The added value of organoid models in the context of pediatric cancer research

Roos T.M. Romanesco (student ID: 6119964)

Plain summary

Organoids hold great potential in advancing cancer research. This review specifically highlights the advantages of using organoids in studies of pediatric eye cancer (or retinoblastoma), kidney cancer and liver cancer. The mechanisms underlying cancer in children are largely unknown and different from cancers mechanisms seen in adults. Therefore, they require their own models to accurately study tumor development and to potentially discover novel drug targets to develop targeted therapies. Organoids are typically created using stem cells, undifferentiated cells that harbor the potential to become various types of cells. Compared to traditional models like cancer cell lines, organoids more accurately replicate the three-dimensional structures and function of human organs, without the use of lab animals.

Organoid models capturing liver cancers in children, including hepatoblastoma (HB), hepatocellular carcinoma (HCC) and fibrolamellar carcinoma (FLC) have shown great potential in cancer research. The models, created from human tumor tissue, have shown to maintain their form, structure and genetic characteristics making them ideal for (personalized) drug screening and drug target discovery. For example, HB organoids have been used to study the role of epigenetic changes in tumor development and to identify potential drug targets. Similarly, HCC organoids have been applied to validate drug specificity. Furthermore, Organoids mimicking types of kidney cancer occurring in children such as Wilms tumor (WT), malignant rhabdoid tumor kidney (MRTK) and translocation renal cell carcinomas (tRCC) provide a reliable platform for drug development and (personalized) drug screening. Kidney cancer organoid models closely resemble the complexity and (genetic) diversity of tumor tissue. For instance, WT and MRTK organoids have been applied to study genetic mutations leading to kidney cancer development and to screen for the efficacy of potential novel therapies. Moreover, retinoblastoma is a rare eye cancer in children. Organoid models have shown to closely resemble the biology of retinoblastoma tumors. The genetic and physiological traits of original tumors were retained, and the models allowed to study early tumor development and effectively screen for potential drug therapies. Among other things, organoids were used to investigate the role of increased transcription of the cancer gene *MYCN* in retinoblastoma and to test the efficacy of drugs like sunitinib and ceftriaxone, showing promising results in drug screening and repurposing.

Organoid models may be created using different type of stem cells, each with their own advantages and disadvantages in pediatric cancer research. Overall, organoid models have provided a more accurate representative of human tumor tissue, closely resembling human tumor biology, compared to other pre-clinical models. Pediatric cancer organoid models may enhance research into mechanisms underlying cancer development and are able to screen and predict patients' personalized response to drug treatment. In addition, organoids may be used to study drug efficacy and toxicity and may enhance the translation of novel targeted drug therapies from pre-clinical models to the market.

Abstract

Pediatric cancers, including retinoblastoma, kidney cancer and liver cancer, present challenges in oncology due to their rarity and distinct underlying carcinogenesis compared to adult cancers. Traditional pre-clinical models, such as cancer cell lines and patient-derived xenografts, often fail to accurately recapitulate the heterogeneity and development of pediatric tumors. Organoid models, derived from iPSCs, hESCs or tumor tissue, offer a promising alternative closely mimicking the three-dimensional structures and heterogeneity of native tumors. This review explores the application of organoid models in pediatric cancer research and drug development. Distinct liver cancer, kidney cancer and retinoblastoma organoid models have demonstrated to maintain phenotypical and genetic features of tumor tissue, facilitating (high throughput) drug screening and studies elucidating cancer mechanisms. Organoids may enhance the accuracy of pre-clinical pediatric cancer studies and accelerate the development of novel treatments, holding significant potential for future fundamental and translational research.

Introduction

Pediatric cancer remains one of the most challenging areas in oncology (1). The rarity of pediatric cancer contributes to the global underestimation of its significant morbidity and mortality (2). The past decades the 5-year survival rate of children diagnosed with cancer has incrementally increased up to 80%, mainly attributed to the development of cytotoxic agents and improved diagnostics (3,4). However, children diagnosed with less common cancer subtypes have poorer prognosis (5). Research into pediatric malignancies has been limited and the pathogenesis of many (rare) subtypes remains unknown (4,5). Moreover, there is a lack of *in vitro* and *in vivo* models accurately recapitulating the heterogeneity of native tumor tissue, for both pediatric and adult cancer (6,7). Novel models closely mimicking native tumor tissue would benefit research into pediatric carcinogenesis and enhance drug development potentially improving children's prognosis.

Childhood neoplasms are fundamentally different from cancers observed in adults. Whereas in adult patients' years of cumulative exposure to external factors, such as smoking and alcohol consumption make up 38% of all cases, the underlying etiology of pediatric malignancies remains largely unknown (8). In general, adult malignant pathogenesis is different from the cancer mechanisms observed in the pediatric population (9). Therefore, potential drug targets are not the same and therapeutic strategies should be focused on the specific underlying tumorigenesis of children. Additionally, harsh treatments like radiotherapy and chemotherapeutics may result in serious adverse late effects and are of stronger impact in children compared to adults (3,6). The risk of long-term events including non-carcinogenic harm like impaired development, organ dysfunction, altered fertility, as well as the development of secondary cancers may outweigh the beneficial effects when treating young patients (6,8). The development of high-precision therapies like immune-based treatments and non-genotoxic treatments would reduce the risk of long-term adverse events in childhood cancer survivors (3).

Models commonly used to perform cancer research *in vitro* include immortalized cell lines. However, these fail to capture tumor heterogeneity and tumor complex three-dimensional architecture (10). Generation of tumor cell lines usually requires intense optimalization of 2D culture during which original tumor heterogeneity may be lost due to genetic selection and alternations (11,12). Pre-clinical *in vivo* patient-derived xenograft (PDX) models have shown to recapitulate the heterogeneity of tumors (13). However, PDX models are expensive, time-consuming and do not allow for high-throughput drug screening (13,14). In addition, the use of laboratory animals may raise ethical concerns and the non-human origin of the microenvironment including immune- and stromal cells may not accurately mimic tumor development and drug response in humans (11,13,15). Organoids, typically derived from stem cells, are self-organizing, complex three-dimensional *in vitro* models and a promising tool in cancer research and drug development (12). Organoids derived from patient tumor tissue, patient derived organoids (PDO), have shown to maintain tumor heterogeneity and may potentially be implemented as a translational tool to predict patient specific drug response (16).

Only 5% of anti-cancer compounds reach market approval after the first-in-human trial, emphasizing the need for more efficient pre-clinical drug development (15,17). Advanced *in vitro* models accurately capturing pediatric tumor characteristics provide a tool to further elucidate pediatric tumorigenesis, identity novel drug targets, enhance (precision) drug development and reveal patient-specific drug sensitivities. Overall, contributing to improved treatment for pediatric cancer patients, potentially increasing the survival rate and decreasing the risk and impact of long-term drug adverse events.
The aim of this review is to provide an overview of organoid models (potentially) utilized in drug development and/or research in the field of pediatric cancer. This overview addresses organoids

modelling cancers typically occurring in pediatric and adolescent patients including renal tumors, retinoblastoma and hepatoblastoma.

Methods

The literature search was conducted in October 2024 utilizing the databases EMBASE and PubMed. Search keywords [MeSH terms] were targeting NEOPLASMS, ORGANOIDS and PEDIATRIC. Duplicates were detected using Rayyan and reviewed by the author based on the title, author and year of publication. If applicable the duplicate version of Embase was deleted and the PubMed version selected for further screening. During both processes, the abstract screening and full text screening, reviewers were blinded to each other's decision. Discrepancies about articles meeting the inclusion criteria between reviewers were evaluated and dissolved in consultation with each other. Articles written in the English language and published from 2009 (first organoid publication by Sato et al. (18)) until the present were eligible for inclusion. Included articles described the development and/or application of organoid models (conform the definition by Reis et al. (19)) derived from human origin in the context of pediatric cancer research; liver cancer, kidney cancer and retinoblastoma.

Results

Liver cancer

Author	**Year**	**Application**	**Organoid origin**	**Models**	**Method**
Saltsman et al. (20)	2020	Drug screening	PDO	HB organoids, HB PDX	Huch et al. (21–23)
Zhen et al. (24)	2023	HB tumorigenesis in cisplatin chemoresistance mechanisms	PDO	HB organoids, cancer cell lines, HB PDX	Huch et al. (22,23,25)
Clavería-Cabello (26)	2023	Drug testing CM272 antitumoral effects	PDO tumor and healthy control	HB Patient-derived cells, PDO, PDX and genetic engineered mouse model	Huch et al. (22,23,25)
Rialdi et al. (27)	2024	Drug efficacy and compound screening including WNTinib	PDO	HCC (genetically engineered) murine organoids, PDO HCC cell lines, HCC PDX	Huch et al. (21–23)
Narayan et al. (13)	2022	Drug repurposing via high-throughput screening	PDO (patient age range 14-36 years old)	FLC organoids, FLC PDX	Huch et al. (21–23)

Table 1. Literature describing the generation and application of pediatric liver malignant organoids.

Liver malignancies occurring in childhood are rare, comprising only 2% of the pediatric cancers (28). One of the most common pediatric liver carcinoma subtypes included in this review is hepatoblastoma (HB), which is usually diagnosed at a young age between 0-5 years old (20). Combining surgery and chemotherapy has been effective in 80% of the patients with HB, resulting in 5-year survival (26). However, a significant amount of HB tumors cannot be removed surgically (post-chemotherapy) or do not respond to chemotherapy and have a poorer prognosis (24,26). Moreover, other subtypes discussed here, hepatocellular carcinoma (HCC) and fibrolamellar carcinoma (FLC), mainly affect the older pediatric population, adolescents and young adults (20,28–30). Patients diagnosed with the aggressive liver cancer subtype HCC have a poor prognosis, around 30% of the patients survives past 5 years after diagnosis (31). Current curative treatment options consist only of complete tumor surgical removal because of common chemo resistance (31). Diagnosis of FLC is often at an advanced stage, because presented symptoms are commonly non-specific. Currently, surgery is the only curative treatment for this rare malignancy but often found not effective in advanced stages (13). The overall poor prognosis emphasizes the need for novel treatment options to cure childhood liver malignancies.

Hepatoblastoma (HB)

Epigenetics are thought to play a huge role in HB tumorigenesis. The most common mutations that have been correlated with HB carcinogenesis are found in the *CTNNB1* gene (which encodes β-catenin) and in *NFE212* (encoding nuclear factor erythroid 2-related factor 2). However, HB is characterized by a remarkable small number of mutations per tumor, indicating (post-)transcriptional regulation play a significant role in the pathogenesis (24,26). Huch et al. (21) established a method for the generation of healthy human adult liver organoids utilizing primary tissue. The liver organoids could be expanded up to 6 months, passaging them between 5-14 days. (22,24–26). In addition, the method has successfully been applied in the generation pediatric HB liver organoids to study HB tumorigenesis and screen for drug sensitivities in HB. For example, Saltsman et al. (20) characterized the HB *in vitro* organoid model, demonstrating the tumoroids accurately recapitulate the mutational profile, tumor architecture and gene expression. Tumor signaling of the Wnt/β-catenin pathway was found indistinguishable comparing human tumor tissue and HB organoids. To identify novel compounds as HB treatment, a medium throughput drug screening assay was performed based on organoid viability. One compound, JQ1, could be identified as potential HB treatment (20). Moreover, to investigate the involvement of the post-transcriptional mechanism alternative splicing (AS) in the development of HB, Zhen et al. (24) generated PDO's using chemo- and radiotherapy naïve tumor tissue. The HB tumor organoids were screened for their chemotherapy resistance via assessing cell viability. Increased

cisplatin sensitivity was observed in organoids with depletion of *PABPN1*, encoding the splicing factor polyadenylate-binding nuclear protein 1. PABPN1 expression was significantly upregulated in organoids resistant to cisplatin demonstrated by western blot assay, indicating PABPN1 plays a role in HB pathogenesis as splicing regulator (24). Furthermore, Clavería-Cabello et al. (26) performed transcriptomic analysis to identify epigenetic drug targets in HB cell lines. They conducted a drug screening with molecules targeting different targets in 2D HB cell lines. Based on this screening, they selected the compound CM272 for further evaluation in HB tumor-derived organoids. Tumor growth inhibition was observed in HB tumor-derived organoids upon treatment with CM272. The viability of control organoids, healthy hepatocyte organoids, was not negatively affected by CM272 compared to cisplatin. A xenograft mouse model derived from a HB tumor cell line was utilized to further assess the anti-cancerous effect of CM272 (26).

Hepatocellular carcinoma (HCC)

Similar to hepatoblastoma, hepatocellular carcinoma (HCC) carries a *CTNNB1* mutation in 30% of the cases. However, a diverse set of mutations in Wnt signaling genes have been correlated with HCC tumorigenesis, displaying a heterogenous group of tumors on histological and molecular levels (31).

Rialdi et al. (27) generated HCC patient-derived organoids harboring *CTNNB1* mutations utilizing the previous mentioned protocol originally established by Huch et al. (21–23). Selected compounds were screened in genetically engineered murine organoids (e.g. CTNNB1-mutated) and identified WNTinib as potential HCC treatment. Additionally, human patient-derived HCC organoids were used to validate the specificity of the drug combined with studies in patient derived HCC cell lines. However, assays elucidating WNTinib mechanism of action were performed in genetically engineered murine organoids and treatment tolerability was assessed in mice allografts (27).

Fibrolamellar carcinoma (FLC)

FLC is characterized by the functional driver mutation DNAJB1-PRKACA, a fusion transcript which is the result of a deletion on chromosome 19. The methods utilized to generate HB and HCC organoids were also applied to generate FLC organoids by Narayan et al. (22,25). Characterization of the FLC organoids was performed using immunohistochemistry and RNA-sequencing, demonstrating the histology and transcriptome of human FLC tissue are recapitulated by FLC organoids. PDO's demonstrated suitability for drug repurposing via a high-throughput drug screening trial evaluating their viability and determining the normalized percentage of inhibition (NPI). In addition, proliferation of FLC organoids was validated by injecting the organoids into mice. The typical fusion transcript DNAJB1-PRKACA was detected and organoid tumors grown in mice histologically resembled FLC tumor tissue (13).

Renal cancer

Author	Year	Application	Organoid origin	Models	Method
Calandrini et al. (6)	2020	Establishment of organoid biobank and drug screening	PDO	Renal malignant organoids (e.g. WT, MRTK and RCC)	Modified Schutgens et al. (32) protocol
Waehle et al. (33)	2021	Studying WT initiation and progression	iPSCs *WT1* wildtype and mutant	WT organoids	Ungricht et al. (34) adapted Morizane et al. (35) protocol
Calandrini et al. (36)	2021	High-throughput drug screening for potential MRTK treatment	PDO	MRTK and WT (control) organoids, MRTK PDX	Calandrini et al. (6)
De Munter et al. (37)	2023	Studying efficacy of CD70-specific nanoCAR T in MRTK	PDO	MRTK and WT (control) organoids, and B-cell lymphoma PDX	Calandrini et al. (6)
Custers et al. (38)	2021	Studying MRTK developmental pathways and exploiting a novel therapeutic approach	PDO	MRTK organoid and normal kidney organoid	Calandrini et al. (6)
Liu et al. (39)	2023	Studying the role of SMARCB1 loss in MRTK development	PDO	MRTK organoids, MRTK cell line	Calandrini et al. (6)
Cao et al. (40)	2022	High-throughput drug and compound screening for (repurposing) potential tRCC treatment	PDO	tRCC	Model establishment

Table 2. Literature describing the generation and application of pediatric renal malignant organoids.

Worldwide, 3.2-11.1% of pediatric cancers are renal tumors (41). Different types of renal tumors may occur in children of which the most common are Wilms tumors (WT), also known as nephroblastoma, in 75% of the new cases. Other renal malignancies occurring in children and discussed in this review are malignant rhabdoid tumors of the kidney (MRTK) and renal cell carcinomas (RCC) (6,7). Children diagnosed with Wilms tumor are usually around the age of 3 years old (6,42) and diagnosis of MRTK is usually between the age of 10-18 months (7). RCC is rare and accounts for 4% of the newly diagnosed pediatric renal cancers, of which the most common form is translocation RCC (tRCC), occurring in childhood and adolescent (43). The survival rate of children diagnosed with renal malignancies differs per (sub)type, with the highest rate of 90% in children diagnosed with Wilms tumor and favorable

histology. The rarer non-WT malignancies have a poorer prognosis and e.g. a survival rate between 20-25% in children diagnosed with the most aggressive type, MRTK (7,42). Furthermore, treatment strategies may depend on histology and the stage of malignancy but usually include a combination of therapies e.g. pre-operative chemotherapy, surgery and post-operative treatment (7,42). Effective treatment options are not available for patients with MRTK and tRCC (36,43).

Calandrini et al. (6) established a biobank of pediatric patient-derived renal tumor organoids through modifying the existing organoid generation protocol (32) to enhance single cell survival. Wilms tumor, MRTK and RCC are all represented in the biobank and in-depth characterization demonstrated organoids to mimic tumor histology and cellular heterogeneity. Whole-genome sequencing, transcriptomic analysis and epigenetic profiling showed high similarities between organoids and their tumor tissues. In addition, Calandrini et al. (6) demonstrated suitability of applying gene editing technologies and high-throughput drug screening in organoids. Biobank pediatric kidney tumor organoid models may be utilized to discover (personalized) drug sensitivities and investigate novel drug targets and tumorigenesis (6).

Wilms tumor

Wilms tumor (WT) is characterized by its specific tri-phasic histological composition, including blastemal-, epithelial- and stromal cells. Wilms tumor may originate from different cell types and display intra-tumor heterogeneity (7). However, relatively few genetic mutations have been linked to originate Wilms tumor (6). In approximately 10-20% of the cases Wilms tumors are initiated by homozygous loss of the Wilms tumor suppressor (WT1), but distinct heterogenous mutations have been identified to initiate WT tumorigenesis, including SIX1, SIX2, CTNNB1, WTX (6,33) of which initiation is thought to occur during embryonic development (7). Calandrini et al. (6) demonstrated various patient-specific sensitivity towards chemotherapeutics in Wilms tumor organoids. In addition, to study initiation and progression WT mechanisms, Waehle et al. (33) modeled and characterized Wilms tumor organoids mimicking patient phenotype through *WT1* deletion in iPSCs. Knocking out *WT1* during different phases of organoid differentiation provided insights on WT1 function in Wilms tumorigenesis (33).

Malignant Rhabdoid tumors of the kidney (MRTK)

MRTK are considered the most aggressive form of renal malignancies. Biallelic inactivation of either *SMARCB1*, in 95% of the cases, or *SMARCA4* are associated with MRTK tumorigenesis. Both are subunits of the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complex (6,7,36,39). Moreover, germline mutations may predispose children to the development of MRTK. However, the tumorigenesis of MRTK is not fully understood (38). To determine (patient specific)

tumor drug sensitivities, Calandrini et al. (36) screened the efficacy of 500 compounds using MRTK patient-derived organoids and identified one as a potential therapeutic agent, MLN4924, through measuring cell viability. The toxicity of MLN4924, a neddylation inhibitor, was assessed using patient-matched healthy kidney organoids, hepatocyte organoids and intestinal organoids. Additionally, the efficacy of MLN4924 was confirmed through their *in vivo* patient-derived MRTK model (36).

To study the efficacy of CD70 specific nanoCAR T cells in MRTK De Munter et. al. (37) generated MRT organoids using patient samples from the Calandrini et al. (6) biobank. MRTK organoid cell lines were selected based on their mRNA levels of CD70 expression, associated with poor prognosis in patients, and confirmed in the organoids through flow cytometry. Furthermore, the efficacy and motility of CD70-specific nanoCAR T cells was assessed in the organoid cultures, utilizing patient derived tumor cell lines lacking CD70 expression as a negative control. Additionally, a B-cell lymphoma patient-derived xenograft model was used to confirm the efficacy of nanoCAR T cells in *vivo*. Discrepancies in demonstrated efficacy between the MRTK organoid model and xenograft model were attributed to enhanced exhaustion of CAR T-cells in the PDX model, potentially due to expression of CD70 on immune cells (37). To further elucidate MRTK tumorigenesis, Custers et al. (38) generated MRT organoids previously established by Calandrini et. al. (6). Earlier studies pointed towards the importance of *SMARCB1* or *SMARCB4* biallelic inactivation as crucial MRTK inducers. However, Custers et al. (38) observed insufficiency of the *SMARCB1* biallelic mutation driving MRT differentiation by itself in patient derived organoid. Single-cell mRNA data demonstrated retained proliferation upon reversing *SMARCB1* loss in MRTK organoids, attributing to the embryonic root hypothesis of MRTK. In addition, Custers et al. (38) demonstrated the efficacy of combining two drugs simulating *SMARCB1* re-expression, HDAC and mTOR, through transcriptomic analysis of treated MRTK organoids. The results identified a potential synergistic treatment option for children with MRTK using patient derived organoids (38). Furthermore, to elucidate the mechanisms of *SMARCB1* loss affecting transcriptional regulation, Liu et al. (39) utilized biobank MRTK organoid models. Multiple assays were performed on MRTK organoids transduced with a control or *SMARCB1* expression, including ATAC-seq to assess chromatin accessibility, ChiP-seq and CUT&RUN to study protein-DNA interactions and 4C-sec and high-resolution in-situ Hi-C experiments to validate changes in the 3D genome. Additionally, to investigate ncBAF inhibition, the morphology, cell growth, *MYC* mRNA levels and RNA-seq data were assessed of MRTK organoids treated with a BRD9 inhibitor. Overall, combined with assays performed on patient tissue samples, the results demonstrate the disruption of super-enhancers due to loss of *SMARCB1* drives MRTK tumorigenesis (39).

Metastatic translocation renal cell carcinoma (tRCC) patient derived organoids

Metastatic translocation RCC (tRCC) is typically characterized by translocations on chromosome Xp11 affecting transcription factor E3 or less common, a translocation of t(6;11)(p21;q12) which is involved with transcription factors EB (TFE3) and EB (TFEB (6,7). To discover potential novel treatment purposes, Cao et al. (40) established metastatic tRCC organoids, the more aggressive subtype of RCC predominantly occurring in children and young adults. The established model was generated using treatment-naïve kidney biopsies from a patient with metastatic tRCC and PRCC-TFE3 fusion, characterization of the model demonstrated conservation of tumor phenotype, three-dimensional structure and cellular composition. In addition, the PDO's were deployed in a high-throughput screening assay for drug repurposing measuring cell viability upon treatment. RNA sequencing data of treated tRCC organoids was analyzed to elucidate the underlying anti-tumor effects of various compounds and identified Critzotinib as a potential treatment for metastatic tRCC based on its effects on autophagy related genes (ARG's) (40).

Retinoblastoma

Author	**Year**	**Application**	**Organoid origin**	**Models**	**Method**
Saengwimol et al. (44)	2018	Screening for therapeutic candidates to treat recurrent RB	PDO	Rb organoids	Model establishment
Saengwimol et al. (45)	2020	Identification and investigation of the role of MYCNOS variants role in RB	PDO (RB170), H9 ESCs	*MYCN*-amplified organoids (PDO) Retinal organoids (ESC-derived)	Saengwimol et al. (44) (PDO) Kaewkhaw et al. (46) (ESC-derived)
Chittavanich et al. (47)	2023	Repurposing Ceftriaxone as potential MYCN-driven Rb treatment	PDO (RB170 and *RB1*-/-)	*MYCN*-amplified organoids and *MYCN*-nonamplified organoids (control)	Saengwimol et al. (44)
Srimongkol et al. (48)	2023	Drug repurposing and screening drug candidates as potential RB treatment through demonstrating efficacy and toxicity	PDO	*RB1*- deficient and *MYCN*-amplified organoids	Saengwimol et al. (44)
Liu et al. (49,50)	2020	Establishment of an RB organoid model to study	hESC CRISPR/Cas9 induced	Organoids with a biallelic *RB1*	Nakano et al. (51)

		RB tumorigenesis and drug (candidate) screening	biallelic *RB1* mutation or ablation	mutation or ablation	
Norrie et al. (14)	2021	Studying cellular origins of RB in patients carrying predisposing mutations	Patient-derived iPSCs CRISPR/Cas-9 inactivation of *RB1,* H9 ESCs *RB1* inactivated, *RB1* wildtype hESCs and iPSCs	Organoids/PDX with *RB1* mutations, RB1 wildtype organoids/PDX,	3D-RET protocol (14) (iPSCs) Modified Nakano et al. (51) protocol (H9 ESC)
Rozanska et al. (52)	2022	Rb model validation for treatment screening and studying the role of pRB in Rb differentiation	hESCs, patient-derived iPSC	*RB1* wildtype (control) and depleted organoids (hESC-derived), *RB1* biallelic mutation organoids (iPSC-derived)	Hallam et al. (53)
Blixt et al. (54)	2022	Investigating the role of MYCN in RB tumorigenesis independently of *RB1* inactivation	RB1-proficient hESCs electroporated with MYCN or $MYCN^{T58A}$ piggyBac vectors	*MYCN*-amplified organoids (hESC-derived), *MYCN*-amplified and wildtype chicken retinas	Zhong et al. (55)
Kanber et al. (56)	2022	Studying Rb development with *RB1* depletion	hESCs *RB1* heterozygous and homozygous mutation with CRISPR/Cas9	*RB1* mutant and wildtype (control) organoids	Döpper et al. (57)
Deng et al. (58)	2020	Studying the effect of homozygous deletion on iPSC maturation and differentiation	*RB1* wildtype (control) and depleted iPSCs using CRISPR/Cas9	*RB1* depleted organoids	Zhong et al. (55)

Table 3. Literature describing the generation and application of pediatric retinoblastoma organoids.

Retinoblastoma (Rb) is a tumor arising during development of the retina, left untreated it may cause death within 1-2 years (14,44). Current treatment strategies focus on ensuring patient survival and preservation of the ocular globe and visual function (44). The onset of Rb is driven through biallelic

inactivation of the *RB1* gene, a tumor suppressor gene, in 98-99% of the cases (type 1). In the other 1-2% the onset is initiated via *MYCN*-amplification and is linked to aggressive recurrences of Rb that are resistant to chemotherapy, referred to as type 2 (14,47,54). Half of the Rb patients carries an allelic *RB1* germline mutation which causes the disease to onset earlier in life compared to patients who do not carry this allelic mutation. Inactivation of the second *RB1* allele in this case is sufficient to induce tumorigenesis (14).

Patient-derived retinoblastoma organoid models

To advance drug testing and drug development in Rb treatment, Saengwimol et al. (44) established and thoroughly characterized a three-dimensional Rb organoid model from patient-derived chemotherapy-naïve tumor tissue. Histological analysis and immunostaining of the Rb organoids demonstrated to mimic the retinal tumor architecture and Rb tissue protein expression. The model maintained the DNA copy number alterations revealed by analysis of PCR sequencing tumor tissue, organoids and blood. Moreover, RNA-seq showed high-expression of genes associated with mesodermal and of cells cone-enriched genes, confirming to retain the gene expression profile of Rb tumor tissue. In addition, in order to validate the application of the Rb model in drug screening, Rb organoids were treated with the clinically treatment combination of topotecan and melphalan. Results showed similar to *in vivo* anti-cancer activity targeting proliferative tumor cones (Ki67+ and RXRγ+ markers) in organoids, indicating the suitability of applying the Rb organoid model to screen for drug sensitives (44). In an additional study, to investigate the potential involvement of *MYCNOS* variants (the opposite strand of *MYCN*) in retinoblastoma, Saengwimol et al. (45) utilized the characterized Rb model (44).

MYCNOS1 was found to be highly expressed in patient-derived Rb tissue (RB170) and maintained in MYCN-amplified Rb patient-derived organoids. However, *MYCNOS1* was downregulated in ESC-derived retinal organoids compared to ESC cells, indicating *MYCNOS1* promotes tumorigenesis in *MYCN*-amplified retinoblastoma, and requires downregulation in retinal differentiation (45). To potentially repurpose ceftriaxone, Chittavanich at al. (47) generated patient-derived *MYCN*-amplified retinoblastoma organoids according the Saengwimol et al. (44) protocol. The antibiotic ceftriaxone was marked as potential treatment for *MYCN*-amplified tumors based on clinical experiences with the drug. Cell viability of organoids upon treatment demonstrated high-potency of ceftriaxone in *MYCN*-driven tumors. Additionally, transcriptomic analysis of treated organoids revealed upregulation of genes associated with Rb regression and whole-actin staining confirmed the decrease of Rb organoids. Lastly, proteomic analysis of treated Rb organoid lysates provided insights on the drug-target and anti-cancer mechanism of ceftriaxone (47). To identify potentially repurposable FDA-approved drugs for Rb treatment, Srimongkol et al. (48) screened 133 chemotherapeutic drugs in Rb patient-derived

organoids (*RB1* inactivated and MYCN-amplified), generated via the previously mentioned established protocol (44). Therapeutic candidates were selected via high-throughput drug screening based on cytotoxicity and growth rate inhibition. Sunitinib displayed advantages over clinically available chemotherapeutics to treat Rb in response consistency, potency, efficacy, inhibition of tumor proliferation and differentiation, tumor cone suppression. In addition, low toxicity of sunitinib treatment was observed in human ESC-derived retinal organoids. Overall, demonstrating a favorable efficacy/toxicity profile in Rb organoids of sunitinib as potential Rb treatment (48).

Stem cell derived retinoblastoma organoid models

To investigate the Rb tumorigenesis, Liu et al. (49,50) genetically edited hESCs using CRISPR/Cas9 to knock-in p.R320X (c.958C>T), a nonsense mutation predisposing patient to the development of Rb, and generated Rb organoids ($RB1^{mut/mut}$). To assess the specific effects of the knock-in, an hESC cell line with *RB1* gene depletion ($RB1^{-/-}$) was taken along. In both, the mutated and depleted *RB1* hESCs, mutagenesis did not affect the expression of genes correlated with cell-cycle, self-renewal and pluripotency, shown through RNA-sequencing. However, at day 75 of differentiation $RB1^{mut/mut}$ and $RB1^{-/-}$ organoids displayed Rb tumor histological features and was confirmed tumorigenesis via transmission electron microscopy (TEM). Furthermore, transcriptomic analysis demonstrated significant upregulation of proto-oncogenes and downregulation of tumor-suppressor genes in *RB1* mutated organoids compared to *RB1* wildtype organoids. Single-cell RNA-sequencing confirmed the heterogeneity of tumor tissue was recapitulated by the Rb organoid model and pointed towards cone precursors as the origin of tumorigenesis. Lastly, the anti-cancerous effect of drugs was screened in Rb organoids through measuring proliferative tumor cone (Ki67+) reduction with flow cytometry (49)

To investigate tumorigenesis upon *RB1* inactivation, Norrie et al. (14) generated an organoid model derived from iPSCs and H9 hESCs. The iPSCs were generated from a subset of patients various *RB1* germline mutations or deletions. As a positive control, CRISPR/Cas9 gene editing was utilized to induce *RB1* inactivation in H9 ESCs and patient-derived iPSCs. Wildtype *RB1* H9 hESCs and iPSCs were taken along as a negative control. According to the method provided by Nakano et al. (51), hESCs organoids were generated and Norrie et al. (14) utilized their own optimized protocol to generate iPSCs derived Rb organoids. After 45 days of differentiation, organoids were dissociated and injected into the eyes of immunocompromised mice to track tumorigenesis. Tumor growth was demonstrated in organoids generated from patient-derived iPSCs (carrying germline mutations) and *RB1* CRISPR/Cas9 edited patient-derived iPSCs and hESCs. Organoids derived from induced *RB1* mutation in wildtype RB1 iPSCs, were found indistinguishable from patient-derived iPSCs (14).

To evaluate the role RB1 in retinal differentiation, Rozanska et al. (52) established two organoid models using patient-derived iPSCs (heterozygous *RB1* depletion) and hESCs (*RB1* wildtype). Using these cell

lines homozygous *RB1* depleted iPSCs and hESCs lines were established using CRISPR/Cas9 gene editing. Homozygous *RB1* depleted human ESC derived organoids and iPSCs displayed tumorgenic phenotype after retinal differentiation including significantly upregulated expression of cone precursors (Ki67+ and RXRγ+) and the lack the presence of amacrine cells, demonstrated with immunostaining and RNA-sequencing. Heterozygous inactivation of *RB1* showed decreased RB1 expression but was found not sufficient to initiate tumorigenesis. Small differences were noticed between the two models in e.g. presence of amacrine cells, but these could be due to epigenetic variations. In addition, in both models a decrease of proliferative cone precursor is seen upon treatment with clinically available chemotherapeutics confirming the suitability of these models in drug screening (52).

To study the underlying mechanism and cell origin of Rb, Blixt et al. (54) generated two novel models utilizing the organoid generation methods by Zhong et al. (55). Studies with mice showed *MYCN*-amplification by itself was not sufficient to drive carcinogenesis and would require biallelic *RB1* depletion. However, Blixt et al. (54) demonstrated *MYCN*-amplification to onset tumorigenesis in hESC-derived organoids and chicken retina with proficient *RB1* expression. *MYCN*-amplification in retinal organoids and chicken retina was initiated via genome integrating piggyBac *MYCN*-expression (54). The progenitors as the cell-of-origin in *MYCN*-driven Rb was supported by selective cell survival of the cone/horizontal cell lineage. Differences observed between the two models were observed in the expression of amacrine cells and retinal ganglion cells of which markers were not expressed by the Rb organoids (54).

Kanber et al. (56) like Rozanska et al. (52) demonstrated homozygous *RB1* mutation to impair retinal differentiation. Human ESC- derived biallelic mutated *RB1* organoids were generated utilizing previously described methods by Döpper et al. (57). *RB1* mutated hESCs were generated via CRISPR/Cas9 gene editing. Differences in gene expression of retinal differentiation comparing wildtype and *RB1* biallelic mutated organoids were significant. There were no differences observed in differentiation gene expression between wildtype and single allelic *RB1* mutation, both resembling fetal retinal tissue. Emphasizing the importance of biallelic inactivation to model Rb *in vitro*. There were no significant differences demonstrated in the number of horizontal cells between wildtype, single and biallelic *RB1* mutated models. The amount of amacrine cells were reduced in biallelic mutated organoids, indicating retina differentiation is dependent on pRB. This is supported by organoids disintegrating after day 130 of culturing. Similar to Blixt et al. (54), Kanber et al. (56) demonstrated cone differentiation was abundant to the loss of pRB, indicating these as the cell-of-origin. (56).

To characterize the associated phenotype of homozygous loss of *RB1* retinoblastoma, Deng et al. (58) utilized the protocol established by Zhong et al. (55). The biallelic mutation of the *RB1* was induced in

human iPSCs (hiPSC) using CRISPR/Cas9 (58). Differentiation of retinal organoids with a single allelic RB1 mutation was not affected. *RB1* expression was demonstrated to incrementally increase during retinal differentiation, suggesting retinal proliferation is dependent on the pRB (58).

Discussion

Distinct sources and approaches are described for organoid generation, and each may have their own benefits and drawbacks. Three different cell type origins are described in methodologies for liver, kidney and retina pediatric cancer organoid generation, induced pluripotent stem cells (iPSCs), human embryonic stem cells (hESCs) and patient-derived primary tumor tissue.

Different studies have investigated the differences between iPSCs and hESCs in organoid differentiation (14,52,59). Human ESCs are derived from blastocytes which could potentially raise ethical questions (60). Whereas iPSCs can be harvested from somatic tissues meaning minimal invasive procedures are required and can be patient-derived to have identical genetics which may be beneficial in clinical studies (61). For instance, patient-derived iPSCs may carry germline mutations that predispose the subject to certain types of malignancies which allows to track the patient-specific spontaneous onset of tumorigenesis (14,33,52). However, in this paper more often healthy iPSCs are utilized in organoid generation of which the establishment does not require patient samples (12). Inducing driver mutations in healthy iPSCs/hESCs allows for the investigation of malignant tissue differentiation and tumor development. Applying gene editing technologies during different timepoints of organoid differentiation to induce a known or suspect driver mutation of tumorigenesis will elucidate malignant mechanisms at different stages of maturation (14,33,52,56,58). Moreover, genetically matching iPSCs to hESCs has revealed that pluripotency and functional differentiation is dependent on the genetic background of the cell rather than cell origin (59). In addition, small differences observed in e.g. expression of amacrine cells between retinoblastoma organoids generated from iPSCs or hESCs may be attributed by differences in epigenetics starting points (52). Indicating that the cell source, iPSCs or hESCs, is not affecting organoid differentiation, but the genetic and epigenetic characteristics of the cell lines are. In contrast to tumor tissue derived organoids, iPSCs and hESCs handling may be more time consuming and expensive as newly established cell lines usually require differentiation optimization, and they cannot be expanded seemingly indefinitely (12). Successful differentiation is described and has been demonstrated with iPSCs (14,52,58) and hESCs (45,49,50,52,54,56) towards retinal organoids and with iPSCs (33) towards kidney organoids, neither have been applied in the differentiation towards liver organoids.

Generating organoids utilizing patient-derived tumor tissue may have translational advantages. The genetic profile of organoids and the patient is identical carrying both germline and somatic mutations,

allowing to study patient-specific malignant characteristics and applying organoids in drug screening to predict patient-specific response (6,16). In addition, PDO matched patient-derived healthy organoids may be utilized as a control to assess drug efficacy and toxicity (26,36). Conversely to non-patient derived organoids, PDO's may potentially reveal genetic driving mutation through whole-genome sequencing (6).

Overall, organoids are a promising tool to study tumorigenesis and to screen and/or validate novel therapeutic approaches in the context of pediatric cancer. Various models described in this review have demonstrated to recapitulate tumor heterogeneity and closely mimic the phenotypical, (epi)genetic and three-dimensional characteristics of human tumor tissue. Moreover, organoids may enhance research into pediatric cancer mechanisms and the prediction of patients' response *in vitro*, potentially minimizing the necessity of *in vivo* models and optimizing clinical results.

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Declaration of generative AI in the writing process

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