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BOOK OF ABSTRACTS

KEYNOTE/INVITED SPEAKERS

Enabling technologies and regulation framework for pollutants toxicity testing: the roadmap for organ models implementation

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INTRODUCTION

Humans are exposed to potentially toxic chemicals in the environment and unintentional combinations with drugs may result in synergistic detrimental effects. Currently standardized toxicological methods do not comprehensively cover potential cardiotoxicity in humans , and regulatory awareness is low for this uncertainty. In view of these observations, the ALTERNATIVE project (https://alternative-project.eu/) is providing systematic reviews and research to improve regulations specifically for cardiotoxicity. To this aim, ALTERNATIVE is developing an innovative platform to detect the cardiotoxicity of chemicals and their mixtures. The platform consists of a three-dimensional (3D) and dynamic *in vitro* model mimicking the young or aged human cardiac tissue, coupled with a reliable, high-throughput monitoring system based on multi-omics analyses and integrated into a Machine Learning (ML) risk assessment tool. ALTERNATIVE is cooperating with the ASPIS cluster to integrate specific approaches for cardiotoxicity into a testing and assessment workflow covering all types of toxicities with New-Approach-Non-Animal-Methods (NAM).

RESULTS AND DISCUSSION

Within the ALTERNATIVE platform, the myocardial tissue model was designed as a biphasic scaffold based on a 3D-printed construct and a photo-responsive hydrogel, working as a structural/mechanical framework and carrier for cell homing, respectively. The construct's forming material was a custom-made poly(caprolactone)-based poly(ester urethane) (PU) with elastomeric-like mechanical properties [1]. The PU was microfabricated into a multi-layered 3D structure by melt-extrusion additive manufacturing and then functionalised with fibronectin by plasma treatment [2, 3]. The hydrogel was obtained by irradiating methacryloyl gelatin (GelMA) solutions under cell-friendly conditions. Different GelMA concentrations were exploited to tune the mechanical properties of gels and simulate the aging process of the native cardiac tissue. The gel was seeded with human-induced pluripotent stem cell-derived cardiomyocytes and human coronary artery endothelial cells. Cells showed viability, contraction ability and modulation of cardiac genes. Omics analyses revealed pathways involved in glycolysis, the Krebs cycle, biosynthesis of amino acids and cardiac contraction, among others.

ALTERNATIVE proof-of-concept validation refers to well-known mixtures of pollutants that have been selected via epidemiological, toxicological and modelling expertise. To this aim, doxorubicin (DOX) has been selected as our positive cardiotoxic control due to its well-documented cardiotoxic effect. A dose-response curve has already been built to evaluate the effect of the drug in both 2D monoculture (100% hiPSC-CMs), considered as a gold-standard model, and 3D cell co-cultures. 2D and 3D environments were compared, showing that the ALTERNATIVE *in vitro* system preserved mitochondrial function for hiPSC-CMs. The same test will be replicated in dynamic cell culture condition to better mimic the native microenvironment. In detail, the 3D cellularised scaffolds will be cultured in a perfused and sensorised bioreactor supplemented with media culture. The bioreactor will be designed basing on the standard dimension of a multiwell plate [preliminary version: 4, *IVTech srl*]. The electrodes will assure the electrical stimulation to recreate the muscle contraction during the experiment. The fluidic circuit will comprise a pressure-driven flow controller with valves, flow sensors and reservoirs. , as well as an electrostimulation system using screen-printed electrodes, to mimic the in vivo conditions. Two sensors, one for pH and one for O_2 concentration, will also be designed in line with the system, similar to the flow sensors.

CONCLUSION

By mixing *in vitro* and *in silico* methods for establishing the toxic profile of chemicals, ALTERNATIVE has the potential to establish a platform able to provide more ethical and close-to-real scenario information on toxicity and lower testing costs, thus resulting in a significant reduction of the associated direct and indirect costs.

ACKNOWLEDGEMENTS

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Towards the 3Rs and beyond: Organs-on-Chip and their potential for human-relevant research

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ABSTRACT
Traditionally, animals have been used in research as models for studying human disease, and for assessing the toxicity of drugs and chemicals. However, this practice has always aroused concerns regarding the ethical aspects associated with animal suffering and their use in biomedical research[1]. In 1959, Russell and Burch proposed the concepts of replacement, reduction, and refinement, better known under the umbrella term of "3Rs Principle", a new applied science that would improve the treatment of laboratory animals while advancing the quality of experimental techniques[2]. In the following years, a number of advanced methods hardly imagined in the mid-20th century have been developed, and the scientific community has adopted a more critical standpoint of the use of animals in research, highlighting the intrinsic limits of the animal model in both human disease modeling and drug discovery field[3,4,5], and urging for strategic actions that prioritizes replacement and (human)-relevant research over refinement and reduction[6]. Recently, the EU Reference Laboratory for alternatives to animal testing of the European Commission's Joint Research Centre carried out a series of studies to produce a robust knowledge collection in which are described in detail non-animal models that are already applied in several biomedical research areas, suggesting that when human tissues are used, they may produce faster, cheaper and more relevant results, providing greater comprehension of biochemical processes of human diseases[7]. Among the emerging advanced *in vitro* models, organ-on-chip platforms are considered the most promising ones, as shown in a landmark study in which human liver-chip was able to correctly identify 87% of drugs that caused drug-induced liver injury to patients despite passing through animal testing[8]. In addition to being useful as tools for understanding toxicity in human tissues, these platforms also provide ways to model disease states *in vitro*, as they are designed to control cell micro-environments and maintain tissue-specific functions to better mimic peculiar functionality and complexity of a living organism's physiological conditions[9].

Particularly, animal models often fail in recapitulating complex diseases which are known to naturally occur only in humans, such as Alzheimer's Disease (AD)[10] or Amyotrophic Lateral Sclerosis (ALS)[11], resulting in repeated failure of drug translation from preclinical to clinical stages. In particular, for ALS, which is a progressive and fatal neurodegenerative disease that caused the loss of neuromuscular junction (NMJ)[12], only two poorly effective drugs have been approved so far by the FDA[13]. Since the discovery of the first ALS-associated gene in 1993[14], ALS research has relied largely on transgenic animal models whose phenotype are distinct from human ALS, thus limiting their validity and muddying the analyses. For instance, some authors suggest that the marked heterogeneity in the cellular and molecular anatomy of NMJ across a range of mammalian species may explain, at least in part, the poor therapeutic translation of clinical trials[15].

Given the crucial role played by the NMJ in ALS, over the past years many different NMJ *in vitro* models have been developed, and the combination of microfluidic and iPSC technologies seems to be one of the most promising avenues for studying NMJ dysfunctions in a range of human diseases, including ALS[16]. In this context, Osaki et al. developed in 2018 an ALS-on-a-chip, one able to recapitulate the human functional NMJ structure within a microfluidic platform in which motor neurons spheroids and 3D muscle fiber bundles are cocultured[17]. The latter is just one of many examples of the important advances that can be achieved in the field of disease modeling and drug screening, showing that scientific and moral progress are sides of the same coin, and that it is our responsibility, as scientists, to promote both.

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CLINICAL USE OF INNOVATIVE BIOTECHNOLOGIES " HUMAN BASED"

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INTRODUCTION

In recent decades, several scientific evidences have gradually grown which have highlighted important critical issues in the predictive capacity, reliability and reproducibility of the "animal model" in the field of translational medicine. This has increasingly revealed the need to aim at "human relevant" biomedical research in order to obtain more effective and precise therapeutic and preventive treatments for patients.

The recent innovative and human biology-based biotechnologies called Organs-on-chip (OOC) and Organoids are the spearhead of this modernization process, currently underway, in biomedical research.

METHODS

A bibliographic search was performed using Pubmed https://pubmed.ncbi.nlm.nih.gov. 4287 scientific publications were collected about clinical setting. Their filtering was related to the last two years in which OOCs and/or Organoids of human origin were used.

Subsequently, six macro-areas of currently very relevant medical interest were identified:

1. improvement / refinement in the development of new drugs and/or complex therapeutic treatments, including personalized ones.
2. identification of the fine pathogenetic mechanisms of human pathologies and refinement of disease modelling.
3. assessment of toxicity for oncological drugs, environmental contaminants, and vaccines.
4. studies about pharmacological microdosing, pharmacokinetic and pharmacodynamic in Humans.
5. biomaterial testing
6. regulatory aspects

For each macro-area, a new bibliographic search was carried out, using the same criteria mentioned above, in order to identify the scientific publications relating to each of them.

RESULTS AND DISCUSSION

Therefore, 80 publications were further selected and divided as follows by macro-area: 27 for macro-area 1, 23 for macro-area 2, 12 for macro-area 3, 14 for macro-area 4, 3 for macro-area 5 and 1 for macro-area 6. From this grouping of scientific articles, some examples were drawn to discuss in the planned oral presentation. (1-8)

CONCLUSIONS

The innovative and recent human-based biotechnologies are already useful in the clinical field today.

Based on the specific human biology, they are able to reveal the fine pathogenetic mechanisms related to our species, and therefore, can be effectively applied in the field of Precision Medicine.

Finally, they can optimize the protection of our health in the therapeutic, preventive, and regulatory fields.

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An Osteochondral Joint-on-Chip to reveal the expression signature of mechanically dysregulated chondrocytes subpopulations

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INTRODUCTION

Osteoarthritis (OA), the most diffused musculoskeletal disorder, is a whole joint degenerative pathology leading to cartilage degeneration and subchondral bone alterations[1]. OA triggering stimuli are however still unknown and no disease modifying treatment is available as a consequence. A correlation between OA and mechanical risk factors suggests the necessity of considering aberrant mechanical stimuli in OA pathogenesis studies. These traits are nonetheless seldom introduced in preclinical *in vitro* models. With the final goal of dissecting the pathways and the cellular subpopulations responsible for OA onset, we aimed at developing an osteochondral unit (OCU) Joint-on-chip model that recapitulates OA phenotype through tissue specific mechanical loading.

EXPERIMENTAL

Through multi-layer soft lithography we coupled the mechanism of our mechanically active OA cartilage-on-chip model[2] with a new vertical burst valve (VBV) system to engineer stacked bi-phasic tissues with an OCU like disposition. The two layers are exposed to different levels of compression (i.e. an hyperphysiological OA inducing 30% for cartilage and a 1% compression for subchondral bone) as estimated through finite element simulations. Healthy human articular chondrocytes (hACs) were used for the cartilaginous layer, bone marrow derived MSCs (coupled with vascular endothelial cells) for subchondral bone. After achieving OCU-like constructs, aberrant mechanical stimuli were applied and tissues analysed in terms of produced matrix (with quantifications of immunofluorescence pictures) and gene expression through RT-qPCR and single cell RNA sequencing (scRNA-seq, N=4 donors).

RESULTS AND DISCUSSION

On-chip osteochondral constructs are characterized by expression of aggrecan and collagen type II (cartilaginous layer) and (HA)hydroxyapatite (bone layer) effectively mimicking OCU stratification (Fig. 1). With the incorporation of HUVECs in the subchondral layer, furthermore, the cartilaginous tissues can be directly interfaced with vascular subchondral layer. Aberrant loading leads to alterations in HA content in the subchondral layer, in accordance to what reported for OA patients. Bulk gene expression analyses of OCU constructs revealed, furthermore, a different loading response with respect to single culture counterparts (e.g with an increase in hypertrophic markers such as IHH but a pro-anabolic response reminiscent of early OA stages). A higher complexity in our modelling strategies might therefore be necessary to achieve representative models. On this regard, scRNA-seq analyses showed that the co-culture is necessary to maintain (or rescue) the major hACs subpopulations highlighted in human OA cartilage[3]. Unbiased clustering demonstrated how co-culture and mechanical loading in our joint-on-chip model modulate the abundance of hACs subpopulations such as fibro-chondrocytes (COL1A1), hypertrophic chondrocytes (COL10A1), homeostatic chondrocytes (JUN) and Regulatory chondrocytes (CHI3LI) which were correlated with OA progression in clinical samples.

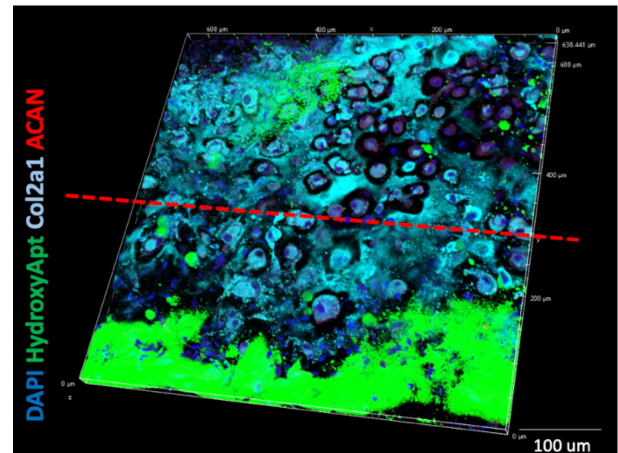

Figure 1. Immunofluorescence images of an osteochondral construct-on-chip at day 14.

CONCLUSION

Our Joint on-chip model paves the way for the *in vitro* recapitulation of phenomena such as cartilage degradation and vascular invasion and the testing of innovative OA drug targets and regenerative therapies. It also represents an instrument to possibly dissect, in a highly controlled environment, the cause-effect mechanism leading to the development of OA.

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In vitro disease models to study pathological mechanosensing

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INTRODUCTION

The onset and progression of aging-associated pathologies is highlighted by continuous local extracellular matrix (ECM) transformation. The contribution of ECM remodelling to disease is largely overlooked when predictive *in vitro* models are proposed.

Following an ischemic insult or in chronic disease, ECM pathological remodeling results in dramatic changes in the chemistry, local stiffness, and nanostructure of the organ scaffold. Deposited by the tissue fibroblasts, the newly produced matrix displays impaired compliance and aberrant signalling, thus hinders cell functionality. The response of tissue cells to the ensuing biomechanical stress leads to the alteration of their transcriptional and post-transcriptional landscape, with the aim of preserving organ function (1). Together with others, our group has been active in the field of pathological mechanosensing for a few years, trying to dissect the processes driving ECM remodelling in disease and unveil the molecular pathways highlighting cell response to the new conditions.

Cardiac diseases represent a paradigmatic example of how ECM pathological remodeling can hinder cell functionality, here affecting muscle contractility and organ pumping function. By using human specimens, induced pluripotent stem cell (iPSC)-derived 3D organoids and a combination of advanced microscopy and molecular techniques, we identified reproducible modifications in the 3D nanostructure, interconnected porosity, and mechanics of heart ECM that contribute to organ failure (2).

We found that the hyperactivation of mechanosensitive Hippo effector in patient-derived cardiac fibroblasts promotes ECM pathological remodelling in a positive loop which eventually favors the fibrotic process and fuels heart failure. Additionally, we showed ECM maladaptive remodelling and YAP reactivation drive dramatic changes in cardiomyocyte transcriptional and post-transcriptional landscape to increase their contractility and preserve organ pumping function (Vinarsky et al, in preparation).

We also highlighted how this process rewires the alternative splicing of numerous genes involved in cardiomyocyte contractility, calcium handling and mechanosensing (3).

ECM remodelling is also key to tumorigenesis, so that altered matrix stiffness is considered predictive of malignancies. We therefore analyzed primary prostate cancer tissues obtained from numerous patients undergoing radical prostatectomy to underline reproducible structural changes in the tumor ECM and promoting the loss of the glandular architecture. Starting from patient cells, we established prostate cancer tumoroids (PCTs) that mimic key features of the native tumor microenvironment (TME) and proceeded to investigate the cooperative role of TGF-β signalling and ECM *desmoplasia* in prompting prostate cell epithelial-to-mesenchymal transition (EMT) and fostering tumor progression and dissemination (4).

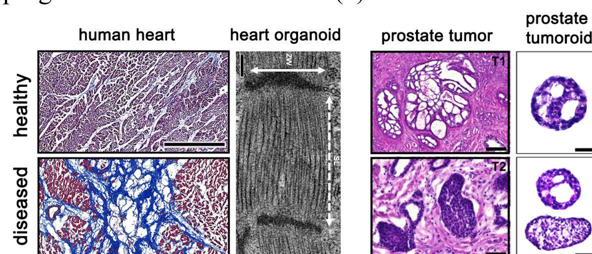

Figure 1. Masson Trichrome staining of ECM in human healthy and diseased heart and Transmission Electron Microscopy image of a single sarcomere unit in iPSC-derived cardiac organoid (left). Haematoxylin-eosin staining of the altered glandular structure in 2 human prostate tumors and in the tumoroids obtained by them.

CONCLUSION

In conclusion, by employing a plethora of *ex vivo* and *in vitro* disease models, molecular and bioengineering techniques we addressed the role of ECM remodelling and mechanosensing in a broad range of pathologies. In particular, our studies contributed to describe characteristic changes in the ECM in tumors and cardiac diseases. Finally, they helped us identify different layers of intracellular mechanosensing activated in response to pathological mechanical turmoil.

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Enhancing the scientific credibility of OoC: standards towards regulatory acceptance

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ABSTRACT

To reach regulatory acceptance of Non-Animal Methods (NAMs) in the chemicals and pharmaceutical domains, standards can be used to support the validation and qualification process, demonstrating scientific reliability and relevance on non-animal methods, thus giving confidence that the data produced can be used to ensure solid, science-based decisions to protect human health and the environment. Many frameworks were proposed to establish scientific credibility of NAMs for regulatory acceptance [1,2], which mainly include elements to assess the human biological relevance, the technical reliability and robustness, the definition of a specific regulatory purpose.

Standards are widely used by companies to support commercial activities on consumer products and are useful in transferring technologies from R&D into applications. In a scientific environment, standards can also be of value, together with publications and patents, to advance the state of the art in many research fields.

Organ-on-Chip (OoC) technologies are examples of how much biology is converging with other fields, such as engineering, physics and mathematics. For these NAMs, standardisation can ensuring proper characterisation of individual devices, benchmarking against appropriate reference elements and supporting efficient communication among stakeholders. Standards can tackle multiple technical aspects of OoC technology, from assessment of expected performance to material characterisation, from reference compound to cell sources, from reporting templates to quality control [3].

In 2021 the European Commission Joint Research Centre and the European Standards Development Organizations CEN-CENELEC organised the "Putting Science into Standards" workshop, bringing together developers, end-users and standardisation experts [4]. To encourage the development of OoC-specific standards, CEN-CENELEC has initiated concrete actions by establishing the OoC Focus Group, a European coordination platform to stimulate and coordinate standardisation efforts. The Focus Group will define a roadmap, set priorities and identify and liaise with Technical Committees to be involved for formal standards development and approval.

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Human microphysiological system for modeling Lung disease

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ABSTRACT

Traditional in vitro and in vivo models are the most potent tools for characterizing the potential of therapeutics during the Drug Discovery process[1]. Differences in organ structure between animal and humans, as well as frequently unclear results when using animal models for drug discovery, highlights the need for in vitro models that can complement animal studies and improve our understanding of human lung physiology. However traditional in vitro system used for the characterization of pharmacological products shows important limits which makes it difficult to recapitulate physiological and pathophysiological functions of drugs. Need to reduce costs and time in the drug development process combined with the increment of regulatory pressure to ban animal use have increased the development of microphysiological systems and novel techniques that are able to better recapitulate the key organ features.

Here, we focus on diseases of the respiratory system and provide an overview of the approach used by pharmaceutical company for applyng this platforms for characterized new therapeutic compounds[2].

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Enhancing the scientific credibility of OoC: standards towards regulatory acceptance

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ABSTRACT

With a budget of €10.1 billion the European Innovation Council has been establised under Horizon Europe to support game changing innovations with the potential to scale up internationally and become market leaders. It support the whole lifecycle of the innovation, from early stage research, to proof of concept, technology transfer, and the financing and scale up of start-ups and SMEs. It focuses mainly on breakthrough, deeptech and disruptive innovation, targeting especially market-creating innovation.

The EIC operates in three different area of intervention: Patfinder, Transition and Accelerator.

EIC Pathfinder: for advanced research, supporting early stage development of future and emerging breakthrough, market-creating and/or deep tech technologies.
EIC Transition: to help researchers and innovators develop the pathway to commercial development for promising research results.
EIC Accelerator: to support individual SMEs, startups and small mid-caps to bridge the financing gap between late stages of research activities and market take-up, to effectively deploy breakthrough, market-creating innovation and scale-up companies where the market does not provide viable financing.

Frontier platforms for experimental cell modelling

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INTRODUCTION

This talk provides the fundamental concepts behind the design of frontier platforms for experimental cell modelling that I have invented in the last two decades.

THE MOAB

One concept is miniaturisation associated to full-thickness optical accessibility[1] to recreate and monitor in real time and in long term culture, the interstitial perfusion process of 3D natural tissues such as brain, bone marrow and lymph node tissues. Cell models based on this concept proved able to recapitulate *in vitro* several slowly-developing biological processes, such as the formation of a bone metastasis by breast cancer cells[2], the instruction of adaptive immune cells in a lymphnode[3] and the neuroprotective effect of mesenchymal stem cell secretome on pathological neurons. I have then connected these models to recapitulate mechanisms involving multiple body compartments, such as the microbiota-gut-brain axis in neurodegeneration[4] and the bone marrow-lymph node axis in leukemic transformation.

THE NICHOID

Another concept is miniaturisation of the 3D cell scaffold. In the aim to better control and monitor stem cell function, I miniaturised the culture substrates in my models by applying a microfabrication technique called two-photon laser polymerization. Using these 3D micro scaffolds, I was able to condition mesenchymal stem cells, neural precursor cells and embryonic stem cells towards maintenance of a greater multipotency or pluripotency, compared to conventional 3D scaffold culture.

THE MICROATLAS

Another revolutionary concept is implanting a cell model in a living organism, to regenerate a microvascular network anastomosed to the host, allowing for studies involving interactions of the cell model with the host immune system. My group has recently replicated the human microvascular niche and relevant druggability *in vivo* in a chick embryo model [5]. These results are opening the way to the application of micro-optics implanted *in vivo* for optical inspection of biological processes[6].

FUTURE DEVELOPMENTS

We will use human breast cancer cells adhering to 3D polymeric micro scaffolds to create arrays of tumour micro environments. We will implant the arrays *in vivo* in the chorioallantoic membrane of an embryonated avian egg, to elicit a foreign-body fibrotic reaction. We will vary the micro scaffolds geometry to condition tumour infiltration by the host’s vessels and cells. We will predict mass transport of solutes and anticancer agents by computational modelling. To validate this platform, we will quantify *in vivo* the dose-dependent efficacy and cancer specificity of therapeutic agents whose success is known to depend on the fibrotic stage of tumours.

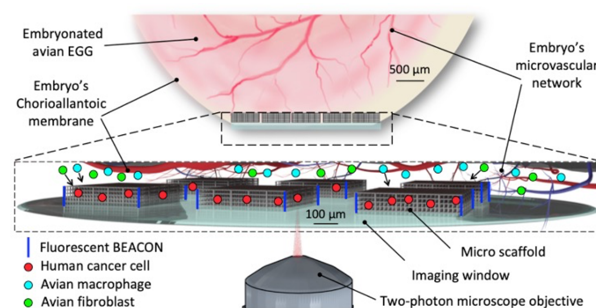

Figure 1: Scheme of idea of exploiting the innate immune system of an avian embryo to induce a foreign-body reaction able to recreate tumour fibrotic micro environments, with variable levels of matrix stiffness and vascularity, to embed human breast cancer cells.

ACKNOWLEDGEMENTS

Alberto Bocconi, Claudio Conci, Emanuela Jacchetti, Chiara Martinelli, Alessandra Nardini, Paolo Ritter and Carolina Testa. ERC-AdG-2021 – G.A. nr 101053122.

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ORAL PRESENTATIONS

A novel tumor-on-a-chip device tracking guided dendritic cell migration through the vascular endothelium upon therapy

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INTRODUCTION

The recruitment of dendritic cells (DC) to tumor microenvironment (TME) is one of the most crucial aspect influencing immune response. Inflammation linked to tumor development allow transmigration through the endothelial barrier of circulating DC, that as the most powerful antigen-presenting cells (APC) capture tumor antigens for induction of T-cell adaptive response. Whether the increased inflammatory context under therapy drives DC migration to TME across the vascular endothelium remains to be elucidated.

EXPERIMENTAL

A novel tumor-on-a-chip device was built with three different compartments hosting the immune component (HD-derived HLA-A2-matched IFN-DCs), the tumor site (SK-Mel-28 melanoma cells) and a blood (HUVEC) vessel-mimicking barrier engineered with a complete lumen characterized by intercellular junctions of endothelial cells ensuring full functionality. By generating this 3D tumor model mimicking cancer growth at the interface of blood vessels, we investigated the guided DC transmigration across the endothelium barrier towards melanoma cells upon drug treatment.

RESULTS AND DISCUSSION

We were able to generate a tumor-on-a-chip device with a central tumor compartment encircled by two independent vascular channels (one for medium supply) interconnected by micro-channels along the circumference and functionalized with flow-perfused HUVEC cells under shear stress reaching full junction maturation to recreate *in vivo* 3D blood microvessel. This device allowed to investigate the physical and biochemical-driven interactions among tumor-immune-endothelial cells. Specifically, by confocal laser scanning microscopy (CLSM) analysis and live-cell imaging, we evaluated: i) the status and interactions between immune-cancer cells at the forefront of vascular barrier; ii) whole vascular channel reconstruction by stitching together confocal images; iii) number of gaps on junctions on the endothelial barrier as marker of tumor activity.

CONCLUSION

The designed tumor-on-a-chip microfluidic platform is a suitable 3D model to investigate the physical and biochemical-driven interactions among tumor-immune-endothelial cells and to monitor the migration of immune cells throughout an endothelium to reach the tumor site.

Efficacy of Sorafenib treatment in a 3D hepatic cancer model

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INTRODUCTION

Nowadays, drug discovery is still a risky, expensive, and time-consuming process. The preclinical research phase relies mainly on the use of *in vitro* and *in vivo* models that even when combined, do not provide a perfect representation of human physiology and function. Besides, in the last years, ethical concerns about the wide use of animals for research purposes have emerged, which promoted the development of *in vitro* models that could better mimic the complexity of tissues and biological systems.

3D cell structures developed in the last years represent very promising models for both healthy and pathological setups. The use of 3D spheroids allows the establishment of cell-to-cell interactions in a more realistic tumor microarchitecture when compared to the 2D tumor cell cultures.

To develop 3D spheroids, hydrogels or commercially available microwell plates are currently used, but with some limitations. These include the lack of homogeneity in spheroid size and shape [1], cost and above all, the limitation on the number of spheroids produced.

To circumvent these limitations, we designed a reusable microcavity insert to generate around 1.500 spheroids per well, in a 48-well plate, and test drugs in thousands of spheroids simultaneously.

EXPERIMENTAL

The microcavity insert was developed to fit a 6-well plate, cut with a 10mm puncher, and attached to the bottom of a well in a 48-well plate, sterilized with ethanol 70%, dried and washed with PBS. A coating to prevent cellular adherence to the microcavities was done before cell seeding. Optimal conditions for spheroid manufacturing were determined with the HepG2 cell line, widely used to develop liver models [2].

Three days after cell seeding, drug treatment was initiated. The drug chosen was Sorafenib, a reference drug used in the treatment of hepatic cancer.

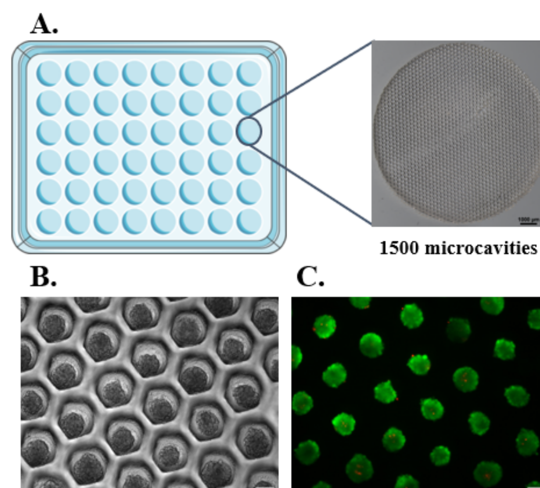
For the Sorafenib treatment, different concentrations of the compound were used and spheroid viability and cytotoxicity were assessed over time, as well as the effect of the compound on the spheroid size and shape.

RESULTS AND DISCUSSION

The microcavity insert was successfully produced and in less than 24h after seeding, cell aggregates were observed. After 48h, the spheroids were compact and homogenous in shape and size. The spheroids remain viable for up to 4 days in culture and can be easily retrieved, as the cells do not attach to the microcavities.

When treated, the spheroids show reduced viability, loss of shape and size reduction with increasing concentration of the compound.

Figure 1. A. Brightfield image of microcavity insert that was attached to the bottom of a well in a 48-well plate (scale bar 1000 µm). B.



Brightfield image of spheroids inside microcavity insert (scale bar 100 µm). C. Viability of spheroids inside microcavity insert (scale bar 100 µm).

CONCLUSION

We were able to design a microcavity insert in which we can generate thousands of spheroids, in a simple and fast manner. The cell aggregates form easily compact spheroids, that can be easily retrieved and used in further experiments.

Thus, this platform is promising to control the number of spheroids produced, as well as their size and shape. We are confident that this platform will be an encouraging technique to perform drug screening faster and on thousands of spheroids simultaneously. The retrieval of the spheroids allows the combination of this 3D model with dynamic systems, to better reproduce the physiological conditions.

ACKNOWLEDGEMENTS

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Exploiting compartmentalization to unravel the contribution of cartilage and synovium to osteoarthritis pathogenesis in a novel joint-on-chip model

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INTRODUCTION

Osteoarthritis (OA) is the most prevalent degenerative joint disease, leading to pain and disability. However, current treatments are only symptoms-relieving, due to the disease complexity and to the lack of knowledge about initial disease mechanisms[1]. In this regard, organs-on-chip can be used to model different joint tissues and investigate the cause-and-effect relationships between the various factors involved in the disease development. To this end, we developed a compartmentalized joint-on-chip model allowing for the co-culture of cartilage and synovium tissues and for the induction of OA traits, aiming at assessing how the breakdown of the natural communication between these tissues contributes to OA development.

EXPERIMENTAL

The here presented microfluidic platform (*Figure 1*) is made up of two distinct culture compartments, dedicated to synovium and cartilage culture, respectively. Each compartment has a central channel to host a 3D micro-construct, and two medium channels on either side. The two compartments are independent at rest and can be put in communication thanks to the presence of normally closed doormat valves. Additionally, mechanical compression can be applied to the cartilage compartment.

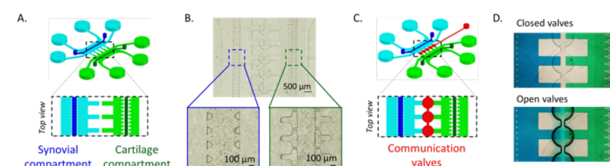

Figure 1. A. Layout of the two culture compartments. B. Picture of the cell culture chamber. C. Layout of the communication valves (red). D. Picture of the platform showing rest configuration (i.e. with closed valves), and configuration with open valves.

Human articular chondrocytes (hACs) embedded in fibrin gel were cultured for two weeks statically. Cartilage formation was assessed through gene expression analysis of relevant ECM proteins (e.g. *COL2A1, ACAN*), as well as through immunostainings. After maturation, a cyclic hyperphysiological compression (HPC) was applied for one week[2] to induce hACs to acquire an OA phenotype, that was investigated through gene expression analysis of inflammatory markers (e.g. *IL6, IL8, MMP13).*
Human synovial fibroblasts (SFBs) and monocytes-derived macrophages (M0s) embedded in a matrix of fibrin gel and collagen type-I were seeded in the corresponding compartment at day 14 and cultured up to seven days, i.e. up to day 21. Synthesis of synovial ECM-relevant proteins, i.e. collagen type-I and lubricin, was assessed. At day 17, communication valves were lifted up, and induction of synovial inflammation was evaluated through immunofluorescence staining of markers indicating macrophage polarization towards pro-inflammatory state M1 (CD80, CD86), MMPs synthesis, as well as changes in the matrix.

RESULTS AND DISCUSSION

Mature cartilage micro-constructs were obtained as proven by a matrix rich in collagen type-II (*Figure 2A*) and by the up-regulation of *COL2A1* and *ACAN* expression at day 14. Additionally, a significant increase in IL6 expression, and an increasing trend of IL8 and MMP13 gene expression in HPC samples compared to static controls at day 21 indicated the induction of OA traits.
In the synovium compartment, enhanced synthesis of collagen type-I and lubricin was shown (*Figure 2B*), suggesting a proper maturation of the tissue. Upon valves opening, synovial inflammation due to communication with HPC OA cartilage tissue was demonstrated by the enhanced synthesis of MMPs (e.g. MMP1, MMP9) operated by SFBs and by macrophage polarization towards pro-inflammatory state M1 (*Figure 2C*). A decreased production of collagen I and lubricin was also detected.

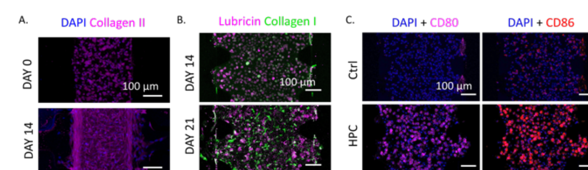

Figure 2: A. Immunofluorescence staining of cartilage constructs. B. Staining of synovium microtissues. C. Staining of synovium at day 21, in communication with static (ctrl) and HPC cartilage constructs.

CONCLUSION

The compartmentalized joint-on-chip model provides a way to grow 3D cartilage and synovial constructs separately, and to induce OA traits in a particular compartment by controlling the communication between chambers over time. The model was exploited to show that injuries caused by mechanical stress to cartilage lead to inflammatory responses in the synovium. More investigations are being held to unravel the role of an inflamed synovium on triggering cartilage degradation and to establish the primary function of the two tissues in the initial stages of OA.

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A Personalized Joint-on-a-Chip to Screen Biological Treatments for Osteoarthritis

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INTRODUCTION

Osteoarthritis (OA) is a highly disabling whole-joint pathology, characterized by synovial inflammation and cartilage degeneration. Among the different therapies proposed for OA treatment, orthobiologics have shown promising results [1], although with some variability between patients. Since animal models cannot be considered as fully reliable, due to differences in pathological mechanisms, particularly for inflammatory diseases, and available in vitro models often fail in recapitulating the joint complexity, we aimed at developing a microfluidic OA model to test their efficacy in a personalized human setting. As a proof-of-concept, we built models with patient-matched cells and synovial fluid, testing the effect of allogeneic mesenchymal stromal cells (MSCs) from bone marrow (BMSCs) and adipose tissue (ASCs) on the production of cytokines and degradative enzymes.

EXPERIMENTAL

To reproduce the joint compartments, we designed a customized chip configuration. The central channel was designed to host the synovial fluid (SynFlu), whilst the two flanking compartments were designed to host hydrogel-embedded articular chondrocytes and synovial fibroblasts. Two external channels were dedicated to the medium. Chondrocytes and synovial fibroblasts were respectively embedded in Hyaluronic Acid with Polyethylene (glycol) Diacrylate crosslinker (HA-PEGDA) and fibrin hydrogel and injected in the microfluidic chip. To evaluate the response of cells to a pathological microenvironment, we injected SynFlu from OA (OA-SynFlu) or healthy donors (H-SynFlu) and assessed the expression of cartilage (Collagen-II and Aggrecan) and synovial (Collagen-I and Lubricin) markers, as well as degradative enzymes. Finally, we generated patient-specific models using donor-matched chondrocytes, synovial fibroblasts and OA-SynFlu. At day 4, ASCs or BMSCs were injected in the central channel, simulating an intra-articular injection of allogeneic MSCs. In the control group, we injected only OA-SynFlu. At day 10, we analyzed cytokine production and the expression of degradative enzymes.

RESULTS AND DISCUSSION

The expression of Collagen-II and Aggrecan, detected in chondrocytes cultured in HA-PEGDA already at day 4, further increased at day 10. Synovial fibroblasts cultured in fibrin expressed Collagen-I and Lubricin both at day 4 and 10. To confirm the generation of an inflamed environment in the joint-on-a-chip model cultured with OA-SynFlu, we quantified inflammatory cytokines released from cells. In our devices, the concentration of all the tested cytokines resulted higher in the models cultured with OA-SynFlu compared to those with H-SynFlu. The cytokines more abundantly expressed in chips cultured with OA-SynFlu resulted to be IL-1α, IL-6, IL-8 and TNF-α. In chondrocytes, Aggrecan expression was slightly decreased by OA-SynFlu, whilst Collagen-II expression remained stable. Interestingly, when cultured with OA-SynFlu, a high number of chondrocytes was positive for Collagen-I, a marker of fibrocartilage. Synovial markers were not significantly affected by OA-SynFlu. Finally, we assessed the expression of matrix metalloproteinases (MMP1 and MMP13), as relevant markers of the matrix degradative process. In both cell types, the production of MMP1 and MMP13 was higher in devices cultured with OA-SynFlu than with H-SynFlu, indicating that the presence of pathological synovial fluid triggers degradative events. Altogether, these results demonstrate that our model recapitulated typical OA hallmarks, such as increased levels of proinflammatory cytokines and degradative enzymes. Lastly, we used our OA joint-on-a-chip model as a screening tool to test orthobiologics. In particular, we evaluated the effects of allogeneic BMSCs and ASCs within patient-specific models, in the perspective of a personalized medicine setting. The quantification of cytokines collected from the devices after MSC injection showed that patient-specific models responded differently to the treatment. Similarly, the analysis of MMP1 and MMP13 expression revealed differences among patients. Indeed, the expected therapeutic effect of MSCs was observed only in some patient-specific models, while other patients were not responsive to the treatment.

CONCLUSION

Our joint-on-a-chip model allowed monitoring changes induced by MSC treatment in inflammatory and degradative profiles, revealing a high variability among patients. These evidences suggest that our joint-on-a-chip model could be used as a platform to stratify the OA patients based on their potential responsiveness to orthobiologics, as well as to test other biological therapies, such as secretome and extracellular vesicles.

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Development of a vascularized osteochondral microfluidic model as a drug screening tool for osteoarthritis

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INTRODUCTION

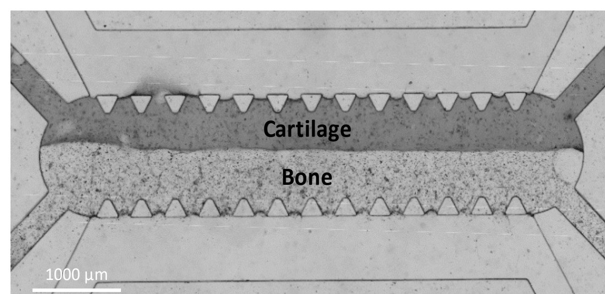
As a whole-joint disease, osteoarthritis (OA) affects all joint components including the articular cartilage and the subchondral bone. Since the current therapies mainly focus on pain relief and symptom suppression, a comprehensive understanding of the interaction between different cell types in cartilage and subchondral bone might pave the way for developing new therapeutic agents counteracting the disease progression. In this context, it is of paramount importance to develop organotypic human-derived in vitro models to study the pathology and screen potential drugs. What follows is an overview of a microfluidic model of osteochondral interface that we developed to mimic OA-like inflammatory conditions and investigate the effects of anti-inflammatory drugs. We looked into the expression of markers involved in cartilage degeneration, bone remodeling and angiogenesis in healthy, OA-like and drug-treated groups.

EXPERIMENTAL

The model contains cartilage and bone compartments in direct contact. The cartilage compartment was realized by embedding healthy human articular chondrocytes in fibrin hydrogel. To model the bone, osteoclasts and osteoblasts as the main actors of bone remodeling, together with endothelial cells and mesenchymal stem cells responsible for vascularization were embedded in fibrin hydrogel enriched with calcium phosphate nanoparticles to mimic the mineralized extracellular matrix of the bone [1]. After a week of maturation, the model was treated with the inflammatory cytokine Interleukin-1 beta (IL-1β), one of the main inflammatory mediators involved in onset and progression of OA, to mimic the disease condition. Afterwards, two different drugs were tested: Interleukin-1 receptor antagonist (IL1Ra), as an inhibitor of IL-1β signalling cascade, and Celecoxib which is a COX-2 inhibitor and is commonly prescribed to modulate the symptoms in arthritis. On day 14, the samples were fixed and immunofluorescence was performed to assess the expression of different markers.

RESULTS AND DISCUSSION

Matrix metalloproteinases (MMP-1 and MMP-13) were selected as markers of cartilage degradation. As expected, MMP1 and MMP13 were upregulated upon IL-1β treatment, while they were not significantly affected by the drug treatment. The pro-angiogenic vascular endothelial growth factor (VEGF), was expressed mainly by endothelial cells in the control group, while it was expressed also by both osteoblasts and osteoclasts in IL-1β treated samples. Interestingly, IL1Ra applied to IL-1β-treated samples induced conditions similar to the control group, where mainly endothelial cells expressed VEGF. Differently, Celecoxib halted VEGF expression altogether. As for bone remodeling, Receptor Activator of Nuclear Factor-kB (RANK) expression by osteoclasts was significantly increased in OA group compared to the control. Both drugs decreased RANK expression, with the effect of Celecoxib being notably more than that of IL1Ra.



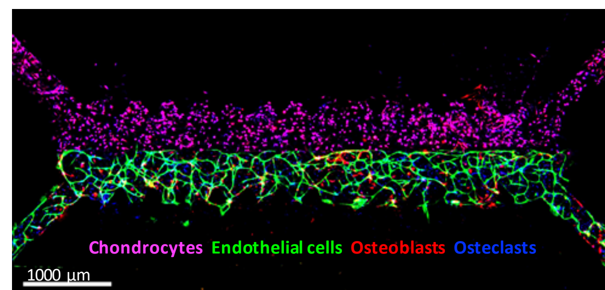


Figure 1. Phase contrast (Top) and immunofluorescence (Bottom) images of the microfluidic osteochondral model. A clear interface is seen between the cartilage and bone compartment while in direct contact. A microvascular network is created in the bone compartment thanks to the self-assembly of endothelial cells.

CONCLUSION

We developed a vascularized microfluidic model of the osteochondral interface, and we demonstrated that our model is responsive to inflammation and can be used to investigate the effect of anti-inflammatory drugs. Remarkably, this model allows studying multiple biological processes, involving both cartilage and subchondral bone, known to have a role in OA onset and progression, as well as dissecting the complex crosstalk between cartilage and bone cells.

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Alginate-Collagen bioink optimization for 3D bioprinted ovarian cancer cell lines and organoids

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INTRODUCTION

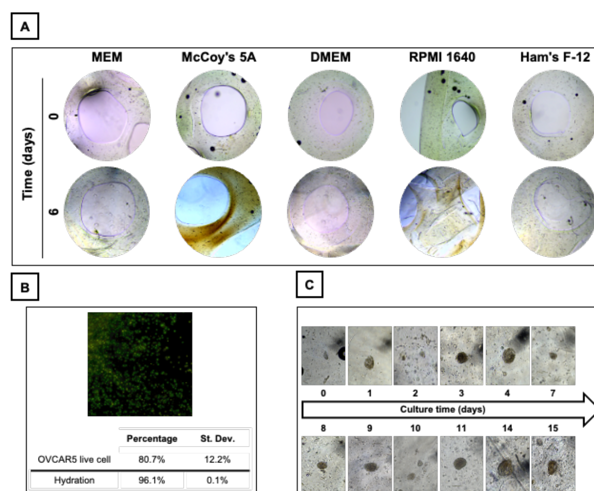
Ovarian cancer (OC) is a heterogeneous disease composed by a complex system of cells that can present spatially and temporally genetic differences and, together with the development of drug resistance, make OC as the most lethal gynaecologic cancer[1]. Current 2D culture methods present a limited ability to mimic OC heterogeneity and various 3D models such as spheroids/organoids are not able to precisely control multiple cell type organization in complex structures. The production of complex, multi-cellular and reproducible constructs are achieved through the three-dimensional bioprinting. The bioink used to print cells and organoids can be adapted globally or locally to provide the more suitable condition (e.g., rigidity) for cells growth[2]. Bioink optimization to successfully bioprint and promote cell viability is a highly active research field. The aim of bioinks consists of providing a suitable environment that supports cell proliferation, and cell differentiation after printing. In this work, we aimed to develop an Alginate-Collagen (SA-COL) bioink that can successfully support OC cell lines and OC mouse ovarian organoids viability, proliferation, and growth.

EXPERIMENTAL

SA-COL bioink was synthetized by dissolving 5% Sodium Alginate in MQ through magnetic stirring for 1 h at 50 °C, then 3% of collagen from rat tail was added. Cells/organoids were added to the bioink and bioprinted using Cellink Inkredible using a 20 mm grid layout to maximise the contact between cells and media. The construct was crosslinked with $CaCl_2$ 50 mM for 1 minute. Live/Dead (L/D) assay was performed to evaluate the extrusion damage. Cells and organoids were cultured for 30 days and pictures were taken. The effects of different media on SA-COL construct were evaluated over 10 days to define which media can efficiently support cell growth without compromising the bioink. Hydration percentage of the printed construct was evaluated by weighting it before and after 24 hours of lyophilization. This model was also evaluated through the treatment of the construct with our PIN1 inhibitor VS10 over 72 hours and L/D assay was performed every 24 hours.

RESULTS AND DISCUSSION

SA-COL bionks are characterized by high transparency and an optimal printability. The behaviour of SA-COL constructs with different media highlighted that McCoy's 5A and RPMI 1640 are not suitable to be used with this bioink as they led to structure lost after only 4 days of contact (Fig. 1A). OVCAR5 cells were successfully printed and cultured in DMEM (supplemented with 10% FBS, 100 U/ml Penicillin and 100 μg/ml Streptomycin) achieving 80.7% of live cells after the extrusion and this result is also due to the low printing pressure. The bioprinted construct showed a great ability to adsorb water reaching 96.1% of hydration (Fig. 1B). Hence, also mouse ovarian organoids were able to grow in SA-COL construct for at least 15 days without passages, becoming a promising long-term model for OC (Fig. 1C).



	Percentage	St. Dev.
OVCAR5 live cell	80.7%	12.2%
Hydration	96.1%	0.1%

Figure 1. *A) Study of the SA-COL bioink structure in contact with different media.* ***B)*** *Live/Dead assay of OVCAR5 bioprinted in SA-COL bioink and hydration percentage of the construct.* ***C)*** *Mouse ovarian organoids culture in SA-COL bioprinted construct.*

CONCLUSIONS

3D bioprinting is a promising technique that will accelerate the understanding of OC by creating always more accurate models of this disease. This work showed that SA-COL bioinks are able to sustain OC cell lines and organoids growth. Hence, this work highlighted that different aspects that must be considered to develop a suitable bioink such as its long-term stability and ability to sustain cellular growth. The success of this technology will require an interdisciplinary approach including microfluidic and organ-on-chip to obtain more accurate models of OC.

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Human mature omental adipocytes used for paclitaxel delivery to ovarian tumor organoids.

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INTRODUCTION

Ovarian cancer (OC) is still the deadliest disease among all gynecological malignancies. The disease is managed by applying aggressive surgery that is followed by chemotherapy. Paclitaxel is still the main chemotherapeutic used in OC treatment, but hydrophobicity and untargeted drug delivery are still important problems to overcome. To face such challenges, we took advantage from the established crosstalk between omental adipocytes and ovarian cancer cells in the tumor microenvironment. Thus, we propose omental mature adipocytes extracted directly from patients undergoing surgery as paclitaxel reservoir for cell-based therapy in OC taken advantages from their ability to release fatty acids and secondly from the cancer cells propensity to migrate towards adipocytes [1]. To achieve this goal, we established a coculture based model of adipocytes and patient derived ovarian tumor organoids to mimic the proximity observed between the two cell types in vivo. To progress in investigating the mechanism behind adipocyte-based paclitaxel delivery to ovarian tumor organoids a microfluidic platform for long term 3D *in vitro* culture of mature adipocytes will be set up.

EXPERIMENTAL

We loaded 2µg/ml of paclitaxel inside 500µl of package volume (PCV) of adipocytes and named them living paclitaxel bullets (LPB). The absolute amount of paclitaxel in the uptake and release experiments was determined by reverse phase liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS). Paclitaxel extraction from adipocytes was performed with 100% acetonitrile solution containing docetaxel used as an internal standard. We further perform AnnexinV vs 7AAD to identify the chemotherapeutic effect of the drug on the SK-OV-3 cell line. In addition, we performed cell cycle analysis to compare the effect of the parental drug and our formulation to arrest cells in the S and G2/M phases. Finally, we established a Live/Dead assay on ovarian tumor organoids cocultured with LPB to show the dead cells inside tumor organoids upon treatment.

RESULTS AND DISCUSSION

The viability of human omental adipocytes was confirmed by using Calcein AM (Figure a,b). LPB show a time dependent drug uptake by reaching near 100% within one hour as demonstrated through LC-HRMS measurements. In addition, we show the intralipid encapsulation of fluorescent paclitaxel inside LPB (Figure c). We demonstrate that mature adipocytes can transfer fluorescently labeled paclitaxel to human ovarian tumor organoids and to human ovarian cancer cell line (OVCAR-5, SK-OV-3, STOSE). We prove that LPB show a non-significant toxicity on mouse derived liver organoids and on human derived fibroblast MRC5. We examine the drug release efficacy using LC-HMRS and show that LPB could release 20% paclitaxel within 48h. The live/dead assay shows a significant increase of PI+ cells in the LPB treated sample respected to the untreated (Figure d). A method to keep adipocytes viable for long term culture could enlighten us on the exact mechanisms behind this drug delivery system.

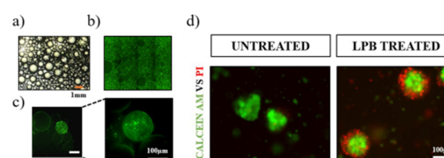

Figure 1. LPB drug effects on ovarian tumor organoids.
a) A phase contrast image of extracted omental human mature adipocytes
b) Calcein AM staining image of adipocytes. Image was acquired using confocal fluorescence microscopy.
c) Intralipid encapsulation of fluorescent paclitaxel inside mature human adipocytes was determined by confocal fluorescence microscopy.
d) Live/dead assay. Calcein AM versus propidium iodide staining of LPB treated ovarian tumor organoids was compared to untreated tumor organoids to assess death after co-colture with LPB.

CONCLUSION

Adipocytes are complicated to handle when cultured *in vitro*. This is due to their fragility and buoyancy in aqueous solution. However, it is still preferable respect to established preadipocytes cell line since this latter do not recapitulate all the physiological conditions of the omental adipocytes. In this direction, some research groups are optimizing 3D adipocytes culture method to keep adipocytes for more days in culture. In this view, our future research aims to establish a more advanced method such as microfluidic system to create a model that mimic better the *in vivo* drug delivery and the distribution of the drug in the tumor microenvironment by using tissue on chip models containing 3D cultured adipocytes and 3D patient derived tumor organoids.

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PDAC-on-chip: modeling the stromal and pancreatic cancer cells crosstalk in vitro

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INTRODUCTION

The pancreatic cancer is the most frequent type of exocrine pancreas tumor and one of the leading causes of cancer-related death worldwide with a five-year survival rate of below 9% [1]. Current research is focusing on better understanding pathologies onsets to tailor effective treatments through the development of pancreatic cancer in vitro models. To date, the tumor-stroma crosstalk remains extremely challenging to be reproduced and monitored in functionally effective models. Therefore, the main purpose of this work is to design and develop an innovative biomimetic 3D in vitro model that replicates the composition of pancreatic exocrine unit in a microfluidic device.

EXPERIMENTAL

A multilayer PDAC-on-chip composed by a top and a bottom layer was developed to culture the PDAC cells (HPDE-KRAS) and the pancreatic stellate cells (PSCs) embedded in a type I collagen gel in the top and bottom layers, respectively. The presence of a biomimetic nanofibrous membrane in the middle of the chip permits to control the interactions between the two cell lines and to easily analyze the effect of the crosstalk on cell behavior. The biomimetic membrane was obtained through electrospinning of a polycaprolactone and gelatin (PCL/gel) solution.

RESULTS AND DISCUSSION

The PDAC-stromal cell relationship was evaluated under co-culture conditions on 24-well inserts including the PCL/Gel electrospun membrane. This simplified model shows that human fibroblasts change their morphology and secrete larger amounts of IL-6 cytokines in the presence of tumor cells, confirming the activation of stromal cells under co-culture. Then, the PDAC-on-chip system was validated by demonstrating that human fibroblasts seeded in a 3D collagen matrix in the bottom microchannel also change to a myofibroblast-like shape with increased expression of α-SMA and secrete larger amounts of IL-6 cytokines. The drug sensitivity of HPDE-KRAS cells cultured in the microfluidic platform was characterized through exposure to different chemotherapeutic agents (bortezomib and gemcitabine) confirming that this microfluidic system is suitable for the evaluation of drug efficacy and serves as a powerful tool for understanding the early evolution steps of PDAC.

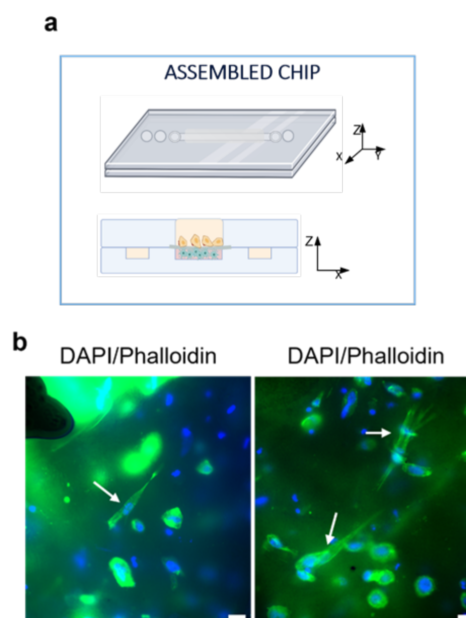

Figure 1. Seeding of PSCs and HPDE-KRAS in the assembled chip under co-culture conditions. (a) Schematic illustration of the assembled chip with the HPDE-KRAS cells seeded in the top and the PSCs in the bottom layers, respectively. (b) Representative confocal images at high magnifications showing the PSCs with elongated spindle shapes, indicated by the white arrows. Scale bars 20 µm.

CONCLUSION

The developed PDAC-on-chip chip design resembles the acino-ductal unit of the exocrine pancreas composed by acinar and ductal epithelial cells surrounded by PSCs which is the anatomical unit where the PDAC mainly develops. Finally, this microfluidic system serves as a powerful tool to mimic evolution steps of PDAC (e.g. fibroblasts activation) and resulted suitable for the evaluation of drugs efficacy.

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3D-printed cell culture system as an *in vitro* platform for non-small cell lung cancer (NSCLC) modelling

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INTRODUCTION

Lung cancer is a heterogeneous disease and represents the leading cause of cancer-related death worldwide(*1*). Disease progression is promoted by a complex microenvironment, which includes several cell types, matrix components(*2*) and pervasive angiogenesis(*3*). Since traditional 2D culture fails at resuming the complexity of *in vivo* model, the aim of this work is the development of a printed *in vitro* device, that mimic NSCLC, which could be use as platform for molecular studies and drug screening.

EXPERIMENTAL

Hydrogel based on methacrylate gelatin (GelMA) (*4*) was used as matrix for the establishment of long term cell culture. Preparation protocol and degree of functionalization (DoF) are depicted in Figure 1. To mimic NSCLC features, two adenocarcinoma lines, A549 and H1299, and normal fibroblasts, MRC-5, were selected. Then, biocompatibility was analyzed by MTT assay, Calcein AM/PI staining and flow cytometry. Matrix production was assessed by qRT-PCR, western blot and immunofluorescence. For 3D printing, an extrusion-based bioprinter system (Regenhu) was used.

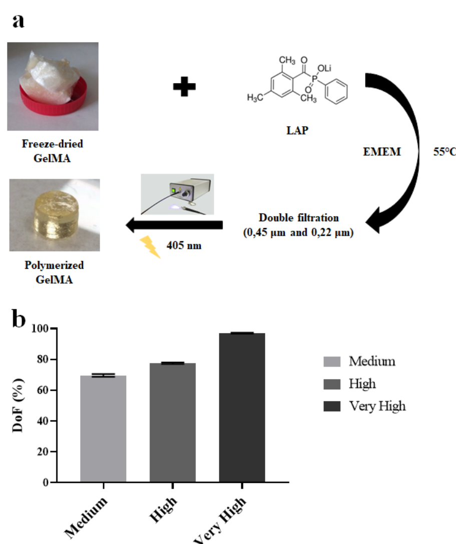

Figure 1. Schematic representation of GelMA hydrogels preparation (a) and methacryloyl substitution of different GelMA formulations determined by fluoraldehyde o-phthalaldehyde assay(b)

RESULTS AND DISCUSSION

Three different GelMA formulations (Medium, High and Very High), were obtained by modulating gelatin degree of functionalization (DoF). These matrices were tests at several concentrations to identify preferable culture conditions. A549 and H1299 cells were grown as mono-culture or co-culture at two tumor: fibroblast ratios (1:1 and 1:2), to determine the cross-talk between them. MTT analysis revealed promising proliferation increase in High and Very High, both at 10%, for A549 model; whereas, for H1299, GelMA percentage must be increased at 12,5% (Figure 2). Good cell viability was demonstrated by Calcein/PI staining. Moreover, the maintenance of the ratio of the two cell types was verified by flow cytometry. Protein and RNA analysis showed a strong fibroblast activation in terms of α-SMA and Collagen I production when cultured in 3D, whereas this modulation was completely lost in the co-cultures suggesting an interplay between the two populations. Further, both models were successfully printed by extrusion without affecting viability.

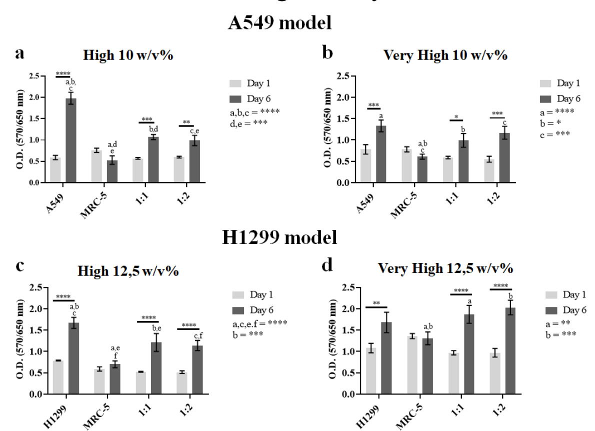

Figure 2. MTT assay of different analyzed model

CONCLUSION

Our results support the feasibility of using GelMA as matrix for 3D long term cell culture. Further analysis will be done to dissect the crosstalk that interplays between the two cell types to understand how tumor cells modulate fibroblasts behaviour. Further, GelMA printability allow the implementation of the model by adding a microfluidic system inside the scaffold to mimic the physiological vasculature. Hence, in the next future, the contribution of the endothelium will be investigated.

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INTESTINAL EPITHELIUM ON CHIP FOR PHARMACOLOGICAL STUDIES

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INTRODUCTION

Discovery of new therapeutics is an expensive and risky process: success rate of candidate drugs is lower than 0.1% with severe waste of investments, in terms of money, time and human safety[1].

In this scenario, the study of absorption, distribution, metabolism and excretion (ADME) is crucial, moreover the absorption efficiency of orally administered therapeutics occurring at the gastrointestinal tract (GUT) level is paramount for their success[2].

Current methods used to assess GUT absorption lack in recapitulating 3D.

Therefore dynamically active microenvironment of intestinal epithelium and Organ on chips (OoC) models have been lately proposed to overcome this limitation[3].

EXPERIMENTAL

We developed an *in-vitro* model of intestinal epithelial barrier in an OoC (uBeat® patented technology[4]) able to provide for mechanical stimulation mimicking the peristaltic motion (Figure 1).

The barrier was recapitulated by culturing CACO2 and HT29 cells mixed in a 9:1 ratio (8 10^6 cells/mL) adhered to a fibrin hydrogel matrix. 10% uniaxial strain stimulation at 0.2 Hz was exerted for 9 days to induce tissue maturation. In particular, the effects of impulse-wave stimulation and sine-wave stimulation were compared to the cells cultured in static conditions.

As readouts, brightfield and fluorescence microscopy analyses were performed in order to compare cell proliferation (Ki-67), epithelial integrity (ZO-1), and specific protein production (villin, mucin) among groups. Moreover, fluorescent dextran (4 kDa) diffusion studies were performed to assess the integrity of the epithelial barrier.

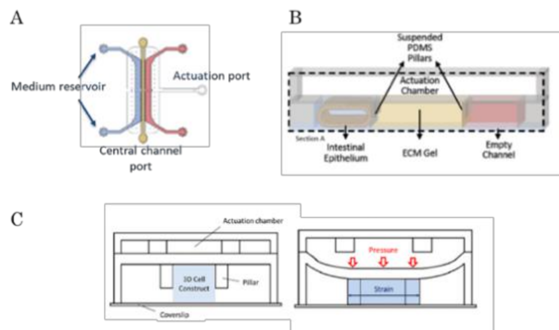

Fig. 1 : Gut on chip model. (A) Top view of the chip showing the ports for medium change and cell seeding, and for ECM (fibrin hydrogel) injection. (B) cross-section of the device showing the biological experiments configuration. (C) Stimulation on the gut on chip through the pressurisation of the pneumatic chamber located on top of the cell culture compartment.

RESULTS AND DISCUSSION

Brightfield microscopy images showed the presence of cells proliferating and self-assembling in 3D villi-like structures. The amount of such structure is higher in both stimulated groups, while the static control mainly showed a 2D cells monolayer (Figure 2).

Mechanically stimulated tissues feature a higher ZO-1 level than static ones. Sine-wave stimulation is also responsible for an increase in villin-positive zones and larger areas hosting 3D-structures, with a statistical difference with respect to both other experimental conditions.

The on-chip epithelial barrier developed with sine-wave stimulation was shown to be impermeable to Dextran even after 1 hour of incubation, therefore proving the functionality of the *in-vitro* model.

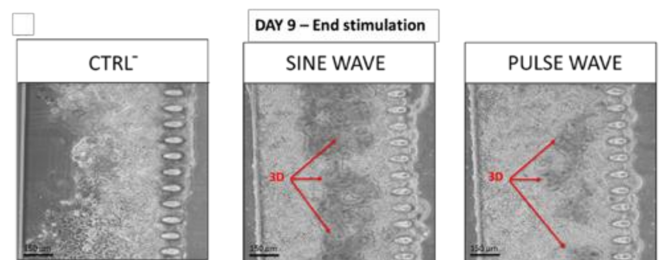

Fig. 2 : Comparison of the intestinal epithelium on chip after 9 days of culture. Cells subjected to two different patterns of stimulation were compared to unstimulated constructs showing that physiological mechanical cues are able to generate 3D villi-like structures (red arrows). Scale bar= 150 µm

CONCLUSION

The system has shown that the mechanical stimulation is a fundamental cue that guides cell behaviour: physiological-like stimulation leads to a massive generation of three-dimensional villi-like structures and improves the stability of the epithelium.

The developed gut-on-chip model represents a powerful tool for drug development that can provide for more accuracy and physiological relevance when it comes to ADME profiling.

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Novel fully dynamic *in vitro* model of Human Blood-Brain Barrier (hBBB)-on-chip with a physiologically relevant structure

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INTRODUCTION

Organ-on-Chip technologies to develop *in vitro* models of the Blood-Brain Barrier (BBB) are considered promising to overcome the limitations of animal models and traditional *in vitro* systems, with the perspectives of accelerating the development of novel drugs and therapies while decreasing the huge expense associated with the failure of preclinical predictions and clinical tests. Focused research is still needed to establish and validate the most convenient design strategy for better mimicking the human BBB [1].

EXPERIMENTAL

Herein, it is reported a novel fully dynamic human BBB-on-a-chip model (Fig. 1) with a double-layer layout and an interposed porous membrane, by culturing an immortalized line of human brain endothelial cells (hCMEC/D3) in the blood side, while primary human pericytes and astrocytes in the brain side.

RESULTS AND DISCUSSION

The developed model resembled the physiological hBBB cellular architecture: i) flow exposure of the endothelial cells (ECs) with the resulting elongation and alignment of the cells in the direction of the laminar flow, ii) pericytes migration for assisting the engineered microvessels organization; and, iii) multilayer and interconnected distribution of astrocytes. Finally, permeability assays and trans-endothelial electrical resistance measurements allowed for the investigation of the barrier integrity and functionality of the model in different conditions.

Human Blood Brain Barrier (hBBB)-on-a-chip

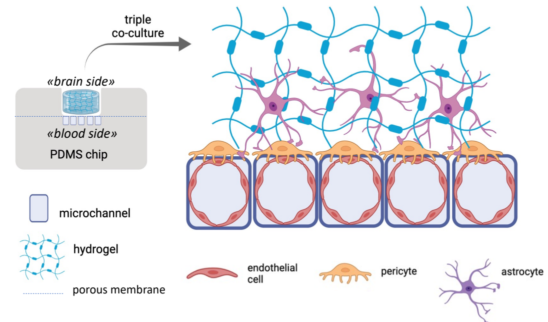

Figure 1. Graphical representation of the proposed in vitro BBB model

CONCLUSION

The results of this study show that the proposed hBBB-on-chip can model the BBB cellular architecture better than other similar OoC-based models, which report neither ECs flow-induced alignment nor pericytes migration [2]. At the same time, this model was able to guarantee full control of the intraluminal flow and better reproducibility than OoCs based on a self-organizing approach, that promotes a 3D physiological but unpredictable network of *ex-novo* microvessels in contact with the brain cells [3].

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A Human Multi-Organ and Dynamic in Vitro Model for Simultaneous and More Predictive Toxo-Efficacy Assays

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INTRODUCTION

The poor rate of success of clinical trials, especially in oncology field, is becoming increasingly evident due to the poor predictability of pre-clinical assays (1). In fact, while in vitro assays fail in recapitulating the complexity of human tissues and organ-organ connection, animal models reveal species-specific drug outcomes.

In this work, we adopted a novel multi-organ microphysiological system (MIVO®) to fluidically connect 3D ovarian cancer tissues with a hepatic cellular model and simulate the systemic cisplatin administration for investigating its anticancer efficacy and simultaneously evaluating potential hepatotoxic effects.

EXPERIMENTAL

Human hepatocellular (Hep-G2) and ovarian cancer (SKOV-3) cell lines were used to realize liver models and 2D/3D tumour models (2). Computational fluid dynamic (CFD) simulations were performed to simulate the capillary blood velocity, that was set-up within MIVO® where 3D ovarian cancer and the liver model were cultured fluidically connected. First, a screening of drug (cisplatin) concentration (10-100µM) was performed by using 2D and 3D single organ models. Then, drug efficacy and toxicity assays were assessed in multi-organ device and results were compared with both static co-cultures and single dynamic organ models. Ovarian and liver cells death, half maximal effective concentration (EC50) and median lethal dose (LD50) for SKOV-3 cells and Hep-G2 cells, respectively, were quantitatively (metabolic analysis) and qualitatively (immunofluorescence) assessed after 48 hours of treatments.

RESULTS AND DISCUSSION

A linear decay of Hep-G2 and SKOV-3 cells viability was observed with increasing cisplatin concentration. This effect was remarkably more evident on the 2D culture with respect to the 3D, which provided cells with a higher drug resistance. Then, no difference in cisplatin response of SKOV-3 cultured in 2D was observed comparing the mono-culture and co-culture, while tumour drug resistance was higher when cells grown in 3D were co-cultured with the liver model; at the same time, a lower toxicity on liver cells was noticed in co-culture with 2D tumours with respect to 3D. Therefore, the dimensional growth of ovarian cancer, more than the presence of the tumour itself, affected hepatic cells susceptibility to cisplatin. In the dynamic single organs setting within the MIVO® device, no differences between liver cells in static and dynamic conditions were observed, while for the 3D ovarian cancer there was a higher drug efficacy in dynamic system. This result was also predicted by the CFD simulations showing that there was a significant increase of the percentage of drug capable of reaching the cells between the 3D static and the dynamic conditions, meaning that the fluid flow enhanced the distribution of cisplatin within the 3D model. Finally, the multi-organ-on-chip was adopted to fluidically connect the ovarian cancer model with the hepatic one. At the clinical plasma concentration (10µM), comparing the results obtained from the static co-cultures, in the dynamic multi-organ system an increase in anti-tumour efficacy of the drug coupled with a decreased toxicity on the liver model were observed, thus representing the best scenario to be associated with what happens during the therapy.

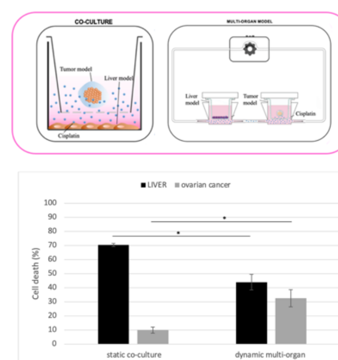

Figure 1. Static co-culture vs dynamic multi-organ model

CONCLUSION

The combination of 3D cell culture, fluidics and multi-organ connections represent the closer scenario to clinics when evaluating the effects of anti-cancer drugs, both in terms of efficacy and toxicity. This indicates the importance of developing and optimizing more predictive platform in pre-clinical tests.

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The epigenetic drug Decitabine co-operates with the IL-33/ST2 axis modifying the tumor microenvironment and promoting anti-tumor activities against melanoma

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INTRODUCTION

IL-33 is an atypical alarmin belonging to the IL-1 cytokine family that plays multiple roles in allergies, autoimmunity and inflammation. Due to its oncologic role recently redefined, IL-33 is an excellent candidate as an immune modulator for the development of new combined therapies, which can lead to encouraging results in the treatment of metastatic melanoma [1]. According to the molecular characteristic of melanoma, its occurrence and progression are due to the accumulation of genetic and epigenetic alterations. In this study, we have explored the combination of IL-33 with the epigenetic drug Decitabine (DAC), a DNA methylation inhibitor that promotes immune recognition by re-activating silenced genes [2]. We evaluated the efficiency of this combined therapeutic approach *in vitro*, *in vivo* and by organ-on-chip (OOC) system.

EXPERIMENTAL

We evaluated the direct effects of DAC and IL-33, singly or in combination, on multicellular spheroids of murine B16.F10 melanoma cells. We next assessed the antitumor effects of DAC/IL-33 in vivo in C57BL/6 mice transplanted with B16.F10 cells. Tumor growth and tumor-immune infiltrates were evaluated at defined times post treatments. We further evaluated immune cell recruitment by a organ-on-chip (OOC) approach in a competitive migration assay [3], in which splenocytes from naïve C57BL/6 mice could preferentially be directed to one of the side chambers containing the B16.F10 not treated or alternatively treated with DAC, IL-33 and DAC/IL-33. We analyzed the role of IL-33 signaling in fostering immune cells migration in our OOC system by using splenocytes from mice deficient for the IL-33 receptor ST2 (ST2 KO) in the device. This analysis was also validated in vivo by comparing the anti-tumor efficacy of DAC in ST2-KO vs WT mice. Given its DNA methylation inhibition activity, we carried out standard and methylation-specific qPCR to see if DAC can modulate the gene expression of IL-33 in B16.F10 cells. Finally to investigate the mechanism of the drug we conducted a DNA methylation assay.

RESULTS AND DISCUSSION

In vitro, DAC exerted direct anti-tumor activity independently of IL-33 inducing the disaggregation of 3D melanoma spheroids. *In vivo*, combined DAC/IL-33 treatment was the most effective in reducing tumor growth in C67BL/6 mice transplanted with B16.F10 melanoma cells, with respect to single treatments or control. Moreover, DAC/IL-33 treatment was the most efficient in promoting T cell recruitment at the tumor site and induced the up-regulation of PD-1 resulting in better anti-tumor response to PD-1 blockade *in vivo*. In the competitive OOC migration assay, exposure of melanoma cells to DAC/IL-33 produced the most powerful chemotactic stimuli for naive syngeneic spleen cells towards melanoma cells, with respect to either single treatment. This trend was maintained or even increased in all competition conditions even at 48 hours from loading. Notably, splenocytes from mice deficient for the IL-33 receptor ST2 (ST2-KO) failed to migrate towards the DAC/IL-33 melanoma chamber, suggesting a role of IL-33/ST2 axis in fostering immune cells migration (Figure 1). Accordingly, *in vivo* treatment with DAC was completely ineffective in reducing tumor growth in melanoma-bearing ST2-KO mice. *In vitro*, DAC dose-dependently increased the expression of IL-33 in melanoma cells through demethylation of a transcription factor binding site located inside the IL-33 gene sequence.

CONCLUSION

Our findings indicate that DAC effectively co-operates with IL-33 against melanoma, promoting immune cell recruitment and modulating the tumor immune microenvironment. The anti-tumor activity of DAC relies, at least in part, on epigenetic regulation of the IL-33/ST2 axis. Targeting the tumor microenvironment with epigenetic drugs that restore the expression of silenced genes with immunotherapies (i.e., IL-33, driving the infiltration of immune effectors) is a promising strategy to overcome therapy resistance in melanoma patients.

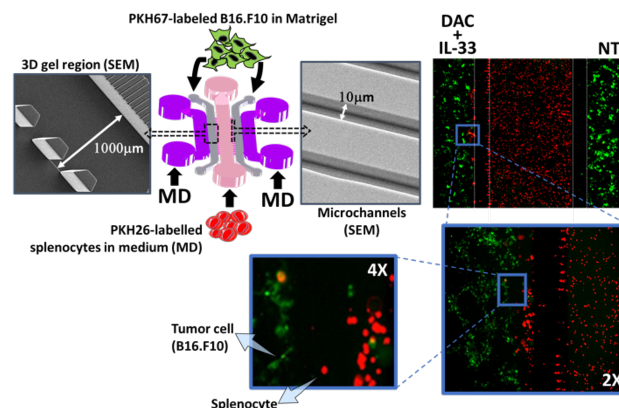

Figure 1. Experimental design of OOC system and results.

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Micro-channels array by two photon lithography for cells migration studies

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INTRODUCTION

The study of tumor cell migration, a key event in cancer metastasis, is of paramount importance in biological and medical research [1]. Microfluidics and microscale technologies can more faithfully represent the *in vivo* environment, and potentially support research on such complex issues. However, standard microfabrication techniques fail to achieve the required single-cell resolution due to the limitation to a range of hundreds rather than tens of micrometers. To gain resolution and nanometric precision we relied on two-photon-polymerization (2PP) to create 3D prints. The combined use of the innovative 2PP technique with microfluidics is a promising tool for studying cell behavior in a specific and controlled microenvironment. We here demonstrate that these devices can be suitable for studying cell migration in the context of Neuroblastoma (NB) metastatic spread at a single-cell resolution, with micro-channels array that recall the shape and size of lymphatic vessels.

EXPERIMENTAL

The master mold for the device was designed with AutoCAD (2021, Autodesk) and comprised 2 lateral cell culture channels connected by a micro-channels array (**Fig.1a**). Each culture channel can be seeded with different cell types, which will interact only via the micro-cannels array. The mold was fabricated using a 2PP-based NanoScribe Photonic Professional GT 3D printer. To verify the quality of the produced master, Scanning Electron Microscopy (SEM, FEI Quanta 400) and 3D optical profilometer (Sensofar S Neox profilometer) analyses were performed. The final device was then produced via standard PDMS replica molding, bound to a glass slide, and sterilized. The platform was first validated using a human NB cell line (SKNAS) and human Mesenchymal Stem Cells (hMSC), chosen to represent the main NB metastatic target site. The device was then tested to observe the migration of both cell lines under different conditions (including the use of NB-derived extracellular vesicles, EVs). Cells were labelled with fluorescent tracers and their displacement along the micro-channels observed for up to 72 hours.

RESULTS AND DISCUSSION

The quality of the produced master was evaluated through SEM (**Fig.1b**) and 3D profilometer analyses. The hydraulic seal of the assembled platform was successfully validated with colored tracers (**Fig.1c**). The biological validation proved the device to be suitable for cell culture, maintaining viabilities >80% for both cell lines after 72 hours (**Fig.1d**). Results of the motility assay experiments, summarized in **Fig1.e**, showed that NB cancer cells increase their motility in the presence of EVs and decrease it when co-cultured with hMSCs, supporting the studies that report a tumor-suppressive behavior for hMSCs. NB-derived EVs have an even stronger influence on hMSCs motility, triplicating the distance that they cover after 72 hours. Cancer-derived EVs transfer their cargo to recipient cells and increase the capability of hMSCs to preferentially migrate towards tumor cells. Our results suggest an effect of NB EVs on hMSCs.

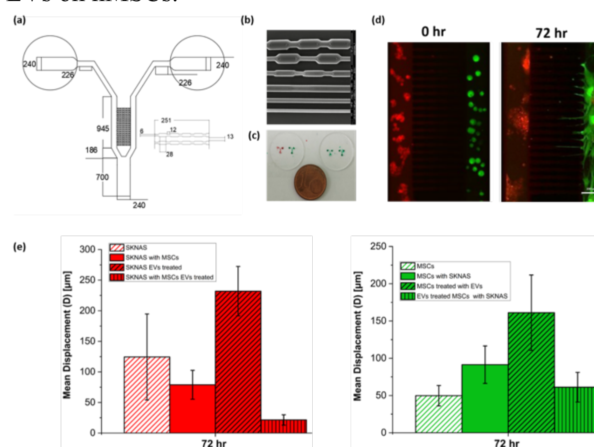

Figure 1. (a) 2D schematic of the device (units in µm); (b) SEM image of different printed micro-channels; (c) Replica of the PDMS device; (d) Fluorescence images of SKNAS (labeled with a red live cell tracker) and hMSC (labeled in green) at seeding time (t=0) and after 72 hours; (e)Results of the migration assays. The plots report the mean distance covered by cells in each experimental condition (SKNAS in the red plot and hMSCs in green).

CONCLUSION

In this work we designed an innovative microdevice that allows studying migration at single-cell resolution. The device was produced using a novel technology, two-photon polymerization, resulting in a reduction of time, costs, and quantities of materials compared to standard microdevices fabrication techniques. 2PP enabled fabrication of micro-channels with a complex geometry resembling that of lymphatic vessels with great repeatability and reproducibility.

The biological validation confirmed that the device was suitable for cell studies. Migration tests highlighted an increased motility of MSCs when cultured with cancer cells and revealed how NB-EVs internalization increased both SKNAS and hMSC motility.

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A novel platform for robust deep learning management of time-lapse videos in lab-on-chip experiments

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INTRODUCTION

One of the major problems in bioimaging is whether the features extracted for a pattern recognition task are robust enough to withstand unpredictable perturbations that may manifest during the image acquisition process. This issue is even more important when it is addressed in the context of deep learning features due to the lack of explainable descriptors (deep features). The increasing plethora of organ-on-chip applications requires a huge amount of video data to be analyzed, and therefore an urgent need of machine learning architectures for data mining and information retrieval. However, the reproducibility of experiments in lab-on-chip devices is still hindered by several aspects related to cell heterogeneity and difficulty in maintaining experimental settings controlled, suffering from a multitude of alterations such as brightness or texture changes, focus shifts, autofluorescence, or photobleaching, to name a few. In this work, we present a platform, called Deep-Manager (DM) [1] to efficiently select those features having lower sensitivity to unspecific disturbances and, at the same time, a high discriminating power. The software is applied to many scenarios from biological investigations, such as video of tumour-on-chip experiments acquired through phase contrast fluorescence microscopy [2]. Results expressed in terms of the accuracy of discrimination of different experimental conditions (drug vs control) demonstrate the superiority of the platform in selecting deep features robust to image degradation effects.

EXPERIMENTAL

The main steps of the DM functionalities are: 1) the user is first asked to select the practical scenario to work (e.g., 2D time-lapse microscopy (TLM), 3D Phase Contrast TLM, or 3D Fluorescence). All the tests available for the selected modality are applied; 2) the user is then asked to select the SETTING text file to load the threshold values for fixing the sensitivity and accuracy of classification bounds of the selected features. In addition, the user is asked to select a deep learning network among pre-trained architectures and the layer to implement *transfer learning*. By applying transfer learning from the perturbed images and from the original images, sensitivity to degradations of the accuracy of classification is computed and features with minimum sensitivity and high level of accuracy of discrimination are selected. An example of a possible outcome from the DM running is plotted in Fig. 1.

RESULTS AND DISCUSSION

To demonstrate the effectiveness of the proposed Deep-Manager software platform, we selected two use cases concerning deep feature extraction in 1) phase contrast time-lapse microscopy videos of immune cells moving in a 3D collagen gel inside microfluidic tumour-on-chip devices that mimic the tumour microenvironment [2] 2) 3D fluorescence microscopy videos of cancer cells going into apoptosis due to killing by cytotoxic T cells in 3D tumour-on-chip [3].

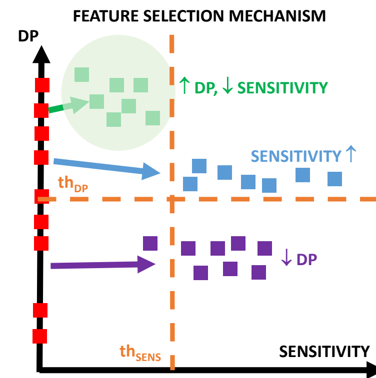

Figure 1. A sketch of the feature selection mechanism using Deep-Manager. The green features are those that verify the criterion of high DP values and low SENSITIVITY values.

By comparing the DM feature selection approach with state-of-the-art methods [1], we obtained an improvement in DP and sensitivity values in the range of 6-10% and 56%-69%, respectively, with the additional strength of automatically providing a reduced set of selected features.

CONCLUSION

The transition from the excellent results obtained in the laboratory to the clinical practice is anything but easy. In the real measurement scenario, many variables are not controlled, and their variations can lead to not acceptable performances. Such an issue is even more critical when it is addressed in the context of deep learning video analysis, unexplainable features, and with the urgent requirement of reproducibility of lab-on-chip experiments. In this work, we have introduced a software platform called Deep-Manager, that counteracts this limitation by analyzing the performance and sensibility of each feature to different disturbances. The potential of the proposed approach has been validated in tumour-on-chip scenarios with different simulated artefacts, evidencing superior performance over standard approaches.

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Parameter estimation of a chemotaxis model as forecasting tool for the dynamics in Cancer-on-Chip experiment

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INTRODUCTION

Immunocompetent Cancer-on-Chip technology enable to recreate complex environments to investigate the cross-talk between tumor and immune infiltrated cells in response to anticancer therapies.
The coupling with live-cell imaging may enable extraction of single-cell tracking profiles which can be processed with advanced mathematical tools.
In this framework, we explore the possibility of bridging the biological in-vitro models and the mathematical-based in-silico models in order to gain further insights into the observed phenomena. Indeed, using data extracted from the OOC biological experiments, in-silico models can be developed and then be applied as predictive models of the observed phenomena.
A macroscopic in-silico model is formulated in [1] and a methodology for estimating model parameters have been proposed and tested on synthetic data in [2].
Our framework is based on parabolic-hyperbolic partial differential equations describing reaction-diffusion-transport phenomena through the evolution of macroscopic quantities such as the density of cells in the chip environment and the spatio-temporal distribution of molecules. This formulation also includes the possibility of drugs administration for drug testing effects.

EXPERIMENTAL

For the development of our methodology we refer to in vitro microfluidic experiments and data in [3], where all the details about the laboratory settings can be found. The immune-oncology chip designed for the experiment consists of a complex geometry composed by cylindrical wells containing, separately, cell culture chambers of tumor and immune cells and allowing circulation of cells and chemical signals in all the chip interconnected areas.

RESULTS AND DISCUSSION

The main issues faced in this work are:
i) the preparation of real data applying Kernel Density Estimation method to transform microscopic data into macroscopic data;
ii) the development of a parameter estimation methodology suitable for data extracted from laboratory experiments, in order to get a forecasting algorithm for different laboratory settings.
iii) the comparison between the results obtained by the model and data extracted from video recordings of laboratory experiment [3] in terms of occupancy of the chip areas,

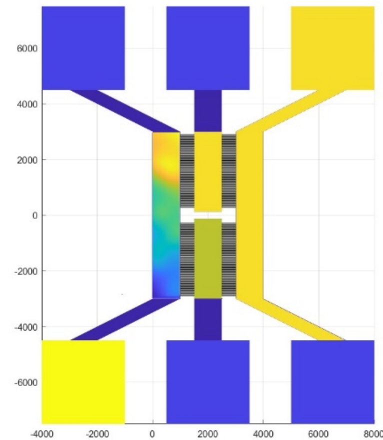

Figure 1. Reconstruction of concentrations of immune cells in microfluidic chip chambers obtained by the in-silico model.

CONCLUSION

Our methodology is tested on laboratory data extracted from [3] and shows the ability of the in-silico model of reconstructing the spatial and temporal distribution of cells and of chemoattractant field in the chip environment The advantage of our approach is the formulation of the problem through macroscopic quantities thus allowing the simulation of large number of cells in a reasonable CPU time.
The calibrated simulation algorithm can be employed both for the optimal design of laboratory experiments and for the drug-testing procedures to see the effects of therapeutic treatments.
Due to the non-trivial dynamics and evolution of the chemical field across time and space in the microfluidic environment, the distribution of chemical gradients is not easily measurable experimentally. Then, suitably calibrated, the simulation algorithm may be employed as a reliable tool of dynamics and interactions between immune and cancer cells and can provide time and space evolution of chemicals in the chip, even at a fine scale.

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POSTER PRESENTATIONS

Passive microfluidic technology helps drug testing in 3D HGSOC organoids

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INTRODUCTION

Every year, worldwide, 140000 women died for ovarian cancer (OC). Indeed, OC is the leading cause of gynecological death in the western countries. It is a silent fast deadly disease, since the lack of specific markers to diagnose it, combined with an absence of specific symptoms, are the cause of a late diagnosis that gives little chance of survival. Treatment consists of debulking surgery or neoadjuvant chemotherapy followed by interval debulking surgery, combined with poly-ADP ribose polymerase (PARP) inhibitors. Even if innovative alternative therapeutic approaches are being tested, the disease remains untreatable. In that scenario, personalized therapy can be of supreme importance. Especially in aggressive tumors such as High grade serous ovarian cancer (HGSOC), it is essential to provide a therapeutic plan as soon as possible to slow down the progress of the disease and increase life expectancy. Unfortunately, to date, there are no models that can faithfully replicate the complexity of ovarian cancer. A novel strategy may be the combination of organoids with microfluidics. Organoids are considered small miniature organs capable of replicating the pathophysiological characteristics of the primary tissue from which they come from. To date, organoids are cultured in a static way, and this leads to the development of necrotic areas in the inner core of the organoids, which reduces their growth and size. This could be overcome by the implementation with microfluidic technology. The controlled, and stable laminar flow (that mimic vessel flow) can better convey nutrients, oxygen and essential metabolites into the inner core of the culture increasing organoids growth[1]. In this way, it is possible to develop 3D cultures that are more representative of the human tumor.

EXPERIMENTAL

Primary tumors and ascites were processed to establish tumoroid cultures (under informed consent for research purposes), then were characterized by IHC showing positivity to PAX8, WT1 and CA-125 (markers of HGSOC) and biobanked. The best Organoflow conditions was refined to culture Organoids in the Mimetas platform. HGSOC organoids were seeded in passive flow and static cultures platforms and cell viability was measured every day for one week in order to establish the growth kinetics. Subsequently, they were treated with carboplatin, paclitaxel, doxorubicin, Caelyx and ATRA (Pin1 inhibitor) for 96h. IC50 was calculated by Resazurin staining, ATP based assay and DAPI/PI colocalization assays. Lastly, FITC-labeled paclitaxel was used to study the drug penetration of ECM in passive flow and static conditions. Figure 1 shows the set up of the experiment.

RESULTS AND DISCUSSION

Under optimized perfusion flow conditions, the cancer organoids growth was faster than static conditions and the number of dead cells was reduced over time. Organoids were treated with the drugs mentioned above for 96 h, dose-response curves were established and IC50 were calculated. The results showed that in the passive flow, the IC50 values were lower than static conditions. Hence, FITC-labelled Paclitaxel were used to show that in passive flow cultures there is a better penetration of the drugs through the ECM compared to static conditions. Indeed, organoids start to die after 48 hours of drug treatment instead of 96 hours, respectively.

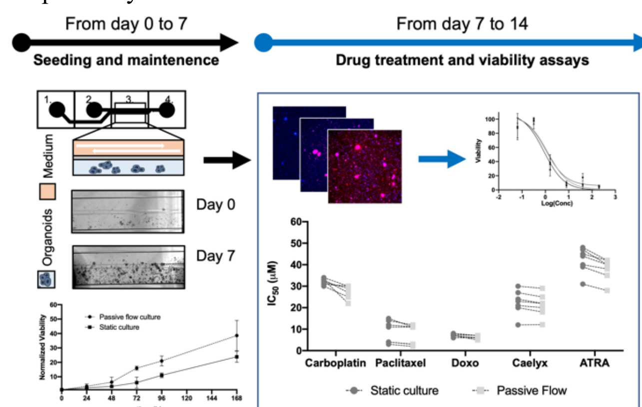

Figure 1. Flowchart: Organoids were seeded both in microfluidic platform and in 96-wells plates (day 0) and were maintained in culture until day7. The viability was calculated, and the growth curve was established. Organoids were incubated with the drugs for 96 hours and then the cell viability was measured; the dose-response curves were established and IC_{50} values were calculated.

CONCLUSION

HGSOC organoids cultures were successfully established in the Mimetas 2-lane OrganoPlate®, showing a higher proliferation rate and a lower death rate compared to static cultures. This can be attributed to the flow helping to carry nutrients, oxygen and to remove toxic substances. In addition, it was demonstrated a better drug penetration in the ECM with microfluidic technology, and a reduction in the IC50 values. This makes this technology a valid tool for drug screening in the personalized medicine, due to its simplicity, easy to use and its compactness. In a perspective in which the tumour progresses quickly and therefore time is essential, a compact platform that provides a result in a short time and which requires the use of a small quantity of sample acquires great importance as a tool for personalized medicine.

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Optimization of advanced setups with integrated readouts for evaluation of cardiac toxicity in a heart on chip device

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INTRODUCTION

Being inserted in the safety screening field, the aim of the present work was to develop and validate an experimental set-up able to predict functional cardiotoxicity of compounds by exploiting 3D engineered cardiac microtissues (uHeart), benchmarked against state-of-the-art 2D Multi Electrodes Array (MEA) based models. The validation was accomplished integrating a combination of readouts (i.e., motion analysis, calcium imaging, viability assays and field potential recordings.

EXPERIMENTAL

uHeart (Biomimx srl, Italy) platforms consist in an upper cell culture layer, where the cells are seeded embedded in a fibrin gel and are feed thanks to two lateral channels filled of medium by four wells per chamber, an intermediate actuation layer which provides a means to mechanically stimulate (i.e., uniaxial strain of 10%, 1 Hz) the 3D microtissue through a pneumatic actuation system and a bottom coverslip. uHeart platforms can additionally hosts electrodes to measure the electrical activities of the cells. Embryonic chicken CMs were isolated from 13 days old embryonic chicken hearts and seed onto the nitrocellulose-coated MEAs with a density of $20x10^3$ cells/µl, while in uHeart CMs were embedded in a fibrin gel (i.e., 10 mg/ml of fibrinogen and 2.5 U/ml of thrombin) at a final density of $100x10^3$ cells/ µl. In uHeart, the mechanical actuation was started at Day 2.

The evolution of microtissues in terms of morphology, viability and functionality was assessed by means of viability assays, contractility analyses to assess standard parameters (i.e., beating period-BP; contraction amplitude-CA; contraction duration–CD; relaxation time–RT), calcium transient analyses, electrophysiological parameters (i.e., RR interval, and the FPD). Finally, the responsiveness of the cardiac samples to a commonly recognized IKr blocker drug (E4031) was assessed.

RESULTS AND DISCUSSION

The 2D culture in MEAs differs from 3D microtissues in uHeart in terms of morphology: 2D CMs well spread at the bottom of the wells, while in the 3D configuration more branched cells were evidenced. Spontaneous beating onset was detected at Day 4 for 2D samples cultured on MEA and at Day 2 for 3D samples in uHeart. Viability assays at Day 1 didn't evidence any difference, while at Day 10 static and dynamic uHeart platform had higher viability (78.02 ± 4.62 % and 77,83 ± 3.72%, respectively) than MEA (60.7 ± 9.71%). Motion analysis (Fig.1) was accomplished to assess the synchronicity of the beating throughout the microtissues, calculated as correlation coefficient (CC) of the effective overlap of all the contraction peaks.

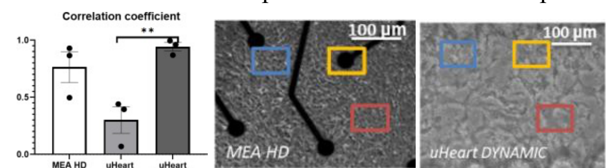

Fig.1: (a) CC computed by analyzing the trace of CA in different zones if the same microtissue in MEA and uHeart. Immunostaiing of MEA (b) and uHeart (c).

The highest values were detected for actuated 3D microtissue (97%), followed by 2D culture (94.6%). The lowest score was obtained in static uHeart, showing a correlation coefficient of about 56.2%. The same trend was obtained by computing the correlation coefficient derived from the calcium transient analysis. MEAs were used to assess the relation between electrical parameters and motional ones. The comparison between RR-interval and the BP carried out on the same day on the same samples yielded to a correlation coefficient of 0.996. The afore mentioned readouts were exploited to assess the effect of E4031 on cardiac microtissue. In all the samples a concentration higher than 5 nM induced an increase in BP and CD with respect to controls without drug, both if analyzed by means of video analysis (Fig.2), or through calcium transient. The same results were obtained also with the computation of RR interval through FP recordings. A further administration of 250 nM induced the arrest of the beat in all the samples, except in 33% of dynamic ones, which were subjected to a further increase in BP and CD.

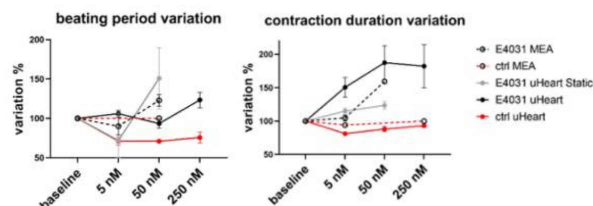

Fig. 2: variation of BP and CD induced by the dose escalation of E4031 in all differently cultured samples.

CONCLUSION

The experiments highlighted the capability of uHeart platforms to be not only a very useful and reliable system in achieving more advanced and highly functional cardiac microtissues, but also in allowing a higher number of combined readouts, potentially evaluated at the same time, with respect to other commercially available systems.

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Development of a liver-heart Multi Organs-on-Chip platform for drug toxicity studies

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INTRODUCTION

The drug development process (DDP) is an expensive and time-consuming route [1]. Most of current methods to assess drug safety in preclinical DDP often fail to detect and predict harmful human side-effects, such as hepatotoxicity and cardiotoxicity, which are the main causes of safety-related drug failures and withdrawals from the market [1]. Multi Organ-on-chip (MOoC) provides a promising solution to develop *in vitro* models enabling effective drug screening studies on target and on off-target tissue simultaneously [2]. In this work, we implemented and validated a liver-heart MOoC ('LivHeart') designed to reproduce the *in vivo* drugs biotransformation and screen their cardiotoxic side-effects upon the hepatic metabolism.

EXPERIMENTAL

The LivHeart (LH, Fig.1a) was designed to host in the same platform the micropatterned co-culture (MPCC) liver-on-chip [3] and the 3D beating heart-on-chip, integrated with a system of electrodes to achieve real-time monitoring of cardiomyocytes' electrical activity. [4]. Systems of normally-closed microfluidic valves were integrated into the platform to finely control the communication and the diffusion between the models, and to avoid convection due to possible hydrostatic pressure differences. Technical characterization was performed either experimentally and/or with finite element model to evaluate valves' working principle, to verify the application of physiological stretching on the cardiac model during the mechanical stimulation and to determine the diffusion dynamics within the platform. The liver model was generated by circular domains of HepG2 surrounded by NIH-3T3 fibroblasts. The cardiac model was generated by previously isolated neonatal rat cardiomyocytes (NRCMs) embedded in a fibrin gel and mechanically stimulated connecting the platform with a custom-made mechanical actuator. For drug toxicity testing, solutions of Terfenadine (TER), Fexofenadine (FEX) or DMSO (10μM each) were incubated first in the liver compartment and then let flow towards the cardiac compartment. Terfenadine and Fexofenadine content in the liver compartment was quantified by multiple reaction monitoring (MRM) mass spectrometry. The effects of TER (i.e., Terfenadine without liver metabolism), liver-metabolized Terfenadine (TER→FEX), FEX and DMSO on both cell types viability were examined and two electrical parameters, namely the beating period (BP) interval and the field potential duration (FPD), were measured from recorded beating electrical signals.

RESULTS AND DISCUSSION

The technical characterization studies confirm the application of a physiological stretching on the cardiac construct and the diffusion of compounds in absence of convective phenomena. Results obtained from the MRM mass spectrometry prove the effective metabolization of Terfenadine into Fexofenadine (FEX 90% vs TER 10% of the total amount of drugs in the liver compartment). MPCC viability is not affected by the incubation and metabolism of Terfenadine (as expected). Conversely, NRCMs viability is affected only by TER incubation (-11.76% compared to the control). The analysis of the electrical signals shows a decrease of the spontaneous beating frequency in all conditions compared to the control (TER -70%, TER→FEX -40% and FEX -77% vs DMSO, Fig.1b) and FPD increased only after the incubation of TER (TER +48% vs TER→FEX +12% vs FEX +6%, Fig.1c), in line with literature data.

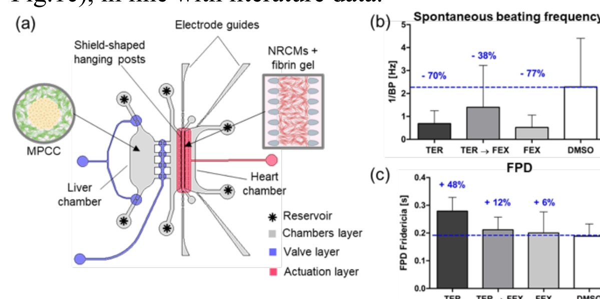

Figure 1. (a) LivHeart (LH) layout. (b) Spontaneous beating frequency and (c) FPD mean values

CONCLUSION

The work here presented proved the successful development of a MOoC platform to investigate the off-target effects of liver-metabolized drugs on cardiac cells functionality in a more physiologic way compared to available single-organ microfluidic platforms. Indeed, the results obtained by monitoring drug-induced alterations in cell viability and cardiac electrophysiology proved the cardiotoxic effects of Terfenadine (i.e., impaired cardiomyocytes viability and prolonged depolarization-repolarization interval) and the non-cardiotoxic effect of Fexofenadine (i.e., both cardiac viability and electrical activity were not altered by the metabolized TER). In conclusion, the LivHeart platform provides a further example of how MOoC may improve drug safety testing in the preclinical phases of DDP.

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Microphysiological systems for the study of neurodegenerative diseases *in vitro*

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INTRODUCTION

Understanding the complex communication between different cell populations and their interaction with the microenvironment is fundamental in neuroscience research. Due to the lack of suitable animal models capable of faithfully reproducing the physio-pathological mechanisms of many human diseases, the development of appropriate *in vitro* approaches and tools, able to selectively analyse and probe specific cells and cell portions (*e.g.*, axons and cell bodies in neurons) has become therefore crucial in this direction. From one hand, the rising technology of organ-on-a-chip (OoCs) offers the possibility to overcome these problems since the possibility to replicate key units of living organs and organisms[1]. From the other hand, the discovery of human induced pluripotent stem cells (hiPSCs) opens new areas for precision medicine[2]. During the past two decades, many platforms have been fabricated in order to study NDDs, focusing the attention on the neuromotor unit that is damaged in these disorders and, in particular, in Amyotrophic Lateral Sclerosis (ALS)[3]. The neuromotor unit is a specialized region composed of presynaptic lower motor neuron, postsynaptic muscle myofiber and terminal Schwann cell, involved in the control of vital body processes, such as voluntary movements and breathing. However, most of the platforms focus on the interaction between motor neurons and muscle cells since, to date, protocols to generate pure populations of terminal Schwann cells still need to be developed. Here, we propose a microfabricated *in vitro* model for studying the neuromotor unit where different neuronal populations, glial and skeletal muscle cells can grow and communicate in a perfusable environment. This platform could be also useful to investigate the ALS pathological mechanism.

EXPERIMENTAL

Microfluidic multi-compartmentalized devices were fabricated by SU-8-based multi-level optical lithography and PDMS replica molding, displaying a series of microchannels that connect three different compartments (hosting three different cell types) and promote neurite elongation unidirectionally from one cell compartment to another one. hiPSC were differentiated toward motor neurons (MNs) and Schwann cells (SCs) and the interactions between them were investigated.

RESULTS AND DISCUSSION

We proposed a microfluidic device with three different perfusable compartments (500 µm wide and 6 mm long) interconnected through a series of narrow microchannels in which the three main components of the neuromotor unit will be hosted[4]. First, we investigated how an accurate choice of device geometrical features and cell culture parameters (*i.e.*, cell density, microfluidic setup and coating procedures) allowed to: i) maximize cell adhesion and proliferation of neuron-like human cells (SH-SY5Y cells) and primary rat Schwann cells, ii) perform on-chip cell differentiation towards more physiologically relevant phenotypes, ii) control the inter-compartment cell migration, iii) execute long-term cell culture studies. Then, we integrated hiPSCs, from both healthy and ALS patient donors, inside the device differentiating them towards MNs and SCs. We co-cultured them to investigate possible interactions such as axonal elongation in the adjacent compartment in the presence of chemical cues (BDNF or CNTF) or other cell types (iPSCs-derived SCs), investigating the potential connections.

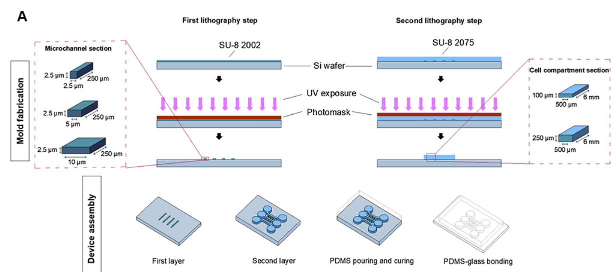

Figure 1: A) Fabrication of the microfluidic device. The mold was realized on a silicon wafer via two-step photolithography process (*top side*). The final PDMS device was fabricated using soft lithography techniques: PDMS mix was poured on the mold, cured, and peeled off. The replica was sealed on a coverslip by means of oxygen plasma treatment and temperature (*bottom side*).

CONCLUSION

We designed a robust microfabricated platform for studying the motor circuit components, performing hiPSCs differentiation on chip towards MNs and SCs and investigating possible interactions between these cell types. This platform could be useful to study the dysfunctions that occur in the neuromuscular junction of patient affected by ALS.

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Digital Light Processing 3D printing optimization of high aspect ratio structures for rapid prototyping of chips towards biomedical applications

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INTRODUCTION

Organ-on-chip and lab-on-chip technologies rely on the use of microfabrication techniques to create small, precise structures that can manipulate and analyze small amounts of fluids and cells. They are traditionally manufactured by optical photolithography and soft lithography. [1,2] Even though this approach offers the possibility to produce features with high resolution (i.e., at the microscale), it is not flexible enough to respond to the necessity of customization and prototyping.

Nowadays, 3D printing technology is used to create microfluidic devices with complex geometries and features. In this study, we propose the development of devices by digital light processing (DLP), which is a vat photopolymerization technology. Studying the process factors by a statistical methodology called Design of Experiment (DoE) [3], we were able to achieve small features (in the order of tens of micrometer) with high aspect ratio (roughly 60).

This optimization was beneficial also in terms of transparency (evaluated by UV-Vis spectrophotometry), and mechanical strength (evaluated by a compression test) of the printed resin. Finally, a proof-of-concept microfluidic device was produced with the optimized parameters. [4]

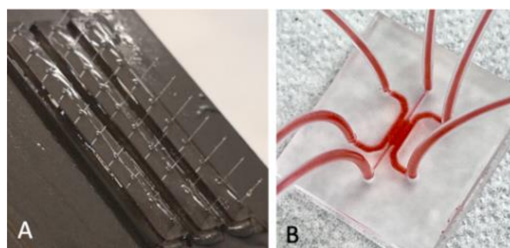
EXPERIMENTAL

A CAD model was developed to optimize the printing process close to the resolution limit of the DLP printer (Asiga MAX X27 UV). The used resin (GR10, Proc3dure) was biocompatible, and the printing parameters were optimized through a statistical approach to obtain high AR pillars (up to 60). The 5 process factors modified during the optimization were the UV layer exposure time (s), the UV curing time (min), the baking temperature (°C), the Sonication Power (%) and the square length (µm) of the printed pillars. The dimensions of the printed pillars were determined by optical microscopy followed by image and statistical analysis. The critical factors in the pillar growth resulted to be the dimensions of the pillar base (and thus the AR), the UV-exposure time of each layer and their mixed effect. Since the dimensions of the object are usually defined by the design, the only choice to modify the outcome was to increase the exposure time. The printed resin was characterized mechanically by a compression test on printed cylinders.

A proof-of-concept chip was finally printed by DLP starting from a CAD file, sealed on a PDMS membrane, and tested under continuous flow against leakages using a microfluidic circuit and coloured water solution.

RESULTS AND DISCUSSION

The most critical aspect in our optimization was the growth of the pillars in the *Z* axis. The difference between the theoretical and measured quotation resulted to be a complex function of the printing factor. Using the empirical model obtained by DOE, we achieved high AR structures (Figure A) tuning the UV exposure time in the 2-4.5 s range. A second DoE optimization revealed that the thickness of the layer was not influential in the variation on the *Z* axis, while the UV exposure time and the nominal AR were significantly important. Following the mechanical characterization, it was noticed that the higher exposure time made the resin mechanically stronger (higher Young's modulus and yield stress), when compared with the printing parameters (0.5 s) suggested by the resin manufacturer. Moreover, moving the UV time exposure from 0.6 s to 2.5 s and 4 s the resin transparency increased, but the color changed to yellowish. Hence, a good compromise was the UV exposure condition at 2.5 s that ensured a high compression modulus without compromising the resin colour. Based on the optimized condition, a proof-of-concept device was printed. After some tests run in dynamic condition to try-out the sealing (Figure B), the microfluidic device was proved to not leak and, thus, to be suitable for dynamic cell culture.



CONCLUSION

In this work we proved the possibility to develop OOCs and LOCs devices with micrometrical features using a DLP methodology. The development of microfluidic devices based on this optimization may represent a substantial improvement to produce innovative OoCs in which the integration with other methodologies (i.e., bioprinting) will require high aspect ratio structures and flexibility in the production process.

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Realization of an Organ-on-chip for the study of gastrointestinal chronic diseases, carcinogenesis and tumors.

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INTRODUCTION

Intestine-on-chip models able to mimic the organ pathophysiological mechanisms and microenvironment have the potential to replace animal experimentation and speed up research and the pharmaceutical development phase [1, 2][3].

Here we report on the development of an Intestine-on-chip model to investigate and support the treatment of both chronic and cancerous gastrointestinal diseases.

EXPERIMENTAL

The device was constructed through photolithography and soft lithography techniques, taking advantage of the biocompatibility and transparency of polydimethylsiloxane (PDMS). The miniature platform consists of two overlapping PDMS layers separated by a porous membrane (polycarbonate or polyester) to recreate the intestinal epithelium-vascular endothelium interface, each containing a channel with an inlet and outlet and two vacuum chambers to mimic the peristaltic movements of the intestine. The device is assembled through oxygen plasma treatment and heat treatment. In addition, to optimize platform closure**,** a mortar layer was used on the PDMS replica as reported by Van der Helm et al [4]. As for the cellular part, a human colorectal adenocarcinoma cell line (Caco-2) was used, which is the most widely used in the literature. These are seeded on the membrane (75 x 10^3 cells/ml), coated with extracellular matrix proteins (e.g., collagen I). The chip was then perfused overnight through a pump syringe at a flow rate of 30µL/h thus ensuring an adequate supply of nutrients and removal of metabolic waste products at all times.[1]

RESULTS AND DISCUSSION

Experiments have shown that the polyester or PET membrane adheres better to the PDMS layer, although it breaks more easily than the polycarbonate membrane. Furthermore, the perfusion system will be optimised to ensure cell growth, and cell experiments will be carried out for devices with different membrane types and different porosity to see where cells grow best.

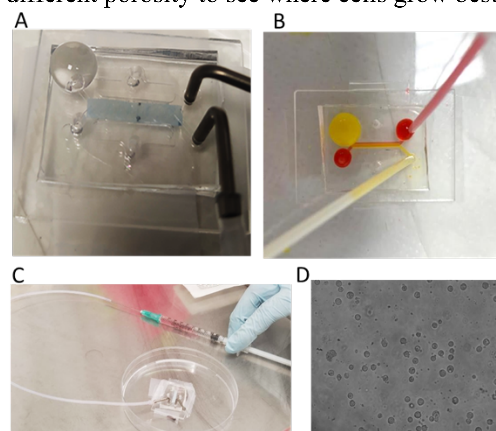

Figure 1. Microfluidic devices with polycarbonate membrane (A) and with PET membrane (B) subjected to flow tests with distilled water (left) and with coloured liquids (right). Seeding of the Caco-2 cells in the upper channel of the device (C) and photo taken from the EVOS Floid Invitrogen microscope of the Caco-2 cells 1 hour after seeding in the chip.(D)

CONCLUSION

This intestine-on-a-chip mimics several physical and functional characteristics of the human intestine and it could therefore be used to carry out pharmacological toxicity studies that could be useful in the discovery of new drugs for the treatment of chronic intestinal diseases and new in vitro disease models.

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Towards physiologically relevant human liver-on-chip as platform for drug screening in metabolic diseases

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INTRODUCTION

To date, high-throughput and physiologically relevant *in vitro* models are increasingly required to improve the predictive value of toxicity testing as valid alternatives to animal experiments. In this regard, 3D Organs-on-Chip (OoC) may fill this gap, allowing scientists to develop miniaturized and complex human disease models holding high pathophysiological relevance for drug screening[1]. Here we present our liver-on-chip platform to mimic the development of a metabolic dysfunction-associated fatty liver disease (MAFLD) and the protective effect of natural polyphenols against disease progression. Besides, we show the further evolution of such microfluidic systems by combining human primary cell-based hepatic organoids for increasing the complexity and predictivity of our OoC platform.

EXPERIMENTAL

Microfluidic devices were fabricated in poly-dimethylsiloxane (PDMS) through a two-layer soft-lithographic process according to a previously validated layout[2,3]. HepG2/C3A cells were expanded in culture, loaded and cultured in a 3D fashion in the chip under microfluidic perfusion. For hepatic steatosis induction, long-chain free fatty acids (FFAs), namely palmitic acid (PA) and oleic acid (OA), were added to the medium, either alone or in combination, at different molar ratios (1:2 and 2:1, respectively) at 1 mM final concentration for 48h[4]. The polyphenols Quercetin (Que) and Hydroxytyrosol (HT) were both used at a physiological concentration of 10 µM for 48h[5,6] (Fig.1 A, B).

Hepatic organoids were derived from primary human hepatocytes (PHH), alone or in combination with non-parenchymal cells (NPCs, endothelial, stellate and Kupffer cells). For MAFLD induction, organoids were cultured for 7 days under FFA overload, namely 133µM OA and 67µM PA. Quercetin was used at a physiological concentration of 10 µM for 7 days. The results of NPC inclusion in hepatic organoids, the steatogenic effects of FFA treatments (i.e., intracellular lipid accumulation, cell viability and oxidative stress) and the role of polyphenols were evaluated *via* confocal microscopy-based high-content analysis (HCA), using fluorescence-based functional assays (Fig.1 C, D).

RESULTS AND DISCUSSION

We observed that the herein developed liver-on-chip platform simulates the chronicity of hepatic steatosis way better than traditional 2D cell cultures, representing a suitable *in vitro* model to recapitulate MAFLD[2]. Moreover, in our microfluidic device, both polyphenols showed a hepatoprotective role against MAFLD, through their lipid-lowering and antioxidant effects, thereby representing a potential therapeutic treatment[3]. We witnessed that the introduction of NPCs supported long-term viability and metabolic functions of hepatic organoids (Fig.1 C). We showed that these co-cultures in hepatic spheroids were representative of native liver tissue. We reported induction of steatosis/steatohepatitis, characterized by triglyceride accumulation, oxidative stress, altered hepatic functions, and fibrosis. Finally, we tested the positive effects of Quercetin, alone or in combination with FFAs, to mitigate the MAFLD manifestation, which significantly increased cell viability, reduced the fibrotic condition and lowered triglycerides content (Fig.1 D).

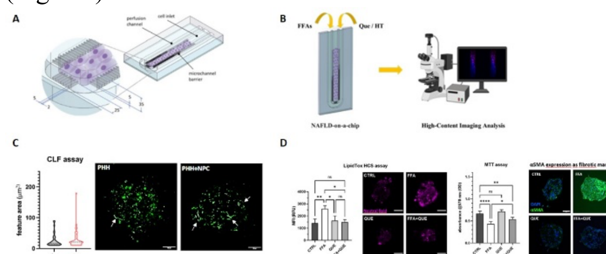

Figure 1 Microfluidic model of the MAFLD-on-a-chip for drug screening. *A) Layout of the liver-on-chip device. B) High-content analysis of MAFLD parameters and polyphenols effect. C) The results of NPCs inclusion in hepatic spheroids. D) The effects of MAFLD induction and Quercetin administration in primary human hepatocyte-derived organoids.*

CONCLUSIONS

Altogether, integration of 3D co-cultures made of PHH- and NPC-derived hepatic organoids into microfluidic devices will help better investigate mechanisms of chronic liver diseases and will move preclinical hepatotoxicity analysis forward. In a future perspective, these cutting-edge technologies may carry future potential for hepatic disease modelling and drug screening in the field of personalized medicine.

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Detection and Characterization of circulating tumor cells (CTCs) using an Optically-induced dielectrophoresis system

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INTRODUCTION

The detection of circulating tumour cells (CTCs) remains a significant challenge in cancer research. These cells are involved in the tumor-metastases cascade and represent a fingerprint of the primary tumor, so the early detection of the CTCs in human liquid biopsies is an alarm bell related to cancer diagnosis, progression, invasiveness, and prognosis [1]. In this regard, we explore the potentiality of a multi-spectral analysis, based on optically-induced dielectrophoresis (ODEP) [2], to distinguish between the human prostatic carcinoma PC3 cells, which mimic prostate cancer-derived CTCs [3], and human leukemia monocytic THP-1 cells, which mimic circulating monocytes [4]. The proposed method highlights a dielectric fingerprint of each cell population, allowing the identification of different subtyping of cells. To collect the response spectra, every single cell was measured and recorded.

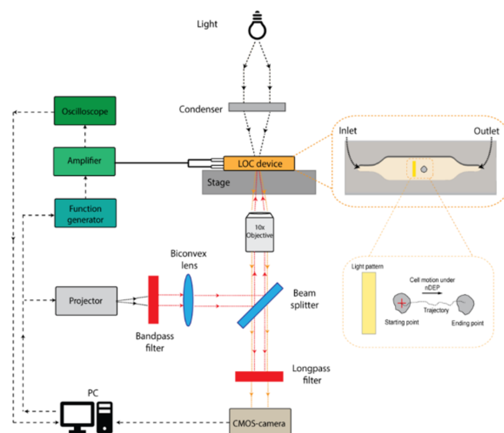

Figure 1. A schematic representation of system set-up.

EXPERIMENTAL

The cell suspension was loaded into a Lab-On-Chip (LOC) integrated with an inverted microscope and a set of instruments connected and controlled with custom software [2]. The entire system setup is shown in fig.1. An AC potential at a specific frequency is applied, and a rectangular light pattern was projected. The generated non-uniform electric field induces a dielectrophoretic (DEP) force on cells by producing three different cell motions: attractive (pDEP), repulsive (nDEP) or steady-state. After that, cell tracking allowed extracting a set of cell kinematic parameters (i.e. the cumulative displacement $CD(f_n)$, the maximum velocity $v_{max}(f_n)$, …) [2]. Finally, a feature selection was performed to build an LDA model for cell classification.

RESULTS AND DISCUSSION

In Fig. 2a, we report the response spectra in the frequency range [25-150] kHz with average and standard deviation of the three parameters for PC3 and THP-1 cells. The frequency in correspondence of the maximum point of the displacement spectrum and the minimum points in velocity and DEP force spectra (fig. 2a) is the crossover frequency, which is 30kHz and 50kHz for PC3 and THP-1, respectively. Fig.2b shows the confusion matrix of the classification model with a total accuracy of 98% achieved using an optimal set of feature set.

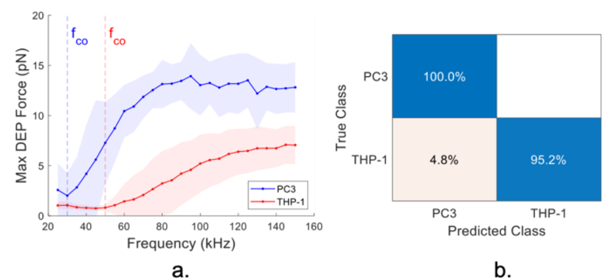

*Figure 2. (**a**)Spectra of maximum DEP force. The average values and standard deviation are reported. Dotted lines indicate the crossover frequency (f_{co}). (**b**) Confusion matrix of the classification model obtained in k-fold cross-validation.*

CONCLUSION

The results obtained suggest the ability of the system to discriminate between the two cell populations on the basis of size, shape, and dielectric properties. Moreover, the exploitation of the spectra can provide the appropriate operating conditions to sort cells and isolate CTCs in a label-free manner under continuous flow.

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Toward a 3D hiPSCs-derived gut-on-chip platform to investigate the intestinal barrier damage

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INTRODUCTION

Organ-on-chip (OoC) models have been proposed as tools to investigate the main organs involved in metabolic pathways such as the intestine (gut-on-chip[1]). These devices allow the intestinal barrier functions to be characterized in an engineered microenvironment[2]. In this scenario, we present a novel gut-on-chip device with biaxial actuation, purposely designed as an advanced *in vitro* drug testing platform in the field of metabolic diseases[3]. Besides, we show the creation of two cellular pathological models of intestinal barrier integrity loss caused by a long-chain saturated fatty acid as Palmitic Acid (PA): a 2D model based on human epithelial colorectal adenocarcinoma cell line (Caco-2 cells)[4] and a 3D model based on human induced pluripotent stem cells (hiPSCs)-derived intestinal organoids. We also tested the effect of a polyphenol, Quercetin (QUE), as a possible therapeutic agent thanks to its anti-inflammatory activity.

EXPERIMENTAL

The device was obtained as a soft-lithographic replica in polydimethylsiloxane (PDMS), as previously described[3]. It was made by a porous membrane in PDMS, placed between two chambers (upper and lower) that can be differentially perfused. The membrane is dedicated to the culture of enterocytes in monolayer and it can be subjected to mechanical stretching thanks to 4 pneumatics micro-actuators integrated into the chip design (Fig. 1A). Caco-2 cells were grown on transwell chambers (12 mm with 0.4 µm pore polyester membrane inserts) in a 12-well plate. Cells were regularly monitored by measuring the epithelial resistance and experiments were performed 21 days after seeding. hiPSCs were differentiated into intestinal organoids according to the protocol by Spence et. al[5], using a temporal series of growth factors mimicking the embryogenic intestinal development and encompassing the steps of definitive endoderm, mid-hindgut and intestinal differentiation (Fig. 1B, C). The effect of PA at a physiological postprandial intestinal concentration (1mM) was tested for 24h to investigate gut epithelium integrity, using both cellular models. QUE was tested at a physiological concentration of 10 µM, using only intestinal organoids. Analysis of cell viability, oxidative stress, cell permeability and immunohistochemistry were conducted by confocal microscopy using fluorescent-based assays. In addition, in intestinal organoids the antioxidant effect of the polyphenol was evaluated by confocal microscopy.

RESULTS AND DISCUSSION

Data obtained from cell viability, oxidative stress and permeability assays showed that the tested concentration of PA affects the intestinal permeability in both 2D and 3D models. Moreover, QUE was demonstrated to restore the ROS levels of PA-treated organoids to the levels of healthy samples. These results, combined with the proof of the modularity and adaptability of our device to provide truly multi-axial strain states, lay the foundations for the integration of our cellular model. Indeed, this device would give the possibility not only to modulate the intensity of cell culture experienced stretching over time, but also to modify its spatial distribution, mimicking, for example, the insurgence of pathological conditions on *in vitro* tissue models.

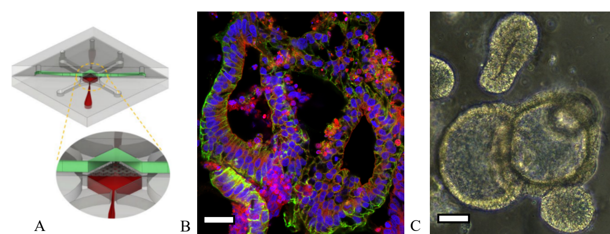

Figure 1. Microfluidic and cellular models for gut-on-chip. *A) Layout of the gut-on-chip device; B) Immunofluorescence image of intestinal organoids; C) Brightfield image of intestinal organoids. Scale bar: 25 µm.*

CONCLUSION

Considering the results, we achieved a further evolution of our models that will be the integration of hiPSC-derived intestinal organoid models into the stretchable microfluidic device to create a platform suitable for disease modelling and drug screening in the precision medicine field.

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Extracellular vesicles as a next-generation drug delivery platform in a more physiological OOC-based microenvironment

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INTRODUCTION

Tumor metastases represent the main cause of mortality in neoplastic patients. Even tumors which are early diagnosed, such as breast cancer, may be characterized by a drastically reduced survival rate in patients with distant metastases. Current therapeutic protocols target cancer cells, but the damage of normal cells and tissues is the main unsolved hurdle. Therefore, the development of innovative targeted therapeutic strategies with minimal side effects and systemic toxicity represents the future clinical challenge. However, the absence of unique markers allowing to specifically target cancer tissues still represent an impediment for the development of new drug delivery strategies. In this scenario, the role of the extracellular matrix (ECM) in tumor growth and progression is gaining growing interest. In particular, an oncofetal variant of fibronectin, termed extradomain B-FN (ED-B), is highly investigated [1], since it is expressed by the newly synthesized perivascular areas of the stromal matrix in a wide range of tumor types. Among novel targeting strategies, extracellular vescicles (EVs) emerged as next-generation drug delivery platforms. EVs have indeed an intrinsic tissue-homing capability, harnessing specific barcodes to find their target both locally and systemically.

EXPERIMENTAL

Metastatic and non-metastatic breast cancer cell lines (MDA-MB-231 and MCF-7, respectively) will be cultured in an alginate-based 3D matrix [2]. Taking advantage of an organ-on-chip (OOC)-based microfluidic platform [3], we will study the expression of ED-B in a more physiological microenvironment, in presence or absence of TGF-beta treatment, a potent epithelial-to-mesenchymal inducer. We also propose an innovative approach to target ED-B with a sequence-specific small peptide exposed on EV surface by click chemistry approach. Functionalized EVs (f-EVs) will be loaded with the chemotherapeutic molecules (*e.g.* paclitaxel) to be specifically delivered toward tumor sites. The 3D tumor model will be cultured physically separated from the circulatory flow, delivering the engineered EVs.

RESULTS AND DISCUSSION

We have successfully set-up EV membrane functionalization (fEVs) and paclitaxel loading (PTX-fEVs). EVs have been isolated from both erythrocytes and plasma samples from healthy donors. The expression of ED-B has been demonstrated in the OOC-based microfluidic platform above described. PTX-fEVs will be dynamically tested for their ability to target chemotherapeutic agent in cancer cells (Figure 1).

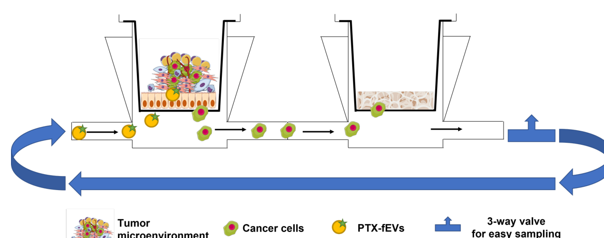

Figure 1. Schematic representation of the experimental design. The 3D tumor model will be cultured in dynamic condition (chamber on the left) in a microfluidic organ-on-chip platform. PTX-fEVs will be add in the circulatory fluid flow and their ability to target chemotherapeutic agent in cancer cells will be evaluated. Their effect on cancer cells migration/extravasation, specificity for tumor tissue and side effects on healthy tissues (chamber on the right) will be also investigated.

CONCLUSION

The novel fluid-dynamic bioreactor (MIVO®) device provides an *in vivo*-like environment, allowing to recapitulate the cell-cell and cell-matrix interactions occurring *in vivo*, providing a more clinically relevant model. It may represent the perfect platform for mimicking systemic drug administration for more predictive drug efficacy assays.

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Lab on Chip for precision medicine: measuring Sorafenib Effectiveness on HCC Cell Proliferation

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INTRODUCTION

A gold standard in the evaluation of the potential anti-cancer drug therapies are cell viability and proliferation assays typically carried out through standardized techniques (e.g. *MTT assay*) refined in the last century which, however, are still not able to satisfy reducing time and cost of drug screening as well as the request to develop therapies best suiting the clinical profile of the patient for a precision medicine.
Here, we report the development of a miniaturized *ECIS (electric cell–substrate impedance spectroscopy)* platform to perform cell proliferation and drug screening assays based on *impedance spectroscopy (IS)* and its application to investigate the time-dependent effect of different concentrations of Sorafenib on *HLF* cells *(hepatocellular carcinoma, HCC)* isolated from biopsy specimens [1].

EXPERIMENTAL

The *Lab on Chip (LoC)* was realised in a clean room according to a bottom-up approach which involved: (i) the construction of the electrochemical transducers by lithographic and lift off processes; (ii) the design of the biocompatible multichambers and its 3D printing; (iii) the final assembly of the individual components (i.e. transducers and microfluidic chambers) through oxygen plasma treatment.
More in detail, the transducer consists of four gold arrays with interdigitated microelectrodes as transducers allowing repeated experiments and statistical analysis and fabricated through photolithography and lift off techniques on a glass substrate with dimensions of 3.5 x 3 cm.
To exploit all the advantages of *LoC* approach, a multiwall (4 chambers) configuration was employed for enabling multi and parallel analysis at low-cost.
After cells seeding, attachment and spreading on the chip, the assays were carried out in real time (about 72 h) by optical imaging and *IS* measurements recording the signals at 40 kHz under 1 mV voltage amplitude allowing a good signal-to-noise ratio without affecting the cell growth to compare the acquired data and gain further insight and validation. We carried out drug tests starting from results of traditional crystal violet dissolution cell proliferation *(MTT)* assay investigating the drug effect at different concentrations (0, 2.5, 5 and 10 μM in DMSO) on cell growth and then, the same experiments were run on LoC.
In addition, we performed multifrequency *EIS* tests (1 Hz - 1 MHz) by LoC to analyse a spectrum of signals derived from cells and, extracting vectors, the results-demonstrated the ability to monitor cell growth, vitality, and motion at immobilization sites in real-time.

RESULTS AND DISCUSSION

Comparing the results between *MTT* assay and *IS* tests, the set of curves exhibited a remarkable dose-dependent behaviour of *HLF* proliferation in both methods **(Fig.1a and b).**

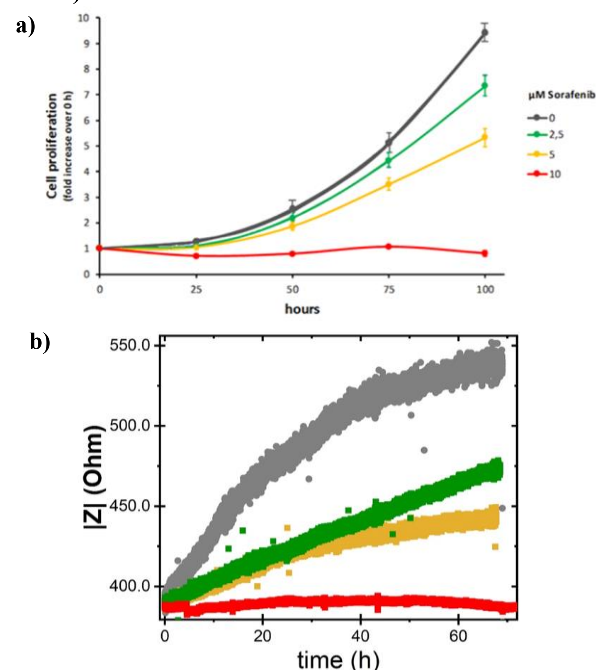

Figure 1.a) Traditional crystal violet cell proliferation assay. HLF cells were seeded and treated with scaled concentrations of *Sorafenib*. At the indicated time points, the cells were fixed and stained with crystal violet. ***b)*** dose-dependent *ECIS* cell proliferation assays showing the influence of the Sorafenib concentration on the proliferation rate.

CONCLUSION

In conclusion, compared to the traditional one, our platform presented several advantages, among these: lesser reagents consumption, operator time, and costs. It could enable high-throughput studies on large drug candidate libraries or on combinations of multiple compounds to assess their putative synergistic effect on patient-derived samples. A further step will be the use of our platform to support *Organ on Chip (OoC)* by *TEER* experiments and biomarker detection supporting research, allowing drug screening and precisione medicine.

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Transcriptomic profiling of *PRKN*-mutant neurons differentiated in a 3D alginate hydrogel matrix

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INTRODUCTION

Parkinson's disease (PD) is the second most frequent neurodegenerative disorder in aging. Mutations in the *PRKN* gene, encoding parkin, represent the most common known cause of autosomal recessive early-onset parkinsonism. The parkin protein is an E3 ubiquitin ligase that has been implicated in several cellular functions including the mitochondrial biogenesis activation, the mtDNA transcription modulation, the mitochondrial genome integrity, the mitophagy pathway, and the control of the cell´s apoptotic response during mitophagy. Furthermore, parkin was found to actively inhibit mitochondria-derived vesicle formation and mitochondrial antigen presentation (MitAP), and in parkin deficiency, inflammatory conditions trigger MitAP in immune cells. To further investigate its role in the pathogenesis of PD, human induced pluripotent stem cells (hiPSCs) were used to generate patient-specific dopaminergic neurons. However, the complexity of the human brain is not fully recapitulated by existing monolayer culture methods. Neurons differentiated in a three dimensional (3D) *in vitro* culture system might better mimic the *in vivo* cellular environment for basic mechanistic studies and represent better predictors of drug responses *in vivo*. In this study, targeted RNA-sequencing analysis in hiPSC-derived dopaminergic neurons cultured in 2D and 3D model systems were performed to investigate disease-linked mechanisms caused by *PRKN* mutations.

EXPERIMENTAL

Human iPSCs from two control individuals and two PD patients carrying a biallelic mutation in the *PRKN* gene (homozygous c.1072Tdel and homozygous Ex3 deletion, respectively) were included in the study. Midbrain dopaminergic (mDA) neurons were generated by using both a conventional 2D protocol (Kriks et al. 2011; Zanon et al., 2017) and a recently established hydrogel-based 3D system (Gilmozzi, Gentile et al., 2021). Samples for RNA isolation were collected at days 0, 10, 20 and 35 of differentiation. Targeted RNA-sequencing libraries were produced using the DriverMapT Human Genome-Wide Expression Profiling Kit, V2 (Cellecta). Differential expression analysis was performed with DESeq2 package (v 1.28.1) using the Wald test for significance testing. Functional annotation of differentially expressed genes (DEGs) was performed on KEGG, Reactome and Gene Ontology databases with ClusterProfiler (v3.16.1) and ReactomePA (v1.32.0).

RESULTS AND DISCUSSION

For both 2D and 3D protocols, a clear transition in gene expression from hiPSC markers (MYC and POU5F1-OCT3/4) to genes associated with mDA differentiation (PTCH1, FZDZ, HES1, OTX2, SLIT1, and LMX1A), and finally to an early expression of mature mDA markers (DCX and DDC) for all cell lines was observed. Overall, 1,426 genes were differentially expressed in *PRKN* mutation carriers compared to healthy individuals at day 35 (351 upregulated and 1,075 downregulated) in the 2D culture condition. Functional annotation analysis of DEGs altered exclusively at each timepoint highlighted cellular response to starvation, protein oxidation, synaptic transmission and axonogenesis as significantly enriched pathways. Additionally, 33 genes were upregulated and 118 genes were downregulated in the *PRKN* mutant cell lines compared to controls at all timepoints. Functional annotation analysis of these genes indicated that the immune response pathways are significantly enriched in the *PRKN* mutants compared to the controls. By analysing the 3D generated transcriptome, 57 genes were differentially expressed in *PRKN* mutation carriers compared to healthy individuals at day 35 (21 upregulated and 36 downregulated). Functional annotation analysis of DEGs altered exclusively at each timepoint showed that immune response pathways, regulation of lipid metabolism, and regulation of insulin-like growth factor were significantly enriched. In this dataset, 12 genes were upregulated and 23 genes were downregulated in the *PRKN* mutant cell lines compared to the controls at all timepoints. Enrichment analysis of these DEGs confirmed a strong correlation between the *PRKN* gene mutation and the alteration of the immune response pathways and inhibition of insulin secretion.

CONCLUSION

Overall, an involvement of dysfunctional parkin in altered axonogenesis, mitochondrial metabolism, and immune response pathways was assessed by using two differentiation approaches. By using the hydrogel 3D model system, we identified *PRKN* mutation-specific gene sets and functional pathways linked to the regulation of insulin-like growth factor and lipid metabolism, thus highlighting the involvement of parkin in these pathways and providing new hypotheses for the development and progression of PD.

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3D bioprinting for skin model tissue engineering

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INTRODUCTION

Skin is the largest human organ and it is the barrier between the human body and external environment[1]. Skin-on-a-chip models are for sure fundamental for in vitro drug testing and they are replacing conventional 2D cell culture and animal models[2]. They can replicate 3D organization and physiological functions of specific tissues or organs at a relatively low cost[3].

EXPERIMENTAL

Methacrylated gelatin (GelMA) was synthesized following Van Den Bulcke et al. protocol[4]. It was then dissolved at 10% w/v in culture medium. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate was used as photo-initiator. The sterilization was performed by filtration. Human fibroblasts were then encapsulated in GelMA and transferred into a printing cartridge. For the 3D bio printing, the 3D Discovery Instrument (RegenHU) was used, equipped with a pneumatic dispensing printhead. The printing was performed directly into hanging cell culture inserts. Immediately after printing the architectures were photopolymerized under UV light and human keratinocytes were seed on top of them. After 3 days of culture, Air Liquid Interface culture started and 3dGROTM Skin Differentiation Medium was supplied and refreshed every two days. The samples will be analyzed in terms of RNA translation and protein transcription after 4, 14, 31 days and 2 months. Live and dead assay and fluorescence microscopy have been already performed.

RESULTS AND DISCUSSION

The skin models are able to survive and continue to mature for at least 2 months and this put them in a good position for long-term testing. The epidermis has been observed to increase a lot its thickness during the model maturation, with good cytokeratin and CD29 expression, becoming more and more similar to human skin.

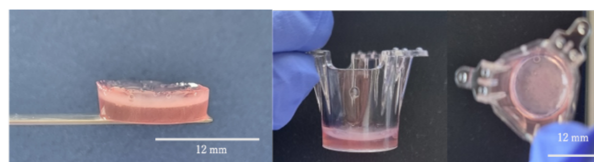

Figure 1 Pictures of the 3D printed models after 31 days of culture.

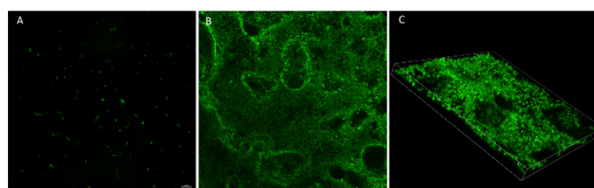

Figure 2 Fluorescence images of Live and Dead assay on Skin models after 1 month of culture A) fibroblast immersed in GelMA (dermal layer) B) epithelial layer and C) Z stack of epithelial layer (height 130 um) (Bar scale 100 µm).

CONCLUSION

We are managing to mimic human skin using 3D bioprinting technology, that mimic the flow of nutrients and waste products that occur in vivo. This put the model in a great position to be an in vitro platform for drug, therapies and cosmetics testing, to have predicting results and to avoid the use of animals in clinical trials.p.

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Copper ions monitoring in cell culture media via anodic stripping voltammetry : from Transwell® to organ-on-chip systems

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INTRODUCTION

Real-time ions monitoring directly in cell culture systems and into organ-on-a-chip platforms represents a significant investigation tool to understand ion regulation and distribution in the body. Copper is an essential trace mineral fundamental for our survival: dietary copper is absorbed by intestinal epithelium and its management and regulation is fundamental to preserve copper homeostasis in the whole body. Imbalance of copper concentrations can be linked to specific pathologies such as Menkes, Wilson's and Alzheimer's diseases. Hence, an interesting process to study is the copper paracellular transport and regulation, monitoring concentration changes in a cell culture medium. Electrochemical methods are cheap, easy and fast and, in particular, anodic stripping voltammetry (ASV) is a powerful technique for trace analysis of metal ions, with low detection limits, good sensitivity, and selectivity [2].

In this study, the performance of ASV for copper detection in the complex cell culture media was evaluated, and a medium pre-treatment protocol was tested to improve the voltammetric signals intensity. First, the potential of the developed acidification protocol was evaluated by performing measurements with a real intestinal epithelial monolayer of Caco-2 cells in a Transwell® culture system. Then, a microfluidic platform that integrates a cell culture and a microfabricated sensor was engineered in order to enable an automatic acidification of the cell culture medium in a separate sensing chamber and a monitoring of copper concentration in real-time.

EXPERIMENTAL

Different cell culture media (MEM, DMEM, F12K) were supplemented with copper solutions (1-20 μM) and measured at physiological pH (pH 7.4) and after acidification at pH 4 via anodic stripping voltammetry. ASV measurements were also performed to monitor Cu^{2+} concentrations in an *in-vitro* system of Caco-2 cells Transwell® culture model, by sampling both the upper and lower media at the beginning of the experiment and after 72 h under physiological and acidic conditions. In order to apply the proposed copper detection protocol in an organ-on-chip system, a proprietary three-electrode integrated sensor was integrated in a commercial microfluidic platform, chambers connected with a microfluidic pressure & flow control instrument. FEA simulations were conducted to identify the best injection conditions to be used for the acidification of the medium. The microfluidic chip includes a cell culture chamber and a separated sensing chamber which allows an automatic acidification protocol and ASV detection of copper ions in chip and real-time, without affecting the cell viability.

RESULTS AND DISCUSSION

ASV measurements of copper ions at physiological conditions (pH 7.4) showed moderate current signals according to the Pourbaix diagram for copper ions and due to the complexity of the culture media. ASV experiments performed after the medium acidification at pH 4 showed higher current values and higher slope values with respect to physiological conditions. A linear correlation between copper concentration and peak current was observed. The acidification protocol was also effective in measuring copper transport in presence of Caco-2 cell monolayer in Transwell® culture system. The microfluidic platform including a cell culture chamber and a sensing chamber with an automatic acidification mechanism (Fig. 1a) was tested with increasing concentration of copper ions and the response results were showed in figure 1b.

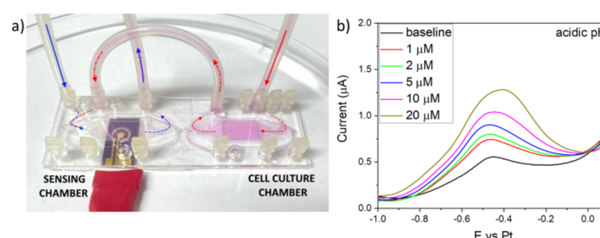

Figure 1. (a) Photograph of the microfluidic chip integrated with ion sensor; (b) ASV for copper determination performed in chip.

CONCLUSION

The results reported in this work represent a first step towards the development of organ-on-chip models that integrate more functionality using advanced sensors, microfluidics and automated biological liquids treatment/detection. Such models could be of great support to study pathologies and complex biological mechanisms.

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Development and validation of a novel MPS platform, towards the standardization and adoption of 3D, dynamic *in vitro* models, replicating tissue barrier functions and physiology.

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INTRODUCTION

Micro Physiological System (MPS) technology is a valuable tool when replicating in vitro organ functions, showing more predictive results than conventional *in vitro* or animal models [1]. However, MPSs integration is still limited by industrial, and standardization hurdles [2]. For these reasons, we developed True Tissue on Platform (TToP), a cartridge-based, scalable, and versatile platform, that aims at replicating tissue barrier functions, enabling a fast integration in laboratory procedures. TToP modular design allows to create barrier tissue models in an open-well static preparation module on tunable substrates. Then, the static module can be plugged in a closed-well perfusion module, enabling an automated perfusion. Finally, the sample can be retrieved easily within the cartridge, to perform advanced end-point analysis (e.g., confocal microscopy (CM), TEM) or further tratments.

EXPERIMENTAL

TToP platform was designed to enable cleanroom-free and PDMS-free fabrication, allowing to obtain up to 300 devices/week, exploiting 3D printing, CO_2 Laser Cutting and multilayer stratification. TToP static module validation have been focused on the generation of a functional intestinal barrier, by culturing Caco-2 cells ($3x10^4$ cells/device) on bare polycarbonate porous membranes, gelatin coated membranes and electrospun gelatin scaffolds. Caco-2 growth rate has been monitored through TEER measurements and live imaging (Hoechst), while Immunofluorescence (IF) endpoint assays evaluated the differentiation by staining tight junctions (ZO-1, nuclei stained with DAPI). TToP versatility allowed to host also organotypic tissues, such as EpiIntestinal™ samples (SMI-100), a human 3D small intestinal model from MatTek™ Corporation (Bratislav, Slovakia) [3]. SMI were hosted in the cartridge and cultured for 12 days in TToP static devices and compared with MatTek™ insert controls. TEER was measured at day 6, 9 and 12, then SMI samples were fixed and stained with Human Epithelial Antigen (HEA, cell surface) and DAPI. In parallel, another batch of SMI samples were incubated at day 12 in the presence/absence of 100µM Lucifer Yellow (LY, 2h). Permeability determined by spectrophotometer as paracellular passage of LY from apical to basal compartment was evaluated and compared between TToP and MatTek™ systems and correlated with TEER values. TEM allowed to evaluate the presence of the brush border and tight junctions. TToP perfusion module (35x80mm), designed to host the static module, was able to apply controlled flow rates in both chambers, allowing live imaging up to 20x. Continuous recirculation of culture medium is generated by embedded piezoelectric micropumps (Bartels), controlled by an Arduino-based unit and a touch screen (NX8048K070). Technical tests have been carried out evaluating the generated flow rates, by inserting 10µm polystyrene (PS) beads in one channel and measuring their velocity by micro particle tracking (MicroPT).

RESULTS AND DISCUSSION

Membrane coated samples and scaffold-based cultures in TToP displayed a faster growth rate, reaching confluence after 9 days (Figure 1a), confirmed also by TEER measures. Bare membrane cultures, on the other side, required of 16 days. Moreover, IF staining demonstrated that the substrate properties promoted cells' differentiation, showing higher cell density and ZO-1 expression.

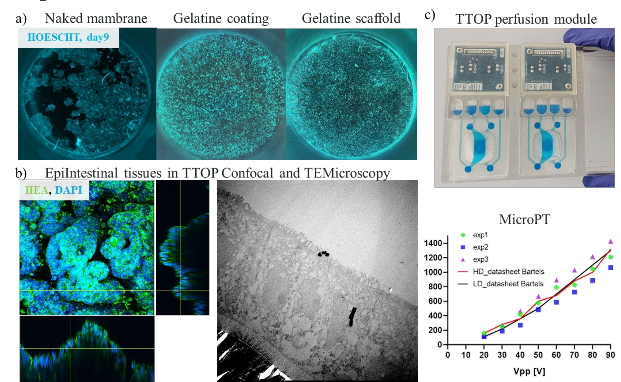

Figure 1. Representative images of Caco-2 (a) or CM and TEM of EpiIntestinal tissues (b) hosted in TToP static modules; c) Perfusion modules and MicroPT curves obtained with PS microbeads.

Similarly, EpiIntestinal™ 3D intestinal tissues demonstrated comparable TEER values in TToP vs. control. Moreover, confocal microscopy and TEM confirmed the presence of the brush border, tight junctions, and allowed to investigate the microtissue 3D morphology (Figure 1b). LY permeability assays showed a high correlation with TEER values (r=0.8285). MicroPT in TToP perfusion modules showed a repeatable generation of flow rates from 50µL/min to 1.5mL/min (Figure 1c). The Arduino-based control unit enabled to control up to 18 perfusion modules.

CONCLUSION

TToP versatile and modular concept was demonstrated by culturing cells on different culture substrates, confirming the importance of the extracellular environment, and integrating in TToP system also organotypic tissues, maintaining the 3D fully differentiated intestinal tissues without morphological or functional alterations. Finally, the compact, tunable, and automated perfusion module showed promising results, allowing, in perspective, to mimic the *in vivo* dynamic microenvironment. Altogether, these features will pave the way for simple standardized and more reliable *in vitro* barrier tissue assays (e.g., drug absorption, drug toxicity, ADME), supporting drug development and limiting animal use.

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Co-culture of hiPSC-CMs and ECs to mimic cardiac tissue

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INTRODUCTION

Human-induced pluripotent stem cells (hiPSCs) have revolutionized the world of basic and translational research. In particular, hiPSCs can be differentiated into cardiomyocytes (hiPSC-CMs), emerging as a reference system for cardiac studies due to their biological similarity to primary CMs (Gisone et al., 2022). However, compared to adult ventricular CMs, the main limitation of hiPSC-CMs is their phenotypic immaturity (Denning et al., 2016). To overcome this limitation and to reproduce the *in vivo* heart microenvironment better, researchers proposed the co-culture of hiPSC-CMs with other cardiac cells, such as endothelial cells (ECs) and fibroblasts (Giacomelli et al., 2020; Campostrini et al., 2021). Besides the influence of cell composition, it is also established that three-dimensional (3D) *in vitro* cell cultures can better mimic the tissue environment of cells in the organism, preserving cells' morphology, phenotype, and polarity, thus allowing for a better study of cells' physiological function and, in turn, obtaining a reliable tissue response (Gisone et al., 2022). This work presents the results of the characterization of hiPSC-CMs + Human Coronary Artery Endothelial Cells (HCAECs) co-culture in a 2D and 3D environment as a cardiac tissue replica for pathophysiological and/or toxicological studies.

EXPERIMENTAL

hiPSCs were cultured in Essential Flex basal medium. The differentiation of the hiPSCs into hiPSC-CMs, after 12 days of culture, was confirmed through immunofluorescence analysis (TNNT2 and NKX2.5 as cardiac cell markers) and Real-Time PCR of the TNNT2 cardiac gene. HCAECs were cultured in Endothelial Cell Basal medium-2 (EBM2). According to the literature on the heart cellular composition (Bai et al., 2018; Giacomelli et al., 2020; Campostrini et al., 2021), two different concentration ratios of cells were tested for co-cultures: 1) 90% hiPSC-CMs + 10% HCAECs; 2) 80% hiPSC-CMs + 20% HCAECs. In addition, 2 different culture media were tested: a) RPMI-1640 supplemented with 2% B27™ Plus Supplement; b) EBM2 + Vascular Endothelial Growth Factor human (VEGF). Cells we co-cultured for two weeks, and the viability (CellTiter-Blue® assay) and the cardiac genes' expression (RT-PCR of TNNT2, CX43, BNP, MYL2, NKX2.5, ACTN2) were evaluated. About the 3D microenvironment, cells were homogeneously dispersed into a Gelatin-Methacryloyl (GelMA, 100% degree of methacryloylation) hydrogel (added with catalytic amount of Lithium phenyl-2,4,6-trimethyl-benzoyl phosphinate as photoinitiator) and then seeded into the wells. Irradiation with UV light (40s, 365 nm, 10 mW/cm^2) was used to polymerize the gels. Cells were co-cultured for two weeks: the CellTiter-Blue® assay was performed to evaluate the viability of the cells.

RESULTS AND DISCUSSION

The immunofluorescence assay confirmed the efficiency of the differentiation protocol of hiPSCs in beating CMs. In addition, the analysis of the expression of the cardiac gene TNNT2 in both cell populations confirmed its presence only in CMs.

In 2D cell co-culture, the CellTiter-Blue® assay did not evidence a significant difference in the viability between the two cell ratios and media, with a trend of higher viability values in EBM2. The Real-Time PCR revealed a significantly higher expression of cardiac genes CX43, ACTN2, and BNP in the co-culture, particularly in the 80/20 ratio than in the hiPSC-CMs monoculture in the different microenvironments. The viability performed on the 3D co-culture models showed no significant differences.

CONCLUSION

The integration of endothelial cells, media culture supporting multi-cellular systems, and microenvironment for the two cell types provide evidence of the maturation process of hiPSC-CMs in terms of cardiac-specific markers (ACTN2, BNP) and intercellular communication (Cx43).

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Design of a bioprinted microfluidic chip as a tumor liver model for drug screening

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INTRODUCTION

Drug discovery is still a risky, expensive, and time-consuming process. The preclinical research phase relies mainly on the use of *in vitro* and *in vivo* models that even when combined, do not provide a perfect representation of human physiology and function.

The liver has a key role in drug metabolism and can be affected by every drug, not only the ones targeting the liver itself. As such, several drugs are removed from the market after liver toxicity is observed. Consequently, developing a better model to screen the new drugs in this organ is of extreme importance.

Microfluidic chips are a promising tool to study complex biological systems, such as drug efficacy and toxicity, by recapitulating the liver 3D microenvironment.

In this case, a microfluidic chip was created by combining 3D bioprinting and a sacrificial template method [1]. Hence, a polydimethylsiloxane (PDMS) microfluidic chip was produced, designed to mimic one-sixth of a hepatic lobule, to study the interaction between endothelial cells and tumor liver spheroids for drug screening.

EXPERIMENTAL

A Bioprinter (BioScaffolder, GeSiM) was used to print a sacrificial material (thermo-responsive Pluronic F-127), according to a pre-designed G-code. Pluronic F-127 40% (w/v) was bioprinted on top of a cured PDMS layer and partially covered by PDMS after bioprinting. After complete curing, the chip was covered with PBS and placed at 4ºC for 48h to remove the sacrificial material. Following sterilization, the PDMS chip was coated and HUVECs were seeded to form an endothelial cell monolayer. In parallel, spheroids from the HepG2 cancer cell line were produced in an ad-hoc PDMS platform and when the HUVECs reached confluence inside the chip, HepG2 spheroids were transferred to the respective chip cavities.

RESULTS AND DISCUSSION

A reproducible microchip production was obtained by tuning the microchip design and bioprinting parameters. HUVECs successfully formed a confluent monolayer 48h after seeding. The tumor spheroid model was inserted in the chip and its interaction with the HUVEC monolayer was assessed.

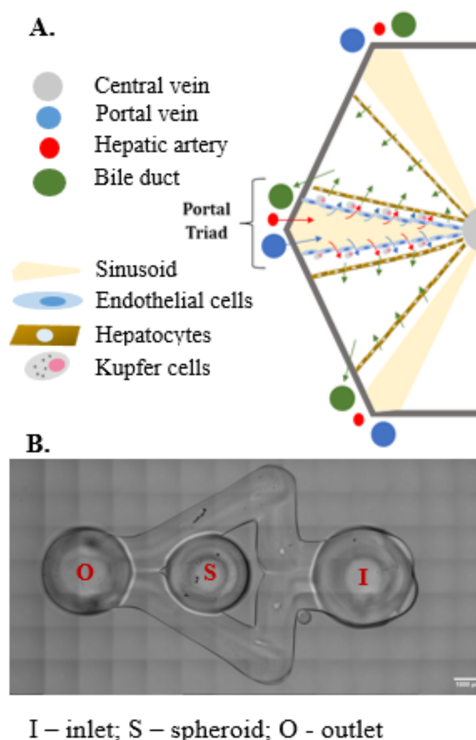

Figure 1. A. Scheme of hepatic lobule structure. B. Inverted brightfield image of microfluidic chip coated with HUVEC cells, with the location of its inlet, outlet and opening for spheroid insertion (scale bar 1000 µm).

CONCLUSION

We were able to design and produce a simple microfluidic chip that can be used to study the interaction between endothelial cells and a tumor model, better resembling the physiological environment than using monoculture systems. This microfluidic chip can be used as a relevant system to assess drug efficacy and toxicity.

ACKNOWLEDGEMENTS

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3D in vitro models for advanced colorectal cancer

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INTRODUCTION

Three-dimensional (3D) cell culture models have emerged as promising tools to bridge the gap between animals and cell culture systems. However, to generate reliable pre-clinical data and identify new effective therapeutics, in vitro models must be integrated with components of the tumor microenvironment (TME). TME has a determinant role in cancer development, it supports the adaptation of disseminated cancer cells required for their survival and homing to distant sites and reduces therapy efficacy [1, 2].

EXPERIMENTAL

We established a collection of organoids and spheroids from liver metastases of 60 CRC patients. We also isolated hepatic Cancer Associated Fibroblasts (CAFs) and normal fibroblasts. Primary cultures were validated by STR identification, tumorigenicity assessment in mice and xenograft comparison with the tumor of origin. Isolated cells were analyzed for the expression of specific markers and by functional assays. Pre-clinical platforms were developed to perform drug screenings and detailed studies on tumor-stroma interactions. 3D co-culture methods were optimized, and custom chips were exploited to mimic interactive dynamics between CAFs and cancer cells. Custom microfluidic chips, consisting of 3D cell culture compartments in hydrogel matrices connected by migration microchannels, were fabricated [3, 4]. They were validated to study the metastatic spread of cancer stem cells (CSCs) towards stroma microenvironment embedded in mixed matrigel/collagen matrices.

RESULTS AND DISCUSSION

A systematic comparison between spheroids and organoids defined specific features of each 3D model. Spheroid cultures are enriched in CSCs [5], endowed with mesenchymal traits while organoids contain a higher percentage of differentiated cells, recapitulating tumor heterogeneity. Consistently, spheroids are more prone to migration/invasion than organoids, when co-cultured with CAFs in a 3D matrix.

The growth rates of spheroids and organoids were compared both in vivo and in vitro, and patient-specific drug sensitivities were screened using the same chemotherapeutic agents used for therapeutical treatment (5 fluoracil, oxaliplatin, irinotecan).

We optimized high-throughput drug screening procedures with spheroids and different co-culture settings of organoids/spheroids and stromal cells to analyze reciprocal spatial organization and how cancer cells are molded by the niche in terms of secretome, specific protein expression and drug response. Chip technology allows to mimic the crosstalk between metastatic CRCs and CAFs and represents a reliable tool to investigate tumor-stroma interactions and the role of the hepatic niche as attractive "soil" for tumor cells homing.

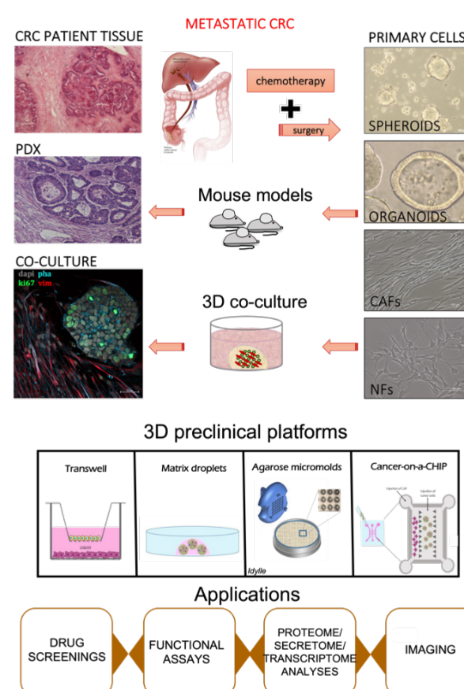

Figure 1. Primary cultures were isolated and banked from ~60 metastatic colorectal cancer patients who underwent hepatic resection upon perioperative chemotherapy: hepatic cancer-associated fibroblasts (CAFs), normal fibroblasts (NFs) derived from the surrounding healthy parenchyma, and metastatic CRC cells propagated as either organoids or spheroids. Left panel: H&E staining of advanced CRC and the corresponding xenograft (PDX) obtained by sub-cutaneous injection of patient-derived cancer cells, showing similar histology and an abundant stroma. 3D in vitro co-cultures mimic the spatial organization of patient's tumor. Organoids are stained with Ki67 and phalloidin antibodies, whereas CAFs express vimentin (red). Lower panel: schematic of 3D pre-clinical platforms developed with primary CRC cells and their applications.

CONCLUSION

We have developed solid 3D co-culture systems with primary cancer cells and CAFs, able to reproduce the structure of the tumor of origin and the response to therapy of patients with metastatic CRC.

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Kinetic detection of apoptosis events via caspase 3/7 activation in a tumor-immune microenvironment on a chip

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INTRODUCTION

The ability to dissect phenomena occurring in complex biological environments is strongly connected with the availability of biological models recapitulating the problem under study, and to the possibility of measuring the processes that develop inside the system.

The development of physiologically relevant microenvironments in a high-throughput/high-content configuration [1,2] and standardized, is proving to be a promising tool to evaluate on one side cancer behavior and evolution [3], on the other drug efficacy and mechanism of action fostering a personalized medicine approach by exploiting patients' biopsies.

EXPERIMENTAL

In this work, we disclose a protocol to identify and characterize the temporal evolution of apoptosis by timelapse fluorescence and confocal imaging in a 3D microfluidic murine co-culture model including cancer and spleen cells.

The MC-38 wild type tumor cells were embedded in a collagen I matrix (14'000 cells/chip); the splenocytes (140'000 cells/chip), recovered from the spleens of C57BL/6 mice, were stimulated with anti-murine CD3.

As a positive control, MC-38 cells were treated with 1mM Staurosporine (STS), a protein kinase inhibitor and apoptosis inducer.

To detect apoptosis events, fluorescence-based caspase 3/7 (CellEvent Caspase-3/7 Green Detection Reagent) assay was used and analyzed by time lapse acquisition under a Nikon A1R+ confocal microscope (FITC channel for apoptotic cells, DAPI channel for Hoechst stained nuclei). High content screening protocol was automated by customized routines through a high-content analysis software suite (Nikon JOBS plugin).

RESULTS AND DISCUSSION

Figure 1a shows the microchip used for this protocol, derived from a previous study [4]. The chip consists of 5 major compartments: a central chamber hosting the immune cells, two side regions for embedding tumor cells in collagen matrix, and two media perfusion chambers. Immune and tumor chambers are connected by two sets of microchannel arrays and are in contact with the media perfusion chambers.

At 6, 9, 12 hours after treatment, representative 3D images (Z stack projections) of apoptotic events were acquired (**Figure 1b**) in three experimental conditions (A. Negative control, B. Splenocytes in co-culture C. Cells treated with STS).

Microscopy datasets were analyzed by quantifying the number of apoptosis events (**Figure 1c**) detected in the green channel normalized to the total number of cells as indicated by the quantification of nuclei based on Hoechst staining, at predefined timepoints or via time-lapse imaging.

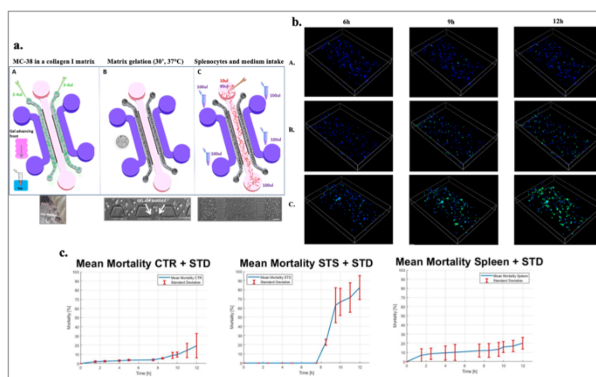

*Figure 1. **a)** Chip Structure; **b)** 3D images (Z stack projections) at 6, 9 and 12 hours from treatment A. Negative control B. Splenocytes coculture C. STS treated cells. Magnification 10X. Blue: Hoechst nuclear stain, Green: Caspase 3/7 activation marker. Each stack is composed of 29 images, spaced 5 µm from each other, for a total thickness of 135 µm, XY imaged dimensions 1000x1273 µm; **c)** Trends of cell apoptosis quantification in the no treatment (CTR), splenocytes coculture and positive control conditions.*

CONCLUSION

The present work highlights the potentiality of on-chip methodologies combined with high content microscopy to measure relevant biological processes in a dynamic manner, with the possibility to exploit Artificial Intelligence-enabled methods for processing high-content data.

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Human Embryonal stem cell-derived cardioids as a model to study the effect of air pollution on developing heart

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INTRODUCTION

Air pollution is a well-known trigger for non-communicable diseases, including cardiovascular diseases as the most life-threatening pathology in urban areas[1]. The Particulate Matter (PM) includes sub micrometer range particles capable of passing physiological barriers (alveolar-pulmonary, placenta, etc.) and reaching the heart by interacting directly with the cardiac tissue. During pregnancy, both the mother and the embryo can be exposed to PM that entered into the circulatory system via inhalation, deteriorating the cardiac performance[2]. Whilst it is possible to constantly monitor heart contractility in adults, little is known about the effect on the embryonic heart. Here we show the effect of $P.M._{10}$ on the cardiac contractility of 3D cardioids differentiated from a human embryonic stem cell line using longitudinal optokinematic incubation system (LOKI).

EXPERIMENTAL

Cardiac spheroids were generated from differentiated Rockefeller University Embryonic Stem Cell Lines (RUES). Cells were thawed in E8 medium (Gibco) with 1% rock inhibitor. The day after the medium was replaced with E8 and changed every 2 days until reaching confluence of 60-70%. Differentiation into cardiomyocytes was performed using PSC cardiomyocyte DIFF Kit from Gibco. Briefly, medium A was added at day 1 of differentiation which was replaced after 48 h with medium B. Medium M was then added at day 5 and changed every 2 days. Beating started after 12-14 days. Cells were then dissociated with Multi Tissue Dissociation Kit 3 (Myltenyi Biotec) and filtrated using magnetic columns (Myltenyi Biotec) after mixing with magnetic antibodies (Myltenyi Biotec) to entrap not differentiated cells. Eluted cardiomyocytes were then counted and seeded in an ultra-low attachment 96-well plate at a density of 50000 cells to form spheroids. DMEM supplemented with 10% FBS was changed every 2-3 days. LOKI system consists of a customized hardware-software platform (a microscope included in a standard cell incubator) capable of monitoring several cardioids over time by acquiring electromechanical activity during acute and chronic exposure. PM_{10} derived from the urban city of Milan was resuspended in medium and administered at increasing dosage, 10, 20, 50 and 100 µg/ml in cardioids. Controls consisted of unstimulated spheroids. The kinematic activities were monitored with LOKI at 4, 24, 48, and 192 hrs. Videos were then postprocessed with Muscle Motion (Fiji)[3] to extrapolate beating parameters such as contraction and velocity profiles, time-to-peak, beating rate.

RESULTS AND DISCUSSION

Cardioids from RUES were successfully generated and maintained for the whole duration of the experiment. Beating and compacting started after 3 days in the ultra-low attachment wells. From video analysis of the stimulated cardioids, it was possible to notice that PM_{10} at 20 µg/ml, 50 µg/ml, and 100 µg/ml affected cardioid mechanics by increasing contraction time after 48 hrs. This was calculated at 90% of contraction overshoot. On the contrary, no changes were noticed in the relaxation time. Also, the half energy was affected with a significant increase after 24-48 hrs after PM_{10} stimulation. Furthermore, it was observed that these 3D embryonal cardioids tended to re-establish contraction duration and contraction velocity for low concentrations of PM after 48 hrs. However, a concentration higher than 50 µg/ml significantly affected both parameters.

CONCLUSION

The developed LOKI customized platform provided a novel low-throughput functional screening approach for the investigation of cardiac toxicology exerted by the PM_{10} in a 3D cardiac model. This system allowed to evaluate the effect of air pollution directly on human embryonal cardiac stage, which was not possible with conventional in vitro and in vivo methods. This platform can be suitable for other high-throughput drug screening whereby a functional investigation is required.

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Selective positioning of different cell types on 3D scaffolds via DNA hybridization

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INTRODUCTION

In the last decade, a great effort has been devoted to the establishment of in vitro platforms mimicking the complexity of cellular and tissue microenvironment. Two-photon lithography (2PL, fig. 1a) has given a major contribution to the field, and several scaffolds for studying cell biology made via 2PL have been reported[1]. Further improvements are represented by techniques for the precise positioning of distinct cell types in a 3D microenvironment. However, current techniques, e.g., antigen-antibody interaction, pose severe limitations for selective single-cell-type tagging as they lack specificity[2]. Here, we report the decoration of 2PL-obtained 3D microscaffolds with single-strand DNA (ssDNA) exploiting light-induced click chemistry.

EXPERIMENTAL

An acrylate-based 2PL resin including an UV-reactive molecule (i.e., photo-enol, PE, fig.1b) was formulated and characterized for photopolymerization parameters (i.e., intensity and dose, fig.1c), for further surface decoration with biomolecules. The aldehyde group of PE enolizes upon UV irradiation and subsequently reacts with a carbon double bond (e.g., of a maleimide molecule) via a Diels-Alder [4+2] cycloaddition click reaction[3]. Therefore, a solution of biotinylated maleimide in DMF was placed on the 2PL-made 2D and 3D microstructures and exposed to a focused 405 nm laser, resulting in the covalent binding of the maleimide to the PE; streptavidin was then incubated, followed by an incubation step with a fluorescent biotinylated oligonucleotide (fig.1d).

RESULTS AND DISCUSSION

The degree of functionalization can be controlled by tuning the laser intensity and exposure time and verified via confocal microscopy.

Moreover, it was possible to repeat the functionalization procedure sequentially for two different oligos both on 2D (fig.1e) and on 3D structures (fig.1f). To test the selective binding affinity of cells to ssDNA, two different cell lines (i.e., NIH3T3 and U2OS) were decorated with cholesterol-TEG complementary strands and incubated on functionalized 3D scaffolds (fig.1g). As expected, cells hybridize more effectively on complementary oligos than on surfaces with non-complementary ssDNA (fig.1h). Selectivity of adhesion on functionalized structures was quantified also with several other cell lines, including stem cells (fig.1i).

CONCLUSION

Our work shows the capability of functionalizing surfaces of 3D scaffolds fabricated via 2PL selectively and precisely. This represents a first step towards controlled micro-environments for lab-on-chip devices hosting multiple cell lines. Current studies focus on (i) studying the biological significance of the selective positioning of cells in 3D platforms (e.g., by investigating paracrine signalling between cells on different areas of the same scaffold), and (ii) scaling up the proposed methodology to the meso- and macroscale by widening the palette of acrylate-based photoresists, thus enabling the fabrication of 3D scaffolds using consumer-grade stereolithographic 3D printers.

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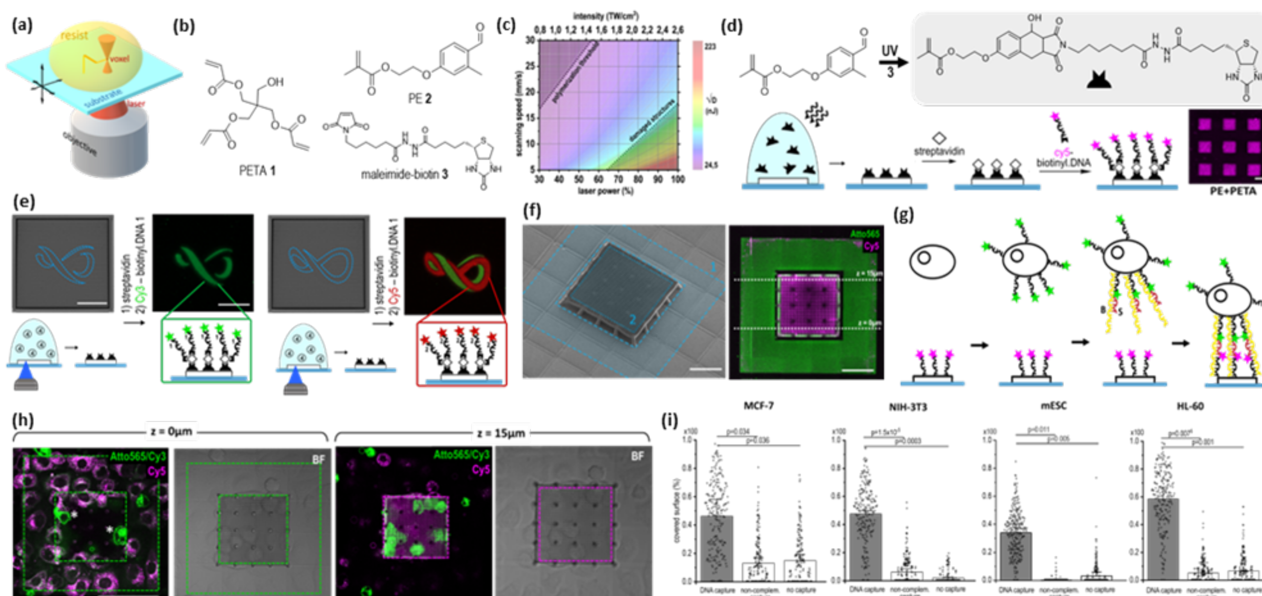

Regulatory issues of Organs-on-chip

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INTRODUCTION

The concept of organ-on-chip (OoC) is gaining success, due to the potentialities of this type of device: OoCs can in principle fulfil the promises of personalised medicine, by recapitulating a specific person's physiology. There are several definitions of OoC available. According to Marx et al. [1], for instance, the term "Organ-on-chip" stands for a fit-for-purpose microfluidic device containing living engineered organ substructures (functional unit(s)) in a controlled microenvironment, which recapitulate one or more aspects of the organ's dynamics, functionality and (patho)physiological responses in vivo under real-time monitoring. This definition allows for a broad range of possible uses of the device, included those having a direct impact on health.
For regulatory compliance, it is essential that each OoC be provided with clear information about the *intended purpose* of the device. From the definition in the MDR [2], the 'intended purpose' means the "use for which a device is intended according to the data supplied by the manufacturer on the label, in the instructions for use or in promotional or sales materials or statements and as specified by the manufacturer in the clinical evaluation". Then, from the intended purpose (or use, synonymously) of the device, the regulatory requirements for OoC can be derived, if applicable.

EU REGULATORY FRAMEWORKS FOR OOC

An OoC with diagnostic functions can be functionally similar to an IVD (in vitro diagnostic) medical device (MD). From the IVD 2017/746 Regulation [3] (specific for this class of MDs) the following definition applies: 'in vitro diagnostic medical device' means "any medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, piece of equipment, software or system, whether used alone or in combination, intended by the manufacturer to be used in vitro for the examination of specimens, including blood and tissue donations, derived from the human body, solely or principally for the purpose of providing information on one or more of the following:
(a) concerning a physiological or pathological process or state; […]
(e) to predict treatment response or reactions;
(f) to define or monitoring therapeutic measures."
From this definition, the similarity of such a device to an OoC with diagnostic capabilities is evident, e.g., for case (a). It must be underlined, though, that an IVD MD, although being a particular type of MD, is still a medical device, as defined in [2]. Hence, it cannot contain cells or tissues of human or animal origin, unless they are made non–viable (see definition in Art. 2 [2]: "'non-viable' means having no potential for metabolism or multiplication"). This renders questionable the applicability of the IVDR [3] to OoCs, at least in the current revision of the former.
An OoC can also have, in principle, a therapeutic intended purpose: the availability of, say, an implantable liver-on-chip, laden with hepatic cells and capable of substituting part of a liver's functions upon implantation, cannot be ruled out, in the future. This would require the implantability of this type of OoC in the human body, with the consequent necessity of demonstrating its safety and efficacy. OoCs of this category are similar functionally to a medical device, according to the definition of the latter [2].
This notwithstanding, the range of application of the MDR excludes the use of viable cells or tissues (either of human or animal origin) in the fabrication of MDs, therefore OoCs are generally not regulated (at least, exclusively regulated) by the MDR.
A more appropriate regulatory framework for such type of OoCs is instead the Regulation (EC) No 1394/2007 on advanced therapy medicinal products (ATMP regulation), allowing the use of viable cells or tissues. In particular, OoC fits well into the definition of 'combined ATMP', since it incorporates, as an integral part of the product, one or more medical devices, besides the cellular component.

DISCUSSION AND CONCLUSION

The analysis of the regulatory status of OoCs, as far as their direct healthcare applications are concerned, has underlined that there are still uncertainties in the pathway of OoCs to the market.
An OoC with diagnostic purpose cannot be regulated solely by the IVDR [3], which does not (yet) foresee devices containing living cells or tissues.
As for OoCs with therapeutic purpose, they may be generally regulated as combined ATMPs, even though limitations arise whenever the cells or tissues used in the fabrication of the device cannot be considered as "engineered", in the sense of Art. 2(c) of the ATMP Regulation.
Hence, an effort to adapt the current regulatory frameworks is probably necessary, in view of the increasing diffusion of OoCs and similar technologies.

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Innovative on-chip technologies for cell biology

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INTRODUCTION

The tumor microenvironment (TME) is the complex and dynamic ecosystem that surrounds a tumor. TME consists of tumor cells, tumor stromal cells including stromal fibroblasts, endothelial cells and immune cells and the non-cellular components of extracellular matrix such as collagen, fibronectin, hyaluronan, laminin, among others. TME components through mutual and dynamic crosstalk are able to influence the growth, invasion, and metastasis of the tumor[1].

Conventional models such us 2D *in vitro* cancer cells cannot mimic the TME such as stroma, nutrients, oxygens, and signaling molecules. Recently, tumor spheroids, 3D cultures of cancer cells, have been developed as model for fundamental biological investigation or for drug screening since they can recapitulate the morphological, genetic, and epigenetic features of a tumors[2]. The traditional methods for spheroids formation present some limits such as poor control on size, uniformity and compositions resulting in differences in nutrient and oxygen distribution inside the 3D structure. In addition, spheroids in liquid medium can differ from the physiological condition in which the mechanical and chemical stimuli of extracellular matrix and TME play a key role in tumor development[3].

Lab-On-Chips (LOCs) technologies have seen a breakthrough burst in translational research, with the aim to mimicking and real-time monitoring of biological events in an always more reproducible environment, thus improving the validity of the bioassays. In this context LOC devices will allow to partially reproduce tumor microenvironment having a disruptive impact on the understanding of disease's features[4].

We developed some tools to mimicking different biological microenvironment. First, we aimed to realize a robust platform able to real time detect cells crossing the membrane (on-chip invasion and migration device). In this way, local invasion events will be investigated, thus correlating biomolecular pathways to real behavior of cells. Second, exploiting the principles of microfluidics we developed a device to generate rapid and uniform tumor spheroids to overcome the limitations of conventional spheroid formation methods (droplet device).

EXPERIMENTAL

Taking advantage of the expertise at CNR Nanotec in monitoring cell adhesion and shape modification through electrochemical methods, the on-chip invasion and migration devices were optimized. In particular, a miniaturized and automatic Boyden chamber, able to detect migrated cells in response to differential membrane functionalization (using ECM proteins) was realized, through electrochemical impedance spectroscopy (EIS) technology[5]. In that case, different cell behavior was detected thanks to the presence of electrodes able to "sense" migrated cells at the endpoint of the experiment. Moreover, a 3D system was implemented, characterized by a lower and upper chamber, separated by a metal porous membrane (with pore size range 8-14µm) obtained by femtosecond laser (fs) ablation[6]. Two compartments are linked to tubes (inlet and outlet), through which it is possible to regulate the flow bioreactor for physiological barriers simulation.

In addition, another device was realized to obtain standardized tumor spheroids by droplet-based microfluidics. The droplet device was fabricated for the production of spheroids with uniform control of size and composition, mimicking a tumour mass in a reproducible way. The device in polymethyl methacrylate (PMMA) substrates that enable optical investigation thanks to its complete transparency, is based on a double T-junction to create a core-shell droplet structure. In this way the core matrigel/hydrogel will be loaded with cancer cells, while the shell will protect cells from shear stress and from contact with mineral oil during droplet generation. The droplets will be collected, after the polymerization process, in the accumulation chamber.

RESULTS AND DISCUSSION

Preliminary data of the on-chip invasion and migration device were obtained in a commercial setup, including a femtosecond laser ablated membrane to separate the two chambers. In particular, we used a passivated microfabricated membrane made of Copper/Kapton/Copper, coated with a nanometric gold layer to make it biocompatible. Membrane pores have a diameter of 8-12 µm, funnel-shaped to enhance the passage from the seeding compartment to the migration side. Prostate cancer cells (PC-3 cell line) were seeded in the device and after 48h they showed both adhesion near pores and ability to cross them, as demonstrated by optical and fluorescent microscope acquisitions[7]. In addition, the possibility to transfer spheroids from commercial cells line onto the functionalized metal membrane through microfluidic channels was explored. After 48 hours, single cells are able to detach from the tumoroid, adhere on membrane surface and migrate towards the pores.

The second device for droplet-based microfluidics generation was theoretical and experimental investigated in order to identify the optimal geometry (in collaboration with Prof. De Tullio) and operating parameters for tumor spheroids growth. The results of the simulations proved that droplet size and droplet regimes depend on the capillary number in the system and on the flow rate ratios of the two phases. In the experimental part of the work, we demonstrated how droplet size depends on the channel dimensions, flow rates and flow rate ratios. Moreover, a standardized protocol to obtain uniform spheroids from breast cancer cell lines is work in progress.

CONCLUSION

The LOCs with microfluidic setup are able to reproduce the tumor microenvironment suitable for cancer migration and invasion processes. Microfluidic devices represent a way to overcome several issues of 2D cell cultures and the absence of TME component and factors.

Available techniques and instruments for microfabrication (micromilling machines, 3D printers), will allow the realization of a fully *plug-n-play* and on demand microfluidic device, including active membrane and related electrical connections, and spheroids-based microfluidics generation device. In a combined approach, spheroids made in a high reproducible manner will be used to test the migration ability of a 3D-culture system following a chemoattractant gradient provided by medium enrichment/exchange or drug delivery into the other side of the membrane mimicking the metastatic ability of tumor cells to invade another tissue.

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Cellular mechanotransduction modelling on micropatterned PDMS substrates

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INTRODUCTION

In their native tissue environment cells are stimulated not only by biological and chemical signals but also by mechanical stimuli. Macroscale topographies are reflected in tissue organization, microscale in the cellular level architecture and nanoscale in the subcellular architectures [1]. Extracellular matrix (ECM) is the principal source of mechanical stimuli for cells and each tissue is characterized by a proper stiffnesses aimed at the maintenance of specific homeostasis and functions of each organ. However, in pathological conditions, the ECM can change its mechanical properties, affecting cell-cell and cell-ECM interaction [2]. In order to replicate stiffness conditions and related variabilities, improvements in micro technology offer the possibility to fabricate micro-patterned substrates characterized by different geometrical and mechanical features that could be exploited to mimic the environment that surrounds cells, influencing their organization in tissues and their functionality. Micropatterned polydimethylsiloxane (PDMS) substrates are designed to offer different stiffnesses to cells cultured in vitro. These microstructures are fabricated by soft-lithography on SU-8 molds, obtained by an optimized protocol of maskless photolithography. Different stiffness is achieved by different geometrical, mechanical and topological features of fabricated micropillars.

EXPERIMENTAL

Substrates were designed and fabricated to obtain distributions of circular micropillars. Considering the behavior of human induced pluripotent stem cell-derived pericytes [3], and the fact that spatial density might affect micropillar shape, different patterns were tested ranging from 4 to 5.5 µm pitches, 4 - 6 µm height range, Ø 3.3 µm. Furthermore, we introduced pillars featuring an oval section (3.5 µm to 7µm) to model heterogeneity versus anisotropy of the substrate stiffness. Double layer monolithic SU8 molds for PDMS substrates were fabricated by means of photolithography techniques, on silicon substrates (selective maskless exposure, Direct Write Lithographer Heidelberg MLA100 www.polifab.polimi.it), by tuning the critical settings: *dose* (exposure energy in terms of mJ/cm^2) and *defocus* (focus position of the laser write head). PDMS micropillars were obtained by molding DOW Sylgard 184 Silicone on the silanized SU8 micropatterned substrates, by changing the ratio of the mix and the curing temperature.
Observation of the SU8 interior shape of cavities and of related PDMS structures, by means of profilometer (3D Optical Profilm3D), were dominated by artefacts so measurements were performed by SEM directly on PDMS sample.

RESULTS AND DISCUSSION

A number of PDMS micropillar substrates, different in height, size, pitch and mechanical properties of the materials were realized, covering an estimated range of stiffness between 140 and 460 KPa. We selected 11 PDMS sample, showing a different condition of pattern and stiffness. PDMS samples were punched, sterilized and positioned in a standard multiplate for cell culture.
Preliminary cellular adhesion assays, conducted on these substrates, revealed a preferential adhesion of endothelial cells on substrates featuring a stiffness in the range of 173 – 381 kPa and featuring a circular cross-section.

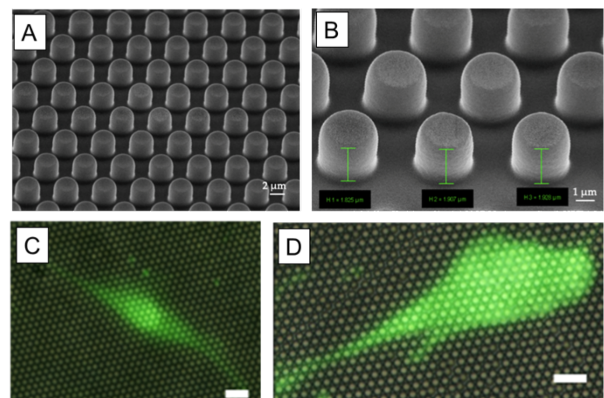

Figure 1. –A-B - PDMS pillars 1.9 µm in height,Ø 3.2µm, spacing 5.5 µm. SEM images C-D Images of endothelial cells on 3 µm height pillars, 4 µm pitch, 10:1 PDMS mixing ratio, and 4 µm height pillars with 4.5 µm pitch, 9:1 PDMS mixing ratio (10 µm scale bar).

CONCLUSION

Preliminary evidences confirmed that cells react to the patterned substrates showing elongation in response to different geometrical/mechanical stimuli.
The optimized microfabrication process here proposed confirmed the possibility of employing these patterns on multilayered structures, mimicking a wide range of stiffnesses conditions for in-vitro tissues and organ modelling.

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Digital Twin of a hESC-derived cardioids-on-a-chip bioreactor

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INTRODUCTION

A digital twin (DT) is a simulation capable of mirroring a system and exchanging information with it. In bioprocesses, a DT is useful to control, optimise and predict the outcome of its physical counterpart. The simulation comprises either mathematical, data-driven or hybrid sub-models [1]. The first group of modelling methods is built on mechanistic knowledge, which describes the system through ordinary differential and algebraic equations. The second group is based on artificial intelligence and the correlation between system quantities. The last one is a mix of the previous two modelling techniques, which is an adequate compromise between model complexity and usefulness. Another fundamental aspect of a DT is the set of sensors and controllers needed to interact with the physical counterpart. Microscopy allowed the study of the world on the micron scale, and organoid-on-a-chips are the result of the advances of this invention combined with machine learning applied to images [2]. Algorithms sped up repetitive image analysis tasks such as classification, recognition, and tracking, allowing the acquisition of substantial amounts of data. Nowadays, high-throughput techniques generate invaluable biological information about the actual system to increase the reliability of the DT [3].

METHODS

The bioreactor comprises hEPSC-derived cardioids cultured on a chip in a temperature and CO_2-controlled chamber. The medium is continuously changed via microfluidic system connected to a pump. The bioreactor includes a microscope equipped with a fast-resolution camera that acquires online data for the postprocessing (see Figure 1). Another work by our group better describes the differentiation of the cardioids phase and provides further details of the developed physical system. The offline measurements of the system are the metabolite concentrations detected by analyser kits. The control variables are the drug concentrations in the medium and the medium flow rate. The substances introduced to the bioreactor are chronotropic or depolarized drugs, such as caffeine and KCl. The contractile profiles acquired and processed by the microscopic camera are used to extract several features, which are fed to multiple machine learning algorithms, such as support vector machine and random forest. These classifiers recognise whether the cardioids are isogenic or mutant and produce performance metrics, e.g., accuracy, true-positive ratio, and F1-score.

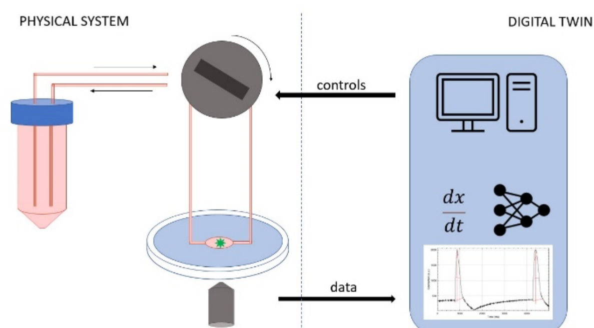

Figure 1. Representation of the physical system with its digital twin. A hybrid model was chosen for the digital twin due to the complexity of representing the cellular culture in a mechanistic way.

RESULTS AND DISCUSSION

The machine learning algorithms were tested on the contractile parameters of the beats of adult mice ventricular cardiomyocytes and cardioids derived from an embryonic stem cell line, which were treated with different drugs and stimulated at different frequencies. The results showed that random forest outperformed the other algorithms in the classification task.

CONCLUSION

A DT in bioprocesses is an excellent way of reducing the operation costs of a bioreactor and it can make experiments more reproducible by monitoring the state and control variables of the biological system. Encouraging results were obtained in our classification task of the cardioid signals. Nonetheless, the aim of our work is not only to classify the beats of the contractile profiles, but also to predict the contractile profiles of different diagnoses by fitting online the parameters of a mathematical function such as a Markovian model.

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Patient-derived endometrial cancer organoids reveal molecular and genomic features of primary tumors

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INTRODUCTION

Endometrial cancer (EC) is one of the most common gynaecologic cancer among women worldwide, where its incidence and mortality rates have been increasing in the last decade. Recently, The Cancer Genome Atlas identified a new set of criteria for the classification of EC based on genomic alterations that categorises endometrial carcinoma into four distinct prognostic molecular subgroups: polymerase ε (POLE) ultra-mutated, microsatellite instability hyper-mutated, copy-number low and copy-number high.

Currently, the gold standard for EC treatment is surgery, radiation and standard chemotherapy.[1]

Patient-derived organoids (PDOs) represent 3D novel advanced preclinical models *in vitro* for studying histological and physiological characteristics of intra-tumour heterogeneity. Moreover, they are emerging as promising tools for evaluating novel therapeutic strategies for promoting personalized therapies for patients. [2]

EXPERIMENTAL

Endometrial cancer tissues derived from surgical resections were placed in tissue storage solution supplemented with penicillin, streptomycin and antimycotic. They were enzymatically minced, cultured in droplets of Geltrex and expanded in an optimized growth medium. Primary tumors and PDOs were characterized by immunohistochemistry using specific prognostic markers and whole-exome sequencing and bulk RNA sequencing. The response of organoids to conventional chemotherapeutic agents was evaluated by the ATPlite assay (Figure 1).

RESULTS AND DISCUSSION

PDOs were generated from ten endometrial cancer patients and were maintained in different optimal culture conditions in accordance with the type of tissue collected by surgical resection: non-tumour (N), peri-tumour (PT) derived from resection at 1 cm from tumour, and the tumour itself (T). During organoid growth, we observed that all kinds of PDOs show considerably different proliferation rates and sizes. Interestingly, we observed that PDOs derived from PT tissue, grew faster in a specific tumour growth medium than in a medium designed for normal tissue.

To verify whether PDOs recapitulate EC patient phenotypes, we performed immunohistochemistry (IHC) with the same markers used on primary tissues.

Furthermore, after DNA/RNA extraction from tissue and PDOs, we compared genetic alterations of tumours with PDOs by Whole-Exome Sequencing (WES) and bulk RNA sequencing.

Interestingly, we found that PDOs reflect histopathological features and genetic profiles of original patient tissues.

Drug sensitivity testing utilising 3D preclinical models is an important and effective method for predicting the efficacy of therapy in individual patients. We verified that PDOs respond to standard therapy at the same extent as patients do, by evaluating PDOs viability upon treatments with clinically relevant doses of several chemotherapeutic agents.

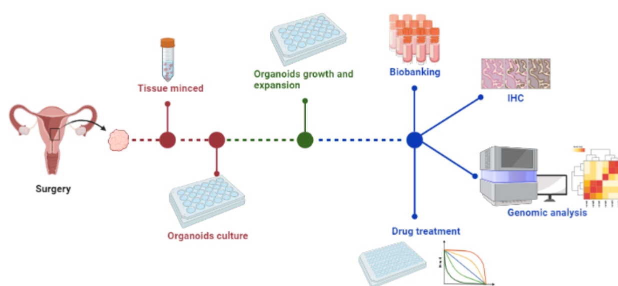

Figure 1. Workflow

CONCLUSION

Here, we suggest a robust preclinical model that mimics endometrial intra-tumour heterogeneity which is a promising tool for clinical application and the assessment of effective therapies. Moreover, the genomic analyses of tumours and matching PDOs could reveal novel therapeutic strategies through personalized treatment appropriate for each patient.

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Bladder cancer organoids: a new frontier for personalized therapy

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INTRODUCTION

Bladder cancer (BC) accounts for 3% of global cancers and has a higher frequency especially in developed countries. Its highest incidence rate arises between the fifth and seventh decades, where it is almost three times more common in men than in women. It is the ninth leading cause of cancer death in Europe. One of the main risk factors is tobacco smoking (1). The standard procedures for treating BC are a transurethral resection of bladder tumor (TURB) and a radical cystectomy. From an histological perspective, bladder cancer is currently classified into three main groups depending on its size and aggressiveness: low-grade non-muscle-invasive bladder cancer (LG NMI), high-grade non-muscle-invasive bladder cancer (HG NMI), and muscle-invasive bladder cancer (MI), which are characterized by high mortality (1). Therapeutic options depend on tumor staging and classification. Re-TURB surgery is carried out within the first 3 months of diagnosis for LG NMI; immunotherapy with intravesical installations of BCG (Calmette-Guerin bacillus) is administered for HG NMI tumors; neoadjuvant chemotherapy followed by radical cystectomy is recommended for MI tumors; and systemic chemotherapy is indicated for metastatic tumors (2).

Three-dimensional bladder cancer patients-derived organoids (PDO) have become very reliable models for the study of cancer heterogeneity, molecular characteristics, and drugs response (3).

The main aim of this study is to create a bladder cancer organoids biobank in order to provide a robust tool for the development of personalized therapeutic strategies tailored to individual patients and tumor features.

EXPERIMENTAL

Bladder cancer tissues from TURBT resection or cystectomy were mechanically and enzymatically digested to obtain single cells suspension. Cells were included in drops of geltrex and cultured with a specific medium supplemented with different factors to promote cell growth. Molecular analysis of PDOs and the original tumor tissues were performed with Whole Exome Sequencing (WES), RNA-sequencing and immunohistochemistry (IHC). PDOs response to different therapeutic agents was assessed by ATPlite assays (Figure 1).

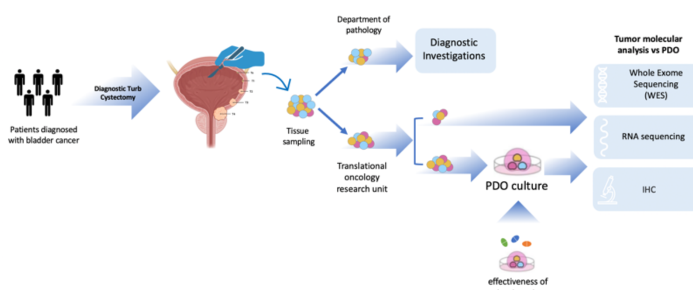

Figure 1. Workflow

RESULTS AND DISCUSSION

PDOs were generated from 10 patients undergoing diagnostic TURB (3 patients) or radical cystectomy (7 patients) at the IRCSS Regina Elena National Cancer Institute (Rome, Italy).

For patients treated with radical cystectomy, PDOs cultures were generated from three different samples: non tumoral tissue (N), peripheric tumour tissue (TP) and central tumour tissue (TC), to recapitulate the heterogeneity of bladder cancer. After establishing the best culturing conditions for generating organoids from bladder cancer cells, we mainly focused on the molecular characterization of PDOs and then matched original tissues to verify their affinity. In particular, by performing Whole Exome Sequencing (WES), RNA-sequencing analysis and immunohistochemistry (IHC), we confirmed that PDOs faithfully recapitulate the original tissue.

After having completed the molecular analysis, we tested the PDOs *in vitro* response to different standard chemotherapeutic approaches to be compared with patient outcome.

CONCLUSION

Nowadays, patient-derived bladder cancer organoids represent faithful experimental models for reflecting true tumour heterogeneity and for testing personalized therapies. We aim to expand the application of bladder PDOs for future preclinical studies to predict *in vitro* the best therapeutic strategy for each patient.

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