A murine mesenchymal stem cell model for initiating events in osteosarcomagenesis points to CDK4/CDK6 inhibition as a therapeutic target

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Osteosarcoma is a high-grade bone-forming neoplasm, with a complex genome. Tumours frequently show chromothripsis, many deletions, translocations and copy number alterations. Alterations in the p53 or Rb pathway are the most common genetic alterations identified in osteosarcoma. Using spontaneously transformed murine mesenchymal stem cells (MSCs) which formed sarcoma after subcutaneous injection into mice, it was previously demonstrated that p53 is most often involved in the transformation towards sarcomas with complex genomes, including osteosarcoma. In the current study, not only loss of p53 but also loss of p16\textsubscript{INK4A} is shown to be a driver of osteosarcomagenesis: murine MSCs with deficient p15\textsubscript{INK4B}, p16\textsubscript{INK4A} or p19\textsubscript{Arf} transform earlier compared to wild-type murine MSCs. Furthermore, in a panel of nine spontaneously transformed murine MSCs, alterations in p15\textsubscript{INK4B}, p16\textsubscript{INK4A}, or p19\textsubscript{Arf} were observed in eight out of nine cases. Alterations in the Rb/p16 pathway could indicate that osteosarcoma cells are vulnerable to CDK4/CDK6 inhibitor treatment. Indeed, using two-dimensional (n = 7) and three-dimensional (n = 3) cultures of human osteosarcoma cell lines, it was shown that osteosarcoma cells with defective p16\textsubscript{INK4A} are sensitive to the CDK4/CDK6 inhibitor palbociclib after 72-hour treatment. A tissue microarray analysis of 109 primary tumour biopsies revealed a subset of patients (20–23%) with intact Rb, but defective p16 or overexpression of CDK4 and/or CDK6. These patients might benefit from CDK4/CDK6 inhibition, therefore our results are promising and might be translated to the clinic.

INTRODUCTION

Osteosarcoma is the most common malignant mesenchymal tumour of the bone in children and adolescents characterized by osteoid formation. Compared to other cancer types driven by a specific mutation, osteosarcomas show highly complex genomes with a relatively high occurrence of chromoaanagenesis, such as chromothripsis. Recurrent alterations are rare, but most often osteosarcomas harbour loss-of-function alterations in TP53 (47–90%) and RB1 (29–47%). The second most common alteration inactivates RBF, a tumour suppressor gene controlling cell cycle progression. Other regulators of the cell cycle pathway are also often affected in osteosarcoma. CDK4 is amplified in 10% of high-grade osteosarcomas, and together with CDK6 directly controls Rb activity by phosphorylation of Rb. Upstream of the Rb pathway, p15\textsuperscript{INK4B} and p16\textsuperscript{INK4A} can inhibit CDK4 and CDK6 activity. p15\textsuperscript{INK4B} is transcribed from the CDKN2B gene, whereas p16\textsuperscript{INK4A} and its alternate reading frame p14\textsuperscript{ARF} (p19\textsuperscript{ARF} in mouse), is transcribed from the CDKN2A gene (a schematic overview of the locus is depicted in Supplementary Fig. S1). p14\textsuperscript{ARF}/p19\textsuperscript{ARF} is involved in activating p53-dependent growth arrest. CDKN2B and CDKN2A are adjacent loci on the genome and are often co-deleted. Somatic alterations in both genes have been identified in 14–19% of osteosarcomas and also often in other tumour types. In particular, deletions of CDKN2A are clinically relevant, as loss of p16\textsubscript{INK4A} is correlated with poor overall survival and poor response to chemotherapy.

We previously demonstrated that murine and canine mesenchymal stem cells (MSCs) spontaneously transform in vitro and can be used to model driver or initiating events involved in the development of sarcomas with complex genomics, including osteosarcoma. We showed that spontaneously transformed murine MSCs harbour point mutations in Trp53 and/or copy number alterations in Cdkn2a and Cdkn2b. Upon inactivation of Trp53, murine MSCs transformed earlier compared to wild-type, confirming the contribution of loss of p53 to spontaneous transformation and development of sarcomas with a complex genome.

In the current study, we investigate the role of the Cdkn2a/Cdkn2b genes in the spontaneous transformation of murine MSCs towards osteosarcoma. We show that murine MSCs with deficient p15\textsuperscript{INK4B}, p16\textsuperscript{INK4A}, or p19\textsuperscript{ARF} transform earlier compared to wild-type MSCs. Furthermore, we demonstrate that the defective cell cycle regulation pathway caused by p16\textsuperscript{INK4A} inactivation can be therapeutically exploited using the selective CDK4/CDK6 inhibitor...
palbociclib in both 2D and 3D in vitro culture models of osteosarcoma cell lines. Our study demonstrates that 20–23% of primary osteosarcoma biopsies showed intact Rb, but defective p16 in >4% or overexpression of CDK4 and/or CDK6, indicating potential benefit from CDK4/CDK6 inhibition in almost one quarter of osteosarcoma patients.

MATERIALS AND METHODS

Cell culture

Murine bone-marrow-derived mesenchymal stem cells (MSCs) were isolated as described previously20, from surplus C57BL/6 J (B6_4, B6_5, B6_7, B6_10), surplus NMRI (NMRI_2, NMRI_3, NMRI_9) mice, or C57BL/6 J mice kindly gifted by Dr. Melissa van Pel (BM42, BM91). Growth curves, differentiation capacity, in vivo growth capacity and a detailed genomic analysis using whole genome sequencing have been described elsewhere for B6_4, B6_5, B6_7, B6_10.14 Additional murine MSCs were isolated from surplus FVB mice and mice with defects in p15INK4b (Ink4b−/−), p16INK4a (Ink4a−/−), p15INK4b and p16INK4a (Ink4ab−/−), or p19ARF (Arf−/−) mice. Mice with deficient p15INK4b (Ink4b−/−) and p16INK4a (Ink4a−/−) were generated as described previously21 (Supplemental Fig. S1). Genetic knockout was confirmed at the protein level by Western blotting and for Ink4a−/− also at the DNA level by PCR using 5′ TGTGGCAACTGATTCAGTTGG 3′ (Gibco) at 37 °C with 5% CO2 in a humidified incubator at 37 °C with 5% CO2 for 4 weeks, after which cells were imaged with GelCount (Oxford Optronix, Milton, UK). Previously transformed cells (B6_10)22 were taken along as a positive control.

Western blotting

For p15, p16, Rb and GAPDH Western blots, whole cell Hot-SDS lysates of murine MSCs or osteosarcoma cell lines were collected as described previously23. For p19 and Histon H3 western blots, nuclear lysates were made by washing cells twice with cold PBS, followed by the addition of PBS-Triton X (0.5%) for 10 min, while shaking on ice. Cells were centrifuged twice, washed with PBS-Triton X (0.5%) and the pellet was resuspended in Hot-SDS buffer (1% SDS, 10 mM EDTA, 10 mM Tris pH 7.4) containing protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (Roche). Protein concentrations of lysates were determined with the Biorad DC25 protein assay kit (Bio-rad, Hercules, CA, USA) according to the manufacturer’s protocol, measured with a microplate reader (Infinite M Plex, Tecan Group Ltd.).

Sample loading, blotting and quantification were performed as previously described19. Blots were stained for p15 (1:500, Abcam, Cambridge, UK), p16 (1:1000, clone JCB, Immunologic, WellMed BV, Duiven, The Netherlands), p19 (1:5000, clone ab80, Abcam), Rb (1:5000, clone G3-245, BD Pharmingen, San Diego, CA, USA), Histon H3 (Invitrogen Life Technologies), GAPDH (AbD Serotec, Kidlington, UK), or GAPDH (1:3000, Cell Signalling). Blots were developed with SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific) using the ChemiDoc Touch Imaging System (Bio-rad).

Immunohistochemistry

Tissue micro array construction. For this study five osteosarcoma tissue micro arrays (TMA) were used, that contain FFPE samples of primary tumour biopsies, primary tumour resections, local relapses and metastases of 158 patients (not all sample types were available for each patient). Clinicopathological details can be found in Supplementary Table S1. Good histological response to chemotherapy was defined as >90% necrosis after chemotherapy24. The construction of one of the TMAs has been described previously (cohorts 1, 2, 25). For the construction of TMAs for cohorts 3, 4, and 4, punches (1.0 mm (cohort 3) or 1.5 mm (cohort 4)) of formalin-fixed paraffin-embedded tumour samples were placed into an acceptor block using the TMA master (3DHISTECH, Budapest, Hungary). For each tumour, three tissue-cores were present in the same block. For cutting sections, the tape transfer system (39475205, Leica Biosystems, Wetzlar, Germany) was used. Each TMA also contained other tissue types as internal controls for immunohistochemistry.

Immunohistochemical staining. Slides of each TMA or slides containing sections collected from paraaffin (10 μm) CellSpheres (LabVision, Fremont, CA, USA) were covered with 3% hydrogen peroxide (H2O2) for 15 min to block endogenous peroxidase activity, washed with PBS, and then blocked with 10% normal horse serum (NHS) for 30 min. Slides were incubated for 1 h with primary antibody against p15INK4b (Cell Signaling), cleaved caspase 3 (Cell Signaling), cleaved caspase 3 (Cell Signalling), cleaved caspase 3 (Cell Signalling), cleaved caspase 3 (Cell Signalling), SATB2 (clone CL0276, Sigma). For CDK6, SATB2, Ki67 and p16INK4a (Ink4a−/−), p19ARF (Arf−/−) mice. Mice with deficient p15INK4b (Ink4b−/−) and p16INK4a (Ink4a−/−) were generated as described previously21. Genetic knockout was confirmed at the protein level by Western blotting and for Ink4a−/− also at the DNA level by PCR using 5′ TGTGGCAACTGATTCAGTTGG 3′ (Gibco) at 37 °C with 5% CO2 in a humidified incubator and were tested regularly for mycoplasma. Each passage cells were trypsinized and counted with a Bürker-Türk counting chamber to calculate population doublings. Human osteosarcoma cell lines 143B, MG63, MDM, SAOS2, ZK58, HAL and KPD were cultured in RPMI 1640 (Gibco), supplemented with 10% FBS, in a humidified incubator at 37 °C and 5% CO2. Human cell lines were retrieved from the EuroBioNet consortium26 and were regularly STR profiled using the GenePrint 10 system kit (Promega, Madison, WI, USA) and tested for mycoplasma. For the generation of multi-cellular tumour spheroids (MCTS) of osteosarcoma cell lines (protocol adapted from ref27), cells were suspended in medium combined with methylcellulose (0.24% w/v) dissolved in DMEM, and seeded into a 1% agarose coated 96-well plate for seven days before the start of an experiment.

Drug treatment

For 2D cultures, human osteosarcoma cell lines or murine MSCs were seeded (between 3000 and 6000 cells per well) into 96-well plates and after 24 h treated with PBS or Palbociclib (dissolved in PBS, PD-0332991, Selleckchemicals, Houston, TX, USA) in concentrations ranging from 0.01 μM to 100 μM. Cells were fixed three days after treatment with 4% formaldehyde and stained with 2 μg/ml Hoechst (Invitrogen Life Technologies, Thermo Fisher Scientific, MA, USA) and nuclei were counted with the Cellomics ArrayScan VTI HCS 700 and HCS Studio Cell Analysis Software (Thermo Fisher Scientific). For MCTS, cells were treated with PBS or palbociclib in concentrations ranging from 0.1 μM to 100 μM. Cells were incubated three days after treatment with PrestoBlue cell viability reagent (Invitrogen Life Technologies) for 90 min and fluorescence was measured using a microplate reader (Infinite M Plex, Tecan Group Ltd., Zürich, Switzerland). After read-out, MCTS were fixed in 4% formaldehyde containing Alcin Blue (1:400) and paraffin embedded. To determine cell viability relative to PBS control, dose response curves were made using Graphpad Prism 8 software, after correcting for background reads and normalized growth rates of each cell line, as described previously23.

Transformation analysis of mesenchymal stem cells

Late passage (> passage 8) murine MSCs were subjected to metaphase analysis and the soft agar anchorage independent growth assay as described previously21. In short, for metaphase harvest murine MSCs were seeded and treated with Calyculin A (80 nM, LC Laboratories, Woburn, MA, USA). Hereafter, cells were washed, incubated with KCl (0.075 M) and fixed with methanol/glacial acetic acid in a ratio of 4:1. The cell suspension was dropped onto a slide and stained with DAPI for microscopic counting of metaphase chromosomes. For the soft agar anchorage independent growth assay, a cell suspension of 50,000 cells were seeded into a top layer of 0.35% agarose, on top of a bottom layer of 0.7% agarose in non-tissue culture treated 6-well plates and incubated in a humidified incubator at 37 °C with 5% CO2 for 4 weeks, after which cells were imaged with GelCount (Oxford Optronix, Milton, UK). Previously transformed cells (B6_10)22 were taken along as a positive control.
between the two observers, slides were reviewed by a third observer (J.V., M.G.B.) to reach a consensus. For quantification of MCTS, the number of positive cells of cleaved caspase 3 and Ki67 stainings was determined using QuPath Software v.0.2.3 on three different sections on each slide.

**RESULTS**

**Loss of CDKN2A/CDKN2B is frequent in spontaneously transformed murine MSCs**

We previously identified a large deletion in the Cdkn2a and Cdkn2b locus in one of three transformed murine MSC cultures (B6_7) that formed sarcoma in vivo, for which the Cdkn2a/Cdkn2b locus is shown in Fig. 1A. To evaluate the relevance of Cdkn2a and Cdkn2b loss we used a larger panel of nine spontaneously transformed murine MSCs, originating from different strains (C57BL/6 J or NMRI), for expression of p15
\(^{\text{Ink4b}}\), p16
\(^{\text{Ink4a}}\), and p19
\(^{\text{Arf}}\) at the protein level. Transformation of six out of nine murine MSC lines has been described previously, for expression of p15
\(^{\text{Ink4b}}\), p16
\(^{\text{Ink4a}}\), and p19
\(^{\text{Arf}}\) at the protein level. Furthermore, loss of p15
\(^{\text{Ink4b}}\), p16
\(^{\text{Ink4a}}\), and p19
\(^{\text{Arf}}\) is a common event, as six out of nine MSCs have lost p15
\(^{\text{Ink4b}}\) expression, and six out of nine MSCs have lost p19
\(^{\text{Arf}}\) expression (Fig. 1B). Eight out of nine MSCs show loss of p15
\(^{\text{Ink4b}}\) expression. However, in this Western blot loss of p16
\(^{\text{Ink4a}}\) protein expression is not always indicative for p16
\(^{\text{Ink4a}}\) deletion on genomic level, illustrated for instance by murine MSC line B6_4 that has intact p16
\(^{\text{Ink4a}}\) based on whole-genome sequencing data (Fig. 1B), but does not show p16
\(^{\text{Ink4a}}\) protein expression.

Murine MSCs deficient for p16
\(^{\text{Ink4a}}\) and for p15
\(^{\text{Ink4b}}\) transform earlier compared to wildtype MSCs

To evaluate whether loss of p16
\(^{\text{Ink4a}}\) and p15
\(^{\text{Ink4b}}\) or both is important in the spontaneous transformation of murine MSCs, MSCs from mice with loss of p16
\(^{\text{Ink4a}}\) (Ink4a
\(^{−/−}\)), loss of p15
\(^{\text{Ink4b}}\) (Ink4b
\(^{−/−}\)), or both (Ink4a
\(^{−/−}\), Ink4b
\(^{−/−}\) mice transformed earlier (after 23 days) compared to wild-type murine MSCs (after 64-76 days) (Fig. 2A). As the Cdkn2a gene also encodes an alternative reading frame, p19
\(^{\text{Arf}}\), murine MSCs with a loss of p19
\(^{\text{Arf}}\) were cultured and also shown to transform earlier (after 23 days) compared to wildtype MSCs (after 61 days) (Fig. 2B). For all knockout mice, the knockout was confirmed by Western blotting and for mice with deficient p16
\(^{\text{Ink4a}}\); p15
\(^{\text{Ink4b}}\) (Ink4ab
\(^{−/−}\)), mice transformed earlier (after 25-46 days) compared to wild-type murine MSCs (after 64-76 days) (Fig. 2A). As the Cdkn2a gene also encodes an alternative reading frame, p19
\(^{\text{Arf}}\), murine MSCs with a loss of p19
\(^{\text{Arf}}\) were cultured and also shown to transform earlier (after 23 days) compared to wildtype MSCs (after 61 days) (Fig. 2B). For all knockout mice, the knockout was confirmed by Western blotting and for mice with deficient p16
\(^{\text{Ink4a}}\); p15
\(^{\text{Ink4b}}\) (Ink4ab
\(^{−/−}\)) mice also at the DNA level by PCR (Supplementary Fig. S3). Transformation of murine MSCs was confirmed by metaphase analysis (Fig. 2C), as late passage MSCs had higher than the normal modal number of 40 chromosomes. However, none of the knockout MSCs showed soft agar anchorage independent growth (Fig. 2D). These results show that loss of p16
\(^{\text{Ink4a}}\) and/or p15
\(^{\text{Ink4b}}\) enhanced proliferation and genomic alterations suggesting transformation.

**Transformed murine MSCs with loss of p16
\(^{\text{Ink4a}}\) and p15
\(^{\text{Ink4b}}\) are sensitive to palbociclib**

Within the Rb pathway, the Ink4 proteins inhibit CDK4/CDK6 activity, thereby inhibiting cell cycle progression. Therefore loss of p16
\(^{\text{Ink4a}}\) and p15
\(^{\text{Ink4b}}\) could make cells more vulnerable to CDK4/CDK6 inhibition.
CDK6 inhibitors to decrease uncontrolled cell proliferation. To investigate this in our murine mesenchymal stem cell model, transformed murine MSCs, in which p16Ink4a and p15Ink4b loss was confirmed, were treated with the CDK4/CDK6 inhibitor palbociclib. Murine MSCs in which whole-genome sequencing previously confirmed loss of p16 Ink4a and p15 Ink4b (B6_7) were more sensitive to palbociclib, with an IC50 of 0.5 µM compared to transformed MSCs that have intact p16Ink4a and p15Ink4b (B6_4 and B6_10), with IC50 values of 3.6 and 10.3 µM, respectively (Fig. 3A).

To determine whether the increased sensitivity was caused by loss of p16Ink4a and p15Ink4b, murine MSCs isolated from Ink4ab−/− mice were treated with palbociclib (Fig. 3B). MSCs from Ink4ab−/− mice showed the highest sensitivity to palbociclib, with IC50 values between 0.8 and 1.2 µM, compared to wild-type MSCs, with an IC50 of 8.3 µM, suggesting that loss of p16Ink4a and p15Ink4b increases sensitivity to palbociclib.

**Human osteosarcoma cell lines are sensitive to palbociclib**

Osteosarcoma cell lines were treated with palbociclib in 2D cultures and IC50 values were determined (Fig. 4A). A highly variable dose-dependent response to palbociclib in all osteosarcoma cell lines was observed. As the efficacy of CDK4/CDK6 inhibition relies on the presence of intact Rb, the Rb status of each osteosarcoma cell line was determined by Western blot. All osteosarcoma cell lines (143B, MG63, MHM, HAL and KPD), except for SAOS2 and ZK58, showed Rb expression (Fig. 4B). SAOS2 and ZK58, with loss of Rb, indeed showed the highest IC50 of palbociclib compared to other osteosarcoma cell lines. However, the difference in IC50 value between ZK58 and other osteosarcoma cell lines with intact Rb was smaller than for SAOS2. The expression status of other proteins involved in the p16-Rb pathway was investigated, including p16INK4A, CDK4 and CDK6. p16INK4A protein expression of each cell line was based on immunohistochemical expression published previously (Fig. 1B). The p16 immunohistochemical status of each cell line was combined with the IC50 values from the current study. Although osteosarcoma cells with loss of p16INK4A showed on average a lower IC50 to palbociclib (1.4 µM) than cells with normal p16INK4A expression (4.1 µM), this difference was not statistically significant (p = 0.3) (Fig. 4C). CDK4 and CDK6 RNA expression levels were
3D cultures of osteosarcoma are also sensitive to palbociclib

Since 2D cultures might be less representative for the in vivo situation compared to 3D cultures, as demonstrated in previous studies, the response to palbociclib in 3D cultures of osteosarcoma cell lines was investigated. Multi-cellular tumour spheroids (MCTS) of three osteosarcoma cell lines were generated (MHM, SAOS2 and MG63) and histological analysis showed the morphological heterogeneity that is also seen in primary tumours (Fig. 5A). MCTS of SAOS2 and MHM show areas suggestive of extracellular matrix deposition and tumour cells were focally positive for SATB2, a marker for osteogenic differentiation (Fig. 5A). MCTS of all osteosarcoma cell lines showed a dose-dependent decrease in cell viability after treatment with palbociclib (Fig. 5B). The IC50 values of 3D cultures were higher compared to 2D cultures (Fig. 5B). In MCTS of osteosarcoma cell lines MHM and MG63, palbociclib treatment significantly reduced the percentage of proliferating cells and increased apoptosis as evident from Ki67 and cleaved caspase 3 staining, respectively (Fig. 5C).

Protein expression of Rb, CDK4 and CDK6 in osteosarcoma patient primary tumour tissue

To estimate which percentage of osteosarcoma patients might be eligible for palbociclib treatment, the expression levels of proteins in the p16-Rb pathway, including Rb, CDK4, CDK6 and p16, was determined in primary tumour tissue of 109 patients using TMA (Table 1; Fig. 6A). As Rb status determines the efficacy of CDK4/CDK6 inhibition, first Rb expression was evaluated. 36.4% of the osteosarcomas have lost expression of Rb. As palbociclib directly inhibits CDK4 and CDK6, protein expression levels of CDK4 and CDK6 were determined. 23.7% of primary tumours were CDK4 positive and 44.8% were CDK6 high. p16 scores have been determined in a previous study in osteosarcoma patients (cohort 2), where 20.5% of osteosarcoma patients showed loss of p16. As a combination of Rb expression and CDK4/CDK6 expression or loss of p16 expression are expected to imply sensitivity to CDK4/CDK6 inhibition, the combination scores were determined (Table 1). Of the primary tumour biopsies, 22.7% of the tumours were positive for both Rb and CDK4, 52.9% were positive for Rb and CDK6, and 43.1% was positive for Rb, and CDK4 or CDK6. In cohort 2, 13.3% of the primary tumour biopsies were positive for Rb, but lost p16 expression. In total, between 20.0% and 23.3% of the tumours are Rbpositive/p16negative, Rbpositive/CDK6positive, or Rbpositive/CDK4positive/CDK6high, which corresponds to the group of patients that might benefit from CDK4/CDK6 inhibitor treatment. A previous study showed that p16 loss was prognostic for poor overall survival in osteosarcoma patients. In the current study, CDK6 scores were found to be prognostic for overall survival, where patients with overexpression of CDK6 have a worse overall survival compared to those patients with low CDK6 expression (Fig. 6B). For Rb and CDK4 expression there was no significant difference in survival (Supplementary Fig. S4). Neither Rb, CDK4, or CDK6 scores are prognostic for metastasis-free survival (Supplementary Fig. S4) or response to chemotherapy (not shown).

DISCUSSION

Transformed murine MSCs provide an excellent model to identify drivers of transformation in osteosarcoma. In addition to p53 alterations, transformed murine MSCs also frequently lose expression of p15Ink4b, p16Ink4a and p19Arf. However, the protein expression status did not always correspond to the genomic status, as is evident for MSC line B6.4 with loss of p16 expression without concomitant genomic alterations at the CDKN2A locus. Alternative mechanisms of p16Ink4a protein expression loss, such as promoter methylation may be active. Nevertheless, the loss of expression of p15Ink4b, p16Ink4a and p19Arf in murine MSCs, reflects the genomic status of human osteosarcoma where alterations in p15Ink4b, p16Ink4a, or p14Arf are also frequently found.

This study shows that loss of either p15Ink4b, p16Ink4a, or p19Arf in the murine MSC model accelerates transformation. Using the
same murine MSC model, we have previously published that these transformed murine MSCs, including those with loss of CDKN2A/CDKN2B, show hallmarks of osteosarcoma with a highly complex genome and many copy number alterations. Although chromothripsis could not be evaluated in the current study, not all human osteosarcomas show signs of chromothripsis either, since this only occurs in ~30% of cases. In another study, we have demonstrated that mice injected with transformed murine MSCs, all with loss of CDKN2A/CDKN2B, formed osteosarcoma, with evident osteoid formation by atypical tumour cells. Thus, our results combined with previously published results, suggest that loss of CDKN2A/CDKN2B is an early driver event in osteosarcoma. Loss of genes in the Rb-p16 pathway is indeed considered as an early event in the transformation towards osteosarcoma. The exact interplay with alterations in the p53 pathway is currently unknown, and since not all late passage murine MSCs with known loss of p16INK4A or p53 could form colonies in soft agar, it is not yet known which combination of genomic alterations are required for transformation. This would warrant further investigation.

Loss of p16INK4A protein expression as a result of homozygous loss of the CDKN2A genomic region has previously been shown to correlate with poor prognosis as well as a poor response to neoadjuvant chemotherapy in osteosarcoma patients. Thus, studying the loss of p16INK4A in osteosarcoma is clinically relevant and gives a rationale for exploring the Rb-p16 pathway as a novel therapeutic option. Cells with loss of p16INK4A are hypothesized to be more sensitive to CDK4/CDK6 inhibition. Therefore, we investigated the sensitivity to CDK4/CDK6 inhibitor palbociclib in osteosarcoma cells. We confirmed that both 2D and 3D cultures of osteosarcoma cells show a dose-dependent decrease in cell viability after palbociclib treatment. This is in line with other in vitro studies showing that pan-CDK or specific CDK4/CDK6 inhibition in osteosarcoma resulted in growth inhibition of cells and increased senescence and/or apoptosis.

In the current study, 2D cultures were more sensitive compared to 3D cultures. The difference in sensitivity is not surprising and could be explained by the formation of tight intercellular contacts within MCTS which hamper drug penetration. In general, the concentrations of palbociclib used in the current study, especially in 3D, are higher compared to concentrations used in the clinic for treatment of HER-2 negative breast cancer and in breast cancer cell lines in vitro.

However, we have treated the cells with a single dose, whereas in the clinic multiple doses are administered. Moreover, the IC50 values determined in the current study are in a comparable concentration range.
Fig. 5  3D cultured MCTS of osteosarcoma cell lines are also sensitive to palbociclib. A Hematoxylin and eosin staining of osteosarcoma MCTS treated with palbociclib for 72 h. Scalebar represents 20 µm. Inset shows SATB2 staining. B Relative cell viability and IC50 values were determined after treatment with palbociclib. C Cleaved caspase 3 and Ki67 staining and quantification of 3D cultured MCTS of OS cell lines treated with 10 µM palbociclib (Palb) or PBS. Scale bar represents 20 µm. n.s. not statistically significant, **p ≤ 0.01, ***p ≤ 0.001.
Table 1. Overview of immunohistochemical expression of Rb, CDK4 and CDK6, combined with previously published p16 protein expression results, in tissue micro-arrays of osteosarcoma.

| RB        | Primary tumour biopsy | Primary tumour resection | Local recurrence | Metastasis |
|-----------|-----------------------|--------------------------|----------------|------------|
|           | Total (n = 66)        | Total (n = 68)           | Total (n = 9)  | Total (n = 38) |
| positive  | 42 (63.6%)            | 42 (61.7%)               | 6 (66.7%)      | 32 (84.2%) |
| negative  | 24 (36.4%)            | 26 (38.2%)               | 3 (33.3%)      | 6 (15.8%)  |
| CDK4      |                       |                          |                |            |
| positive  | 18 (23.7%)            | 15 (19.5%)               | 4 (44.4%)      | 7 (18.4%)  |
| negative  | 58 (76.3%)            | 62 (80.5%)               | 5 (55.6%)      | 31 (81.6%) |
| CDK6      |                       |                          |                |            |
| high      | 26 (44.8%)            | 32 (57.1%)               | 6 (85.7%)      | 12 (46.2%) |
| low       | 32 (56.2%)            | 24 (42.8%)               | 1 (14.3%)      | 14 (53.8%) |

Combination status

| Combination status | |
|--------------------|------|
| Rb\textsuperscript{positive} and CDK4\textsuperscript{positive} | 15/66 (22.7%) |
| Rb\textsuperscript{positive} and CDK6\textsuperscript{positive} | 27/51 (52.9%) |
| Rb\textsuperscript{positive} and CDK4\textsuperscript{positive} and CDK6\textsuperscript{positive} | 6/51 (11.8%) |
| Rb\textsuperscript{positive} and CDK4\textsuperscript{positive} or CDK6\textsuperscript{positive} | 22/51 (43.1%) |

P16 (combination) status

| P16 (combination) status | |
|--------------------------|------|
| Rb\textsuperscript{positive} and p16\textsuperscript{negative} | 4/30 (13.3%) |
| Rb\textsuperscript{positive} /p16\textsuperscript{negative} or Rb\textsuperscript{positive} /CDK4\textsuperscript{positive} | 6/30 (20.0%) |
| Rb\textsuperscript{positive} /p16\textsuperscript{negative} or Rb\textsuperscript{positive} /CDK6\textsuperscript{positive} | 7/30 (23.3%) |

Fig. 6 Immunohistochemistry of osteosarcoma tissue micro array. A Example of immunohistochemical staining of Rb, CDK4, or CDK6, scored as positive or negative in tissue micro-arrays of osteosarcoma. Scale bar represents 50 µm. B Kaplan–Meier curves of overall survival in osteosarcoma patients, based on CDK6 scores.
range when compared to other in vitro studies in osteosarcoma cells using palbociclib. Thus, our data support previous findings that CDK4/CDK6 inhibition might be a new targeted treatment strategy for osteosarcoma patients. However, more research is needed to investigate the efficacy of palbociclib for osteosarcoma patients, and whether there are any adverse effects. For the treatment of breast cancer, it was reported that the adverse effects of palbociclib include neutropenia, leukopenia or anemia.

Currently, two phase 2 clinical trials are ongoing which include advanced cases of sarcoma that overexpress CDK4, including osteosarcoma, for palbociclib treatment or similar CDK4/CDK6 inhibitors. One of these studies was also designed to test the utility of CDK4 expression in predicting tumour response to CDK inhibitors, but this study did not evaluate whether patients show loss of p16, or intact Rb. In the current study sensitivity to palbociclib did not correlate with CDK4 expression in osteosarcoma cells.

Not only CDK4, but also CDK6 was evaluated as a prognostic and predictive biomarker in our study. Interestingly, about half of the osteosarcoma patients (44.8%) showed overexpression of CDK6 which was associated with a worse overall survival. In this group of patients palbociclib could be beneficial to improve outcome. Palbociclib is most effective when Rb is intact, which was the case in approximately half (52.9%) of the patients with CDK6 overexpression. The expression status of CDK4 and CDK6 is important to consider, since it was recently published that palbociclib selectively dissociates p21 from cyclin D1-CDK4-p21 complexes and not of cyclin D1-CDK6-p21 complexes, which could affect drug sensitivity and resistance.

Another subgroup of osteosarcoma patients that might benefit from CDK4/CDK6 inhibition, include patients with intact Rb, but loss of p16 expression. The in vitro study confirms that osteosarcoma cell lines with intact Rb and/or loss of p16 are more sensitive to palbociclib compared to osteosarcoma cell lines with loss of Rb and/or intact p16. Furthermore, osteosarcoma cell lines with loss of p16 staining by immunohistochemistry showed a trend towards higher sensitivity to palbociclib compared to cells without loss of p16. The immunohistochemical study on the tissue microarray indicates that 13.3% of the patients falls within this category of intact Rb and loss of p16 that may benefit from palbociclib treatment. Taken together, our results illustrate that there is a clear subset of osteosarcoma patients (20–23%) for which CDK4/CDK6 inhibition might be promising and that loss of p16 protein expression or overexpression of CDK6, combined with intact Rb, may serve as a biomarker to select eligible patients.

In conclusion, this study demonstrated that our model of transformed murine MSCs provide a valuable tool to identify targets for therapy and the identification of biomarkers for osteosarcoma patients. Our results illustrate that loss of CDKN2A and/or CDKN2B are early events in the development of osteosarcoma, and that these events can be targeted by CDK4/CDK6 inhibition, which might be used as a novel therapeutic option in approximately 20–23% of the patients.

DATA AVAILABILITY
All data generated or analyzed during this study are included in this published article and its supplementary information files.

REFERENCES
1. Behjat, S. et al. Recurrent mutation of IGF signalling genes and distinct patterns of genomic rearrangement in osteosarcoma. Nat. Commun. 8, 15936 (2017).
2. Stephens, P. J. et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell 144, 27–40 (2011).
3. Cortes-Criana, I. et al. Comprehensive analysis of chromothripsis in 2,658 human cancers using whole-genome sequencing. Nat. Genet. 52, 331–341 (2020).
4. Perry, J. A. et al. Complementary genomic approaches highlight the PI3K/mTOR pathway as a common vulnerability in osteosarcoma. Proc. Natl Acad. Sci. USA 111, E5564–E5573 (2014).
5. Kovac, M. et al. Exome sequencing of osteosarcoma reveals mutation signatures reminiscent of BRCA deficiency. Nat. Commun. 6, 8940 (2015).
6. Weinberg, R. The retinoblastoma protein and cell cycle control. Cef 81, 323–330 (1995).
7. Sayles, L. C. et al. Genome-informed targeted therapy for osteosarcoma. Cancer Discov. 9, 46–63 (2019).
8. Mejia-Guerrero, S. et al. Characterization of the 12q15MDM2and 12q13–14CDK4amplics and clinical correlations in osteosarcoma. Genes Chromosom. Cancer. https://doi.org/10.1002/gcc.20761, NA-NA (2010).
9. Burkhart, D. L. & Sage, J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat. Rev. Cancer 8, 671–682 (2008).
10. Sherr, C. J. & Weber, J. D. The ARF/p53 pathway. Curr. Opin. Genet. Dev. 10, 94–99 (2000).
11. Tsujiya, T. et al. Analysis of the p16INK4, p14ARF, p15, TP53, and MDM2 Genes and Their Prognostic Implications in Osteosarcoma and Ewing Sarcoma. Cancer Genet. Cytogenet. 120, 91–98 (2000).
12. Mohseny, A. B. et al. Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of CDkn2. J. Pathol. 219, 294–305 (2009).
13. Righi, A. et al. p16 expression as a prognostic and predictive marker in high-grade localized osteosarcoma of the extremities: an analysis of 357 cases. Hum. Pathol. 58, 15–23 (2016).
14. Kosemehmetoglu, K., Arcic, F., Karsilogo, Y., Kandemir, O. & Ozcan, A. p16 expression predicts neoadjuvant tumor necrosis in osteosarcomas: reappraisal with a larger series using whole sections. Hum. Pathol. 50, 170–175 (2016).
15. Bu, J. et al. P16/nk4a overexpression and survival in osteosarcoma patients: a meta analysis. Int. J. Clin. Exp. Pathol. 7, 6091–6096 (2014).
16. Borys, D. et al. P16 expression predicts necrotic response among patients with osteosarcoma receiving neoadjuvant chemotherapy. Hum. Pathol. 43, 1948–1954 (2012).
17. Malra, A., Roberts, H., Weinberg, A. & Geradts, J. Loss of p16ink4a expression correlates with decreased survival in pediatric osteosarcomas. Int. J. Cancer 95, 34–8 (2001).
18. Franceschini, N. et al. Transformed murine and canine mesenchymal stem cells as a model for sarcoma with complex genomics. Cancers 13, 1126 (2021).
19. Krupenfort, P. et al. p15Ink4b is a critical tumour suppressor in the absence of p16ink4a. Nature 448, 943–946 (2007).
20. Ottaviano, L. et al. Molecular characterization of commonly used cell lines for bone tumor research: a trans-European EuroBoNet effort. Genes Chromosom. Cancer. 49, 40–51 (2010).
21. Zhang, W. et al. Optimization of the formation of embedded multicellular spheroids of MCF-7 cells: how to reliably produce a biomimetic 3D model. Anal. Biochem. 515, 47–54 (2016).
22. Hafner, M., Niepel, M., Chung, M. & Sorger, P. K. Growth rate inhibition metrics correct for confounders in measuring sensitivity to cancer drugs. Nat. Methods 13, 521–527 (2016).
23. WHO Classification of Tumours of Soft Tissue and Bone 5th edn, Vol. 3 (WHO Classification of Tumours Editorial Board, 2020).
24. Mohseny, A. B. et al. Small deletions but not methylation underlie CDKN2A/p16 loss of expression in conventional osteosarcoma. Genes Chromosom. Cancer. 49, 1095–1103 (2010).
25. Buddingh, E. P. et al. Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents. Clin. Cancer Res. 17, 2110–2119 (2011).
26. Schrage, Y. M. et al. Central chondrosarcoma progression is associated with pRB pathway alterations: CDK4 down-regulation and p16 overexpression inhibit cell growth in vitro. J. Cell Mol. Med. 13, 2843–2852 (2009).
27. Gong, W. et al. Cyclin-dependent kinase 6 (CDK6) is a candidate diagnostic biomarker for early non-small cell lung cancer. Transl. Cancer Res. 9, 95–103 (2020).
28. Barkhead, P. et al. QuPath: open source software for digital pathology image analysis. Sci. Rep. 7, 16878 (2017).
29. Kresse, S. H. et al. Integrative analysis reveals relationships of genetic and epigenetic alterations in osteosarcoma. PloS ONE 7, e48262 (2012).
30. Chow, T. et al. Creating in vitro three-dimensional tumor models: a guide for the biofabrication of a primary osteosarcoma model. Tissue Eng. Part B 8 https://doi.org/10.1089/ten.teb.2020.0254 (2020).
31. De Luca, A. et al. Relevance of 3D culture systems to study osteosarcoma environment. J. Exp. Clin. Cancer Res. 37, 2 (2018).
32. Gao, S., Shen, J., Hornicek, F. & Duan, Z. Three-dimensional (3D) culture in sarcoma research and the clinical significance. Biofabrication 9, 032003 (2017).
33. Sherr, C. J., Beach, D. & Shapiro, G. I. Targeting CDK4 and CDK6: from discovery to therapy. Cancer Discov. 6, 353–367 (2016).
35. Guha, M. Cyclin-dependent kinase inhibitors move into Phase III. *Nat. Rev. Drug Discov.* **11**, 892–894 (2012).
36. Kovac, M., et al. The early evolutionary landscape of osteosarcoma provides clues for targeted treatment strategies. *J. Pathol.* https://doi.org/10.1002/path.5699 (2021).
37. Mohseny, A. B., Hogendoom, P. C. & Cleton-Jansen, A. M. Osteosarcoma models: from cell lines to zebrafish. *Sarcoma* **2012**, 417271 (2012).
38. Tang, Y., et al. P16 protein expression as a useful predictive biomarker for neoadjuvant chemotherapy response in patients with high-grade osteosarcoma. *Medicine* **96**, e6714 (2017).
39. Zhou, Y. et al. Expression and therapeutic implications of cyclin-dependent kinase 4 (CDK4) in osteosarcoma. *Biochim. Biophys. Acta Mol. Basis Dis.* **1864**, 1573–1582 (2018).
40. Perez, M., Galván, S. M., García, M. P., Marin, J. J. & Camero, A. Efficacy of CDK4 inhibition against sarcomas depends on their levels of CDK4 and p16ink4 mRNA. *Oncotarget* **6**, 40557–74 (2015).
41. Gong, X. et al. Generation of multicellular tumor spheroids with microwell-based agarose scaffolds for drug testing. *PloS ONE* **10**, e0130348 (2015).
42. Cristofanilli, M. et al. Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *Lancet Oncol.* **17**, 425–439 (2016).
43. Delmore, J. E. et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **166**, 904–917 (2011).
44. Finn, R. S. et al. Palbociclib and letrozole in advanced breast cancer. *N. Engl. J. Med.* **375**, 1925–1936 (2016).
45. Trial of Palbociclib in Second Line of Advanced Sarcomas With CDK4 Overexpression, https://clinicaltrials.gov/ct2/show/study/NCT03242382 Accessed 18-01-2021.
46. Abemaciclib for Bone and Soft Tissue Sarcoma With Cyclin-Dependent Kinase (CDK) Pathway Alteration, https://clinicaltrials.gov/ct2/show/NCT04040205 Accessed 18-01-2021.
47. Pack, L. R., Daigh, L. H., Chung, M. & Meyer, T. Clinical CDK4/6 inhibitors induce selective and immediate dissociation of p21 from cyclin D-CDK2 to inhibit CDK2. *Nat. Commun.* **12**, 3356 (2021).

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**AUTHOR CONTRIBUTIONS**

N.F., J.V.M.G.B. and A.M.C.J. designed the study. N.F. performed all MSC culture experiments. R.G. and N.F. scored all tissue micro-arrays, with J.V.M.G.B. scoring any discrepancies. A.K. and L.B. performed Western blotting and immunohistochemistry experiments. P.K. provided all FVB mice for this study. N.F., J.V.M.G.B. and A.M.C.J. analyzed the data. All authors contributed to and approved the final version of the manuscript.

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**COMPETING INTERESTS**

The authors declare no competing interests.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

All samples were handled according to the ethical guidelines described in “Code for Proper Secondary Use of Human Tissue in the Netherlands” in a coded (pseudonymized) manner, as approved by the Leiden University Medical Center ethical board (B17.036).

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