A physiologically relevant 3D collagen-based scaffold-neuroblastoma cell system exhibits chemosensitivity similar to orthotopic xenograft models.

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A R T I C L E   I N   P R E S S


Full length article

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A B S T R A C T

3D scaffold-based in vitro cell culturing is a recent technological advancement in cancer research bridging the gap between conventional 2D culture and in vivo tumours. The main challenge in treating neuroblastoma, a paediatric cancer of the sympathetic nervous system, is to combat tumour metastasis and resistance to multiple chemotherapeutic drugs. The aim of this study was to establish a physiologically relevant 3D neuroblastoma tissue-engineered system and explore its therapeutic relevance. Two neuroblastoma cell lines, chemotherapeutic sensitive Kelly and chemotherapeutic resistant KellyCis83 were cultured in a 3D in vitro model on two collagen-based scaffolds containing either glycosaminoglycan (Coll-GAG) or nanohydroxyapatite (Coll-nHA) and compared to 2D cell culture and an orthotopic murine model. Both neuroblastoma cell lines actively infiltrated the scaffolds and proliferated displaying >100-fold increased resistance to cisplatin treatment when compared to 2D cultures, exhibiting chemosensitivity similar to orthotopic xenograft in vivo models. This model demonstrated its applicability to validate miRNA-based gene delivery. The efficacy of liposomes bearing miRNA mimics uptake and gene knockdown was similar in both 2D and 3D in vitro culturing models highlighting the proof-of-principle for the applicability of 3D collagen-based scaffolds cell system for validation of miRNA function. Collectively, this data shows the successful development and characterisation of a physiologically relevant, scaffold-based 3D tissue-engineered neuroblastoma cell model, strongly supporting its value in the evaluation of chemotherapeutics, targeted therapies and investigation of neuroblastoma pathogenesis. While neuroblastoma is the specific disease being focused upon, the platform may have multifunctionality beyond this tumour type.

Statement of Significance

Traditional 2D cell cultures do not completely capture the 3D architecture of cells and extracellular matrix contributing to a gap in our understanding of mammalian biology at the tissue level and may explain some of the discrepancies between in vitro and in vivo results. Here, we demonstrated the successful development and characterisation of a physiologically relevant, scaffold-based 3D tissue-engineered neuroblastoma cell model, strongly supporting its value in the evaluation of chemotherapeutics, targeted therapies and investigation of neuroblastoma pathogenesis. The ability to test drugs in this reproducible and controllable tissue-engineered model system will help reduce the attrition rate of the drug development process and lead to more effective and tailored therapies. Importantly, such 3D cell models help to reduce and replace animals for pre-clinical research addressing the principles of the 3Rs. © 2018 Acta Materialia Inc. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

In the native microenvironment, tumour cells are surrounded by three-dimensional (3D) essential physical scaffolding called extracellular matrix (ECM). The ECM composition is shaped by proteoglycans and fibrous proteins that are secreted locally by cells and remain closely connected [1]. The ECM shapes cellular architecture and maintains tissue homeostasis. The tumour ECM can influence disease progression, patient prognosis and response to treatment [2–4]. A major challenge today is to distinguish the relative contributions of structural, molecular and microenvironmental changes to cancer progression. Traditional 2D cultures do not completely capture the 3D architecture of cells and ECM leading to a gap in our understanding of mammalian biology at the tissue level and may explain some of the discrepancies between in vitro and in vivo results [5–8] leading to only 1 in 10 drugs reaching clinical trials and approval by the FDA [9].

3D scaffold-based in vitro cell culturing is a new innovative approach in cancer research to bridge the gap between conventional 2D culture and in vivo tumours [5–7]. The use of scaffold-based cell culturing would help to reduce and/or replace animals for pre-clinical research aligning with the guiding principles for the care and use of animals in biomedical research known as the 3Rs (Replacement, Reduction and Refinement [10]) and increase the potential for a strong economic impact and patient benefit. The use of such 3D culture systems allows for the precise manipulation of cell and ECM components of the microenvironment. The analysis of their contribution to the structure and function of a cell or tissue is vital in our understanding of disease progression and discovery of new effective drugs [6,8,9,11].

Scaffolds provide a 3D structural matrix which offers the necessary support for cells to proliferate, migrate, differentiate, deposit ECM and respond to stimuli, similar to in vivo biological systems. Collagen is a very attractive material for tissue-engineering and regenerative medicine applications because of its natural occurrence in the human body. Collagen triggers the driving force underpinning cell adhesion, migration, chemotaxis, and tissue resistance to multiple chemotherapeutic drugs thus emphasising an imminent need for new treatment options. Current neuroblastoma studies employ either 2D cell culture systems, murine models or alternatively a mix of both, increasing the risk of inconsistencies between these two research models [27,37], and thus highlighting the limited translational efficacy of the results obtained in 2D models and requiring new pre-clinical models.

In order to establish and characterise physiologically relevant tissue-engineered 3D neuroblastoma in vitro models capable of recapitulating elements of a native tumour tissue microenvironment, we aimed to examine cisplatin sensitive and resistant neuroblastoma cells on different collagen-based scaffolds, collagen-glycosaminoglycan (Coll-GAG) and collagen-nanohydroxyapatite (Coll-nHA). If successful, this model could be used further to gain insights into mechanisms of disease pathogenesis and improve the translational efficiency between results obtained in vitro, in vivo and in the clinic. Both Coll-GAG and Coll-nHA scaffolds have been successfully used to study primary tumour microenvironment in breast cancer [19] and metastasis to bone in prostate cancer [23], respectively. Therefore, they may represent attractive matrices for modelling of metastatic neuroblastoma as bone marrow (70.5%) and bone (55.7%) are the most common sites for metastases [38]. Both types of scaffolds have controllable physical and biological properties and consist of a porous, collagen-based layer fabricated using freeze-drying techniques that were originally developed and extensively studied for bone tissue engineering applications [12,14–17,39,40]. GAGs, negatively charged carbohydrates, are commonly found in the ECM involved in cell attachment, migration, proliferation and differentiation [15]. Nano-hydroxyapatites (nHAs), a calcium phosphate, are common elements of the mineral composition of the human bone tissues and extensively used as a biocompatible material for the bone replacement and regeneration [41]. Coll-nHA scaffolds have been extensively characterised for their biocompatibility, toxicity and the osteoconductive and osteoinductive features [12,14,16,17]. Thus GAGs and nHA are attractive composites for reconstructing primary and metastatic bone/bone marrow tumour microenvironment.

Having characterised the cell response to chemotherapeutic in the proposed 3D in vitro cell model, we then used this model to evaluate miRNA-mediated gene regulation. MiRNAs are a class of small, noncoding RNAs that regulate gene expression at translational level [42–44]. These molecules control the expression of a great variety of genes driving cell cycle, migration, differentiation, development, apoptosis, and metabolism [33,43,45,46]. Numerous studies describe dysregulation of miRNA expression in tumours, including neuroblastoma emphasising their potential in the generation of new drugs for therapeutic intervention. In neuroblastoma, some miRNA were found to be over- or under expressed demonstrating their complex role as either “oncomirs” or tumour-suppressors, respectively [29,33,46–48]. These functions can be exploited to repair signaling pathways essential for normal cellular function and block those upregulated in the pathological conditions. Unsurprisingly, the development of miRNA therapeutics is under extensive investigation by several companies for the variety of health conditions, including cancer [49–51]. Here, we explored the 3D scaffold-based in vitro cell culturing platform for neuroblastoma, however, it may have multi-functionality beyond this tumour type.

2. Materials and methods

2.1. Scaffold fabrication

Collagen-glycosaminoglycan (Coll-GAG) and composite collagen-nHA scaffolds (Coll-nHA) were manufactured as described previously [12,15,16]. Briefly, collagen slurry (0.5% w/v) was fabricated by blending fibrillary collagen I (Integra Life Sciences, Inc.) with 0.05 M acetic acid.

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Coll-GAG scaffolds were produced using a freeze-drying technique described previously [52]. The collagen slurry was combined with chondroitin-6-sulphate isolated from shark cartilage (Sigma-Aldrich, Germany) in a 0.05 M glacial acetic acid solution. For Coll-nHA scaffolds, nHA particles were synthesized as previously described [12,14] and mixed with the collagen slurry during the blending process yielding a 2:1 ratio of nHA:collagen scaffold. Slurries were degassed, freeze-dried and all scaffolds were cross-linked and sterilized using a dehydrothermal (DHT) treatment. Cylindrical scaffolds (6 mm diameter, 4 mm height) cut by a biopsy punch were further cross-linked with 14 mM N-(3-Dimethylamino propyl)-N’-ethylcarbodiimide hydrochloride and 5.5 mM N-Hydroxysuccinimide (EDAC/NHS) in dH2O to improve the mechanical properties of the constructs.

2.2. Cell lines

The Kelly cell line is an MYCN amplified neuroblastoma cell line with a 17q chromosomal gain [53]. The KellyCis83 cell line was derived from the Kelly cell line by pulse exposure to cisplatin and extensively characterised by array Comparative Genomic Hybridization (aCGH), mass spectrometry, proliferation and toxicity assays [54]. KellyLuc and KellyCis83Luc cell lines contain a luciferase and neomycin phosphotransferase genes encoded by pGL4.51 (luc2/CMV/Neo) (Promega) (Supplementary Methods). Cell lines were authenticated by aCGH and Short Tandem Repeat-Polymerase Chain Reaction (STR PCR, SOP ECACC/047). Mycoplasma testing was routinely performed using MycoAlert Mycoplasma Detection kit (Lonza).

2.3. Cell plating densities

Cells were plated at different densities depending on the cell culturing platform due to different growth rates in 2D and 3D. In 2D cell culture experiments, KellyLuc and KellyCis83Luc cells were plated at 10^6 cells/well into 24-well plates in 1 ml of complete media. For 3D cell culture, 2 x 10^5 cells were plated in 24 well non-adherent tissue culture plates onto the scaffolds. After 15 min incubation at room temperature (RT), 2 ml of complete growth media was added to each well and incubated at 37 °C and 5% CO2. Culture media was changed twice every week.

2.4. 3D cell proliferation assay

KellyLuc and KellyCis83Luc cells were plated in 2D in 24 well tissue culture plates or on scaffolds as detailed in 2.3. and maintained under normal growth conditions for analysis at each time point (day 1, 7, 14 and 21). Cells were lysed with 1% Triton-X in 0.1 M carbonate buffer and subjected to 3 freeze-thaw cycles at 80 °C, followed by DNA quantification using the Quanti-itds dsDNA assay kit (Invitrogen) per manufacturer’s instructions. Fluorescence intensity was quantified using a Perkin Elmer Victor2 1420 fluorescent plate reader (excitation 485 nm, emission 535 nm).

2.5. 3D cell viability assay

The cell viability was examined using a Live/Dead®Viability/Cytototoxicity Kit for Mammalian Cells (Invitrogen) according to manufacturer’s instructions. Stained cells were analysed by microscopy using a fluorescent and phase contrast inverted microscope Sony-Lennox Optika4083.CLS coupled with Optika Vision Pro Software (Lennox Ireland).

2.6. Cell metabolic activity – Chromogranin A secretion

Cell metabolic activity was investigated for Chromogranin A (CgA) secretion by neuroblastoma cells into conditioned media and serum were measured on day 1, 7, 14 and 21 by a commercial ELISA kit (Abcam #ab196271) according to the manufacturer’s instructions. Only tumour-derived CgA was detected since the assay distinguishes between human and murine CgA. Results were expressed as absolute values.

2.7. Cell transfection

Mature miR-324-5p mimics and scrambled oligonucleotide controls (Ambion) were transiently transfected into cells grown in 2D and 3D at a final concentration of 10 nM and 30 nM respectively using Lipofectamine® RNAiMAX (Invitrogen). For 2D cell culture, neuroblastoma cells were seeded at 3 x 10^4 cells/well of a 96-well plate and cell viability was assessed using acid phosphatase assay as per the protocol described previously [55].

For 3D cell culture, neuroblastoma cells were seeded and incubated as in Section 2.3 before transfection. Briefly, seven days after seeding the media was removed from wells and replaced with 1 ml growth media without serum and antibiotics, but containing the miRNA duplex-Lipofectamine® RNAiMAX at a final miRNA concentration of 30 nM. The plates were mixed gently and incubated for 6 hr. After 6 hr, the transfection mixture was removed from the cells and replaced with the fresh medium and incubated for 7 days at 37 °C in 5% CO2.

2.8. Gene expression analysis by qPCR

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer instructions and quantified spectrophotometrically. RNA was reverse transcribed followed by quantitative analysis of gene expression using specific Taqman gene expression (VA1C1, Hs01631624_gH) and miRNA assays (has-miR-324-5p, 000539) (Applied Biosystems). 18S rRNA (Hs99999901_m1), RNU44 (ID: TM001094) and RNU48 (ID: TM001006) were used for normalization in gene and microRNA expression studies, respectively. A relative fold change in expression of the target gene transcript was determined using the comparative cycle threshold method (2−ΔΔCT).

2.9. Animal procedures

All animal experiments were approved by the Animal Ethics Committee, Royal College of Surgeons in Ireland (REC no. 784) and by the local scientific animal regulatory authority, the HPRA (P002/AE19127) and were conducted in accordance with European Union directive on the subject of animal rights.

6–8-week-old female Hsd:Athymic Nude-Foxn1nu mouse were purchased from Harlan (UK). Mice were housed under pathogen-free conditions and received autoclaved water and food. Mice were anaesthetised using 1.5% isoflurane inhalation (0.2 L/min).

For the orthotopic adrenal gland injection, the skin and the mesentery membrane were cut. 100 μl of the neuroblastoma cell suspension (1 x 10^6 cells) were injected using a 27-G needle with 1-cc syringe by the insertion under the renal capsule and towards the superior pole of the kidney. The muscle layer was closed with a single Safil 5/0 absorbable suture using surgical knots. Following this, the skin was closed with a single Reflex 7 mm stainless steel wound clip. The animal was placed in a clean cage with a heated pad for recovery and observed for 15 min to ensure complete recovery from anaesthesia.

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All xenografted mice were sacrificed on day 35 post-surgery or before if any signs of morbidity were detected by injecting 150 mg/kg sodium pentobarbital (Euthanal, 200 mg/ml) and waiting for the loss of all reflexes before extracting up to 1 ml whole blood via cardiac puncture. The blood was placed in a tube pre-coated with K2 EDTA (Fisher Scientific). The tumours were resected and removed from the contralateral kidney. The weight of a primary tumour and the contralateral kidney were measured. The tumour weight was determined by subtracting the weight of the contralateral kidney from a tumour. After weight determination, the tumour was snap-frozen in liquid nitrogen.

2.10. Bioluminescence imaging

For in vitro bioluminescence, Luciferase-expressing KellyLuc and KellyCis83Luc cells were plated in the full growth media in a 96-well culture plate (Corning) at an initial concentration of 10^5 cells per well and serially diluted to 500 cells per well in hexaplicate. 4 h after plating when cells were adherent to the well bottom, culture media was replaced and with 200 μl D-Luciferin (Xenogen) (150 μg/ml in sterile phosphate-buffered saline). Cells were incubated at 37 °C in humidified air containing 5% CO₂ for 5 min and then read for 30 s using Victor V5 plate reader (Perkin Elmer), with an identical range of interest used to measure bioluminescence of cells in each well. In vivo bioluminescence images were obtained using an IVIS Imaging System (Perkin Elmer). Luciferase-expressing KellyLuc and KellyCis83Luc cells were visualized in vivo after intra-peritoneal injection of 150 mg/kg D-Luciferin substrate (Sigma-Aldrich). Animals were anaesthetised, placed inside the IVIS Imaging System (Perkin Elmer) specimen chamber and imaged 10–12 min after D-luciferin injection. Bioluminescent images of the tumours in the mice were digitised and electronically displayed as a pseudo-colour overlay onto a grey scale image of the mouse on the Living Image software (Perkin Elmer), with an identical range of interest used to measure bioluminescence of cells in each well.

2.11. Histology

Tissue-engineered constructs of cells grown on scaffolds were removed from the cultured media, washed in PBS and fixed in 4% paraformaldehyde for 30 min. The constructs were dehydrated and paraffin embedded using an automated tissue processor (ASP300, Leica) and cut into 10 μm sections (RM2255, Leica). Cellular colonization and matrix deposition were performed on deparaffinised sections using standard Hematoxylin & Eosin (H&E) staining. Histological assessment of collagen distribution was examined using Picro-Sirius Red. Sections were imaged on a digital microscope (NIS Elements, Nikon).

Cellular infiltration on scaffolds was assessed by nuclear fluorescent DAPI (Invitrogen) staining on a Carl Zeiss 710 laser scanning microscope equipped with a W N-Achroplan 10× (NA 0.3) & 20× (NA 1.0) objective. Subsequent image preparation was conducted using the open-source software, Fiji [56]. The high-intensity DAPI fluorescence allowed the sufficient contrast for simultaneous imaging of cellular nuclei and collagen scaffolds which had their own background fluorescence.

2.12. Cisplatin chemotherapeutic treatment

2.12.1. Cisplatin treatment in vitro

KellyLuc and KellyCis83Luc cells were seeded in 3D as in 2.3. Cells were incubated for 1 or 7 days before cisplatin treatment began. Cisplatin was added to the media to obtain a working concentration of 20 μM or 200 μM for an additional 7 days. The media with cisplatin was replaced every 3–4 days.

2.12.2. Cisplatin treatment in vivo

Xenografted KellyLuc (16 animals) and KellyCis83Luc (16 animals) mice were divided randomly into two groups (8 treated (I) and 8 untreated (II) mice per group in each cell line). The chemotherapeutic treatment regime was started 2 weeks after surgery (or when the tumours reached 1.5 cm³ size). Cisplatin (CIS) was injected intraperitoneally every 3 days (on days 14, 17, 20, 23, 26, 29 and 32) at a concentration of 3.5 mg/kg in 100 μl of sterile PBS in group I, while group II received the same volume of sterile PBS alone. The size of a tumour was measured every 4 days by palpation and by bioluminescence imaging as above on days 7, 14 and 21 or as required.

2.13. Immunodetection

Total protein was analysed by western blotting using primary antibodies anti-CGA (Abcam, #ab15160), anti-VDAC1 (Abcam, #ab15895), anti-TUBA (Abcam, #ab18251) followed by anti-mouse (CST, #7076) or anti-rabbit (CST, #7074) secondary antibody. Chemiluminescence was analysed using the Amersham Imager 600 followed by densitometry and quantitative analysis using Image J (version 1.8 Java).

2.14. Statistical methods

All the data were processed in GraphPad Prism 5 for Windows (GraphPad Software Inc.). Statistical significance was determined for experimental data by using the unpaired Student’s t-test. In all cases, error bars are representative of the standard deviation of the mean of three biological experiments unless otherwise stated. Differences between tumour volumes were analysed with Mann-Whitney U test. A P-value of <0.05 was regarded as statistically significant (*p < 0.05; **P < 0.01; ***P < 0.001).

3. Results

3.1. Development of a 3D tissue-engineered model for neuroblastoma

Disease recurrence with the appearance of drug-resistant tumour cells is the primary obstacle to effective treatment of high-risk neuroblastoma patients. Therefore, for this study, we selected cisplatin sensitive KellyLuc and resistant KellyCis83Luc which were developed and extensively characterised by aCGH, mass spectrometry, proliferation and toxicity assays [54]. Both cell lines were plated on Coll-GAG and Coll-NHA scaffolds and were grown for 21 days (Fig. 1A, B). Time points for analysis were days 1, 7, 14 and 21. The ability of both collagen-based scaffolds to support the growth of two neuroblastoma cell lines, cisplatin sensitive KellyLuc and resistant KellyCis83Luc, over a period of 21 days is demonstrated in Fig. 1. In contrast, the same cell lines grown in conventional 2D monolayer cell culture reached confluence by day 7 forcing us to exclude the results comparing the proliferation rates in 2D beyond this time point (Fig. 1C). Both cell lines exhibited slower proliferation on scaffolds than that of the cells cultured in 2D monolayer (Fig. 1F). The data were in agreement with previously published reports demonstrating slower proliferation of can-
The data presented here indicate that the active proliferation of both cell lines on each scaffold occurred by the day 7 (relative proliferation 2.5–2.6 fold change) before starting to slow down (0.9–1.5 fold change) (Fig. 1G). A statistically significant reduction in cell proliferation was observed for neuroblastoma cells grown on Coll-GAG scaffolds from day 14 to day 21 for KellyLuc (p < 0.05) (Fig. 1G) and from day 7 to day 21 for Kelly-Cis83Luc (p < 0.01) (Fig. 1I). Both cell lines were found to maintain similar proliferation rates on both scaffolds suggesting that both scaffolds promote adhesion and growth of the neuroblastoma cells. Interestingly, Kelly-Cis83Luc demonstrated higher proliferation rates when compared to KellyLuc grown on Coll-nHA (Fig. 1I). The reduced doubling time of Kelly-Cis83 in 2D in vitro from 51 to 33 hrs and altered pathways such as Cell Death and Survival and Cell Growth and Proliferation may be responsible for the higher proliferation rates [54]. These features were acquired during the development of cisplatin resistance and may contribute to the better compatibility of these cells with Coll-nHA promoting cell growth and colonisation.

3.2. Characterisation of neuroblastoma cell viability and infiltration on collagen-based scaffolds

To confirm that the 3D scaffold-based neuroblastoma cell model maintains cell viability, both neuroblastoma cell lines grown on the Coll-GAG and Coll-nHA scaffolds were assessed with the Live/Dead Viability/Cytotoxicity Kit. This procedure has previously been used for examination of cell viability on scaffolds of

![Figure 1](image-url)
different nature [23,58,59]. Fig. 2 demonstrates high numbers of viable cells stained with Calcein AM (green fluorescence, Fig. 2A) and fewer dead cells stained with EthD-1 (red fluorescence, Fig. 2B) confirming that the majority of the neuroblastoma cells retained an intact cell membrane.

Once the capacity of cells to proliferate on the scaffolds was confirmed, the cells were further assessed for their ability to infiltrate the Coll-GAG and Coll-nHA scaffolds on day 14 (Fig. 2C). As demonstrated by H&E staining neuroblastoma cells colonised the scaffolds but exhibited an altered adhesion pattern depending on the cell line. After 24 hrs of cell seeding, both cell lines resided along the scaffold edge (Supplementary Fig. 1). By day 14 KellyLuc cells formed small cell colonies and infiltrated more deeply with a wider dispersion profile throughout the matrices when compared to KellyCis83Luc, which had much larger colonies evident on both scaffold types with minimal single cells presentation. The confocal imaging within the core of the scaffold further confirmed that the cells were able to colonise into the scaffolds in a cell type-dependent manner (Supplementary Fig. 1).

Additionally, 2D monolayer cells were also stained with crystal violet to visualise their attachment profile which demonstrated over confluence by day 7 (Fig. 2D) further supporting the limitations of the 2D cell cultures on and beyond 7 days of culturing.

3.3. Characterisation of CgA secretion as surrogate marker of neuroblastoma cell viability and growth in vitro and in vivo

Chromogranin A (CgA) is a non-specific marker of neuroendocrine tumours including neuroblastoma. It may affect various components of the tumour stroma and regulate tumour growth. CgA serum levels can be used to predict neuroblastoma progression and treatment efficacy [60–62]. Our previous proteomic study identified that cisplatin-resistant cell line, KellyCis83Luc, had three-fold higher CgA expression when compared with cisplatin sensitive Kelly [54]. Investigation of CgA secretion by these two cell lines 24 h after plating in 2D in vitro also demonstrated significantly higher levels of CgA by KellyCis83Luc (2.7 fold change, p = 0.037) when compared with KellyLuc (Fig. 3C). The similar levels of CgA evident on day 7 in 2D are due to overgrowth of both cell lines on plastic (Fig. 3A). Blood and tissue derived from KellyCis83Luc and KellyLuc tumours were profiled for expression of CgA using Western blot and ELISA, respectively (Fig. 3B and C).

![Fig. 2. Neuroblastoma cell viability and infiltration on collagen-based scaffolds. Live/dead staining of neuroblastoma cells grown on collagen-based scaffolds 14 days after plating with Calcein AM (green fluorescence) labelled live cells (A) and EthD-1 (red fluorescence) labelled dead cells (B). H&E staining of neuroblastoma cells grown on collagen-based scaffolds 14 days after plating (C). Crystal violet staining of neuroblastoma cells grown on 2D monolayer 7 days after plating (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
expression in KellyCis83Luc tumours was significantly higher than in KellyLuc tumours further confirming the proteomics profiling data of our recent study [54]. Furthermore, levels of circulating CgA in blood showed correlation with the tumour size (Fig. 3B).

Having confirmed the CgA secretion as a surrogate marker of neuroblastoma cell viability and growth in 2D in vitro and in vivo, we further investigated how 3D culturing impacts CgA secretion by neuroblastoma cells. Both KellyLuc and KellyCis83Luc cells were grown on the two collagen-based 3D scaffolds (Coll-GAG and Coll-nHA) over 21 days and the conditioned medium was analysed by ELISA for CgA (Fig. 3D and E). As anticipated, cisplatin-resistant KellyCis83Luc cells secreted CgA at significantly higher levels than sensitive KellyLuc regardless of the matrix. The CgA secretion by cells grown on both Coll-GAG and Coll-nHA scaffolds exhibited similar patterns which mirrored DNA content distribution (Fig. 4D and E). The overall trend in both 3D cell culture models (Coll-GAG and Coll-nHA) of higher CgA secretion with increasing neuroblastoma cell numbers is consistent with clinical data (Fig. 3A and B) [60,61] and were in agreement with those observed in vivo reiterating the usefulness of the 3D model system to mimic more closely the in vivo scenario.

3.4. Modelling chemotherapy response in 3D in vitro

Previous studies reported that 3D culturing increases cancer cell resistance to chemotherapy when compared to their 2D monolayer counterparts [23,57,63]. Therefore, we examined the sensitivity of KellyLuc and KellyCis83Luc cells cultured in 3D to cisplatin. Cisplatin and other platin-containing drugs are routinely used in neuroblastoma treatment where it forms cross-links with DNA and blocks cell division leading to apoptotic cell death [64,65].

In this study, both neuroblastoma cell lines grown on both scaffolds were treated in two different ways. In the first instance, the cisplatin treatment started on day 1 and continued for 7 days reflecting the well-established approach for drug challenge in 2D traditional cell culturing to obtain IC50 values [54]. Cisplatin at 200 μM was in line with clinically recommended dosage (40–120 mg/m² or 1.33–3.99 mg/kg) [66–68]. As expected the cell growth was significantly inhibited in the presence of 200 μM cisplatin both on plastic and on 3D collagen-based scaffolds (Fig. 4A–C). The other approach included mimicking physiological relevance for tumour treatment; neuroblastoma cells were grown on both scaffolds for 7 days to allow cells to form a critical mass of tumour cells prior to cisplatin treatment at 20 and 200 μM. These doses are in the range of 8–14 and 80–140 times higher than cisplatin both on plastic and on 3D collagen-based scaffolds (Fig. 4A–C). The other approach included mimicking physiological relevance for tumour treatment; neuroblastoma cells were grown on both scaffolds for 7 days to allow cells to form a critical mass of tumour cells prior to cisplatin treatment at 20 and 200 μM. These doses are in the range of 8–14 and 80–140 times higher than the IC50 of Kelly (1.4 ± 0.25 μM) and Kelly Cis83 (2.45 ± 0.40 μM) grown in 2D respectively [54] (Fig. 4C and D). Cisplatin treatment continued to day 14. Cell response to cisplatin was examined by assessment of DNA content and nuclei staining with DAPI. Fig. 4 demonstrates that DNA content levels of cells treated with 20 μM cisplatin over the 7 days stayed the same as untreated controls (Fig. 4D and E) while DAPI staining displayed some degree of nuclei damage (Fig. 4G). The DNA content measurement allows analysis of the entire sample, while the cell imaging heavily depends on the selection of the inspection area resulting in some bias and may not represent the full picture. A significant reduction in cell proliferation was observed for neuroblastoma cells grown on both scaffold types treated with 200 μM cisplatin for 7 (Fig. 4D and E) and 14 (Supplementary Fig. 2) days. Seven days after treatment, a statistically significant increase in resistance to cisplatin by 37% and 30% was displayed by KellyCis83Luc when compared to KellyLuc grown on Coll-GAG and Coll-nHA, respectively (Fig. 4F). The observation confirmed the preservation of...
Fig. 4. Neuroblastoma cell response to cisplatin treatment in 2D monolayer compared to 3D collagen-based scaffolds. Cisplatin treatment at 200 μM started on day 1 and continued to day 7 on 2D monolayer (A) and on Coll-GAG (B) and Coll-nHA scaffolds (C). Effect of cisplatin treatment on cell DNA content at 20 μM and 200 μM doses delivered on day 7 and continued to day 14 on Coll-GAG (D) and Coll-nHA scaffolds (E). Comparison of cell response to 200 μM cisplatin between cisplatin sensitive KellyLuc and KellyCis83Luc grown on both scaffolds after 7 days treatment (F). Untreated, UNT, Cisplatin, CIS. Asterisks indicate statistical significance obtained using a paired Student’s t-test. (‘p < 0.05, “p < 0.01, “”p < 0.001, results are mean ± S.D (n = 3)). Confocal imaging of DAPI stained nuclei of KellyLuc and KellyCis83Luc cells grown on collagen-based scaffolds at day 14, 7 days after treatment with cisplatin at 20 μM and 200 μM (G). Scale bar 20 μm.
drug-resistant properties acquired in 2D by KellyCis83Luc when cultured in 3D in vitro. The results are consistent with chemosensitivity in vivo (Fig. 5), further demonstrating the physiological significance of the 3D scaffold-based cisplatin-resistant neuroblastoma model.

3.5. Modelling chemotherapy response in 3D in vivo

We further validated the proposed 3D in vitro tissue-engineered cell system using a murine orthotopic xenograft model of neuroblastoma injecting both KellyLuc and KellyCis83Luc cell suspensions in the adrenal gland (Fig. 4A and B) as described previously [48,69]. Tumour growth was then assessed by bioluminescent imaging on days 7, 14, and 21 revealing an increase in the bioluminescent intensity over the course of several weeks (Fig. 4C and D).

We then evaluated sensitivity to cisplatin of neuroblastoma tumours using the KellyLuc and KellyCis83Luc orthotopic xenograft models by systematic administration of cisplatin at 3.5 mg/kg (≈200 μM) 14 days after cancer cell suspension inoculation. Chosen cisplatin dosage was in line with clinically recommended dosage (40–120 mg/m² or 1.33–3.99 mg/kg) [66–68]. Measurements of post-mortem volumes and weights (day 35) confirmed the highly significant reduction in tumour growth in cisplatin-treated mice (Fig. 4E). Cisplatin sensitive KellyLuc average tumour weight was 3.84875 g before and 0.36 g after treatment, demonstrating 10 fold decrease in tumour mass. Cisplatin-resistant KellyCis83Luc average tumour mass was 4.065 g before and 1.5 g after treatment, demonstrating 2.7 fold reduction in tumour mass. It is very likely that tumour microenvironment contributed to the overall tumour growth and response to treatment displaying greater tumour mass reduction as a result of treatment. Notably, the data confines the maintenance of drug-resistant properties in KellyCis83Luc that were acquired in 2D.

3.6. Evaluating miRNA gene delivery in 2D and 3D in vitro

To further enhance the therapeutic relevance of this 3D in vitro tissue-engineered cell model, we tested the ability of the system to predict responses to gene-targeted approaches such as miRNA interference. The ability of a single miRNA to downregulate many genes belonging to multiple cellular pathways at once makes them attractive in developing miRNA-based therapeutic strategies [48,51,70,71]. Despite the intensive research in miRNA-mediated therapeutics, the successful application of miRNAs as a cancer
therapy is very limited partially due to the lack of suitable in vitro pre-clinical models that allow more detailed functional research and as such reduce risks and attrition rates at a later stage [33,50,51,71,72]. The concept of targeted therapies requires target identification and validation. The development of miRNA-based therapies follows the same path. With this in mind, we have selected miR-324-5p as a potential tumour suppressor. MiR-324-5p has been used in the 37 miRNA expression signature predictive of neuroblastoma patient survival [29]. Firstly, we validated clinical significance of miR-324-5p as an independent predictor in a cohort of 328 neuroblastoma tumours (Supplementary Fig. 3A). Firstly, we validated clinical significance of miR-324-5p as an independent predictor in a cohort of 328 neuroblastoma tumours (Supplementary Fig. 3A). The low expression of miR-324-5p was significantly associated with poor prognosis (raw p = 1.4E06; p = 4.5E04, Bonferroni correction). Given the significant association, we investigated the effects of miR-324-5p ectopic expression in neuroblastoma cells on viability.

Fig. 6. Proof-of-concept for the proposed 3D in vitro tissue-engineered model in the evaluation of miRNA-based therapeutic. (A) Ectopic over-expression of miR-324-5p mimics transfection into Kelly cells grown in 2D significantly reduced cell viability (p = 0.016), indicating a potential tumour suppressive function. Expression of mirRNA (B) and VDAC1 mRNA (C) 48hr after transfection with miR-324-5p mimic or scrambled negative control in neuroblastoma Kelly cell line in 2D as determined by RT-qPCR. (D) Effect of miR-324-5p mimics transfection on cell DNA content 7 days after transfection on Coll-nHA scaffold. Expression of mirRNA (E) and VDAC1 mRNA (F) 7 days after transfection with miR-324-5p mimic or scrambled negative control in neuroblastoma Kelly cell line in 3D as determined by RT-qPCR. (G) Assessment of viability of neuroblastoma cells grown on collagen-based scaffolds 7 days after transfection with mir-324-5p mimic or scrambled negative control using DAPI labelled cell nuclei, Calcein AM (green fluorescence) labelled live cells and EthD-1 (red fluorescence) labelled dead cells. Labelling mir-324-5p mimic, mir-324-5p, scrambled negative control, NC, positive control siKinesin, KIFF. Asterisks indicate statistical significance obtained using a paired Student's t-test. ( *p < 0.05, **p < 0.01, ***p < 0.001, n = 3 for all experiments). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
and the predicted targets for this miRNA. Ectopic overexpression of miR-324-5p mimics in Kelly neuroblastoma cell line grown in 2D in vitro significantly inhibited cell viability and proliferation (Fig. 6A and B) and inversely correlated with the expression of its direct target – the voltage-dependent anion channel 1 (VDAC1) on mRNA (Fig. 6C) and protein levels (Supplementary Fig. 3D). At this stage, miR-324-5p has successfully passed the initial screen in the 2D traditional culturing model as a candidate for miRNA-based therapeutics.

At the next stage, miR-324-5p was tested in our 3D neuroblastoma model. To mimic physiological relevance of this miRNA-based strategy for tumour treatment: the same neuroblastoma Kelly cells were grown on the Coll-nHA scaffold for 7 days to allow cells to form a critical mass of tumour cells prior miRNA transfection. The Coll-nHA scaffold was selected for further miRNA validation based on its more robust and consistent performance in pair with Kelly cell line. Cell response to transfection with liposome bearing miR-324-5p mimics was examined by assessment of DNA content, nuclei staining with DAPI and Live/Dead cytotoxic assays. Fig. 6D demonstrates no reduction in neuroblastoma cell proliferation grown on scaffolds 7 days after transfection with miR-324-5p mimics when compared to scrambled negative control. Notably, the miR-324-5p mimics successfully penetrated cell membrane (Fig. 6E) and repressed expression of VDAC1 mRNA as validated by RT-qPCR at the same time point (Fig. 6F). The efficacy of cellular uptake and gene knockdown was similar in both 2D and 3D in vitro culturing models highlighting the proof-of-principle for the applicability of 3D collagen-based scaffolds cell system for validation of miRNA function.

Although ectopic overexpression of miR-324-5p on day 7 displayed high levels it did not affect cell viability in 3D (Fig. 6G) as was demonstrated in 2D (Fig. 6A). This observation can be explained by the different cellular context of 2D and 3D models which, in turn, elicit a different miRNA functioning. Based on the evidence, miR-324-5p did not demonstrate tumour suppressor function in 3D in vitro. This discrepancy highlights the limitations of the traditional 2D in vitro screening in the successful translation of discovery made in 2D into clinic [5,6,22,57]. Our data support the importance of the 3D architecture of cells and cell environment for validation of targeted miRNA-based therapies and technological advances provided by 3D tissue-engineering.

4. Discussion

Unlike research for adult cancer, paediatric cancer research has very few disease-relevant preclinical models capable of generating effective patient-tailored models to guide clinical development of paediatric drugs. Current neuroblastoma studies employ either 2D cell culture systems, murine models or alternatively a mix of both [37]. The use of 3D models for neuroblastoma is limited to growing cells as spheroids for either research of neuroblastoma stem cells [73–77] or drug screening [78–81]. To the best of our knowledge, a 3D scaffold-based neuroblastoma cell model has not been previously evaluated, thus highlighting the existing gap for a validated and physiologically relevant 3D in vitro bioengineered tumour platform, which has controllable and adaptable properties.

Here, we have confirmed the capacity of collagen-based scaffolds to support the growth of two neuroblastoma cell lines, chemotherapeutic (cisplatin) sensitive Kelly and resistant Kelly-Cis83 cells [54]. Both neuroblastoma cell lines robustly penetrated and proliferated on the scaffolds and displayed >100-fold increased resistance to cisplatin treatment when compared to 2D cultures exhibiting chemosensitivity to cisplatin, a cytotoxic drug commonly used in neuroblastoma treatment, at a concentration similar to that used in vivo (3.5 mg/kg =198 μM when using in mice models) and in the chemotherapy induction regimen in the clinic (1.3 –3.99 mg/kg) [66–68]. The concentration of cisplatin at 200 μM is >100 times higher than the IC50 values of the same cell lines Kelly (1.4 ± 0.25 μM) and Kelly Cis83 (2.45 ± 0.40 μM) grown on 2D monolayer [54]. Importantly, KellyCis83 Luc demonstrated the preservation of cisplatin-resistant properties acquired in 2D both in 3D in vitro and in vivo. The data is in agreement with previous studies reporting a significant increase in cancer cell resistance to chemotherapy when grown in 3D when compared to their 2D monolayer counterparts in various cancer types [23,57,63]. The majority of drug screening on neuroblastoma cells grown in 3D spheroids demonstrated similar results. SK-N-SH neuroblastoma spheroids demonstrated higher resistance to increasing 15-deoxy-PGJ2 treatment when compared to the monolayer model [79]. Doxorubicin IC50 values of SH-SYSY spheroids were also higher than that of 2D [78]. On the contrary, sensitivity to cisplatin in 2 neuroblastoma cell lines NASS and SJNB8 grown on plastic and as multicellular tumour spheroids demonstrated similar results of therapeutic sensitivity in 2D and 3D [82]. This discrepancy can be explained by a different approach in drug treatment such as duration of treatment and starting time. Importantly, our data are consistent with chemosensitivity exerted by the same cell lines in vivo and therefore further reiterating the usefulness of the 3D collagen-based scaffold cell culturing system to mimic more closely the in vivo scenario. The scope of this cytotoxic assessment was limited to a single drug cisplatin, consequently, testing other drugs or their combination would validate further the relevance of this model as a new pre-clinical drug testing platform.

Cell growth patterns for KellyLuc and KellyCis83Luc were cell type-dependent reflecting their acquired changes during the development of cisplatin resistance [54], leading to altered expression of cell surface signaling molecules. The histological assessment found that neuroblastoma cells infiltrated both scaffold types and cells grew in clusters as well as coating the collagen fibres and pores of both. KellyLuc demonstrated widespread cell penetration and more dispersion throughout the scaffold from the surface while KellyCis83Luc cells grew in large clusters on both scaffold types. Further testing of other neuroblastoma cell lines with distinct migratory and biological features coupled with a characterisation of cell surface receptors responsible for cell adhesion and attachment to the ECM (e.g. integrins) will give a more comprehensive understanding of neuroblastoma cell behaviour in the described 3D in vitro model. It would be valuable to explore co-culturing of neuroblastoma cells with other cell types of tumour microenvironment (e.g. tumour-associated macrophages, fibroblasts or mesenchymal stem cells) to advance this model creating a more physiologically relevant setup.

The results suggest that both scaffolds have great potential to be used to model neuroblastoma ECM and a more complex tumour microenvironment depending on the research question. As bone marrow (70.5%) and bone (55.7%) [38] are the most common sites for neuroblastoma metastases, the Coll-nHA scaffolds may represent attractive matrices for metastatic neuroblastoma research due to its similarity to the inorganic component of human bone [12,14,16,83] and therefore would better mimic the in vivo bone/ bone marrow neuroblastoma microenvironment. In follow up studies, it would be invaluable to dissect the ECM composition of clinical samples of bone/bone marrow origin and compare to the ECM secreted by neuroblastoma cells grown on collagen-based scaffolds. This would provide us with crucial information for future developments to even more closely recapitulate the local native microenvironment.

The identification of the most promising miRNA candidates and/or its targets for each health condition, including cancer, is the greatest challenge in the field of miRNA-based therapeutics.
partially due to the relatively small number of preclinical models that can mimic the disease at the tissue level [5,11,22,57]. Importantly, our understanding of basic miRNA functions is far from complete. To the best of our knowledge, the direct comparison of miRNA efficacy in 2D and 3D disease models has not been previously investigated. Although gene knockdown using RNA silencing was demonstrated for the 3D prostate cancer model using collagen-based scaffolds [20,23]. As demonstrated in this report, we showed the difference in chemosensitivity between 2D and 3D, evidencing usefulness of the model for miRNA validation. Neuroblastoma Kelly cells grown both in 2D and 3D in vitro were treated with liposomes bearing mimics of clinically relevant mir-324-5p. The initial identification and validation of this miRNA in 2D in vitro screening has not been confirmed by 3D in vitro. This data illustrates the complexity of cell biology and the activation of different pathways in 2D and 3D. Validation of targets and miRNA candidates selected in 2D in vitro for therapy needs to be investigated with care because the functioning of some miRNAs can be highly dependent on the cell microenvironment. The acknowledgement of these challenges is essential in the correct interpretation of the research and assessment of the promising drugs and targets. This study provides a proof-of-concept study showing the enormous potential of the described 3D in vitro cell model for preclinical screening of targeted drugs. Further exploration of the miRNA or RNA silencing biology using the proposed 3D in vitro cell model will produce a better quality of information that will be therapeutically useful in various types of cancer and health conditions. This system can be used to advance drug chemistry and delivery technologies, including nanoparticle systems before its moves to the animal testing [20,21,23].

5. Conclusion

Collectively, this data showed for the first time, the successful development and characterisation of a physiologically relevant, 3D tissue-engineered neuroblastoma cell model using collagen-based scaffolds. This 3D in vitro model demonstrates a physiological similarity in vivo models, making evident the potential of this model to serve as a tool to elucidate neuroblastoma pathogenesis and for the development of new drugs. The principal advantages of 3D culture systems are the ability to precisely manipulate components of the microenvironment and subsequently analyse the effect of its structure and function on cellular and tissue levels [6,9,11].

The 3D tissue-engineered neuroblastoma cell model described herein represents a platform for molecularly dissecting the biological cues that underlie disease progression. This model will furthermore allow development of the system by increasing its complexity through the incorporation of other cell types, such as fibroblasts, mesenchymal stem cells or cells of the immune system creating a more physiologically relevant in vitro scenario. The microenvironment within the scaffolds can be modified through cell transplantation, presentation of ECM proteins, or delivery of proteins or genes using delivery vectors. The described 3D platform may have explored for other tumour types and disease.

The ability to test drugs in this reproducible and controllable tissue-engineered model system will help reduce the attrition rate of the drug development process and lead to more effective and tailored therapies. Importantly, such 3D cell models help to reduce and replace animals for pre-clinical research addressing the principles of the 3Rs [84]. This will confer significant patient benefit and should be of major commercial interest to pharmaceutical companies.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.actbio.2018.02.004.

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