Tumor organoids: a new, promising model to test the efficacy of cancer immunotherapies

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Abstract
Cancer is the second leading cause of death worldwide. Recently, a new promising therapy has been developed and is already a standard treatment for some forms of cancer: cancer immunotherapy. Because immunotherapy is expensive and can have adverse events it would be important to analyse if patients indeed can benefit from immunotherapeutic approaches.
Biomarkers can be used to predict the outcome of the therapy, but also a model that can represent the disease in vivo as well as in vitro might be useful. Yet, currently used disease models are limited with respect to the reflection of tumor heterogeneity and tumor microenvironment.
A promising new model to study the efficacy of cancer immunotherapy could be based on organoids. Patient's tumor tissue can be used to generate 3D tumor organoids. In this essay the organoid model will be discussed as a potential model for testing the efficacy of immunotherapies and the model will be compared to currently used models. The tumor organoid model is a promising model, because it could provide additional insight in tumor heterogeneity by growing multiple organoids of multiple biopsies from a tumor. Moreover, the tumor microenvironment can be partially simulated by co-culturing these tumor organoids with immune cells or other cell types.
However, further research needs to be done to overcome the still existing limitations, mostly concerning the insufficient simulation of the tumor microenvironment. Nevertheless, all together the tumor organoids are a promising new model that can be used for testing the efficacy of immunotherapy.

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Introduction

According to the World Health Organization (WHO) in 2018, cancer is the second leading cause of death worldwide. Accounting for approximately 9.6 million deaths per year. Because of this high mortality number, a proper treatment for cancer is important to improve survival rates. Until a few years ago, non-surgical cancer treatment consisted of chemotherapy and radiation. However, the problem of these treatments is low target selectivity, drug resistance, severe side effects, and for chemo incapability to successfully address metastases [1]. In contrast, immunotherapy has a higher selectivity and because of that might has more potential as an anti-cancer drug.

The National Cancer Institute defines immunotherapy as "A type of therapy that uses substances to stimulate or suppress the immune system to help the body fight cancer, infection, and other diseases. Some types of immunotherapy only target certain cells of the immune system. Others affect the immune system in a general way" (www.cancer.net).

When tumor formation takes place, an immune response may be induced. One of the reasons an immune response is induces is that the mutations in the tumor cells may result in neo-antigens. However, the intensity the immune response towards the tumor is insufficient [2]. This is probably because tumor cells cause the inhibition of antitumor immune responses, this may happen by activating immune checkpoints. Immunotherapy inhibits these immune checkpoints and thereby can re-activate antitumor responses [3]. These immune checkpoint inhibitors modulate the interaction of T cells with antigen-presenting cells (APCs) or tumor cells [4].

Different kinds of immunotherapies are currently being developed: monoclonal antibodies, vaccines, engineered oncolytic viruses, adoptive cellular therapy, cytokine-based adjuvant therapies, and small molecule targeting drugs [1]. Of these different immunotherapies the monoclonal antibodies are currently used in clinic.

Although immunotherapy seems a promising way to fight cancer, only a minority of cancer patients benefits from immunotherapy, if a patient reacts to the therapy depends on the tumor micro-environment. Concerning the infiltration of immune cells there are three phenotypes of the tumor micro-environment: immune cells are integrated in the tumor, surrounding the tumor or aren't at the tumor side at all. The first and second phenotype will respond better to immunotherapy than the third [5]. Furthermore, immunotherapy has adverse events, it can cause autoimmunity. This happens because the immunotherapy inhibits the checkpoints that normally helps forming barriers against autoimmunity [3] Because of the adverse events of immunotherapy, it is important to test whether a person could benefit from the therapy.

Currently there are cancer models available on which immunotherapies can be tested, however because these models are not accurate enough a lot of drugs working on these cancer models fail in clinical trials and are therefore rejected [6].

Organoids are three dimensional structures that can be grown from adult or pluripotent, embryonic or induced, stem cells and organize into an organ-like structures, which, in the case of adult stem cells, is specific for the tissue of origin [7,8]. Organoids might be a more promising model for testing the efficacy of immunotherapy, than the models that are available now. In this essay will be discussed if patient derived tumor organoids are a good model for testing the efficacy of immunotherapy.

Immune checkpoints

Immune checkpoints are negative regulatory pathways, which can be activated by immune cells but also by tumor cells. These pathways are associated with immune homeostasis [5]. The immune checkpoints can be used for immunotherapy, by inhibiting them using monoclonal antibodies [1]. The most common checkpoints inhibitors used for immunotherapy are monoclonal antibodies specific for cytotoxic T-lymphocyte protein 4 (CTLA-4) and programmed cell death 1 (PD-1) [9].

CTLA-4 and PD-1 are both members of a family of immunoglobulin related receptors. Both receptors have an inhibitory role on T-cell function [10]. However, their pathways operate at different stages of the immune response [11].

The CTLA-4 immune checkpoint inhibitor stops potentially autoreactive T cells in the beginning of the naive T cell activation, this process happens in the lymph nodes [11]. Multiple stimulatory signals are necessary for T cell activation, one of them is binding of the T cell receptor (TCR) to the major histocompatibility complex (MHC) on the antigen presenting cell (APC). A second stimulatory signal is binding of B7 on the APC with CD28 on the T cell (Figure 1A). when the T cell is activated this will lead to increased T cell survival, proliferation of T cells, and differentiation of T cells through interleukin-2 production. CTLA-4 is a competitive receptor towards CD28, with a higher binding affinity for B7. However, binding of B7 and CTLA-4 will not stimulate the T cell activation [11]. The immunotherapy ipilimumab, which is an anti-CTLA-4 can be used to inactivate CTLA-4 and thereby increase the activation of T cells [3].

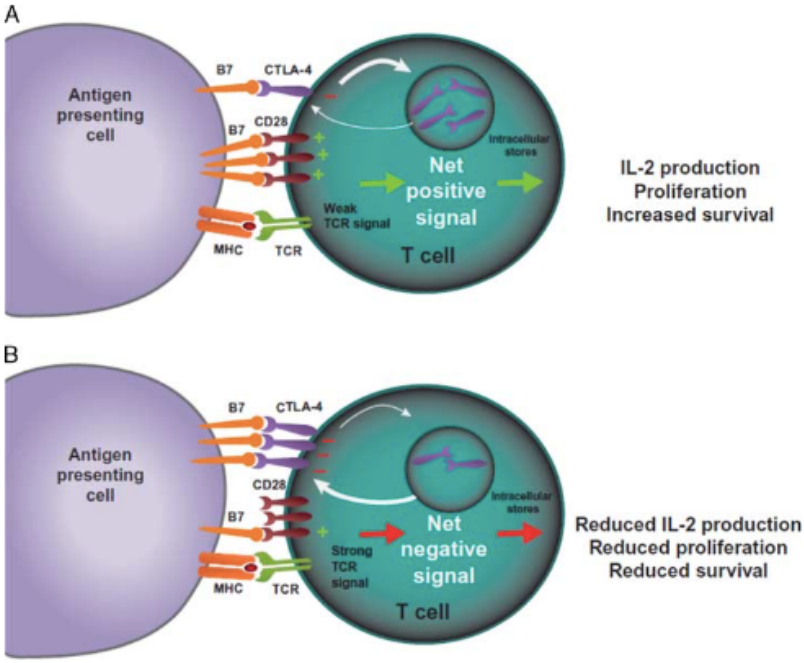

Figure 1. CTLA-4 pathway [11]. A: binding of MHC to TCR and binding of B7 mostly to CD28, which are both stimulatory signals will lead to a posite signal and thus T cell activation. B: binding of MHC to TCR (stimulatory signal), but binding of B7 mostly to CTLA-4 (no stimulatory signal) will lead to a negative signal and thus T cell inactivation.

The PD-1 pathway regulates the activated T cells, this happens in later stages of the immune response, in the peripheral tissue. PD-1 binds to PD ligand 1 (PD-L1), these bindings inhibit the T cell proliferation, cytokine production and survival of T cells (Figure 2). PD-1 being present on the T cell is a characteristic of T cell exhaustion and will lead to T cell dysfunction [11]. The immunotherapies pembrolizumab and nivolumab, which are both anti-PD-1, can be used to inactivate PD-1 and thereby keep the T cells activated [3].

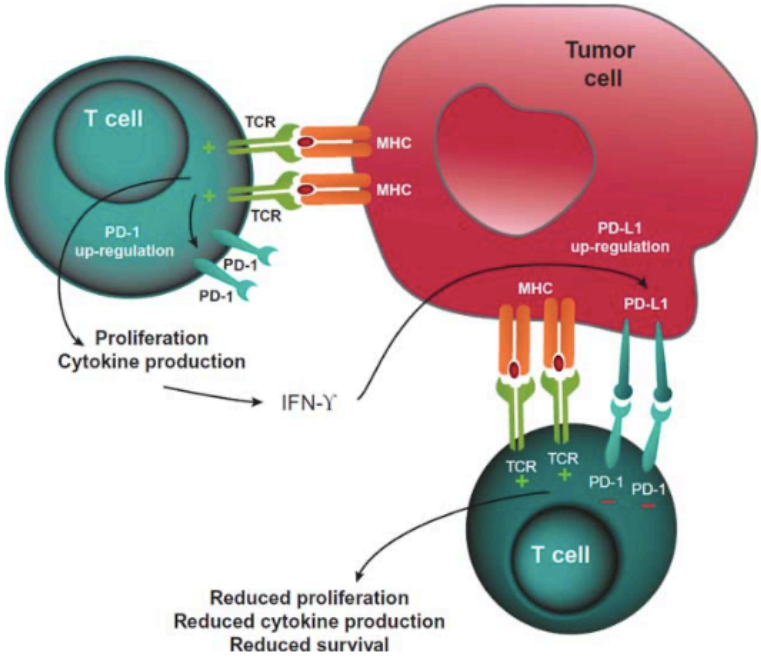

Figure 2. PD-1 pathway [11]. MHC on tumor cells, presenting neo-antigens can activate T cells by binding to the TCR. Long TCR stimulation causes an upregulation of PD-1 which can bind to the PD-L1 and PD-L2 on tumor cells leading to T cell inactivation.

Biomarkers for testing immunotherapy efficacy

Biomarkers are medical signs that can test the medical state of a patient [12]. Using these biomarkers, the outcome of the immunotherapy can be predicted [3]. There are a lot of factors that can influence the response to immunotherapy, in this essay only a few will be discussed.

One of the many biomarkers is the absolute lymphocyte count (ALC). The study of Ku et al. showed that 48% of melanoma patients with elevated ALC at base-line responded to treatment with ipilimumab, while in patients with low ALC only 23% of benefitted from the same therapy [13].

Another biomarker is the abundance of memory T cells, this can predict the outcome for anti-CTLA4 therapy, whereas the abundance of natural killer cells can predict the outcome for anti-PD-1 therapy. Both biomarkers showed a positive correlation with the outcome of the therapy [14].

A third biomarker that can be analyzed using immunofluorescence is PD-L1 overexpression in the tumor tissue, that is already treated with an anti-PD-1 therapy. The study of Reck et al. showed that the survival of patients treated with pembrolizumab was higher when they had PD-L1 expression in over 50% of tumor cells [15].

The amount of neoantigens is another biomarker for predicting immunotherapy response. With new sequencing techniques it now is possible to identify mutations in the exome of a tumor. The more neoantigens a tumor has, the more the immune system recognizes the tumor cells as foreign and thus more antigens will lead to a bigger response of immune system to immunotherapy [16].

Following up the neoantigens, mismatch repair (MMR) deficiency can also be a predictive biomarker, MMR-deficient tumors will have an increased rate of somatic mutations [17].

There are many more possible predictive biomarkers, however it remains a challenge to find new biomarkers that have both a high sensitivity and high specificity. Also, very few

biomarkers are clinically widespread, this because there is a lot of mutational differences between different tumors. But also within each tumor, the mutational spectra is different, the previously discussed heterogeneity [18]. Therefore, it would be helpful to also have an accurate disease model, to test the efficacy of the immunotherapy.

Monolayer cancer cell line model

Currently, different models are used for testing the efficacy of immunotherapies. The first model, that was used for in vitro tumor modeling is a monolayer tumor-derived cell line [19, 20]. Although tumor-derived cell lines are easy to work with there are some major disadvantages when using them for immunotherapy or drugs in general screening. The first disadvantage is that the cancer cell lines have been maintained in growth-promoting cocktails, in monolayer instead of a three-dimensional (3D) culture, causing the cultured cells to grow much faster than cancer cells in vivo [21]. Another disadvantage is that the cell lines do not have the tumor heterogeneity that is present in a primary cancer, the heterogeneity in the tumor is caused by the large number of cell divisions [22]. Because of this heterogeneity the tumor has cells with different characteristics and thus the cells will have different levels of sensitivity to the treatment [23]. Since the cancer cell line does not have this heterogeneity the outcome of the drugs essay will not be accurate for the patient's tumor. The third disadvantage of a monolayer cancer cell line is that this cell line model does not have the components of the tumor microenvironment [20]. This microenvironment consists of the extracellular matrix, the tumor's blood and lymphatic vessels, and the stromal cells, consisting of angiogenic vascular cells, cancer-associated fibroblastic cells, and infiltrating immune cells (figure 3) [1,24]. The responsiveness of the tumor to the immunotherapy depends a lot on the microenvironment, especially on the infiltrating immune cells. So, when an immunotherapy is tested on a cancer cell line the result will not be accurate.

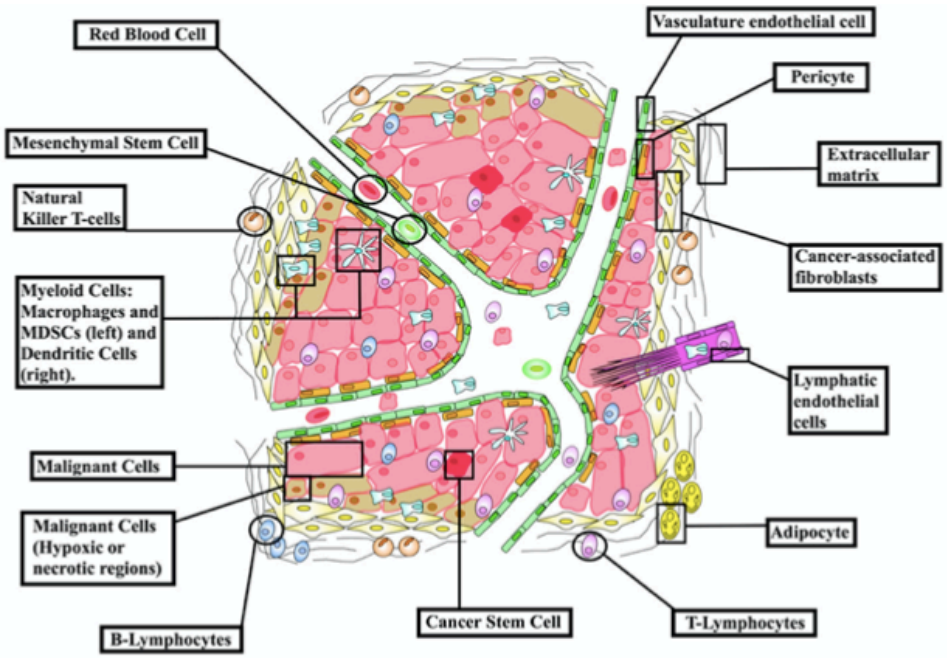

Figure 3. The tumor microenvironment [1]. Tumor cells surrounded by other cell types needed for the tumor cells to live, proliferate, and form a tissue. Including: immune cells, vascular and blood cells, fibroblasts, mesenchymal cells and extracellular matrix.

3D tumor model

This model is a 3D tumor structure that is derived from a cancer cell line and that can also be cocultured with other cell types [19]. These so-called tumor spheroids were created by providing conditions in which the tumor's cell-cell adhesion was greater than the adhesion of cells to the substrate the cells were plated on [25]. Because of this 3D conformation it represents the original tumor more accurately; unlike monolayer cultures, 3D cultures have diffusion-limited distribution of nutrients, oxygen, metabolites, and signaling molecules [26]. It seems like a promising model for testing immunotherapy efficacy, however there are some challenges. These challenges are how to culture the 3D structures and the inability to mimic the tumor microenvironment accurately, in particular the tumor immune cell interaction [19]. Also, just like the monolayer cancer cell line, there are differences between cell line-based models and the original tumors. Especially for rare mutations it can be hard to find a comparable cancer cell line.

PDX-tumor model

A third model is a patient derived xenograft (PDX) model. In contrast to the cancer cell line models, a PDX model is generated out of a patient's tumor tissue (figure 4). These tumor cells are transplanted into an immune-deficient animal. The transplantation happens in an orthotopic or a subcutaneous manner [27]. Then, after the tumor is expanded in the mice, the candidate drug (in this case the immunotherapy) can be tested on the mice [28]. This model ought to be a better representation of the tumor heterogeneity and would also represent the relevant components of the tumor microenvironment [20]. A PDX model can mimic the interactions of tumor cells with stromal cells and extracellular matrix, however it cannot mimic the interaction of tumor cells with the immune system [19]. Furthermore, because the mice immune system is absent the transplanted tumors grow faster, which makes the outcome of immunotherapeutic drugs assay unreliable [29]. Also, studies suggest that transplanting the tumor cells into a mouse causes alterations and rearrangements in the genome [30]. So both, the heterogeneity and the tumor microenvironment, in a PDX model are still not equal to the original tumor. Furthermore, not all tumors qualify for transplantation; most PDX models were generated from metastatic tumors, nonmetastatic tumors often showed engraftment failure [19]. Another disadvantage of the PDX model is that it takes four to eight months to develop a PDX model. Most patients cannot wait this long for therapy, therefore for testing the efficacy of immunotherapy the PDX model cannot be applied [29].

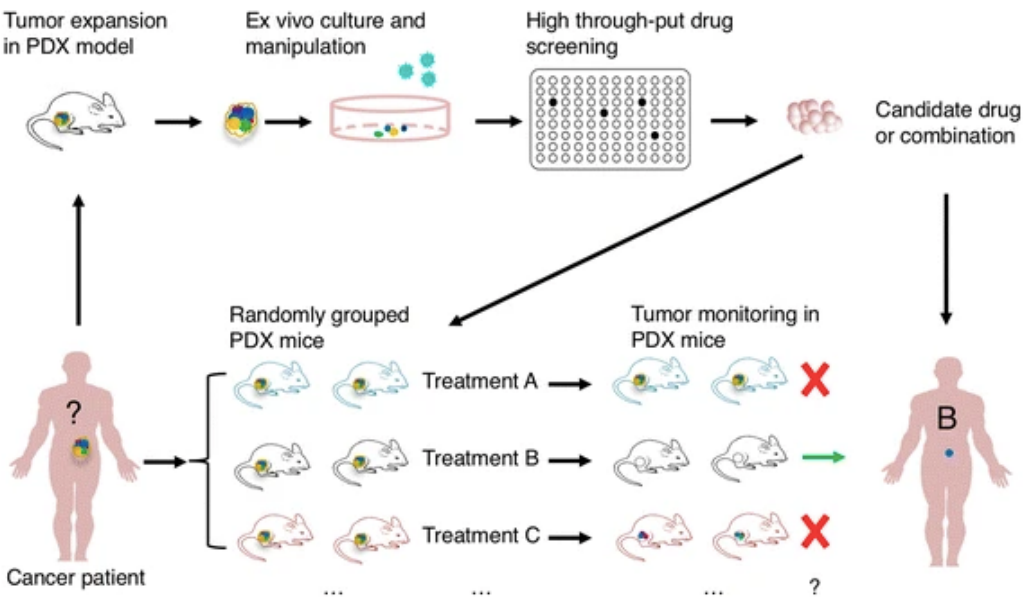

Figure 4. Use of PDX model in testing immunotherapy efficacy [28]. The tumor is (partially) removed by surgery and the tissue is transplanted into immunosuppressed mice. After expanding of the tumor in mice, therapies can be tested in vitro by removing the tumor and then the candidate drugs can be tested on mice.

Tumor organoid model

As previously mentioned, organoids are 3D structures that resemble the organ of origin in terms of structure and function [7, 8]. The organoid has three main features: it functions as the corresponding organ, it has multiple cell types of the corresponding organ, and the cells organize like the primary tissue [31].

In the case of patient-derived tumor organoids, the organoids will be generated out of adult stem cells (ASC) (figure 5). Organoids are generated by providing the appropriate nutrients and growth conditions in the medium specific for the type of tissue/organ, these growth conditions mimic signals that control tissue repair or maintenance [33]. The organoids are embedded in Matrigel, which is an extracellular matrix mixture isolated from living cells, containing the proteins laminin, collagen IV, entactin, and proteoglycans and can be enriched with growth factors [34]. The most common growth factors are R-spondin, Wnt, bone morphogenetic protein antagonist Noggin, and epidermal growth factor [19]. Matrigel is used to mimic the in vivo environment of the tissue [34].

In general, the use of organoids has improved the in vitro organogenesis and disease modeling and it has created new possibilities for the development of drugs [35]. Tumor organoids are currently used for modeling infection-cancer development, genetic carcinoma, and mutation-tumorigenesis processes. Furthermore, these tumor organoids also have potential in testing drug's efficacy, toxicity and new therapeutic compounds [36]. Patient-derived tumor organoids were already generated for many different types of cancer, including gastric, colon, pancreatic, breast, and prostate cancer [19].

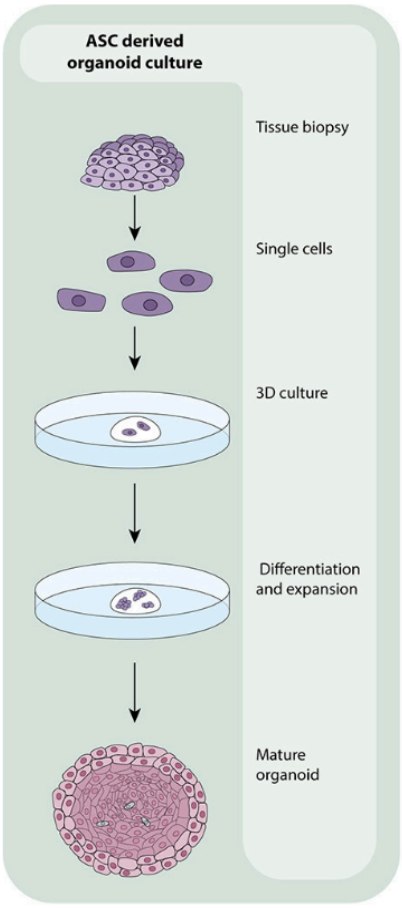

Figure 5. Formation of an organoid model [32]. A tissue biopsy is taken of the tumor. These cells are made single cell and are then plated in Matrigel with medium containing the right nutrients and growth factors. Eventually the single cells will form organoids.

Relative to the PDX model, the organoid model has some big advantages. One is that the organoid models only needs a small sample size of the patient's tumor, derived from a needle biopsy, whereas the PDX requires larger sample sizes, derived from surgery [37]. A second advantage of the organoid model is that is not that time consuming to generate the organoids, unlike the PDX model where it takes four to eight months [29]. Another advantage of the organoid model in contrast to all the other cancer models is that the organoid model can better mimic the tumor heterogeneity, by generating multiple organoids from different areas of the tumor [38]. For testing the efficacy of immunotherapy, the most important advantage of the organoid model is that immune cells can be co-cultured with the tumor organoid [36]. This means that now for the first time the response of immune cells in the tumor can be seen in vitro. For example, in the study of Nozaki et al. they co-cultured intraepithelial lymphocytes (IELs) with intestinal epithelial cell (IEC) organoids to analyze the expansion and motility of these lymphocytes [39]. Figure 6 shows that the IELs indeed nicely migrate through the organoid and are highly motile.

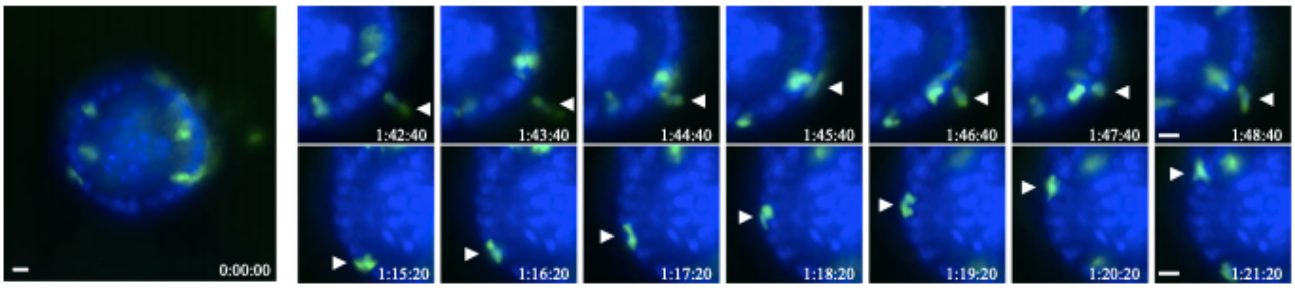

Figure 6. migration of IELs co-cultured with IEC organoids [38]. Time-lapse imaging of EGFP + IELs co-cocultured with wild-type IECs. Nuclei is stained using Hoechst 33342. IELs are migrating around in the IEC organoid (top images) and moving around in the basal side of the IEC organoid (bottom images). Imaging was performed after two days.

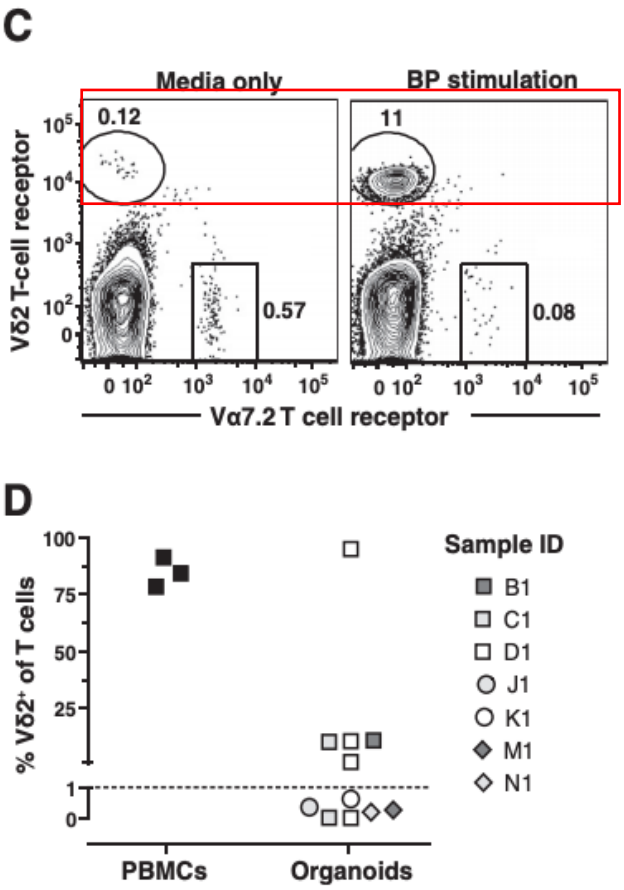

Figure 7. Primary breast epithelium organoids containing T lymphocytes that respond to BP [39]. C: flow cytometry showing Vδ2⁺ T cell expansion in the presence of BP. D: quantification of percentage Vδ2⁺ T cells from organoids compared to the positive control of PBMCs.

The same year Zumwalde et al. identified a subset of T lymphocytes, Vδ2⁺ T cells, which were already present in preparations of primary breast epithelium organoids, that can proliferate in these organoids when targeted by the FDA approved aminobisphophonate (BP) drug (figure 7) [40]. Exposure to BP led to an increase in Vδ2⁺ T cells (figure 7C). overall, in 45% of the organoid samples, BP exposure resulted in a detectable Vδ2⁺ T cells expansion compared to the positive control of peripheral blood mononuclear cells (PBMCs) (figure 7D).

Finally, the study of Takahashi et al. developed a system for assaying immune checkpoints [41]. In this study the immune checkpoint inhibitors nivolumab and pembrolizumab were evaluated using F-PDOs, which are patient-derived tumor organoids from the Fukushima Translational Research Project, co-cultured with PBMCs. In this experiment RLUN16, which is a lung F-PDO, was used as target and PBMCs, which were treated with the bacterial superantigen SEB to induce the expression op PD-1, were used as effector cells. The results of this study, showed in figure 8, were that the percentage of cytolysis of RLUN16 cells were similar between the treatment with nivolumab or pembrolizumab alone and RLUN16 + PBMC(+SEB). Also, the presence of nivolumab or pembrolizumab together with PBMC (+SEB) led to a significant increase in cytolysis.

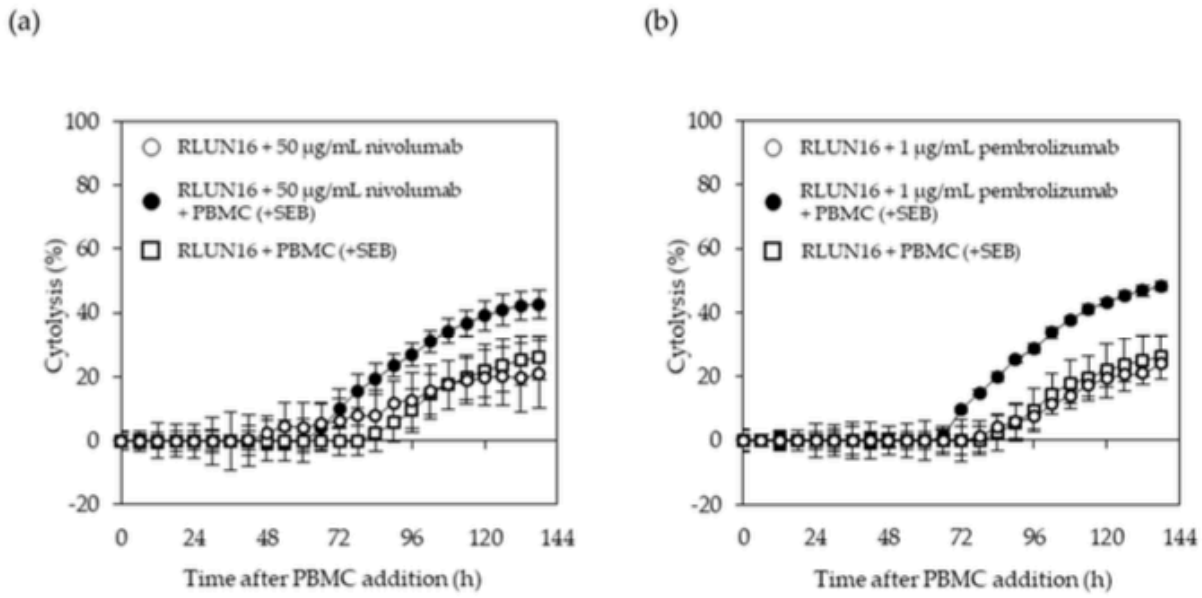

Figure 8. Measurements of RLUN16 cytolysis by nivolumab (a) and pembrolizumab (b) [40]. Open squares: RLUN16 with PBMCs + SEB alone, open circles: anti-PD-1 antibody alone, and closed circles: anti-PD-1 treatment in combination with PBMCs + SEB. Nivolumab and pembrolizumab were used at concentrations of 50 and 1 μg/mL respectively.

Unfortunately, although in the future the tumor organoid model has a lot of potential applications, for now there are still some limitations regarding the organoid model. Even though the tumor organoids can be co-cultured with other cell types it still is not perfectly similar to the original tumor microenvironment [19]. In the studies of Zumwalde et al. and Nozaki et al. they only co-cultured one type of immune cell with the organoids, while there are a lot of different immune cell types that play a role in the anti-tumor response. However, in the study of Takahashi et al. they already co-cultured multiple immune cells together with the organoid, by using PBMCs. Nevertheless, other tissues like nervous system, blood vessels and muscle layer should also be included to get an accurate tumor microenvironment. For this the co-culture method needs to be further developed. Another aspect that is still a limitation is that some cell types still cannot be expanded for a long time as an organoid, therefore the culturing method should be optimized [35]. Additionally, the current tumor organoids are mostly derived from epithelial cells, so further research should be done to allow the generation of tumor organoids derived from other types of cells. One last limitation is that the growth factors in the culture medium have an effect on the gene expression of the tumor cells [42]. This may have an effect on the response to immunotherapy and therefore the influence of these growth factors on the gene expression of tumor cells should be tested.

Discussion

The research in immunotherapy is quite developed. Two important immune checkpoints are already well studied, CTLA-4 and PD-1. CTLA-4 and PD-1 are both receptors on T cells, which can inactivate the T cell proliferation, survival, and differentiation. CTLA-4 is important in the early immune response, in the lymph nodes, while PD-1 is expressive in later stages of immune response, in the peripheral tissue. Also, at least three working immune checkpoint inhibitors, ipilimumab as an anti-CTLA4 and pembrolizumab and nivolumab as an anti-PD-1 immunotherapy are used in the clinic. Yet, research is still working on finding new promising immune checkpoints and immune checkpoints inhibitors that suppress these immune checkpoints.

Furthermore, there are a lot of biomarkers available for testing the immunotherapy efficacy in an individual patient, such as the amount of neoantigens and the ALC. But still it is hard to find a biomarker that works for the whole tumor of a patient, due to heterogeneity, let alone multiple individuals with different types of tumors. Therefore, it would be useful to have an accurate disease model, that can be used for immunotherapy research as well.

There are already some great cancer models that can be used for immunotherapy research, a monolayer cancer cell line model, a 3D model based on a cancer cell line and a PDX model. However, there are some major limitations of these models, a limitation specific for the cancer cell line models is that these models are based on a cell line instead of on the patient's tumor, so the mutations are not accurate enough. Furthermore, the disadvantages of all the currently used models mentioned above are that they do not represent the tumor heterogeneity and the tumor microenvironment of the original tumor accurately, while both features are important for the response to the immunotherapy. The last disadvantage for the PDX model specifically is that it takes a long time for the tumor to engraft. A model that could potentially resolve all of these disadvantages is the organoid model, which is a 3D model based on a patient's tumor tissue. This model is easier and faster to grow than the PDX model and by growing different organoids of different biopsies of the tumor, the tumor heterogeneity could be mimicked. Besides, by co-culturing these tumor organoids together with immune cells, which was already done by Nozaki et al., Zumwalde et al., and Takahashi et al. the tumor microenvironment could be partially simulated. Nevertheless, there are still limitations regarding this organoid model mostly concerning the tumor microenvironment. Because, though co-culturing of one or a couple of different cell type(s) with the tumor cells is possible, it still is not representing the tumor microenvironment, which consists of many different cell types, accurately. Another limitation is that not all cell types can be expanded for a long time as organoids yet, which limits the research done on these cell types. Furthermore, the growth factors, used in the Matrigel and medium have an effect on gene expression of the tumor cells and this may have an effect on the response to immunotherapy.

Concluding, the tumor organoid model has great potential for immunotherapy research, in specific for testing the efficacy of immunotherapy of individual patients. However further research is needed on co-culturing these tumor organoids with multiple different cell types. As well as on optimizing the protocol for growing organoids of different cell types. And finally, on what the influence of growth factors is on the gene expression of tumor cells and how this influences the response to immunotherapy.

References

[1] Marshall, H. T. & Djamgoz, M. B. A. Immuno-oncology: Emerging targets and combination therapies. *Frontiers in Oncology* 8(315), (2018). doi: 10.3389/fonc.2018.00315.

[2] Rizvi, N. A., Hellmann, M.D., Snyder, A., Kvistborg, P., Makarov, V., Havel, J.J., Lee, W., Yuan, J., Wong, P., Ho, T.S., Miller, M.L., Rekhtman, N., Moreira, A.L., Ibrahim, F., Bruggeman, C., Gasmi, B., Zappasodi, R., Maeda, Y., Sander, C., Garon, E.B., Merghoub, T., Wolchok, J.D., Schumacher, T.N. & Chan, T.A. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 348(6230), 124-128 (2015). doi: 10.1126/science.aaa1348.

[3] Darvin, P., Toor, S. M., Sasidharan Nair, V. & Elkord, E. Immune checkpoint inhibitors: recent progress and potential biomarkers. *Experimental and Molecular Medicine* 50(12), 1-11 (2018). doi: 10.1038/s12276-018-0191-1.

[4] Steven, A., Fisher, S. A. & Robinson, B. W. Immunotherapy for lung cancer. *Respirology* 21(5), 821-833 (2016). doi:10.1111/resp.12789.

[5] Chen, D. S. & Mellman, I. rug . *Nature* 541(7637), 321–330 (2017). doi: 10.1038/nature21349.

[6] Zhou, J., Su, J., Fu, X., Zheng, L. & Yin, Z. Microfluidic device for primary tumor spheroid isolation. *Experimental Hematology & Oncology* 6(22), 1-7 (2017). doi: 10.1186/s40164-017-0084-3.

[7] Kurmann, A. A., Serra, M., Hawkins, F., Rankin, S.A., Mori, M., Astapova, I., Ullas, S., Lin, S., Bilodeau, M., Rossant, J., Jean, J.C., Ikonomou, L., Deterding, R.R., Shannon, J.M., Zorn, A.M., Hollenberg, A.N. & Kotton, D.N. Regeneration of Thyroid Function by Transplantation of Differentiated Pluripotent Stem Cells. *Cell Stem Cell* 17(5), 527–542 (2015). doi: 10.1016/j.stem.2015.09.004.

[8] Bar-Ephraim, Y. E., Kretzschmar, K. & Clevers, H. Organoids in immunological research. *Nature Reviews Immunology* (2019). doi:10.1038/s41577-019-0248-y.

[9] Topalian, S. L., Taube, J. M., Anders, R. A. & Pardoll, D. M. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. *Nature Reviews Cancer* 16(5), 275–287 (2016). doi: 10.1038/nrc.2016.36.

[10] Rowshanravan, B., Halliday, N. & Sansom, D. M. CTLA-4: A moving target in immunotherapy. *Blood* 131(1), 58–67 (2018). doi: 10.1182/blood-2017-06-741033.

[11] Buchbinder, E. I. & Desai, A. CTLA-4 and PD-1 Pathways Similarities, Differences, and Implications of Their Inhibition. *American Journal of Clinical Oncology* 39(1), 98-106 (2015). doi:10.1097/COC.0000000000000239.

[12] Strimbu, K. & Tavel, J. A. What are biomarkers? *Current Opinion in HIV and AIDS* 5(6), 463–466 (2010). doi:10.1097/COH.0b013e32833ed177.

[13] Ku, G. Y., Yuan, J., Page, D.B., Schroeder, S.E.A., Panageas, K.S., Carvajal, R.D., Chapman, P.B., Schwartz, G.K., Allison, J.P. & Wolchok, J.D. Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting lymphocyte count after 2 doses correlates with survival. *Cancer* 116(7), 1767–1775 (2010). doi: 10.1002/cncr.24951.

[14] Subrahmanyam, P.B., Dong, Z., Gusenleitner, D., Giobbie-Hurder, A., Severgnini, M., Zhou, J., Manos, M., Eastman, L.M., Maecker, H.T. & Hodi, F.S. Distinct predictive biomarker candidates for response to anti-CTLA-4 and anti-PD-1 immunotherapy in melanoma patients. *Journal for ImmunoTherapy of Cancer* 6(1), 1-14 (2018). doi: 10.1186/s40425-018-0328-8.

[15] Reck, M., Rodríguez-Abreu, D., Robinson, A.G., Hui, R., Csöszi, T., Fülöp, A., Gottfried, M., Peled, N., Tafreshi, A., Cuffe, S., O'Brien, M., Rao, S., Hotta, K., Leiby, M.A., Lubiniecki,

G.M., Shentu, Y., Rangwala, R., Brahmer, J.R. & KEYNOTE-024 Investigators. Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. The *New England Journal of Medicine* 375(19), 1823–1833 (2016). doi: 10.1056/NEJMoa1606774.
[16] Schumacher, T. N. & Schreiber, R. D. Neoantigens in cancer immunotherapy. *Science* 348(6230), 69–74 (2015). doi: 10.1126/science.aaa4971.
[17] Viale, G., Trapani, D. & Curigliano, G. Mismatch Repair Deficiency as a Predictive Biomarker for Immunotherapy Efficacy. *BioMed Research International*, 1-7 (2017). doi:10.1155/2017/4719194.
[18] Music, M., Prassas, I. & Diamandis, E. P. Critical Reviews in Clinical Laboratory Sciences Optimizing cancer immunotherapy: Is it time for personalized predictive biomarkers? *Critical Reviews in Clinical Laboratory Sciences* 55(7), 466–479 (2018). doi: 10.1080/10408363.2018.1499706.
[19] Aboulkheyr Es, H., Montazeri, L., Aref, A. R., Vosough, M. & Baharvand, H. Personalized Cancer Medicine: An Organoid Approach. *Trends in Biotechnology* 36(4), 358–371 (2018). doi: 10.1016/j.tibtech.2017.12.005.
[20] Wilding, J. L. & Bodmer, W. F. Cancer cell lines for drug discovery and development. *Cancer Research* 74(9), 2377–2384 (2014). doi: 10.1158/0008-5472.CAN-13-2971.
[21] Gillet, J.P., Varma, S. & Gottesman, M. M. the Clinical relevance of Cancer Cell Lines. *Journal of the National Cancer Institute* 105(7), 452–458 (2013). doi: 10.1093/jnci/djt007.
[22] Marusyk, A. & Polyak, K. Tumor heterogeneity: causes and consequences. *Biochimica et Biophysica Acta* 1805(1), 105-117 (2009). doi:10.1016/j.bbcan.2009.11.002.
[23] Dagogo-Jack, I. & Shaw, A. T. Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews Clinical Oncology* 15(2), 81–94 (2018). doi: 10.1038/nrclinonc.2017.166.
[24] Gkretsi, V., Stylianou, A., Papageorgis, P., Polydorou, C. & Stylianopoulos, T. Remodeling components of the tumor microenvironment to enhance cancer therapy. *Frontiers in Oncology* 5(214), (2015). doi: 10.3389/fonc.2015.00214.
[25] Weiswald, L. B., Bellet, D. & Dangles-Marie, V. Spherical Cancer Models in Tumor Biology. *Neoplasia* 17(1), 1–15 (2015). doi: 10.1016/j.neo.2014.12.004.
[26] Pampaloni, F., Reynaud, E. G. & Stelzer, E. H. K. The third dimension bridges the gap between cell culture and live tissue. *Nature Reviews. Molecular Cell Biology* 8(10), 839–845 (2007). doi: 10.1038/nrm2236.
[27] Tentler, J. J., Tan, A.C., Weekes, C.D., Jimeno, A., Leong, S., Pitts, T. M., Arcaroli, J. J., Messersmith, W. A. & Eckhardt, S. G. Patient-derived tumour xenografts as models for oncology drug development. *Nature Reviews Clinical Oncology* 9, 338–350 (2012).
[28] Lai, Y., Wei, X., Lin, S., Qin, L., Cheng, L. & Li, P. Current status and perspectives of patient-derived xenograft models in cancer research. *Journal of Hematology and Oncology* 10(1), 1–14 (2017). doi: 10.1186/s13045-017-0470-7.
[29] Hidalgo, M., Amant, F., Biankin, A.V., Budinská, E., Byrne, A.T., Caldas, C., Clarke, R.B., de Jong, S., Jonkers, J., Mari Maelandsmo, G., Roman-Roman, S., Seoane, J., Trusolino, L. & Villanueva, A. Patient-Derived Xenograft Models: An Emerging Platform for Translational Cancer Research. *Cancer Discovery* 4(9), 998-1013 (2014). doi:10.1158/2159-8290.CD-14-0001.
[30] Reyal, F., Guyader, C., Decraene, C., Lucchesi, C., Auger, N., Assayag, F., De Plater, L., Gentien, D., Poupon, M. F., Cottu, P., De Cremoux, P., Gestraud, P., Vincent-Salomon, A., Fontaine, J. J., Roman-Roman, S., Delattre, O., Decaudin, D. & Marangoni, E. Molecular profiling of patient-derived breast cancer xenografts. *Breast Cancer Research* 14(1), 1-14 (2012). doi: 10.1186/bcr3095.

[31] Lancaster, M. A., Knoblich, J.A. Organogenesis in a dish: modeling development and disease using organoid technologies. *Science* 345(6194), 1-9 (2014). doi: 10.1126/science.1247125.
[32] Iakobachvili, N. & Peters, P. J. Humans in a Dish: The Potential of Organoids in Modeling Immunity and Infectious Diseases. *Frontiers in Microbiology* 8(2402), 1-7 (2017). doi: 10.3389/fmicb.2017.02402.
[33] De Souza, N. *Organoids*. *Nature Publishing Group* 15(23) (2018). doi:10.1038/nmeth.4576
[34] Hughes, C. S., Postovit, L. M. & Lajoie, G. A. Matrigel: a complex protein mixture required for optimal growth of cell culture. *Proteomics* 10(9), 1886–1890 (2010). doi: 10.1002/pmic.200900758.
[35] Yin, X., Mead, B.E., Safaee, H., Langer, R., Karp, J.M. & Levy, O. Engineering Stem Cell Organoids. *Cell Stem Cell* 18(1), 25-38 (2016). doi:10.1016/j.stem.2015.12.005.
[36] Xu, H., Lyu, X., Yi, M., Zhao, W., Song, Y. & Wu, K. Organoid technology and applications in cancer research. *Journal of Hematology and Oncology* 11(1), 1-15 (2018). doi: 10.1186/s13045-018-0662-9.
[37] Boj, S. F., Hwang, C. I., Baker, L. A., Chio, I. I. C., Engle, D. D., Corbo, V., Jager, M., Ponz-Sarvise, M., Tiriac, H., Spector, M. S., Gracanin, A., Oni, T., Yu, K. H., Van Boxtel, R., Huch, M., Rivera, K. D., Wilson, J. P., Feigin, M. E., Öhlund, D., Handly-Santana, A., Ardito-Abraham, C. M., Ludwig, M., Elyada, E., Alagesan, B., Biffi, G., Yordanov, G. N., Delcuze, B., Creighton, B., Wright, K., Park, Y., Morsink, F. H. M., Molenaar, I, Q., Borel Rinkes, I. H., Cuppen, E., Hao, Y., Jin, Y., Nijman, I. J., Iacobuzio-Donahue, C., Leach, S. D., Pappin, D. J., Hammel, M., Klimstra, D. S., Basturk, O., Hruban, R. H., Offerhaus, G. J., Vries, R. G. J., Clevers, H. & Tuveson, D. A. Organoid models of human and mouse ductal pancreatic cancer. *Cell* 160(1-2), 324–338 (2015). doi: 10.1016/j.cell.2014.12.021.
[38] Baker, L. A., Tiriac, H., Clevers, H. & Tuveson, D. A. Modeling Pancreatic Cancer with Organoids. *Trends in Cancer* 2(4), 176–190 (2016). doi: 10.1016/j.trecan.2016.03.004.
[39] Nozaki, K., Mochizuki, W., Matsumoto, Y., Matsumoto, T., Fukuda, M., Mizutani, T., Watanabe, M. & Nakamura, T. Co-culture with intestinal epithelial organoids allows efficient expansion and motility analysis of intraepithelial lymphocytes. *Journal of Gastroenterology* 51(3), 206-213. doi:10.1007/s00535-016-1170-8.
[40] Zumwalde, N. A., Haag, J.D., Sharma, D., Mirrielees, J.A., Wilke, L.G., Gould, M.N. & Gumperz, J.E. Analysis of immune cells from human mammary ductal epithelial organoids reveals Vδ2+ T cells that efficiently target breast carcinoma cells in the presence of bisphosphonate. *Cancer Prevention Research* 9(4), 305–316 (2016). doi: 10.1158/1940-6207.CAPR-15-0370-T.
[41] Takahashi, N., Hoshi, H., Higa, A., Hiyama, G., Tamura, H., Ogawa, M., Takagi, K., Goda, K., Okabe, N., Muto, S., Suzuki, H., Shimomura, K., Watanabe, S. & Takagi, M. An In Vitro System for Evaluating Molecular Targeted Drugs Using Lung Patient-Derived Tumor Organoids. *Cells* 8(5), 481 (2019). doi: 10.3390/cells8050481.
[42] Liu, S., Kam, W. R., Ding, J., Hatton, M. P. & Sullivan, D. A. Effect of Growth Factors on the Proliferation and Gene Expression of Human Meibomian Gland Epithelial Cells. *Investigative Ophthalmology Visual Science* 54(4), 2541–2550 (2013). doi: 10.1167/iovs.12-11221.