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SPATIAL MODELING OF TUMOR MICROENVIRONMENT

BARIŞ ALTUNKARA

A THESIS SUBMITTED FOR THE DEGREE OF

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

TÜMÖR MİKROORTAMIN UZAYSAL MODELLENMESİ

BARIŞ ALTUNKARA
YÜKSEK LİSANS TEZİ
BİYOMÜHENDİSLİK ANABİLİM DALI

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GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 19/07/2023 tarih ve 2023/40 sayılı kararıyla oluşturulan jüri tarafından 16/08/2023 tarihinde tez savunma sınavı yapılan Barış Altunkara'nın tez çalışması Biyomühendislik Anabilim Dalı Biyoinformatik ve Sistem Biyolojisi Programında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

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ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun ...../...../…… tarih ve ……/…..... sayılı kararı.

İMZA/MÜHÜR

SUMMARY

Cancer is one of the diseases that humanity suffered for a long time. We understood many aspects of this disease via rigorous medical and molecular research, but there is still much to learn. In this scope, investigating the microenvironment of tumors and molecular interactions can reveal fundamental mechanistic features of cancer.

One of the major fields to focus on when investigating tumor cell properties is the communication between cells and their microenvironment. Cancer initiation, progression, and patient prognosis are all influenced by interactions between tumor cells and their microenvironment. Recent researches include microenvironment composition, stromal cell proportions or activation states of cells to define the diverse structure of cancer. With the help of spatial mathematical modeling, we can understand how cells in the tumor microenvironment behave and we can discover more effective drug or treatment targets in the tumor microenvironment.

In this thesis, PhysiCell/BioFVM was used as the modeling environment. 2D or 3D agent-based models of tissues can be constructed and simulated using this tool.

In this work, tissue-like multicellular structures were designed and simulated. It is shown that simulation of tissues using spatial mathematical models developed in PhysiCell mimic the tumor initiation, progression, and response to factors such as hypoxia and drug treatment.

Key Words: Tumor Microenvironment, Spatial Modeling, Hypoxia, HIF.

ÖZET

Kanser insanoğlunun uzun süredir cebelleştiği bir hastalıktır. Bu hastalığın birçok yönünü, kapsamlı tıbbi ve moleküler çalışmalar neticesinde anlamış olmamıza rağmen, daha çok fazla öğrenilmesi gereken şey var. Tümör mikro çevresini ve içeriğindeki etkileşimleri incelemek kansere yönelik daha fazla özellikleri bize gösterecektir.

Tümör hücrelerinin özelliklerini incelerken dikkat etmemiz gereken önemli alanlardan biri hücrelerin birbirleri arasındaki ve mikro çevreleriyle olan iletişimleridir. Kanser başlangıcı, ilerlemesi ve prognoz gibi durumlar tümör hücrelerinin kendi aralarındaki ve mikro çevrelerindeki iletişimlerinden etkilenmektedirler. Kanser daha önce anormal mutasyonlar neticesiyle ortaya çıkan heterojen hastalık olarak düşünülürken, buna artık mikro çevre yapısı ve içeriği, çevresel hücre oranları ve aktivasyon durumları gibi özellikler de eklenmektedir. Modelleme sayesinde, tümör mikro çevresindeki hücrelerin davranışlarını anlayabilir ve tümör mikro çevresindeki hedeflere yönelik daha etkili ilaçlar ve tedaviler geliştirilebilir.

Bu tezde, PhysiCell/BioFVM modelleme ortamı kullanıldı. 2D veya 3D ajan bazlı doku modelleri bu yapıyı kullanarak geliştirilebilir.

Bu çalışmada, doku benzeri çok hücreli bir yapı tasarlandı. PhysiCell platformunda uzaysal matematiksel modeller aracılığıyla yapılan simülasyonlarla, tümör başlangıcı, gelişimi ve hipoksi ve ilaç tedavisi gibi çeşitli faktörlere yanıtlar taklit edilebilir.

Anahtar Kelimeler: Tümör Mikroçevresi, Uzaysal Modelleme, Hipoksi, HIF.

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I am grateful to my parents for their love and support.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
Ang	:	Angiopoietin
APC	:	Antigen-Presenting Cells
Bcl2	:	B Cell Lymphoma 2
Bid	:	BH3 Interacting Domain Death Agonist
BMDC	:	Bone Marrow-Derived Cell
BMP	:	Bone Morphogenetic Protein
CAF	:	Cancer Associated Fibroblast
CCL	:	Chemokine (C-C motif) Ligand
CCR	:	Chemokine (C-C motif) Receptor
CD8	:	Cluster of Differentiation 8(Transmembrane Glycoprotein)
CSC	:	Cancer Stem Cell
CSF	:	Colony Stimulating Factor
CSF1R	:	Colony Stimulating Factor 1 Receptor
CTLA4	:	Cytotoxic T-lymphocyte Associated Protein 4(CD152)
CXCL	:	Chemokine (C-X-C motif) Ligand
CXCR	:	Chemokine (C-X-C motif) Receptor
ECM	:	Extra Cellular Matrix
EGF	:	Epidermal Growth Factor
EGFR	:	Epidermal Growth Factor Receptor
EMT	:	Epithelial-to-Mesenchymal Transition
EndMT	:	Endothelial-to-Mesenchymal Transition
ERK	:	Extracellular Signal-Regulated Kinase
ET-1	:	Endothelin 1
FBA	:	Flux Balance Analysis
FGF	:	Fibroblast Growth Factor
Glut-1/2	:	Glucose Transporter 1/2
HIF	:	Hypoxia Inducible Factor
HRE	:	Hypoxia Response Element
ICI	:	Immune Checkpoint Inhibitor
IFN	:	Interferon
IGF1	:	Insulin-like Growth Factor 1
IL	:	Interleukin
JAK2	:	Janus Kinase 2
MCP1	:	Monocyte Chemotactic Protein 1
MCT	:	Monocarboxylate Transporters
MDSC	:	Myeloid-Derived Suppressor Cell
MET	:	Mesenchymal-to-Epithelial Transition
MHC	:	Major Histocompatibility Complex

MMP	:	Matrix Metalloproteinase
MSC	:	Mesenchymal Stem Cell
NK	:	Natural Killer
NO	:	Nitric Oxide
ODDD	:	Oxygen-Dependent Degradation Domain
p21	:	Cyclin-Dependent Kinase Inhibitor 1
PD1	:	Programmed Cell Death Protein 1
PDGF	:	Platelet-Derived Growth Factor
PDL1	:	Programmed Death Ligand 1
PGE2	:	Prostaglandin E2
PHD	:	Prolyl-4-Hydroxylase Domain
pVHL	:	Von Hippel Lindau Tumor Suppressor Protein
ROS	:	Reactive Oxygen Species
SBML	:	Systems Biology Markup Language
Sox2	:	Sex Determining Region Y-box
STAT6	:	Signal Transducer and Activator of Transcription 6
TAM	:	Tumor Associated Macrophage
TCR	:	T Cell Receptor
TGF-β	:	Transforming Growth Factor-Beta
Th1	:	T Helper 1 Cell
TME	:	Tumor Microenvironment
VEGF	:	Vascular Endothelial Growth Factor
Wnt	:	Wingless and Int-1(Group of signal transduction pathways)
XML	:	Extensible Markup Language
YAP	:	Yes-Associated Protein

LIST of FIGURES

1. INTRODUCTION

Cancer is likely to be an ancient disease; our knowledge of ancient documents indicates existence of similar diseases that have close phenotypic behaviors. We understood many molecular aspects of cancer in more than hundred years of research, but there are still various aspects of cancer and relevant processes to be discovered. Cancer cells have strong influence on many other cells, organs, processes in human body. When we focus on only cancer cells in treatments or drug applications, we ignore effects of distinct cellular components, interactions, processes, metabolites, chemokines and cytokines, on the patients. With this approach we can kill some of the cancer cells, or we can cure some of the abnormal processes caused by cancer cells, but this will not cure the disease itself. To be able to cure cancer, we need to target distinct aspects of carcinogenesis. For example, angiogenesis, metastasis, entry to circulatory system, survival in the circulatory system, immune evasion, immune suppression, cell differentiations and many others are all features of carcinogenesis. All of these processes require distinct cell types, products that came from stromal cells or cells that respond to signaling. In this scope, we need to investigate tumor microenvironment and its interactions to reveal fundamental features of cancer.

To be able to establish tumor related models, we need to understand tumor microenvironment very well. Interactions and signaling between tumor cells and stromal cells, between tumor cells and immune system cells or between stromal cells and immune system cells are really important for well-established tumor microenvironment models. Further, cancers have capability to gain resistance to different treatment approaches. Therefore, we need to understand communications in tumor microenvironment and develop combine treatments to cut alternative signaling or processes utilized by the tumor. For example, immune checkpoint inhibitors (ICI) are widely used treatments that inhibit PD1-PDL1 binding. But when we look at results of treatments, we can see acidic environment, abnormal shape of vasculature system that caused by VEGF and activation of T-regulatory and myeloid derived suppressor cells (MDSCs) prevent contents of this treatment to reach the relevant regions of the tumor. Therefore, we need to develop ICI treatments that will not be affected by acidic environment and also perhaps combine this treatment with anti-

VEGF and T-regulatory or MDSC suppressor treatments. Once all molecular mechanisms are revealed, to deal with cancer, we have two main options, one of them is to develop personal medicines for each cancer patient or we can target common properties of all cancer types. Mathematical modeling of tumors may pave the way for realizing either of these options.

In cancer model used in this thesis, cell types, chemokines, cytokines or metabolites that have the most impact in tumor microenvironment were chosen to be represented. Every cell type or molecular product that take part in carcinogenesis were not represented in this model, because every addition to model increase simulation time dramatically. Adding important components one by one after the initial model setup was undertaken with the objective of vigilantly identifying and addressing potential errors that may arise. In upcoming chapters, some biological information about components that are included in the model are going to be given.

1.1 Significance of Tumor Microenvironment

The tumor microenvironment (TME) is a complex and dynamically changing medium that contains both cellular and acellular components with relative activities and functions in cancer progression [Lebleu, 2015]. There are nonmalignant and malignant cells, immune system cells and other soluble molecules such as chemokines and cytokines in the TME medium that influence tumor growth, survival and dissemination [Del Prete et al, 2017]. The TME is one of the main components in cancer development and progression. Properties of the TME have huge effects on cell behavior, metastatic potential, and cancer stem cell development and differentiation [Spill et al, 2016; Arneth, 2019].

Cancer is not just a mutation derived disease, as recent research highlighted, we can separate tumors according to their microenvironmental composition, stromal cell proportions or activation states [Hanahan et al, 2011; Hanahan et al, 2012]. In response to evolving environmental conditions and oncogenic signals from tumors, TME continually changes over time to promote cancer progression [Quail et al, 2013]. Tumors interact closely and continuously with the surrounding microenvironment and organs via the lymphatic or circulatory system. Thus, tumor cells can influence the microenvironment through the release of extracellular signals,

such as paracrine signals, to induce peripheral immune tolerance, support tumor angiogenesis and tumor invasion [Korneev et al, 2017].

1.2 Significance of Modeling

In complex diseases such as cancer, it is hard and expensive to put every detail of the disease into laboratory experiments. Gathering data from cell lines, proteins, metabolites is a difficult task to do, if we aim to get all of these profiles. We have to do many experiments on cell-cell environment, then we need to establish animal models to test our combinations to measure effectiveness of our drugs or treatments. Further, we do not have the guarantee that successful animal experiments will translate to humans. Only after clinical trials on humans, we can be sure about the efficacy of our drugs and treatments. With modeling, we can establish similar environments in computers. With the help of this approach, if we have well established model and model platform, we can eliminate unsuccessful laboratory attempts.

2. BIOLOGICAL BACKGROUND

2.1 Tumor Microenvironment

TME includes many different types of cells such as fibroblasts, macrophages, aerobic-anaerobic tumor cells, cancer stem cells, natural killer (NK) cells, T-cytotoxic and T-regulatory cells, dendritic cells, neutrophils, mesenchymal stem cells (MSCs), MDSCs, bone marrow derived dendritic cells (BMDCs). Concentrations of these cells and their products in microenvironment vary according to location of the cancer, unique properties of the host body, immune system cells availabilities, etc. There is extensive signaling between tumor cells and cells that are in tumor microenvironment with the help of extracellular matrix (ECM) structure. With the help of stromal cells, especially fibroblasts, tumor cells modify ECM structure to support tumor progression. Modified ECM structure increase protumor related signals, prevent anti-tumor signaling and also, prevent anti-tumor related cells to invade inside of primary tumor. During tumor development, stromal cells that are surrounding the cancer cells, growth factors or other transcription factors modify the phenotypic behavior of the stromal cells to promote tumor development [Semenza, 2012b; Al Tameemi et al, 2019].

The dynamics of TME can be basically divided into two different phases. Although TME exists in a continuum of functional states. According to the simplified classification, in early tumor stages, immune system and stromal cells are in anti-tumor phenotypes. We have NK cells that recognize antigens on tumor cells and then kill them with granzyme B and perforin. Also, anti-tumor microenvironment has normal macrophages and dendritic cells that recognize tumor cell-specific antigens and present them to cytotoxic T cells. Normal fibroblasts and macrophages contribute to a growth-suppressive state (Fig. 2.1) [Quail and Joyce, 2013]. After some time and signaling, tumor cells acquire immune evasion, immune suppression, angiogenesis and macrophage polarization switches. With the help of “education processes” led by tumor cells, fibroblasts and macrophages obtain pro-tumorigenic functions. Tumor associated macrophages (TAMs) bring pro-tumorigenic phenotypes, such as growth, angiogenesis, and invasion, to the fore within the primary tumor by secreting plethora of pro-tumorigenic proteases,

cytokines and growth factors (VEGF, CSF-1, EGF, etc.). As the tumor grows, these TAMs participate in mobilization of suppressor cells for immune system such as MDSCs and T-regulatory cells into circulation in response to activated cytokines induced by tumorigenesis (TGF-β, CXCL5-CXCR2). These cells disrupt antigen presentation by other antigen presenting cells (APCs), inhibition of B and T cell proliferation or activation and inhibition of NK cell mediated cytotoxicity. Cancer associated fibroblasts (CAFs) are activated by factors (TGF-β, FGF, PDGF, etc.) that are supplied by the tumor, and they secrete ECM proteins and basement membrane components, regulate differentiation, modulate immune responses and contribute to deregulated homeostasis. Hence, fibroblasts are important regulators of ECM structure, and they contribute to ECM structure dynamics according to tumor progression. This ECM structure and polarization status of stromal cells highly depend on chronic inflammation and hypoxia. Effects of these conditions and related processes converts the antitumor microenvironment to protumor microenvironment as shown in Fig. 2.1 [Quail and Joyce, 2013]. M1-phenotype macrophages and dendritic cells cannot enter tumor microenvironment and make antigen presentation to T cells because of Treg and MDSC cells prevention. NK cells and T cells cannot infiltrate into tumor structure and cannot release their cytotoxic particles. Also, acidic microenvironment and competition towards glucose hinder their infiltration.

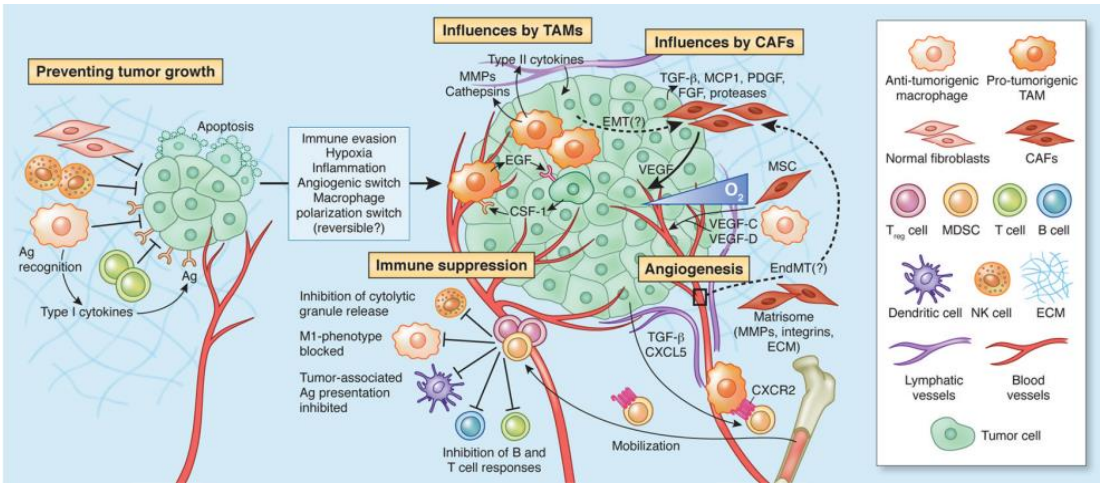


Figure 2.1: Anti-tumor and protumor TME structure.

2.2 Hypoxic Tumor Microenvironment

Hypoxia and chronic inflammations are main causes of changes in the TME structure. Chronic inflammation and tumorigenesis have strong links [Balkwill et al, 2001]. High chronic inflammation corresponds to increased cancer risks. With the impact of chronic inflammation, some immune system cells, especially macrophages, infiltrate into the TME which increases protumor phenotypes of related immune system cells. One of the main properties of solid tumors is hypoxia. Hypoxia is a condition when the oxygen concentration is lower than %1 in microenvironment. As shown in Fig. 2.2, proximity to vessels determine hypoxia regions [Al Tameemi et al, 2019]. If proximity to vessel is in between 100μm-150μm, we can call these regions as hypoxic regions. Beyond these distances, places are called as necrotic regions. In our body, we can face transient hypoxia states that can be caused by sport activities, temporary circulatory problems, etc. Transient hypoxia leads some cells such as muscle cells to die or microenvironment in those areas produce angiogenesis related factors. After some dense physical activities, muscle energy demands exceed the energy sources provided by oxygen supply. Muscle cells make metabolic switch and start to produce lactate from glycolysis. Cancer cells use same processes. Persistent and recurrent hypoxia is not optimal for biological functions, therefore multicellular organisms have been compelled to regulate their activities at both organism-wide and cellular-level to adapt hypoxia [Al Tameemi et al, 2019]. Selection processes lead to the survival of some tumor cells with genetic compatibility to hypoxic regions [Vaupel et al, 2004].

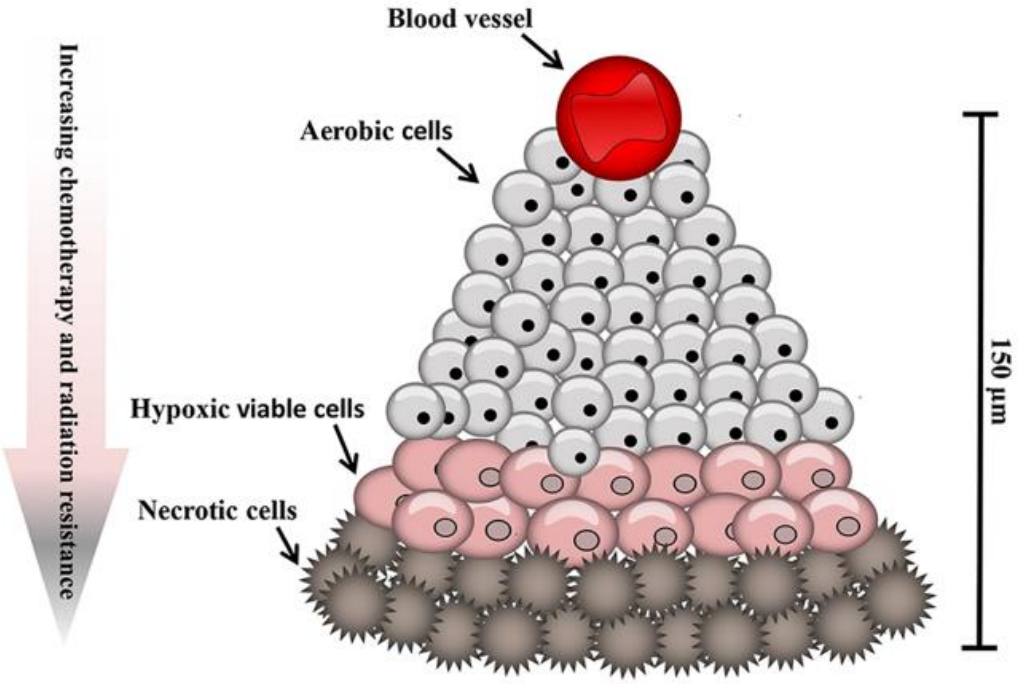


Figure 2.2: Hypoxic regions in solid tumors.

Hypoxia leads to poor prognosis in cancer patients [Vaupel, 2008]. Hypoxia causes metastasis, angiogenesis and metabolic switch related genes to be overexpressed and drive stromal cells to be educated to support cancer progression [Vaupel et al, 2001]. Tumors can overcome the challenges posed by a harsh and hypoxic microenvironment by stimulation of production of novel blood vessels via release of some hypoxia-inducible angiogenic factors such as VEGF [Seo et al, 2014].

In hypoxic regions, all cells can produce hypoxia inducible factors (HIFs). These transcription factors have huge influence on many different genes and pathways. HIFs are important regulators that tumor cells use to adapt to hypoxic stress. In response to insufficient oxygen levels, these transcription factors accumulate in hypoxic regions [Schito et al, 2017; Wolff et al, 2017]. Humans have three types of HIFs (HIF-1, HIF-2, HIF-3). HIF-1 is commonly found in tumor cells and HIF-2 is frequently overexpressed in TAMs [Talks et al, 2000; Weidemann et al, 2008; Robinson et al, 2017]. We have so much information about HIF-1 and HIF-2 and their properties, but we do not have detailed information about HIF-3 and related processes. HIF-1 frequently plays a role in metabolism and growth-related processes, while HIF-2 mostly influence cell differentiation related processes. All of the HIFs include heterodimer structure composed of alpha and beta subunits and these

heterodimers are dissociated in normoxic conditions as shown in Fig. 2.3 [Al Tameemi et al, 2019].

In tumor microenvironment, we mainly focus on HIF-1alpha, because this subunit is well characterized when compared to other subunits, and it has huge influence on TME structure dynamics. HIF-1alpha is an oxygen-sensitive subunit. In normoxic conditions, they are immediately degraded by ubiquitin-mediated process. Activation of ubiquitinization causes by von-Hippel-Lindau (pVHL) protein. Hydroxylation of proline residues in oxygen dependent degradation domain (ODDD) by prolyl-4-hydroxylase domain (PHD) enzyme acylation of lysine make HIFs target for ubiquitination processes [Dengler et al, 2014]. Hydroxylated or acetylated HIF-alpha can be identified by tumor suppressor pVHL. This results as poly-ubiquitination by E3 ubiquitin-ligase and this complex degraded by proteasome 26S [Masoud et al, 2015]. There are more hydroxylation processes on different regions that limits activation of HIF.

In hypoxia conditions, HIF-1alpha is not degraded and translocated from cytoplasm into the nucleus to bind HIF-1beta to form HIF-1 as shown in the left side of Fig. 2.3 [Zimna et al, 2015]. HIF-1beta subunit is not affected by oxygen concentrations and it is constitutively present in the nucleus. Absence of oxygen in TME, results in blocked hydroxylation processes and prevent HIF-alpha and pVHL binding because PHDs and other hydroxylation enzymes use oxygen as substrates [Arsenault et al, 2015; Maxwell et al, 2017]. This leads to HIF-alpha translocation to nucleus and generation of HIF dimer. Low oxygen concentration is not the only condition that inhibits PHD activation. Nitric oxide (NO), reactive oxygen species (ROS) and other conditions can inhibit PHD activation either in hypoxic and normoxic conditions [Maxwell et al, 2017]. ROS(e.g. hydrogen peroxide) can trigger stress-responsive gene expression (e.g. HIF-1alpha) [Gao et al, 2007; Chandel, 2010]. Research shows that hypoxia is not the only reason for HIF stabilization. We can see HIF can be stabilized in aerobic conditions if ROS and NO are present and are not degraded by cellular mechanisms. It shows the importance of HIF production in carcinogenesis.

HIF-1 can regulate expression of more than 100 genes by binding to hypoxia response elements (HRE) in their promoter regions. Some of them are; p21, glucose transporter 1 (Glut1), glucose transporter 3 (Glut3), insulin-like growth factor binding protein-1 and -3, insulin-like growth factor 2, transforming growth factor

beta (TGF-beta) and VEGF [Maxwell et al, 2001; Masoud et al, 2015]. On the right side of Fig. 2.3, some processes that these HRE regions have effects on are shown [Al Tameemi et al, 2019]. Most of these genes are responsible for metabolism, angiogenesis, metastasis and survival related processes.

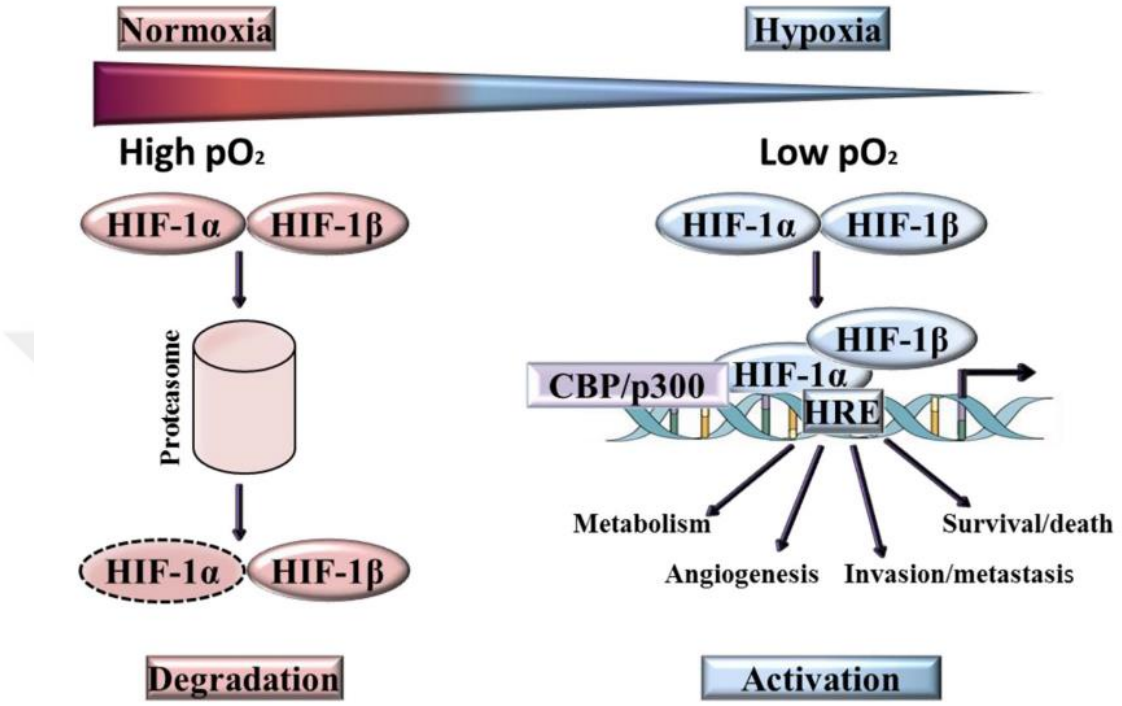


Figure 2.3: HIF subunits in normoxia and hypoxia.

HIF-1 can reduce apoptosis by inhibiting Bid expression which is in pro-apoptotic Bcl2 family, stimulates survivin expression [Erler et al, 2004; Peng et al, 2006]. With the effect of these expression differences, cancer cells can protect themselves from hypoxia related apoptosis procedures and effects of drug applications [Erler et al, 2004].

Hypoxia condition has high influence on extracellular matrix (ECM) structure. In hypoxic regions, we have more aligned and stiff ECM structure as shown in Fig. 2.4 [Petrova et al., 2018]. Stromal cells have different phenotypes in those regions and we can also see more glycolytic metabolites such as lactate. Because of changed glycolysis metabolism, lactate is produced and this decreases the pH in those areas. Low pH can make cell selection that allow only resistant cells and has huge influence on phase differentiations. Some immune system cells that have cytotoxic properties have difficulty to enter those areas. This contributes to immune system evasion abilities of tumor cells. As shown in Fig. 2.4, ECM has normal structure and normal

pH values in close regions to the veins [Petrova et al., 2018]. This demonstrates us the discrepancy between these two regions.

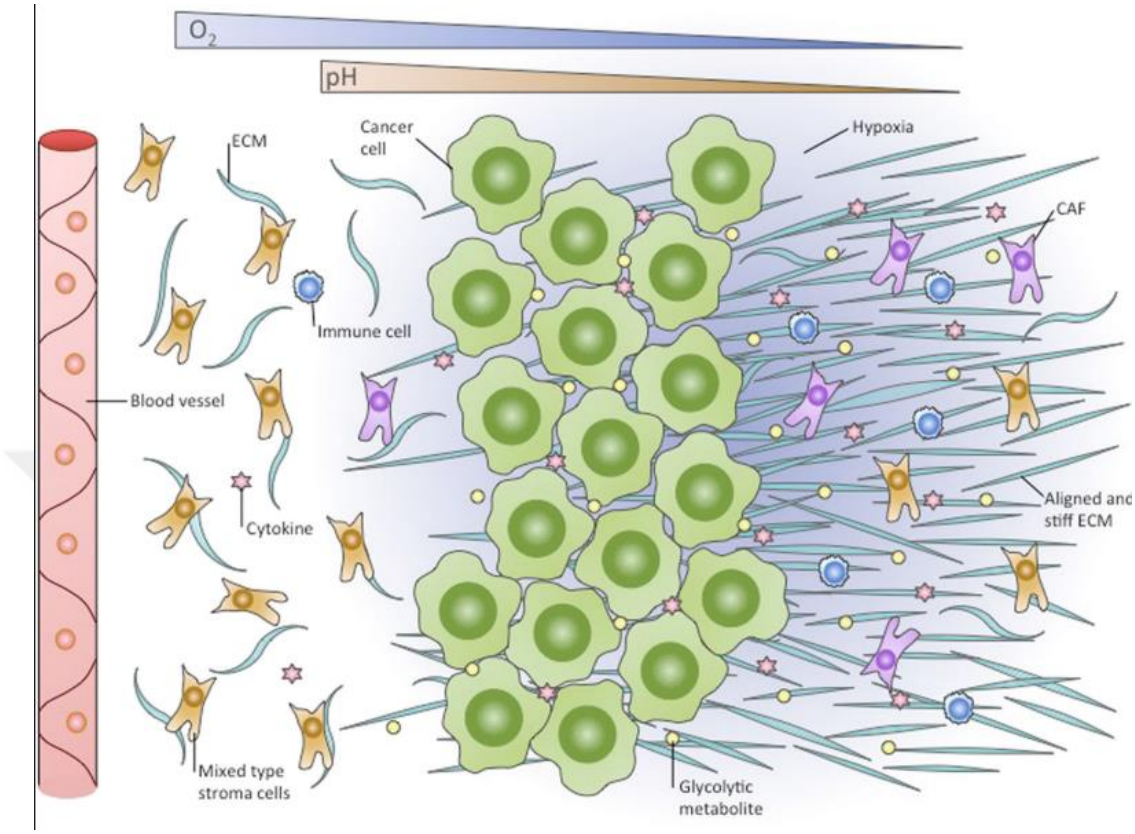


Figure 2.4: ECM structure in hypoxia.

Extent of neo-vascularization that is induced by HIF induced angiogenesis implicates HIF-mediated expression of some important angiogenic factors, VEGF-A and Ang-2 are two of them [Brahimi-Horn et al, 2007].

2.3 Cells In Tumor Microenvironment

There are a number of different types of stromal cells in TME. In my model, I used the most important ones. To emphasize their importance, in the next sections, informations about them and their influence on the TME are provided.

2.3.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are bone marrow derived cells. They can differentiate into various cell types such as fibroblasts, epithelial cells and

endothelial cells [Shi et al, 2010; Zhang et al, 2014; Jiang et al, 2015; Sai et al, 2019]. These cells make chemotaxis towards tumor microenvironment with the effect of chemokines that cells in the TME produce [Karnoub et al, 2007; Krueger et al, 2018]. Normally, MSCs are recruited to wound healing site to repair damaged tissue [Papaccio et al, 2017]. Since cancer is considered as "wounds that never heal", MSCs are recruited to tumor site for healing. Human body recognize tumor site as wound and recruit MSCs to repair it.

In TME, MSCs are educated by taking signals from cancer cells and tumor promoting microenvironment structure. These cells are important tumor microenvironment components. They have abilities to differentiate into fibroblasts, endothelial cells and some other cells that reside in TME. With this differentiation processes, they contribute to TME shaping. They can differentiate into cancer stem cells (CSCs) as well. In addition, MSCs have immunomodulation functions [Wang et al, 2015; Sugino et al, 2017; Wang et al, 2018]. They recruit MDSCs and Treg cells to the TME to suppress immune system with cytotoxic effects. MSCs also contribute to epithelial-to-mesenchymal transition (EMT), angiogenesis, resistance to drug therapies and metastasis [Papaccio et al, 2017].

MSCs can contribute to angiogenesis by differentiating to endothelial cells and releasing plethora of pro-angiogenic signals and factors. In the case of the harsh hypoxia situation within the tumor, several growth factors such as PDGF and VEGF are overexpressed and represent potent chemotactic and mitogenic factors for MSC. MSCs migrate towards to tumor site and promote vasculogenesis by an autonomous VEGF production and strengthen the pro-angiogenic potential of tumors. MSCs that produce VEGF, Ang-1 and other pro-angiogenic factors could differentiate into pericytes and endothelial cells to support tumor vascularization and growth. MSCs can secrete pro-angiogenic factors such as interleukin-6 (IL-6) and Ang-1. Also, MSCs can induce cancer cells to produce endothelin-1 (ET-1) to promote tumor angiogenesis.

2.3.2 Macrophages

Macrophages are one of the most significant cell types in innate immune system and also in TME. Macrophages coordinate specific immune responses through pathogen phagocytosis, antigen presentation, wound healing, tissue repair

and immune homeostasis [Chen et al, 2015; Hinshaw et al, 2019]. Normal macrophages produce pro-inflammatory cytokines, take part in antigen presentation and show anti-tumor behaviors [Biswas et al, 2010]. Inflammation response for pathogens and subsequent works orchestrated by macrophages. As we know cancer is wound that never heals, it sends signals that are used in wound healing consistently to recruit stromal or other macrophages to tumor microenvironment. With the effects of chronic inflammation status, macrophages are continually called to TME. Some chemokines are responsible for monocyte recruitment such as CCL1-7. Monocytes that are from bone marrow are one source for macrophages. Monocytes differentiate into macrophages with effects of some chemokines such as CSF-1 [Qin et al, 2014; Peiseler et al, 2018]. Also, MSCs can recruit tumor associated macrophages (TAMs) into tumor site via CCL2 ligand and its receptor CCR2. TAMs can be recruited to hypoxic regions of tumors with the effects of macrophage chemoattractants such as endothelin-2 and VEGF [Lewis et al, 2005]. VEGF is also responsible for vessel formation and angiogenesis, demonstrating the important relationship between TAMs and angiogenesis [Escribese et al, 2012].

In tumor microenvironment, macrophages with different phenotypes and behaviors can be found. Macrophages are functionally plastic cells. Due to many different environmental effects and physiological conditions, they can change their behavior. Hypoxia has huge influence on macrophage polarization as hypoxia enhance the expression of cytokines or chemokines that are responsible for macrophage polarization and decrease expression values of anti-tumor related ones [Petrova et al, 2018]. With the help of interleukin-4 (IL-4), transforming growth factor-beta (TGF-beta) and some other chemokines secreted in TME, M2(M1 for normal macrophages) polarized macrophages or tumor associated macrophages (TAMs) arise [Hagemann et al, 2008; Biswas et al, 2010; Flavell et al, 2010; Wang et al, 2010; Pyonteck et al, 2013; Cook et al, 2013]. IL-4 is responsible for initiation of STAT6 signaling in macrophages and these signaling drive them to polarization. Cancer associated fibroblasts are one type of stromal cells that have influence on macrophage polarization by contributing to hypoxic tumor microenvironment structure, secreting tumor related chemokines or cytokines and changing ECM structure to support tumor survival. To be able to do these processes macrophages play critical role. TAMs secrete immune suppressive chemokines to help tumor cells.

TAMs are incontrovertible regulators in tumorigenesis. They can be tissue resident, or they can come from bone marrow or spleen. We call them tumor associated because they take part in multiple aspects of tumor progression [Qian et al, 2010]. TAMs that are around the edge of the tumor give invasiveness ability to tumor cells with the help of paracrine signaling loop involving tumor-derived colony stimulating factor-1 (CSF-1) and macrophage-derived EGF [Goswami et al, 2005; Condeelis et al, 2006; Coniglio et al, 2012]. TAMs can produce proteases, especially cysteine cathepsins to support tumor progression and also give protein degradation ability to tumors. This ability is important for tumor cells, because they need to degrade proteins in ECM to make invasion or they can degrade proteins that cover vessels to enter circulatory system. Epithelial cells have E-cadherin binds between them. This hinders their movement and also force them to stop their proliferation. TAMs secrete interleukin-6 (IL-6) to degrade these bindings. This process starts metastasis related processes as now tumor cells are free to move. Following that, TAMs-derived and CAFs-derived TGF-beta causes tumor epithelial-to-mesenchymal transition (EMT) [Bonde et al, 2012]. Degradation of E-cadherin bindings and EMT are early and the most important steps of metastasis. Production of other pro-migratory chemokines and cytokines such as EGF, CXCL12-CXCR4 and ECM related proteins such as collagens to reshape ECM are next steps to accelerate tumor motility and invasion rate [Condeelis et al, 2003; Wyckoff et al, 2007; Gocheva et al, 2010].

TAMs also have influence on tumor cell survival processes. They can inhibit antitumor T cell activities and they can mediate and reduce effectiveness of treatments such as chemotherapy, immune checkpoint inhibitors and drugs with cytotoxic abilities.

2.3.3 Fibroblasts

Fibroblasts are important stromal cells. They can produce components that are in ECM structure and collagen. Maintaining structural framework, wound healing, regulation of epithelial cell differentiation, immune response modulation and maintaining homeostasis balance are their primary roles [Tomasek et al, 2002; Kalluri et al, 2006]. Besides these, fibroblasts are source for growth factors, proteases, greater contractile force and proliferation activities [Tao et al, 2017].

Fibroblasts are the most abundant connective tissue cells. With their roles in human body and their high numbers in critical regions make them really important for carcinogenesis. Interactions between tumor cells and fibroblasts are of indisputable importance.

Cancer cells can educate fibroblasts to become CAFs by secreting some signaling molecules such as basic fibroblast growth factor (bFGF), IL-6, platelet-derived growth factor (PDGF) and TGF-beta [Tao et al, 2017]. HIF-1 is also another contributor to CAF transformation. There are some studies on CAFs that they can be generated by endothelial-to-mesenchymal transition (EndMT). It is also shown that some CAFs in tumors were originated from endothelial cells [Zeisberg et al, 2007]. Endothelial cells come from blood vessels to differentiate into mesenchymal cells. Also, CAFs production can be promoted by EMT, because we know that mesenchymal-like tumor cells with epithelial origin express CAF markers [Petersen et al, 2003; Orr et al, 2012]. In solid tumors, CAFs accumulation into TME is a common feature. This accumulation occurs via effect of some growth factors and cytokines in the TME. CAF activation and accumulation can be mediated by TGF-beta, monocyte chemotactic protein (MCP1), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and some proteases [Olumi et al, 1999; Kalluri et al, 2006; Dumont et al, 2013; Marsh et al, 2013]. As mentioned before, CAFs have significant role in ECM remodeling to support tumor progression. YAP transcription factor is also an important component in this process [Calvo et al, 2013]. YAP induction modulates the cytoskeleton and matrix stiffness by factors that YAP regulates. CAFs are major source for growth factors. Cancer use homeostasis related CAF abilities on its own advantage. Secretion of growth factors to maintain homeostasis if there is tissue loss in environment is one of the core missions of CAFs. Besides normal growth factors that increase cell proliferation rates, CAFs can produce VEGF to induce vascular permeability and angiogenesis [Fukumura et al, 1998]. CAFs can produce CXCL12 cytokine to contribute tumor cell migration by inducing CXCL12-CXCR4 binding. CAFs participate in TGF-beta/PDGF signaling to support EMT and help tumor cells to acquire invasive phenotype [Van Zijl et al, 2009]. By secreting pro-invasive factors and ECM structure remodeling, CAFs are important metastasis contributors.

Cancer associated fibroblasts express lactate and pyruvate monocarboxylate transporters MCT1 and MCT4 [Koukourakis et al, 2006]. Also, they can consume

glucose and produce lactate in hypoxic conditions. By doing these, they help tumor cells to overcome harsh hypoxic conditions and facilitate glycolysis metabolism. Acidic microenvironment that caused by lactate production and secretion of immune suppressive cytokines that polarize macrophages to TAMs by fibroblasts results in cytotoxic T cell activity inhibition or deletion of these cells in the TME [Lakins et al, 2018].

2.3.4 T-Cytotoxic Cells

Cytotoxic T cells are a specific and important member of T cells. They have abilities to kill pathogens such as cancer cells, viruses or bacteria and also, they can drive damaged cells to apoptosis [Al-Shura, 2020]. T-cell receptors (TCRs) are main component in specific antigen recognition. Class I MHC molecules are responsible for antigen transportation. Cell specific antigens bind to these molecules and then they are brought to the surface of the cell. T cells recognize them when they reach out to the surface. If this antigen is specific for TCR of T cell, T cell binds to MHC I class complex and destroys the cell. To be able to do this process, T cells require CD8 glycoprotein that binds to complex. Therefore, we can call cytotoxic T cells as CD8+ T cells.

Cytotoxic T cells are crucial immune system cells that fight against cancer cells. As mentioned before, cytotoxic T cells can kill tumor cells by recognition of a specific antigen that presented on membrane of tumor cells and stimulate an immune response [Klebanoff et al, 2006]. They form the main defense line against cancer cells. Cytotoxic T cells have abilities to drive cancer cells to apoptosis. To gain resistance to cytotoxic abilities of these cells, cancer cells develop many immune suppression and immune evasion related processes.

Cytotoxic T cells and their cytotoxic effects in the TME are usually supported by T helper 1 (Th1) cells that release interferon-gamma (IFN-gamma) and interleukin-2 (IL-2) [Wang et al, 2004]. With the help of these molecules, cytotoxic T cells mediate programmed death processes in normal microenvironment conditions. Normal macrophages produce CXCL9/CXCL10 cytokines. When these cytokines bind to CXCR3 receptor on T cells, recruitment of T cells to the tumor occur. But in tumor educated microenvironment, there are many obstacles ahead of T cells and their cytotoxic activities. As shown in Fig. 2.5, hypoxia and hypoxia-related

factors such as HIFs can block activation of cytotoxic T cells in many ways [Petrova et al., 2018]. As mentioned before, HIFs can bind to promoter regions of more than 100 genes in our cells, immune suppression related genes are top priority for tumor cell survival. Cancer cells and cancer educated immune system cells express programmed-death ligand 1 (PD-L1) on their surface to bind with programmed-death protein 1 (PD-1) in hypoxic conditions. This binding decreases cytotoxic activities of cytotoxic T cells. IFN-gamma receptor and IFN-gamma binding and some cancer related signaling are other causes of PD-L1 and PD-1 binding. Also, HIFs can bind to promoter region of CTLA-4 in cytotoxic T cells and make them express CTLA-4. Binding between CTLA-4 on T cells and CD80/CD86 ligands on antigen-presenting cells (APCs) inhibits cytotoxic T cell activities.

Acidic microenvironment and structurally changed ECM are other inhibitors of cytotoxic T cell cytotoxicity. T cells with cytotoxic abilities have troubles entering structurally changed TME. Dense and complex structure of newly formed ECM prevent cells to infiltrate unless they secrete proteases or cathepsins that degrade proteins on their way. Cytotoxic T cells do not have ability to produce these kinds of molecules. Also, T cells in acidic microenvironment cannot present their cytotoxic effects. Acidic microenvironment and competition for nutrition are other reasons for cytotoxic T cells to not prefer to infiltrate these regions.

As mentioned in the Macrophage section, macrophages and some other cells such as MSCs produce cytokines and chemokines to recruit Tregs to TME. These Tregs suppress cytotoxic T cells in the TME. Tregs form a barrier at regions that are close to tumor margins to prevent invasion of T cells and do not let them infiltrate. Besides recruitment of Tregs to TME, macrophages can induce differentiation of monocytes to Tregs instead of differentiation to cytotoxic T cells, leading to high Treg ratio in TME.

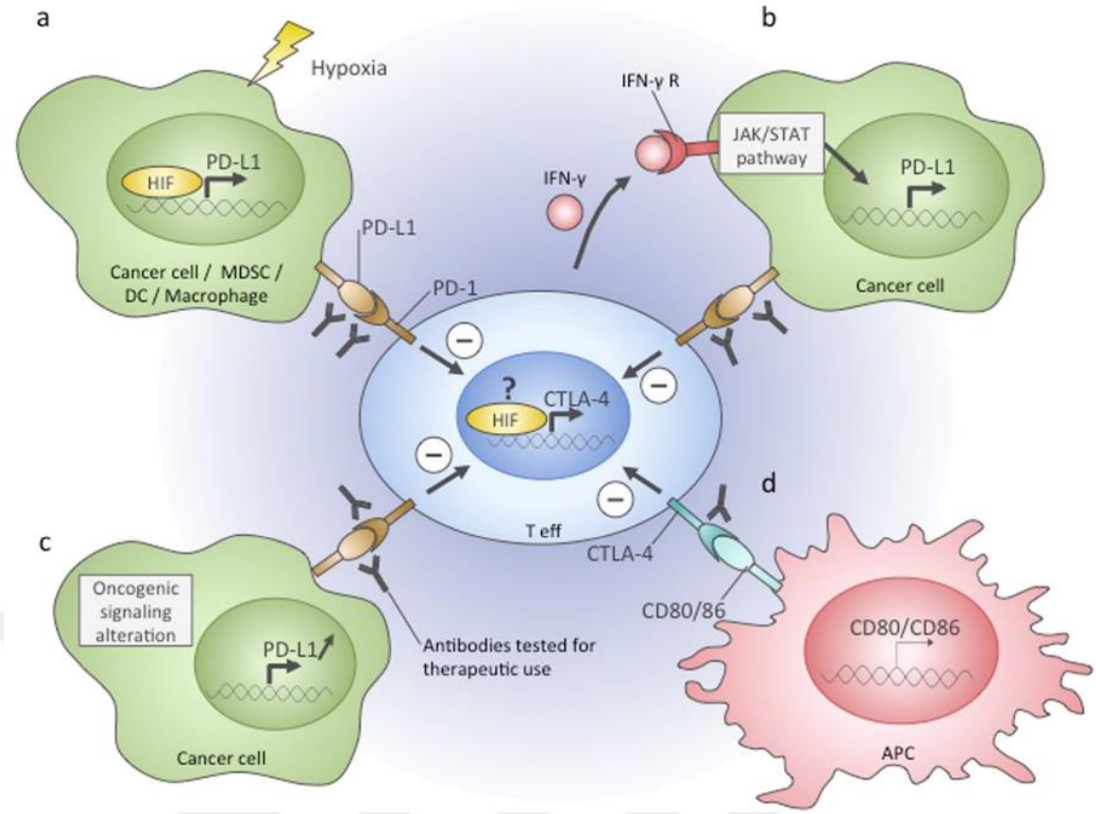


Figure 2.5: Cytotoxic T cell cytotoxicity inhibition.

2.3.5 T-Regulatory Cells

One of the critical steps in carcinogenesis is immune suppression. Treg cells are immune system cells in the TME that have diverse immune modulatory functions [Flavell et al, 2010; Whiteside et al, 2012]. In normal conditions, Treg cells regulate the activation and expansion of B cells or T cells and play key role in maintaining homeostasis of innate lymphocytes [Gasteiger et al, 2013]. It means that, excessive immune responses to antigens are regulated by these cells. This regulation prevents allergic reactions and in extreme cases autoimmune responses that results in targeting of the host cells by its own immune system. Cancer cells use this mechanism for its own advantages.

In cancer, increased Treg cell numbers correlate with reduced survival in patients, but this varies in some specific cancer types [Bates et al, 2006; Fu et al, 2007; Hsieh et al, 2012]. As mentioned before, Treg cells are important cell for immune suppression. They can have suppressive functions by preventing antigen presentation and interfering with cytotoxic effects of CD8+ T cells and their functions by inhibiting cytolytic granule release [Von Boehmer et al, 2013].

Recruitment and proliferation of Tregs are influenced by some immune system cells and other stromal cells. TAMs, cancer educated dendritic cells, MDSCs that are differentiated from monocytes via signals from MSCs reside in the TME are responsible cells for recruitment and proliferation.

2.3.6 Natural Killer Cells

NK cells are another type of significant cytotoxic lymphocytes in innate immune system [Perera, 2021]. NK cells are responsible for rapid responses to pathogens such as tumor cells, viruses, etc. They play critical role in cancer surveillance and pathogen defense due to their unique abilities. Normal cytotoxic cells recognize MHC complex like cytotoxic T cells and produce related cytokines and then drive these pathogens to apoptosis or lysis. But NK cells can kill pathogens without antibodies or MHC structure on them instantly leading to faster immune reaction. This role is especially important in cancer tissues because, cytotoxic T cells can not kill pathogens if they do not have MHC complex and some cancer cells such as cancer stem cells (CSCs) are missing MHC I markers and this gives them immune evasion ability.

NK cells can secrete inflammatory cytokines and show cytotoxic functions based on CD56 expression [Stabile et al, 2017; Glasner et al, 2018]. NK cells are responsible for death receptor mediated apoptosis and perforin/granzyme B mediated cytotoxicity to target pathogens such as tumor cells [Imai et al, 2000]. This cytotoxic effect can limit tumor growth.

Tumors develop many mechanisms to evade NK-mediated destruction. This includes coating themselves in collagen that is highly expressed in tumor educated TME by the influences of CAFs to be attached to inhibitory NK receptors and utilizing platelets as a shield to evade from detection of tumor cells by NK cells [Maurer et al, 2018]. Tumor cells that have ability to do metastasis are coated by platelets in circulation to form protective aggregates. This formation interferes with NK cell-mediated cytotoxicity by fibrin accumulation and prevents recognition of tumor cells by NK cells [Palumbo et al, 2008; Felding-Habermann et al, 2011]. Also, cancer has a coagulation system that is not only related with circulation, but also in metastasis related processes. Tissue factor (TF) is a coagulation protein that take

critical responsibility in NK-cell mediated lysis interfering [Palumbo et al, 2008; Gil-Bernabe et al, 2012].

In the TME, phenotype of NK cells varies, acidic and hypoxic microenvironment and changed ECM structure play huge role in this phenotype changes. NK cells cannot infiltrate into TME after tumor related education processes such as recruited Treg cells and MDSCs related immune suppression. These cells prevent NKs infiltration into the TME and stop or inhibit their cytotoxic effects. NK cells with impaired cytotoxicity are major bone marrow-derived dendritic cells (BMDCs) contributors in hypoxia condition. BMDCs have interactions with angiogenesis related processes indicating the importance of NK cell phenotype changes in angiogenesis [Lyden et al, 2001; Conejo-Garcia et al, 2004; Gao et al, 2008; Peinado et al, 2012].

2.3.7 Cancer Stem Cells

CSCs are undifferentiated and self-renewing cells that can take part in heterogeneous tumor structure. Because of their self-renewal and differentiating capacities, they can play critical role in initiation and progression of tumors [Papaccio et al, 2017]. A number of studies on solid tumors reveal that CSCs are general feature of many types of cancer. As shown in Fig. 2.6, they can be derived from stem cells, mostly from MSCs, by some oncogenic hits caused by the instable TME and lack of DNA repairing systems [Papaccio et al, 2017]. Also shown in the Fig. 2.6, some types of chemotherapies, radiotherapies are ineffective against these cells because of their mesenchymal like phenotype [Papaccio et al, 2017]. Immune system cells cannot make antigen recognition because CSCs do not have specific antigens on their surface, hence CSCs are chemo, radio-and also immune-resistant cells. Further, they do not represent human leukocyte antigen class 1 that used in T-cell mediated antigen recognition on their membrane, leading to escape from the killing process mediated by the T lymphocytes. Self-renewing capacities of CSCs combine with the resistance to treatments to make them act as reservoir cells and also make them to be the cause of recurrence [Clarke et al, 2006; Holohan et al, 2013; Schöning et al, 2017]. Also, CSCs adopted to slow cycling growth rates(quiescence), enabling these cells to avoid processes that target cells with high proliferation rates [Clarke et al, 2006].

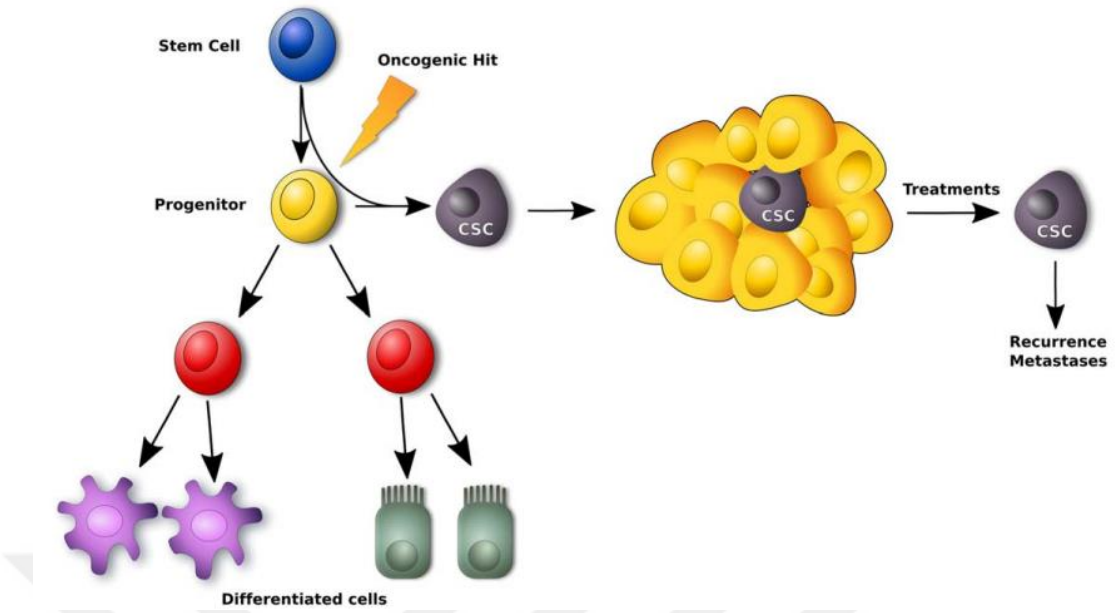


Figure 2.6: Model of CSCs theory.

Stem cells lose their stem-related phenotypes under atmospheric oxygen conditions (%21) [Simon et al, 2008]. Stem cells are stabilized in hypoxic conditions that have %1 oxygen concentrations. For example, some stem cells operate in the hypoxic endosteal regions of bone marrow, which also applies to CSCs [Kubota et al, 2008]. Recruitment of CSCs to the tumor site occurs when favorable hypoxic conditions are met in the TME [Farnie et al, 2015; Doğan et al, 2017]. CSCs favor hypoxic sites within the tumor to maintain their undifferentiated state hence CSCs population best protected in hypoxic conditions [Das et al, 2008]. In the case of harsh conditions within the core regions of the tumor, cancer cells can escape from these regions due to hypoxia related aggressive selection pressure for resilient stem-like tumor cells that have ability to migrate away to margin of the tumor. With the help of HIF-1alpha, VEGF and some other proangiogenic factors, CSCs can survive in hypoxic conditions. HIFs influence on cancer cell stemness is well documented by researchers with studies that show HIF-1alpha and HIF-2alpha are key figures for CSC stemness. HIF-1alpha upregulation is essential for CSC survival in hypoxic conditions because it increase CSC proliferation rate [Lau et al, 2017]. As mentioned before, HIF-2 is included highly in differentiation related processes and stemness properties may be influenced more by HIF-2alpha [Covello et al, 2006; Keith et al,

2012]. HIF-2alpha targets stem cell upkeep genes, including Notch, octamer-binding transcription factor 4(Oct4) and the sex determining region Y-box (Sox2).

CSCs generally interact with the TME and its components through the activation of self-renewal and stemness-related pathways such as Notch-1 and PI3K pathways [Mao et al, 2013; McAndrews et al, 2015]. TME has direct effect on development and survivability of CSCs via molecules such as MMP-3 and Wnt ligands [Mills et al, 2016; Hu et al, 2017]. By these TME-mediated signaling processes CSCs are involved in immune modulation and the formation of inflammatory niches [Motz et al, 2011; Bayne et al, 2012; Bussard et al, 2016]. CAFs that reside in the TME sustains CSCs' stem-related properties [Forsberg et al, 1993; Giannoni et al, 2010]. Population of CSCs is maintained and kept in control by MSCs as MSCs are responsible for generation of CSCs. MSCs can increase number of CSCs via bone morphogenetic proteins (BMPs), especially via BMP2 and BMP4 signaling. These signaling events contribute to maintaining CSCs properties and survival of CSCs. MSCs produce prostaglandin E2(PGE2) after IL-1alpha and IL-1beta secretions stimulation by cancer cells. This stimulation leads to secretion of IL-6, CXCL1 and CXCL8 by MSCs to increase stemness of cancer cells and finally strong expansion of CSCs.

2.3.8 Dendritic Cells

Dendritic cells (DCs) are one other important type of immune system cells. DCs have roles in pathogen-specific T-cell responses, they are important supporters for protective immunity. DCs can support protective immunity by making recognition of antigen source, capturing, presentation of antigens to relevant cells, up-regulate co-stimulatory molecules, producing inflammatory cytokines and travelling to secondary lymphoid organs for presentation of antigens. DCs act as messenger cells and they are like bridges between the adaptive and innate immune system properties. When they do their normal functions by patrolling between tissues and body parts, they can also encounter tumor structures in cancer patients. In these TME structures, some immune suppressive factors such as IL-10, TGF-beta, PGE2, VEGF and other cytokines inhibit DC maturation into immunogenic cells and promote tolerogenic phenotypes [Motta et al, 2016]. When they leave the TME site they can regain their normal abilities. Normal DCs act as tumor suppressive cells in

early stages of tumor, but they became tumor promoting cells as the tumor progresses.

2.3.9 Extra Cellular Matrix

ECM contains many different macromolecules, including collagens, glycoproteins and some enzymes that have biomechanical activities and functions [Grivennikov et al, 2010; Hanahan et al, 2011]. Proteoglycans, minerals, fibrous proteins, active tissue components that maintains cell adhesion, proliferation and communications and lastly water constitute the ECM structure [Anderson et al, 2010; Feig et al, 2013; Korneev et al, 2017; Walker et al, 2018]. The ECM structure has huge impact on the production of laminin, elastin, collagen and other different fibrous proteins [McAndrews et al, 2015; Korneev et al, 2017]. These properties make them crucial components in cancer development. Cancer cells need adhesion related processes and signals or communication with other cells to develop proliferation, angiogenesis and metastasis related processes. By altering its physical, density, morphological and composition properties, the ECM influences migration of cancer cells in the TME [Walker et al, 2018]. As shown in Fig. 2.4, the TME has more differentiated, aligned and stiff structure [Petrova et al, 2018].

With the help of ECM proteinases, the ECM structure undergoes a remodeling process that modify and degrade its primary components. This remodeling processes are highly influenced by cancer cells. Influences of cancer cells on ECM can be seen in Fig. 2.7 [Petrova et al, 2018]. With the help of hypoxia condition in the TME, produced HIFs can bind to some genes that are related to ECM structure dynamics and adhesion related proteins. HIFs can bind to promoter regions of integrin genes to produce integrin proteins. These integrins then migrate onto cell membrane to communicate with laminin and fibronectin on ECM to make cancer cells to be able to move and make some signaling processes that are important for cancer development. Changed ECM composition by cancer cells has a significant impact on integrin signaling, pathways such as Hippo pathway and EMT related pathways and processes that influence cancer development [Korneev et al, 2017]. Integrins also influence and mediate many other aspects of cellular functions such as differentiation, proliferation and survival [Keely et al, 2011]. Also, HIFs can bind to promoter regions of genes that are taking part in collagen fiber production.

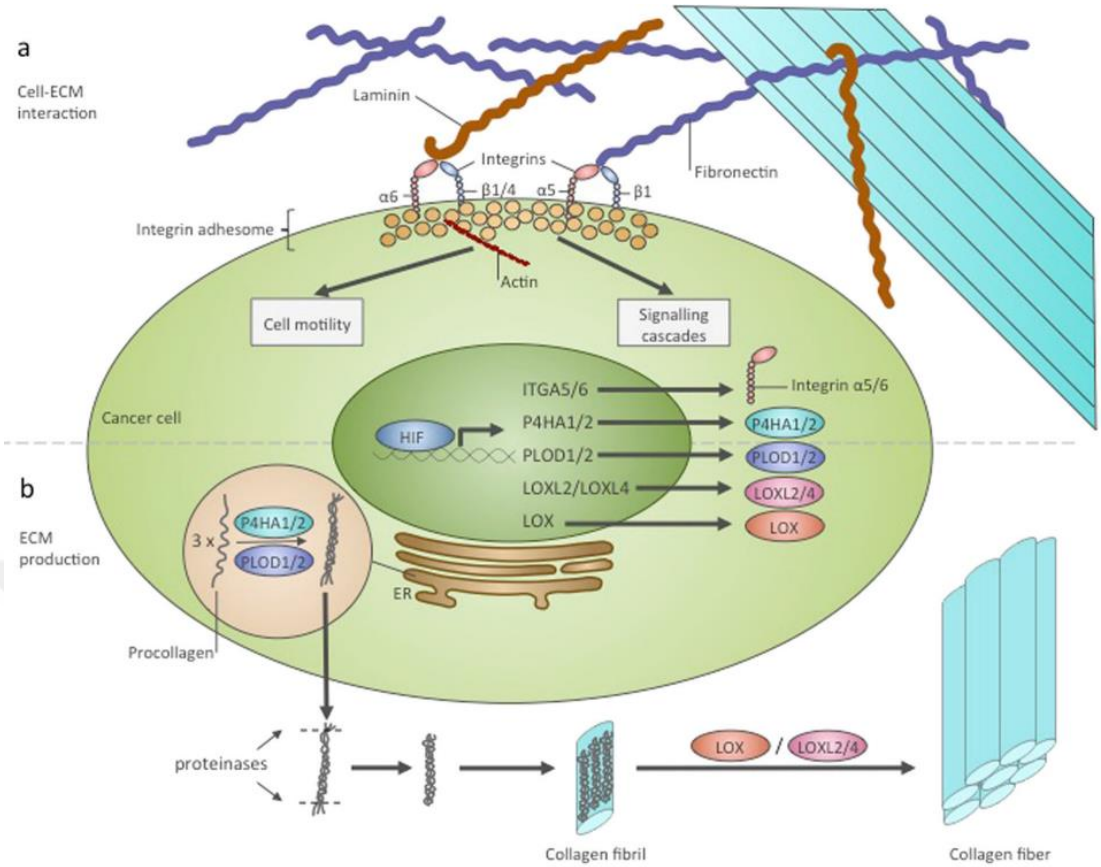


Figure 2.7: HIFs effects on ECM biosynthesis and cell motility.

Tumor cells prefer dense ECM structure. High concentrations of collagen, proteoglycans, proteins and glycoproteins are properties of tumor preferred ECM structure [Hu et al, 2017]. Effectivity of biologics, immunotherapeutics, chemotherapy and some other anticancer agents hindered by remodeled dense ECM structure. Production and accumulation of collagen and hyaluronan in the TME increase tension in the ECM and increase solid stress induced by growth and put significant pressure on stromal cells [Mills et al, 2016]. Stromal cells, blood cells and immune system cells that cannot deal with stress and pressure are eliminated from the TME and also their infiltration to media prevented by these mechanisms.

Cells tend to migrate away from the low-density ECM regions to ECM regions that have high density as a result of the adhesion gradient [Korneev et al, 2017].

In normal stroma, fibroblast cells are predominant cells. Normally they secrete ECM related molecules and contribute to ECM structure to form natural barrier against tumor progression. Fibroblasts are useful for depositing ECM and basement membrane components [Tomasek et al, 2002; Kalluri et al, 2006]. Tumor cells can

educate ECM to support and promote tumor progression. CAFs are important figures in ECM education. They can produce fibronectin, alpha-smooth muscle actin, collagen and other proteins to alter the ECM architecture. Remodeling the ECM to support tumorigenesis by CAFs need induction of YAP transcription factor [Calvo et al, 2013]. Cancer cells with the help of CAFs use proteases, matrix metalloproteinases and cathepsins to migrate easily in new formed ECM structure. Cells that are not able to reach these products are challenged by the stiff structure of newly formed ECM.

2.3.10 Chemokines/Cytokines/Metabolites

Cytotoxic T cells, T helper I and NK cells express CXC-chemokine receptor 3 (CXCR3), a receptor for CXC-chemokine ligand 9 (CXCL9) and CXCL10. The cells can migrate into tumors in response to these chemokines. Increased levels of CXCL9 and CXCL10 are associated with high tumor infiltration by cytotoxic T cells and correlate with decreased metastasis and improve survival rates in patients [Zhang et al, 2003; Pages et al, 2005; Sato et al, 2005; Galon et al, 2006; Kryczek et al, 2009; Zhao et al, 2016]. Also, CXCL9 and CXCL10 are tumor angiogenesis inhibitors [Charmeliet et al, 2000; Romagnani et al, 2001]. They can prevent basic fibroblast growth factor (bFGF)-induced angiogenesis [Strieter et al, 1995; Angiolillo et al, 1995].

There are two main signaling processes that recruit Treg cells to the TME. One of them is CCR4-CCL22 signaling. Treg cells express CCR4 and get recruited into the TME in response to CCL22 that is mainly produced by macrophages and tumor cells [Curiel et al, 2004]. Second signaling is between CCR10 and CCL28. With the help of CCL28 that is produced by the TME cells in hypoxic conditions, Treg cells express CCR10 in response and migrate towards it [Facciabene et al, 2011].

CCL2-CCR2 signaling is used for recruitment of macrophages to the TME [Qian et al, 2011]. CCL2 expression correlates with high TAM numbers and also associated with poor patient prognosis [Pollard et al, 2004]. CCL2 also induces production of some chemokines to promote macrophage retention in the TME [Kitamura et al, 2015]. CCL2 targets vascular endothelial cells via the Janus kinase 2(JAK2)-STAT5 and some other protein kinase pathways to affect tumor vascularization and tumor metastasis [Goede et al, 1999; Wolf et al, 2012; Bonapace

et al, 2014]. CCL2 also can promote proliferation, survival, motility, EMT and stemness of cancer cells by its own activities and by inducing matrix metalloproteinase 9 (MMP9) secretin [Azenshtein et al, 2002; Stamenkovic et al, 2000; Robinson et al, 2002; Fang et al, 2012; Tsuyada et al, 2012; Long et al, 2012; Zou et al, 2015; Long et al, 2015]. HIF-1alpha can interact with promoter regions of CCL2 to induce its expression [Mojsilovic-Petrovic et al, 2007].

CXCL12 targets vascular endothelial cells with VEGF to promote tumor angiogenesis [Kryczek et al, 2005; Kryczek et al, 2007]. CXCL12 can also promote tumor cell proliferation and survival [Scotton et al, 2001; Scotton et al, 2002]. Furthermore, CXCL12 and its receptor CXCR4 signaling pathway promotes tumor cell invasion, metastasis, tumor proliferation and stemness [Müller et al, 2001; Murakami et al, 2002; Helbig et al, 2003; Zeelenberg et al, 2003; Kang et al, 2003; Darash-Yahana et al, 2004]. Hypoxia triggers CXCL12 expression in tumor cells, fibroblasts and stem cells by HIF-1 binding to promoter regions of CXCL12 gene [Hitchon et al, 2002; Ceradini et al, 2004; Kryczek et al, 2005]. Also, hypoxia promotes receptor of CXCL12, CXCR4, expression in TAMs and tumor cells [Schioppa et al, 2003; Staller et al, 2003]. CXCR4 expressed tumor cells can migrate towards CXCL12 with the help of HIF-1alpha activation.

Cancer cells can produce CXCL16 which take part in recruitment of bone marrow-derived MSCs with the help of CXCL16-CXCR6 receptor-ligand binding. MSCs that have CXCR6 receptor migrate into tumor site by CXCL16 signals.

CCL28 is a chemokine that is produced by TME cells in hypoxic regions. This chemokine induce chemotaxis of CCR10 expressing T-regulatory cells into these hypoxic regions [Facciabene et al, 2011].

CSF-1 and its receptor CSF-1R are used for polarization processes of macrophages and promoting metastasis related processes [Qian et al, 2009; Pyonteck et al, 2013]. CSF-1 is an important component in preventing recruitment of immune system cells and accumulation of TAMs [Qian et al, 2009; Qian et al, 2011; DeNardo et al, 2011; Pyonteck et al, 2013].

Interferon-gamma (IFN-gamma) is a cytokine that plays a significant role in regulating the immune response of its target cells [Tau et al, 1999]. IFN-gamma has important roles in both innate and adaptive immunity. IFN-gamma is an activator of macrophages and MHC class II molecule expression inducer. IFN-gamma has immunostimulatory and immunomodulatory effects. T helper cells, cytotoxic T cells,

macrophages and NK cells can produce IFN-gamma. Produced IFN-gamma promotes NK cell activity [Konjevic et al, 2019], increases antigen presentation and lysosome activities and causes normal cells to increase MHC class molecules expression. IL-12, IL-15, IL-18 and other interferons are associated with IFN-gamma expression upregulation [Castro et al, 2018] and IL-4, IL-10 and TGF-beta and glucocorticoids are responsible for IFN-gamma downregulation [Bhat et al, 2018]. Cytotoxic T cells are supported by cells that release IFN-gamma [Wang et al, 2004]. IFN-gamma increase cytotoxic effects of immune system cells and acts against tumor progression.

IL-6 is a cytokine with a pleiotropic effect on immune response, inflammation and blood cell production. IL-6 decreases the production of albumin, fibronectin and transferrin. IL-6 controls some inflammation related processes in chronic inflammation situations. Also, IL-6 has anti-adhesive effects. IL-6 cytokines that produced by macrophages can reduce E-cadherin bindings between cancer cells with epithelial phenotype. These E-cadherin bindings keep the epithelial cells tied together to prevent their movement and keep epithelial tissues intact. Hence, E-cadherin binding breakage results as EMT and invasion related phenotypes of epithelial based tumor cells.

Lactate is an important metabolite in tumor microenvironment. Pyruvate is an end-product of anaerobic glycolysis, and it reaches high concentrations especially in hypoxic conditions. Cells produce lactate from pyruvate metabolism, but lactate production is not only restricted in anaerobic glycolysis. Activation of aerobic glycolysis pathway that produce lactate from glucose in the presence of oxygen is called as Warburg Effect. In aerobic glycolysis, rate of lactate production from glucose is 10 to 100 times greater than total oxidation of glucose in mitochondria [Al Tameemi et al, 2019]. Acidic structure of the TME is highly caused by lactate. We can encounter low intracellular pH values at hypoxic regions that caused by production of lactate from glucose. Secretion of lactate is the following step and this results in acidification of stroma and consequent suppression of anticancer immune response [Locasale et al, 2010]. CAFs express monocarboxylate co-transporters MCT1 and MCT4. As shown in Fig. 2.8, MCT1 and MCT4 are used to transport lactate to inside and outside of cells [Lyssiotis and Kimmelman, 2017]. Cancer cells and CAFs in hypoxic regions produce lactate from glucose and secrete them via MCT4. Cancer cells in the normoxic regions uptake lactate by the help of MCT1.

They use lactate in redox balance, nitrogen balance, biosynthesis and angiogenesis related programs. With the help of lactate effect, MSCs can differentiate into CAFs and macrophages can change their polarization state into M2 macrophages status that are called as TAMs.

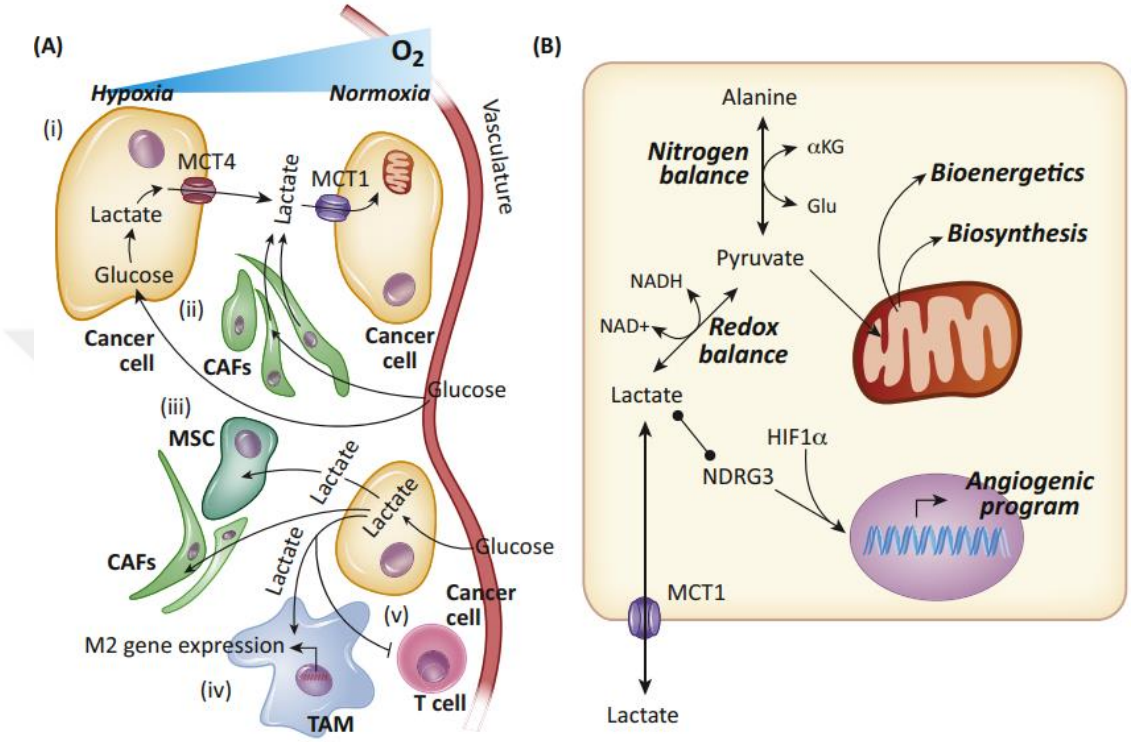


Figure 2.8: Lactate metabolisms in the TME.

2.4 Invasion/Metastasis

Metastasis is a concept that differentiate a tumor from cancer. When tumor cells gain metastasis ability, they can be called as cancer cells and disease can be called as cancer disease. Invasion and invasive abilities are first step of metastasis. Tumor cells need to be able to invade other tissues. They can be attracted by high oxygen concentrations and vessels. To reach oxygen-sufficient areas, they invade into basal structures that reside under epithelial tissues to vessels. This is one of the solutions that tumor cells developed to obtain energy sources. Another solution is the development of new vessels from existent vessels into the core of tumor structure with the process of angiogenesis. Invasion of cancer cells to other tissues and metastasis are strongly promoted by hypoxia. In hypoxic regions we have

heterogeneous tumor cells. Some of them try to maintain their aerobic energy production by migrating towards vessels and some of them stay in the hypoxic regions of tumor and change their metabolism to anaerobic energy production structure. Hence one can easily say that hypoxia is crucial in metastasis. Hypoxia increase the likelihood of metastasis in cancer patients [Höckel et al, 2001; Vaupel et al, 2008]. Main factor in gaining invasive phenotype is EMT. EMT is used by cancer cells to lose their epithelial markers to gain mesenchymal traits and acquire an invasive phenotype that finally leads to metastasis [Mani et al, 2008]. Normal epithelial cells cannot move because of the restrictions that they have. One of them is E-cadherin bindings. Macrophages produce IL-6 to cut them free from these bindings to make them able to move. This event marks an initial stage. CAFs, TAMs, MSCs and others produce more chemokines and cytokines to contribute EMT formation. For example, CXCL12-CXCR4, CSF1-CSF1R, EGF-EGFR bindings are important for formation of EMT. Also, TGF-beta plays crucial roles in EMT formation as well. With the help of EMT formation, cancer cells change their phenotype into stem-like mesenchymal structure and this give them abilities to move freely.

As we know, fibroblasts are the predominant cells in the stroma. They can secrete extracellular matrix related products. CAFs in the TME produce proteins such as collagen, fibronectin, etc. to alter ECM structure. With the help of cancer cell and stromal cells effects, morphology and phenotypes of cancer cells changed to become more metastatic and invasive. Cancer cells that produce important proteases, cathepsins or matrix metalloproteinases can degrade ECM on their way to invade other tissues. This mechanism gives cancer cells the ability to move and also restrict cells that do not have these proteases to infiltrate into tumor structure. Metastasis abilities of both malignant and premalignant epithelial cells are enhanced by CAFs that confer mesenchymal-like phenotype with the help of CXCL12 and IGF1 [Zhang et al, 2013]. TAMs have a significant role in preparing cells to get invasive cellular phenotypes at the tumor margin [Condeelis et al, 2006]. TAMs do this process by Wnt and some other paracrine signaling loops involving TGF-beta, CSF1 and EGF [Goswami et al, 2005; Condeelis et al, 2006; Coniglio et al, 2012].

Fig. 2.9 shows the general concept of metastasis. There is a developed tumor structure in the left side of the figure [Quail and Joyce, 2013]. With the help of hypoxia effects, TAMs, CAFs and MSCs, tumor cells change the TME according to their own advantage. ECM has fibrillar collagen accumulation and promote invasive

phenotypes. Tumor cells that have invasive phenotypes and EMT migrate towards tissues with high oxygen concentrations by invasion or migrate towards vessels through passages that TAMs opened by degrading structure that composed of endothelial cells and pericyte cells. In the vessel, cancer cells are vulnerable to NK cell cytotoxicity. NK cells that are circulating in vessels recognize mesenchymal-like cancer cells because they do not have MHC class antigens on their surfaces. In this case, platelets take part and coat cancer cells to prevent NK cell cytotoxicity and also antigen recognition and presentation processes. Platelet coated cancer cells have immune evasion and can migrate to regions that are near to premetastatic niche and extravasate through passages that they opened with the help of platelets and leave platelets at those points. Premetastatic niches include crucial components for secondary tumor progression. When cancer cells reach those points, they do opposite of EMT, mesenchymal to epithelial transition (MET), to be able to proliferate and increase their numbers.

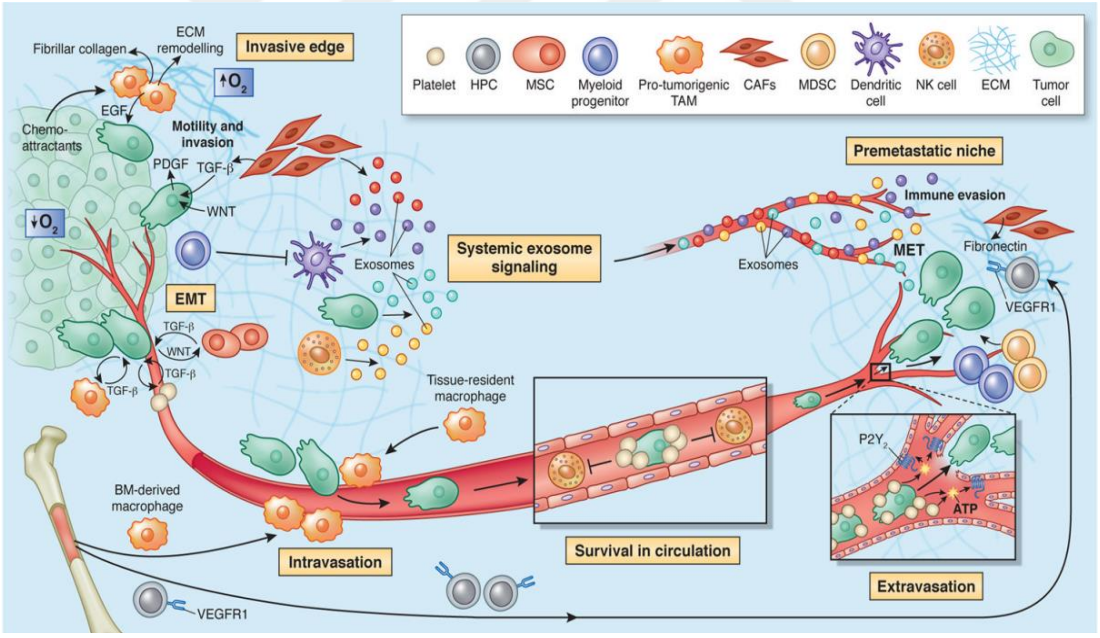


Figure 2.9: Processes that are took place in metastasis.

2.5 Angiogenesis

Angiogenesis is one of the main processes that take place in carcinogenesis. With the help of this process, tumor cells can overcome nutrition deficiency in the TME by inducing angiogenesis. By making new blood vessels to develop new blood

supply via the release of VEGF and other hypoxia-inducible angiogenic factors, tumor cells contribute to overcoming of the proliferation limitations that caused by stressful TME [Seo et al, 2014]. Tumor cells can make their own vascularization with the help of re-modeling of pre-existing vessels, angiogenesis, bone marrow-derived endothelial cell recruitment, vascular mimicry, differentiation and some other associated processes. Tumor structures either use pre-existing vessels to branch new vessels outwards or they can contribute to making them by using endothelial progenitor cells [Hanahan et al, 2011]. Epithelial-like tumor cells tend to migrate away from hypoxic regions to oxygen rich tissues, but CSCs tend to stay in the center of the hypoxic region to contribute to the vessel development. They are maintained by growth factors and proangiogenic factors that are secreted by endothelial cells that are in hypoxic conditions and this situation is called as hypoxic niche hypothesis. Hypoxia activated HIF1-alpha regulates alternative signaling processes for angiogenesis [Mills et al, 2016; Hu et al, 2017]. CSCs and MSCs are both responsible for production of VEGF, angiopoietin-1 (Ang-1) and other pro-angiogenic factors and also, they can differentiate into pericytes and endothelial cells. Association between pericytes and endothelial cells is required for angiogenesis and occurs in a disorganized structure that leads to convoluted blood flow. Endothelial cells are main vessel structure cells and pericytes cover them and keep them intact. MSCs secrete IL-6 and Ang-1, also induce cancer cells to produce endothelin-1 (ET-1) and thereby promoting tumor angiogenesis. Induction of tumor angiogenesis is strongly influenced by ERK and Akt pathways in endothelial cells that are activated by ET-1. In severe hypoxia situation, VEGF, PDGF and several other growth factors are strongly expressed and related crucial chemotactic and mitogenic factors expressed for MSCs. MSCs migrate towards hypoxic tumor sites and promote vasculogenesis by an autonomous VEGF production and then enhance the pro-angiogenic potential of tumors. Cancer makes itself channels for fluid transport in vascular mimicry that is independent of typical modes of angiogenesis. Production of VEGF-D and VEGF-C by macrophages increase inflammation, angiogenesis and lymphangiogenesis in cancer [Schoppmann et al, 2002]. CAFs and MDSCs are also took place in angiogenesis related processes. Finally, TAM accumulation correlates with angiogenesis at hypoxic regions [Lewis et al, 2005].

3. PHYSICELL

PhysiCell is a flexible open-source framework for building agent-based multicellular models in 2-D or 3-D tissue environments. It aims to provide the ideal virtual laboratory for multicellular systems that simulates both the biochemical microenvironment and large numbers of mechanically and biochemically interacting cells [Ghaffarizadeh et al, 2018]. PhysiCell provides both the stage and the players for studying interacting cells in dynamic tissue microenvironments. It includes biologically-driven submodels for cell cycling, apoptosis, necrosis, solid and fluid volume changes, mechanics and motility. It is a C++ based platform and benefits from simplicity and execution speed of C++. PhysiCell has been parallelized with OpenMP and its performance scales linearly with the number of cells. PhysiCell runs on a variety of platforms (Linux, macOS and Windows) with some software dependencies.

In PhysiCell models, each cell (agent) can dynamically update its phenotype based on its microenvironmental conditions. There are “intracellular” and “non-intracelluar” sample models in default PhysiCell folder, intracellular models include intracellular processes such as reactions or activation/inhibition events in the cells, whereas non-intracellular models lack such features. There are a number of differences between intracellular and non-intracellular models based on their behaviors, features and codes. By manipulating, adding some features or deleting some of them on the sample models, users can develop their own models, using knowledge of C++, XML and SBML. Intracellular models were developed after the initial release of PhysiCell, in intracellular models, methods such as FBA (flux balance analysis), Libroadrunner (library that is written in C/C++ language to support simulation of SBML based models) can be used to investigate intracellular dynamics. Libroadrunner make calculations that are related with reactions and formulas inside of the assignment part and send parameters that are found after these processes to PhysiCell. By making these calculations behind working PhysiCell simulations in every time step, Libroadrunner contributes to dynamic structure of intracellular models. The utilization of intracellular models, particularly Libroadrunner models, necessitates an awareness of their inherent limitations, which stem from the reliance on default functions or properties distinct from those present

in non-intracellular models. Intracellular models use SMBL models for each cell type and non-intracellular models do not use them. Users have the opportunity to undertake the development of more intricate models that encapsulate diverse facets of cancer through the utilization of PhysiCell. The overarching objective of this thesis is to initiate these initial strides towards achieving this aim.

4. MATERIALS & METHODS

4.1 Setup and Configuration of PhysiCell Tool

PhysiCell tool can be downloaded from Github repository (https://github.com/MathCancer/PhysiCell) or can be downloaded manually from the PhysiCell website (http://physicell.org/). In July 2022 developers published a python file in their hackathon to allow download of files via running this python file. In another Github repository (https://github.com/physicell-training/ws2022/tree/main/setup), users can download mentioned Python file named as "get_physicell.py" to download PhysiCell files via typing "python get_physicell.py" in command line. To be able to use Python to download files and then in PhysiCell related processes, users need to download and install Anaconda/Python. Once all tools are installed, users can reach files that are shown in Fig. 4.1.

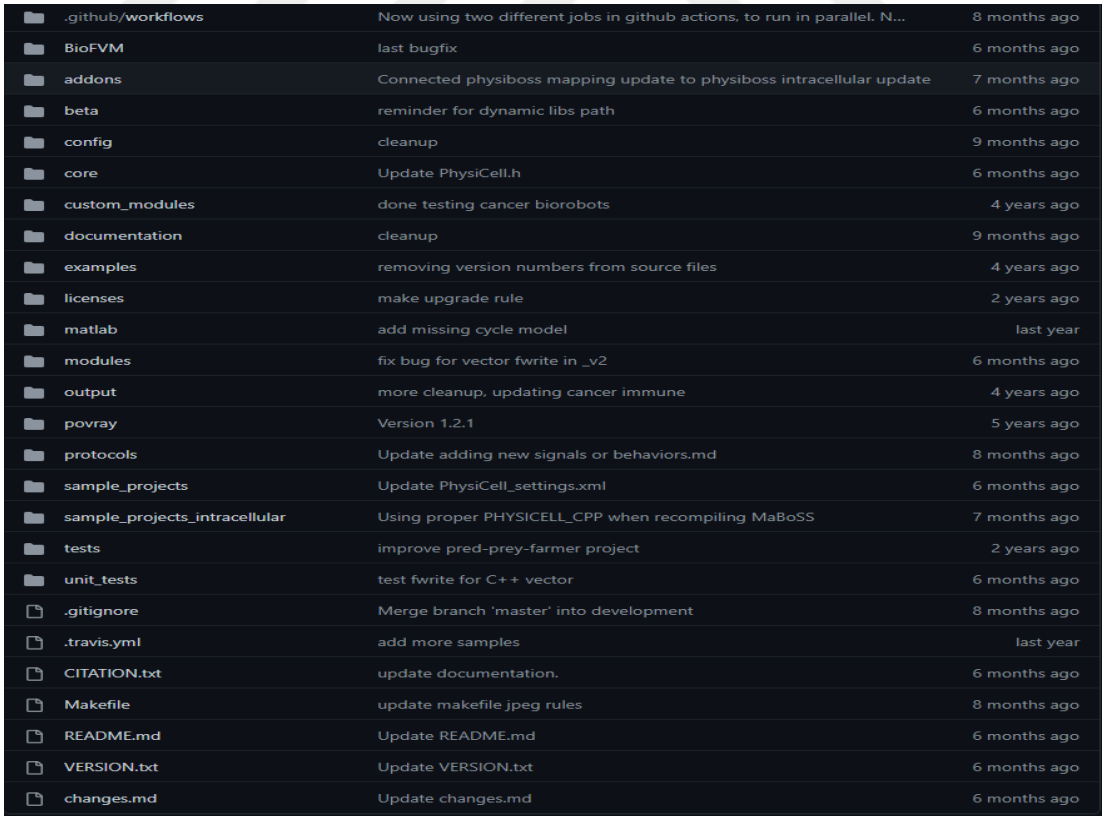


Figure 4.1: PhysiCell folder contents.

To be able to use PhysiCell in command line, users need to install some additional tools like make, gcc and others. MSYS2 is used to download these tools. MSYS2 can be downloaded from "https://www.msys2.org/". After MSYS2 installation, users need to add the line given in Fig. 4.2 inside the prompt to install required libraries to system.

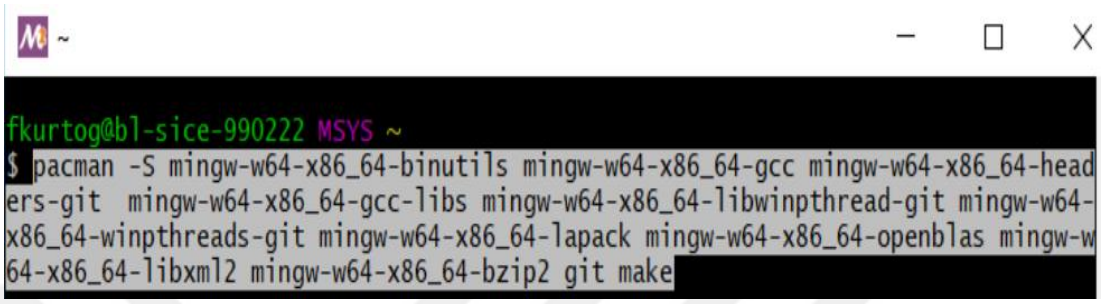


Figure 4.2: Line for installing required libraries in MSYS2.

After these installation processes, users need to add some folders and files to PATH in Windows OS. In Linux or MacOS systems, these steps vary. Users need to install these libraries in terminal of Linux one by one by using "apt-get install {package}" by putting required package name in "{package}" part. In Linux, users can use "export PATH=/file/path" commands to put required file paths to system. Following paths needed to be added to path in Windows OS;

- C:\msys64\mingw64\bin
- C:\msys64\usr\bin
- .\addons\libRoadrunner\roadrunner\bin

Users only need to add the third path to system in Linux and MacOS, because by using "apt-get install" users can add required libraries to system directly in Linux and MacOS.

Using Anaconda Prompt or CMD in Windows OS and terminal in Linux, users need to proceed into PhysiCell folder. If users successfully install "make, gcc" and some other libraries, they are able to use "make list-projects" command to see names of samples that PhysiCell team generated. Users can see the result of this command in Fig. 4.3.

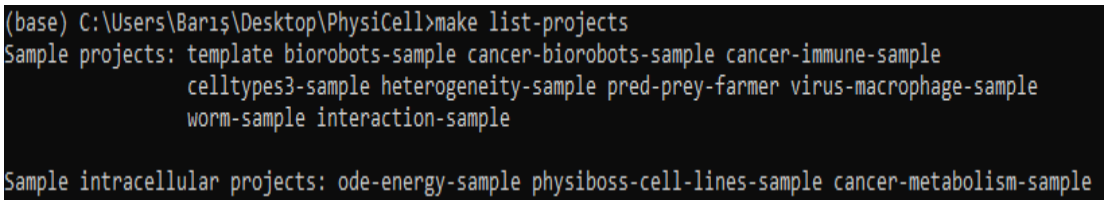


Figure 4.3: "make list-projects" command result.

There are models with non-intracellular and intracellular features as can be seen in the result. In this thesis, Libroadrunner was used to develop the TME model. In PhysiCell “ode-energy-sample” sample model is a template structure for Libroadrunner models. By using “make ode-energy-sample” in command prompt, users can get these sample files from “sample_projects_intracellular” folder. After simply writing “make” in the command prompt, users can actuate “Makefile” file to get other files that are related to Libroadrunner. Final output folder can be seen in Fig. 4.4. Core files in BioFVM and core folders are brought into the default PhysiCell folder after the make process. As can be seen in Fig. 4.4, there is an “ode_energy.exe” file in the folder. In Linux “exe” files do not exist, so that application name is simply “ode_energy”. By clicking this file, users can run the model, but this is not recommended. In command prompt, by typing “ode_energy” in CMD or “./ode_energy” in Powershell users can run the model inside the command prompt. This is the ideal way to run models because if our models include some errors, we will not be able to see error messages and we need to open terminal screen over and over. After running the model, results of the model are generated in the output folder for each time interval that users set in XML files. For every time interval, “.mat”, “.xml”, “.svg” files are produced. Results of default model can be seen in Fig. 4.5. In 2 minutes of running time, model was able to produce representation of a tissue model for 1 day period. This working time and elapsed time in each interval vary according to cell numbers, cell behaviors and cell properties. If users have cells with excessive numbers of reactions, running time is dramatically increased.

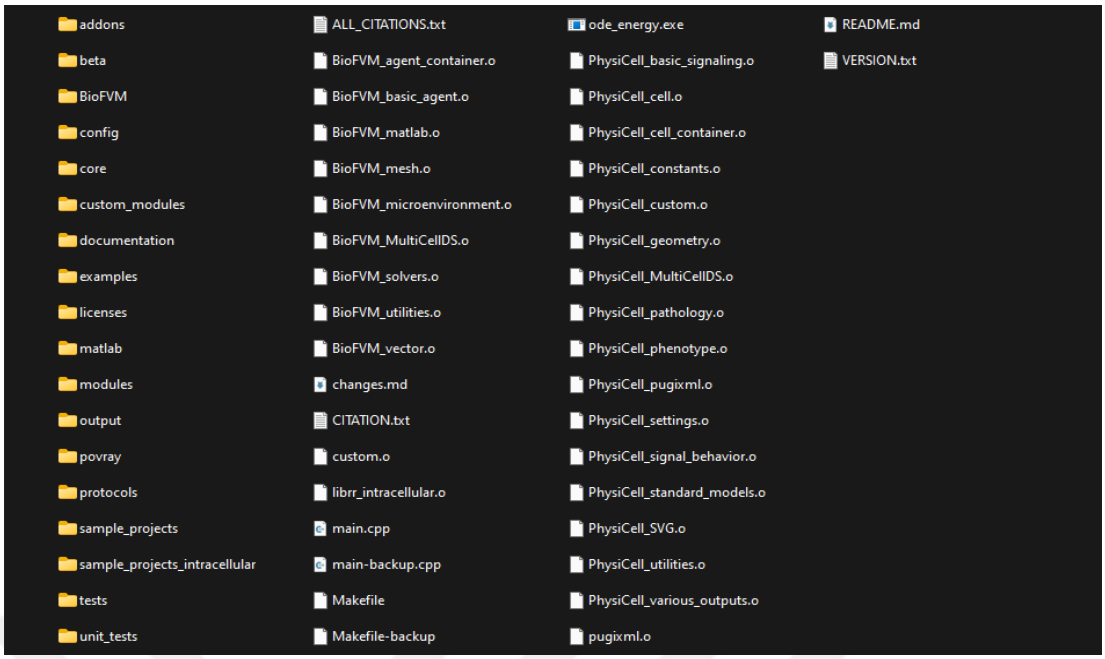


Figure 4.4: Final folder contents after make commands.

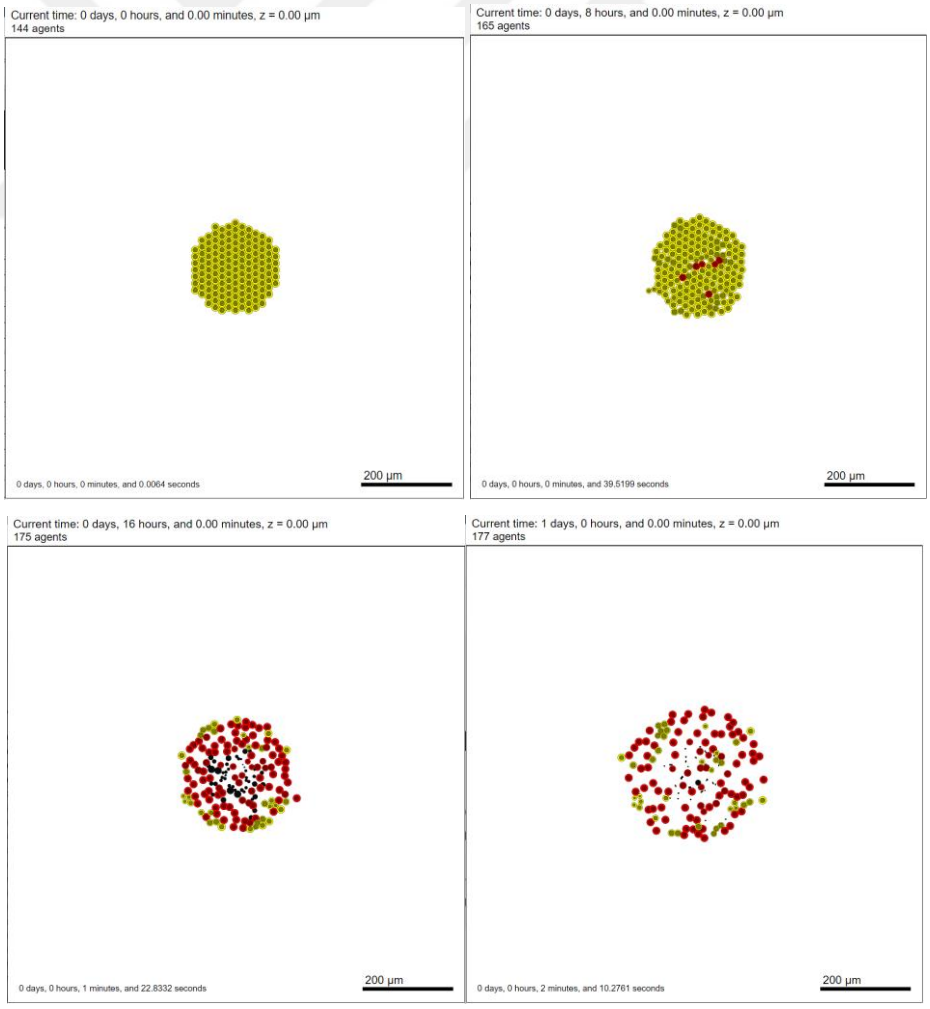


Figure 4.5: Results from default ode-energy-sample model for every 8 hours.

If users want to run other sample models, they need to type “make reset”-“make clean”-“make data-cleanup” in PhysiCell folder by using command prompt to return PhysiCell folder structure to default version. “make reset” deletes files that we get from sample file, “make clean” deletes files that we get from BioFVM (diffusive transport solver for 2D and 3D space), core, custom_modules and config folders and finally “make data-cleanup” deletes results of the model from output file [Ghaffarizadeh et al, 2016].

4.2 Making Customized Models

One has to make changes on “custom.cpp” file in “custom_modules folder, “PhysiCell_Settings.xml” file in “config” folders and SBML files that are in “config” folder to develop their own models. We need to add every species, metabolites, chemokines etc. to these cpp, xml and SBML files to develop models.

“PhysiCell_Settings.xml” file includes parameters of metabolites, chemokines, cell types and parameters of their own properties like motility, migration bias, transition rate, apoptosis rate etc. We have to set these parameters inside xml file to enable PhysiCell to reach them with cpp files. Also, parameters that maintain simulation area sizes, 2D or 3D options, running and interval times, parallelization options, etc. need to be set by the user. In microenvironment setup part, we need to add every metabolite and chemokine by defining its properties such as diffusion coefficient, decay rate and initial concentration. Also, we need to give all of them unique index values to make PhysiCell to be able to distinguish them from each other in simulation. In Fig. 4.6 general structure of component addition process can be seen. After completing the microenvironment setup section, we begin to add cell types in cell definitions sections. We set cell types names and give them indices. Cell definition includes cell cycle properties, death related properties, volume related properties, motility properties, secretion and uptake sections and finally intracellular sections that setup communications with SBML species. In intracellular section, we need to provide folder path of the SBML file and substrate names that are used in XML file and subtrate names that are in SBML files. These fields are case sensitive, hence we need to write substrate names exactly the same way that was written in each file. Fig. 4.7 shows a screenshot of this intracellular part. After cell definitions

section we need to add CSV file folder path to get coordinates for each cell. We can make the CSV file manually for few cells or use Python scripts to add large number of cells. As can be seen in Fig. 4.8, every line corresponds to coordinate of one cell. It includes x,y,z,index numbers respectively. Index numbers should be same with cell definition indices that we used in XML file.

```
<microenvironment_setup>
    <variable name="oxygen" units="mmHg" ID="0">
        <physical_parameter_set>
            <diffusion_coefficient units="micron^2/min">100.0</diffusion_coefficient>
            <decay_rate units="1/min">0.0</decay_rate>
        </physical_parameter_set>
        <initial_condition units="mmHg">38</initial_condition>
        <Dirichlet_boundary_condition units="mmHg" enabled="false">0</Dirichlet_boundary_condition>
    </variable>

        <variable name="glucose" units="mM" ID="1">
        <physical_parameter_set>
            <diffusion_coefficient units="micron^2/min">300.0</diffusion_coefficient>
            <decay_rate units="1/min">0.0</decay_rate>
        </physical_parameter_set>
        <initial_condition units="mM">50</initial_condition>
        <Dirichlet_boundary_condition units="mM" enabled="false">0.0</Dirichlet_boundary_condition>
    </variable>

    <variable name="lactate" units="mM" ID="2">
        <physical_parameter_set>
            <diffusion_coefficient units="micron^2/min">300.0</diffusion_coefficient>
            <decay_rate units="1/min">0.0</decay_rate>
        </physical_parameter_set>
        <initial_condition units="mM">0</initial_condition>
        <Dirichlet_boundary_condition units="mmHg" enabled="false">0.0</Dirichlet_boundary_condition>
    </variable>
```

Figure 4.6: Microenvironment setup part of XML file.

```
<intracellular type="roadrunner">
    <sbml_filename>./config/epitel.xml</sbml_filename>
    <intracellular_dt>0.01</intracellular_dt>
    <map PC_substrate="oxygen" sbml_species="Oxygen"></map >
    <map PC_substrate="HIF1" sbml_species="HIF1"></map >
    <map PC_substrate="glucose" sbml_species="Glucose"></map >
    <map PC_substrate="lactate" sbml_species="Lactate"></map >
    <map PC_substrate="PD1" sbml_species="PD1"></map >
    <map PC_substrate="PDL1" sbml_species="PDL1"></map >
    <map PC_substrate="TGF" sbml_species="TGF"></map >
    <map PC_substrate="IFN" sbml_species="IFN"></map >
    <map PC_substrate="perforin" sbml_species="Perforin"></map >
    <map PC_substrate="granzymeB" sbml_species="GranzymeB"></map >
    <map PC_substrate="IL" sbml_species="IL"></map >
    <map PC_substrate="CXCR4" sbml_species="CXCR4"></map >
    <map PC_substrate="CXCL12" sbml_species="CXCL12"></map >
    <map PC_substrate="EGF" sbml_species="EGF"></map >
    <map PC_substrate="EGFR" sbml_species="EGFR"></map >
    <map PC_substrate="CSF1" sbml_species="CSF1"></map >
    <map PC_substrate="CSF1R" sbml_species="CSF1R"></map >
    <map PC_substrate="CXCL16" sbml_species="CXCL16"></map >
    <map PC_substrate="CXCR6" sbml_species="CXCR6"></map >
    <map PC_substrate="GF" sbml_species="GF"></map >
    <map PC_substrate="CCR2" sbml_species="CCR2"></map >
    <map PC_substrate="CCL2" sbml_species="CCL2"></map >
    <map PC_substrate="CXCR3" sbml_species="CXCR3"></map >
    <map PC_substrate="CXCL9" sbml_species="CXCL9"></map >
    <map PC_substrate="CXCL10" sbml_species="CXCL10"></map >
    <map PC_substrate="CCR10" sbml_species="CCR10"></map >
    <map PC_substrate="CCL28" sbml_species="CCL28"></map >
    <map PC_substrate="Ecadherin" sbml_species="Ecadherin"></map >
    <map PC_phenotype="da" sbml_species="apoptosis_rate"></map>
    <map PC_phenotype="mms" sbml_species="migration_speed"></map>
    <map PC_phenotype="ssr_lactate" sbml_species="Lactate_Secretion_Rate"></map>
    <map PC_phenotype="ssr_HIF1" sbml_species="HIF1_Secretion_Rate"></map>
    <map PC_phenotype="ssr_PD1" sbml_species="PD1_Secretion_Rate"></map>
    <map PC_phenotype="ssr_PDL1" sbml_species="PDL1_Secretion_Rate"></map>
    <map PC_phenotype="ssr_TGF" sbml_species="TGF_Secretion_Rate"></map>
    <map PC_phenotype="ssr_IFN" sbml_species="IFN_Secretion_Rate"></map>
    <map PC_phenotype="ssr_perforin" sbml_species="Perforin_Secretion_Rate"></map>
    <map PC_phenotype="ssr_granzymeB" sbml_species="GranzymeB_Secretion_Rate"></map>
    <map PC_phenotype="ssr_IL" sbml_species="IL_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CXCR4" sbml_species="CXCR4_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CXCL12" sbml_species="CXCL12_Secretion_Rate"></map>
    <map PC_phenotype="ssr_EGF" sbml_species="EGF_Secretion_Rate"></map>
    <map PC_phenotype="ssr_EGFR" sbml_species="EGFR_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CSF1" sbml_species="CSF1_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CSF1R" sbml_species="CSF1R_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CXCL16" sbml_species="CXCL16_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CXCR6" sbml_species="CXCR6_Secretion_Rate"></map>
    <map PC_phenotype="ssr_GF" sbml_species="GF_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CCR2" sbml_species="CCR2_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CCL2" sbml_species="CCL2_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CXCR3" sbml_species="CXCR3_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CXCL9" sbml_species="CXCL9_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CXCL10" sbml_species="CXCL10_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CCR10" sbml_species="CCR10_Secretion_Rate"></map>
    <map PC_phenotype="ssr_CCL28" sbml_species="CCL28_Secretion_Rate"></map>
    <map PC_phenotype="ssr_Ecadherin" sbml_species="Ecadherin_Secretion_Rate"></map>
    <map PC_phenotype="ctr_0_0" sbml_species="Transition_Rate"></map>
```

Figure 4.7: Intracellular part of XML file.

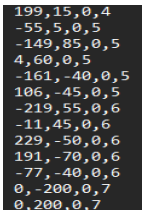


Figure 4.8: Coordinates of some cells in CSV file.

Custom.cpp file is the main C++ file that control the intracellular model for Libroadrunner. In includes create_cell_types, setup_microenvironment, setup_tissue, update_intracellular and finally a coloring function and also a create_cell_circle_positions function. These are functions declared in custom.cpp file but, it also uses functions and classes are declared in other PhysiCell C++ files.

In default PhysiCell intracellular models, we can not make cell placements with CSV file. Hence, I added a function to custom.cpp file to make it able to get cell coordinates. Developers of the tool used create_cell_circle_positions and some other processes in setup_tissue function to make tissue placement. But, I needed to add each cell by using CSV file to be able to eliminate cell placement process that in the setup_tissue function and add load_cells_csv_intracellular function to make cell placement by using CSV file. In custom.cpp file, we need to add each substrate to relevant fields to make them to be processed by PhysiCell. Fig. 4.9 shows one of the fields that substrates names placed in. There are lots of this kind of fields that we need to add substrate names and all of them have different writing schematic and different purposes.

I also improved the coloring function in custom.cpp file because default one could not deal with more than one cell coloring process and also coloring functions in other sample models gave random colors to each cell. Index based coloring process was made to give unique color to each cell by using RGB color codes. With the help of this new function we are able to make models that look similar to real tissues.

```
180         int my_type = (int) data[3];
181         Cell_Definition* pCD = find_cell_definition( my_type );
182         if( pCD != NULL )
183         {
184             std::cout << "Creating " << pCD->name << " (type=" << pCD->type << ") at "
185             << position << std::endl;
186             Cell* pCell = create_cell( *pCD );
187             pCell->assign_position( position );
188
189             // do intracellular stuff - this will vary depending on cell type:
190             int i_Oxy_i = pCell->custom_data.find_variable_index( "intra_oxy" );
191             int i_Glu_i = pCell->custom_data.find_variable_index( "intra_glu" );
192             int i_Lac_i = pCell->custom_data.find_variable_index( "intra_lac" );
193             int i_PD1_i = pCell->custom_data.find_variable_index( "intra_PD1" );
194             int i_PDL1_i = pCell->custom_data.find_variable_index( "intra_PDL1" );
195             int i_HIF1_i = pCell->custom_data.find_variable_index( "intra_HIF1" );
196             int i_TGF_i = pCell->custom_data.find_variable_index( "intra_TGF" );
197             int i_IFN_i = pCell->custom_data.find_variable_index( "intra_IFN" );
198             int i_Per_i = pCell->custom_data.find_variable_index( "intra_per" );
199             int i_Gra_i = pCell->custom_data.find_variable_index( "intra_gra" );
200             int i_IL_i = pCell->custom_data.find_variable_index( "intra_IL" );
201             int i_CXCR4_i = pCell->custom_data.find_variable_index( "intra_CXCR4" );
202             int i_CXCL12_i = pCell->custom_data.find_variable_index( "intra_CXCL12" );
203             int i_EGF_i = pCell->custom_data.find_variable_index( "intra_EGF" );
204             int i_EGFR_i = pCell->custom_data.find_variable_index( "intra_EGFR" );
205             int i_CSF1_i = pCell->custom_data.find_variable_index( "intra_CSF1" );
206             int i_CSF1R_i = pCell->custom_data.find_variable_index( "intra_CSF1R" );
207             int i_CXCL16_i = pCell->custom_data.find_variable_index( "intra_CXCL16" );
208             int i_CXCR6_i = pCell->custom_data.find_variable_index( "intra_CXCR6" );
209             int i_GF_i = pCell->custom_data.find_variable_index( "intra_GF" );
210             int i_CCR2_i = pCell->custom_data.find_variable_index( "intra_CCR2" );
211             int i_CCL2_i = pCell->custom_data.find_variable_index( "intra_CCL2" );
212             int i_CXCR3_i = pCell->custom_data.find_variable_index( "intra_CXCR3" );
213             int i_CXCL9_i = pCell->custom_data.find_variable_index( "intra_CXCL9" );
214             int i_CXCL10_i = pCell->custom_data.find_variable_index( "intra_CXCL10" );
215             int i_CCR10_i = pCell->custom_data.find_variable_index( "intra_CCR10" );
216             int i_CCL28_i = pCell->custom_data.find_variable_index( "intra_CCL28" );
217             int i_Ecadherin_i = pCell->custom_data.find_variable_index( "intra_Ecadherin" );
218             int energy_vi = pCell->custom_data.find_variable_index( "intra_energy" );
219
220             pCell->custom_data[i_Oxy_i] = parameters.doubles("initial_internal_oxygen");
221             pCell->custom_data[i_Glu_i] = parameters.doubles("initial_internal_glucose");
222             pCell->custom_data[i_PD1_i] = parameters.doubles("initial_internal_PD1");
223             pCell->custom_data[i_Lac_i] = parameters.doubles("initial_internal_lactate");
224             pCell->custom_data[i_PDL1_i] = parameters.doubles("initial_internal_PDL1");
225             pCell->custom_data[i_HIF1_i] = parameters.doubles("initial_internal_HIF1");
226             pCell->custom_data[i_TGF_i] = parameters.doubles("initial_internal_TGF");
227             pCell->custom_data[i_IFN_i] = parameters.doubles("initial_internal_IFN");
228             pCell->custom_data[i_Per_i] = parameters.doubles("initial_internal_perforin");
229             pCell->custom_data[i_Gra_i] = parameters.doubles("initial_internal_granzymeB");
```

Figure 4.9: Substrate names in custom.cpp file.

As mentioned before, intracellular LibroadRunner model was used in this thesis. In models with intracellular features, users need to generate an SBML file for each cell type. One can use SBML files that are generated as a genome scale metabolic network or they can make simpler SBML files manually. SBML files of genome scale metabolic network include lower and upper boundary values of reaction fluxes rather than kinetic expressions or cellular events and they can only be used in FBA. Hence, in this thesis, SBML files manually crafted for each cell that wanted to add. In my model, there are 8 different types of cells in the TME and therefore 8 different SBML files were made to represent them and their mechanics. There are different programs and platforms to make SBML files and also, one can make SBML files with using Python and R programming languages. But, COPASI (application for analysis and simulation of biochemical dynamics) was used to make these files [Hoops et al, 2006].

4.3 Making SBML Files by Using COPASI

At first, we need to add ourselves and our mail to COPASI by using tools-preferences tabs. Then, in model tab we need to press “add me” button to add our informations to SBML files. Without this step, when we try to export SBML files, some warning and error messages can arise. As we know, inside and outside of cells, we have some compartments. Extracellular, intracellular and mitochondrial compartments are some examples of them. In our model we only used intracellular compartment inside SBML files. If we did not make other compartments, every species and others are going to be in intracellular compartment. As we saw in the Fig. 4.7, XML file includes token parts that are responsible for the communication between SBML and XML files. In SBML files, we need to write each species name same way we wrote in these token parts. These are case sensitive parts. We add subtrates names in species part of SBML files. We need to represent each substrate in SBML files to ensure proper communications between SBML and XML files. Species can be declared as taking part in reactions, having a fixed value or value set by an assigned equation. In fixed type, we can give it a value and this value can not be changed by simulations. In reactions option, we can give it a value like in fixed option, but this value can be changed by reactions that we declare in reactions part of SBML. We also have assignment option. In this option, we can write an equation based on concentrations of substrates. In PhysiCell, if cell uptake substrates other than glucose and oxygen from outside of the cell by using uptake processes, we noticed that these substrates cannot be used in reactions and events parts in SBML files due to an unknown technical problem in the tool. Hence, if we have these kinds of substrates, now we only use assignment option to represent tissue environment. As we can see in Fig. 4.10, 59 species are listed on the left side of the figure. In the right side of the figure, we can see cancer cell’s migration speed set as an assignment type with an equation. In this equation, substrates that are produced by other cells in the TME such as macrophages, fibroblasts, etc. were used. There are both reaction and assignment types in SBML files that used in these models. Combination of these was used to represent designed tissues and processes. As can be seen in Fig. 4.10, we can set complex equations that includes substrates from different cells to make some connections between the TME. By this way, cells in the TME can contribute to

phenotypic behaviour of cancer cells in a sophisticated way. We have enough mechanistic knowledge to set these equations to represent biology more accurately. But, PhysiCell is still in development phase, hence, it limits our ability to design more complex tissue models.

Currently, assignment equations for migration speed, apoptosis rate and substrates are declared in each cell type's SBML files. Uptake rates in Fig. 4.10 have different production profiles in different cells. For example, macrophages produce CSF1R and secrete it to the TME, cancer cells take these CSF1R from the TME by species uptake rate to use it in its own processes. In some SBML files, there are both uptake and secretion rates. As mentioned before, PhysiCell is in development phase and production or uptake processes of some substrates can make simulation problems. For example, GranzymeB in this SBML file had this kind of problem. By making both uptake and secretion rate, also setting secretion rate parameter of GranzymeB to 0 solved this issue.

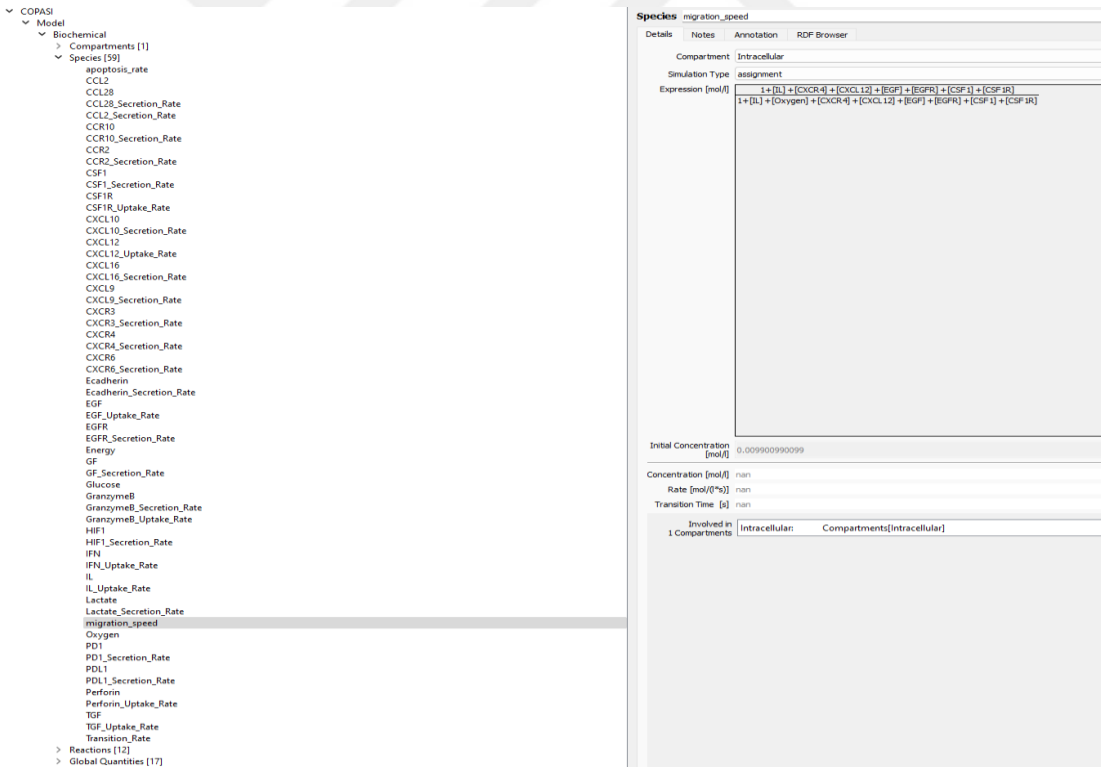


Figure 4.10: Species part of SBML file of cancer cell in COPASI.

Fig. 4.11 shows reactions in the cancer SBML file. It includes reactions of some of substrates that are in the TME. We have to give separate names to each

reaction and these reaction names can not be overwriten in other places in the SBML files. In reaction tab, I wrote different reaction formula to each substrate that need to be produced by cancer cells. We use “+” sign to add different substrates, “*” sign to multiply substrate concentrations and “->” sign to make irreversible reactions. In these reactions, we need k values to represent the reaction rate constants in the formulas. If we do not assign k values for reactions in global quantities, reactions use default k value which is 0.1. As we can see, glucose was used to produce other substrates. This is not an ideal way to represent protein production but, in PhysiCell, we have some structural problems. Producing substrates from another substrates that taken from other cells or from environment is problematic. Only glucose can be used to make substrates by reactions currently. Also, glucose and oxygen are fed into the environment by default by PhysiCell. PhysiCell use Dirichlet Boundary structure to give oxygen and glucose into simulation media from boundries of the simulation spaces.

#	Name	Reaction	Rate Law	Flux [mol/s]	Noise Expression
1	Aerobic	Glucose + 6 * Oxygen -> 76 * Energy	Mass action (irreversible)	nan	
2	Energy_Usage	Energy ->	Mass action (irreversible)	nan	
3	Anaerobic	Glucose -> Lactate + 4 * Energy	Mass action (irreversible)	nan	
4	PDL1prod	Glucose -> PDL1	Mass action (irreversible)	nan	
5	HIF1prod	Glucose -> HIF1	Mass action (irreversible)	nan	
6	CXCR4prod	Glucose -> CXCR4	Mass action (irreversible)	nan	
7	EGFRprod	Glucose -> 10 * EGFR	Mass action (irreversible)	nan	
8	CSF1prod	Glucose -> 10 * CSF1	Mass action (irreversible)	nan	
9	CXCL16prod	Glucose -> CXCL16	Mass action (irreversible)	nan	
10	CCL2prod	Glucose -> 10 * CCL2	Mass action (irreversible)	nan	
11	CCL28prod	Glucose -> CCL28	Mass action (irreversible)	nan	
12	Ecadprod	Glucose -> Ecadherin	Mass action (irreversible)	nan	
	New Reaction				

Figure 4.11: Reactions part of the cancer SBML file.

In Fig. 4.12, global quantities can be seen. k values and some threshold values are declared in this part. As mentioned before, k values are used in reactions as rate constants. Threshold values are used in events part of the SMBL. If concentration level of a certain substrate reaches this threshold, we can make simulation to change some of its properties, such as secretion rates of some substrates. Parameters in

global quantities can be changed in the Events section. For example, if HIF levels in this SBML reach beyond HIF_thresh value, some k values are going to be changed from 0 to chosen positive values to start producing substrates via reactions.

#	Name	Type	Unit	Initial Value [Unit]	Transient Value [Unit]	Rate [Unit/s]	Initial Expression [Unit]	Expression [Unit] or [Unit/s]	Noise Expression
1	k_aer	fixed	?	0.01	nan	0			
2	energy_prolif_thresh	fixed	?	400	nan	0			
3	k_usage	fixed	?	0.0023	nan	0			
4	k_anaer	fixed	?	0	nan	0			
5	k_PDL1	fixed	?	0	nan	0			
6	k_HIF1	fixed	?	0	nan	0			
7	k_EGFR	fixed	?	0	nan	0			
8	k_CSF1	fixed	?	0	nan	0			
9	k_CXCR4	fixed	?	0	nan	0			
10	k_CXCL16	fixed	?	0	nan	0			
11	k_CCL2	fixed	?	0	nan	0			
12	k_CCL28	fixed	?	0	nan	0			
13	oxygen_HIF	fixed	?	0.5	nan	0			
14	HIF_thresh	fixed	?	0.3	nan	0			
15	HIF_die_thresh	fixed	?	0.1	nan	0			
16	Oxygen_move_thresh	fixed	?	0.35	nan	0			
17	k_Ecad	fixed	?	0	nan	0			
	New Quantity	fixed		0					

Figure 4.12: Global quantities part of cancer SBML file.

10 events were declared in cancer SBML file. Fig. 4.13 shows names of these events. Aer_stop is used to stop aerobic metabolism if oxygen levels are below threshold value. HIF1 production also depends on oxygen levels to be produced. Other substrates can be produced if HIF1 levels reach required levels. In Fig. 4.14, CCL2_prod event is shown. We have trigger expression part on top of the figure. If this HIF1 depended trigger expression satisfied, it is going to change k_CCL2 value from 0 to 0.1 and CCL2_Secretion_Rate from 0 to 1.

With these control mechanisms, we can try to shape the TME. PhysiCell will process these events, reactions and assignments parts and make the calculations on concentration levels and cell placement parameters to demonstrate dynamic tissue environment. In this model, I tried to make TME model but, one can make models for other diseases by following some steps that mentioned in materials and methods chapter. In intracellular models, COPASI can be helpful. But there are some problems in using COPASI to make SBML files for PhysiCell. If a user wants to make SBML files for first time, making SBML files with using COPASI will not

make problem. But, editing already existing SBML files can introduce really frustrating and difficult to detect bugs and prevent simulation to run. ID conflict is a main problem if we want to edit SBML files using COPASI. It could give different ID name to parameter if we made a change on it. This will cause simulation errors, because, PhysiCell processes these ID names to match them to the tokens inside the XML file. These ID names should be same with names that are used in XML file. To prevent this kind of conflicts, I suggest using code editors like Notepad++ and Visual Studio to open these already existing SBML files and make changes on them manually. However, making new SBML files by using these code editors is not recommended.

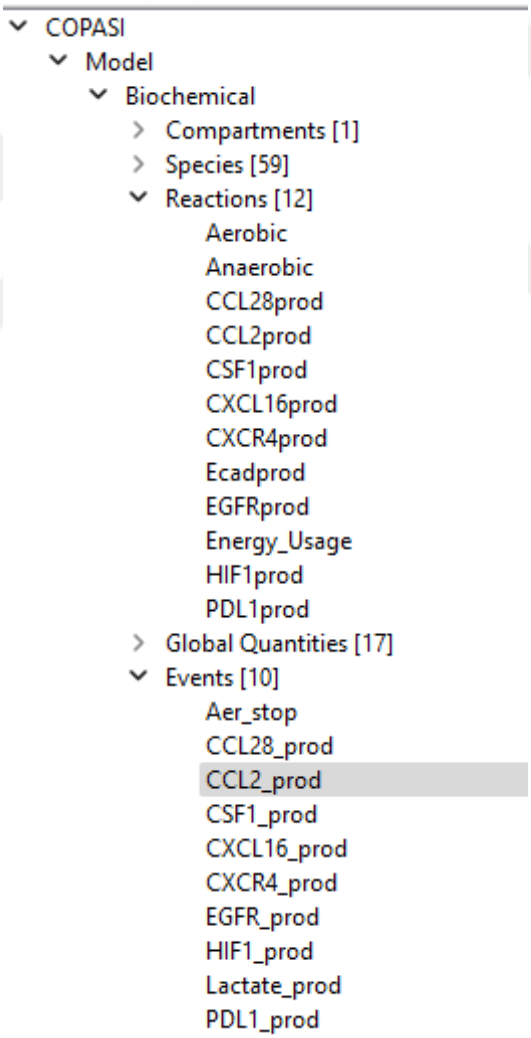


Figure 4.13: Events part in cancer SBML file.

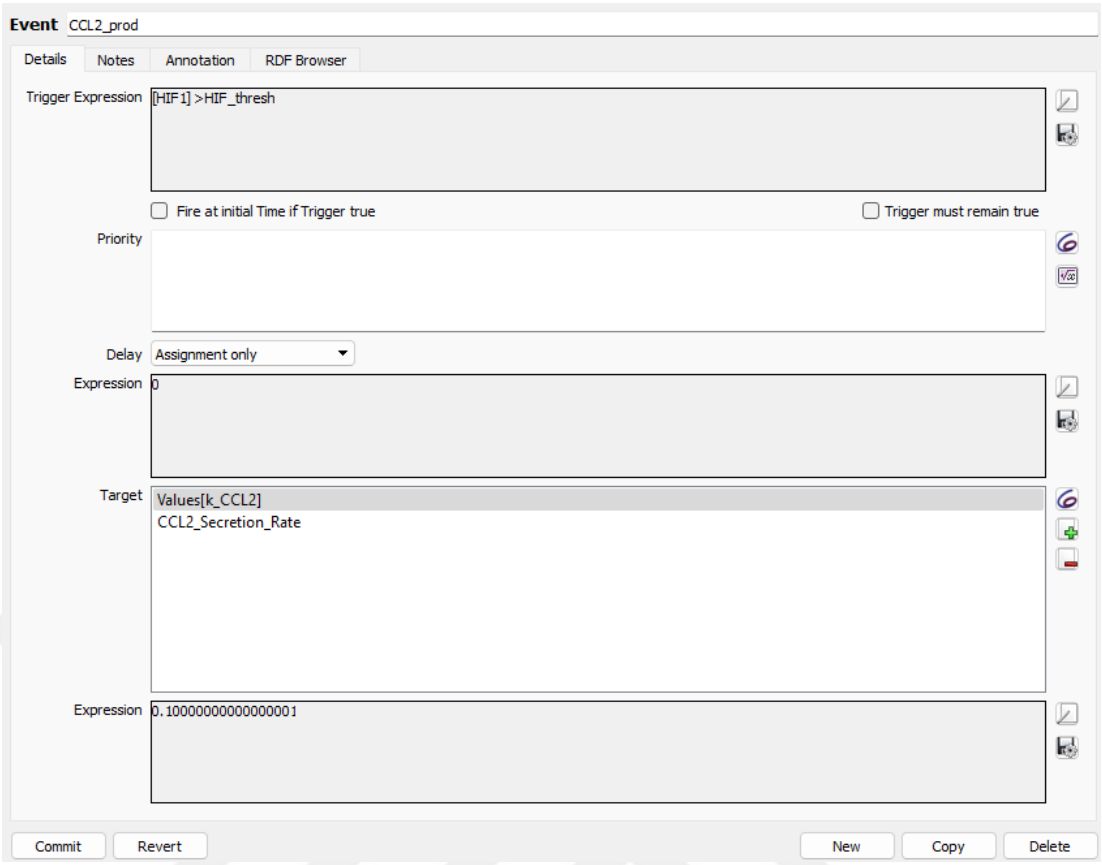


Figure 4.14: CCL2_prod event in cancer SBML file.

To conclude, PhysiCell is still in development phase and inevitably it has many bugs and problems. I am working on source C++ codes to solve some of the problems I encountered. In a future projects, some methods that I used in this thesis can be improved to more reliable and meaningful versions.

5. RESULTS

In scope of this thesis study, TME, healthy lung, healthy liver, and lung with cancer models were made. Properties of TME model were already described in Materials and Methods section. Other models have limited properties and they do not include complex SBML structures. For example, there are 28 different substrates in the TME model but, there are only 3 different substrates in other models. Other models are made to represent real tissue structures that are taken from microscopic images.

5.1 Healthy Lung Tissue Model

In Fig. 5.1, a microscope image of healthy lung tissue is seen. This figure contains one bronchiole and several alveolar structures that surround it. This figure was used as a template to develop a model that visually looks similar to healthy lung tissue. Fig. 5.2 contains some results that are produced by simulation of this model. 4 panels represent different time intervals, 1 hour, 3 days, 6 days and 10 days of simulation time are presented. Results were produced in 3.5 hour of real simulation runtime (Intel Xeon W-2245 processor, 64 Gb DDR4 RAM, 1 TB SSD, Nvidia Quadro RTX 4000 graphics card), there are 826 cells in this model. I tried to represent a bronchiole and an alveolar structure to test making models look similar to real 2D tissue can be done or not. Epithelial cells, muscle cells and laminar structures are represented in this model. Cells take oxygen and glucose from microenvironment to produce energy. Apoptosis rates are calculated by using produced energy from cells as threshold constants. To prevent accumulation of energy, each cell has energy usage reaction that continually releases energy. Muscle cells and laminars do not have motility and proliferation abilities to maintain tissue structure in this model. Keeping this tissue in intact structure is important. We also have healthy tissues in TME structure. To represent these tissues, we need know how to make tissues that do not change their structure, homeostasis status, immobility and other behaviors unless there are factors that affect these properties. Only epithelial cells have a small proliferation and apoptosis rate. Fig. 5.3 shows oxygen concentrations in different time intervals. As we can see, oxygen levels change with time. It means that, model

can take oxygen from microenvironment and use it in its own reactions. Oxygen levels are low in parts that are dense in cells. This kind of oxygen depletion is not normal in healthy tissues, but I wanted to test and show how oxygen or substrate usage can be represented with PhysiCell simulations.

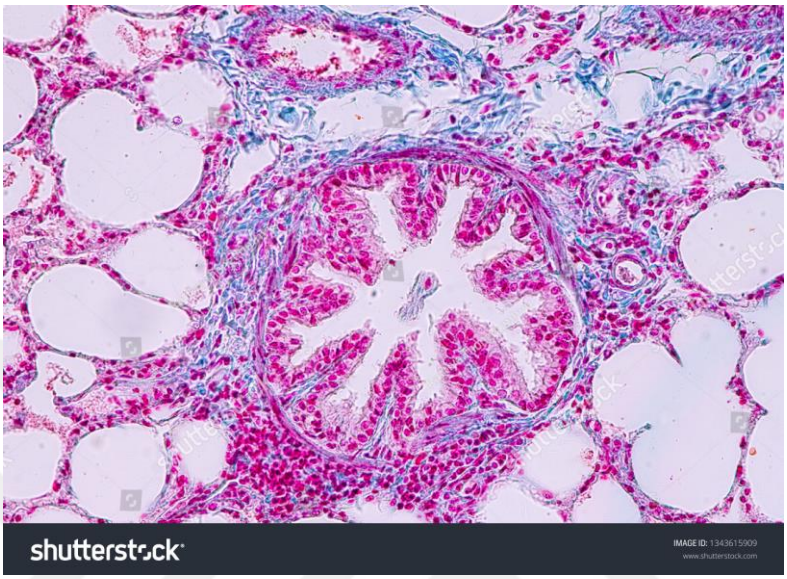


Figure 5.1: Lung tissue image under microscope.

Figure 5.2: Lung tissue model that has bronchiole and alveolar.

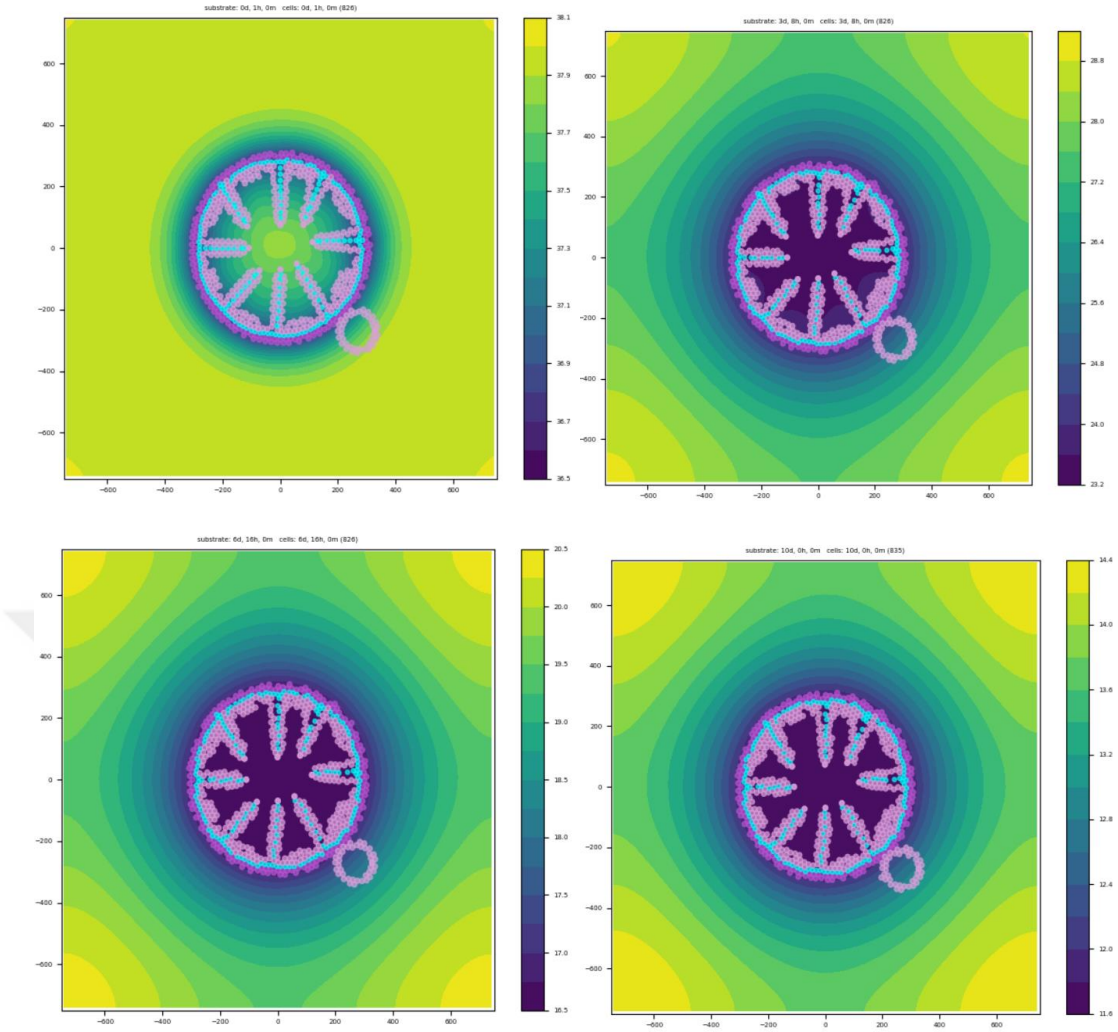


Figure 5.3: Lung tissue model. Change in oxygen concentration over time.

5.2 Healthy Liver Tissue Model

There is another healthy tissue model where, healthy liver tissue model was demonstrated. Fig. 5.4 shows microscopic image of an animal liver tissue. In this figure, there are some lobules that contain central veins in the middle of them and epithelial cells that surround. Each of these lobules are outlined by fibrous connective tissue. These connective tissues separate lobules from each other. From this template image, model that we can see in Fig. 5.5 was produced. Two lobules were added with epithelial cells and connective tissue in this model. One big cell was put to represent central vein in the middle, which provides oxygen to the tissue by diffusion. In the XML file, there are options for cells to make them big or small by changing volume parameters. By using this feature, I made the central vein structure bigger than other cells to represent a cross-section of the vein. Motility, proliferation

and apoptosis rates of all these cells were set to 0 to keep their structural integrity. This is important because I want to disrupt this integrity after introducing cancer cell. Hence, healthy tissue should not break its integrity if there are no other forces in microenvironment. In Fig. 5.6, we can see oxygen levels in different time intervals. We can easily see oxygen levels were differentiated.

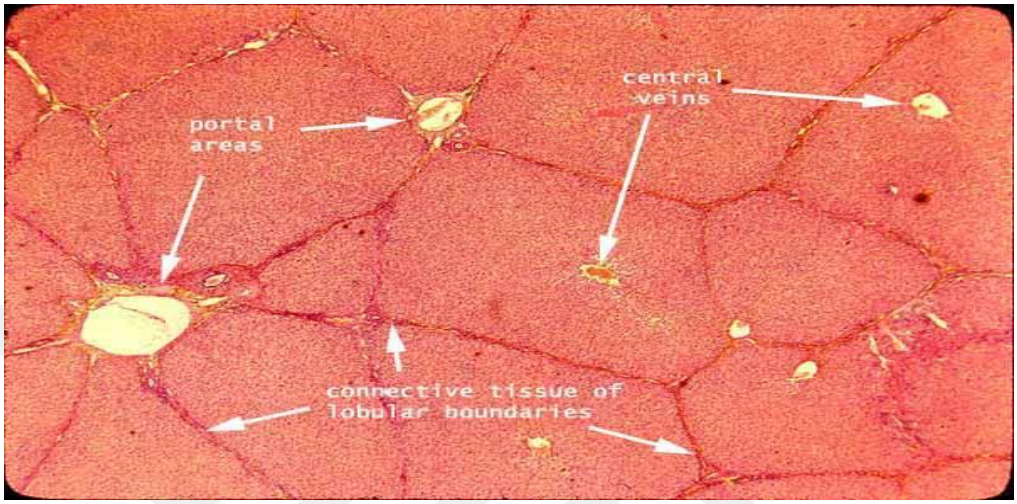


Figure 5.4: Liver tissue image under microscope.

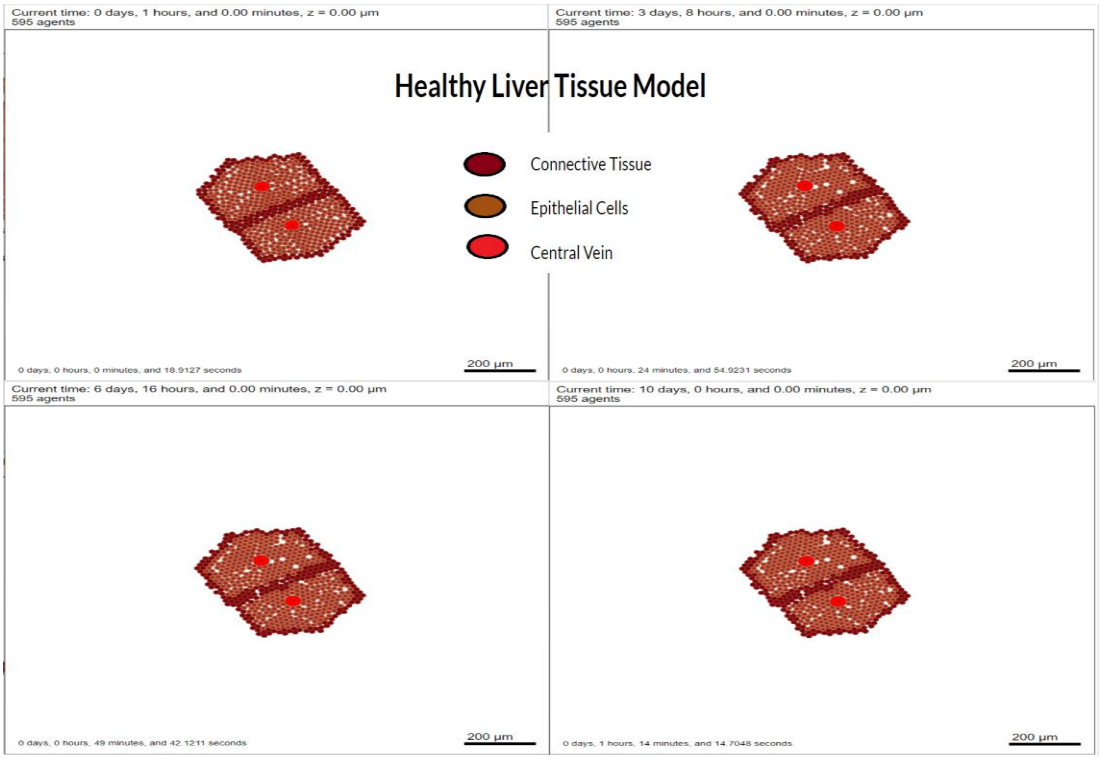


Figure 5.5: Liver tissue model in PhysiCell.

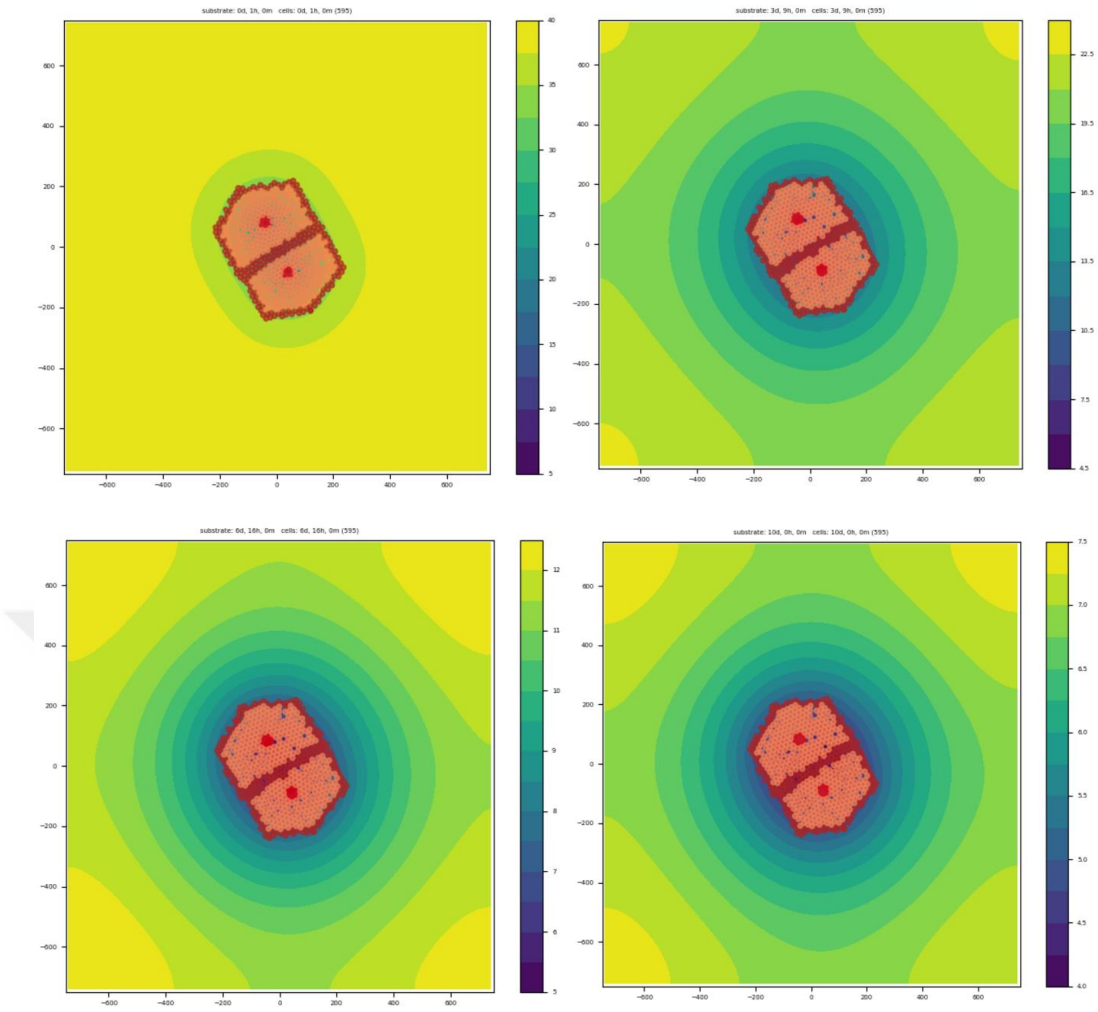


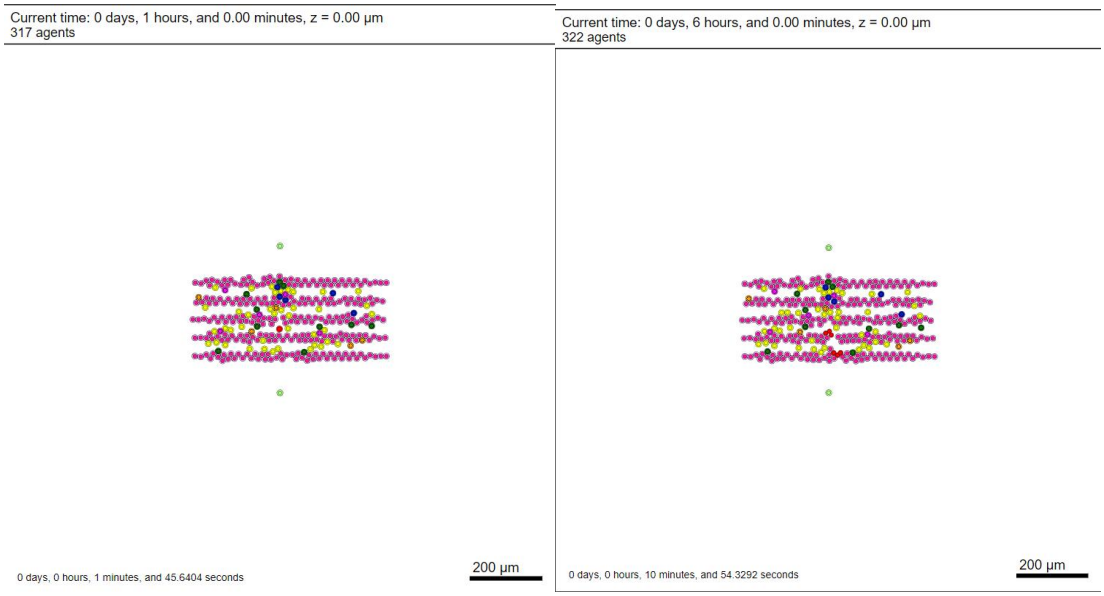
Figure 5.6: Liver tissue model. Oxygen concentration differentiation over time.

5.3 Complex TME Model

The main model developed in this thesis study is the tumor microenvironment model, representative parts of input files of the main model were presented in the Materials and Methods section. TME model is a relatively complex model, and it includes complex interactions between the cells. Products of cells in the TME can change properties of cancer cell such as ability to move, migration speed, transition rate, apoptosis and production of some other products.

Fig. 5.7 shows results of 4 different time intervals. As it can be seen in this figure, the TME model includes high number of epithelial cells. Every epithelial cell layer are positioned in parallel to other epithelial layers. There are macrophages, T-cytotoxic cells, T-regulatory cells, natural killer cells, fibroblasts and mesenchymal stem cells in this TME. This model has only one cancer cell in the middle of the

TME at the beginning. This cancer cell has proliferation, migration and apoptosis properties and these properties depend on signals or products that are produced by other microenvironmental cells. Cancer cells increase their numbers over time, and they have ability to move. Also, these cancer cells have ability to die after taking signals that trigger apoptosis. All these abilities are important when we deal with TME models because all these abilities depend on signals that are produced by other cells in the TME. As we can see from this figure, cancer cells can divide, move and die. It means that, I made a basic TME structure that has capabilities to represent tumor formation, immune system cells-cancer cell signaling and metastasis. In this model, cancer cells stop their oxygen dependent metabolism when oxygen levels are too low and shift their metabolism to glycolysis after producing HIF1. In this state, they seek oxygen to make chemotaxis. Cancer cells in this model migrate to the nearest place that has high oxygen concentration. Oxygen chemotaxis is an important step of metastasis. To be able to represent oxygen chemotaxis in this model, making metastasis models are now more realistic than before. Also, when we look at this figure deeply, we can see movement of other cells. Other cells in the TME have motility abilities too. They have their own chemotaxis profiles but, their chemotaxis targets were produced by other cells and this situation limits their motility distance because they are close to these chemotaxis targets.



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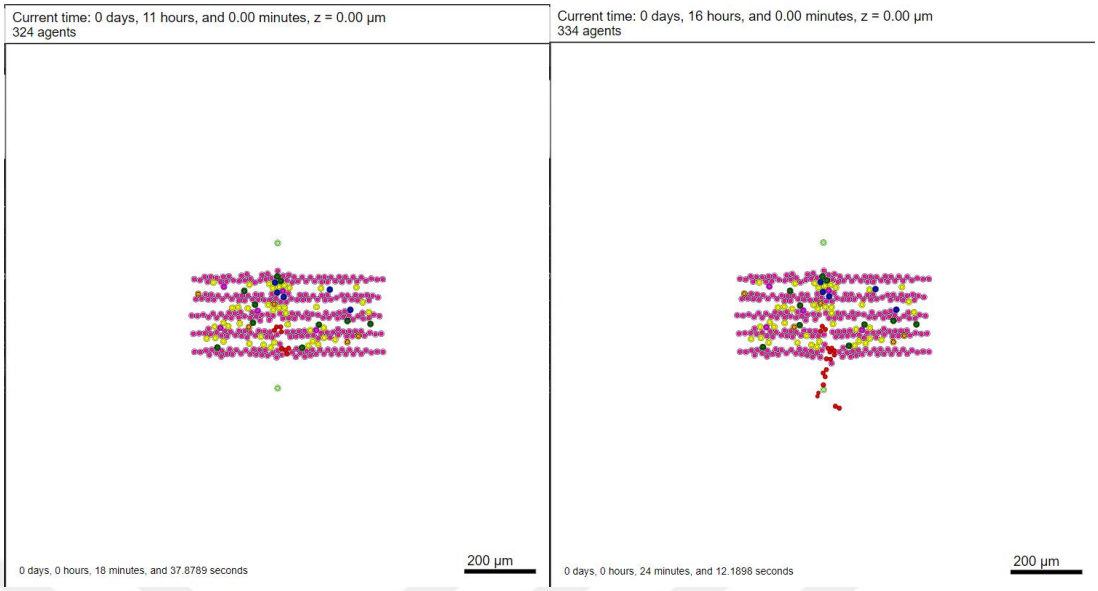


Figure 5.7: TME model results in different time intervals.

Fig. 5.8 shows HIF1 concentration levels in different time intervals. All cells can produce HIF1 if oxygen levels are smaller than the threshold. Hence, cells in the core of tissue were able to produce HIF1, while cells that are at the perimeters of the tissue did not produce HIF1. This demonstrated dynamic structure of this model. All production abilities depend on levels of other molecules in the TME. We can clearly see HIF1 production follows moving cancer cells. This shows us that, movement is not an obstacle for production. I also defined a decay rate to HIF1 in XML file. Because of this decay rate, HIF1 production rate reaches an equilibrium state that maintains oxygen concentration at 4.2 mmHg value in dense areas. HIF1 has a critical trigger role in this model. Every cancer related product and phenotypic behaviour is based on availability of HIF1 in the microenvironment. It triggers and controls glycolytic switch, apoptosis (both initiation and termination processes), stops production of immune system related molecules and cells, cancer cell motility and chemotaxis, chemotaxis of other cells, production of tumor-related molecules, differentiation of macrophages and fibroblasts (by changing production rates of some molecules at different HIF1 concentrations) and proliferation rate of cancer cells.

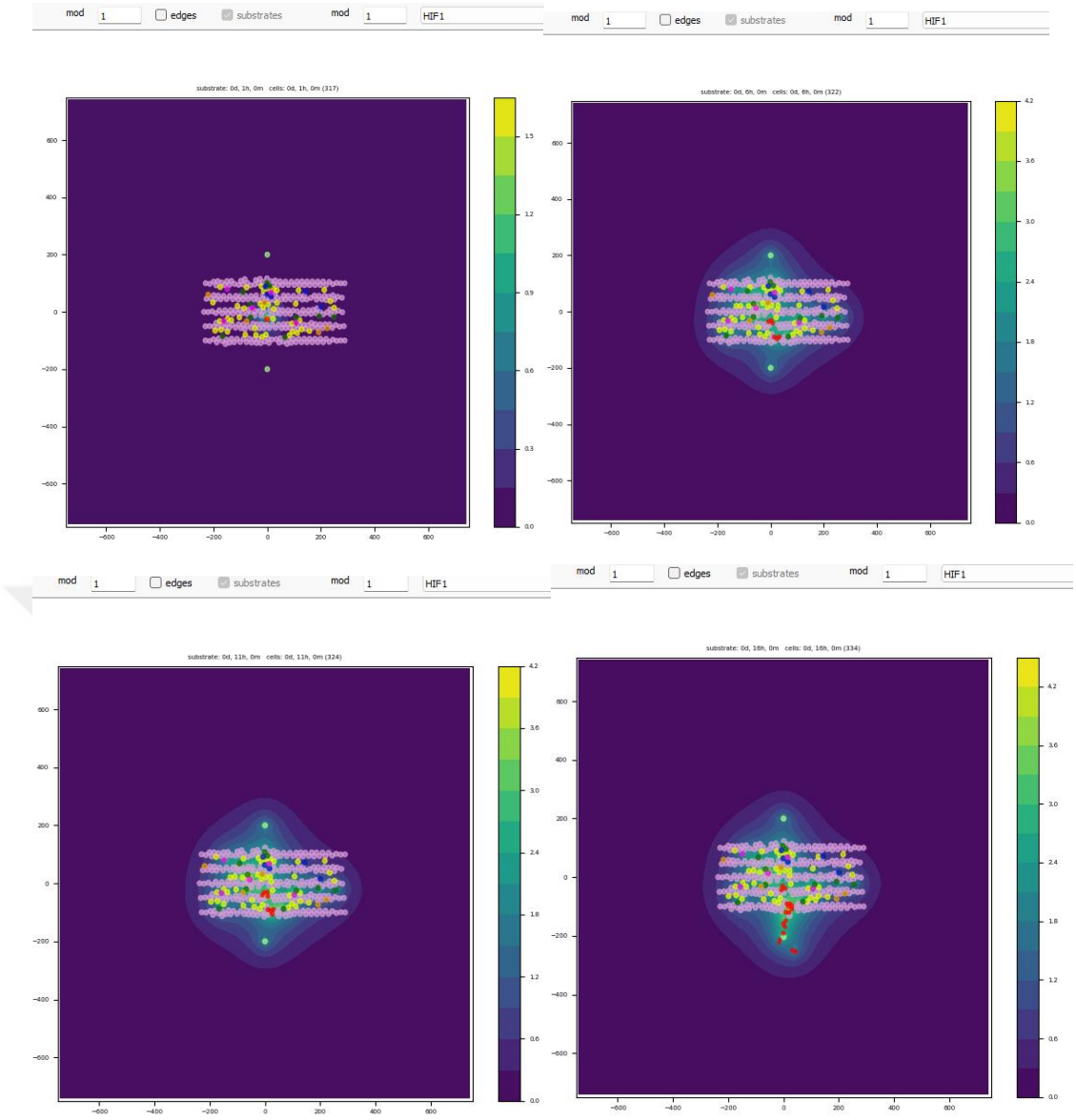


Figure 5.8: HIF1 concentration differentiation.

I made epithelial cells to produce E-cadherin molecules. With the help of these molecules, movement of epithelial cells stopped when E-cadherin concentration levels are higher than the threshold that was set. Also, I prevent their proliferation abilities by using same threshold. Epithelial cells will sense the E-cadherin levels to proceed to proliferation. I aimed to make epithelial cells to proliferate if there are not any epithelial cell around them by using E-cadherin concentration levels.

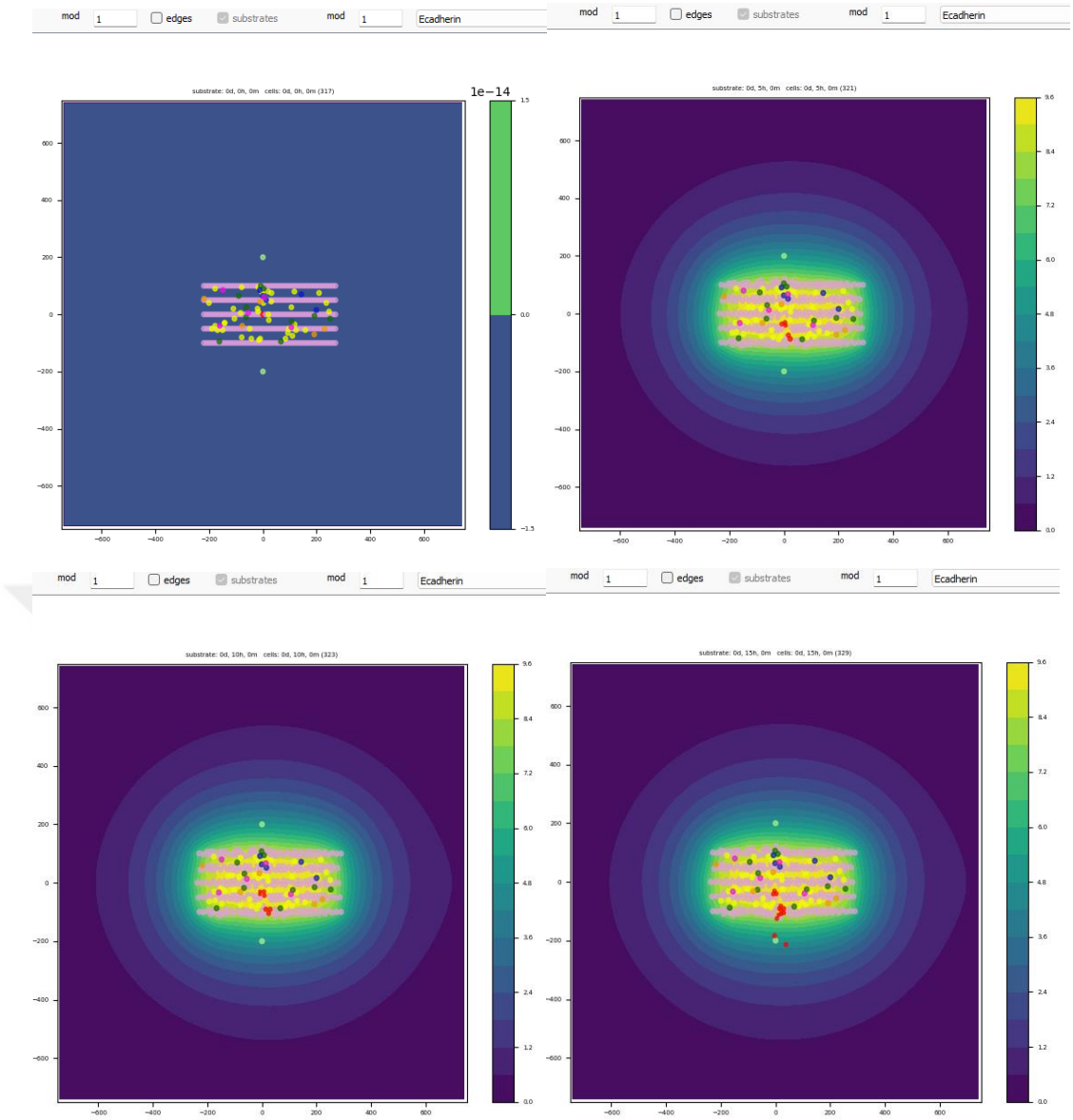


Figure 5.9: E-cadherin production from epithelial cells.

As we know, PD1-PDL1 binding has a significant effect in immune suppression. There are lots of approved drugs that target this binding and they are called as immune checkpoint inhibitors (ICI). I wanted to add this feature in my TME model to represent communication between cancer cells and cytotoxic T cells via production of perforin and granzyme B. HIF1 concentration impacts PDL1 production in cancer cells. PDL1 molecules induce PD1 production in T-cytotoxic cells. PD1 and PDL1 concentration levels decrease or increase to regulate the perforin and granzyme B production status in T-cytotoxic cells. This binding only impacts T-cytotoxic cells and does not affect NK cells. In Fig. 5.10, it shows that PD1 concentrations only appear around T-cytotoxic cells.

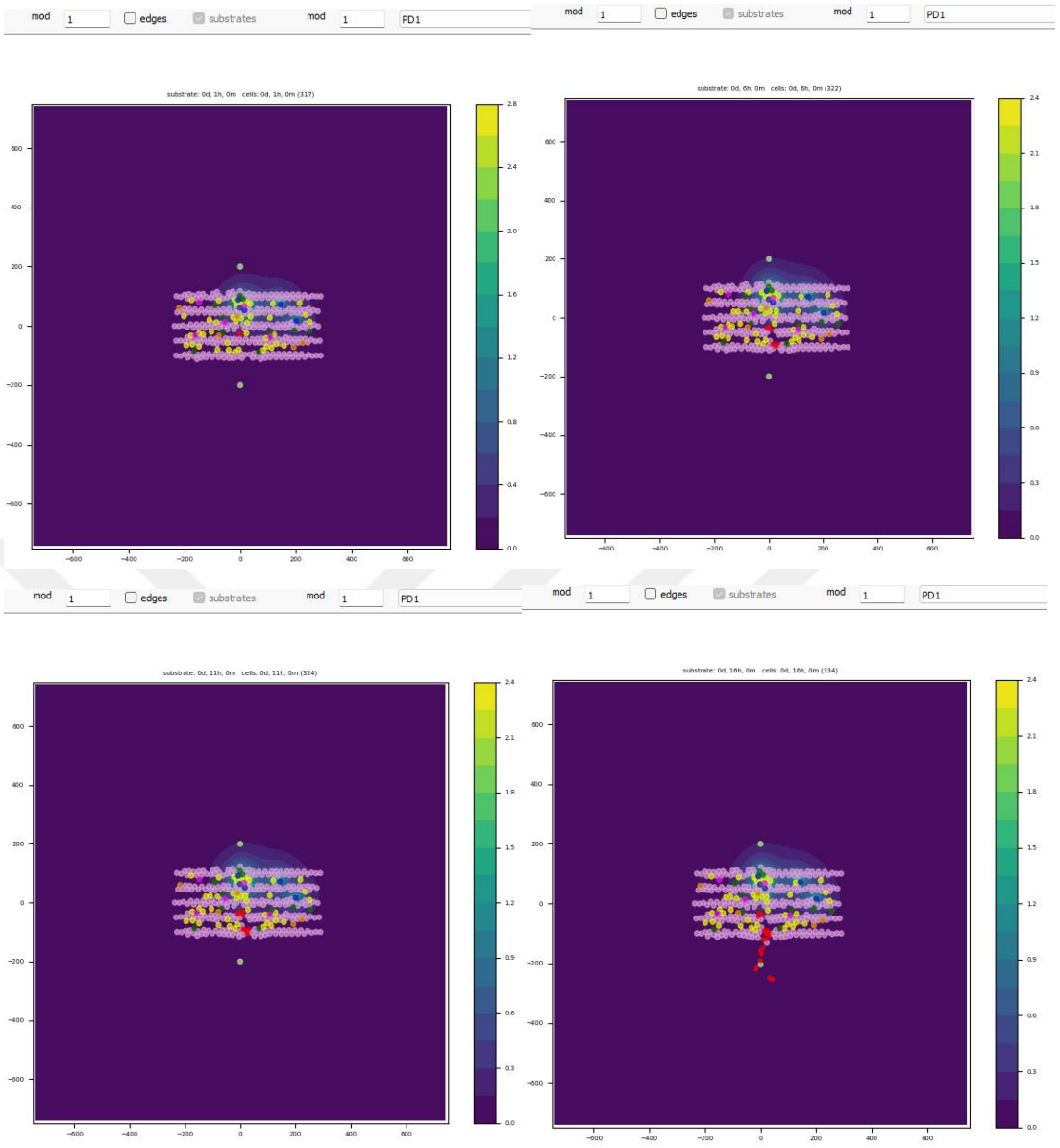


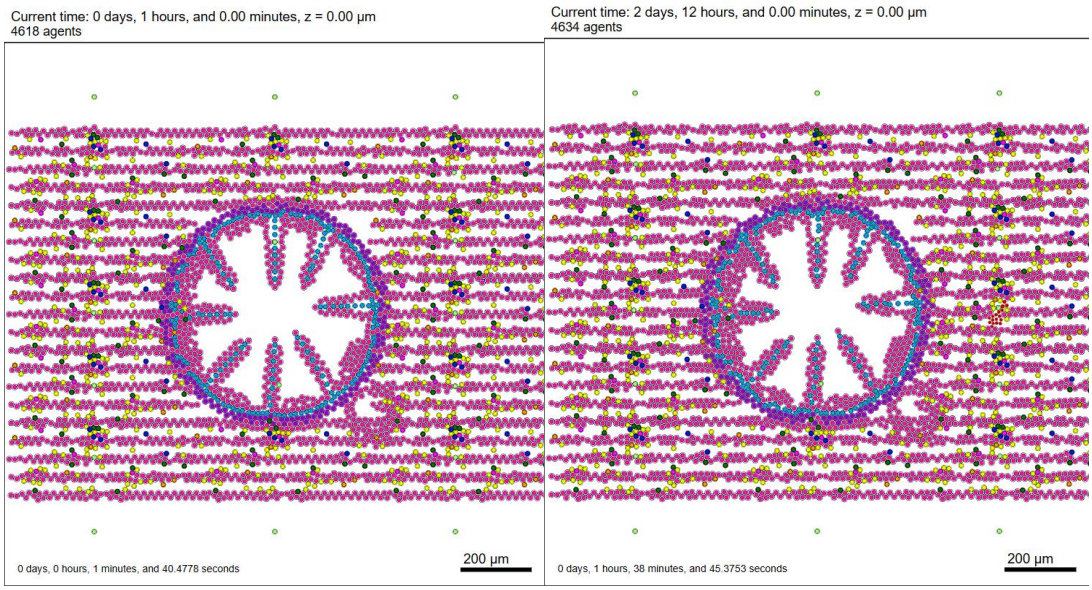
Figure 5.10: PD1 production from T-cytotoxic cells.

5.4 Healthy Lung and TME Combined Model

After constructing and simulating these healthy tissue structures, I wanted to merge TME model with healthy lung tissue model to make lung tissue with cancer. Result of this simulation is in Fig. 5.11. As we can see, there are 4618 cells in this model, but SBML contents of these cells are not complex. All of these cells have only 3 different substrates and reactions are related with these substrates. I encountered problems when I fully integrated the TME model to healthy lung tissue model. It is hard to deal with this many cells when combined with complex SBML

structures for computers and simulation of this model requires long run-times. Fully maintaining and running this model with complex SBML contexts is my future work.

Initially, there is only one cancer cell at the middle-right side of the TME structure (red color). Cancer cell has high proliferation rate but other cells do not have high proliferation rates. Result of this high proliferation rate can be seen at simulation time of 7 days and 12 hours. After some time, we can clearly see one cancer cell led to a tumor structure at the middle-right side of the TME. As it can be seen at this figure, after some time epithelial cells died because of low energy production that was caused by low oxygen and glucose concentrations. There are images of oxygen, glucose and lactate levels that were taken at 20th hour of simulation time in Fig. 5.12. Oxygen and glucose levels are depleted at cell-dense areas, and this shows us that cells can take oxygen and glucose from microenvironment. When we look at lactate levels in this figure, we can see high lactate levels in dense areas. Cancer cells and fibroblasts can switch to glycolysis if oxygen levels are under the threshold value. In glycolysis switch process, these cells stop producing energy via oxygen consumption and start to produce energy and lactate by taking only glucose from the TME. Lactate concentration is zero at the beginning. Hence, lactate levels in Fig. 5.12 are result of this glycolysis switch and it also shows that, I successfully represented this switch in this model.



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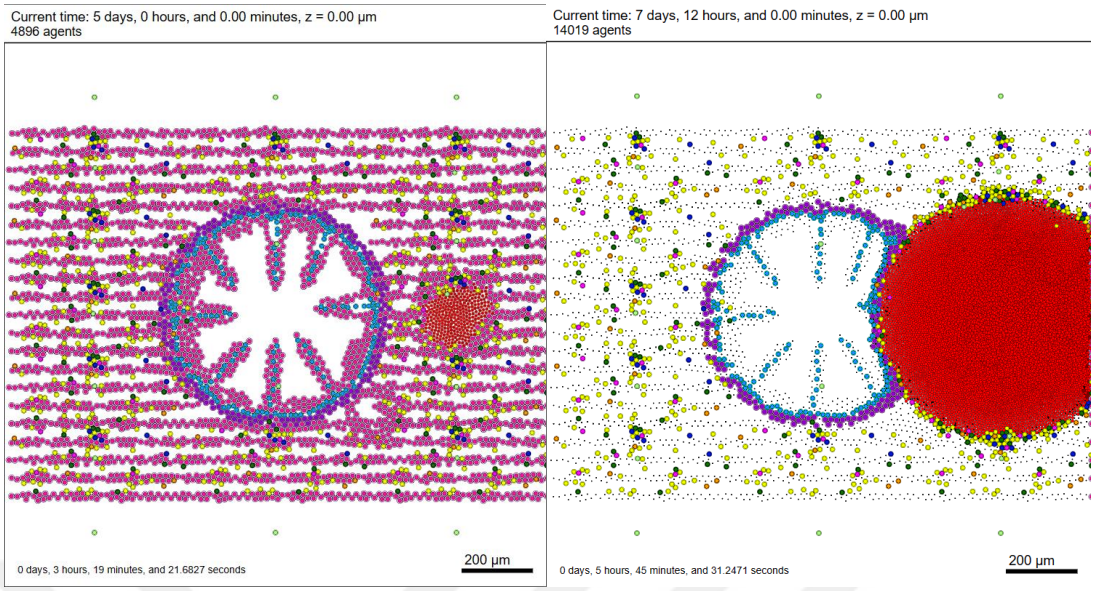


Figure 5.11: Model of lung tissue with cancer.

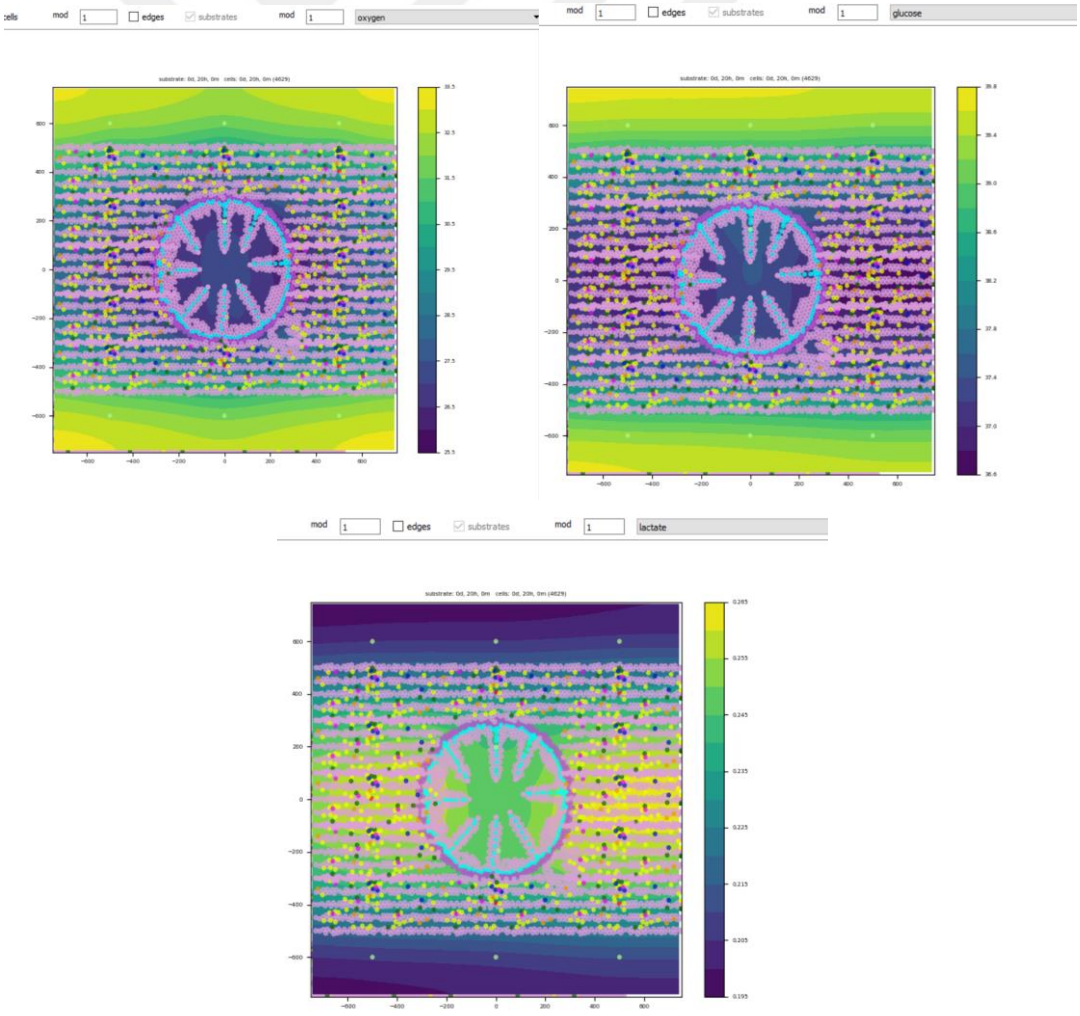
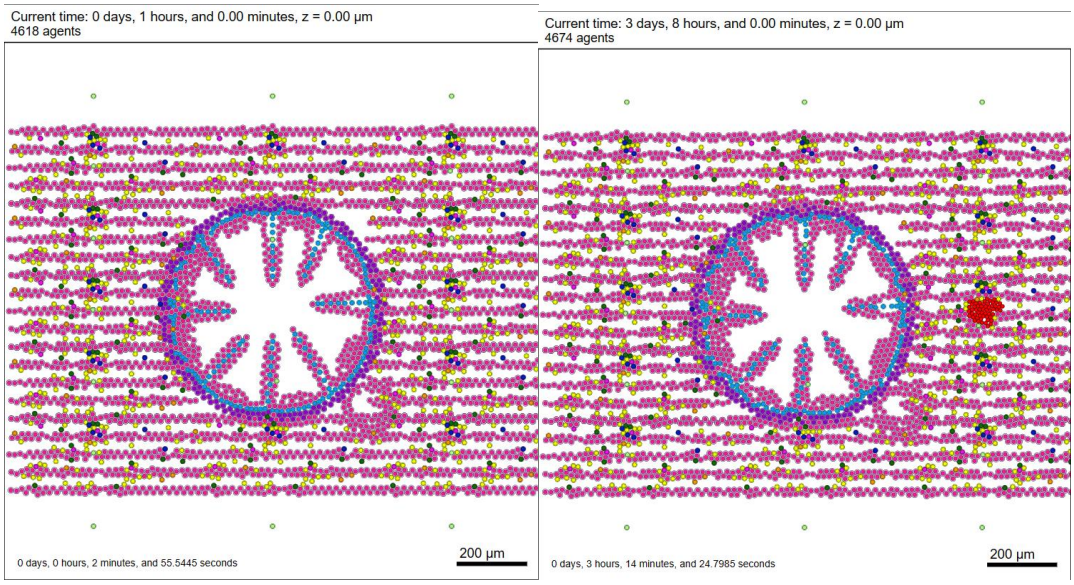


Figure 5.12: Concentration levels of oxygen, glucose and lactate.

High proliferation rates of tumor cells decreased the oxygen concentrations in the TME. Because of this condition, epithelial cells in the TME died after size of tumor structure reached considerable size as shown in Fig. 5.12. Actually, I wanted to prevent apoptosis of epithelial cells and also, I wanted to kill tumor cells that are in the core of tumor structure to represent necrotic tissue that commonly found in all solid tumors. To do that, I manually optimized some parameters related with oxygen uptake, apoptosis rate and some threshold values to keep these processes under control. After the parameter tuning, the results shown Fig. 5.13 were produced. As it can be seen in this figure, epithelial or other stromal cells survived the low oxygen concentration condition at the core of the tumor. Also, tumor cells at the core of the tumor structure died because of low oxygen concentration and they formed necrotic tissue in the core of the tumor. Black colored cells represent dying cells. When they are completely dead, they become invisible and removed from the environment. Growing tumor push other cells from their original places to near tissues. This situation disrupts tissue structure and integrity as we commonly see in tumors of cancer patients. Oxygen concentration levels are showed in Fig. 5.14 where we can see oxygen concentrations are changing across regions of the tissue. Epithelial cells and stromal cells that include different immune system cells have lower oxygen consumption than tumor cells. We have high oxygen concentration at left side of the tissue results, but high oxygen consumption and high proliferation rates of tumor cells decreased oxygen levels to 0 and took oxygen from left side as well.



Continuation of the figure

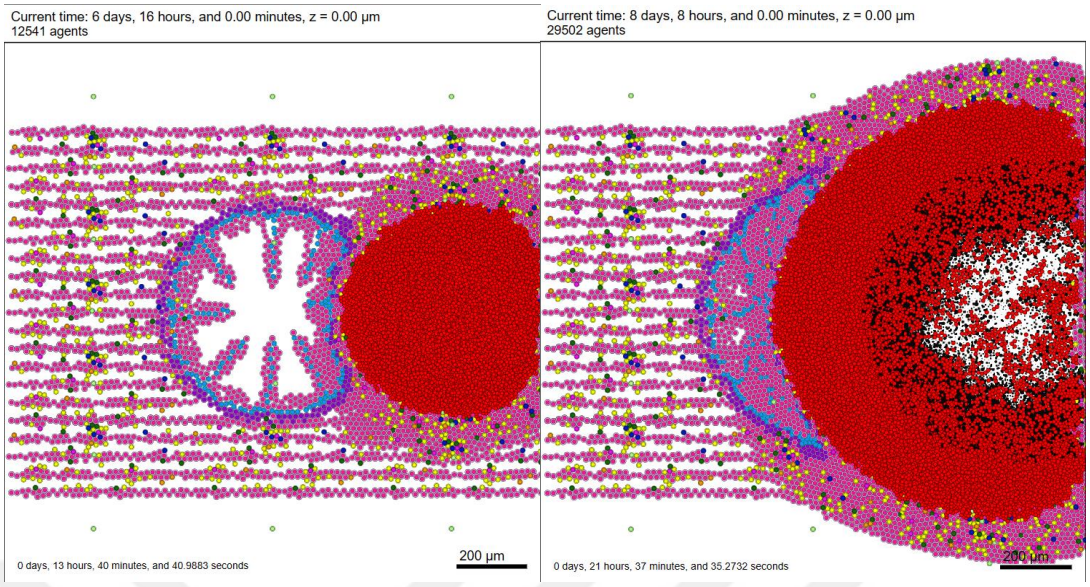


Figure 5.13: Updated lung tissue with cancer model.

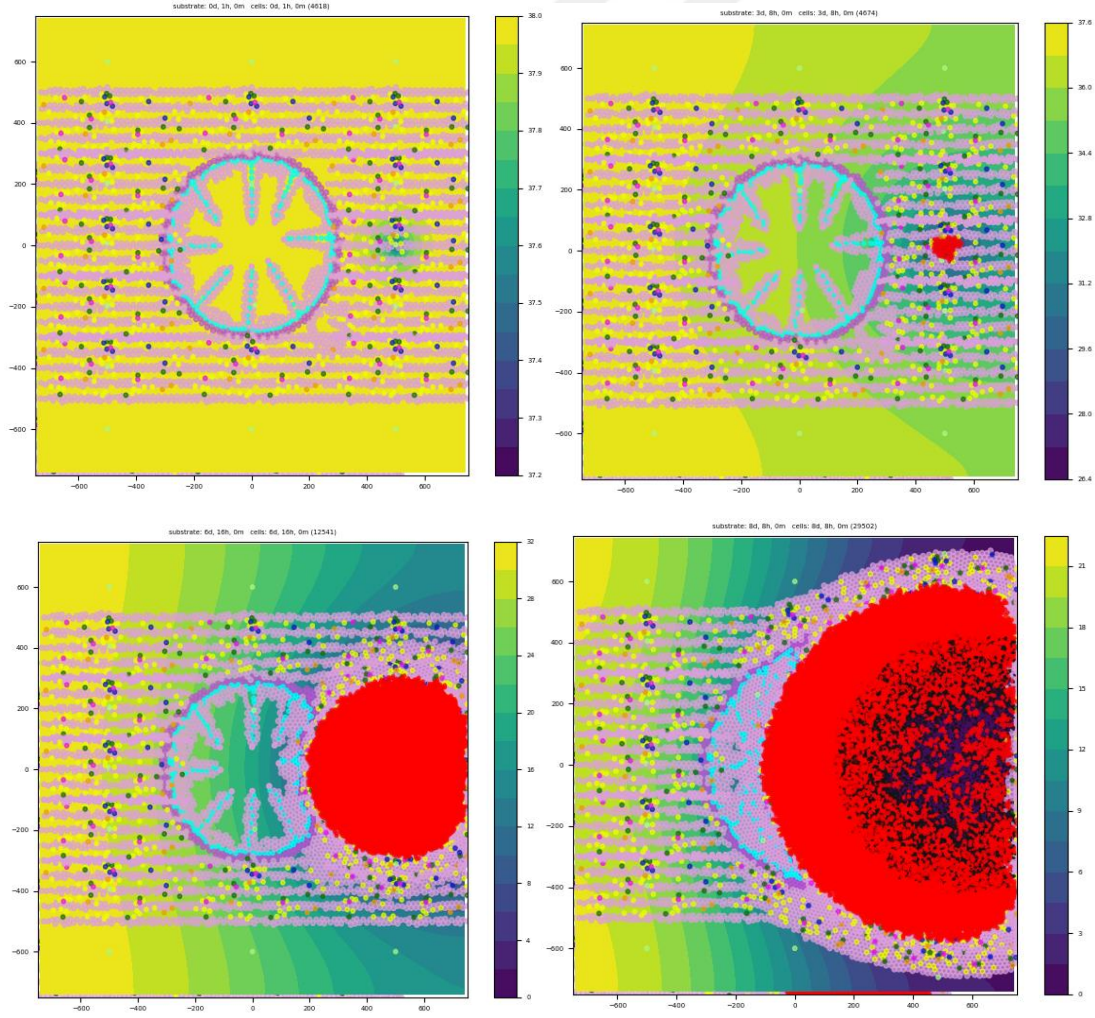


Figure 5.14: Oxygen concentrations at different time intervals.

6. CONCLUSION

In this work, different tissue models were made in 2D using PhysiCell. In some models, simulations were able to represent motility, chemotaxis, apoptosis, proliferation rate changes, and also secretion and uptake rate changes by modifying some parameters and setting relative thresholds to concentration changes of products in SBML files. Some equations are made in assignments part of SBML files to dynamically change concentration of some substrates. With these properties, invasion, immune evasion, immune suppression and different proliferation rates for different cells were modeled. In this work, it is shown that many biological properties can be represented by a simulation platform. By using files that are made in scope of this thesis, researchers can model tumor development by using different proliferation rates, secretion rates, uptake rates, production capacities and death rates. We have an option to set different parameters for cancer cells and other cells to observe their expansion conditions and equilibrium status depending on death-proliferation ratios. We have ability to represent high proliferation rate of cancer cells with these models. One can easily apply drugs that target proliferation rate of cancer cells to measure their effectiveness by changing parameters that are related with transition rate in SBML files. Also, that is shown that cells in the TME can move if they have required parameters and properties. Stromal cells and cancer cells can move to places that their chemotaxis targets reside. This means, all cells in the TME can move if their motility permissions are equal to “true” value in the XML file. This condition allows us to represent models that prioritize movement. Apoptosis abilities of cells in these models are successfully represented. This is a crucial ability for biological tissue models. Cells in the TME die because of low oxygen concentration and other substrates have huge impact on apoptosis of cancer cells. Transition rates, motility rates and apoptosis rates are controlled in events and assignments parts of SBML files. By using events, secretion rates and production rates that depend on thresholds can be changed. These thresholds generally look up to energy levels or oxygen concentrations in the TME. Also, tissue models that are similar to real tissue images successfully made by using Python programming language to generate CSV files that contain cell coordinates and cell types.

In these simulation trials, there were many PhysiCell caused problems. In complex and more cell-to-cell interactions depended models, there were lots of crashing problems that needed to be solved. For example, in the TME model that has moving cancer cells, when one cell dies, simulation crashes. This situation only appears in the complex TME model and did not show up in other simulations. By looking at core C++ files and changing responsible parts that caused this error can solve this issue. There are also some production and uptake problems. Some of these problems were solved but still several of them remain in the complex TME model. Some cells produce substrates that depend on other substrates irrespective of whether they are absent in the environment or not. Secretion rates of substrates can be controlled in events part of SBML. But, in some cells, when starting secretion rate is 0, they do not produce any substrates. After starting secretion rates are manually changed, they start to produce these substrates. Another problem is that substrates that produced by substrates that taken by using an uptake rate constant, cannot be used in the events part of SBML files. In some simulation, "Convergence Failure" error that is caused by "cvode" files that are in Libroadrunner files can be encountered. When this error occurs in command prompt screen, it slows down the simulation and after some time is passed, it shuts simulation down.

In conclusion, making 2D or 3D tissue models is an important biological objective. These models can accelerate biological studies and inventions. By making and using these models, researchers can lift the weight on laboratory works. Laboratory works will be used only as final tests to certify developments. In this thesis, I contributed to development of simulation environments for tissue modeling. I was able to represent some biological properties of cancer development in different simulation scenarios. Interactions of immune system and other stromal cells with cancer cells, effects of hypoxia and drugs, and results of different apoptosis and proliferation rates tried to be shown in simulations. More importantly, by changing files and codes inside of them, template structures that can be used for cancer and other diseases are developed and they can be used by any other researchers.

In future works, firstly, I want to make TME model that has battleground-like properties. I want to represent fight of cancer against immune system structure dynamically. Proliferation and apoptosis rates will change dynamically due to influences of the TME. Then, I will add new cells and products that take part in cancer development, angiogenesis and metastasis. I already showed combination of

healthy organ tissue model and the TME model. I want to represent different organ models with cancer development. As I said, I want to add angiogenesis related structures to my models. Also, reconstruction of blood vessels and blood cells is another work to be done. Finally, I want to test effects of chemicals and nutrition on tumor growth and metastasis using my models to finalize my cancer model development. Actually, this work can be used as a template work. One can use properties that I defined to represent the TME structure for modeling other diseases. I want to develop models for other diseases as well in the future.

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