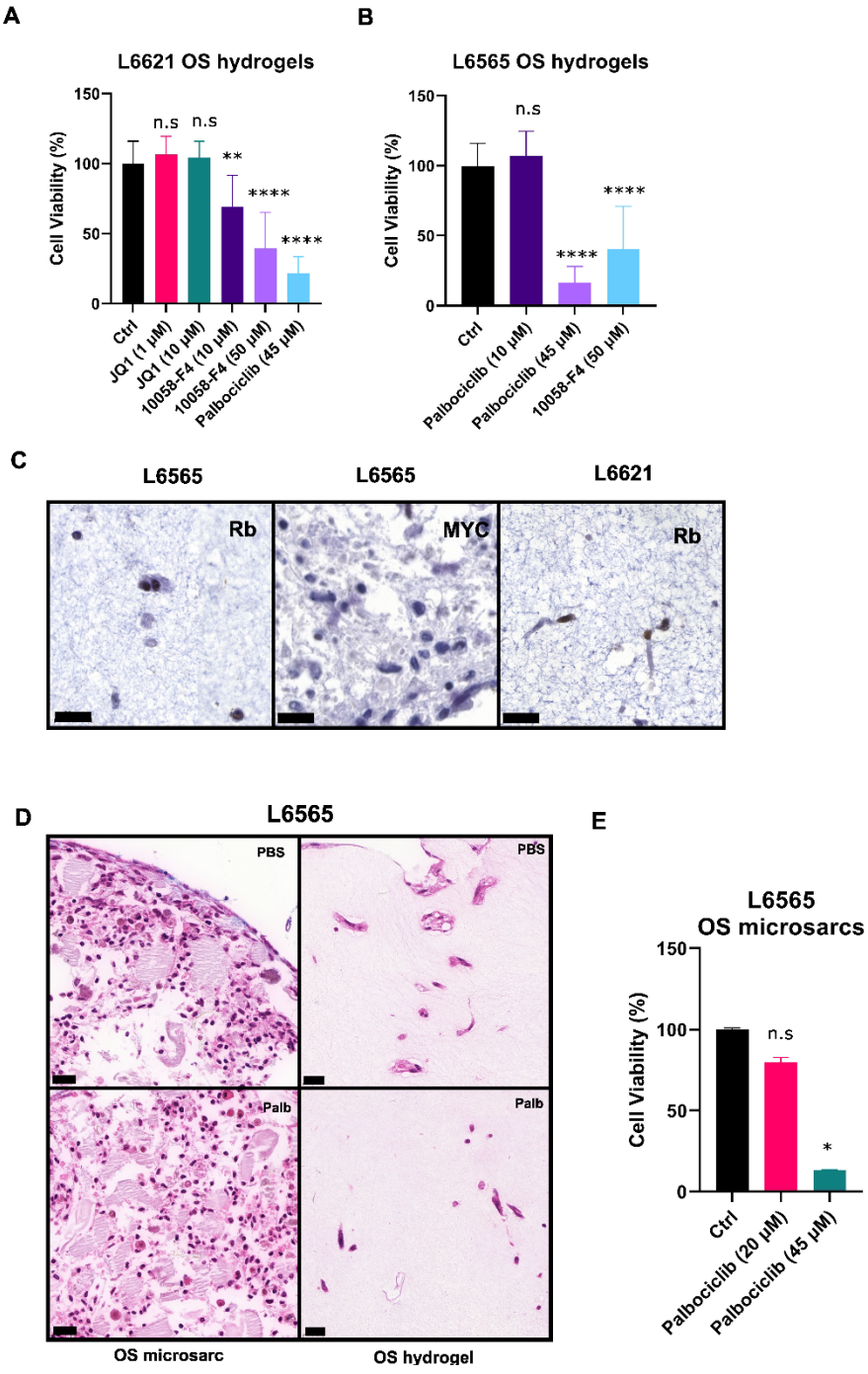
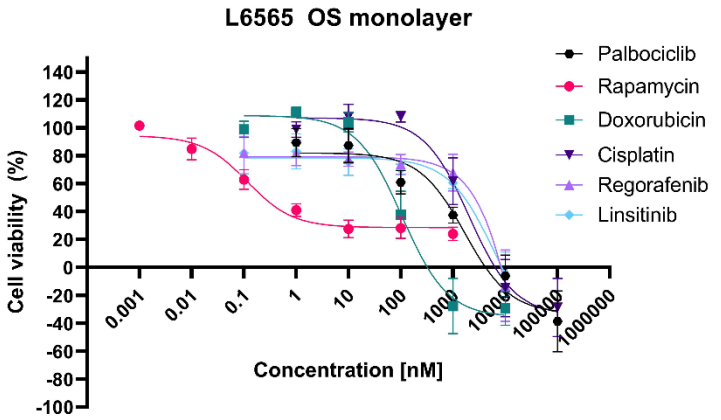


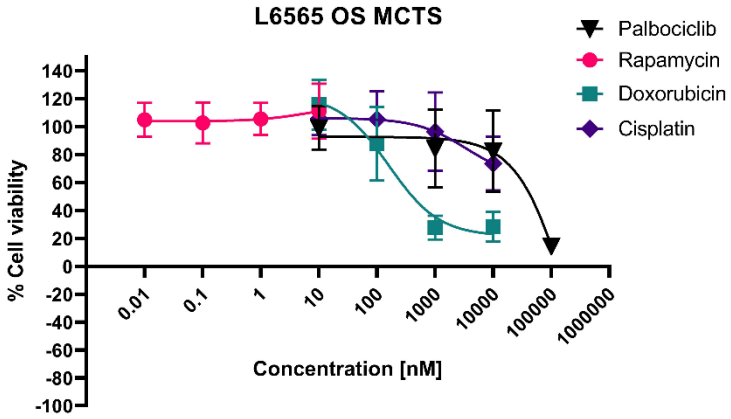
Figure 4. Targeted treatment for L6621 and L6565. (**A**) L6621 osteosarcoma hydrogels, with a MYC amplification, were treated with MYC inhibitors JQ1 and 10058-F4, or palbociclib for 72 hours, after which cell viability was determined. Bars represent the average of seven experiments performed In triplicate, with the standard deviation. Drug treated cells were compared to non-treated controls. ** = P ≤ 0.01; **** = P ≤ 0.001; n.s = not statistically significant. (**B**) L6565 osteosarcoma hydrogels with a loss of *CDKN2A* were treated with palbociclib or 10058-F4 for 72 hours, after which cell viability was determined. Bars represent the average of seven experiments performed in triplicate, with the standard deviation. Drug treated cells were compared to non-treated controls. **** = P ≤ 0.001; n.s = not statistically significant. (**C**) L6565 osteosarcoma hydrogels or microsarcs were stained for Rb and MYC respectively. L6621 osteosarcoma hydrogels were stained for Rb. Scalebar represents 20 µm. (**D**) L6565 osteosarcoma hydrogels and microsarcs did not show difference in morphology between treated (45 µM) and untreated conditions. Scalebar represent 20 µm (**E**) Osteosarcoma microsarcs were treated with palbociclib for 72 hours, after which cell viability was determined. Bars represent the average of one experiment performed in triplicate, with the standard deviation. Drug treated cells were compared to non-treated controls **** = P ≤ 0.05; n.s = not statistically significant

A



Drug	IC50 (nM)
Palbociclib	611
Rapamycin	0.19
Doxorubicin	71
Cisplatin	1473
Regorafenib	3013
Linsitinib	2166

B



Drug	IC50
Palbociclib	45 µM
Rapamycin	N.A.
Doxorubicin	410 nM
Cisplatin	N.A.

C

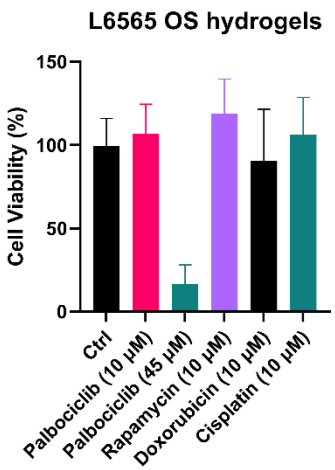


Figure 5. Literature-informed focused drug treatment for L6565. (**A**) 2D cultured cells were treated with each drug for 72 hours, after which cell viability and IC50 values were determined. Graph represents the average of three experiments performed In triplicate, with the standard deviation. (**B**) L6565 MCTS were treated with each drug for 72 hours, after which cell viability and IC50 values were determined. For drugs rapamycin and cisplatin no IC50 value could be determined (N.A.). Graph represents the average of three experiments performed in triplicate, with the standard deviation. (**C**) Osteosarcoma hydrogels of L6565 were treated with each drug for 72 hours. Bars represents the average of seven experiments performed in triplicate, with the standard deviation.

Discussion

Osteosarcoma is currently treated by a combination of (neo)adjuvant chemotherapy and surgery, which has not improved patients' survival over the last decades. Thus, novel therapies are urgently needed for osteosarcoma patients as survival rates remain disappointingly below 60%. To translate pre-clinical findings to the clinic, potential novel therapies should be tested in suitable, representative *in vitro* models. For this purpose, we have successfully established osteosarcoma hydrogels, long-term 3D cultures of primary tumours, derived straight from osteosarcoma patient resection material.

Of the established osteosarcoma hydrogels, 100% of the cultures were propagated until at least seven passages. Not all tested osteosarcoma hydrogels were observed to harbour the same genetic alterations as the original tumour, indicating that these osteosarcoma hydrogels did not contain tumour cells and were probably overgrown by fibroblasts, or that key alterations were lost at higher passages. Nevertheless, three out of seven (43%) osteosarcoma hydrogels tested contained tumour cells with identical genetic alterations as compared to the original tumour, irrespective of tumour status (primary/recurrence/metastasis) or treatment. Long-term (<6 months) growth of lung metastatic osteosarcoma was demonstrated previously by He and colleagues, however these methods did not succeed in establishing long-term non-metastatic osteosarcoma cultures (11). Osteosarcoma hydrogel production methods used in the current study are tailored to mimic the osteosarcoma microenvironment (collagen hydrogels and osteogenic growth factors), single-cell seeded and utilize less additional supplements compared to culture methods frequently used for organoid culture of epithelial cancers. Supplementation in a sarcoma context leads to a more representative and cost-effective culture of osteosarcoma patient samples and allows study of primary tumours as well as metastases. Additionally, the level of antibiotics used for culture of microsarcs should be as low as possible in future experiments, as they may have an effect on response to therapy (32).

Of all osteosarcoma cultures, L6565 was most successful, both in 2D as well as in 3D, although this osteosarcoma is slightly unusual due to the absence of the typical complex genome with a plethora of copy number changes. In fact, the patient presented with a pathological fracture of the left humerus and a lesion was seen in the left clavicle. Biopsies of the clavicle as well as the humerus revealed similar morphology displaying a low-grade undifferentiated spindle cell sarcoma without deposition of osteoid, and MDM2 amplification as determined by FISH was lacking. First, the 6 cm lesion in the clavicle was resected, showing a heterogeneous morphology, including an intermediate grade area with deposition of osteoid, resulting in the diagnosis of osteosarcoma. Resection of the humerus was performed, revealing a 20 cm tumour with similar morphology but also including areas of conventional high-grade osteosarcoma in addition to high grade spindle cell areas without deposition of osteoid. No fusions were detected using Archer fusionplex sarcoma analysis in routine diagnostics.

It was shown that L6565 carried a homozygous loss of *CDKN2A,* with intact Rb, thereby suggesting vulnerability to CDK4/CDK6 inhibitors. L6565 osteosarcoma hydrogels were indeed sensitive to CDK4/CDK6 inhibitor palbociclib, and showed no sensitivity towards drugs that did not inhibit the p16 pathway, including linsitinib, regorafenib and rapamycin. This result is in line with previous studies that a subset of osteosarcomas are vulnerable to palbociclib (16, 29, 33, 34). However, the dosage used in the current study was relatively high as compared to the dosage currently administered to patients with HER2 negative breast cancer (31). Combination with other drugs including rapamycin, doxorubicin or cisplatin could not increase sensitivity to palbociclib and therefore the dose could not be lowered. Nevertheless, our previous study and other studies using osteosarcoma cells have used a dosage in a similar (high) concentration range as the current study and showed promising results (33, 35). Moreover, there are currently two clinical trials ongoing that test CDK4/CDK6 inhibitors in osteosarcoma patients with a known alteration in the CDK4/CDK6 pathway (36, 37).

The primary tumour and patient-derived osteosarcoma hydrogel line L6621 had a *MYC* amplification, suggesting potential sensitivity to treatment with MYC inhibitors. *MYC* amplification occurs in 9% of the osteosarcoma patients (38) and correlates with worse overall survival (30). Moreover, MYC was shown to be a potential driver of osteosarcoma, since mouse bone marrow stromal cells with overexpression of MYC and loss of CDKN2A transformed towards osteosarcoma (39). In the present study, we have tested a bromodomain and extra terminal domain (BET) inhibitor, JQ1, which inhibits MYC activity indirectly by transcriptional repression of *MYC* (29). JQ1 was previously shown to inhibit growth of osteosarcoma cells (34, 40). In osteosarcoma hydrogels, L6621 did not display a reduced cell viability after JQ1 treatment. In contrast, a direct MYC inhibitor, 10058-F4, which was previously shown to decrease cell proliferation in osteosarcoma cells both in 2D and 3D culture models (30), reduced cell viability in L6621. The presence or absence of MYC amplification did not predict response to 10058-F4 in our small series, since also line L6565 – lacking *MYC* amplification - showed a decrease in cell viability after treatment. This suggests that treatment with 10058-F4 might be further explored as novel treatment strategy for osteosarcoma, irrespective of the MYC amplification status.

Drug responses were compared between osteosarcoma 2D culture, culture as MCTS, and a hydrogel culture of the L6565 line. The sensitivity to palbociclib in our study was independent of the 3D culture method, since both osteosarcoma hydrogels and osteosarcoma MCTS showed sensitivity. This indicates a successful inhibitor effect regardless of physiochemical environment and higher cellularity. Comparing all culture methods, as expected, 2D cultured cells were most sensitive to the tested therapeutics. The difference in sensitivity between 2D and 3D cultured cells is not surprising and can be explained by the increase in tight intercellular junctions which occur in 3D cultures and reduce drug penetration (41). However, the difference in sensitivity in 3D osteosarcoma hydrogels compared to 2D propagated cells transitioned to a 3D environment (MCTS) might be explained by additional changes occurring

upon continuous culturing on a 2D plastic surface (8, 9). Further investigation into benefits of 3D primary culture models that have never been in contact with a plastic surface, is required to determine increased representativity over 2D propagated 3D models.

The slow growth rate and continuous monitoring of culture conditions required for osteosarcoma hydrogels does not allow for patient-specific personalized treatment at this moment. Before these models can be used for personalized treatment, culture conditions would need optimization to allow short-term assessment of each patient-derived culture without 2D propagation. Nevertheless, the current study is a proof-of-principle that osteosarcoma hydrogels can be used for pre-clinical precision medicine. Currently, most often a drug discovery pipeline involves screening in 2D cultured cells, followed by *in vivo* testing, and finally clinical trials. Our results suggest that the inclusion of drug testing on 3D cultured cells prior to *in vivo* testing is feasible and may provide valuable information about drug response. In particular 3D primary cultures that have never been in contact with a 2D plastic surface, such as the ones used in this study, may be more representative of *in vivo* and should be studied further in order to determine optimal *in vitro* models for drug discovery pipeline integration.

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Supplementary Figures

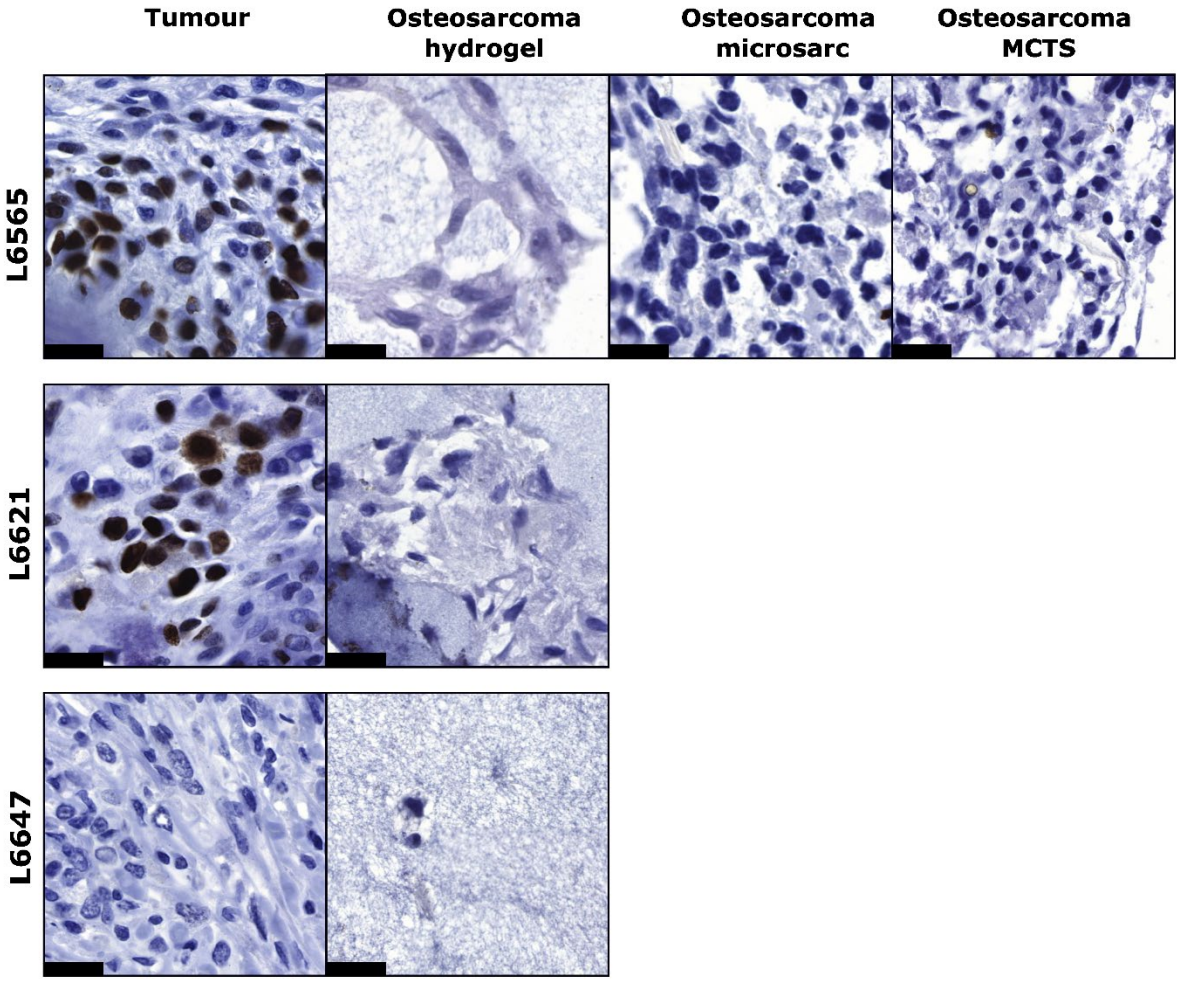

Figure S1. SATB2 staining of osteosarcomas L6565, L6621 and L6647 and the corresponding 3D cultures (hydrogel, microsarc or MCTS). Only these patient samples were stained for SATB2. Scalebar = 20 µm.

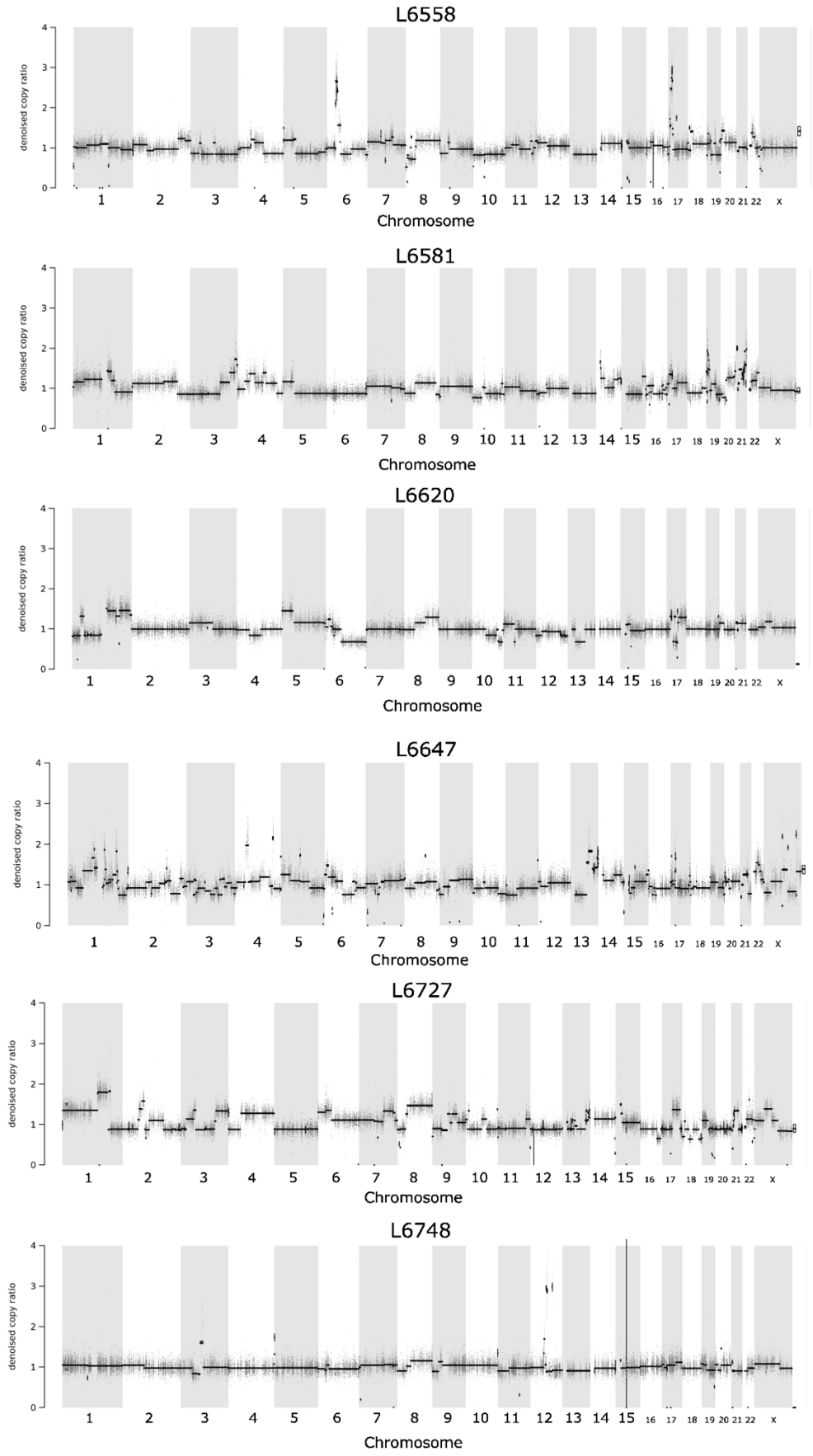

Figure S2. Copy number profiles of L6558, L6581, L6620, L6647, L6727 and L6748.

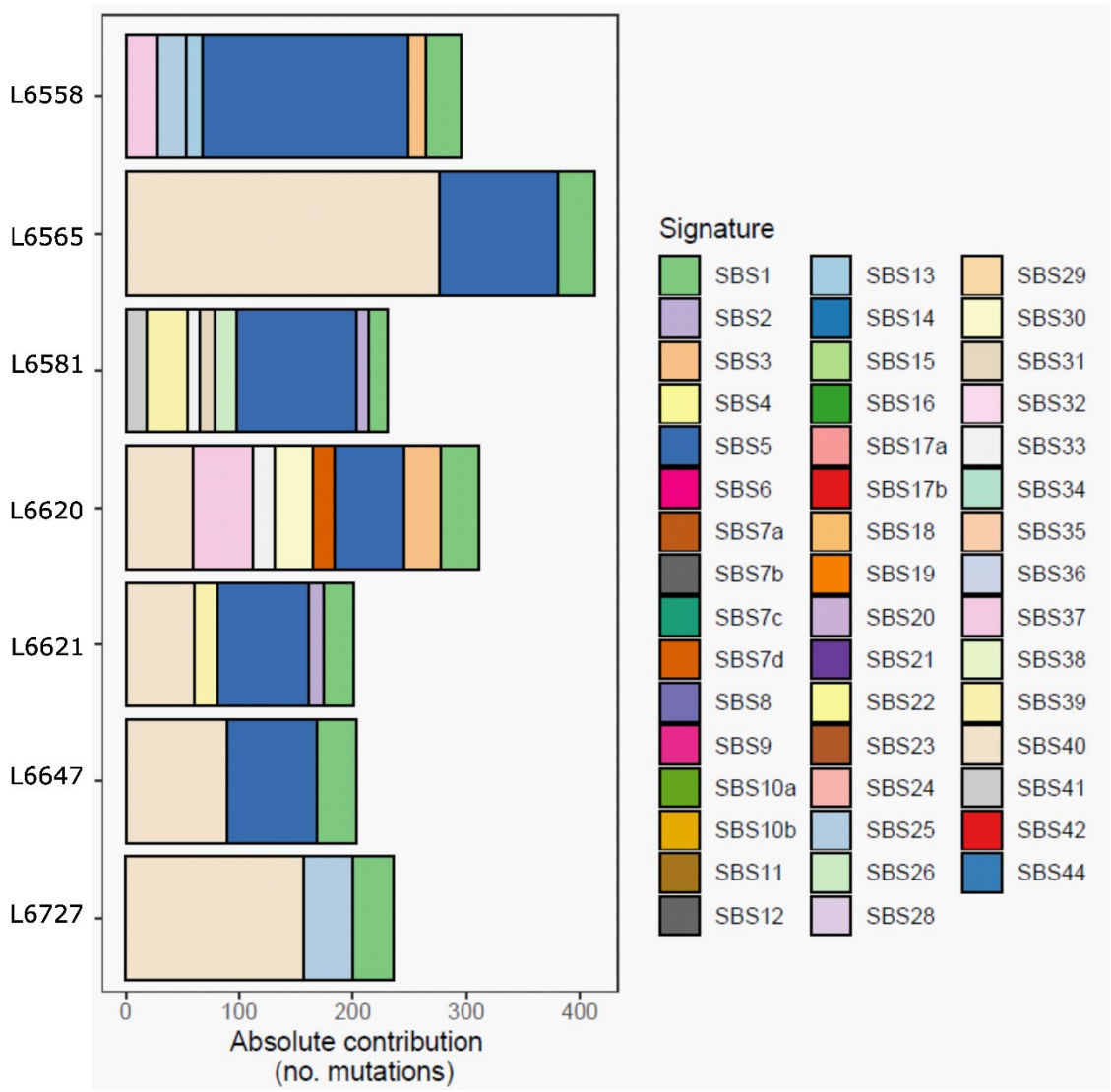

Figure S3. Mutational signatures for all osteosarcoma samples, based on SBS reference signatures.

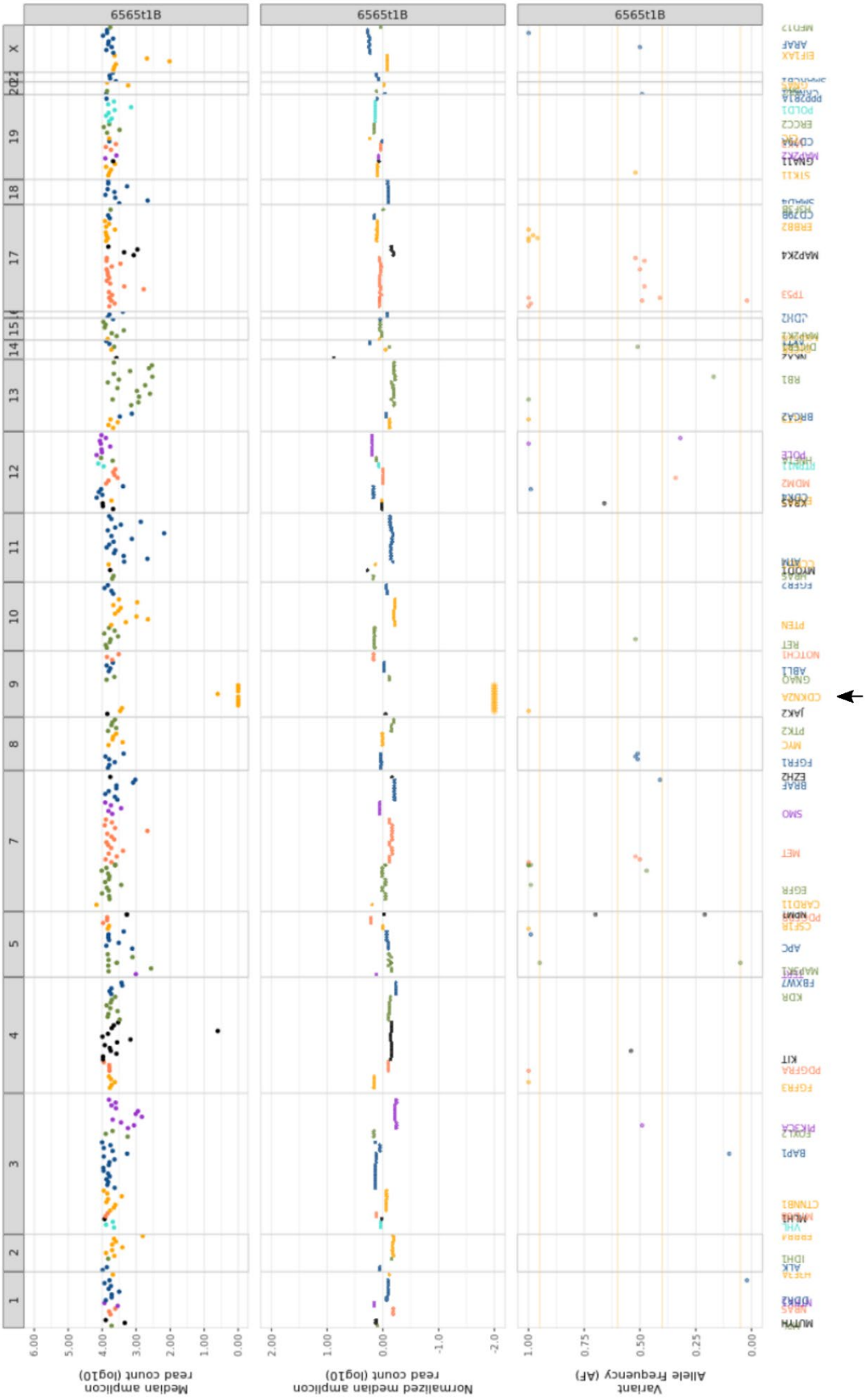

Figure S4. Copy number variation analysis based on Cancer hot spot panel sequencing of L6565. Upper panel: logarithmic scale, each dot represents the median read count per amplicon. Middle panel: normalized read counts. Lower panel: variant allele frequency. Arrow indicates the copy number loss of *CDKN2A*.

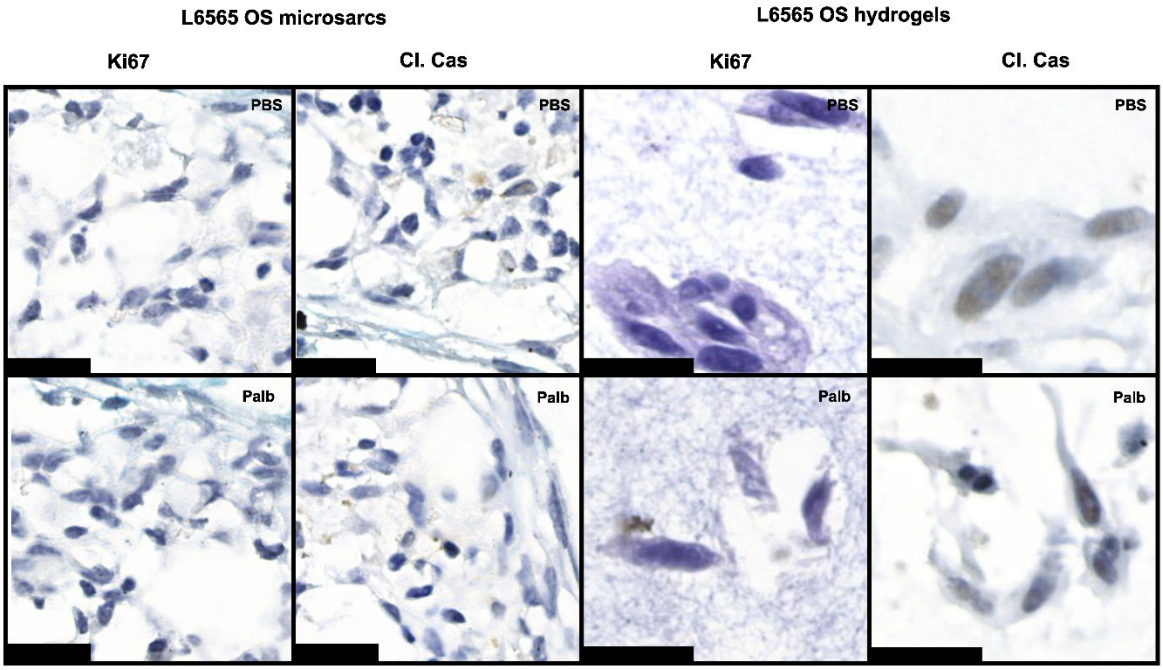

Figure S5. Ki67 and cleaved caspase 3 staining for L6565 osteosarcoma hydrogels and microsarcs. Scalebar = 20 µm

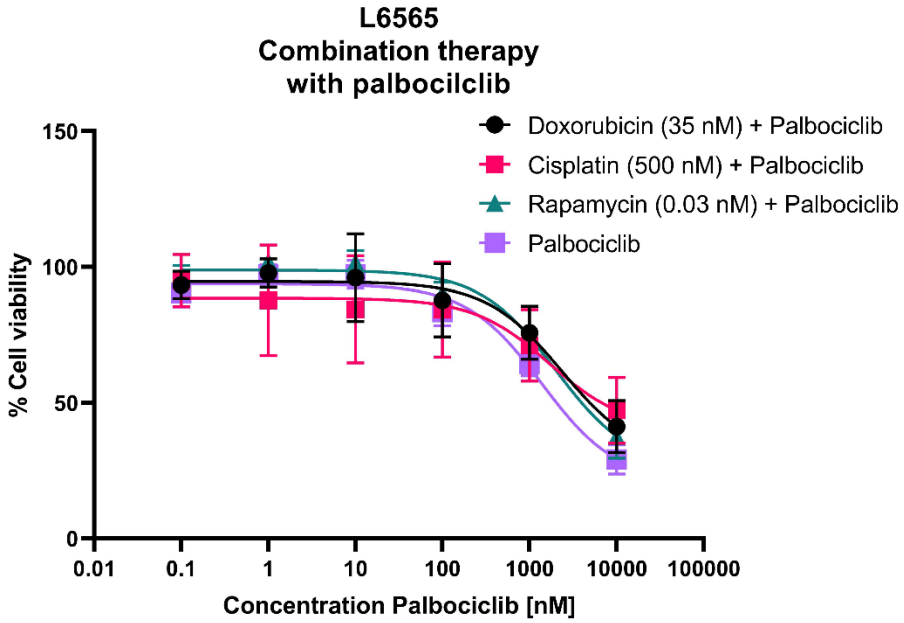

Figure S6. L6565 2D cultured cells were treated with different drugs combined with palbociclib. The x-axis depicts the concentration of palbociclib. Cells were pre-treated with doxorubicin (35 nM) and cisplatin (500 nM) for 24 hours, after which palbociclib was added for 72 hours in total. Rapamycin (0.03 nM) and palbociclib were administered simultaneously for 72 hours. Graph represents the average of three experiments performed In triplicate, with the standard deviation.