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Patient-derived models: Advanced tools for precision medicine in neuroblastoma

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Neuroblastoma is a childhood cancer derived from the sympathetic nervous system. High-risk neuroblastoma patients have a poor overall survival and account for ~15% of childhood cancer deaths. There is thus a need for clinically relevant and authentic models of neuroblastoma that closely resemble the human disease to further interrogate underlying mechanisms and to develop novel therapeutic strategies. Here we review recent developments in patient-derived neuroblastoma xenograft models and *in vitro* cultures. These models can be used to decipher mechanisms of metastasis and treatment resistance, for drug screening, and preclinical drug testing. Patient-derived neuroblastoma models may also provide useful information about clonal evolution, phenotypic plasticity, and cell states in relation to neuroblastoma progression. We summarize current opportunities for, but also barriers to, future model development and application. Integration of patient-derived models with patient data holds promise for the development of precision medicine treatment strategies for children with high-risk neuroblastoma.

KEYWORDS

drug screening, neuroblastoma, patient-derived models, patient-derived xenograft, pediatric cancer, precision medicine, tumor organoids

1 Introduction

Despite significant academic, industrial, and clinical efforts, successfully translating preclinical findings to clinical trials and practice remains challenging (1–4) and less than 10% of drugs entering oncology clinical trials are eventually approved for clinical use (1, 5). Furthermore, these efforts and failures come at high financial and ethical costs. Pediatric malignancies have special considerations, since clinical drug testing is even more restricted by the relatively small number of patients and the ethics related to long-term side-effects in children. Involvement of multiple stakeholders is important to address the lack of childhood-specific drug development in the pharmaceutical sector (6, 7). Thus, there is an urgent need for clinically relevant and biologically accurate

preclinical models to minimize these current bottlenecks to drug development and implementation (8).

Neuroblastoma (NB) is the most common solid extracranial pediatric tumor, accounting for ~15% of pediatric oncology deaths (9, 10). NB can be regarded as an aberration of neural crest development, and although it can arise anywhere along the sympathetic nervous system, most primary tumors are found in the adrenal gland (11). NB is biologically and clinically heterogenous, and patients are stratified into different risk groups based on tumor characteristics and disease presentation (12, 13). Clinical responses vary from spontaneous regression to metastatic and drug-resistant disease despite intensive treatment (13). Furthermore, patients often suffer from severe therapy-related long-term adverse effects (14).

NB is a copy number-driven disease with few targetable somatic mutations found at diagnosis, especially when compared with adult malignancies (15). The *MYCN* oncogene is amplified in ~20% of cases and is strongly correlated with aggressive phenotypes and unfavorable clinical outcomes (16). Other common chromosomal copy number changes, including 11q loss and 17q gain, are also poor prognostic features (17). Recurrent mutations are rare in NB but include *ALK* (9%), *ATRX* (7%), and *PTEN* (3%) mutations (15). In relapsed tumors, genome-wide sequencing has revealed a higher prevalence of recurrent mutations in targetable pathways, such as RAS-MAPK (18, 19), but at relatively low frequencies. Recent transcriptional and epigenetic analyses suggest that NB cells can adopt at least two phenotypic cell states, known as adrenergic (ADR)/differentiated and mesenchymal (MES)/immature (20–24). These findings highlight the heterogeneous and dynamic nature of NB.

Reliable, predictive, and authentic NB models are important because: (i) NB is uncommon, so sufficiently powered clinical trials are challenging and patient material is scarce for molecular studies (25), placing extra weight on the translatability of preclinical results; (ii) preclinical models that accurately recapitulate known clinical, genetic, and transcriptional intra- and inter-tumor heterogeneity are important for the identification of effective therapeutic targets; and (iii) patient-derived (PD) models resemble the clinical scenario better than conventional models and can therefore be used to screen for and test the most promising and safe novel therapies.

Here we discuss recent progress in patient-derived xenografts (PDXs) and PD *in vitro* cultures as preclinical NB models. We summarize the development of different PD models, their utility in studying biological mechanisms and treatment responses, and how they can be utilized for preclinical drug testing to improve treatment strategies against NB (Figure 1).

2 PD NB models

Conventional cell lines have been used as laboratory models for decades, and they have provided valuable knowledge about

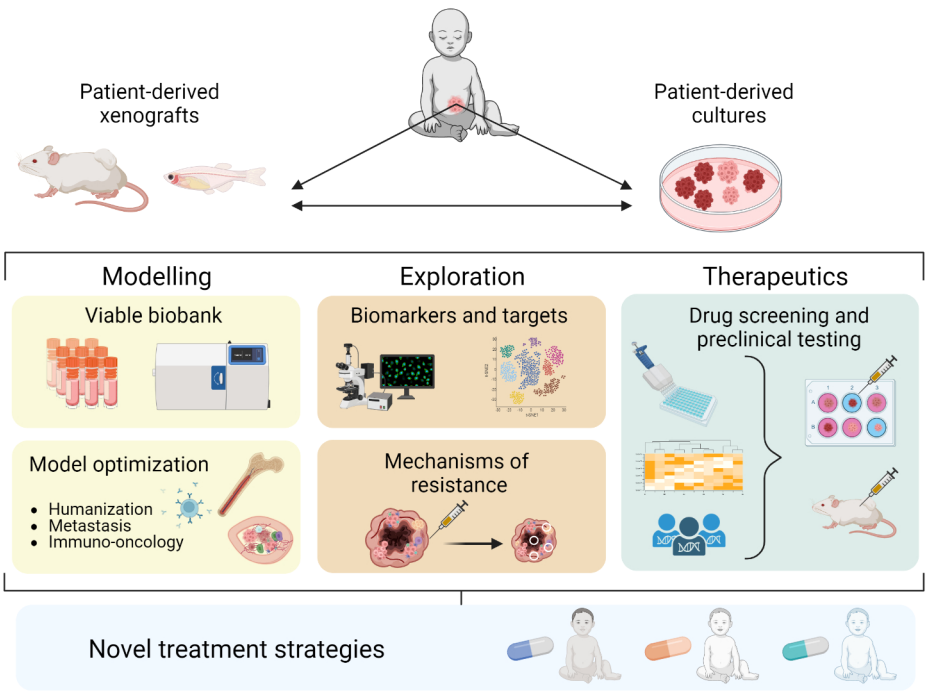

FIGURE 1
Establishment and application of PD models in NB contributing to novel treatment strategies.

tumor biology and drug efficacy in many cancer types. However, cancer cell lines are usually passaged under serum-containing conditions for years and thus their molecular profiles often differ from the original patient tumor (26, 27). This matters in terms of model fidelity, especially when considering clinical applicability; for example, clinically important features such as drug resistance might be lost after long-term *in vitro* passaging (27). PD NB models are established directly from tumor material obtained from children after parental informed consent. PD models have been shown to better reflect the features (e.g., treatment response) of their original tumors, compared with conventional models (28–31). PDXs have now been established from many diverse tumor types of adult and pediatric cancers including NB (32–35). Over the last few decades, the cancer research community has gradually turned towards PD model systems (8), and the US National Cancer Institute recently decided to replace its panel of human cancer cell lines (NCI-60) with well-characterized PDX models (36) for drug screening.

2.1 Establishment of NB PDXs *in vivo*

NB PDXs have been established in immunocompromised mice, mainly by implanting tumor samples or cells obtained from patients next to the adrenal gland (orthotopic implantation) or subcutaneously (ectopic/heterologous implantation). Established orthotopic PDX tumors can be monitored by clinical imaging techniques such as FDG-PET or MRI (37), and they have been shown to retain important patient tumor characteristics such as invasive growth patterns into surrounding tissues and spontaneous metastatic capacity to the bone marrow, lungs, and liver (37–39). PDX models retain NB-specific molecular features, including cellular differentiation status, protein marker expression (synaptophysin, chromogranin A, NCAM/CD56), chromosomal copy number changes (including 1p loss, *MYCN* amplification, 17q gain), mutational profiles, and DNA methylation status (32, 34, 37–41). Transcriptional analysis of orthotopic NB PDXs has shown that they also retain a certain degree of patient-specific gene expression, indicating transcriptional stability, from the corresponding NBs (32, 39, 40). Thus, although a PDX is established from only a fragment of the original patient tumor, data from multiple laboratories have shown that NB PDXs represent the main and clinically relevant features of NB patient tumors. There are now several sources of NB PDX tumors (detailed in (42)), including the US Pediatric Preclinical *In Vivo* Testing Consortium (PIVOT) and the European ITCC-P4 - Pediatric Preclinical Proof of Concept Platform.

The site of implantation affects the PDX model: orthotopic implantation has a higher engraftment rate and tumors grow faster than those implanted subcutaneously (32, 41). The human tumor microenvironment (TME) is gradually lost *in vivo*. Instead, orthotopic NB PDXs have been shown to contain a murine TME including for example vascularization, pericytes, macrophages, and extracellular matrix resembling the architecture in the parental NB (38). Potential functional differences between human and mouse TMEs are not fully elucidated and this uncertainty is important to consider (43). It has been debated whether the use of mice as hosts leads to murine-specific tumor evolution during PDX engraftment and propagation (44, 45). However, serial *in vivo* passaging of orthotopic NB PDXs for up to two years has shown that PDXs retain key genetic aberrations (e.g., 1p loss, *MYCN* amplification, and 17q gain) and acquire only minor genetic changes over time, as would be expected from their natural evolution (39). Clonal dynamics studies during tumor progression in PDXs have shown the presence of branched evolution, clonal sweeps, and convergent evolution of specific small deletions in potentially tumor-associated genes (46), a pattern similar to tumor evolution in NB patients (47).

There have been cases where human lymphomas have developed at the site of NB-cell injection (41, 48), or when murine-derived tumors have replaced the human PDX (49, 50), so thorough and frequent characterization of PDXs is necessary. Setting up robust biobanks for storage of well-characterized, early passage PDX-tumors will be of great benefit to the research community (8).

While most PDX models have been established in mice, zebrafish are increasingly used as hosts for implantation of PD tumor cells, including NB. Zebrafish allow for rapid and low-cost preclinical drug screening in an intact organism that may inform about precision medicine strategies in NB (51, 52). Furthermore, genetically-modified strains are available and tumors can be visualized from an early stage and followed dynamically. However, challenges in translating drug testing findings to patients include limited toxicity and pharmacodynamic data (51), temperature differences, and the non-mammalian TME.

PD cells and tumor biopsies have also been implanted into chick embryos with a high engraftment rate, forming metastases only from tumor cells from patients with metastatic NB and not from localized disease (53). NB cells migrated along the embryonic aorta and along peripheral nerves, demonstrating these as major routes for metastatic dissemination. This model allows for investigation of tumor progression and metastasis in an embryonic environment *in vivo* (53). However, the clinical relevance of the models remains uncertain.

2.2 PD cultures *in vitro*

PD tumor cultures are established *in vitro* directly from patients and can be grown as tumor organoids (PDOs), spheroids, or as semi-attached or attached cultures. PD cultures provide an opportunity to test potential therapeutics in a faster, high-throughput manner compared to PDXs.

PD NB cultures are isolated directly from primary or metastatic tumors from patients and are cultured in serum-

free medium with defined growth factors to avoid neurospecific differentiation. This is best achieved on low-attachment plastics, with or without Matrigel or other scaffolding materials. Several groups have shown that PD NB cultures retain the copy-number profiles, mutation patterns, and other genetic and phenotypic characteristics of the tumor of origin (54–57) in both Matrigel and as free-floating spheres, but PDOs in Matrigel have better self-organization (56). Establishing PD cultures from different stages and subgroups of NB has been challenging. In general, more aggressive, *MYCN*-amplified, and metastatic tumors are easier to propagate *in vitro*. Recent advances in 3D scaffolding with hydrogels and porous scaffolding (reviewed in (58)) together with further optimization of culture conditions might increase the probability of successful establishment. Characterization of culture conditions is also important to understand how different transcriptional cell states might be maintained *in vitro*. Notably, it is important to verify NB identity and lack of contamination with other cells, which can otherwise overtake PD NB cultures (59, 60).

Since the limited number of NB patients restricts the number of models, a complementary approach is to use PDX-derived *in vitro* cultures after expansion of patient material *in vivo* (37, 40, 57, 61–63). Similar to PD cultures, PDX-derived NB cells can be grown adherent or as free-floating 3D cultures, and they retain patient-specific genomic aberrations as well as tumorigenic and metastatic capacity *in vivo* (62). Drug responses between NB PD- and PDX-derived cultures are highly correlated, suggesting that these models can be used interchangeably for drug testing (41). Biobanking of PD- and PDX-derived NB cultures will be a very important tool for future drug screening and larger preclinical drug testing (8).

3 Applications of PD NB models

Conventional cell lines, cell-line derived xenografts, and genetically engineered mouse models have been the main preclinical tools used to study resistance mechanisms and for drug testing. By using PD models that retain the main characteristics and heterogeneity of the original tumors, patient tumors and their treatment response can be better represented in the laboratory, thereby bridging the gap between preclinical models and the clinic.

3.1 Identification of treatment resistance mechanisms and biomarkers

Treatment resistance, relapse after therapy, and metastasis are urgent clinical problems in NB. A few studies have used PD models to identify diverse mechanisms implicated in NB invasion, migration, metastasis (64, 65), and resistance to specific chemotherapies (66). Using a clinically relevant treatment protocol (COJEC-induction therapy), NB PDXs show similar chemotherapy responses to their corresponding patients, suggesting that NB PDXs are useful for modelling chemoresistance and relapse (46). The models showed that chemoresistant NBs have a lower ADR signature and enrichment for an immature MES-like phenotype, suggesting an association between the MES cell state and relapse (46). These results are consistent with recent findings in the clinical setting (24, 67). The ability to accurately model treatment responses and their association with phenotypic cell states make *in vivo* PDXs a very promising tool to explore NB phenotypes in a reproducible manner, as well as characterizing the role of phenotypic plasticity in acquired and intrinsic resistance.

Reliable biomarkers for monitoring tumor responses are important for longer-term studies of relapse and resistance, and in clinical diagnostics. NB PDXs reproduce the patient's relative levels of circulating metanephrines (68). Given that metanephrines are tumor progression biomarkers [plasma levels correlate with tumor volume (69)], this could pave the way for a minimally invasive method of monitoring tumor response/resistance in orthotopic PDX models. Another approach for monitoring responses is with gene signatures as recently optimized and used in a therapeutic study for high-risk relapsed NB in PD models (70).

3.2 Drug testing

3.2.1 Application in the preclinical setting

Preclinical PD model testing is now highly recommended for proof-of-concept studies of new drugs and drug combinations aiming for clinical trials in the pediatric population (8). Many NB targets identified in patients have been tested in PD models *in vitro* and *in vivo*, allowing the evaluation of specific responses in tumors harboring different underlying, molecular alterations. Some known genetic vulnerabilities in NB are still under investigation, while others, for example ALK, have been clinically tested (71, 72). Table 1 presents an overview of recent preclinical drug investigations of established and novel NB targets that were identified and tested in PD models.

High-throughput screens (HTS) *in vitro* can facilitate the discovery of specific targets and/or drugs using CRISPR/siRNA or phenotypic response. Most screening approaches still use conventional cell lines, but more recently PDX-derived cultures of high-risk NB have been used for the initial identification, for example for a KSP inhibitor (77). Compounds identified in drug screens can be further verified *in vivo* in PDXs.

In our experience, PD models show high intra-model variability in drug response (46, 75, 77) and are often less responsive to different treatments than conventional cell lines and xenografts [discussed also in (29)]. The lower sensitivity of PD models could indicate an even smaller effect in patients, thus

TABLE 1 Selected preclinical drug testing studies using NB patient-derived models.

Target	Description	PD *in vivo*	PD *in vitro*	References
Small molecules				
CNR2, MAPK8	TargetTranslator tool for drug discovery	✓	✓	Almstedt et al., 2020 (52)
SHP2	Targeting tumors with low expression of NF1	✓	✓	Cai et al., 2022 (73)
ROS (ferroptosis)	Antioxidant pathways inhibition	✓	✓	Floros et al., 2021 (74)
PIM/PI3K/mTOR	New triple inhibitor	✓	✓	Mohlin et al., 2019 (75)
RAS/antimitotic	Antimitotic effects of rigosertib	✓	✓	Radke et al., 2021 (76)
KSP (Eg5)	HTS identifying new inhibitors, complete response in PDXs	✓	✓	Hansson et al., 2020 (77)
KSP (Eg5)	New oral inhibitor, liver metastasis model	✓	–	Masanas et al., 2020 (78)
Antimitotic	New inhibitor in taxane- and chemoresistant models	✓	–	Grohman et al., 2021 (79)
PARP, ATM	Targeting DNA damage, ATRX mutant NB	✓	–	George et al., 2020 (80)
PP2A	New PP2A activators	✓	–	Bownes et al., 2022 (81)
TOP2B	HTS, redefining MoA of an inhibitor	✓	–	Pan et al., 2021 (82)
CHK1	Prexasertib with chemotherapy in NB	✓	–	Lowery et al., 2019 (83)
ALK, TRK, JAK2/STAT, Src/FAK	Multikinase targeting	✓	–	O´Donohue et al., 2021 (84)
PHGDH	LC-MS-based proteomics, MYCN-associated targets	✓	–	Arlt et al., 2021 (85)
PGDB5	DNA transposase inhibition impairs DNA repair	✓	–	Henssen et al., 2017 (86)
ALK	New molecule: lorlatinib	✓	–	Infarinato et al., 2016 (87)
CAIX/CAXII	New inhibitor, organotypic slice culture	–	✓	Huo et al., 2022 (88)
Drug combinations				
CHK1+RRM2	Synergistic effects on replication stress	✓	✓	Nunes et al., 2022 (89)
ALK+chemo	Crizotinib combination with chemotherapy	✓	✓	Krytska et al., 2016 (90)
ALK+CDK4/6	Combination screen identifying new synergistic targets	✓	✓	Wood et al., 2017 (91)
ALK+PIM1	CRISPR screen, targeting ALK resistance	✓	✓	Trigg et al., 2019 (92)
BCL2+MDM2; BCL2+ CYCLO/TOPO; BCL2 +MCL1	Venetoclax in combinations with clinically relevant agents	✓	✓	Dalton et al., 2021 (93)
BCL2+ferentidine	Synergistic effects of venetoclax and ferentidine	✓	✓	Nguyen et al., 2019 (94)
MGMT+TMZ+TOP2;	Combination of drugs targeting DNA damage	✓	✓	Hindle et al., 2021 (95)
HDAC+DOXO	Rapid zebra fish screen	✓	✓	Wrobel et al., 2020 (96)
PLK1/BRD4	Screen of dual inhibitors	✓	–	Timme et al., 2020 (97)
BCL-2+Aurora A	Screen of new venetoclax combinations	✓	–	Ham et al., 2016 (98)
TBX2	TF addiction is targeted by BETi + CDK7i	–	✓	Decaester et al., 2018 (99)
Immunotherapy & other				
ALK	Antibody-toxin conjugate directed towards ALK	✓	✓	Sano et al., 2019 (100)
Oncolytic therapeutics	oHSV expressing mIL-12	✓	✓	Quinn et al., 2022 (101)
aNK cells+anti-GD2	Residual disease targeting	✓	–	Barry et al., 2019 (102)

(Continued)

TABLE 1 Continued

Target	Description	PD *in vivo*	PD *in vitro*	References
IL-15+anti-GD2	Substitution of IL15 for IL2 to limit toxicities	✓	–	Nguyen et al., 2019 (103)
IL-15/21+anti-GD2	GD2-targeted IL delivery in orthotopic models	✓	–	Nguyen et al., 2022 (104)
TOP1+anti-GD2	GD2-targeted nanoparticle delivery of SN-38	✓	–	Monderrubio et al., 2017 (105)

ALK, anaplastic lymphoma kinase; ATM, ataxia-telangiectasia mutated serine/threonine kinase; BCL, B-cell lymphoma; BET, bromodomain and extra-terminal domain; BRD4, bromodomain containing 4; CA, carbonic anhydrase; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; CNR, cannabinoid receptor; CYCLO, cyclophosphamide; DOXO, doxycycline; FAK, focal adhesion kinase; GD2, disialoganglioside; HDAC, histone deacetylase; IL, interleukin; JAK, Janus kinase; KSP, kinesin spindle protein; MAPK, mitogen-activated protein kinase; MCL, induced myeloid leukemia cell differentiation; MDM, E3 ubiquitin-protein ligase; MGMT, O-6-methylguanine-DNA methyltransferase; oHSV, oncolytic herpes simplex virus; mTOR, mammalian target of rapamycin; PARP, poly (ADP-ribose) polymerase; PGBD5, PiggyBac transposable element derived 5; PHGDH, phosphoglycerate dehydrogenase; PI3K, phosphoinositide 3-kinases; PIM, Pim-1 proto-oncogene, serine/threonine kinase; PLK, polo-like kinase; PP2A, protein phosphatase 2; ROS, reactive oxygen species; RRM2, ribonucleotide reductase regulatory subunit M2; SHP2, protein tyrosine phosphatase; STAT, signal transducer and activator of transcription; TBX, T-box transcription factor; TF, transcription factor; TOP2, topoisomerase II alpha; TOPO, topotecan; TRK, tropomyosin receptor kinase.

providing important information with respect to optimal clinical implementation.

3.2.2 Application for precision medicine in the clinic

The possibility of identifying actionable genetic alterations in pediatric cancers has contributed to optimism that the approach is useful for clinical trial design and target identification for high-risk and relapsed pediatric tumors, including high-risk NB (71, 72). Langenberg et al. thoroughly summarized current pediatric precision medicine programs around the world (106). Many of the programs/consortia [Pediatric MATCH (US) or INFORM (Europe)] have enabled patients to receive treatments tailored to the individual tumor's molecular profile (107–109). However, relatively few identified mutations (<30%) have led to targeted therapies (106, 107, 110). This highlights the need for molecular profiling of patients to be backed up by real-time functional testing of drug sensitivities in PD models.

Both the PIVOT (US, earlier PPTC) and ITCC-P4 (Europe) repositories hold PDXs. Considering that PDXs take time to establish, co-clinical avatar studies are generally very difficult. Nevertheless, the rarity of pediatric cancers and scarcity of models representing specific subtypes within pediatric tumors makes those repositories a valuable resource for the accelerated development and translation of novel therapeutics into early phase trials (34, 111, 112). Lau et al. developed a pediatric precision medicine platform (including a few high-risk NBs) of PDX models and HTS in PDOs, observing a correlation between PDX results, HTS-PDOs, and the clinical responses in patients (113). Importantly, the addition of functional drug testing to a genome-only analysis increased the number of patients with drug options by also identifying drug sensitivities not associated with molecular hallmarks (113).

Real-time drug testing for the immediate benefit of the patient is likely to be more feasible in PDO models where the time for establishment is much shorter and the readout can be performed with a higher throughput. The COMPASS consortium (Clinical implementation Of Multidimensional PhenotypicAl drug SenSitivities in paediatric precision oncology) is a large-scale effort to implement HTS in PD models. This European collaborative platform aims to implement PDO screening for individualized drug sensitivity assessment and therapy (114). Recently, the network also standardized drug scoring tools and developed machine learning approaches (115, 116).

4 Current and future model optimization

Although the successful establishment of PD NB models is encouraging, certain aspects can still be improved. For example, the distribution and function of the extracellular matrix (ECM) has been shown to influence NB progression in patient samples (117, 118). Consistently, modulation of ECM components induces specific cell behaviors of PD NB cultures (63). Optimization of ECM conditions could thus contribute to improved NB modelling.

The lack of a complete immune system is a limitation of most PDX models, since PD tumors are generally implanted in immunocompromised mouse strains (e.g., NSG) to permit tumor engraftment. Reconstitution of a humanized immune system, for example by injection of human hematopoietic stem cells into sub-lethally irradiated mice, could improve the immune status of the models (119). A technically advanced humanized mouse strain (MISTRG) supports the intrinsic development of human natural killer (NK) cells after bone marrow transplantation (120). When combined with orthotopic NB PDXs, these mice have allowed the identification of immune modulating functions in common between PDXs and patient tumors and suggest that the model is useful for immuno-oncology studies in general and in NB in particular (121). The use of PDO and stromal/immune cell co-cultures can be applied *in vitro*, where it has been very

challenging to optimize culture conditions for multiple cell types over longer periods (122, 123). Co-cultures of NB organoids and peripheral blood mononuclear cells (from a healthy donor) were recently used to test a novel immunotherapy (124).

Innovative technological advances have suggested that microfluidics (lab-on-a-chip) and bioprinting may provide future systems for studying tumor cell and stromal/immune cell interactions. Functional short-time cultures of both tumor cells and immune cells in a microfluidic system have been reported for adult cancers (125, 126) and might be applicable also to pediatric cancers. Very recently, the first bioprinted, vascularized NB microenvironment on a fluidic chip was reported (127). Implantation of cell line-derived NB spheroids led to NB cell survival for two weeks and successful micro-vessel infiltration of the spheroids (127). A different study managed to establish PD NB organotypic slice cultures that could potentially preserve an intact NB tumor microenvironment (88). This study used a perfusion-based bioreactor to force medium through the tissue, thereby providing continuous nutrient delivery to the whole tumor.

Orthotopic NB PDXs retain spontaneous metastatic capacity *in vivo* to the lungs, liver, and bone marrow, mimicking the entire process from primary tumor growth to invasion and metastasis (37, 39). However, the TME is generally murine (38) and there are uncertainties about cross reactivity between human NB cells and the mouse TME. The presence of human mesenchymal stem cells can increase growth and metastasis of NB cells *in vivo* (128), suggesting species preference. Recent advances in tissue engineering have produced *in vivo* models of humanized bone (so-called ossicles) in mice. Implanted PDX-derived NB cells form osteolytic tumor lesions in the ossicles and display higher and faster engraftment rates than in mouse bone (129). This model could thus be valuable for the investigation of human NB growth and treatment responses in a humanized metastatic niche.

Further optimization of PD models to account for more patient-like microenvironmental factors both *in vivo* and *in vitro* is ongoing and will likely contribute to improved translatability.

5 Conclusions

The use of clinically relevant preclinical models is of immense importance in childhood cancers, such as high-risk NB, where the access to patient material is limited. PD models reflect the characteristics of the tumor of origin better than conventional *in vivo* and *in vitro* models. Nevertheless, ongoing efforts may further optimize their translational relevance. Existing NB PDXs and PD cultures have been – and continue to be – used to decipher therapy resistance and for target identification and drug testing. Future studies will need to investigate how PD models can be used to exploit phenotypic plasticity and NB cell states in preclinical studies to better benefit NB patients.

Author contributions

KA, KR, AA, AS, AM and DB wrote and reviewed the manuscript. KA and DB supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Publisher's note

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