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A bioengineering approach to investigating the possible role of fibronectin in neuroblastoma dormancy and relapse

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PREFACE

This master thesis has been written in the context of the 2020 global pandemic caused by SARS-CoV-2. Originally, the thesis was designed as practical work based in the study of neuroblastoma vascularization and differentiation through the use of biomaterial engineering. However, in mid-March (after three weeks of starting my work) exceptional situation and lockdown measures were taken by the Spanish Government, making impossible further laboratory work. This forced a change in the practical scope of the thesis towards a more theoretical approach.

To diverge from a purely bibliographical and systematic review work I, at the proposition of my tutor, Dr. Aranzazu Villasante, accorded to implement the use of practical cases as the basis for a thesis in the shape of a research proposal. For 6 weeks after the lockdown measures started, I produced five practical cases based on the critical analysis of 5 scientific publications related to neuroblastoma and/or biomaterial engineering. The cases consisted of answering a set of questions proposed by Dr. Villasante. This set of questions included at its end a section demanding a new hypothesis based on the knowledge created by the discussed publication, as well as the necessary experiments to test that hypothesis. Once each case was finished, Dr. Villasante and I would discuss it via videoconference.

With some modifications and refinements, these hypotheses became the backbone of the research proposal here presented. In consequence, the proposed experimental design is in part inspired by the techniques and methods utilized in these articles. Given the fact that the Master in Biomedicine and Biotechnology is oriented towards academic research, I have designed the project to fit the timescale of a doctoral project, being achievable in approximately three years.

This document contains said research proposal, while the annex contains the five practical cases.

Last, but not least, I would like to thank Dr. Villasante for her patience, guidance, and goodwill even through these strange times. I would also like to thank and acknowledge Prof. Gabriel Moncalián's flexibility, support, and understanding in the previous, particular circumstances that I had to confront.

Thank you, Arantza, and thank you, Gabi. Thank you very much.

To my sister, Sara, without whom this master's thesis work would have never taken place. Thank you for pressing that button for me.

CONTENTS

ABSTRACT

Neuroblastoma (NB) is the most common extracranial solid tumor in children and is frequently diagnosed at a metastatic stage. Despite therapy, tumor relapse occurs for around 50% of high-risk NB patients. At relapse, these patients have a very poor prognosis and overall survival rate decreases. The mechanisms behind NB relapse are not well understood, but recent studies suggest that chemoresistant dormant cells are in part responsible for tumor recurrence.

Fibronectin (Fn) is an extracellular matrix protein (ECM) shown to promote breast cancer cell dormancy in a TGFβ1-dependent manner. Fn presents three regions of alternative splicing: extra domain A (EDA), extra domain B (EDB), and type III constant segment (IIICS). The presence of EDA-containing Fn (EDA+ Fn) in the ECM generates a positive feedback loop by activating the TGFβ1 signaling pathway, which in turn promotes the expression of EDA+ Fn.

Besides its role in dormancy, the activation of the TGFβ1 signaling pathway promotes the differentiation of stromal fibroblasts towards cancer-associated fibroblasts (CAFs), which are known to promote tumor progression and chemoresistance.

Altogether, we hypothesized that the ECM protein fibronectin, and specifically EDA+ Fn, could play a critical role in neuroblastoma dormancy, chemoresistance, and relapse by activating the ERK signaling pathway in NB cells and the TGFβ1 signaling pathway in fibroblasts of the tumor environment.

To validate our hypothesis here we propose three specific aims; (i) to demonstrate that fibronectin induces cancer cell dormancy and relapse in neuroblastoma; (ii) to elucidate the role of the EDA domain in Fn-induced dormancy and relapse; (iii) to demonstrate how EDA+ Fn plays a crucial role in CAF activation, initiating an ECM-remodeling process that promotes tumor growth and progression.

SPECIFIC AIMS

Cancer cell dormancy is a clinically undetectable state of tumor cells underlying minimum residual disease, metastasis, and cancer relapse among others (Jahanban-Esfahlan et al., 2019). **Neuroblastoma (NB)**, on the other hand, is the most common extracranial solid tumor in children and often has a poor prognosis (Modak and Cheung, 2010). More than half of the patients have a metastatic disease at diagnosis, and approximately 50% of children with high-risk NB that complete consolidation therapy will suffer an early or late relapse (Reynolds, 2004).

Despite its **key importance in relapse and metastasis**, cancer cell **dormancy** is poorly understood. However, a very recent publication by Barney et al. (2020) used an *in vitro* model of cell dormancy, showing how **extracellular matrix (ECM)** composition is a key contributor to this process in breast cancer cells, demonstrating how **tumor-cell secreted fibronectin (TCFn)** matrixes can improve very significantly **cancer cell survival during long dormancy periods**.

While **TCFn** matrixes strongly **enhance cell dormancy**, cells grown on plasma fibronectin (pFn)-coated surfaces exhibit diminished survival rates. To date, the mechanism causing this **drastic reduction between cells grown in TCFn and pFn** remains unknown. **We propose a driving mechanism**: the **fibronectin** gene displays **alternative splicing**, and up to 20 variants exist in the human organism. Through extensive bibliographical research, we have identified a specific Fn domain, **Extra Domain A (EDA)**, which is present in TCFn and not in pFn, and possesses unique characteristics.

EDA-containing fibronectin (EDA+ Fn) has a 5-fold higher ability to attract and bind **latent TGFβ binding protein (LTBP1**; Klinberg et al., 2018**)** than pFn, and is a necessary element of **TGFβ1-dependent** myofibroblast differentiation (Serini et al., 1998), potentially facilitating the activation of cancer-associated fibroblasts (CAFs).

Biomaterials and tissue engineering offer interesting tools to study **cell-ECM interactions** in both **2D** and **3D cultures** where **ECM composition** and **mechanical** constraints can be defined at will; as well tools to research **interactions** between **different cell types**, using techniques such as **micropatterning** to study cell interactions in a spatially controlled manner while maintaining a defined ECM.

The information gathered led us to hypothesize that **EDA+ Fn** expression by cancer cells in **neuroblastoma** enables **cancer cell dormancy** when tumor cells face adverse conditions, while at the same time **triggering** the activation of local fibroblasts leading to the **appearance of CAFs**. **Our long-term** goal is to **validate this hypothesis** using techniques based on biomaterial and tissue engineering.

We are proposing to accomplish this hypothesis with the following **specific aims**:

1. To study **cancer cell dormancy in neuroblastoma**, we will reproduce some of the experimental approaches done in breast cancer cell lines by Barney et al. using **serum deprivation** as a mean to **induce dormancy**, testing a variety of NB cell lines with varying capacity to produce metastases and relapse.
2. To verify the impact of **fibronectin's EDA domain** in dormancy, we will study the **expression** and localization of **Fn splice variants** and different **integrins** along the set of NB cell lines, **comparing** their ability to enter **dormancy** with their Fn and integrin **expression and distribution patterns**.
3. To study the interactions between **NB cells** and **fibroblasts** with special attention to **EDA+ Fn** and **CAF** differentiation, we will **use** different **co-culture methods** including **micropatterning** and 3D **porous scaffolds**.

INTRODUCTION

Neuroblastoma

Epidemiology

Neuroblastoma (NB), is a highly vascularized cancer of the sympathetic nervous system, and the most common extracranial solid tumor of infancy. It is a clinically heterogeneous malignancy that accounts for 15% of cancer deaths in children, with an incidence of 10.2 cases per million children under 15 years of age (Park et al., 2010). Neuroblastoma prognosis correlates with the patient's age at the time of diagnosis and the degree of differentiation, with outcomes varying from high rates of survival to recurrence and mortality. Grave prognosis, however, is common. The difficulty at diagnosing NB causes that more than half of the cases are detected at the metastatic stage (Reynolds, 2004). Besides, it is estimated that 50-60% of children in the high-risk group who complete consolidation therapy experience an early or late relapse (Maris, 2010). The clinical relevance of metastases and relapse in neuroblastoma, being both processes governed by the ability of the tumor to survive during extended periods of adverse conditions and capacity to alter its microenvironment to a more favorable one, makes especially relevant the study of cancer cell dormancy and its relationship with the extracellular matrix (ECM) in this kind of tumor.

Biology

The degree of differentiation of a tumor measures the phenotypical variation of the tumor's cells respect their original tissue or organ; being very differentiated those cells closely resembling their healthy tissue counterparts, and poorly differentiated those cells whose phenotype has drastically shifted from these. The process through which tumor cells undergo a phenotypic change, losing their original characteristics, is called de-differentiation. De-differentiated cells suppose a major risk for cancer patients, as they possess enhanced plasticity and multipotency, granting them higher possibilities of invading new environments different from their original niche, producing metastases. When studying neuroblastoma differentiation is a subject of great relevance, both clinically and biologically.

From a clinical point of view, NB de-differentiation correlates with a bad prognosis. Interestingly, it is sometimes reverted spontaneously by a re-differentiation event, causing a sudden regression of the disease (Nakagawara, 1998; Nickerson et al. 2000). This discovery has led to therapies based on the induction of re-differentiation using isotretinoin (13-cisretinoic acid) which have been incorporated into multimodal treatments for patients with high-risk neuroblastoma (Brodeur and Bagatell, 2014; Brodeur, 2018).

From a biological point of view, and especially for those studying neuroblastoma *in vitro*, it is of key importance to note the findings done by Lam et al. (2010; practical case 1). In their article, they described how the stiffness of the extracellular matrix alone can induce neuroblastoma cell redifferentiation. ECM stiffness refers to the elasticity of the substrate in which cells are embedded, or in the case of two-dimensional (2D) *in vitro* cultures, to the stiffness of the culture substrate utilized. It is measured through

Young's elastic modulus (*E*) using pascals (Pa) as the unit. According to Lam et al., neuroblastoma cell lines grown on low stiffnesses of 0.1 to 1 kPa will stay in their natural dedifferentiated, multipotent state, while they shift their phenotype to a neuron-like one when grown in substrates with stiffnesses over 50 kPa, thus suffering a redifferentiation process. Classical culture surfaces such as polystyrene tissue culture plates and glass culture surfaces lay in the MPa range (Pelham and Wang, 1997). This represents a challenge when studying most neuroblastoma cell lines *in vitro*. The stiffness-induced re-differentiation process makes it necessary for researchers to implement biomaterial engineering culture methods in their experimental designs. By culturing NB cells in surfaces with a tailored stiffness, this artifactual re-differentiation process can be avoided.

Another important characteristic of neuroblastoma's biology is cancer cell dormancy. It is one of the possible mechanisms behind chemoresistance and common relapses (Veschi et al., 2019). Cancer cell dormancy is the process through which a tumor cell enters a state of quiescence, suffering cell cycle arrest and becoming insensible to the cytostatic drugs used in chemotherapy (Ranganathan et al., 2006). A recent article by Barney et al. (2020; practical case 4) describes one possible mechanism behind cancer cell dormancy: tumor cell-secreted fibronectin (TCFn) strongly promotes cell survival during long periods of serum deprivation in breast cancer cells. Importantly, they found that fibronectin purified from human plasma (pFn) fails to produce this same effect. The authors also described how when cells are incubated with transforming growth factor-β1 (TGFβ1) during the serum starvation assays, TCFn secretion was significantly increased. Opposingly, TGFβ receptor inhibition it shut off almost completely, implying that fibronectin-induced dormancy dependent on TGFβ1 signaling

Cancer-associated fibroblasts

Today, the consensus is that tumors are composed of both cancer cells and stromal cells (i.e. immune cells, fibroblasts, etc) and that their interactions have crucial importance in cancer biology (Hashimoto et al., 2016). Like many other cell types found in tumors, cancer-associated fibroblasts (CAFs) present an altered phenotype induced by cancer cells. This new phenotype, similar to that of myofibroblasts, grants them a key role in ECM regulation in the tumor niche. Given their similarity, both CAFs and myofibroblasts can be identified using α-Smooth muscle actin (α-SMA) as a molecular marker, since its expression is highly upregulated as a result of their activation.

Although normal non-activated fibroblasts are present too in the tumor stroma, the majority of present fibroblasts are CAFs presenting this secreting phenotype. Importantly, these CAFs do not return to a normal state as myofibroblasts do under physiological conditions, maintaining a perpetual fibrogenic activity (Tomasek et al., 2002). The ECM remodeling caused by CAFs promotes cancer proliferation, invasiveness, angiogenesis, and progression, resulting in an increased overall tumor ability to proliferate and produce metastases (Vong and Kalluri, 2011).

Tissue and biomaterial engineering

Surface stiffness and cellular biology

Pelham and Wang (1997) first described how substrate stiffness could affect cellular motility and morphology in fibroblasts. Since then, substrate stiffness has been shown to affect adhesion, migration, cell differentiation and proliferation, angiogenesis, tumor metastasis, embryonic development, and other processes (Wells, 2008; Xia et al., 2017). In this context, it is worth recalling how substrate stiffness drives neuroblastoma cell differentiation with the consequent loss of "stemness" as explained above (Lam et al., 2010).

The elastic modulus of the ECM varies greatly across the different human tissues and organs, being the brain's E the lowest in the human body at several hundred pascals (Pa). Muscle's E is around 12 kPa, while tendons and cartilages lay in the megapascal range (Levental et al., 2007). Bone, the hardest tissue, has an average E of 10 to 20 GPa (Rho et al., 1993). It is key to point out how ECM-remodeling processes such as fibrosis can drastically change the stiffness of a tissue. While a normal liver has a stiffness slightly higher than that of the brain, around 500 Pa, the changes to the ECM caused by fibroblast pathological activation can increase its E to 20 kPa (Georges et al., 2007). ECM-remodeling processes are also of importance in tumors and can be associated with malignancy (Wullkopf et al., 2018).

The relevance of substrate stiffness in cell biology has led to the development of several methods for cell culture under controlled mechanical conditions. These are based on the fabrication two-dimensional (2D) and three-dimensional (3D) substrates where the ratio of a polymer vs. a crosslinker agent is used to tune the stiffness. Higher ratios of the crosslinker agent allow for the formation of more covalent bonds in the material, resulting in a higher elastic module (Yan et al., 2019). The materials utilized to manufacture the substrates vary depending on their applications and their dimensionality, being polydimethylsiloxane (PDMS, a kind of silicone) a common compound used for 2D applications. Conversely, ECM proteins such as collagen-I or inert proteins such as silk fibroin are the among most common materials used for 3D applications.

Two-dimensional substrates and micropatterning

PDMS is routinely used in biomaterial engineering to produce 2D cell culture substrates of determinate stiffnesses with ease. It is a commercially available, elastic, non-toxic, transparent, biocompatible, and hydrophobic polymer, making it an interesting material to implement in cell culture (Raczkowska et al., 2016). Given its hydrophobic nature, surface functionalization is necessary for cell adhesion. Approaches such as UV-ozone treatment can be used to ionize the silicone surface improving its protein adherence and wettability. However, this modification produces a surface capable only of forming ionic bonds with a coating, which are not desirably durable. To avoid this, carbodiimide crosslinking chemistry can be used to form covalent bonds between PDMS and a coating molecule, strongly stabilizing the interaction (Zhou et al., 2011).

Other compounds can also be used to produce substrates with controlled stiffness. Lam et al. (2010; practical case 1) used polyacrylamide to produce gels of stiffnesses ranging from 0.01 kPa to 1000 kPa. However, a complex manufacturing process was necessary to modify the polyacrylamide surface to reduce its cytotoxicity (Ulrich et al., 2009).

The coating process can be further tweaked with the use of antifouling layers or microcontact printing to produce micropatterned surfaces where one or more proteins of interest are inlaid in a spatially controlled manner (Martinez-Rivas et al., 2017). These techniques allow for the study on how ligand disposition, cell cluster shape, and inter-cellular tension affect cell biology (McBeath et al., 2004). They can also be used for the implementation of co-culture methods where different cell types are confined in discrete, spatially controlled patterns to study their interaction (Dickinson et al., 2012; practical case 2). When using microcontact patterning for the study of cellular interactions among two cell types, it may occur that a valid couple of cell type-specific ligands cannot be found. This may make it impossible to have two different cell types exclusively bind a single type of ligand molecule in the pattern. In these cases, it is possible to combine different patterning strategies to circumvent the lack of ligand-cell type specificity.

When using materials like PDMS for micropatterning, the coated areas of the pattern can be surrounded by non-coated, non-adherent surfaces, strictly confining cell growth to the coated areas. By altering the shape and size of these coated areas, the number of cells per cell cluster can be adjusted. Altering these parameters can alter cell proliferation and behavior (Mammoto et al., 2004). This technique has also been used to determine mesenchymal stem cell (MSC) differentiation fate, as bigger cell groups can establish stronger cell-ECM and cell-cell adhesions. These adhesions allow cells to subject each other to higher tensions than cells confined in smaller clusters, leading to a mechanosensing-dependent differentiation process where the MSCs differentiate into the three mesenchymal lineages depending on cell cluster size (McBeath et al., 2004).

Another article by Shimizu et al. (2014; practical case 3) represents an impressive advance in patterning technology and its potential, shifting our technical ability to produce patterns from the micro- to the nanoscale (Shimizu et al., 2014). The researchers were capable of deploying a quasi-hexagonal pattern of 10 nm gold particles coated with the cyclic peptide cRGD (capable of binding a wide range of integrins) where the size of the gold nanoparticles allows for a single integrin dimer to bind a single nanoparticle. Furthermore, they used photocleavable PEG12K as an antifouling agent in the nanoparticles, allowing them to photoactivate the surface. This way only the UV-irradiated areas are cell-adhesive, enabling for the performance of clean, high-resolution cell migration assays where integrin ligand availability and spacing can be tightly controlled.

Three-dimensional cultures

Most cell types in the human organism, if not all, grow and thrive in heterotypic three-dimensional environments where cells are completely embedded in the ECM. This is not restricted to physiological tissues and organs: it is also common to pathological structures such as tumors. However, since the advent

of cell culture, methods for the culture of cells have mainly focused on homotypic 2D monolayers grown in either glass or polystyrene surfaces. These conditions are unfortunately distant from the physiological environments that cells usually inhabit. Today, it is well known that cells grown on traditional 2D culture surfaces behave differently as they would do in a 3D culture environment or *in vivo* (Benton et al., 2009; Fraley et al., 2010).

During the last two decades, different strategies for the development of three-dimensional culture systems have been used. The use of spheroids and porous scaffolds are among the most relevant techniques (Haycock, 2010). Spheroids exploit the tendency of most cell types to aggregate naturally, using techniques such as hanging drop or concave wells to concentrate suspended cells around the apex of the droplet or well thanks to gravity (Froehlich et al., 2016). Porous scaffolds, conversely, are based on the manufacturing of protein-based 3D matrixes. These allow for the study of cell-ECM interactions in an environment where the matrix mechanical and molecular properties can be controlled by the researcher, in opposition to spheroids where cells freely assemble their 3D matrix (Carletti et al., 2010). Porous scaffolds can be manufactured utilizing a vast amount of techniques: solid free-form fabrication technologies (such as 3D printing), electrospinning of nanofibers into random patterns, or freeze-drying fabrication.

Fibronectin biology and alternative splicing

Fibronectin is a 440-kD dimeric glycoprotein widely distributed in plasma and in the ECM (Kurkinen et al., 1980). Since the '80s, it's been known that the fibronectin gene presents alternative splicing. Ruoslahti (1988) published an extensive review on fibronectin biology and biochemistry, where a report of the up to 20 described fibronectin splice variants is included. Fibronectin has three domains that present alternative splicing: extra domains A (EDA), extra domain B (EDB), and the type-III constant domain (IIICS). Domains EDA and EDB are mutually exclusive, and are never present in plasma Fn, while the IIICS domain can be present in it.

Both EDA+ and EDB+ fibronectin are oncofetal isoforms, given the fact that their expression patterns are restricted to development, but re-gained in tumors (Kumra and Reinhardt, 2016). Therefore, both isoforms can be present in TCFn. Besides its localization in tumors, EDA+ Fn can be found in wound healing and in fibrotic lesions, where it is secreted by fibroblasts causing their activation and differentiation towards the myofibroblast phenotype (White and Muro, 2011). Conversely, EDB+ Fn can also be found in proliferative diabetic retinopathy (PDR). It is of interest to note that EDB+ Fn localization in tumors and PDR is mainly restricted to blood vessels, and plays a direct role in tumor vasculogenesis (Khan et al., 2005).

EDA Fibronectin

The presence of the EDA domain grants ECM fibronectin improved functionality. EDA+ Fn potentiates cell cycle progression and ERK signaling more strongly than EDA- Fn (Manabe et al., 1999). This correlates with the results obtained by Barney et al. (2020), where inhibition of ERK signaling of tumor cells growing

in TCFn reduces cell survival rates to levels found in cells growing in neutral collagen I coating. Additionally, the presence of this domain is necessary for TGFβ1-dependent myofibroblast differentiation (Serini et al., 1998). This could link the expression of EDA+ Fn to CAF activation and ECM remodeling.

Serini et al. (1998) described three key factors underlying TGFβ1-dependent myofibroblast differentiation. First, EDA+ Fn expression is increased when fibroblasts are incubated with TGFβ1, and the expression of EDA+ Fn precedes that of the myofibroblast differentiation marker α-SMA is expressed. This hints that this Fn isoform may mediate a step of the differentiation process. Second, the incubation of fibroblasts with IST-9 (an antibody specifically binding the EDA domain of Fn) shuts down TGFβ1 activation and therefore fibroblast differentiation towards the myofibroblast phenotype. Antibodies targeting other Fn domains do not have the same effect, highlighting the necessity of EDA's functionality for this activation process. Third, given that active TGFβ1 increases EDA+ Fn expression, and that EDA+ Fn increases latent TGFβ1 activation, both molecules act synergistically forming a positive feedback loop that ultimately leads to fibroblast differentiation.

Later in time, EDA+ fibronectin was shown to be essential for the association of latent TGFβ binding protein 1 (LTPB1) to the ECM (Zilberberg et al., 2012). It was also found that EDA+ Fn binds LTBP1 twice as efficiently as its pFn counterpart, and EDA blockage with the IST-9 antibody reduced the ratio of active TGFβ1 10-fold as a result of LTBP1 binding competition (Klinberg et al., 2018). These findings synergize with those of Serini et al (1998), partially unveiling the mechanisms behind TGFβ1-dependent fibroblast differentiation.

Integrin biology

Fibronectin, as well as many other ECM proteins, interacts with cells by binding integrins. Integrins are heterodimeric transmembrane proteins with intra- and extracellular domains that link the cytoskeleton to the ECM. Their function is not merely structural: they also activate signaling pathways, acting as a biomechanical sensor. They are composed of two subunits: one of the α type and one of the β type. 18 different α and 8 β subunits exist, which can combine to form at least 24 identified heterodimers *in vivo* (Kechagia et al., 2019).

Fibronectin contains a vast amount of integrin-specific binding motifs that enable cell adhesion. More specifically, the EDA domain contains a unique motif (residue sequence EDGIHEL) which binds integrins α4β1, α4β7, and α9β1. Both α4β1 and α4β7 can bind other domains of fibronectin, but these binding sites are composed of a different motif, which may have consequences over binding and function (Yalak et al., 2019). Interestingly, Horwacik and Rokita (2017; practical case 5) published the results of a partial integrin screening over the neuroblastoma cell lines IMR-32, CHP-134, LA-N-5, and Kelly. With it, they were able to show how only the cell lines expressing high levels α4 and β1 can attach to and grow in fibronectin matrixes, being both processes reversible by using the α4β1 inhibitor BIO1211. These findings highlight the

importance of integrin expression patterns when studying the effect of cell-ECM interactions and exhibit the utility of using integrin inhibition for such studies.

Additionally, and as a note of importance, EDA+ Fn has been shown to induce the aforementioned fibroblast differentiation process by binding to the α4β7 integrin in lung stromal fibroblasts (Kohan et al, 2010), one of the three integrins shown to bind EDA. This gives further importance to the study of the role of integrin expression in fibronectin-dependent cell dormancy.

HYPOTHESIS

Summarizing the key concepts of the introduction, our research hypothesis is based on the following published research data and assumptions:

1. Cancer cells under serum starvation express TCFn which in turn promotes cancer cell dormancy and survival. However, pFn fails to produce the same effect.

2. Expression of TCFn in serum-starved cancer cells can be increased with exogenous TGF β 1 and inhibited with a TGF β R inhibitor (Barney et al. 2020).

3. EDA+ Fn expression is increased in the presence of TGF β 1 (Serini et al., 1998). These findings point towards the possibility that the TCFn produced by dormant breast cancer cells is in fact of the EDA+ isoform.

4. EDA+ Fn is necessary for fibroblast activation and phenotype switch, a process that leads to increased TGF β 1 expression with the resulting positive feedback loop (Serini et al., 1998).

5. The ECM-remodeling process triggers the transition from healthy fibroblasts to the CAF phenotype and also plays a role in tumor progression (De Wever et al., 2008).

Taken all together we can establish the following hypothesis:

(i) Neuroblastoma cells express EDA+ Fn when facing adverse conditions such as serum starvation, what activates (ii) a short-term autocrine signaling pathway capable of enabling cell dormancy mediated by integrin and ERK signaling, and (iii) long-term paracrine signaling that favors the differentiation of stromal fibroblasts towards a CAF phenotype mediated by integrins and TGF β 1 signaling.

The combination of autocrine and paracrine signaling could give NB-EDA+ cells the tools to survive to processes like chemotherapy by entering dormancy and to recover thereafter by altering their ECM and microenvironment. Given the clinical relevance of relapse in neuroblastoma, we believe that the discovery of the involvement of EDA+ Fn in this phenomenon would open the door for better therapies.

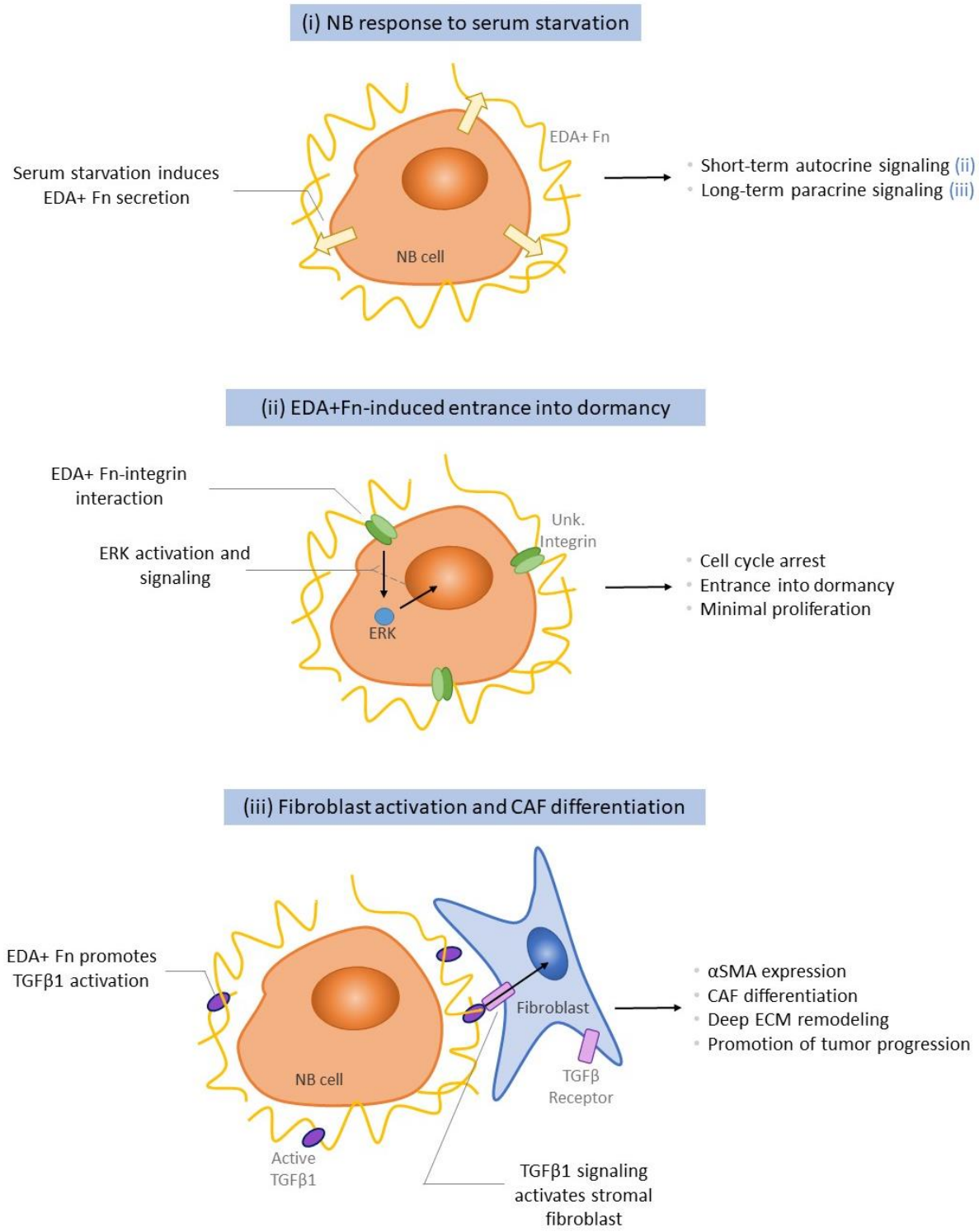

Fig 1. Graphical model illustrating the three sections of our proposed hypothesis. For illustration comprehensibility, the interactions between integrins and the cytoskeleton have not been represented. This note is for clarifying that, despite how integrins have been depicted, they are not to be confused with common transmembrane receptors such as the TGFβ receptor.

OBJECTIVES

In order to validate the proposed hypothesis, the following objectives will be pursued:

1. To successfully translate the use of serum starvation as an inducer of fibronectin-dependent cancer cell dormancy as described by Barney et al. (2020) to study cancer cell dormancy in neuroblastoma.
2. To verify that the EDA domain is a necessary element for fibronectin-dependent dormancy, elucidating the role of integrin signaling and the TGFβ1 - EDA+ Fn positive feedback loop in this process.
3. To study the effects of EDA+ fibronectin secretion over normal fibroblast populations, carefully examining their possible differentiation towards a pro-fibrotic, cancer-associated fibroblast phenotype with the subsequent remodeling of the ECM, promoting a pro-tumorigenic microenvironment.

MATERIALS AND METHODS

Preparation of functionalized 2D substrates of physiological stiffnesses

To produce 2D substrates with controlled mechanical and biochemical properties, we will utilize PDMS and carbodiimide chemistry. We will fabricate these substrates in multi-well plates to augment the capacity of our experimentation throughput to be able to test a wide range of NB cell lines.

To produce substrates of a desired stiffness, first we will need to calibrate the stiffness that a specific PDMS-to-curing agent ratio yields (PDMS mass to Curing agent mass; hereby P:C ratio). For this, we will produce PDMS cylinders of 10 mm diameter and 7 mm height using molds and the P:C ratios for each type of PDMS established in Table 1. The conventional type of PDMS used for the fabrication of substrates, Sylgard 184, cannot reach stiffnesses of 1 kPa and below. To reach these lower stiffnesses we will use Sylgard 527 PDMS, which can reach elastic moduli of down to 0.1 kPa (Moraes et al., 2015). The different PDMS and curing agent mixtures will be prepared, cast in the molds, and cured in a dry oven at 60 ºC for 4 hours.

Table 1. Ratios of PDMS to curing agent used to produce the samples for the study of P:C ratio impact over substrate stiffness, based on published methods (Moraes et al., 2015). Small variations in the ratio cause drastic variations in Sylgard 527 after curing, for what the differences between P:C ratios are smaller.

PDMS type	P:C ratio (m:m)
Slygard 184	70:1 50:1 25:1 10:1 (~1000 kPa)
Sylgard 527	5:4 (~1 kPa) 1:1 (~0.1 kPa) 4:5 2:3 1:2

The cured PDMS cylinders will then be subject to compressive tests using a texture analyzer in the style of XT-PLUS (TA instruments). This device produces a deformation in the material and measures the resulting force-displacement curve, which is used to calculate the elastic modulus. The *E* of cylinders of each P:C ratio will be measured in triplicate, and the average measurements used to perform a regression curve. This curve will be used to calculate the P:C ratios required to produce substrates of each experiment's desired stiffnesses.

The 2D soft substrates will be fabricated in multi-well well plates by pouring the desired mixture of PDMS and curing agent in each well and curing it at 60 ºC for 4 hours. Once cured, the substrates will be subject to UV/ozone activation for 5 minutes and incubated in a crosslinking solution containing 0.05 M MES buffer, 0.5 M NaCl, 0.1 M 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and 0.1 M N-Hydroxysuccinimide (NHS) for 15 minutes in the dark. The wells will then be washed repeatedly with PBS buffer, letting the PBS sit for 5 minutes during each wash and avoiding letting the wells dry between washes.

At this point, the PDMS surfaces can be coated by incubating a concentrated solution of the desired protein or polypeptide at 4 ºC in the dark for 16 hours. After incubation with the desired protein, several PBS washes must be performed given that EDC and NHS are cytotoxic compounds. At this point, the plates are ready to use in experiments.

For the scope of this project, two different kinds of coatings will be used; one based in poly-D-lysine (PDL), used to study the capacity of cells to produce a dormancy-inducing ECM themselves; and one based in EDA+ fibronectin to study whether this isoform is responsible for the dormancy-inducing effect. Cellular fibronectin (supposed to include the EDA domain) can be outsourced from two companies producing and selling cellular Fn (ScienCell, cat. no. 8488; Sigma-Aldrich, cat. no. F2518). We will request information about the splicing variants of their products to verify whether they do include the EDA domain, and source our EDA+ Fn from any of them in case their product is EDA positive.

Preparation of 3D porous scaffolds

Some of the experiments of this project will require the use of 3D culture methods. For this, we will use freeze-drying fabrication, which allows for batch manufacturing of scaffolds. This technique starts with a highly concentrated solution of a given protein which is placed in a mold and frozen at -40 ºC (fig. 2). The ice crystal formation during the freezing process is exploited to produce gaps in the sample. The frozen material is sublimated in a lyophilizer, leaving hollow pores where the ice crystals were, forming a dry protein scaffold. To resist dissolution when re-wetted, the scaffold must be subject to carbodiimide crosslinking. The covalent bonds formed in the crosslinking step make the structure stable in water.

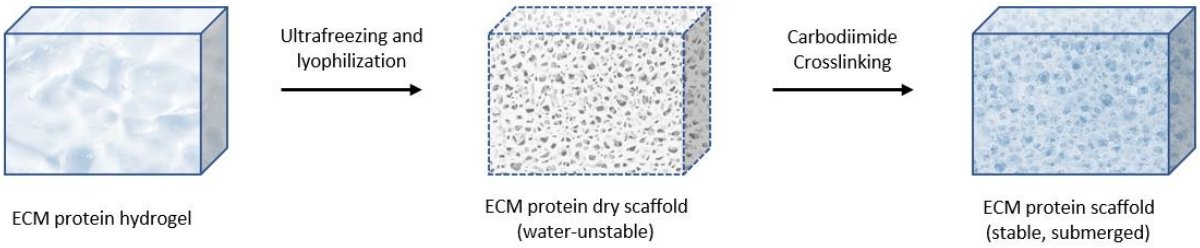

Figure 2. Schematic representation of porous scaffold fabrication using freeze-drying fabrication.

To produce our 3D scaffolds, we will use the freeze-drying technique as described by Villasante et al. (2016). To fabricate these porous scaffolds, 200 µL of a highly concentrated solution of silk fibroin (7 – 10 mg/mL; Sigma-Aldrich) will be pipetted in cylindrical molds of 8 mm diameter and 5 mm height and frozen at -40 ºC for 4 hours. After freezing, the samples will be lyophilized at -40 ºC for 16 hours. Once lyophilized, the materials will be submerged in a crosslinking solution containing 95% v/v ethanol, 33 mM EDC, and 6 mM NHS for 4 hours at room temperature. The scaffolds will then be washed 5 times in distilled water, 5 minutes each time, re-frozen at -40 ºC for 4 hours, and re-lyophilized at -40 ºC for 16 hours. The proposed protocol produces scaffolds with an elastic modulus of approximately 1 kPa. At this point, dry scaffolds can be stored for days. Before their utilization, they must be sterilized by submerging them in 95% ethanol for 1 hour, washed 5 times in PBS buffer, and coated by submerging the scaffolds in a sterile PDL solution (0.1 mg/mL) for 1 hour at 37 ºC.

Cell lines

A set of 16 NB cell lines displaying different degrees of tumoral progression stemming from less aggressive malignancies to more aggressive will be studied in different experiments (Table 2). These cell lines have been chosen to cover all tumor stages, 1 to 4, including the stage 4S, a special classification for neuroblastoma. Stage 4S neuroblastomas have a higher spontaneous regression rate and a better prognosis than stage 4 patients (Taggart et al., 2011). The amplification state of MycN, which strongly correlates with prognosis and tumor aggressivity has also been assessed. Certain NB lines have been selected given specific characteristics that make them interesting for the scope of the study. These characteristics can be consulted in the Remarks section of Table 2.

Table 2. Neuroblastoma cell lines selected for cell dormancy screening using the serum starvation method. BM = bone marrow, LN = lymph node, Unk. = unknown.

Cell line	Stage (Thiele, 1998)	Metastasis (Thiele, 1998)	MycN amplification (Harenza et al. 2017; Thiele, 1998)	Integrin expression (Horwacik and Rokita, 2017)	Remarks
CLB-Pe	1	No	Yes	-	-
STA-NB-3	2	No	Yes	-	-
NLF	3	No	Yes, low	-	-
WSN	3	No	Yes	-	-
NBL-S	3	No	No	-	-
CHP-134	4	LN	Yes, medium	α1, α4, αv, β1, β3	-
LA-N-5	4	BM	Yes, high	α1, α2, αv, β1, β5	
SK-N-DZ	4	BM	Yes, medium	-	Differentiates in stiffnesses over 50 kPa (Lam et al., 2010)
SH-N-SH	4	BM	No	-	Can induce VEGF-dependent angiogenesis (Li et al., 2013)
LA-N-6	4	BM, bone	No	-	-
NGP	4	BM, lung	Yes, high	-	-
CLB-Ga	4	BM, LN, bone, mediastinum	Yes	-	-
NB69(2)	4	Lung, liver, sternum	No	-	-
NBL-W	4S	Liver	Yes	-	Stage 4S neuroblastoma exhibits a higher spontaneous regression rate (Taggart et al., 2011)
IMR 32	Unk.	Unk.	Yes, low	α1, α4, β1	-
Kelly	Unk	Unk.	Yes, high	-	-

Serum starvation assays

To study dormancy in neuroblastoma cells, we will use the *in vitro* cell dormancy protocol described by Barney et al. (2020), based on the use of serum starvation to induce dormancy. This method will be used routinely to study dormancy in a wide range of varying conditions. Serum starvation assays will be performed by seeding cell cultures onto a 2D substrate/3D scaffold depending on the experiment. Cells will be seeded at a concentration of 30,000 cells/cm^2 in the multi-well plates. Once seeded in the substrate or scaffold, cells will be cultured in each cell line's specific culture medium with no serum. The serum starvation assays will be performed, unless stated otherwise, using the following temporal schemes: 7-day serum-free culture, 28-day serum-free culture, and 28-day serum-free culture followed by 7 days of culture with serum (fig. 3), being the last used to study cell recovery after dormancy. The culture medium will be changed every 2 to 3 days.

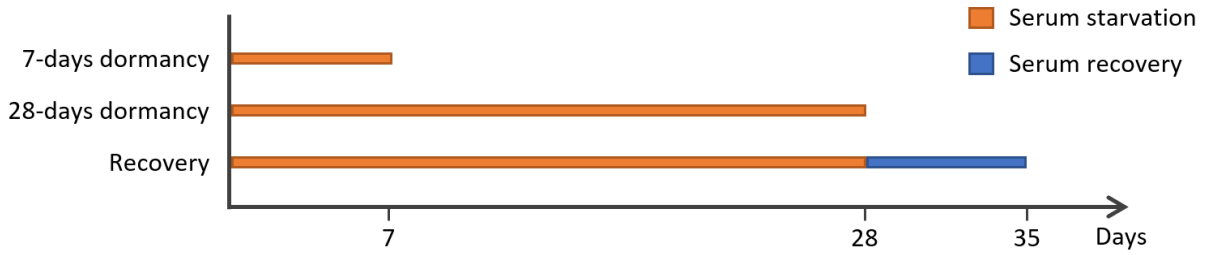

Figure 3. Schematic representation of culture conditions and periods used to determine NB cell line ability to enter and exit cell dormancy.

Cell dormancy analyses

Cell proliferation

To assess cell proliferation in the serum starvation assays, we will use the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo-Fisher). This assay measures the amount of double-stranded DNA (dsDNA) found in a present sample, which correlates with cell density. It can be used to quantify cell populations both 2D and 3D cultures.

First, samples will be homogenized by incubating them in a digestion solution containing papain (125 µg/mL), L-cysteine (2 mM), and EDTA (0.333 M) in PBS buffer. Incubation will take place in the multi-well plates used for the starvation protocol, with mild shaking at 65 ºC for 16 hours. Once homogenized, 100 µL of each sample will be transferred to a 96-well plate and combined with 100 µL of the PicoGreen™ reagent, previously diluted 20X with TE buffer (included in the kit). The samples will then be incubated at room temperature for 5 minutes and their fluorescence will be analyzed in a plate reader.

To approximate the number of cells that corresponds to a given fluorescence intensity, each serum starvation assay will be complemented with homogenization and fluorescence analysis of a standard curve. The standard culture will be prepared by performing serial dilutions of a cell culture of known cell density. The dilutions will be digested and read in parallel together with the experimental samples.

Cell cycle

To gain insight into the cell cycle status of different NB cell populations, we will use flow cytometry (FC) to assess the cellular DNA content and p21 and Ki67 expression. To study cell cycle arrest, we will use propidium iodide (PI), p21 as a marker for cell cycle arrest at the G0/G1 stage, and Ki67 as a marker for actively proliferating cells.

For this, we will adapt the protocol published by Kim and Sederstorm (2015). First, we will harvest and fix cells by incubating them in a 70% ethanol solution at -20 ºC for 2 hours. Then we will rinse cells repeatedly with FC buffer (1X PBS supplemented with 2% fetal bovine serum and 1 mM EDTA) and incubate them with a FITC-conjugated anti-Ki67 antibody (Santa Cruz Biotechnology) or an Alexa-647-conjugated anti-p21 antibody (Abcam) for 30 minutes. One set of samples will be used for each antibody. After this, samples will be rinsed with FC buffer and incubated with 50 µg/ml PI (Sigma-Aldrich) for 20 minutes. At this point, flow cytometry can be performed using the adequate lasers and filter settings to collect data.

Immunostainings

Certain experiments will evaluate the expression of a protein using immunofluorescence. In table 3, we compile the different proposed primary antibodies and the secondary antibodies needed for fluorescence microscopy imaging. For the staining protocol, cell cultures will be devoid of culture medium and washed with warm PBS.

Table 3. Commercial antibodies destined to the study of fibronectin, EDA+ fibronectin, and αSMA expression using immunostainings and fluorescence microscopy.

Protein	Primary antibody, species of origin	Secondary antibody
Fn	EP5, mouse mAb (Santa Cruz Biotechnology)	Goat Anti-Mouse IgG-Alexa Fluor® 790 (Abcam)
EDA domain of Fn	IST-9, mouse mAb (Abcam)	
αSMA	1A4, mouse mAb (Thermo Fisher)	

The cultures will then be fixed and permeabilized with a PBS solution containing 4% paraformaldehyde and 0.2 Triton X-100 for 10 minutes. The samples will then be incubated with a blocking buffer containing 2% bovine serum albumin (BSA) in PBS for 30 minutes. Once blocked, the samples will be incubated with the primary antibody at the concentration recommended by the supplier for 2 hours at room temperature. Once this period ends, the samples will be washed three times with PBS and incubated with the secondary antibody for 1 hour. Finally, the samples will be washed again for three times, being ready to observe using fluorescence microscopy at this point.

Manufacturing dormancy-inducing decellularized matrixes

To study the effects caused by ECMs produced by neuroblastoma cells exposed to serum starvation, some of our experiments will use decellularized matrixes. The ECM of cells grown in a coated culture substrate can be decellularized using the protocol developed by Castelló-Cros and Cukierman (2009). Following it, we will culture a cell line exhibiting high capacity to enter dormancy on soft 2D PDMS substrates coated with poly-D-lysine in multi-well plates. This hypothetical cell line will be referred to as the HDC (High Dormancy Capacity) cell line from now on. The HDC cells will be seeded at a density of 30,000 cells/cm^2 in serum-free medium for 28 days. This is the period required for cells to produce a dormancy-inducing ECM (Barney et al. 2020). After this, the culture will be exposed to phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 20 mM NH_4OH at 37 ºC for 10 minutes. This incubation should destroy cytoplasmic membranes, therefore eliminating the cells from the matrix. The cell debris will be washed repeatedly with PBS, after which the cells will be kept for 1 month at 4 ºC. The decellularized matrixes are ready to use after this period (Castelló-Cros and Cukierman, 2009).

EDA+ inhibition and competition assays

To test the relevance of EDA+ Fn signaling in dormancy, we will study the effects of its inhibition. For this, we will use the antibody IST-9 (Abcam), which specifically targets the EDA domain of fibronectin, and peptides composed the EDA domain sequence ordered from a company producing custom peptide (i.e. Thermo Fisher). Both the antibody and the peptides will be used in serum starvation assays by being added in the culture medium at the concentration required by the product. If no recommended concentration is stated in the product's specifications, we will perform titration assays to decide a working concentration.

Signaling pathway inhibition assays

To study the implication of different signaling pathways involved in serum starvation-induced dormancy, we will use small molecule inhibitors. To study ERK signaling, we will use the small molecule inhibitor FR180204 (Sigma-Aldrich); for the focal adhesion kinase (FAK), we will use FAK inhibitor 14 (Sigma-Aldrich; and for the MAPK kinase (MEK), we will use PD0325901 (Sigma-Aldrich).

These inhibitors will be added to serum starvation assays to see how the inhibition of the different signaling pathways affects entrance into dormancy and cell survival. ERK, FAK, and MEK phosphorylation states using the antibodies listed in Table 4 to perform Western Blot analyses based on chemiluminescence.

Table 4. Commercial antibodies destined to the study of ERK, FAK, and MEK signaling pathway activation states using Western blot in different experiments throughout the project.

Protein	Primary antibody	Secondary antibody
ERK	M5670, rabbit mAb (Sigma-Aldrich)	Goat Anti-Mouse IgG Antibody HRP conjugate (Sigma-Aldrich)
FAK	SAB4502495, rabbit mAb (Sigma-Aldrich)	
MEK	SAB4502404, rabbit mAb (Sigma-Aldrich)	
pERK (p-204)	2D11, mouse mAb (Santa Cruz Biotechnology)	Goat Anti-Rabbit IgG Antibody HRP conjugate (Sigma-Aldrich)
pFAK (p-397)	E-4, mouse mAb (Santa Cruz Biotechnology)	
pMEK (p-218/p-222)	7E10, mouse mAb (Santa Cruz Biotechnology)	

Analysis of mRNA levels of fibronectin and integrins

We will study the expression of different fibronectin splicing variants and integrins to gather expression profiles and use them to analyze the relevance of the different proteins on cell dormancy. For this, we will study the expression of the different genes listed in table 5 by RT-qPCR. For this, we will seed the 16 different cell lines listed in table 2 at a density of 30,000 cells/cm^2 in 24 well-plates with soft 2D culture substrates coated with PDL. Each cell will be cultured in triplicate. They will be cultured without serum in their respective culture mediums for 7 days, as this period should be enough for cells to adapt their expression pattern to the serum starvation condition. At this point, RNA will be extracted using the PicoPure™ RNA Isolation Kit (Thermo Fisher). Once extracted, the samples will be transferred to 96-well plates and the SYBR® Green Quantitative RT-qPCR Kit (Sigma-Aldrich) will be used to measure the expression of the target genes. GAPDH will be used as the control housekeeping gene. To study fibronectin alternative splicing, we will design exon-exclusive primers.

Table 5. Target and control genes for the RT-qPCR analysis. This screen includes the different isoforms of fibronectin and different integrin subunits. Some integrin subunits have been selected given their ability to bind specifically the EDA+ motif, as explained in the "Integrin biology" section.

Integrin α subunits	Integrin β subunits	Fibronectin splicing variants	Housekeeping gene
α1, α2, α3, α4, α5, α7, α9, αv	β1, β2, β3, β5, β7	EDA+, EDB+, ED-, IIICS	GAPDH

Integrin inhibition assays

To study integrin adhesion inhibition in a heterodimer-specific manner we will use small molecule inhibitors such as AF1864 (inhibits α5β1; R&D Systems) and BIO 1211 (inhibits α4β1; Tocris Bioscience, Biokom) among others. There are no commercially available small molecule inhibitors of α4β7, an integrin of potential interest for our project, for what we will use Vedolizumab biosimilar monoclonal antibodies such as MAB10078 (R&D Systems). These inhibitors have been selected for integrins of interest given the studied literature, although the protein expression assays performed in the project may result in the finding of other integrins worthy of study. Were this the case, small molecule inhibitors or antibodies will be obtained and tested too. The different inhibitors or antibodies will be added to the culture medium of cells undergoing serum starvation assays in the required concentration.

If we find an integrin with a probable role in dormancy through our screenings, but no dimer-specific inhibitors or antibodies can be acquired commercially, we will design a siRNA against its gene and transfect it to perform the integrin inhibition assays. However, the results obtained would have to be studied carefully, as this strategy necessarily targets the gene of either one or both of the subunits in the integrin dimer. This could potentially cause off-target effects by inhibiting the formation of other integrin dimers and affecting their signaling pathways.

Micropatterning neuroblastoma on soft 2D substrates

In this project, we will use micropatterning techniques to study the impact of intercellular forces and cluster size on NB dormancy and to compare the differential effects of EDA- and EDA+ Fn. To study intercellular forces and cluster size, we will use a microcontact printing method where we inlay an EDA+ Fn pattern of circles of varying sizes in a 2D soft PDMS substrate (Shen and Kam, 2008; fig. 4).

This process starts with the fabrication of a silicon mold with the desired pattern using photolithography. This process requires specific training and equipment, for what a collaboration with a group with expertise would be of interest. If this was not a possibility, molds can be obtained from specialized companies. Once a mold is obtained, a PDMS mixture with a P:C ratio of 10:1 is poured on the mold and baked at 60 ºC for 4 hours. Once cured and semi-rigid, the PDMS stamp is peeled from the mold and activated using UV-ozone for 5 minutes. The stamp is then loaded with a coating solution of EDA+ Fn (50 μg/ml), and the excess is dried carefully using a technical paper wipe. The loaded stamp is then inverted and placed on top of a 2D PDMS culture substrate previously activated and exposed to the EDC/NHS crosslinking solution. The mold then is pressed lightly for the solution to be transferred to the culture substrate, and then retired. The micropatterned culture substrate is then washed thoroughly and repeatedly with PBS to remove protein solution and EDC/NHS (step not depicted in fig. 4). Once washed, the micropatterned substrates are ready for cell seeding and experimentation.

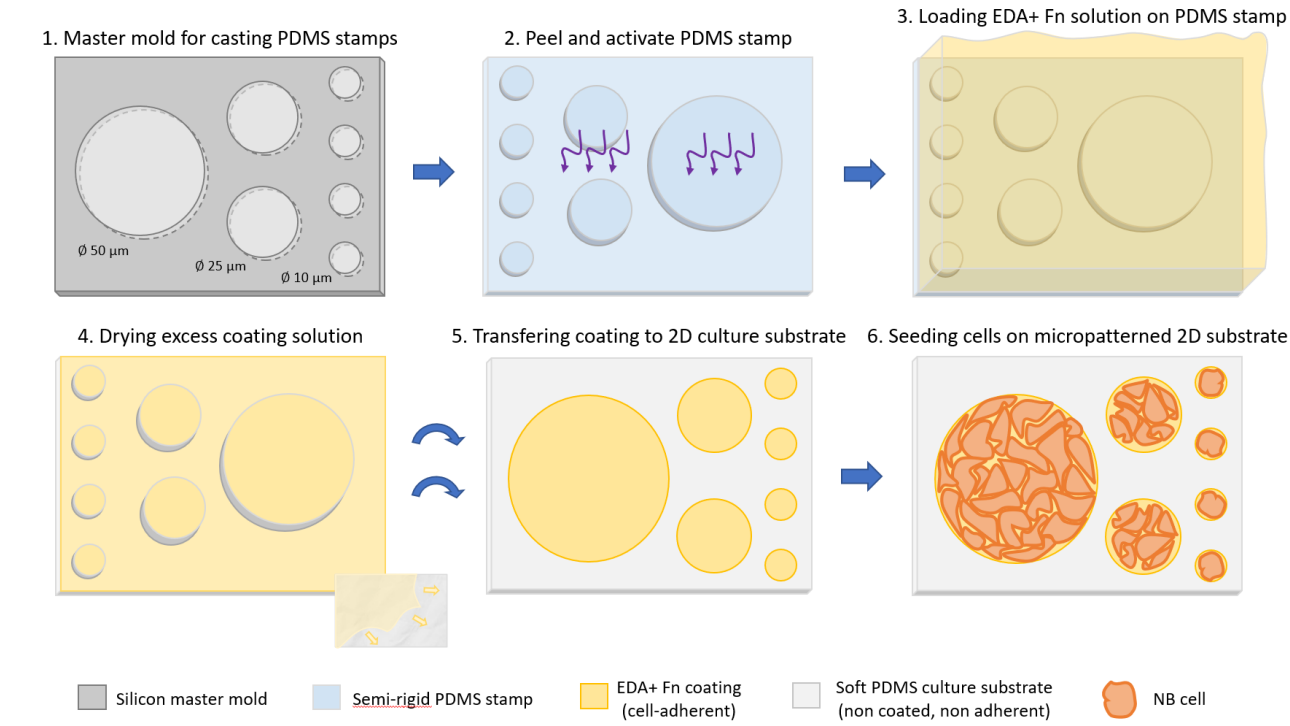

Figure 4. Schematic representation of the microcontact printing process utilized to create micropatterned 2D soft PDMS substrates with EDA+ Fn. For a clearer depiction, only a partial representation of the micropattern has been illustrated. An iteration of this pattern will be used, resulting in more spots on the pattern and reaching a total stamp size of 4 mm. Including bigger patterned circles for bigger cell clusters can be optional. A washing step between steps 5 and 6 has been excluded for a clearer process depiction.

Figure 5. Schematic representation of the sequential microcontact printing process used to produce 2D soft PDMS substrates co-patterned with EDA- and EDA+ Fn. The numbers in the scheme stem from the numeration used in figure 4. Only a fraction of 1 mm of the stamp has been represented – the actual pattern would be repeated horizontally and elongated vertically to fill a 5 mm square stamp. A washing step between steps 5 and 6 has been excluded for a clearer process depiction.

To study the differential effects of EDA- Fn and EDA+ Fn, we will produce substrates with a combined micropattern in which both Fn isoforms are alternatively patterned. To do this, we will use a new mold with rectangular shapes and follow the same procedure to cast, load, and dry PDMS stamps above explained (Fig 4: sections 1-4). In this case, we will produce two different stamps and coat one with EDA- Fn (50 μg/ml), and the other with EDA+ Fn (50 μg/ml). Then, the transferring process will be performed sequentially, first with one stamp and then the other inverted horizontally (Fig 5). After this, the micropatterned substrate will be washed repeatedly with PBS (step not depicted in fig. 5). Once washed, the substrate will be ready for cell seeding and experimentation.

Fibroblast and CAF characterization

To study fibroblast activation and differentiation in presence of EDA+ Fn and/or dormant neuroblastoma cells, we will utilize the Human Adrenal Fibroblast cell line (ScienCell) for cell cultures and co-cultures with NB cells. To assess the differentiation state towards the CAF phenotype, we will study the expression of αSMA using the 1A4 antibody for immunostainings (Thermo Fisher). Additionally, in some experiments we will study matrix metalloproteinase (MMP) expression. We will use the MMP Activity Assay kit (Abcam) to quantify MMP activity and the Human MMP Antibody Array (Abcam) to assess specific MMP expression.

Neuroblastoma and fibroblast co-cultures

To study the interaction between neuroblastoma cells and fibroblasts in the context of dormancy, we will perform co-cultures of these cell types and perform serum starvation assays. To study the possible role of fibroblasts during NB entrance and exit from dormancy we will use two different culture regimes.

First, to evaluate if fibroblasts can contribute towards NB cells entrance into dormancy, we will seed a mixture of both HDC cells and Human Adrenal Fibroblasts in soft 2D substrates coated with PDL. They will be submitted to a serum starvation assay by culturing them without serum for 28 days, and then with serum for a 28-day recovery period. We have designated a longer recovery period to account for the timescale in which fibroblasts react to fibronectin, commence the activation and differentiation process, and start remodeling their ECM.

Second, we will study if fibroblasts play a role in the recovery process after serum is added to the culture medium. Because fibroblasts will most possibly not survive the serum starvation period, we will first culture the HDC line under serum starvation for 28 days. At the end of this period, fibroblasts will be seeded at the time that serum is added to the culture medium again, starting a 28-day recovery period.

In both regimes, three different numbers of fibroblasts will be seeded to choose the best initial density: 5,000, 10,000, and 20,000 fibroblasts per well. With this, we hope to find an adequate number of fibroblasts so that this cell type can exert an effect over dormancy while not taking over the cell culture and outgrowing neuroblastoma cells.

Because the previously used cell quantification technique cannot discriminate between cell types, it is impractical for the scope of this co-culture-based experiment. For this, we will transfect the HDC cell line with a plasmid containing the GFP gene and the fibroblast line with mCherry. We will study both cell lines' population density using cell sorting every 7 days. Additionally, we will use the sorted populations to study their expression profiles by performing an RT-qPCR for the genes listed in table 5 and several MMP genes, plus the αSMA gene for fibroblast populations.

Among the different conditions and time points, we will select those exhibiting results of interest and perform a more in-depth analysis using immunostaining and fluorescence microscopy. We will study the distribution of EDA+ Fn in the ECM, as well as the location of those integrins found to correlate with EDA+ Fn-induced dormancy.

EXPERIMENTAL DESIGN

Objective 1 – Translation of the serum starvation-induced dormancy *in vitro* model to neuroblastoma

First, we will fabricate 2D soft culture substrates and use them to validate the use of serum starvation as a model of neuroblastoma cell dormancy *in vitro*. We plan to use PDL-coated 2D substrates of 1 kPa stiffness to conduct a first dormancy screening by culturing the 16 NB cell lines under serum starvation conditions. We will determine cell proliferation by using the Picogreen™ technique and analyze cell cycle using flow cytometry at the different time points of the assay (day 0, 7, 28, and 28 + 7 days recovery). To implement positive and negative controls, breast cancer cell lines HCC 1954 and HCC 202 will be used, for they present high and low ability to enter dormancy, respectively. Each condition will be run in triplicate using 24-well plates will be used in this screening. Based on the results obtained, we will assess each cell line's ability to enter dormancy.

Additionally, and in parallel with the first starvation assay, we will perform anti-Fn immunostaining on all 16 NB cell lines plus two controls cultured for 28 days without serum. We will use two different antibodies, one against the constant region of Fn (EP5) and one against the EDA+ domain (IST-9). With this, we aim to preliminarily assess whether the presence of Fn and EDA+ Fn is also associated with dormancy.

To study the effect of the ECM over dormancy, an EDA+ Fn-expressing NB cell line with prominent ability to enter cell dormancy will be used to produce decellularized matrixes. A second serum starvation screening will be performed using decellularized matrixes of HDC cell cultures and using only the 7-day serum starvation condition. Because the cell lines will be seeded on a dormancy-inducing, EDA+ Fn-containing matrix the cells do not need an ECM-remodeling and adaptation period.

We will also study how ECM stiffness affects neuroblastoma cell dormancy by seeding HDC in 2D substrates coated with PDL of an elastic modulus of 0.1, 1, 50, 300, and 1000 kPa. We will analyze cell

proliferation and cell cycle to assess the impact of stiffness over dormancy. Besides, we will also evaluate if the dimensionality of the cell culture substrate affects dormancy. For this, we will seed the HDC cell line in silk-based, PDL-coated 3D culture substrates and submit it to a serum starvation assay. The number of cells seeded will be the same as in the first serum starvation assay, and the proliferation rates of both will be compared.

Finally, we will analyze ERK, FAK, and MEK signaling in dormancy by performing a Western blot analysis after performing a serum starvation assay on a subset of three cell lines with high, medium, and low ability to enter cell dormancy.

Expected results

We hope to find that our set of selected NB cell lines exhibits varying capacities to enter cell dormancy and to survive in our first serum starvation screening. Optimally, we would find a cell line with a high survival rate (i.e. the HDC line) whose decellularized matrix, produced during 28 days of serum starvation, contains EDA+ fibronectin and is capable of inducing dormancy in some of the cell lines of the second serum starvation assay. We also expect that dormancy will be affected by the stiffness of the substrate, since harder stiffnesses induce NB cell differentiation in a process which could potentially alter the cell's ability to enter dormancy. Among the different signaling pathways studied, we expect that ERK will be upregulated in dormant cultures, as previously described by Barney et al (2020).

Contingency plan

Although we hypothesize that serum starvation-induced cell dormancy will occur in cell lines of a wide variety of cancer types, it is possible that none of the selected NB cell lines is capable of fibronectin-dependent cell dormancy. If this is found to be true, it would imply the failure of objective 1 because the serum-starvation *in vitro* model would not be of use to study dormancy in neuroblastoma. This would hinder the possibility of investigating Fn-dependent dormancy in neuroblastoma. However, if this scenario were to be faced, objectives 2 and 3 could still be pursued in their totality using the breast cancer cell lines described by Barney et al. (2020) and modifying some of the protocols.

Objective 2 – Studying the relevance of the EDA domain of fibronectin in neuroblastoma cell dormancy

To evaluate if the EDA domain does have a crucial role in cell dormancy, we will utilize the HDC line to perform a series of serum starvation assays under different conditions. First, we will perform a serum starvation assay using different 2D soft substrates: PDL-coated, decellularized matrixes, pFn-coated, and EDA+ Fn-coated. We will perform a second assay in EDA+ Fn-coated substrates where we test how adding the IST-9 antibody (anti-EDA) or the EDA peptide in the culture medium affects cell dormancy. In this second assay, we will also analyze survival rates and ERK, MEK, and FAK phosphorylation states. We will

finally perform a third assay where we will study how the shutdown of ERK, MEK, and FAK signaling pathways affect cell dormancy using small molecule inhibitors.

To gain a deeper understanding of the gene expression profile of the different cell lines and how it affects their ability to enter dormancy, we will study the integrin expression pattern and the fibronectin splicing profile of the different cell lines. We will use these results to evaluate which integrins are linked to dormancy and further study their role by implementing integrin inhibition assays.

Finally, we will use the micropatterned substrates with circular patterns of varying sizes to study the relevance of cell cluster size and intracellular tension over dormancy using HDC cells. Additionally, we will use the substrates micropatterned sequentially EDA- Fn and EDA+ Fn to study if HDC cells have a differential affinity for these substrates under normal conditions or under serum starvation.

Expected results

We expect to find, as our hypothesis states, that the EDA+ domain is necessary for the dormancy-inducing effect of TCFn. We believe that we will find how cell populations seeded in EDA+ Fn substrates exhibit higher proportions of dormant cells than those exposed to EDA inhibitors or those seeded in pFn. We hope to find specific integrins (possibly $\alpha4\beta7$) that are linked to dormancy and implicated in the transduction of EDA+ Fn signaling into the cell. We also expect to find that cell cluster size and intercellular tension will positively affect EDA+ Fn-dependent dormancy. Similarly, we expect to find that cells grown on sequentially patterned substrates survive only in the areas coated with EDA+ Fn, possibly influencing cell migration when under serum starvation.

Contingency plan

Although we expect that at least one of the cellular fibronectins commercialized by the companies ScienCell and Sigma-Aldrich will contain the EDA domain, none of them has tested this. In case we found that none of the products contains the EDA domain, it would be necessary to generate a cell line destined for EDA+ Fn production to express and purify the protein. It is also a possibility that we fail to find integrins whose expression correlates with dormancy. If this happened, it would not be possible to perform the integrin inhibition assays. Conversely, we may find a specific integrin that is linked to dormancy for which no commercial antibodies or inhibitors exist. If this were to happen, we would use siRNAs to perform gene knockdown studies. On a different note, it is possible that substrates coated with EDA+ Fn do not induce dormancy as decellularized matrixes containing fibronectin have been shown to do. If this were the case, the microcontact printing methods could not utilize this protein and would require the testing of other molecules such as PDL or possibly pFn.

Objective 3 – Evaluating the effects of EDA+ fibronectin over stromal fibroblast populations

To study the possible effect that EDA+ Fn secreted by dormant neuroblastoma cells could have over the fibroblasts found in the tumor stroma, we will utilize an adrenal fibroblast cell line. First, we will assess whether seeding the fibroblasts in different substrates affects fibroblast activation and differentiation TGFβ1 is added to the culture medium. For this, we will seed fibroblasts in 2D soft PDMS substrates of the following kinds: PDL-coated, HDC decellularized matrixes, EDA+ Fn-coated, and pFn-coated. We will culture them with or without TGFβ1, and we will study differentiation towards the CAF phenotype by studying αSMA and MMP expression. Second, we will study how the blocking of EDA and/or the TGFβ receptor affects fibroblast differentiation when incubated in EDA+ Fn-coated 2D substrates in the presence and absence of TGFβ1.

To observe and evaluate the direct interactions between dormant neuroblastoma cells and fibroblasts, we will use the described co-culture system. We will study how fibroblasts interact with NB cells in the entrance into dormancy and during exit and recovery. To better fit the fibroblast differentiation and ECM remodeling process timescales, we will use recovery periods of 28 days instead of 7 days. We will use cell sorting technologies together with RT-qPCR to study the genic expression of fibronectin isoforms, integrins, and MMPs at different time points, as well as αSMA, to track fibroblast differentiation. Complementarily, we will use immunostainings and fluorescence microscopy to image the ECM in those time points of interest, carefully studying possible interactions between Fn and integrins and MMP expression.

Expected results

We expect to find that TGFβ1-induced fibroblast activation and differentiation is dependent on the substrate that the fibroblasts are cultured on. We expect that fibroblasts seeded in decellularized matrixes and EDA+ Fn-coated substrates will be more active: a higher proportion of the fibroblasts in the culture would then be transitioning to the CAF phenotype and expressing the specific marker αSMA. We also predict that this behavior will be reverted by adding the IST-9 antibody or the EDA peptide to the culture medium, confirming the relevance of the EDA domain in CAF differentiation. Lastly, from our co-culture experiments we hope to find that when the HDC line is cultured under serum starvation, it is capable of inducing fibroblast activation. We believe that while EDA+ Fn expression may be increased in the short term, it could be degraded and replaced with other ECM components in the long term, leaving no trace of its relevance in NB cell dormancy.

Contingency plan

The experiments proposed for this objective are end-point studies with no other experiments depending on their results. Whether their results correspond to our expected results or not, they will provide valuable information for the goals of the study. However, some of the experiments could be modified to avoid any

problems that could arise. For example, the NB-fibroblast cocultures under serum starvation could fail to activate fibroblast differentiation because none of the cell types express TGFβ1 in these conditions. If this happened, the experiment could be repeated adding exogenous TGFβ1 to promote differentiation and assess how this molecule would affect the interactions between both cell types in a pro-fibrotic and pro-inflammatory molecular context.

Chronogram

Table 6. Chronogram depicting the 3-year schedule planned for this project.

		Year 1				Year 2				Year 3			
	Experimental methods	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
Objective 1	Developing 2D and 3D substrates												
	Screening for dormancy in NB - PDL substrates												
	Developing the matrix decellularization protocol												
	Screening for dormancy in NB - Decellularized matrixes												
	Impact of substrate stiffness over dormancy												
	Impact of culture dimensionality over dormancy												
	Studying ERK, FAK and MEK signaling												
Objective 2	Studying NB dormancy in different substrates												
	Impact of blocking the EDA domain over dormancy												
	Impact of signaling pathway inhibition over dormancy												
	Genic expression studies												
	Integrin inhibition assays												
	Micropatterning studies												
Objective 3	Studying fibroblast activation in different substrates												
	Impact of blocking EDA over fibroblast activation												
	Co-culturing of micropatterned NB cells with fibroblasts												

DISCUSSION

Understanding the mechanisms underlying cancer relapse is one of the open frontiers in oncology. Because neuroblastoma is a pediatric tumor where relapse is not only frequent but usually lethal, reaching this goal is especially important. Not only to reduce the mortality associated with this type of cancer but especially because neuroblastoma relapse tends to occur in the first decade of life, severely truncating the life expectancy of those with this condition (Modak and Cheung, 2010). For this, we believe that understanding the mechanisms driving NB relapse is key to obtain a better understanding of this detrimental process. By knowing the causes of relapse, new therapies targeted towards it could arise and significantly improve the outcome of neuroblastoma patients.

Cancer cell dormancy is widely considered to be one of the biological processes contributing to relapse. While it remains unclear how cells enter and exit the dormant state, there is growing evidence pointing towards the role of the ECM in the regulation of this process. More specifically, fibronectin has been directly linked to dormancy in at least two different types of cancer (Aguirre-Ghiso et al., 2017; Barney et al., 2020). For these reasons we believe that a project investigating whether fibronectin plays a role too in neuroblastoma dormancy and relapse is highly interesting.

From a more biological point of view, we have pointed out the importance of fibronectin alternative splicing regarding the EDA domain. It is doubtlessly relevant in processes related to tumor progression such as CAF differentiation and, as previously mentioned, may enable fibronectin to induce dormancy. With the methods and experiments proposed, we hope to shine light on the latter issue and to verify whether this domain is necessary for Fn-induced cancer cell dormancy.

We also expect to find that EDA-containing fibronectin will not only enable NB dormancy and survival over extended periods of adverse conditions but also drive CAF differentiation. An interesting feature of fibronectin-dependent cell dormancy is that the Fn-rich matrix produced during the dormant state needs to be degraded for cells to exit it. The differentiation of fibroblasts towards the CAF phenotype would certainly contribute to this degradation, acting as a key accomplice for tumor cells by starting a deep ECM remodeling process. This way, NB and CAFs could potentially collaborate to produce a microenvironment that promotes tumor progression and growth.

Interestingly, the mechanism we propose could enable cells to survive harsh conditions such as chemotherapy and to recover once these have passed in an untraceable manner. Once a population of dormant NB cells exits dormancy and reorganizes its ECM composition with help of newly activated CAFs, it needs to degrade the same matrix that enabled its survival. This would strictly narrow the spatial and temporal window in which EDA+ fibronectin can be detected, allowing this hypothetical mechanism to remain unobserved and unknown. This possibility further highlights the need for the development of an *in vitro* model for neuroblastoma cell dormancy.

All of the above strengthens the importance of meeting our three specific aims; to generate a preclinical *in vitro* model of cell dormancy for neuroblastoma; to study the possible role of fibronectin, and specifically its EDA+ isoform on NB cell dormancy; and to investigate the potential contribution of this protein towards CAF activation and differentiation.

In the future, the neuroblastoma dormancy model developed for this study could be used to screen drug libraries for compounds capable of inhibiting entrance and exit from dormancy. Drugs capable of doing so could potentially be used as a combined treatment with or after chemotherapy to combat minimal residual disease more efficaciously, therefore minimizing relapse frequency.

Additionally, we would like to point out the relevance of the EDB-containing isoform of fibronectin. Our study, once completed, could be followed up by investigating the role of the alternatively spliced EDB domain over tumoral angiogenic and vasculogenic processes. Neuroblastoma is a highly vascularized tumor that can induction the creation of new blood vessels utilizing several different mechanisms, some of which are still not well understood.

Concluding, we believe that our proposed hypothesis and project are in line with the latest findings regarding the role of the ECM in cancer cell dormancy. We also believe that if this investigation is fruitful, it would represent a crucial piece of knowledge towards the fight against relapse in neuroblastoma. The validation of

our hypothesis would be a very relevant contribution towards our understanding of NB dormancy, chemoresistance, and relapse, topics of utmost importance in pediatric oncology.

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2020

Annex

A bioengineering approach to investigating the possible role of fibronectin in neuroblastoma dormancy and relapse

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Contents

Practical case 1

Extracellular matrix rigidity modulates neuroblastoma cell differentiation and N-myc expression

Lam, W. A., Cao, L., Umesh, V., Keung, A. J., Sen, S., & Kumar, S. (2010). Molecular Cancer, 9(1), 35. https://doi.org/10.1186/1476-4598-9-35

1. What is the biological and/or clinical question that the article seeks to answer?

In the article, previous findings are noted (19, 22-30) showing how ECM stiffness and other biophysical properties of it have direct influence over neuroblastoma stem cell differentiation, neuronal maturation, neurite growth and extension, malignancy, and tumoral phenotype, and also how the signalling pathways influenced by ECM stiffness coincide, at least partially, with those of retinoic acid (RA) induced differentiation. Thus, the biological question the authors propose and try to answer is:

Do mechanical cues from the ECM influence spontaneous neuroblastoma differentiation, and if so, do they share signalling pathways with retinoic acid?

2. What is the working hypothesis?

To answer the questions the following hypothesis was proposed: mechanical cues from the ECM have an impact on neuroblastoma, affecting spontaneous and RA-induced differentiation, as well as altering its phenotype in means of clinically relevant and markers of proliferation and differentiation.

3. What does this article show? Is it of utility in the short or long term? What kind of work is it (Basic science, molecular biology mechanism, method, or device development)?

The study evidences how ECM stiffness, a simple biophysical parameter, is able to exert changes in biological markers of differentiation and proliferation of clinical relevance in neuroblastoma prognosis. They also show how this signalling acts synergistically with RA in the induction of differentiation.

It's a basic science article with a clear short and mid-term utility: the effect that surface culture rigidity exerts on neuroblastoma cells may also affect other cells, altering the results obtained in experiments studying differentiation and proliferation effects of pharmaceuticals, for example.

4. What are the critical experiments and principal methods utilized to answer the biological/clinical questions and confirm (or not) the working hypothesis?

1. Culturing of SK-N-DZ cells on collagen I-laminated polyacrylamide (PA) gels of stiffnesses varying from 0.01 kPa to 10000 kPa, both in absence and presence of RA. Measurements: neurite length.
2. Ki67 (proliferative marker) immunostaining of SK-N-DZ cells cultured on two different stiffnesses (0.1 kPa and 1000 kPa), both in absence and presence of RA. Measurements: Ki67 immunofluorescence.
3. WST-1 (metabolic activity reporter) staining of SK-N-DZ cells cultured on three different stiffnesses (1 kPa, 50 kPa, and 300 kPa), both in absence and presence of RA. Measurements: WST-1 absorbance.
4. RT-PCR quantification of N-myc expression in SK-N-DZ cells cultured on two different stiffnesses (1 kPa and 300 kPa). Measurements: N-myc relative expression.

5. Identify the controls used in the experiments and explain their utility or function for the analysis of the results.

Given the nature of the experiments performed, the definition of a control is slightly difficult – when the effect of surface rigidity is studied, all surfaces will have a determinate, discrete rigidity which will affect cell behaviour, making the implementation of a "negative control" impossible.

The use of glass in the first experiment could be deemed a control, since it is a standard surface for cell culture with which the researchers can use to compare how the cells behave in the PA gels vs. a common culture surface. However, when it comes to quantification and measurements of the effect that surface rigidity exerts over neuroblastoma differentiation and proliferation, it should be taken as another point in the studied range of stiffnesses, and not a standard, "outsider" control.

The rest of experiments do not present controls, since both the absence of RA and its presence are conditions of study, and thus not controlled measurements to be used as reference.

6. What are the conclusions?

The authors conclude that their hypothesis has been proved right, that surface rigidity by itself can directly alter neuroblastoma differentiation and proliferation, as well as biomarkers of clinical interest such as N-myc.

7. Do you believe that the experiments and the interpretation of the results are adequate to answer the biological/clinical questions and confirm (or not) the working hypothesis?

I believe the performed experiments are well based, conducted, and interpreted in order to answer the biological question, but I believe more could have been done to prove the answer. Using only neurite length and N-myc expression decay as differentiation markers may fall short of what was possible to do for testing and proving differentiation, since other relevant biomarkers could remain unaltered. Further experiments could strengthen the claim.

8. Do you believe that the results are well interpreted and that the conclusions are well-founded?

I believe that the results are well interpreted, and that the conclusions are well funded, but as just stated, they could have been supported with more data and experiments.

9. Is the working hypothesis confirmed to a 100% with the obtained results?

Almost 100%, yes, but as stated in answers 7 and 8, it could be more solid.

10. Write a critical analysis of the article stating its strong and weak points

The article describes a novel factor that can promote differentiation of neuroblastoma cells, and most importantly, describes a purely mechanical one. For decades biophysical factors have been completely overlooked, being biochemical cues the ones thought to produce most, if not all, changes in cellular biology. This work, as others have done before it, describes a key process by which most preclinical research is involved: the own rigidity of the culture plaques and flasks can cause changes in cell differentiation and proliferation. If this process is overlooked, assays testing pharmacological compounds or novel therapies will fail to properly show the effect these therapies have, as stiffness-induced differentiation will happen as an artefact, distorting the results. With very simple experiments, the authors have been able to describe a

process which may concern to all researchers of the field. The use of "classical" culturing surfaces such as glass and plastic may bias results.

On the downside, the simplicity of the experiments may at the same time take a bit of strength off the claims of the researchers.

11. What experiments do you believe should have been performed to improve the article and make the conclusions more robust?

In the first place, and after the first experiment results were observed, I would choose two determinate stiffnesses, a low one where differentiation should not occur and a high one where it should, and culture them with different concentrations of RA. Retinoic acid, a known pro-differentiating agent, would allow me to observe the effects that RA exerts over the cells in terms of proliferation and differentiation, as to have a sort of control experiment with which I could compare the results obtained with the alteration of surface rigidity.

Then, later in time, maybe in parallel with experiments 3 and 4 where differentiation and proliferation are assessed, I would perform experiments to study the differentiation process more closely, by using immature and mature neuronal markers like doublecortin and NeuN. With these experiments, I would expect to see the same results as with previous ones (i.e. neuroblastoma cells differentiate), only with more "resolution power" on the process.

12. Suggest a different working hypothesis based in the same biological/clinical question posed in this article

Two separate hypotheses are proposed to not surrender individual success of any of them to the other, although they could be combined, as they will in the abstract on question 14.

1. Mechanical cues from the extracellular matrix affect neuroblastoma cell differentiation and proliferation in irreversibly.
2. Mechanical cues from the extracellular matrix affect cellular differentiation and proliferation in different cell types.

13. What would be the critical experiments that you would need to perform to confirm (or deny) the hypothesis that you have proposed in question 12? What controls would be needed?

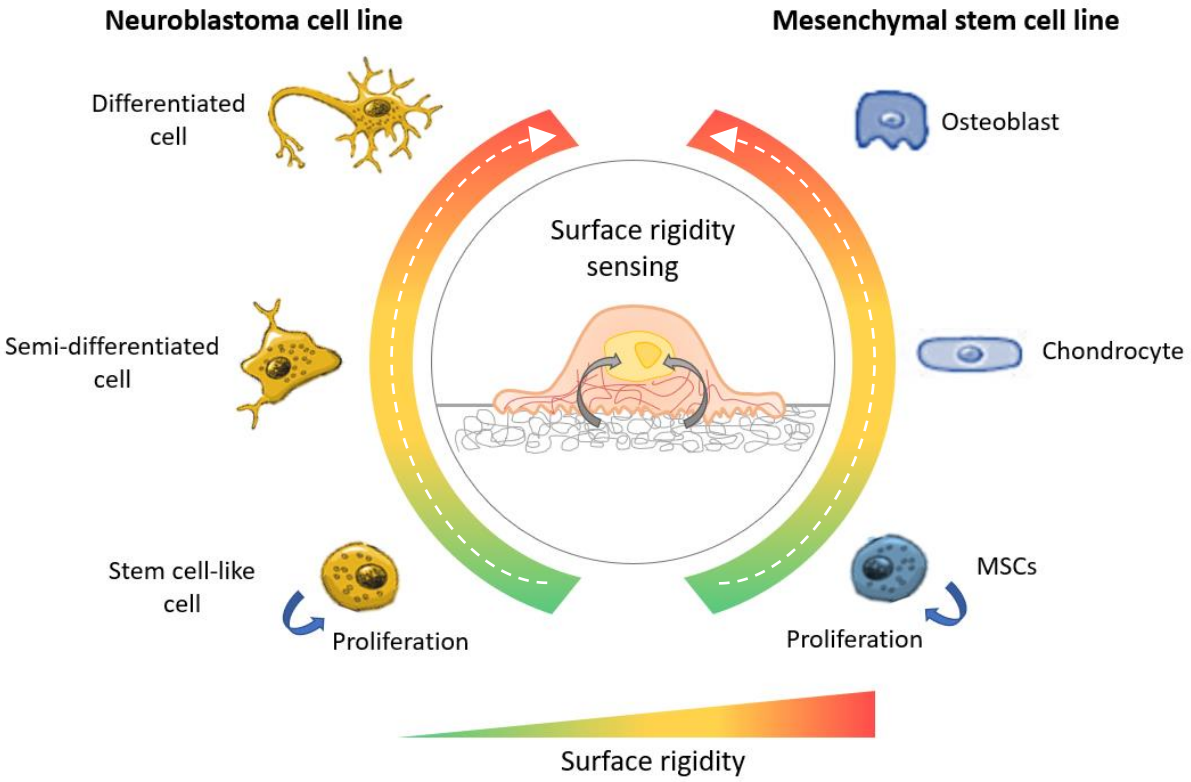
In order to test the first hypothesis, I would use the two following experiments – the first to track behaviour over time, the second to check the effect of a change in the stiffness the cells are cultured on.

a. Culturing of SK-N-DZ cells on 3 different stiffnesses (0.1, 50, and 300 kPa), one in each range set in experiment number 1. Measurement of neurite length and Ki67 immunostaining in timepoints 0, 1, 2, 3 and 4 weeks.
b. Culturing of SK-N-DZ cells on 3 different stiffnesses (0.1, 30, and 300 kPa). After 3 days, the culturing period of the original article, cells from each stiffness will be harvested and split in 3 cultures of 0.1, 50, and 300 kPa stiffnesses, being cultured for another 3 days. Measurement of neurite length and Ki67 immunostaining of all 9 cultures.

To test the second hypothesis, I would repeat the experiments 2 and 3 performed in the original article for the results obtained to be comparable to SK-N-DZ. Experiments 1 and 4 are neuroblastoma specific (neurite outgrowth and N-myc expression measurement), so they need to be adapted. I would test a multipotent stem cell line, more specifically a mesenchymal stem cell (MSC) line, which would for sure be of interest when testing the effect of surface stiffness. Mesenchymal stem cells can differentiate into osteoblasts, chondrocytes, and adipocytes, three cell types which inhabit very different environments with

three very different stiffness which could account for their differentiation processes. In order to assess their grade of differentiation, as done in experiments 1 and 4 in the original paper, I would qualitatively study their morphology and behaviour to try to discern if there's differentiation to any MSC-derived cell type. If these qualitative parameters were to show differentiation processes, I would attempt to use immunofluorescence against markers of the cell type that the MSCs have differentiated to.

14. Design a graphical abstract for the hypothetical article that your proposed hypothesis and experiments would yield.



Practical case 2

Patterning microscale extracellular matrices to study endothelial and cancer cell interactions in vitro

Dickinson, L. E., Lütgebaucks, C., Lewis, D. M., & Gerecht, S. (2012).
Lab on a Chip, 12(21), 4244. https://doi.org/10.1039/c2lc40819h

1. What is the biological and/or clinical question that the article seeks to answer?

The article, being a technical paper, answers a technical question rather than a biological one:

Can we produce a spatially controlled cell co-culture system with which to study interactions between cancer cells (BCCs) and endothelial cells (ECFCs), and among these cells and the composition of the extracellular matrix (ECM)?

2. What is the working hypothesis?

Again, taking the nature of the paper into account, the question might need a small twist. In this article they test a technical design (two, actually) rather than a hypothesis.

1. The design consisting of sequentially patterned fibronectin (Fn) and hyaluronic acid (HA) will allow for the discrete and spatially controlled co-culture of ECFCs and BCCs, each growing respectively in one of the patterned molecules.
2. The design consisting of a combination of 2D patterning of Fn for ECFCs and encapsulation of BCCs in micromolded HA hydrogels will allow for the discrete and spatially controlled co-culture of ECFCs and BCCs.

However, they do present a minor biological hypothesis which, while not the basis of the study, is the base of the first design: they hope that BCCs will specifically bind to HA, while ECFCs will specifically bind to Fn. This proves to be wrong as BCCs do not bind only HA but are actually capable of binding Fn and even PEGylated surfaces.

3. What does this article show? Is it of utility in the short or long term? What kind of work is it (Basic science, molecular biology mechanism, method, or device development)?

This work demonstrates the capacity of the second co-culturing method for the study of cell-cell and cell-ECM interactions in a spatially and biochemically controlled manner. It presents a powerful tool for the study of how tumour cells interact with endothelial cells, taking also into account the composition of the ECM.

The article is, as previously mentioned, a methodological or technical paper.

4. What are the critical experiments and principal methods utilized to answer the biological/clinical questions and confirm (or not) the working hypothesis?

1. Surface co-patterning of fibronectin and hyaluronic acid using microcontact printing and silane chemistry (OTS and APTMS respectively). Measurements: immunofluorescence measuring of Fn and HA intensity and spatial disposition on the surface after the process.

2. Culturing of ECFCs on Fn-HA patterned substrates. Control: culture in Fn-Collagen I patterned substrates (no preference expected). Measurements: bright field and fluorescent microscopy observation of cell location.
3. Culturing of three different BCC lines; nontumorigenic MCF10A; tumorigenic, non-metastatic MCF7; and metastatic MDA-MB-231; on the Fn-HA patterned surfaces. Measurements: bright field and fluorescent microscopy observation of cell location.
4. Co-culture of ECFCs and the three different BCC lines on Fn-HA patterned surfaces. Measurements: bright field and fluorescent microscopy observation of cell location.
5. Encapsulation of the three BCC lines in HA hydrogels. Measurements: cell survival rate at days 0, 1, and 3.
6. Encapsulation of BCC cell line MDA-MB-231 in micromolded HA hydrogels. Control: culture in standard conditions. Measurements: Ki67 immunostaining.
7. Co-culture of encapsulated MDA-MB-231 cells in HA micromolded hydrogels in contact with ECFCs cultured in 2D-patterned Fn. Measurements: bright field and fluorescent microscopy observation of cell location.

5. Identify the controls used in the experiments and explain their utility or function for the analysis of the results.

Experiments 2 and 6 present controls. In experiment 2, a control is used where ECFCs are cultured in Fn-Collagen I patterned substrates. It was previously known that ECFCs show no preference between Fn and collagen I molecules in terms of adhesion, and that they would grow in both when co-patterned. They used this knowledge to compare how ECFCs behave once they are placed in Fn-HA patterned surfaces, where a differential adhesion is expected.

In experiment 6, standard culture conditions are used as a control in the assessment of BCC proliferation using the measurement of Ki67 immunofluorescence. This control is compared with BCCs encapsulated in HA matrixes to assess whether the cells' proliferative capacity was diminished when embedded in HA.

6. What are the conclusions?

Authors conclude that BCCs do not specifically attach and adhere to HA, rendering the first co-culture technical design failed. They also conclude that when using the combined HA micromolding and Fn patterning co-culture method, BCCs and ECFCs are contained in their spaces, allowing for a stereoscopically controlled study of their interactions.

7. Do you believe that the experiments and the interpretation of the results are adequate to answer the biological/clinical questions and confirm (or not) the working hypothesis?

There's an ample set of experiments supporting their claims. It must be noted how the authors even published negative results (i.e. their first design not working), showing the evidence for it. The expermients seem adequate to answer their questions, as they're fairly simple and can be answered with the sole location and attachment preference of the cultured cells.

8. Do you believe that the results are well interpreted and that the conclusions are well-founded?

Yes, I believe the claims are fair and the conclusions well based. If an objection can be proposed, it would be against the time period in which the cells remain where originally designed, as in their second method ECFCs are only contained in the Fn pattern for 24 hours, time after which they replace the Fn with their own ECM as BCCs will eventually do too. This limits experiments to 24 hours unless ECFC migration can be overlooked for the purpose of one's experiment, reducing the utility of the co-culture method.

9. Is the working hypothesis confirmed to a 100% with the obtained results?

Following the answer to question number 2, I would say that the second co-culture method has been properly validated. The first design would also have been properly invalidated.

10. Write a critical analysis of the article stating its strong and weak points.

The authors show two methods with great potential to study cell-cell interactions in a spatially controlled manner. As a note, even though the first design may have not worked with BCCs and HA, other ECM components could work for other cell types. The second study is doubtlessly a powerful tool to study how cancer cells can interact and affect the cells in their environment. Even if the cells and polymers used in their example are limited to 24 hour studies, this could be enough time to see interesting results, and, as in the first method, other cells and polymers could possibly be used with much longer and more adequate culture times.

11. What experiments do you believe should have been performed to improve the article and make the conclusions more robust?

I believe that the experiments performed and reported are well suited to back their claims and conclusions, and in that sense, I would not perform any experiments. I would, however, add experiments aimed at expanding the knowledge and utility resulting from the work.

In experiment 7, ECFCs and BCCs need different time periods to break through their designated location on the combined method co-culture (24 and 48 hours respectively). After seeing the results of experiments 3 and 4, where BCCs migration and survival are clearly not restricted by the molecule which they bind, it would be logic to question whether the encapsulation process itself is the one responsible for holding these cells for longer (48h) than the Fn patterned ECFCs.

I would propose a third technical design based on the hypothesis that containment time in the 7th experiment stems from the technique used to pattern the cells rather than from the molecule used to pattern them.

Being ECFCs (cultured on a 2D Fn-patterned surface) the cells with the shorter containment time, this third technical design would be fully based on the alternative micromolding technique. This way I'd be able to compare this design with the second one from the original article, allowing me to see whether this difference stems from the way in which cells have been set "in place" (i.e. by using a 2D pattern vs. embedding them on a hydrogel), or from the molecule which theoretically does this (Fn and HA).

I would thus develop a method to distribute the micromolded HA and Fn patterns by designing a mold allowing for the sequential deployment of the micromolded hydrogels in the desired distribution. In case this wasn't possible, an approach similar to that in the original second technical design could be used, where two surfaces are laden with the micromolded HA/Fn and the embedded BCCs and ECFCs respectively, to later be put in contact by inverting one in top of the other.

I would finally repeat the observations in experiment number 7 (cell location and ECM degradation over 48 hours at least, going up to 72h) in this third setting.

12. Suggest a different working hypothesis based in the same biological/clinical question posed in this article.

The authors mention in the conclusions how this co-culture method could potentially be used with most cell types. This would doubtlessly be an interesting model for studying neuroblastoma (NB) vasculogenesis, given that this kind of tumour is frequently highly vascularized. This model could possibly enable us to

study the way in which cells in the outer layers of a tumour, where most vessels are formed by "host" cells and not tumour endothelial cells (TECs) interact with.

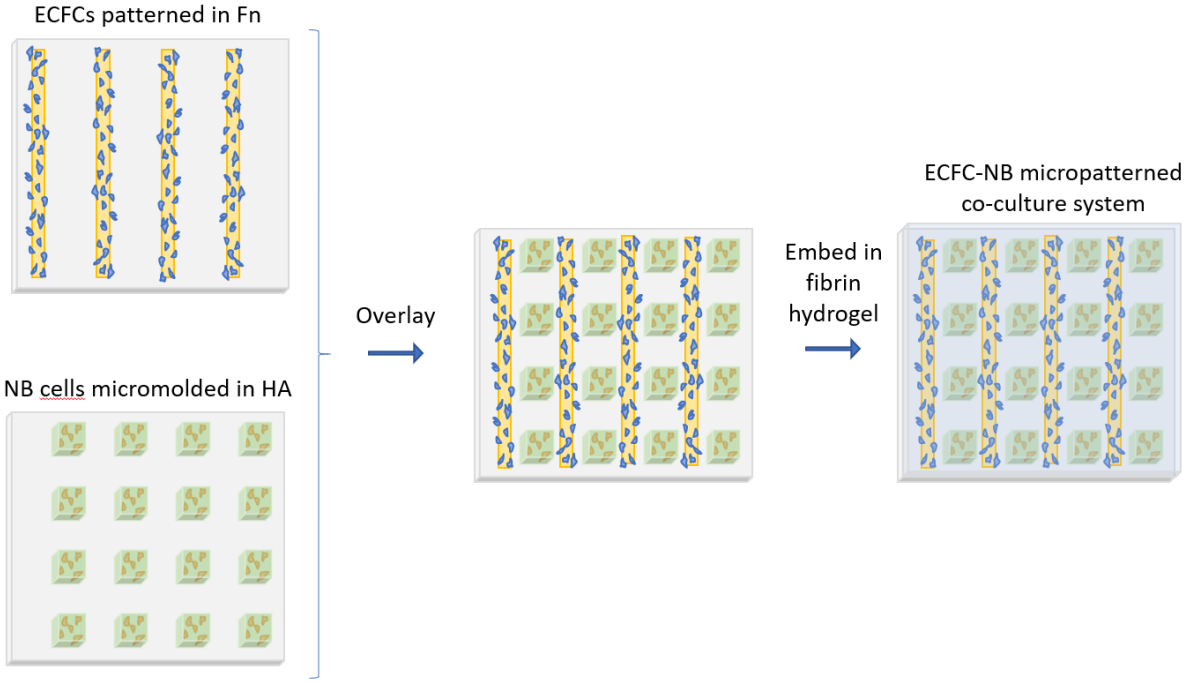
I would thus present the hypothesis that the second co-culture method developed in the article can be used too with neuroblastoma cell lines, becoming a new preclinical model for the study of vasculogenesis in NB.

13. What would be the critical experiments that you would need to perform to confirm (or deny) the hypothesis that you have proposed in question 12? What controls would be needed?

In order to assess whether the second co-culture method designed by the researchers could be adapted to neuroblastoma studies, I would first perform experiment 5 using a three distinct NB cell lines showing the characteristics that the BCCs did in the original article (a non-malignant line; a malignant, non-metastatic line; and a metastatic line). Once their viability and proliferation rate are well described, I would choose the most suitable line (that with higher viability and proliferation rates) and perform experiments 6 and 7. With the results, I'd expect to see that NB cell lines behave similarly as the BCC cell lines, staying in their micromolded matrixes for long enough to study their interactions with ECFCs. As a control, the cell lines used in the paper could be used in all 3 experiments.

This model could then potentially be used to test pharmaceuticals which block vascularization induction in normal cells, to see if impairing common vasculogenic pathways inhibits the interactions between NB cells and ECFCs, or to test different conditions shown to drive TEC differentiation, such as hypoxia, to see what role normal ECs would play in such an environment.

14. Design a graphical abstract for the hypothetical article that your proposed hypothesis and experiments would yield.



Practical case 3

A Photoactivatable Nanopatterned Substrate for Analyzing Collective Cell Migration with Precisely Tuned Cell-Extracellular Matrix Ligand Interactions

Shimizu, Y., Boehm, H., Yamaguchi, K., Spatz, J. P., & Nakanishi, J. (2014). PLoS ONE, 9(3), e91875. https://doi.org/10.1371/journal.pone.0091875

1. What is the biological and/or clinical question that the article seeks to answer??

The question which the authors aim to answer would be as follows: Does extracellular matrix (ECM) geometry affect the individual/collective behaviour of cell migration?

2. What is the working hypothesis?

The researchers propose the following hypothesis: The use of substrates with spatially and geometrically well-defined cell-adhesive ligands affects the collectiveness of HeLa cell migration.

3. What does this article show? Is it of utility in the short or long term? What kind of work is it (Basic science, molecular biology mechanism, method, or device development)?

This work describes a novel culture substrate for studying cell migration in a spatially, geometrically, and biochemically controlled setting, at the same time that it describes how this substrate affects FAK phosphorylation and function, ultimately altering migration profiles of HeLa cells. It is an article which combines the description of a new technical development with a basic science discovery.

4. What are the critical experiments and principal methods utilized to answer the biological/clinical questions and confirm (or not) the working hypothesis?

1. Nano-patterned photoactivatable substrate preparation and validation using gold-thiol and silane chemistries and testing before and after UV photoactivation. Measurements: scanning electron microscopy imaging of UV-irradiated and non-irradiated surfaces.
2. Seeding of HeLa cells in nano-patterned photoactivatable substrates (NPS) irradiated with UV in varying intensities (between 0 and 10 J/cm^2). Measurements: Optical microscopy for the assessment of adhered cell numbers.
3. SEM imaging of HeLa cells on the nano-patterned photoactivated substrate.
4. Seeding of HeLa cells in photopatterned stripes in NPS. Control: Seeding of HeLa cells in photopatterned NPS with only PEG12K functionalization (no cRGD, no adherence expected). Measurements: Assessment of cell location with optical microscopy.
5. Seeding of HeLa cells in photopatterned circles (150 μm diameter) in NPS, with a second irradiation after 9h for the induction of cell migration. Control: Seeding of HeLa cells in photoactivatable homogeneous gold surfaces. Measurements: time-lapse microscopy imaging over a 9-hour period.
6. Seeding of HeLa cells in photopatterned circles (150 μm diameter) in NPS, with a second irradiation after 9h for the induction of cell migration. 3 hours after induction, cells were fixed for immunostaining. Control: same experiment in homogeneous surfaces. Measurements: fluorescence microscopy of fixed cells immunostained for N-cadherin, F-actin, and vinculin.

7. *In silico* analysis of cell migration behaviour based on the time-lapse movies taken in experiment number 5. Measurements: cell morphology over time, cell migration tracking, individual cell migration rates, directional persistence of individual cell migration.
8. Seeding of HeLa cells following the same proceeding as in experiment number 6. Measurements: fluorescence microscopy of fixed cells immunostained for FAK, pY379 FAK and pY861 FAK.

5. Identify the controls used in the experiments and explain their utility or function for the analysis of the results.

Several controls are used in most of the experiments in order to compare how the cells behave in the NPS compared to other surfaces. Experiments 4, 5, 6, and 8 have controls.

Experiment 4

In the control, cells are seeded in a substrate which has been photopatterned just like the normal one, but in this case, the substrate does not have cRGD, so after photoactivation (and thus cleaving of the only functionalizing molecule PEG12K) the nude gold should be exposed, rendering the whole substrate, photoactivated or not, non-adhesive.

The control thus allows the researchers to see if and how the cells adhere in the normal NPS, in comparison to a similar substrate which has undergone the same photoactivation process but is not adhesive.

Experiments 5, 6 and 8

Cells are seeded both in the normal NPS and in a control substrate which is homogeneously laden with the gold nanoparticles and thus also homogeneously functionalized with PEG12K and cRGD. This control is used in these three experiments studying cell migration after the release of cells from their confinement in the 150 μm circles through photoactivation.

These controls are essential to see how a nanoscopically defined adhesion ligand geometry influences cell migration versus a normal, homogeneous surface.

6. What are the conclusions?

The authors conclude that their hypothesis is valid, and that the use of their NPS alone is enough to alter the manner in which HeLa cells migrate: they do so in an independent, individual manner when in the nano-patterned surface, but migrate collectively in the homogeneous gold surface. They also conclude that the driver of this different migration profile is the defect in Y861 phosphorylation of FAK found in cells seeded in the nano-patterned surface. Taking these conclusions into account, the authors also state that the traditional consensus surrounding the thought that cells stablish more cell-cell interactions the less cell-ECM interactions they can set up, is not valid for the assayed specific geometry and chosen cell line.

7. Do you believe that the experiments and the interpretation of the results are adequate to answer the biological/clinical questions and confirm (or not) the working hypothesis?

Although the experiments are well chosen and conducted, I believe that they lack another control. The authors state that the controlled geometry their substrate provides is a key factor in the differential migration profile that the cells demonstrate in their experiments. However, they only compare the migration behaviour of the cells in the NPS to a homogeneously functionalized surface, failing to show how their system does against a non-geometrically controlled, stochastically nano-patterned surface. I believe their claims need to be proven too against such surfaces in order to state that the geometry is a key factor driving the difference, as other factors are also present in their experiments (cRGD abundance, lack of any PEG12K in the homogeneous photopatterned surfaces, etc).

8. Do you believe that the results are well interpreted and that the conclusions are well-founded?

I believe that their conclusions, as I have interpreted them in question number 6, are well based, and that that the data presented backs these claims. The critique in the answer to question 7 refers more to disperse conclusions or claims found in the introduction and discussion sections, which are not found in the main conclusions.

9. Is the working hypothesis confirmed to a 100% with the obtained results?

Yes, it is confirmed if we consider my interpretation of the hypothesis of the article to be correct (no clear hypothesis is stated in it): HeLa cells have been shown to migrate differently in their substrate, at least if compared to a homogeneous one.

10. Write a critical analysis of the article stating its strong and weak points.

From the technical point of view, the methodology to produce the substrate and how successfully it's able to harbour cell adhesion and proliferation while at the same time being photoactivatable and geometrically controlled is impressive. It is doubtlessly a very interesting platform for studying cell adhesion and interaction with the ECM, as well as cell migration, in which we can control the adhesion ligands, the size of the adhesion "spots", and the distance among those in order to see how cells behave. This can be done too at the same time as molecular factors are also added in the system in order to further alter cell migration. The authors even provide the first cornerstone of the signalling pathway which might be transducing the information obtained from the substrate to the cytoplasm: FAK and its pY861 form.

The weaknesses the work presents are in the lack of study on how different geometries affect migration. Being one of the main points of interest of the article, and reportedly, one of the benefits of the methodology developed by the researchers, it would be logic to expect them to show how different distances between the nanoparticles affect cell mobility, and also, how different geometries (i.e. a less organized, more stochastic organization of the nanoparticles) make the cells behave.

11. What experiments do you believe should have been performed to improve the article and make the conclusions more robust?

As previously mentioned, I would add a second control using a less precise technique for the deposition of the nanoparticles, based in dilution alone, in order to compare how the substrate performs in comparison to other substrates with uncontrolled geometries. I would implement these controls in experiment 5, 6 and 8, also performing the *in silico* studies of experiment 7 on the results of the time-lapse of this novel control condition.

12. Suggest a different working hypothesis based in the same biological/clinical question posed in this article.

First hypothesis

Megison et al. (2013) have described how inhibiting FAK using siRNAs or small molecule inhibitors reduces neuroblastoma (NB) migration. Although they did study Y397 phosphorylation, they did not address Y861 phosphorylation, which would have been of interest in relation to the discussed article.

This, together with the nano-patterned photoactivatable surfaces described in the article sets a very interesting base for research. I would propose a hypothesis stating as follows:

The collectiveness of neuroblastoma cell migration is altered when adhered to the nano-patterned photoactivated substrate, being the individual migration pattern favoured in respect to the homogeneous gold surface, but it is restricted to collective migration in presence of a FAK small molecule inhibitor.

Second hypothesis

As a second consideration, the platform would be also very interesting if it could be adapted into PDMS surfaces of stiffnesses around 0.1 to 2 kPa. It would be of great interest to study how ECM geometry would affect neuroblastoma differentiation. Neuroblastoma cells exhibit a certain lability, being able to spontaneously differentiate into cells exhibiting hallmarks of neuronal lineage (i.e. neurite outgrowth), while also losing their proliferative capacity. It has already been shown how surface stiffness alone can induce this spontaneous differentiation (Lam et al., 2010), and cell ligand geometry could also induce it or in turn, stall the cells in their stem cell-like stages.

Supposing that this adaptation would be a possibility from the technical point of view, and taking into consideration how the nano-patterned surface highly promotes extension of cell protrusions, I would propose a second hypothesis:

Neuroblastoma spontaneous differentiation is favoured when cells are seeded in nano-patterned photoactivated PDMS substrates of stiffnesses of 0.1 to 2 kPa.

13. What would be the critical experiments that you would need to perform to confirm (or deny) the hypothesis that you have proposed in question 12? What controls would be needed?

First hypothesis

In the first place, it would be necessary to test the survivability and proliferative capacity of a neuroblastoma cell line in the NPS. Once this point is set out by assessing factors such as cell adhesion, Ki67 expression, cell growth and division, etc., the hypothesis could start to be tested. In order to do so, I would repeat experiments 5 to 8 but adding two new conditions to all of them: The NPS with the small molecule inhibitor PF-573,228, used by Megison et al. in their article, and the homogeneous gold substrate, also with PF-573,228. I would use as controls the versions without the molecule. I would expect similar results as in the discussed article, where the cells' migration pattern effectively switches to individual when seeded in the NPS vs. the homogeneous gold surface but is reverted to collective in presence of PF-573,228.

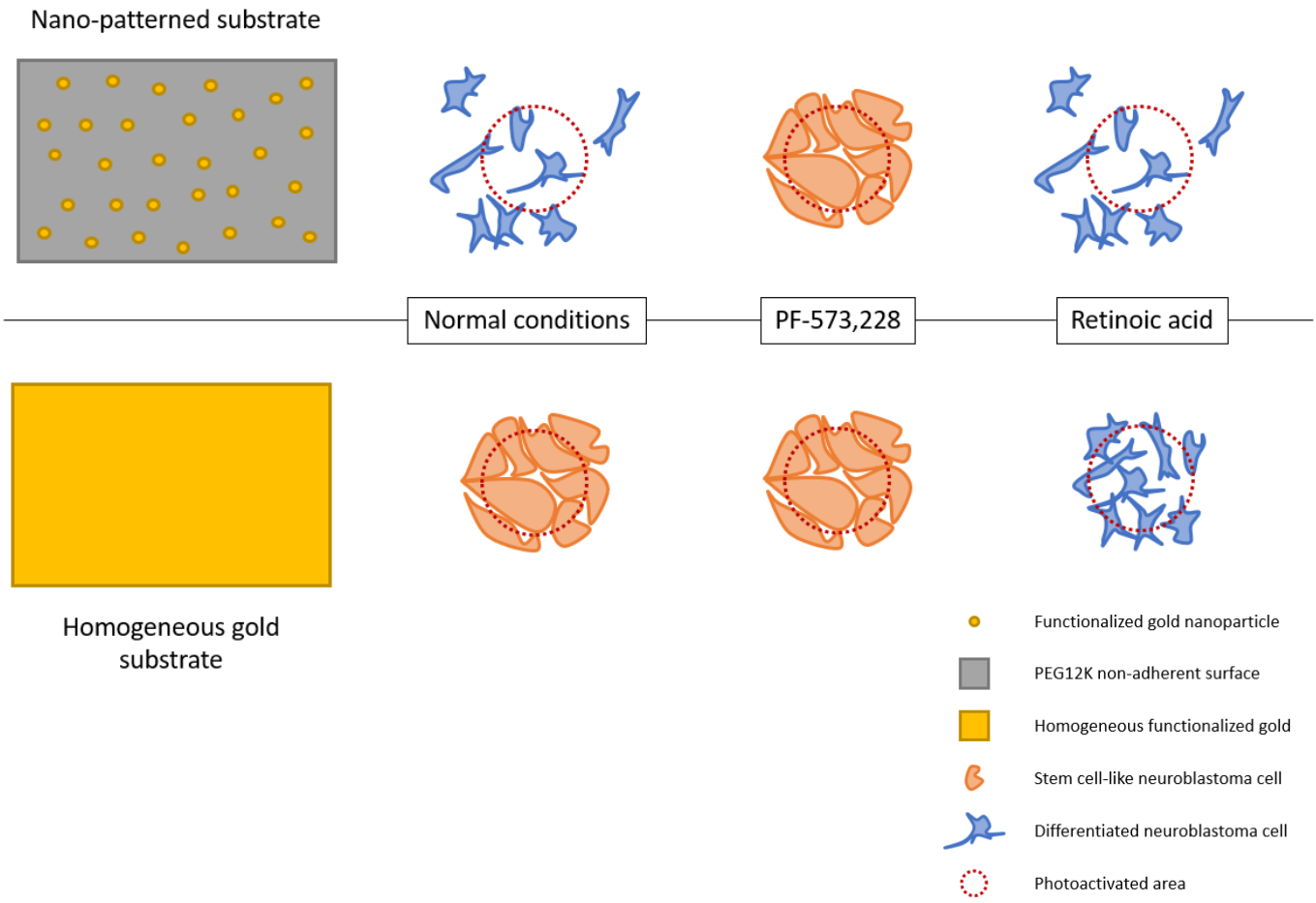
Second hypothesis

It is important to recall that this hypothesis is based on the premise that it is possible to manufacture a low-stiffness PDMS substrate that is nano-patterned and photoactivatable, as well as a low-stiffness PDMS substrate with photoactivatable homogeneous gold surface in the same way that the substrates from the discussed article are possible to produce, and that neuroblastoma survivability in the NPS has been already tested for the first hypothesis. In order to test this second hypothesis, I would conduct the following experiments:

1. Seeding of NB cells in photoactivated PDMS nano-patterned and homogeneous substrates. Controls: fibrinogen-coated PDMS substrates of 1 kPa and 300 kPa. Measurements: Observation and measurement of neurite extension using optical microscopy and image analysis software.
2. Same experimental design and measurements as experiment number 1 but in presence of retinoic acid.

Based on the results obtained from these experiments, I would decide whether to keep studying the molecular basis behind them. If they were promising, I would repeat the immunostainings (N-cadherin, F-actin, and vinculin; and FAK, pY397 FAK, and pY861 FAK) performed in the discussed article, in order to see if FAK signalling also has a role in NB spontaneous differentiation.

14. Design a graphical abstract for the hypothetical article that your proposed hypothesis and experiments would yield.



Practical case 4

Tumor cell–organized fibronectin maintenance of a dormant breast cancer population

Barney, L. E., Hall, C. L., Schwartz, A. D., Parks, A. N., … Peyton, S. R. (2020). Science Advances, 6(11), eaaz4157. https://doi.org/10.1126/sciadv.aaz4157

1. What is the biological and/or clinical question that the article seeks to answer?

What is the role of the ECM in the regulation of cell dormancy, survival during the long periods of dormancy and regrowth and proliferation after these periods?

2. What is the working hypothesis?

Cancer cells are capable of secreting and assembling ECM proteins which in turn promote cell survival over dormancy periods but need to be degraded in order for the cells to regain their ability to proliferate when the dormancy-inducing conditions have passed.

3. What does this article show? Is it of utility in the short or long term? What kind of work is it (Basic science, molecular biology mechanism, method, or device development)?

The discussed article is a basic science work, which demonstrates the key importance of ECM composition and structure in cell dormancy, as well as their tight regulation by cancerous cells. Their *in vitro* model of dormancy is uniquely interesting as it allows for the study of heterogeneous dormant cell populations over time, in contrast to end-point *in vivo* studies. They also show how fibronectin is a prominent ECM protein regarding dormancy, and how TGFβ, ROCK, ERK and MEK signaling are tightly involved in the regulation of ECM deposition and assembly, ultimately affecting cell dormancy too.

This information is of great utility, as it purveys new, very important information on one of the main challenges in oncology: targeting cancer cell dormancy and metastasis.

4. What are the critical experiments and principal methods utilized to answer the biological/clinical questions and confirm (or not) the working hypothesis?

1. Culturing of 23 breast cancer cell lines during 8 weeks of serum starvation in tissue culture plates, collagen I-laden glass coverslips, and bone marrow-recapitulating coverslips, followed by 7 days of recovery in serum-containing culture media. Measurements: Optical microscopy for the assessment of confluence and cell proliferation.
2. Immunofluorescence staining of HCC1954 cells cultured in serum starvation during 2 and 28 days with antibodies for fibronectin, pan-laminin, collagen I, vitronectin, and osteopontin. Additional condition of 7 days recovery after 28 days starvation with fibronectin staining. Controls: immunofluorescence staining of fibronectin in a culture constantly exposed to serum-containing culture media. Measurements: fluorescence microscopy to study immunofluorescence.
3. Immunoassay for determining MMP activity in HCC1954 cells cultured in serum-containing media, in serum starvation for 28 days, and in after a 7-day serum recovery after 28 days of serum starvation. Measurements: fluorescence microscopy.
4. Culturing of HCC1954 cells in a decellularized fibronectin-rich matrix produced by dormant HCC1954 cells during 28 days of serum starvation. Controls: culturing of HCC1954 cells in

fibronectin coverslips and collagen I coverslips. Measurements: Fluorescence microscopy for the assessment of nucleuses count per imaging field (DAPI staining).

5. Culturing of HCC1954 cells in presence of TGFβ1, TGFβ2, and LY-364749, a pan-TGFβ inhibitor, during 7 and 28 days of serum starvation. Controls: no TGFβ or inhibitor addition. Measurements: Fluorescence microscopy for the assessment of nucleuses count per imaging field (DAPI staining), and visualization of immunostaining of fibronectin.
6. Culturing of HCC1954 cells in presence of anti-α5 integrin antibodies, or ROCK inhibitor Y-27632 in various concentrations (10 μM, 1 μM, 0.1 μM) and different matrixes (collagen I and decellularized matrixes) according to the scheme in figure S1. Controls: grey bars in scheme (no inhibitor). Measurements: fluorescence microscopy for the assessment of nucleuses count per imaging field (DAPI staining) and visualization of fibronectin immunostaining.
7. Culturing of HCC1954 cells in presence of anti-β1 integrin antibodies, FAK inhibitor 14, PD0325901 (MEK inhibitor), FR180204 (ERK inhibitor), and cilengitide in different matrixes (collagen I and decellularized matrixes), according to the scheme in figure S1.

Figure S1. Scheme for the time schedule of experiments number 6 and 7. Extracted from the discussed paper.

5. Identify the controls used in the experiments and explain their utility or function for the analysis of the results.

Experiment 2 and 3

Controls are used where cells do not undergo serum starvation, and thus are not induced to enter dormancy.

Experiment 4

In order to assess how the culturing of cells in the decellularized matrix affects cell dormancy and survival, they use two controls where the cells are cultured in fibronectin or collagen I-lined coverslips.

Experiment 5, 6, and 7

In these experiments, different molecules, antibodies, and inhibitors are presented to the cells growing or in dormancy-inducing conditions. All 3 of these experiments run parallel experiences where the studied molecule is not presented to the cells, which are used as controls.

6. What are the conclusions?

The authors conclude that the dynamics of fibronectin assembly and degradation regulate breast cancer cell dormancy in a TGFβ-dependent manner. They consider their hypothesis to be correct, while also giving important insights in the mechanisms underlying this fibronectin-dependent cell dormancy; they show how α5β1, ROCK, and TGFβ are necessary for fibronectin matrix assembly; how reactivation after dormancy strongly depends on MMP degradation of the fibronectin matrix; and how long-term survival depends on FAK-ERK signalling. They finish their conclusions stating the importance of these findings in the treatment of metastases: they show how targeting dormant metastasis through cell-ECM adhesion and its dependent signalling mechanisms could possibly be used as a therapeutic strategy, supporting the studies using cilengitide as an anti-metastatic drug with incredibly valuable information.

7. Do you believe that the experiments and the interpretation of the results are adequate to answer the biological/clinical questions and confirm (or not) the working hypothesis?

Yes. The researchers have performed a myriad of experiments of which I have only gathered the ones I deem to be the most important for question number 4. These range from proteomic analysis using LC-MS to more common immunostainings. They screen for an incredibly wide range of cell lines and ECM components, and for an abundant amount of small molecule inhibitors and antibodies to test the mechanisms underlying cell dormancy and survival.

8. Do you believe that the results are well interpreted and that the conclusions are well-founded?

Yes, I believe their conclusions are well funded, and their data well interpreted.

9. Is the working hypothesis confirmed to a 100% with the obtained results?

Yes. Not only the hypothesis is perfectly confirmed – the authors provide much more information than fits in that hypothesis, by unravelling the mechanisms that cause it to be true.

10. Write a critical analysis of the article stating its strong and weak points.

The discussed article is an impressive research effort which makes use of a simple, yet very utile and efficient strategy: the induction of dormancy through mere serum starvation. In comparison to other articles discussed in former cases of study, this article makes use of a very simple technique and exploits it efficaciously, obtaining high amounts of valuable data which the authors have turned into interesting statements and claims. The experiments are in fact run in a "high-throughput" manner, testing up to 23 cell lines in three different conditions for a single experiment, but going further with other time and resource-consuming experiments which can even be found in the supplementary material, such as the experiment studying 13 different ECM-component compositions for their coverslips in 3 cell lines, testing even different concentrations for some of the compositions. With proper execution and with an appealing use of data visualization to show and explain their results, the authors have skilfully produced a scientific publication of great quality.

11. What experiments do you believe should have been performed to improve the article and make the conclusions more robust?

As said, the authors have successfully answered their initially proposed questions, having their research ideas yielded much information as well as some new questions. One of them is specifically interesting; in experiment number 4, they see how cells survive dormancy on a decellularized fibronectin matrix produced by HCC1954 cells much more efficiently than on a coverslip functionalized with collagen I; while they have a lower survival rate when seeded in a coverslip functionalized with fibronectin. They then hypothesize that this happens due to a differential fibronectin assembly and architecture, resting at ease with this idea as it would be out of the scope of the article. However, they completely overlook the possibility that the fibronectin used in the coverslip functionalization may not be exactly the same as the one produced by the cancerous cells. I would thus perform an LC-MS on the decellularized ECM produced by the HCC1954 cells and on the functionalized fibronectin matrix using the same procedures, in order to see how both compare.

12. Suggest a different working hypothesis based in the same biological/clinical question posed in this article.

The idea that a molecular difference between the fibronectin used in the coverslips and the one produced by the HCC1954 cells could cause the difference in the survival rate between the two matrixes, rather than a differential architecture, caused me to perform an extensive bibliographical research on human fibronectin. I will summarize the hallmarks of this search which I deem necessary show the base for my hypothesis.

First and foremost, it is key to point out how there exist several splice variants of fibronectin whose abundancy varies through development and, in the adult organism, among tissues. there are up to 20 variants of fibronectin in humans, and regarding adults two main types exist: soluble isoforms found in plasma (plasma fibronectin, or pFn) and insoluble isoforms which form fibrils, deposited as part of the ECM (cellular fibronectin, or cFn) (Ruoslahti, 1988; White and Muro, 2011). Fibronectin presents three regions of alternative splicing, known as extra domain A (EDA), extra domain B (EDB), and type III constant segment (IIICS). Plasma Fn lacks EDA and EDB, which in contrast are present in different proportions in cFn.

The fibronectin used to functionalize the coverslips, obtained from Millipore, happens to be extracted from human plasma and therefore is composed of pFn isoforms, thus lacking EDA and EDB domains. Tumour cells, in exchange, produce EDA- and EDB-containing fibronectin (cFn; Infusa et al., 1995), which strongly supported my suspect that the fibronectin produced by HCC1954 cells could be different from the one used by the researchers, giving foot to a hypothesis explaining the difference between both approaches which seems more plausible and concrete than a differential assembly and architecture.

Furthermore, the authors showed in the discussed paper how exogenous TGFβ1 highly promoted cell dormancy and improved survival rates at 28 days of serum deprivation. White and Muro (2011) described how, TGFβ induces EDA-Fn expression, and as a very relevant note, also show how while total TGFβ production is not altered in EDA Fn-deficient mice in respect to WT mice, TGFβ activation appears to be significantly impaired. This implies a positive feedback loop where TGFβ induces EDA-containing fibronectin expression, and EDA Fn then allows for the activation of latent TGFβ, which was later confirmed by Ding et al. (2012). This feedback loop can in fact be seen in the results of the discussed paper (fig. S2). The addition of TGFβ1 not only increases cell survival at 28 days, but it also causes an increased deposition of fibronectin. The addition of a TGFβ receptor inhibitor severely reduces both cell survival and fibronectin deposition at day 28.

All these data led me to produce the following hypothesis:

Fibronectin-induced cancer cell dormancy requires the presence of the EDA-containing fibronectin splicing variant to induce TGFβ1 activation and consequent signalling, which causes a signalling cascade inside the cancer cell leading to increased EDA Fn secretion and increased cell survival rates during long periods of dormancy.

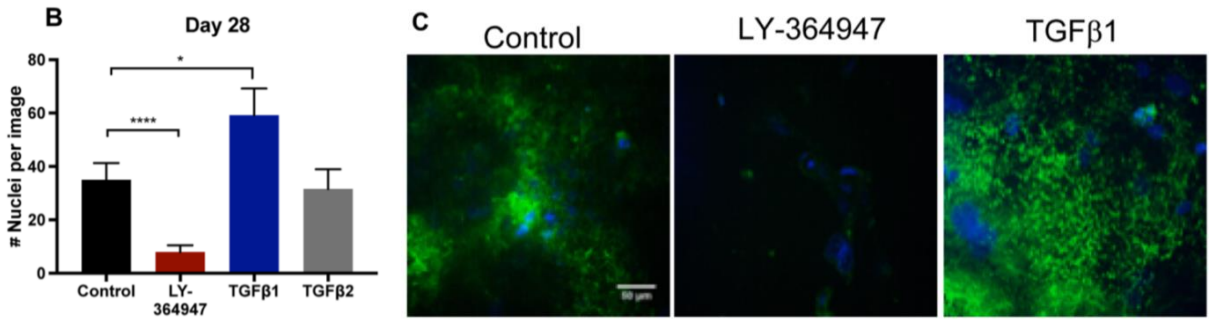


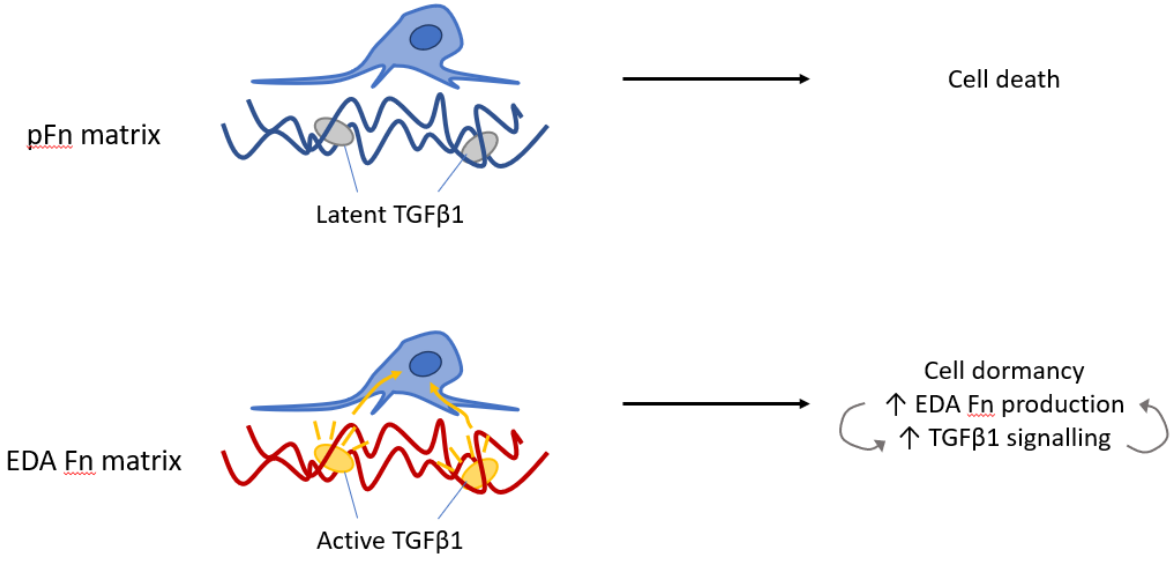
Figure S2. Extracted from the discussed article. Following its nomenclature and numeration: (B) Number of viable HCC1954 cells after 28 days of serum deprivation-induced cell dormancy in presence of TGFβ inhibitor LY-364947, TGFβ1, TGFβ2 and control conditions. (C) Fibronectin immunostaining (green, DAPI in blue) after 28 days of dormancy, in presence of LY-364947, TGFβ1, and control conditions.

13. What would be the critical experiments that you would need to perform to confirm (or deny) the hypothesis that you have proposed in question 12? What controls would be needed?

To test my hypothesis, I would perform the following experiments:

1. LC-MS on the decellularized matrix produced by HCC1954 cells after 28 days of serum starvation-induced dormancy. I would expect to find the EDA-containing splicing variant of fibronectin in the ECM.
2. RT q-PCR on samples of HCC1954 cells at days 0, 7, and 28 of serum starvation, screening for the expression of different Fn splicing variants using exon-exclusive primers. With this I would expect to determine the splice variants expressed by cancer cells during dormancy
3. In order to study localization of the different variants, I would purchase mAbs targeting the most interesting variants (EDA-containing, EDB-containing, pFn), and would do immunostainings of the cells growing in their matrixes.
4. If EDA Fn can be purchased from a producer, I would functionalize coverslips with it and see if positively affects entrance into cell dormancy and survival rates as the HCC1954-produced ECM.

14. Design a graphical abstract for the hypothetical article that your proposed hypothesis and experiments would yield.



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Ding, Y., Gelfenbeyn, K., Freire-de-Lima, L., Handa, K., & Hakomori, S. I. (2012). Induction of epithelial-mesenchymal transition with O-glycosylated oncofetal fibronectin. FEBS letters, 586(13), 1813-1820.

Inufusa, H., Nakamura, M., Adachi, T., Nakatani, Y., Shindo, K., Yasutomi, M., & Matsuura, H. (1995). Localization of oncofetal and normal fibronectin in colorectal cancer. Correlation with histologic grade, liver metastasis, and prognosis. *Cancer*, *75*(12), 2802-2808.

Practical case 5

Modulation of interactions of neuroblastoma cell lines with extracellular matrix proteins affects their sensitivity to treatment with the anti-GD2 ganglioside antibody 14G2a

Horwacik, I., & Rokita, H. (2017). International Journal of Oncology, 50(5), 1899–1914
https://doi.org/10.3892/ijo.2017.3959

1. What is the biological and/or clinical question that the article seeks to answer?

Is antiGD2 14G2a-induced neuroblastoma cytotoxicity influenced by inhibition of integrin binding to proteins in the extracellular matrix?

2. What is the working hypothesis?

The authors have published a previous work (Kowalczyk et al, 2009) showing how presence of fibronectin or collagen IV augmented ATP levels of cells treated with 14G2a mAb, which led them to hypothesize that integrin binding to ECM proteins and subsequent signaling modulates neuroblastoma cells' sensitivity to 14G2a-induced cytotoxicity.

3. What does this article show? Is it of utility in the short or long term? What kind of work is it (Basic science, molecular biology mechanism, method, or device development)?

This is a basic science article, showing how the use of inhibitors for integrins α4β1, αVβ3, and αVβ5 affects the efficacy of 14G2a mAb cytotoxicity in different neuroblastoma cell lines, as well as the behavior of said cells, including changes in their morphology, migration, and survival. The results published have a limited utility, as the experiments performed are focused in further investigating the cross effects that the studied integrins may have in the cytotoxicity induced by the 14G2a antibody, although some important conclusions can be taken apart from that scope.

4. What are the critical experiments and principal methods utilized to answer the biological/clinical questions and confirm (or not) the working hypothesis?

1. Culturing of IMR-32 and CHP-134 cell lines in presence of absence of 14G2a mAb (20 μg/ml, the same concentration is used in all further experiments) and a GD2 mimic peptide (0.23 mM), and in presence of both combined. Controls: culturing of cells in presence of a peptide with the scrambled version of the GD2 mimic (0.23 mM), in presence and absence of 14G2a mAb. Measurements: optical microscopy for the assessment of cellular morphology and aggregation or spreading; relative ATP levels.
2. Culturing of IMR-32, CHP-134, LA-N-1, LA-N-5, and Kelly cell lines in adherent and non-adherent culture plates, in presence or absence of 14G2a mAb. Measurements: optical microscopy for the assessment of cellular morphology and aggregation or spreading; relative ATP levels; relative caspase-3/-7 activity.
3. Seeding of IMR-32, CHP-134, LA-N-1, LA-N-5, and Kelly cell lines on a cell adhesion array using antibody-coated wells screening for integrin subunits α1-α5, αV, β1-β4, and β6, and integrins αVβ3, αVβ5, and α5β1. Control: plates without antibody coating. Measurements: 560 nm absorbance of

the cells in each well stained with trypan blue, relativized against the negative control in non-coated wells.

4. Culturing of IMR-32, CHP-134, and Kelly cell lines in culture plates in presence of integrin α4β1 inhibitor BIO1211, 14G2a mAb, or both combined. Culturing of IMR-32 in fibronectin-coated plates under the same conditions. Controls: cells cultured with no inhibitor or mAb. Measurements: optical microscopy for the assessment of cellular morphology and aggregation or spreading; relative ATP levels.
5. Culturing of LA-N-5, CHP-134, and Kelly cell lines in vitronectin-coated plates in presence of the αVβ3 and αVβ5 inhibitors cilengitide (CGT) or SB273005 (SB) alone or in combination with 14G2a mAb. Controls: cells cultured with no inhibitor or mAb. Measurements: optical microscopy for the assessment of cellular morphology and aggregation or spreading; relative ATP levels; caspase-3/-7 activity.

5. Identify the controls used in the experiments and explain their utility or function for the analysis of the results.

Experiment 1

A control peptide containing the scrambled sequence of the anti-14G2a peptide is used to assure the peptide's specificity for the mAb.

Experiments 2, 4, and 5

These experiments use common negative controls where no compound (integrin inhibitor) or mAb is used.

Experiment 3

In this adhesion assay, plates with no antibody coating are used as a negative control.

6. What are the conclusions?

They conclude that their experiments verify that the inhibitors tested induce changes over neuroblastoma cell line appearance, attachment, and survival. Their conclusions are rather short and does not reflect their hypothesis, as they limit their claims to the point where they simply state they have broadened the knowledge on factors influencing 14G2a-induced cytotoxicity. They fail to conclude how the different inhibitors synergize with or modulate 14G2a cytotoxicity.

7. Do you believe that the experiments and the interpretation of the results are adequate to answer the biological/clinical questions and confirm (or not) the working hypothesis?

Although most of the experimental designs seem to be well suited to answer the questions set by the researchers, I believe that the measurements made in the experiments could have a deeper insight. The use of ATP levels can be a good manner of estimating the effect a certain molecule or mAb might have over a cell culture, but other quantitative measurements can be done which more robustly and reliably portray similar information (cell viability). Counting the number of cells per area after different periods of time or the use of Ki67 staining besides the measurement of ATP levels would give us a more precise insight on the state of the cells under the different conditions tested.

On the other hand, the authors rely too often in the use of qualitative measurements such as cell morphology of estimations of confluence, when more interesting measurements could be done for both of these parameters to complement the information that the qualitative analysis brings, such as immunofluorescence addressing actin filaments or focal adhesions, or migration assays as simple as scratch-wound healing.

8. Do you believe that the results are well interpreted and that the conclusions are well-founded?

I believe they did not dedicate enough importance to their conclusions in the article. Where they only set out the vague claims commented in question 6, there are other interesting claims which can be extracted from their information. First, they could have mentioned how their data hint to two axis for the different types of cell tested; one consisting of fibronectin-α4β1-BIO1211 for IMR-32 cells; and a second one vitronectin-αVβ3/αVβ5-SB273005 for CHP-134, LA-N-5 and Kelly cells. They could have concluded that the effects of inhibitors for the most prevalent integrin type in each axis resemble those caused by 14G2a mAb (affectation of cell morphology, culture confluence, aggregation, etc). These claims would better adapt to a justification or validation of their hypothesis than their short concluding remarks, which have not even been redacted in a separate, dedicate section but are instead included in the discussion part.

9. Is the working hypothesis confirmed to a 100% with the obtained results?

I would not consider that it is. In order to prove that binding of the different integrins to ECM proteins modulates 14G2a-induced cytotoxicity one would need to unravel a mechanism that leads to an increase or decrease of said cytotoxicity in a synergistic or antagonistic manner. The results obtained by the researchers show how in some of the cell lines the combination of an integrin inhibitor and 14G2a have a stronger effect than the inhibitor or the mAb alone, but the results do not sort out that this increase or decrease in the measured parameter is caused simply by mere addition of two different effects (that of the inhibitor, plus that of the mAb). It could be possible then to argue that they act in the same way: by simply blocking sterically the capacity of cells to attach to the ECM. Oppositely these results could in fact be used to justify that the cytotoxicity caused by 14G2a is independent of protein adhesion, as the inhibition of a certain integrin does not "consume" the pathway exploited by 14G2a for its cytotoxicity, allowing for an increased cell death in presence of both.

10. Write a critical analysis of the article stating its strong and weak points.

The article lacks a clearly stated hypothesis, and thus, lacks the most relevant part of the conclusions in an article: the assessment of whether the initial hypothesis has or not been validated. Despite this vagueness surrounding the hypothesis, which probably stems from the inconclusiveness that the results have yielded surrounding the relationship between inhibition of integrin binding and 14G2a, the article does present relevant amounts of interesting data, especially regarding cell viability in presence of the different inhibitors. Albeit the authors fails to discern how the cytotoxicity of 14G2a relates to that of the integrin inhibitors, they have published valuable information regarding the importance of differential integrin expression for the interaction with fibronectin versus vitronectin in neuroblastoma cells, as well as the possibility of taking advantage of this blocking this interaction using specific integrin inhibitors.

11. What experiments do you believe should have been performed to improve the article and make the conclusions more robust?

Previously discussed articles, such as that by Shimizu et al. (2014; case 3), have showed the possibility of seeding and culturing cells in surfaces functionalized with the peptides which compose the specific integrin binding sites found in ECM proteins such as fibronectin. Shimizu et al. used the peptide RGD, present in fibronectin, which binds several integrins such as αVβ3 or αVβ5. Other peptide sequences such as KLDAPT, EDGIHEL or REDV could be used to functionalize culture surfaces and study how 14G2a affects cytotoxicity in experimental settings where a limited set of integrins (or even a single type) can interact with their environment, instead of seeding the cells in ECMs presenting several binding sites for over 10 different types of integrins and then inhibiting specific types. With this approach, the researchers could have had studied positive effects of specific integrin binding on 14G2a cytotoxicity (i.e. abundance of a single type of interaction) instead of the negative effects of integrin binding (i.e. integrin inhibition),

granting a new scope on the way that integrin binding and signaling interferes or synergizes with 14G2a-induced cytotoxicity.

12. Suggest a different working hypothesis based in the same biological/clinical question posed in this article.

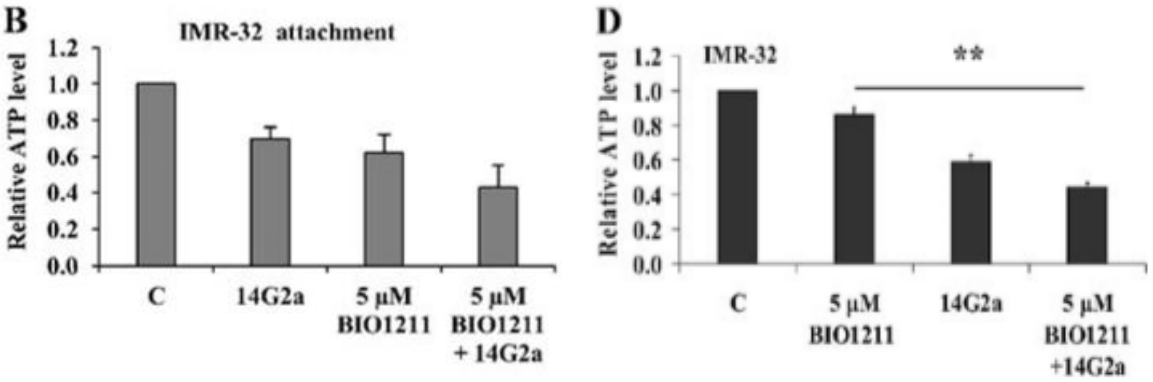


Figure S3. Extracted from figure 5 of the discussed article; follows its numeration. (B) Relative ATP levels of IMR-32 cells seeded in fibronectin-coated wells after 2 hours. (C) Relative ATP levels of IMR-32 cells cultured for 3 days in fibronectin-coated wells.

When IMR-32 cells are seeded on fibronectin-coated wells in presence of the α4β1 inhibitor BIO1211, their attachment to the wells is reduced compared to cells not exposed to the inhibitor (Fig. S3B). Similarly, if the IMR-32 cells are cultured for 72 hours in the fibronectin-coated wells, their viability is lower when in presence of BIO1211 (Fig S3C). This informs us of the importance of α4β1 binding to fibronectin for the survival and viability of the neuroblastoma cell line IMR-32.

These experiments set a very interesting starting point for translating the experiments from case 4 using decellularized matrixes to study cell dormancy in breast cancer cell lines to neuroblastoma cell lines. They also hint at the importance of α4β1 binding in cell adhesion and viability, remarking the how the EDA domain in fibronectin may play an important role in this aspect. The EDA domain has a specific binding site for α4β1 containing an EDGIHEL motif which could be key in the regulation of cell dormancy and survival over long periods of time in self-produced micro-tumoral environments. A third point of interest can be brought from the relationship of both studies: it would be of great interest to perform the integrin attachment screening assay performed in the discussed article among the screenings performed in case 4 and study whether the expression of determined integrins correlates with survival in dormant cell-produced fibronectin matrixes. It could be that only the cell lines expressing determinate integrins can enter dormancy after being seeded in the decellularized matrixes produced by HCC1954 cells.

Combining the information of cases number 4 and number 5 I would present 3 interconnected hypotheses.

1. The studies on serum starvation-induced cell dormancy in breast cancer cell lines conducted by Barney et al. (2020) can be translated to neuroblastoma. Thus, some neuroblastoma cell lines can also enter cell dormancy under serum starvation if seeded in a HCC1954 decellularized fibronectin cell matrix. Certain cell lines may in fact be capable of producing such dormancy-promoting matrixes.
2. The capacity of cancer cells to survive long periods of cell dormancy when embedded in tumor cell-produced fibronectin matrixes depends on the expression and function of determinate integrin subtypes.
3. One of the main integrins involved in the fibronectin-mediated induction of cell dormancy may be α4β1, effecting its role in the induction of cell dormancy through binding to the EDGIHEL motif present in the EDA domain of cellular fibronectin, allowing through this interaction the activation

of latent TGFβ1 and subsequent TGFβ1 signaling, ultimately leading to cell survival over dormancy periods.

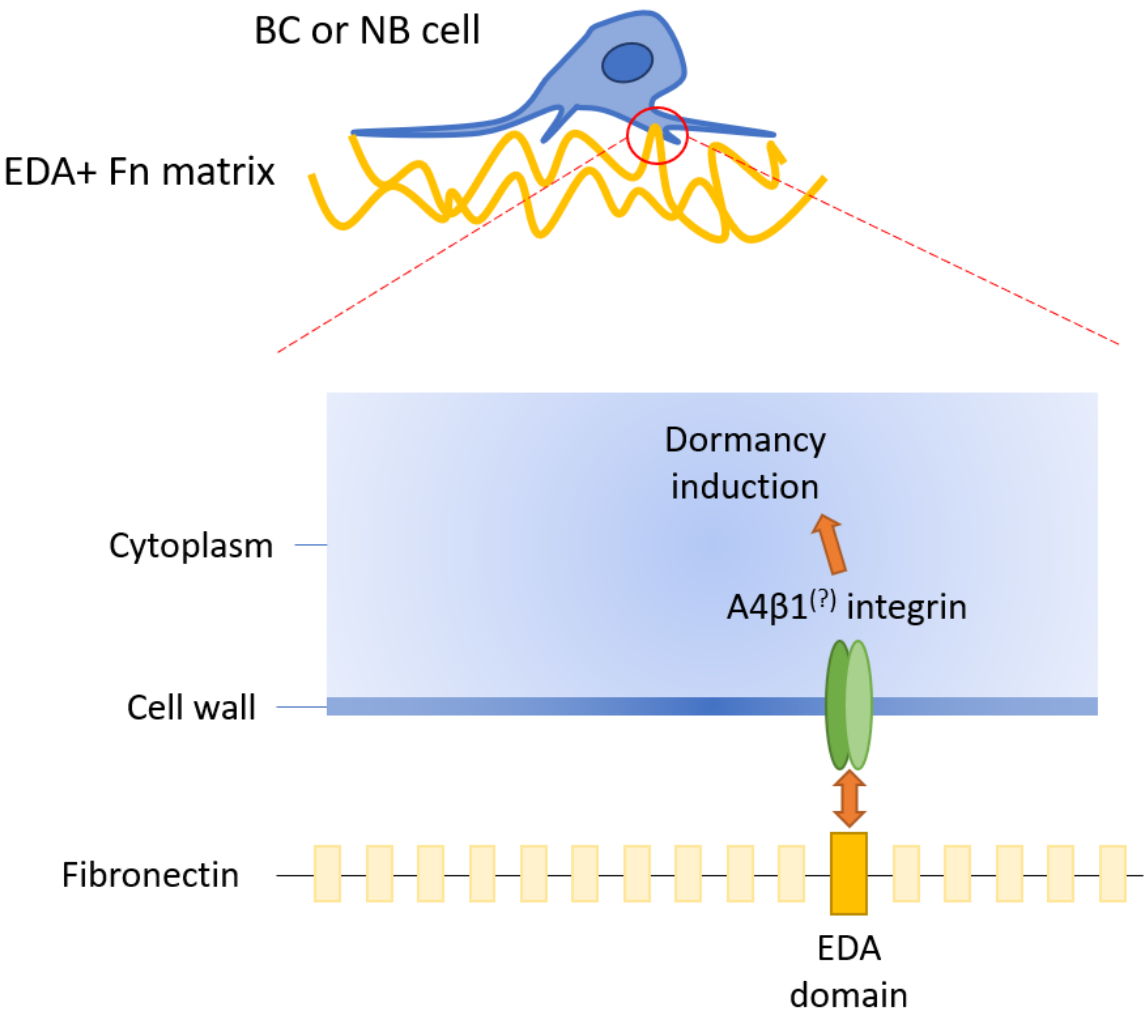
13. What would be the critical experiments that you would need to perform to confirm (or deny) the hypothesis that you have proposed in question 12? What controls would be needed?

For the first hypothesis, it would be necessary to select a number of neuroblastoma cell lines of a certain diversity, and repeat some of the main experiments performed by Barney et al., starting with the screening for capacity of entering serum starvation-induced cell dormancy in common culture plates and in fibronectin and collagen I coverslips; following with the determination of whether cell lines experiment an improved survival over cell dormancy when grown in decellularized ECMs produced by HCC1954 cells; and continuing with the characterization of the importance of TGFβ signaling, ERK, MEK and ROCK pathways. It would be of interest to test whether the cell lines which perform best in tissue culture plates and functionalized coverslips also produce an ECM which improves survival during cell dormancy of cells cultured in their decellularized matrixes.

For the second hypothesis, select breast cancer cell lines of the cell dormancy survival spectrum described by Barney et al. can be used to perform an integrin attachment screening assay such as the one performed in the discussed article, in order to stipulate if there's a relationship between the expression and binding of certain types of integrin with the capacity to enter cell dormancy when in presence of decellularized fibronectin ECMs.

Lastly, for the third hypothesis, several different approaches would be needed to validate such a complex hypothesis. In the first place, the presence and relative quantity of EDA-fibronectin and other splicing variants of fibronectin in the decellularized ECMs produced by HCC1954 cells would need to be analyzed. Secondly, the affinity of α4β1 for the different binding domains present in fibronectin should be addressed in order to confirm that the EDGIHEL domain present in EDA is relevant and key although other domains such as KLDAPT are present in plasma fibronectin too (which could explain the reduced adhesion of IMR-32 cells to the pFn-functionalized wells used in fig. S3). It would then be necessary to validate that latent TGFβ1 activation is dependent in the presence of this domain. If these points could be validated, it would mean that a mechanism explaining the differential capacity of cells to enter dormancy and survive long periods of serum-deprivation when cultured on decellularized cellular fibronectin matrixes versus coverslips functionalized with plasma fibronectin has been elucidated.

14. Design a graphical abstract for the hypothetical article that your proposed hypothesis and experiments would yield.



Additional bibliography

Kowalczyk, A., Gil, M., Horwacik, I., Odrowąż, Ż., Kozbor, D., & Rokita, H. (2009). The GD2-specific 14G2a monoclonal antibody induces apoptosis and enhances cytotoxicity of chemotherapeutic drugs in IMR-32 human neuroblastoma cells. *Cancer letters*, *281*(2), 171-182.