

T.C.
DOKUZ EYLÜL UNIVERSITY
İZMİR INTERNATIONAL
INSTITUTE OF BIOMEDICINE
AND GENOME

**INDUCIBLE PLURIPOTENT STEM CELL
(IPSCs) DERIVED HEPATIC ORGANOIDS
FOR MODELING LIVER CANCER**

MUSTAFA KARABIÇICI

MOLECULAR BIOLOGY AND GENETICS

DOCTORAL THESIS

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student Mustafa KARABIÇİCİ successfully completed his doctoral thesis entitled '**Inducible
Pluripotent Stem Cell (IPSCs) Derived Hepatic Organoids in Modeling of Liver Cancer**' on
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PREFACE

In this thesis, it is aimed to model fibrosis, which is the early stage of liver cancer, with the mutation to be induced in the CRISPR/Cas9-mediated p53 gene from inducible pluripotent stem cell (iPSC)-derived hepatic organoids obtained from healthy humans. This study was supported by the TUBITAK 1001 program with the code 117Z858.



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ABBREVIATIONS

AddMEM/F12	Advanced Dulbecco's Modified Eagle Medium/Ham's F-12
AML	Acute myeloid leukemia
APC	Adenomatous Polyposis Coli
BMP7	Bone morphogenetic protein 7
CAFs	Cancer-associated fibroblasts
CCL18	Chemokine (C-C motif) ligand 18
CCL22	C-C motif chemokine 22
CHIR	CHIR99021 (GSK3B inhibitor)
COL1A1	Collagen type I alpha 1 chain
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
crRNA	CRISPR RNA
CSCs	Cancer stem cells
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butyl ester
DM	Differentiation medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eHEPO	Hepatic Organoid
EM	Expansion medium
EpCAM	Epithelial cellular adhesion molecule
FACS	Fluorescence-Activated Cell Sorting

FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF10	Fibroblast growth factor 10
FGF19	Fibroblast growth factor 19
GAPs	Ras GTPase-activating protein
GEFs	Ras guanine nucleotide exchange factors
GPC3	Glypican 3
gRNA	guide RNA
HCC	Hepatocellular Carcinoma
HDR	Homology-directed repair
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HMGA2	High Mobility Group AT-Hook 2
HSC	Hepatic stellate cells
IHC	Immunohistochemistry
IL 4	Interleukin 4
IL10	Interleukin 10
IPSCs	Inducible Pluripotent Stem Cell
MCP1	Monocyte Chemoattractant Protein-1
MUC1	Mucin 1, cell surface-associated
NAC	N-acetylcysteine
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis

NF1	Neurofibromin
NHEJ	Non-homologous end-joining
PAM	Protospacer adjacent motif
PAS	Periodic acid-Schiff
PCR	Polymerase chain reaction
PDGFRB	Platelet-derived growth factor receptor beta
PEI	Polyethyleneimine
scRNA-seq	Single cell RNA-seq
Sox 9	SRY-Box Transcription Factor 9
TAMs	Tumor-associated macrophages
TGF- β	Transforming growth factor-beta
THP1	Human monocytic cell line
TICs	Tumor initiating cells
TME	Tumor microenvironment
TP53	Tumor protein P53

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Pluripotent Stem Cell (IPSCs)-Derived Hepatic Organoids for Modeling Liver Cancer

ABSTRACT

Liver cancer is a heterogeneous disease with very limited treatment options, which ranks 4th in the world in terms of death. Hepatocellular Carcinoma (HCC), the most common type, is cancer that usually develops on the basis of cirrhosis and is characterized by late diagnosis and drug resistance. The most important limitation in understanding the early molecular pathophysiological changes and identifying new treatment targets in HCC is lack of the in vitro and in vivo models mimicking human tumorigenesis. In this context, in this study, the interactions of the microenvironment with stemness and senescence morphology in hepatocytes produced from IPSCs were investigated (Karabicici, 2021) and it was aimed to develop a long-term and large-scale 3D cell culture model that models the formation and development of pre-malignant stages of liver cancer such as fibrosis and cirrhosis.

Here we utilize CRISPR/Cas9 technology for targeted gene modification of three of the most commonly mutated liver cancer genes (p53, APC, and NF1) in Induced Pluripotent Stem Cell derived healthy organoids (eHEPO). Although the CRISPR/Cas9 strategy to make these driver genes knockout has been worked successfully in HEK293 cells, we only continued with p53 knockout on eHEPOs. For this, we firstly transfected px458 plasmids containing p53 specific gRNA to the eHEPOs via optimized Nucleofector conditions. After enrichment of the GFP⁺ cells by FACS, we manually selected p53 KO-eHEPO clones under Nutlin-3a treatment.

To mimic pro-carcinogenic TME activated LX2 and M2 polarized cell secretoms were added to growing medium of p53 KO-eHEPOs as well as WT controls. P53-KO highly proliferative (Ki67) and all displayed features of pro-carcinogenic pathological marks of pre-malign stages including increased nuclear–cytoplasmic ratio, pleiomorphic and hyperchromatic nuclei, pseudo glandular and tubular rosettes and inflammatory-like cell morphology. In addition, mason trichome staining area and fibrotic gene expressions have been shown to increase significantly.

As a result, it has been shown that a fibrotic organoid model can be created that will make significant contributions to the understanding of tumor formation mechanisms in a carcinogenic microenvironment by using p53 KO-eHEPOs, which is one of the driver genes at the onset of HCC.

Keywords: Organoid, CRISPR/Cas9, HCC, p53, Fibrotic Organoid

Karaciğer Kanseri'nin Modellenmesinde İndüklenebilir Pluripotent Kök Hücre (İPSCs) Kaynaklı Hepatik Organoidler

ÖZET

Karaciğer kanseri, dünyada ölüm sıralamasında 4. sırada yer alan, tedavi seçenekleri çok sınırlı heterojen bir hastalıktır. En sık görülen tipi olan Hepatosellüler Karsinoma (HCC) genellikle siroz temelinde gelişen, geç tanı ve ilaç direnci ile karakterize bir kanserdir. HCC'de erken moleküler patofizyolojik değişikliklerin anlaşılmasında ve yeni tedavi hedeflerinin belirlenmesinde en önemli sınırlılık, insan tümör oluşumunu taklit eden in vitro ve in vivo modellerin eksikliğidir. Bu kapsamda İPSC lerden üretilen hepatositlerde mikroçevrenin stemness ve senesens morfolojisindeki etkileşimleri incelenmiş olup (Karabicici, 2021) karaciğer kanserinin fibroz ve siroz gibi malign öncesi evrelerinin oluşumunu ve gelişimini modelleyen uzun vadeli ve büyük ölçekli bir 3D hücre kültürü modeli geliştirmek amaçlanmıştır.

Bu kapsamda, İndüklenmiş Pluripotent Kök Hücre (İPSCs) kaynaklı sağlıklı organoidlerde (eHEPO) en yaygın olarak mutasyona uğradığı bildirilen 3 driver genin (p53, APC ve NF1) hedeflenen gen modifikasyonu için CRISPR/Cas9 teknolojisi kullanılmıştır. Bu driver genleri hedefleyen CRISPR/Cas9 stratejisi, HEK293T hücrelerinde başarılı bir şekilde çalışmış olsa da, bu tez kapsamında yalnızca p53 KO eHEPO ların kanser tümör mikroçevresindeki yanıtları incelenmiştir. Bunun için p53'e özgü gRNA içeren px458 plazmitleri optimize edilmiş Nucleofector koşulları aracılığıyla eHEPO'lara transfekte edilmiştir. Sonrasında elde edilen GFP+ hücreler FACS ile ayrıldıktan sonra, Nutlin-3a selektif ortamı içerisinde büyüeyebilen p53 KO eHEPO klonları manuel olarak seçilmiştir. Pro-kanserojenik TME yi taklit etmek için aktive edilmiş LX2 ve M2 polarize hücre sekretomları, WT kontrollerinin yanı sıra p53 KO-eHEPO'ların büyüyen ortamına ilave edilmiştir. Bu işlem sonrasında P53-KO eHEPOLarda artmış proliferasyonun yanısıra (Ki67) artan nükleer-sitoplazmik oran, pleiomorfik ve hiperkromatik çekirdekler, psödo glandüler ve tübüler rozetler ve inflamatuvar benzeri hücre morfolojisi dahil olmak üzere, prokarsinogenik tümör markerları gözlenmiştir. Ayrıca mason trichome boyanan alan ve fibrotik gen ekspresyonlarının önemli ölçüde arttığı gösterilmiştir.

Sonuç olarak, HCC başlangıcındaki driver genlerden biri olan p53 KO eHEPO kullanılarak pro kanserojen bir mikroçevrede tümör oluşum mekanizmalarının anlaşılmasına önemli katkılar sağlayacak fibrotik bir organoid modelinin oluşturulabileceği gösterilmiştir.

Anahtar Kelimeler: Organoid, CRISPR/Cas9, HCC, p53, Fibrotic Organoid

1. INTRODUCTION AND AIM

1.1. Definition and Significance of the Problem

HCC is a type of cancer characterized by its late prognosis and drug resistance. Sorafenib, a multikinase inhibitor, was used for a long time, the only FDA-approved drug used in advanced cases, but Sorafenib treatment has been shown to prolong the life of patients by only 3 months (Cheng et al., 2009). Fluoro-Sorafenib (Regorafenib), which was approved by the FDA in April 2017, is recommended to be used in Sorafenib-resistant patients. However, it is not preferred due to its very serious side effects (Bruix, Cheng, et al., 2017; Bruix, Qin, et al., 2017). The biggest obstacle to the development of new treatment options is the lack of models similar to human tumor microenvironments. There are transgenic mouse models, however, their resemblance to human scenarios is quite limited due to the inability to establish premalignant processes (Dusabineza et al., 2012). On the other hand, conventional 2D grown HCC cell lines used in most studies undergo phenotypic changes due to long-term culture and most importantly other has been shown to be contaminated with cell lines (Qiu et al., 2016; Rebouissou, Zucman-Rossi, Moreau, Qiu, & Hui, 2017). Again, 3D models of spheroids have been made using very few HCC cell lines. However, since the HCC cell lines used in this study already contain a wide variety of mutational microsatellite instability and chromosomal abnormalities, this model poses a major obstacle to elucidating the roles of driver genes in the carcinogenesis process. **As a result, there is no good in vivo and in vitro model for liver cancer to work the interaction of driver genes with TME interaction and tumor initiation process related with it.**

However, organoid technology, which is formed by growing cells in a three-dimensional matrix, from adult stem cells or pluripotent stem cells in the primary tissue, by imitating key elements of real embryogenesis such as self-assemble and self-renewal, in real tissue. They are mini-organs that can be stably cultured for a long time, in which spatial cell distribution is visible and preserving their function and physiology in real tissue (Llovet et al., 2021). Organoids, which can be formed from one or more cells originating from an adult tissue, embryonic stem cells or induced pluripotent stem (iPSC) cells, have been used for many purposes, from elucidating developmental biology and the mechanisms of self-renewal of healthy tissues, modeling of metabolic and single gene diseases, and in recent years, modeling tumorigenesis (Clevers, 2016; Dutta, Heo, & Clevers, 2017; Fatehullah, Tan, & Barker, 2016; Schutgens & Clevers, 2020; Sun et al., 2019).

In one of the first studies on this subject, it was shown that as a result of consecutive deletions and mutations in the driver genes (K-Ras, APC, p53 and SMAD4) in colorectal adenocarcinoma using CRISPR-Cas9 technology, the development of colorectal cancer was created similar to the real scenario (Drost et al., 2015; Matano et al., 2015). In this way, by imitating the mechanisms of the onset and progressive processes of cancer, it paves the way for the elucidation of molecular mechanisms and the development of new treatment strategies.

1.2. Aim of Study

In this study, it was aimed to model the developmental process of Hepatocellular Carcinoma (HCC) by causing mutations with CRISPR-Cas9 technology in p53, APC and NF1 driver genes, respectively, in healthy human liver organoids. For this purpose, within the scope of this thesis, it is aimed to model early fibrosis, which is induced in a pro-carcinogenic microenvironment in p53 KO organoids, which is one of the possible first hits.

1.3. Research Hypothesis

This study aims to address the lack of an in vivo-in vitro model similar to the human tumor microenvironment, which is the biggest obstacle to elucidating the possible mechanisms at the onset of HCC, which has limited drug success characterized by its late prognosis, drug resistance and heterogeneity, and to develop new treatment options for these mechanisms.

2. INTRODUCTION

2.1 Molecular Mechanisms of Liver Cancer

Liver cancer is the 6th most common cancer in the world, 4th in mortality rates, and is characterized by its poor prognosis and drug resistance. It is known that external environmental factors such as Aflatoxin B, Hepatitis B, Hepatitis C viruses, alcohol or genetic mutations that cause mitogenic effects are effective in the emergence of the disease (Singal, Lampertico, & Nahon, 2020). Depending on the chronic damage caused by these factors, an over-stimulated tissue renewal (regeneration) process is stimulated in the liver. Following this, collagen and extracellular matrix (ECM) accumulating in the environment trigger the formation of fibrotic tissue. Dysplastic nodules, in which the ECM is more concentrated, may regress depending on the amount of mitogen in the tissue, or they may progress and form cirrhotic tissue, which constitutes 90% of liver cancer (Figure 1) (Farazi & DePinho, 2006).

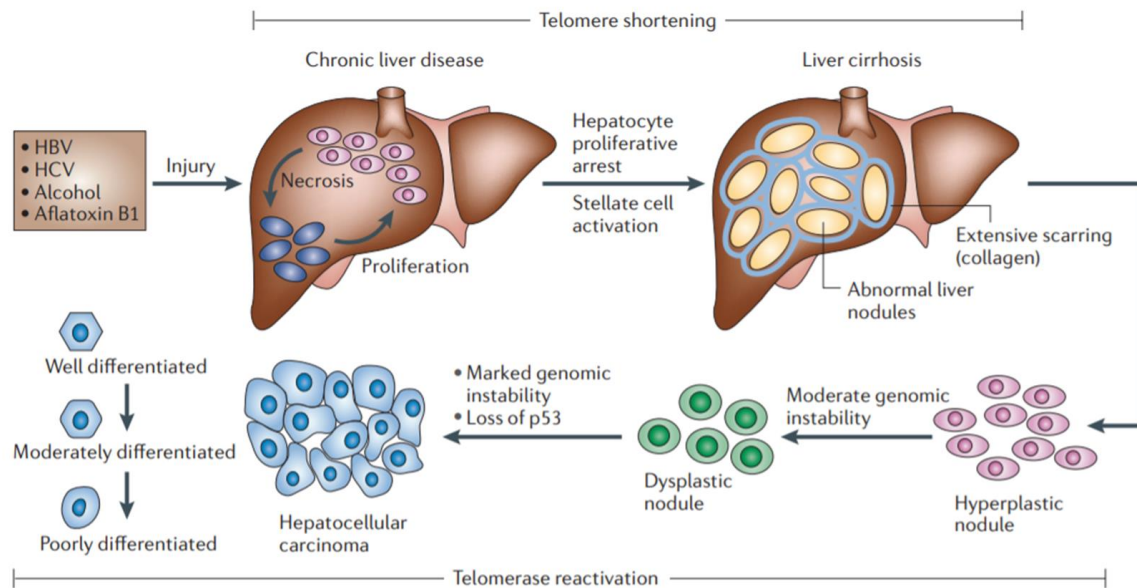


Figure 1. Histopathological progression of hepatocellular carcinoma

The first and most effective mitogenic change seen in the development of HCC is the increase in telomerase activity due to hTERT promoter mutations, and it has been reported that mutations in genes such as TP53, CTNNB1, VEGFA, FGF19/CNND1, CDKN2A in the later stages cause the development of clones that can multiply indefinitely, are resistant to apoptosis and are invasive. Studies have shown that there are 35-80 somatic mutations in an average HCC (Totoki et al., 2014; Zucman-Rossi, Villanueva, Nault, & Llovet, 2015). It is known that most of these genetic changes are in passenger genes without functional carcinogenic consequences,

and only a few (probably 4-8) mutations are seen in genes belonging to key signaling pathways that play a role in HCC development. The main pathways that gain importance with the effect of these mutations in HCC are; Telomere protection, p53-cell cycle pathway; Wnt/b-catenin pathway; Epigenetic regulations, oxidative stress pathway; PI3K/AKT/mTOR and RAS/RAF/MAPK pathways (Figure 2) (Craig, von Felden, Garcia-Lezana, Sarcognato, & Villanueva, 2020).

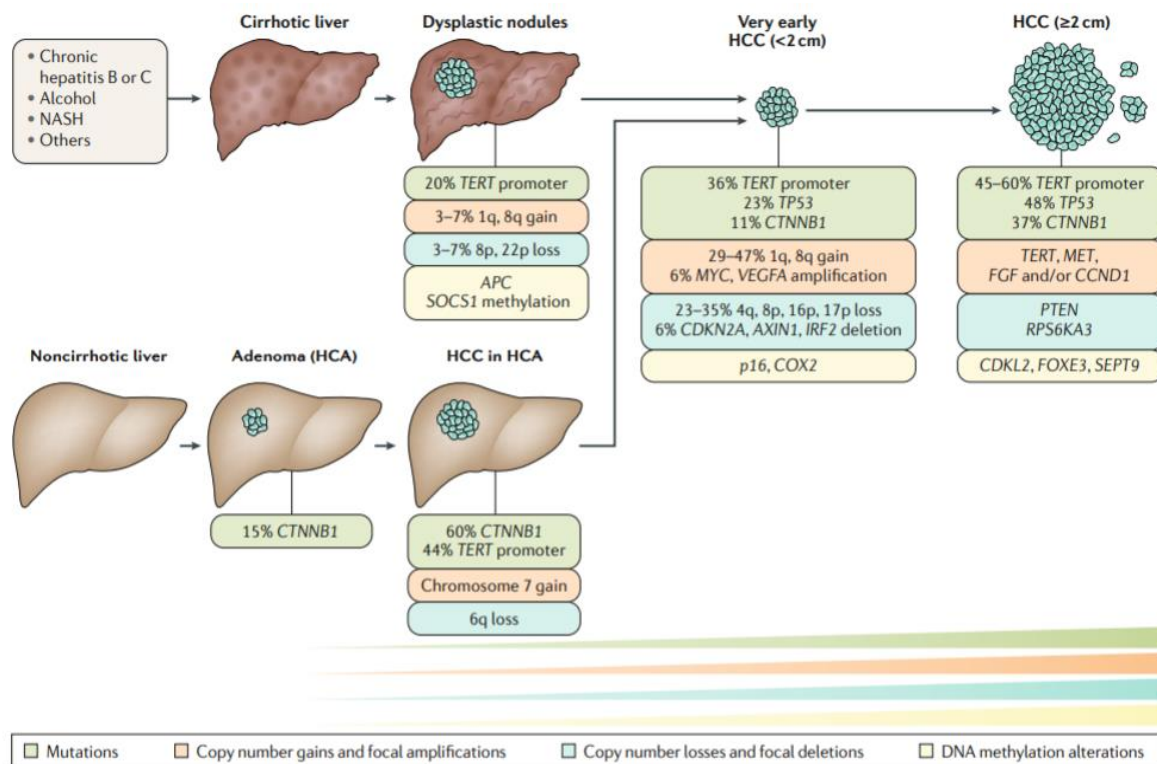


Figure 2. The Process of Hepatocarcinogenesis

Genetic changes that occur in the carcinogenesis process in HCC have been determined by important studies, including numerous case studies, thanks to high resolution and efficient omics technologies made in the last ten years (Llovet & Villanueva, 2016; Schulze et al., 2015; Zucman-Rossi et al., 2015). In molecular classification based on genomic profiling, HCC is divided into two major subgroups. The first is the “proliferative class”, which is rich in signals related to cell proliferation and cell cycle and has a more aggressive phenotype, and the other is the “non-proliferative class” which usually reflects normal hepatic physiology. Interestingly, it is known that progenitor cell markers such as EpCAM are also positive in the proliferative class in which activation of AKT/mTOR, MET, TGFβ, IGF and Ras/MAPK pathways is observed, and AFP levels and the risk of recurrence in this group of patients are very high. In

the non-proliferative class characterized by immune response, it was found that the Wnt signaling pathway was mostly active and was observed in HCV and alcohol-related HCC (Figure 3) (Llovet & Villanueva, 2016).

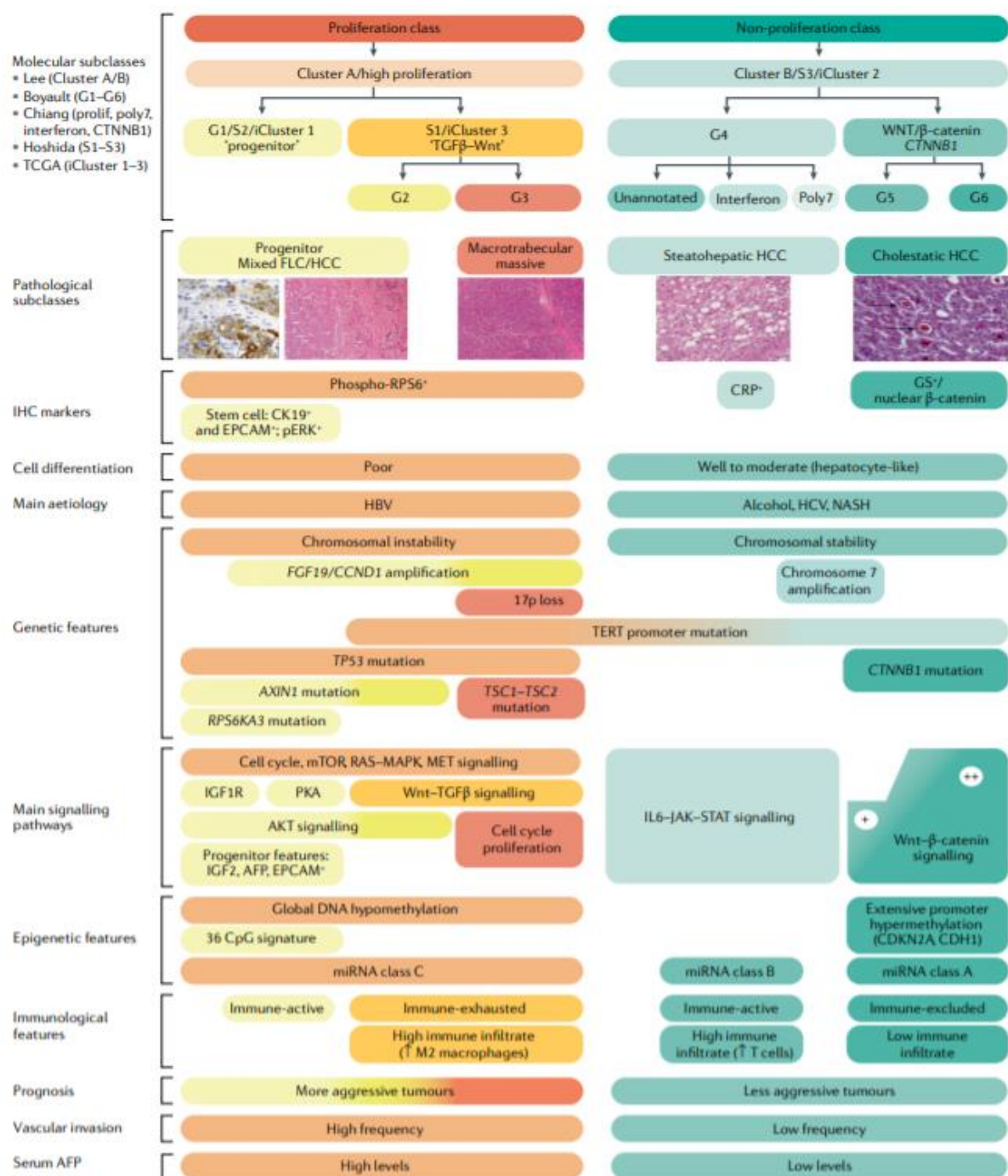


Figure 3. Proliferative and non-proliferative HCC

Sorafenib, a multi-kinase inhibitor used in advanced human HCC patient cases and which has been the only FDA-approved drug for quite some time, can only prolong survival of patients

up to 3 months after treatment. It is reported that as a result of this period, patients develop various drug resistance mechanisms and the prognosis of the disease worsens (Cheng et al., 2009). In cases where Sorafenib fails, Fluro-Sorafenib (Regorafenib), which has been FDA approved since April 2017, is recommended to be used in Sorafenib resistant patients. However, it is not preferred due to its very serious side effects (Iavarone et al., 2021). In addition, in studies conducted by our group, it has been shown that Regorafenib increases the cancer stem cell population in HCC and that Regorafenib develops metastatic properties via the TGF β pathway in the progressive stage (Karabicici, Azbazdar, et al., 2021). While new searches continue, drugs such as lenvatinib, cabozantinib, ramucirumab as well as immune checkpoint inhibitors such as nivolumab and pembrolizumab were approved by the FDA between 2017-2020 (Luo, Wu, & He, 2021). However, although these newly approved drugs have clinical trials, their molecular mechanisms of action are not fully known. Therefore, there is a great need to develop new and effective treatment strategies, and especially to elucidate the mechanisms of tumor initiation and development. At this point, the biggest obstacle in the development of new treatment options is the lack of realistic models that can mimic the physiological changes in human hepatocarcinogenesis processes. The lack of premalignant processes in transgenic mouse models in the literature (Dusabineza et al., 2012) and the phenotypic changes seen in conventional 2D grown HCC cell lines due to long-term culture (Qiu et al., 2016; Rebouissou et al., 2017) are the most important limitations of these models. It has been shown that 3D spheroid models created by single and co-culture with cell lines cannot overcome the limitations of conventional cell lines and are not suitable models for elucidating the important and specific roles of the genes to be examined in the carcinogenesis process, due to the wide variety of microsatellite instability and chromosomal abnormalities that cell lines currently contain (Takai et al., 2016). In conclusion, a reliable platform is needed to understand the pathogenesis of HCC and the phenotypic and physiological changes during development.

2.2 p53 gene role in HCC:

p53 is a tumor suppressor gene with a 53 kDa protein structure with six major domains required for transcriptional activation. These are: two amino-terminal transcriptional activation domains (TADs); a proline-rich domain (PRD); a sequence-specific DNA binding domain (DBD); an oligomerization domain and a C-terminal domain (CTD). The transcriptional activity of p53 is tightly controlled by the negative regulatory molecule Mdm2. After the signal from the

environment, p53 is activated in the tetrameric form where four identical 393 residues come together. These activation and activity controls of p53 are regulated by specific regions located at the N and C terminus. Stress conditions such as DNA damage, oncogene signaling, and hypoxic microenvironment trigger the tetramerization of p53, inducing nuclear translocation and target p53 response element binding. As a result, it has been reported that p53 can regulate gene sets related to cell differentiation-dedifferentiation, metabolism and TME modification, as well as functions such as apoptosis, cell cycle, and DNA repair (Figure 4) (Boutelle & Attardi, 2021; Joerger & Fersht, 2008)

(A)



(B)

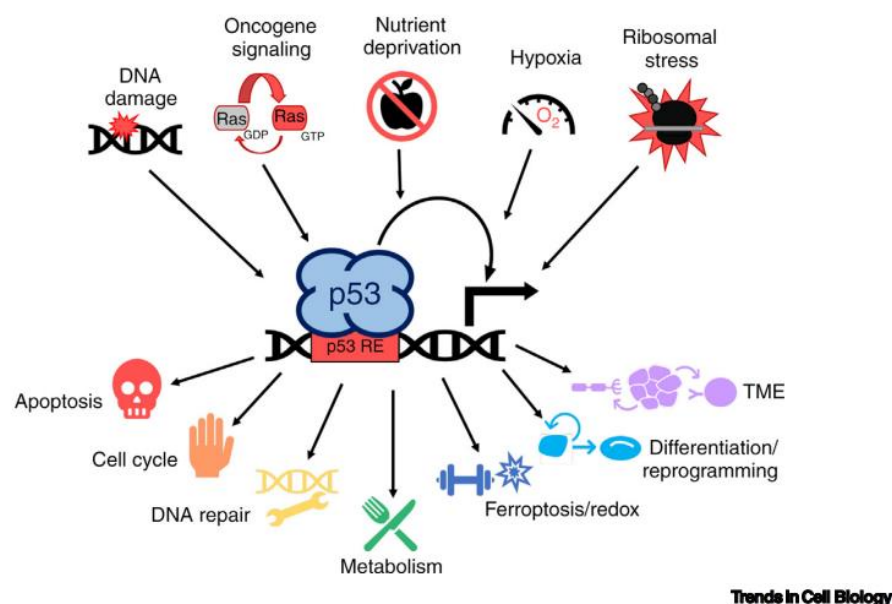


Figure 4. P53 structure and function.

p53 is known to be lost in an average of 50% of liver cancer (Figure 5). Known as the guardian of the genome, p53 is the leading player in the basic mechanisms of carcinogenesis (hallmarks), such as detection of damage, cell cycle arrest, and escape from apoptosis (Hussain, Schwank, Staib, Wang, & Harris, 2007). In addition, it is known to play a central role in the control of the senescence (cellular aging) mechanism in disease development. Regenerative nodules formed in the liver due to chronic inflammation are resolved by the elimination of activated stellates or parenchymal cells by apoptosis mediated by immune cells, after completing a certain replication cycle and entering senescence. However, loss of function due to any mutation in the

p53 gene may cause the progression of the disease and the formation of a cirrhotic background, with the continuous accumulation of cells in the inflammation environment and the continuous release of chemokines and cytokines that create proliferative signals (Lujambio et al., 2013).

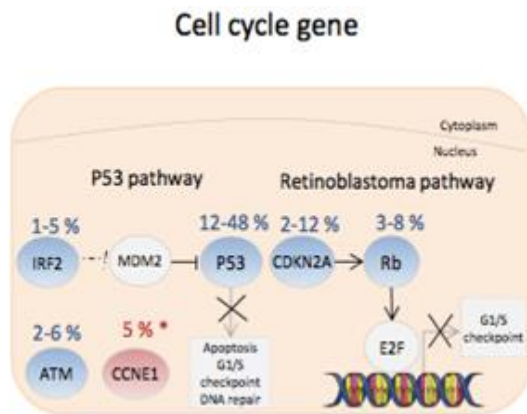


Figure 5. P53 signaling pathway and mutation rates

It has been reported that an increase in tumor formation can be seen up to 4.5 months in P53 KO mice, and that they are more sensitive to some environmental chemical stimulants, and the amount of neoplasm formation in the relevant tissue increases (Donehower, 1996; Yamamoto et al., 2000). In addition to this there is an increase in the amount of plasma alanine transferase and hepatic damage formation is induced in p53 KO mice (Yahagi et al., 2004). Studies have reported that P53 mutation or disruption of its regulation by Mdm2 is associated with spontaneous liver inflammation, steatosis, and fibrosis in the liver, as well as altered glucose metabolism (Cao et al., 2020). Similarly, it plays a role in modifying the TME, recruiting immune suppressive CD11b⁺ cells to the region, keeping CD4⁺ T helper 1 and CD8⁺ T tumor infiltrating cells away, and controlling the accumulation of COL4A1, which is associated with a fibrotic background, in the TME (Boutelle & Attardi, 2021).

In recent years, it has been reported that P53 plays a direct role in the formation of hepatic progenitor cells at the onset of tumor in the liver via the CD44-AKT pathway (Dhar et al., 2018). As stated in this study, another reason why P53 is so effectively involved in the tumorigenesis process arises from its task of maintaining the balance between the maintenance, self-renewal and differentiation capacities of embryonic and stem cells (Figure 6) (Ghatak, Das Ghosh, & Roychoudhury, 2020)

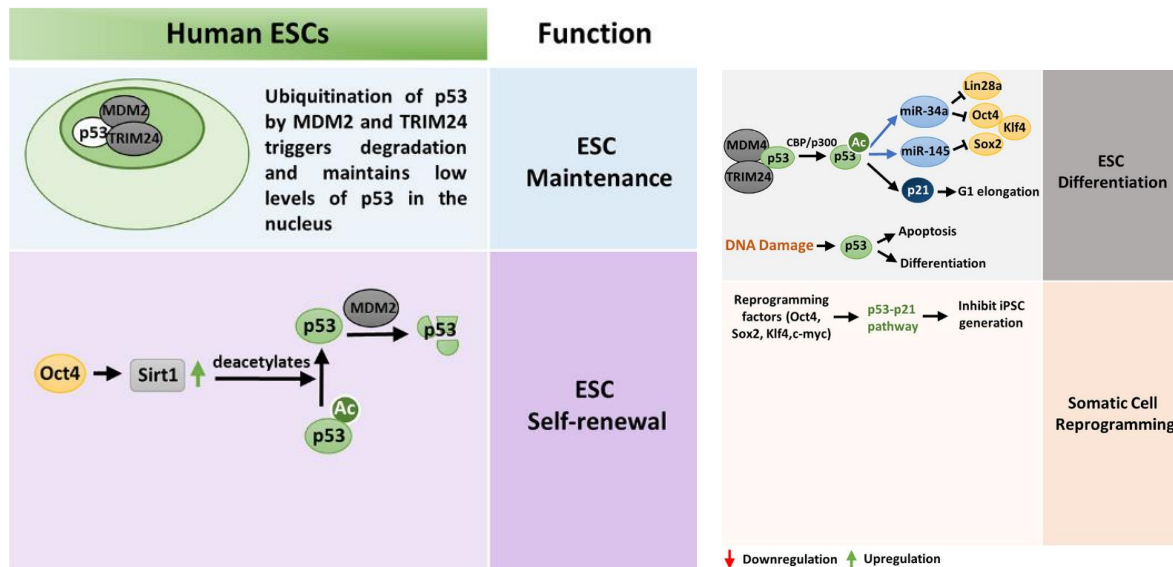


Figure 6. P53 function in cell plasticity

In the study of Dhar D., 2018 it is shown that tumor-initiating hepatic progenitor cells are localized in the pericentral region where the progenitor cells of the liver are located (with stem cell feature). Interestingly, a recent study showed that iPSCs produced by Yamanaka factors from somatic cells are formed approximately 100 times more effectively in conditions where p53 is KO. Again, it has been reported that in recent years, especially the expression levels of cancer stem cell markers are controlled by p53 and the absence of p53 in the tumorigenesis process or the increase in the expression of the mutant form of markers such as CD133, ALDH1, CD44, LGR5 support stemness (Figure 7) (Ghatak et al., 2020).

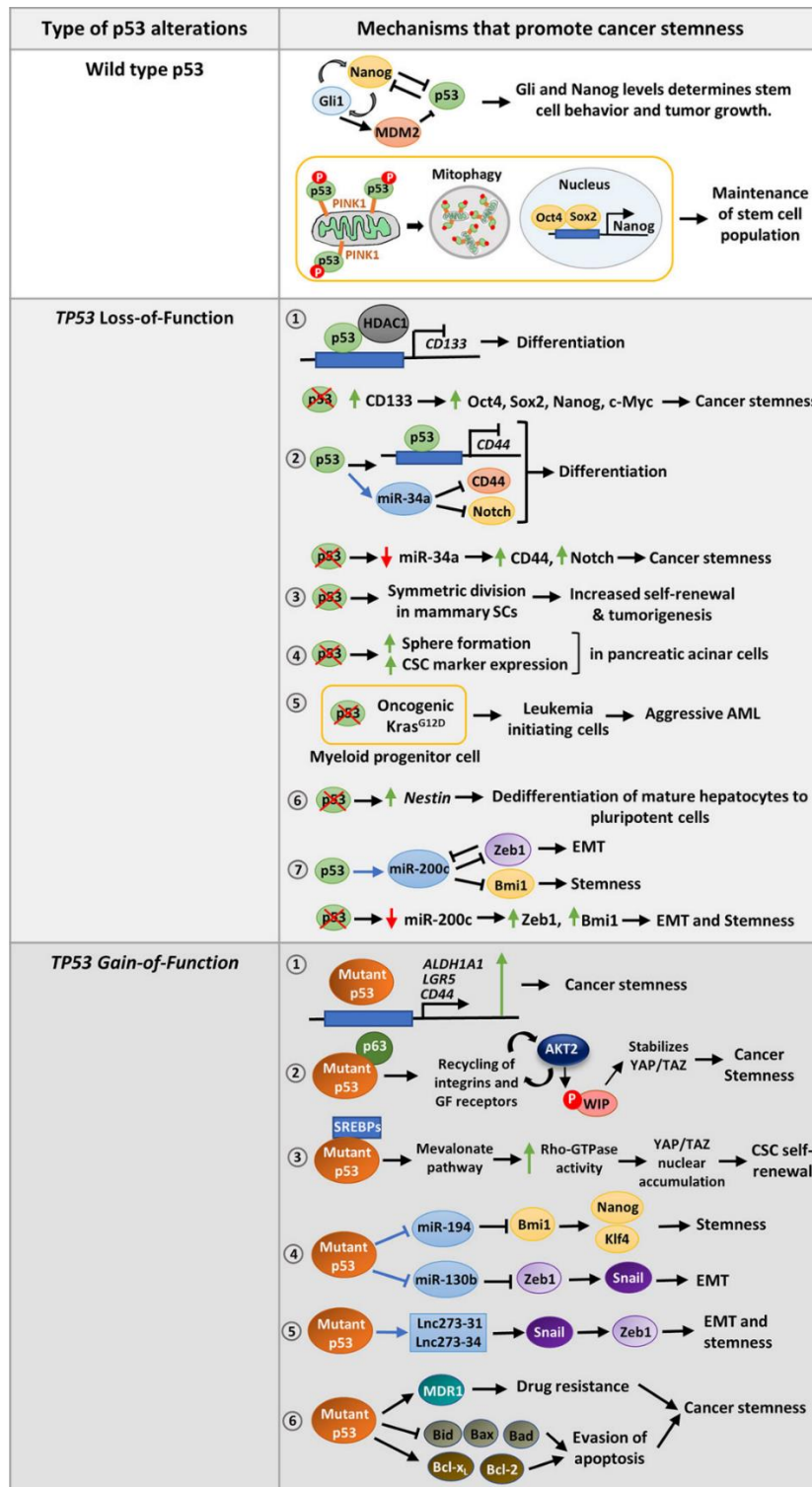


Figure 7. P53 mediated stemness mechanisms.

P53 and stemness and senescence trilogy:

In our previous study, the role of the microenvironment in the control of stemness was investigated in hepatocytes produced from iPSCs. According to the results we obtained, it was

observed that microenvironment components (SASP) collected from cell groups with and without cancer stem cell characteristics significantly formed senescence morphology in hepatocytes and increased the stemness feature. Similar results were observed in the HuH7 ancestral cell line, where p53 accumulates in the cell due to the conformational mutation it carries. However, different responses were observed in cell lines with mutational status or null characteristic of p53 (Karabici, Alptekin, Firtina Karagonlar, & Erdal, 2021). From this point of view, it is aimed to create a fibrotic organoid model that develops on the basis of senescence, where we see different responses related to p53 that better reflects the real physiology. Thus, pro-carcinogenic microenvironment (condition medium: secretome) collected from stellate and macrophage cells, which play a role in the formation of the fibrotic process, was added to WT and P53-KO eHEPO (CRISPR-Cas9 derived) cultures. As a result of the results we obtained, it was observed that a p53-dependent fibrotic organoid model could be formed and fibrotic markers were highly expressed.

As a result, a good understanding of P53's mechanisms in tumor initiation and development is very important in the development of promising treatment strategies against cancer. The p53KO-eHEPOs we obtained from healthy eHEPOs in our study are very valuable in terms of reflecting the real physiology and eliminating the lack of in vitro and in vivo models necessary to understand the tumorigenesis mechanisms, especially in a pro-carcinogenic microenvironment. In this sense, a tumor onset-fibrotic liver model has been brought to the literature through this thesis study.

2.3 APC gene role in HCC:

The second gene we targeted in our study is the Adenomatous Polyposis Coli (APC) gene, whose biallelic genetic inactivation has been shown to induce tumor, which is involved in the canonical Wnt/b-catenin signaling pathway, the second most frequently mutated signaling pathway in liver cancer development. It is known that the canonical Wnt/b-catenin signaling pathway is one of the key mechanisms in liver development, proliferation, migration and maintaining the stem cell population. In mutation analyzes performed in HCC patients, it has been reported that point mutations that inhibit B-catenin degradation are approximately 30%, while Axin1 and APC mutations that trigger B-catenin accumulation in the cell without Wnt signaling are found around 15% (Figure 8). Therefore, it was evaluated as a very ideal candidate gene for our study (Kato et al., 2006).

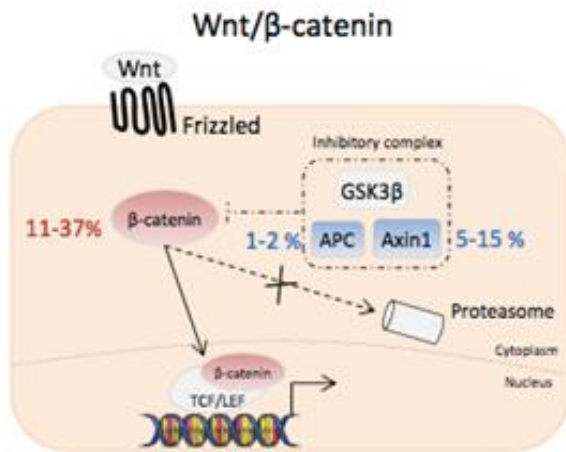


Figure 8. Wnt/b-catenin signaling pathway and mutation rates

2.4 NF1 gene role in HCC:

Neurofibromin (NF1), another gene that we targeted in our study, is a Ras GTPase-activating protein (GAPs) family member, which function as negative regulators of Ras. Ras proteins are the control buttons of a multitude of signaling pathways that control cell biology. There are two main conformations of Ras proteins, GDP-linked inactive and GTP-linked active. Proteins that determine the transition between these two forms are Ras guanine nucleotide exchange factors (GEFs) and Ras (GAPs). Ras GEFs trigger the activation of Ras by increasing the change from GDP to GTP, while Ras GAPs increase the intrinsic Ras GTPase activity which causes Ras inactivation by converting from GTP to GDP. The mutant oncogenic form of the Ras genes (Ha-, K-, and N-Ras) is expressed in approximately 30% of all cancers. Constitutive activation is observed in this oncogenic form due to insensitivity to Ras GAPs. Inactivation of Ras inhibitors, or suppression of Ras GAPs, are pathways that are responsible for the excessive upregulation of the Ras signaling pathway during the cancerization process, apart from mutant Ras (Figure 9) (Calvisi et al., 2011; Song et al., 2017). Therefore, targeting the NF1 pathway in HCC is very important for understanding the tumorigenesis process.

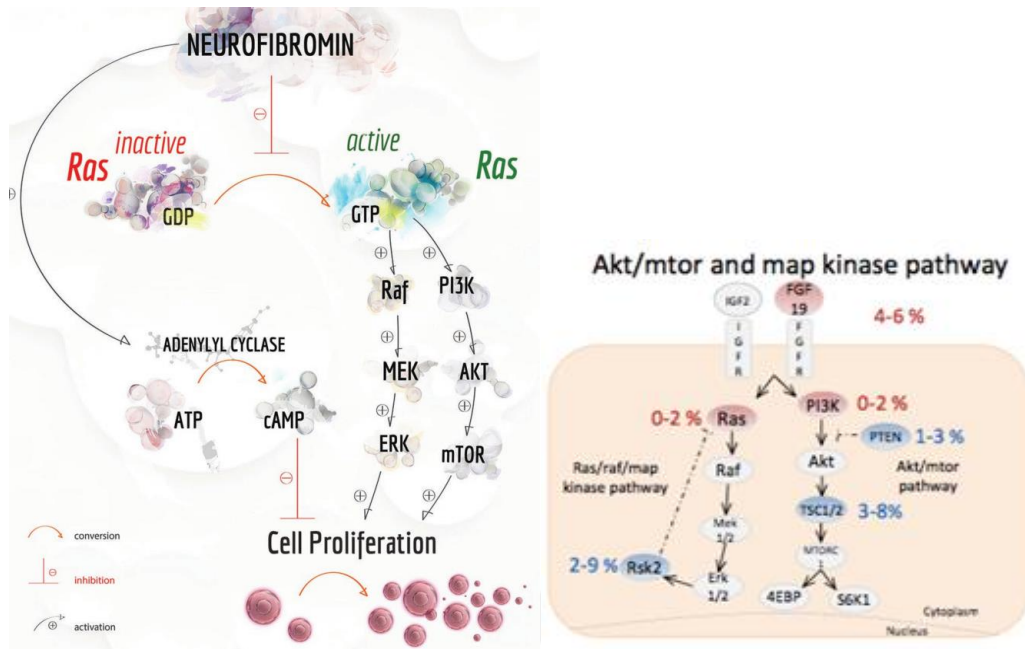


Figure 9. The importance of NF1 in the RAS pathway and mutation rates in the RAS signaling pathway

2.5 Hepatic progenitor cells and cancer stem cells in tumor initiation and development

Today, although the exact starting point of cancer is not known, it is known that the processes of "sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis" develop as a result of a single type of mutation or accumulation of mutations. At this point, studies with mouse models and primary human tissues have shown that solid tumors are usually triggered by driver mutations such as P53, APC, and Kras that occur in stem/progenitor cells of normal tissues (Bajaj, Diaz, & Reya, 2020; Hanahan & Weinberg, 2011). Studies in the literature have shown that tumors are induced, especially in the region where LGR5⁺ stem cell-like progenitor cells are located, especially in the pericentral region of the liver (Ang et al., 2019; Dhar et al., 2018). At this point, in vitro-in vivo models that mimic the mutations of progenitor cells are very important for understanding the early stage mechanisms of the tumor and developing treatment strategies.

It is known that progenitor cells, which have a role in cancer initiation, have specific surface markers and some characteristic features. These cells are subpopulations of chemotherapy-resistant cells that have self-renewal capacity and the capacity to differentiate into different cell types according to the needs of the cancer cell, and are called tumor initiating cells (TICs) and cancer stem cells (CSCs). It was determined that cancer stem cells, first identified in acute

myeloid leukemia (AML), were characterized by CD34⁺ CD38[−] surface markers. Over time, these specific rare cell populations, which are characterized by certain markers in each cancer cell population, have been defined and they have become possible treatment targets in the fight against cancer. Among the prominent markers for Liver cancer, intracellular markers such as EpCAM, CD133, CD24, CD90, CD44, as well as AFP, Nanog, Sox2, Oct 3-4, Notch and signal pathways such as TGFB, Wnt-b catenin pathway come to the fore (Figure 10) (Mohan, Raj Rajan, Mohan, Kollenchery Puthenveetil, & Maliekal, 2021; S et al., 2016; Walcher et al., 2020).

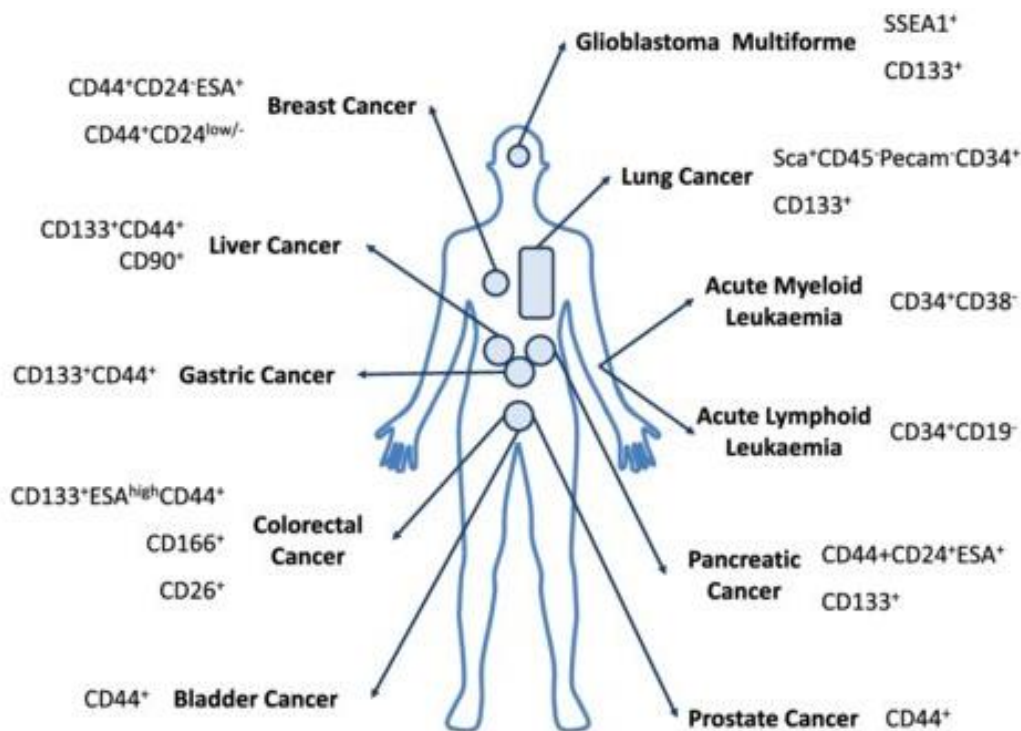


Figure 10. The most widely known cancer stem cell surface markers

One of the early studies of these surface markers was shown to induce the formation of hyper vascular tumors in as little as 4 weeks when EpCAM⁺ cells were injected into NOD/SCID mice. Again, in the same study, it was reported that cells carrying CD133 surface marker, which are expressed in parallel with EpCAM⁺ cells, also have high tumor initiation capacity (Yamashita et al., 2009). In addition, when tumors collected from early-stage cancer patients were examined in a study, it was shown that patients with homogeneous EpCAM-expression had a worse prognosis (Krause et al., 2020). In a recent study, the tumor-initiating capacity of EpCAM⁺ cells were evaluated in non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), which are common liver diseases. In this study, EpCAM⁺ cells injected

into immunocompromised mice induced by NASH liver microenvironment were reported to cause tumor initiation at a significantly higher rate compared to the control group (Pandit et al., 2020).

Another surface marker, CD133 (Prominin-1), is a surface protein of approximately 120 kDa, containing two extracellular loops and five transmembrane domains. It exhibits an increased expression pattern in progenitor or cancer stem cells, usually blunt to the EpCAM surface marker. It has been reported that CD133⁺ cells have a higher proliferation capacity compared to the control group, are more resistant to apoptosis, are resistant to chemotherapy, and have a high metastatic capacity, thus being associated with an aggressive tumor progression (Glumac & LeBeau, 2018). In recent studies, it has been reported that CD133 activates the Wnt pathway via the PI3K/AKT pathway and supports the formation of tumor-initiating cells. It has been shown that the depletion of CD133 and the disappearance of CSC features, while its overexpression causes an increase in stemness character (Mohan et al., 2021). Similarly, it is reported to trigger stabilization of the EGFR/AKT pathway and promote stemness (F. Liu & Qian, 2021). In the another study reported that CD133⁺ cells mediate the regulation of tumorigenesis of TICs via neurotensin, IL-8, CXCL1 and MAPK signaling pathway (Tang et al., 2012).

It has been reported that CD24 positive cells, a mucin-like cell surface glycoprotein, discovered in a study to mimic the responses of patients after chemotherapy in immunocompromised mice and to examine the behavior of TICs, have self-renewal and high tumor initiation capacity via STAT3/Nanog pathway (T. K. Lee et al., 2011). Interestingly, it has been reported that HGF and IL-6 secreted from CAFs, which have an important role from the early stage to the advanced stages of cancer, support the self-renewal and stemness properties of CD24⁺ positive cancer cells through a mechanism regulated by STAT3 (Y. Li et al., 2019). It has been reported that this mechanism comes to the fore in tumors that are observed to be particularly aggressive, where CD24 creates a "don't eat me" signal to macrophages and cytotoxic T cells, allowing cancerous cells to escape and grow from the tumor (C. Li et al., 2021). In addition, it has been reported that CD24 inactivates p53 by disrupting the ARF-NRP interaction, which stabilizes p53, and also prevents macrophages from targeting cancer cells (L. Wang et al., 2015). Based on this, especially in the early stage, CD24 may have a role in hepatic progenitor cells escaping from the tumor and gaining stemness.

Another surface marker is the CD90 molecule in the glycoprotein structure, which is a cell adhesion molecule. It has been reported that this molecule is highly expressed in CSCs in different cancers, including HCC, and it regulates cell adhesion, migration, apoptosis, axon growth, cell-cell and cell-matrix interactions, T-cell activation, and fibrosis (Kumar, Bhanja, Bhattacharyya, & Jaganathan, 2016; T. Liu et al., 2019; Zhou, Zhang, Zhang, & Li, 2021). CD90 is generally not expressed in well-differentiated hepatocytes, but is expressed in hepatic stem/progenitor cells. In addition, it has been reported that CD90⁺ cells may be associated with CAF, α -SMA and vimentin positive cells are also CD90⁺ and CD90⁺ may increase myofibroblast activation (Sukowati et al., 2013). It is also reported that these cells can modify the tumor microenvironment and induce angiogenesis (Conigliaro et al., 2015). From this point of view, it is seen that CD90 may have a role in the early stage of cancer.

CD44, another cell surface glycoprotein with a size of approximately 90 kDa, is a transmembrane protein associated with the ECM and hyaluronic acid (HA). It has two isoforms, standard CD44s and variant CD44v. CD44 is involved in mechanisms such as organ development, diverse immune functions and hematopoiesis, T cell differentiation, branching morphogenesis, proliferation, adhesion and migration. In addition, it is also a well-defined marker of CSCs for many cancer types. It has been reported that CD44 reveals the character of CSCs, especially through the TGFB/Stat3 pathway. In recent years, studies have shown that CD44 plays a central role in HCC, especially at tumor initiation. It has been shown that CD44 suppresses p53 by stimulating the nuclear translocation of Mdm2, which is a natural inhibitor of p53, via Akt signaling pathway in the beginning of cancer in the liver and leads to the transformation of hepatocytes into HCC progenitor cells (Dhar et al., 2018). In another study, it has been shown that CD44 has a role in the formation of clonal aggregates, which have a role in tumor growth and heterogeneity of melanoma cells in 3D matrix, and that cells that do not carry CD44 do not have this feature (Wessels et al., 2017).

Based on all these studies, in the early stages of liver cancer, single or cumulative accumulations of changes in surface markers in addition to driver genes such as p53 can induce tumor development in the cancerization of stem/progenitor cells. For this reason, within the scope of this thesis, the changes in these surface markers were examined in the WT and p53 KO Organoid model and the possible effects of surface markers on the development of the fibrotic process were investigated.

2.6 Role of tumor microenvironment in tumor initiation and development

The main cell types in the liver are hepatocytes, hepatic stellate cells (HSCs), Kupffer cells, and endothelial cells. 70-80% of liver cells are composed of parenchyma and the remaining 20-30% are non-parenchymal cells (Si-Tayeb et al., 2010). Hepatic stellate cells, which are non-parenchymal cells, constitute 5-8% of the liver tissue (Yin, Evason, Asahina, & Stainier, 2013) while Kupfer cells constitute approximately 20% of the non-parenchymal cells and approximately 6% of the liver tissue in total (Duarte et al., 2015). Stellate and macrophages can support or suppress the effects of each other under certain conditions.

In a healthy liver, the pro-fibrotic environment created by stellate cells due to inflammation is dissolved by macrophages and the affected cells go to apoptosis. On the other hand, the immune response is suppressed and HCC development is supported in the tolerogenic tumor microenvironment (TME), which causes dysfunctional tissue repair caused by chronic liver inflammation and ultimately supports tumor formation. Among many cells in the TME, cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs), which have an anti-inflammatory and pro-tumorigenic secretome that suppress the anti-tumor immune response, are reported to support tumor development.

CAFs modify the ECM around the tumor and restrict the entry of tumor-fighting CD8⁺ T cells into the region. Similarly, the entry, activities and polarization of dendritic and M1 macrophages, which fight against inflammatory and tumorigenic cells, are suppressed. In addition, Treg cells support the formation of a TGFB-mediated immunosuppressive microenvironment. Similarly, CAFs effect the polarization of macrophages, creating a microenvironment in which cancer cells can grow and develop (Figure 11) (Raskov, Orhan, Gaggar, & Gogenur, 2021).

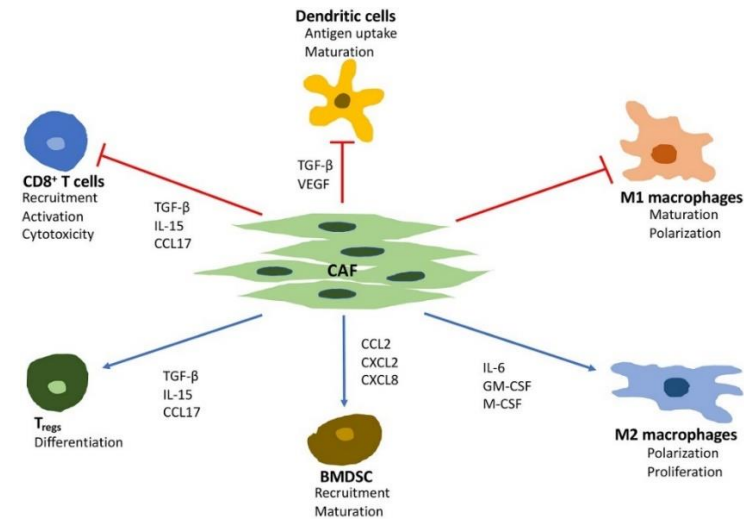


Figure 11. Cancer associated macrophages

Depending on the polarity of the macrophages, whose polarity can change depending on the different cytokines and chemokines in the TME secreted primarily from the CAFs, their roles and functions change significantly. It has been reported that pro-inflammatory macrophages that support pro-apoptotic responses and fight tumors are in the M1 polarization, whereas the polarization that has roles such as wound healing, immune suppressive, and angiogenesis is called tumor-supporting M2 (Figure 12) (L. Li et al., 2020; C. Liu et al., 2010; Lujambio et al., 2013; Pradere et al., 2013; C. Wang et al., 2021)

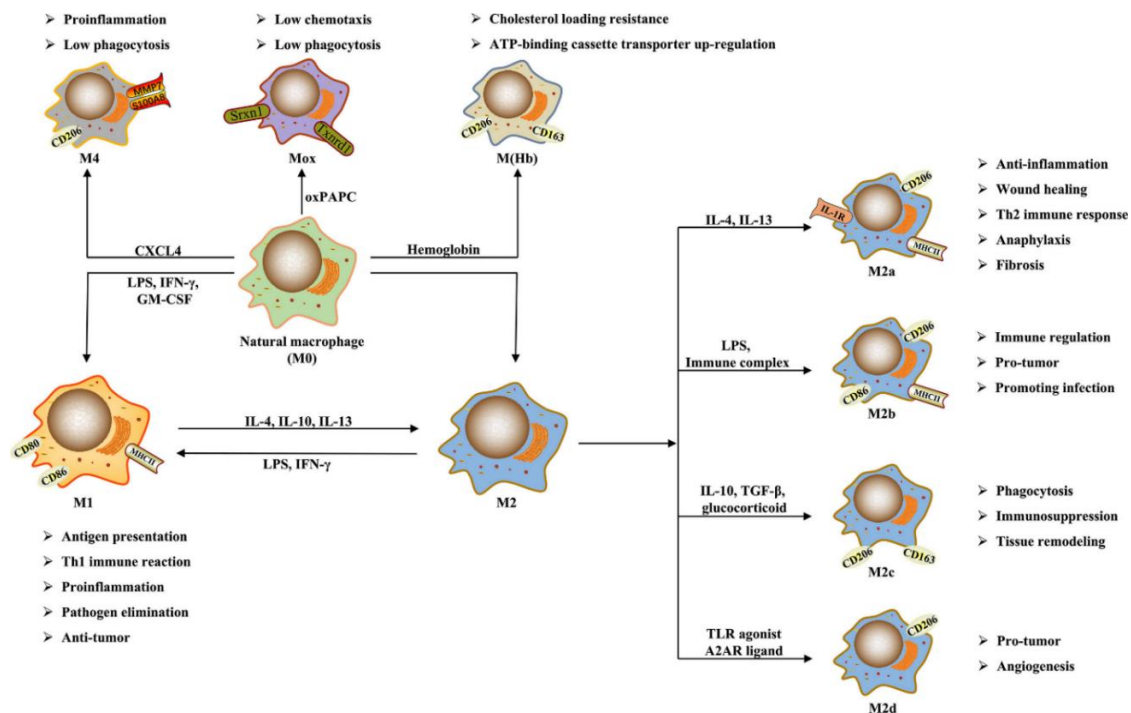


Figure 12. Macrophage polarizations

In particular, M2 macrophages, which are activated by cytokines such as IL4, IL10, secrete transforming growth factor beta (TGF- β), and chemokine ligands such as CCL18, CCL22, and proliferation, angiogenesis, migration and invasion mediated by signal pathways such as TGFB, Wnt/b-catenin, Stat3 and NFkB. It is reported that they support and accelerate tumor development (Figure 13) (Arvanitakis, Koletsa, Mitroulis, & Germanidis, 2022; Duan & Luo, 2021; N. Li et al., 2021; Tian, Hou, Liu, Han, & Wei, 2019; C. Wang et al., 2021; Yao, Li, Zhang, Chen, & Wang, 2018)

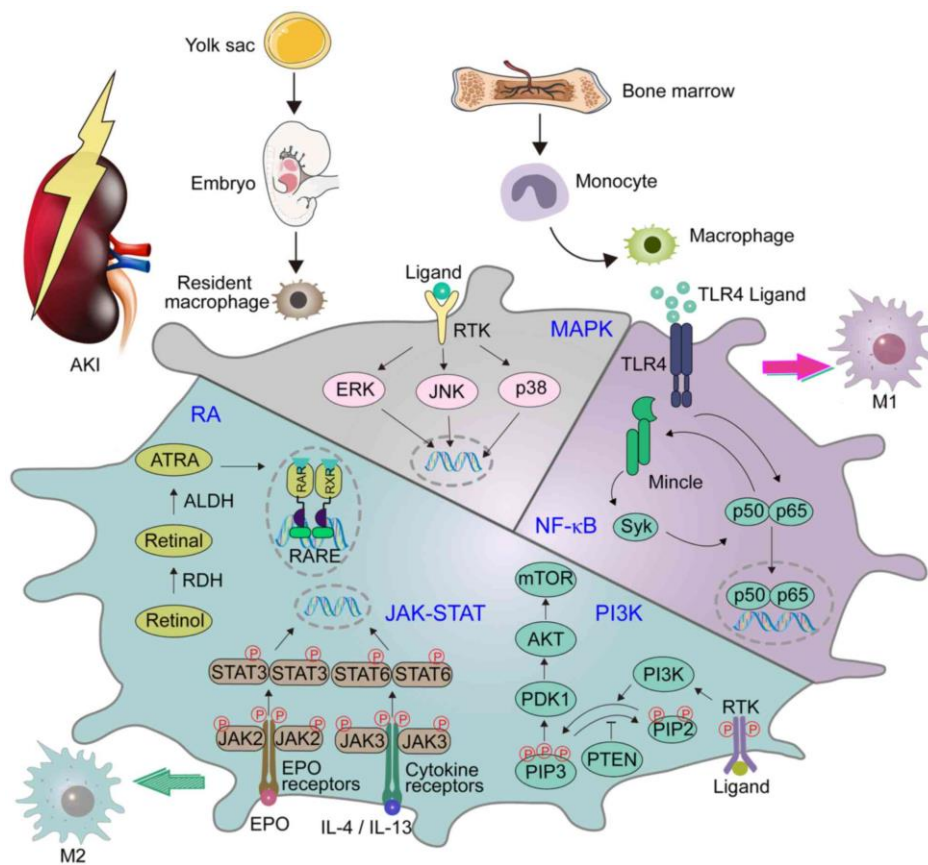


Figure 13. Macrophage associated signaling pathways

In addition, macrophages have been reported to be responsible for the regulation of uncontrolled activated hepatic stellate cells (HSC), which causes extracellular matrix (ECM) accumulation at the onset of fibrosis. It has also been shown that after activation, HSCs support M2 polarization of macrophages. Thus, these two cell groups undesirably create a stable tumor microenvironment and support tumor initiation and progression. In our study, the secretomes (cell media containing cytokines, chemokines and the growth factors they secrete) of these two cell groups were collected and added to the WT and p53 KO organoid culture, and the responses of p53 KO organoids in the pro-carcinogenic microenvironment were investigated.

2.7 Organoid-mediated cancer models:

Cancer models using organoid technology have become very common in recent years, and organoid culture can be made from metastatic lesions, tumor cells obtained from liquid effusions, most commonly from primary tumor tissue. One of the greatest advantages of this technology is that it can be studied by preserving the physiological properties of cells with very low amounts, especially circulating tumor cells. There are two main sources of cells that make up the organoid culture. First, tissues obtained more commonly from solid or liquid biopsies are mechanically or enzymatically minced and cultured in a 3D matrix. In the second method, the driver genes such as P53, APC, NF1, KRAS, TGFB known to play a role in the onset and progression of cancer are mutated with the help of CRISPR-like tools in healthy organoids. This approach is a very valuable model for the study of rare tumors and tumors that cannot be obtained by biopsy and has promising potential for individualized treatments (Figure 14) (LeSavage, Suhar, Broguiere, Lutolf, & Heilshorn, 2022).

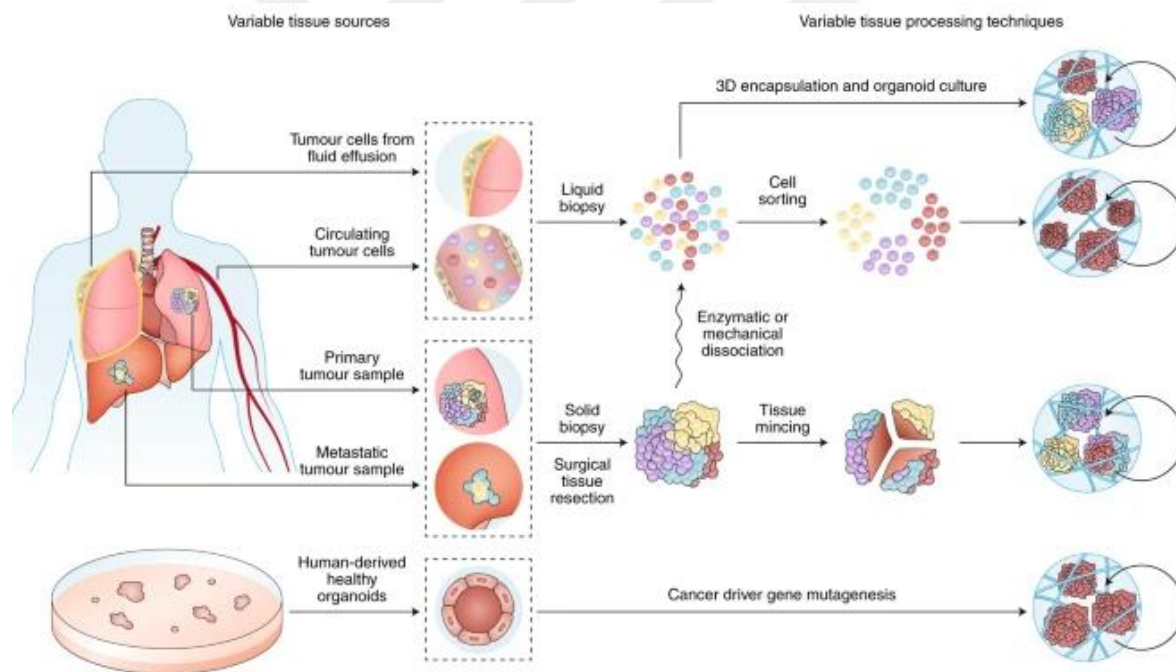


Figure 14. Cancer organoid models

In recent years, models of human tumors such as small intestine, colon, pancreas and colonic carcinomas have been created by using organoids obtained in culture in a three-dimensional matrix of adult stem cells or pluripotent stem cells, and it has been reported that these models are the most realistic in vitro models (Kim, Koo, & Knoblich, 2020). Briefly, these models are obtained by establishing organoid cultures from patient tumors or by establishing relevant

cancer-inducing mutations in organoids obtained from healthy volunteers. Organoid briefly, is a mini-culturable stably long-term culture, where the spatial cell distribution in the real tissue appears and preserves its function and physiology in the real tissue, which is formed by imitating the key elements of real embryogenesis such as self-assemble and self-renewal. They are organ-like 3-dimensional structures (J. Lee & Koehler, 2021; Sato et al., 2009). In 2015, Hans Clevers and Toshiro Sato demonstrated for the first-time in vitro modeling of a cancer disease using organoid technology together with CRISPR-Cas9 technology, a revolutionary technology in genome editing. In this study, consecutive deletions and mutations in driver genes (K-Ras, APC, p53 and SMAD4) in colorectal adeno-carcinoma were created by CRISPR-Cas9 technology in human intestinal organoids. With this model, they showed that multistep carcinogenesis of colorectal cancer and they can create this cancer model similar to the real scenario. Again, in these important studies, the researchers used selection strategies based on growth factors used in organoid cultures, since the actual efficiency of the CRISPR-Cas9 system is low. Accordingly, they removed ligands such as Wnt3a/R-spondin, Noggin, and EGF, which activate WNT, TGF β , BMP and EGF signaling pathways, which are vital for the formation and maintenance of organoid cultures of tissues such as the colon and liver. Thus, the selection of mutant organoids in terms of these signaling pathways was achieved through growth advantage (Drost et al., 2015; Matano et al., 2015). In this study, after p53 was "knocked out" with the CRISPR-Cas9 system, a chemical called Nultlin-3, which inhibits the Mdm2 molecule and stabilizes the p53 protein, was added to the culture medium, causing cells without p53 knockout to enter into apoptosis, and selection was carried out in this way.

Using this approach, within the scope of our project, it was aimed to model HCC by generating mutations in the p53, APC and NF1 driver genes, respectively, in healthy human liver organoids with CRISPR-Cas9 technology. It is predicted that this model will be the most realistic model that can be used to elucidate possible mechanisms in the early development process of HCC and to develop new treatment options for these mechanisms. In addition, the model has the potential to be used as a model that will form the basis for the determination of other genes that have an activating role in the early HCC process. Again, within the scope of this study, the role of p53 in the development of an early stage fibrotic background and the potential to develop a fibrosis organoid model were predicted.

2.8 CRISPR/Cas9 Mediated Gene Regulations:

The CRISPR (Clustered Regularly Interspaced Palindromic Repeats) system, which will be used to obtain mutant organoids within the scope of the study, is defined as a genome engineering technology. This revolutionary technology enables genome editing in various fields of research (Jinek et al., 2013). CRISPR was first discovered as a defense mechanism of prokaryotes against foreign nucleic acids and functions as RNA-directed endonuclease complexes (Ratner, Sampson, & Weiss, 2016; Shabbir et al., 2016). The defense genome of prokaryotes consists of a CRISPR region and surrounding palindromic repeats. During defense, this DNA region is transcribed and spacer and single-stranded CRISPR RNAs (crRNA) are encoded. These sequences are then complementary matched to their target foreign nucleic acids (Barrangou et al., 2007; Barrangou & van Pijkeren, 2016). At this stage, the foreign nucleic acid is identified and degraded by the Cas (CRISPR associated) enzyme, which has the endonuclease activity of the defense mechanism. Studies by Feng Zhang and George Church have shown that the CRISPR/Cas9 system can also be applied in eukaryotic cells (Cong et al., 2013; Mali et al., 2013). Genome editing with the Cas9 enzyme occurs in two different ways. First, after DNA is cut by double helix Cas9, cells repair this DNA damage by NHEJ (non-homologous end joining) mechanism. Because the NHEJ repair system is a mechanism that requires specific polymerases, nucleases, and ligases, it is an error-prone process. Therefore, in the repair process that takes place in this way, nucleotide addition (insertion) or loss (deletion) in the damaged area results. If the repaired site contains a protein-coding sequence, this type of repair mechanism exhibits a frameshift due to the appearance of premature stop codons and/or nucleotide insertion. This leads to loss of the function of the targeted gene.

The second mechanism is that the special enzymes of the cells place a template DNA sequence (donor template) surrounded by homologous sequences to the site of the damage on the DNA. This type of repair is used to insert a sequence of interest into the genome and is defined as homology directed repair (HDR). Eukaryotic cells often prefer the NHEJ mechanism for DNA damage repair because the probability of finding a template sequence in the other method is very low (unless it is given synthetically from the outside) (Figure 15) (Nambiar et al., 2019).

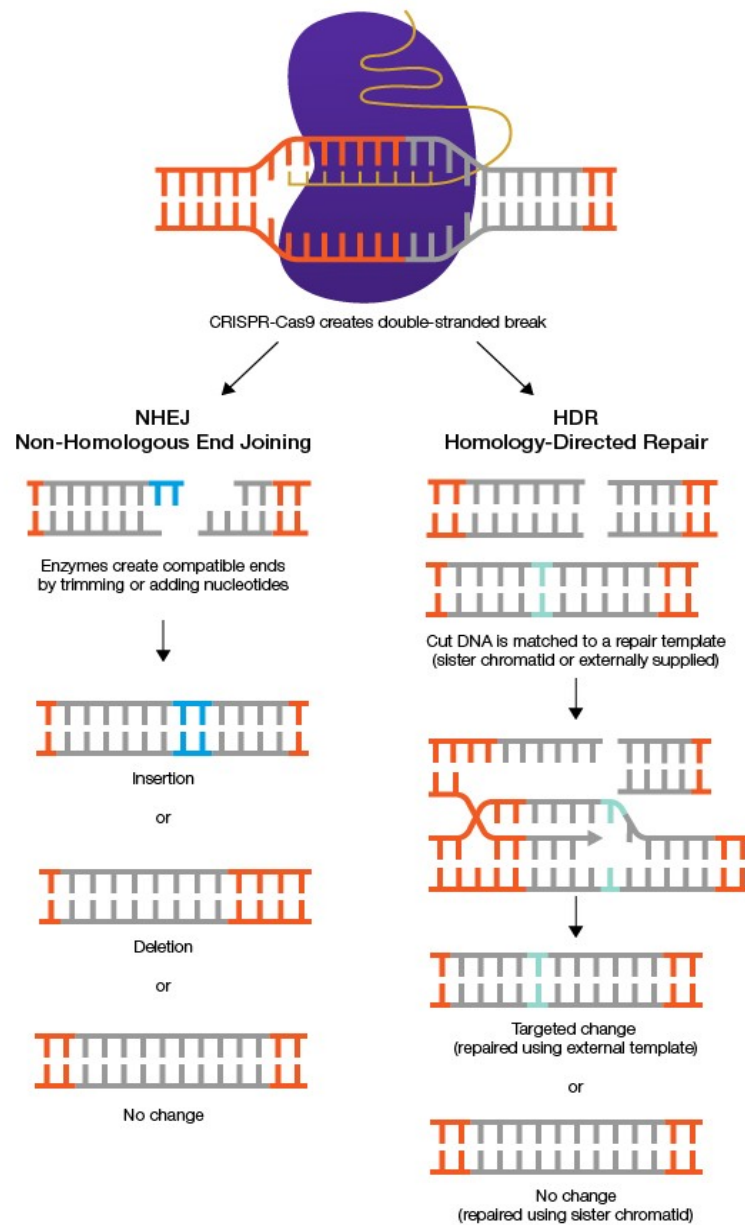


Figure 15. CRISPR-Cas9 editing

In this way, gene deletions and frameshift mutations can be successfully performed in cell culture with guide RNA (gRNA) designed to specifically bind to the DNA sequence of the gene of interest (Barrangou & van Pijkeren, 2016; Jasin & Haber, 2016; Paquet et al., 2016).

3. MATERIALS AND METHOD

3.1. Type of research

The related study is descriptive and experimental.

3.2. Place and time of research

The related study was carried out between 07/09/2017 and 05/31/2022 in İzmir Biomedicine and Genome Center.

3.3. The Universe and Sample of the Research

In the related study, no human material was used directly, instead cells converted to iPSC from fibroblasts from a healthy donor were the source of the study. These iPSC cells were maintained as hepatic organoid culture (eHEPO), which was shown to have functional liver functions within the scope of a written project, and these cells were used in our study.

3.4. Study material

In the study, healthy liver organoids produced from iPSC cells differentiated from fibroblast-derived cells obtained with the consent of the patient within the scope of the ethics committee were used.

3.5. Variables of the study

In our study, fibrotic responses of WT and p53 KO organoids in a pro-carcinogenic microenvironment were investigated. The independent variables are incubation of WT and p53 KO organoids for the same times as the pro-carcinogenic microenvironment. The dependent variable is that the resulting microenvironment due to incubation increases the expression of fibrotic markers.

3.6. Data collection tools

3.6.1. Material

Table 1. Type and model of Equipment

Type and Model of Equipment	Name of place or Cat no:	Purpose of Use
Class II biological safety cabinet (Thermo Scientific Holten Safe)	IBG	For cell culture studies
CO ₂ incubator (memmert)	IBG	For cell culture studies

Cell culture centrifuge (Eppendorf Centrifuge, 5702)	IBG	For cell culture studies
Spectrophotometer (Thermo Nanodrop 2000)	IBG	For quantity/quality measurement of DNA and RNA
Centrifuge (Thermo Scientific MicroCL 17R)	IBG	For centrifugations
Liquid nitrogen tank (Thermo Cryoplus 2)	IBG	For storage of organoids
+4C Refrigerator (Bosh)	IBG	For storage of organoids
-20 Refrigerator (Bosh)	IBG	For storage of organoids
-86 Refrigerator (Thermo scientific)	IBG	For storage of organoids
-140 Nitrogen Tank (Thermo scientific)	IBG	For storage of organoids
Lonza 4D nucleofector	IBG	Transfection of organoids
BD FACS Aria™	IBG	Cell sort
Fortessa flow cytometry	IBG	Cell analysis
Sterile serological pipette	IBG	Cell Culture
Cell culture dishes 96 well plate	IBG	Seeding cells
Cell culture dishes 48 well plate	IBG	Seeding cells
Cell culture dishes 24 well plate	IBG	Seeding cells
Cell culture dishes 12 well plate	IBG	Seeding cells
Cell culture dishes 6 well plate	IBG	Seeding cells
Eppendorf (200ul-500ul-1.5ml-2ml-5ml)	IBG	Qpcr, medium collecting
Mesh (100-40µm)	IBG	Before cell sort separate double cells
Filter 0.2 and 0.45 um	IBG	Secretom filtering
Syringes (10-20-50)	IBG	Secretom filtering
Filtered types(10ul-100ul-200ul-1000ul)	IBG	Cell culture
Cryo vial tubes,	IBG	Freezing of the cells
Flow tubes	IBG	Cell sort and flow analysis
Slide	IBG	OCT staining's
15 ml Falcon	IBG	Cell culture

50 ml Falcon	IBG	Cell Culture
Advanced DMEM F12	IBG	Cell culture
B27	IBG	Cell Culture
N-acetylcysteine	IBG	Cell culture
Gastrin	IBG	Cell Culture
epidermal growth factor (EGF)	IBG	Cell culture
R-spo1 CM	IBG	Cell Culture
fibroblast growth factor 10 (FGF10)	IBG	Cell culture
hepatocyte growth factor (HGF)	IBG	Cell Culture
Nicotinamide	IBG	Cell culture
A83.01	IBG	Cell Culture
Forskolin	IBG	Cell culture
BMP7	IBG	Cell Culture
FGF19	IBG	Cell culture
DAPT	IBG	Cell Culture
Dexamethasone	IBG	Cell culture

3.6.2 Methods

3.6.2.1 iPSCs culture and Hepatocytes differentiation

Human-inducible pluripotent stem cells (hiPSCs) were cultured with mTESR1 medium in a Matrigel- coated culture dish. After the cell density reached 70%, the hiPSC medium was replaced with RPMI/ B27 medium containing 50 ng/mL Wnt3a ,100 ng/mL Activin A, and 10 ng/mL HGF for 3–5 days to induce endoderm cells. Subsequently, endodermal cells were differentiated to progenitor cells by changing RPMI medium with the knockout [KO]/DMEM containing 20% knockout serum replacement, 1 mM L-glutamine, 1% nonessential amino acid, 0.1 mM 2-mercaptoethanol, and 1% dimethyl sulfoxide for further 4 days. In the final stage, cells were cultured with Iscove's modified Dulbecco's (IMDM) medium containing 0.5 uM dexamethasone, 20 ng/mL oncostatin M, and 50 mg/mL ITS premix for 5 days in order to obtain mature hepatocytes

3.6.2.2 SA-B galactosidase assay

SA-bGAL activity was measured by using the Senescence Detection Kit (BioVision; Cat #K320-250, Milpitas, USA) per manufacturer's instructions. Briefly, cells were seeded into triplicates in 6-well plates, washed twice with 1X PBS, and then fixed with formalin for 10 min at room temperature. Next, cells were washed twice with 1 X PBS and incubated overnight at 37 °C with b-gal substrate in an acidic buffer (pH 6.0). Next day, cells were examined with an inverted microscope and photographed (Olympus BX41).

3.6.2.3 QPCR

Total RNA was isolated using the GeneJET RNA purification kit (Thermo Fisher Scientific Baltics UAB; Cat #K0732, Vilnius, Lithuania) according to manufacturer's instructions, and the RNA concentration was detected using NanoDrop. One microgram of RNA was then converted to cDNA using a Maxima First Strand cDNA Synthesis Kit or Thermo RevertAid cDNA Synthesis Kit. For RT-qPCR experiments, expression levels were determined in quadruplicate on a 7500 Fast RT PCR System using the TaqMan Universal Master Mix or Promega Gotaq. The relative gene expression was normalized to the RPL41 gene and calculated by using the $2^{-\Delta\Delta CT}$ method. The primers are given in Table 2.

Table 2. Taqman and Gotaq specific primers for qPCR.

GENE ID	FORWARD PRIMER	REVERSE PRIMER	PROP NO
AFP	tagcgctgcaacaatgaag	tctgcaatgacagcctcaag	6
AXIN2	gatatccagtgatgcgctga	actgccacacgataaggag	56
CK19	agtaccagcggctcatgg	cttcctgtccctcgagca	15
COL1A1	atgttcagctttgtggacctc	ctgtacgcaggtgattggtg	15
CTNNB1	tggttaattcttggtattacgaca	ccaccactagccagtatgatga	8
EPCAM	ccatgtgctggtgtgtgaa	tgtgttttagttcaatgatgatcca	3
HNF4A	acggacagatgtgtgagtgg	gcaggagcttatagggtcag	27

IL-6	gatgagtacaaaagtcctgatcca	ctgcagccactgggtctgt	40
KLF4	cgttccagtgcacaaaatg	catgtgtaaggcgaggtggt	85
RPL41	ggccttagcgccattttt	ttggacctctgcctcatctt	41
TGFB-RI	gcagacttaggactggcagtaag	agaacttcaggggccatgt	5
GENE ID	FORWARD	REVERSE	Gotaq
CCL22	GCCTACTCTGATGACCGTGG	GAGAGTTGGCACAGGCTTCT	No prop
CD206	ACTCCACTTTCAGTGCCTGG	TCCATCCGTCCAAAGGAACG	No prop
COL3A1	TGGAGGATGGTTGCACGAAA	ACAGCCTTGCGTGTTTCGATA	No prop
MCP1	AGATCTGTGCTGACCCCAAG	GGAGTTTGGGTTTGCTTGTCC	No prop
MMP9	GTACTCGACCTGTACCAGCG	AGAAGCCCCACTTCTTGTGCG	No prop
PDGFRB	GTCGGAGACCCGGTATGTG	CTTAGCTCCAGCACTCGGAC	No prop
SERPINA1	CCACCGCCATCTTCTTCTGCCTGA	GAGCTTCAGGGGTGCCTCCTCTGTG	No prop
TNFA	TCAGAGGGCCTGTACCTCAT	GGAGGTTGACCTTGGTCTGG	No prop

3.6.2.4 Organoid Culture

The creation and maintenance of organoids was carried out by our group as outlined in the study published in 2019 (Figure 16). Briefly, the organoids already in the nitrogen tank were dissolved and centrifuged with Advanced DMEM F12, and the pellet was mixed with 60% matrigel and 40% medium and inoculated into 30-50 µl droplets. Plates were turned upside down and placed in the incubator for 15-30 minutes to freeze. Then, 1% B27, 1.25 mM N-acetylcysteine, 10 nM gastrin, 50 ng/mL epidermal growth factor (EGF), 10% R-spo1 CM, 100 ng/mL fibroblast

growth factor prepared in Advanced DMEM F12 necessary for their growth. EM (Expansion medium) medium containing 10 (FGF10), 25 ng/mL hepatocyte growth factor (HGF), 10 mM nicotinamide, 5 μ M A83.01 and 10 μ M forskolin was added. Passaging was performed after the growth of the organoids. After approximately 6-8 days, the cells were removed from the plate with the help of cold medium and the pelleted organoids were fragmented by pipetting and passaged at a ratio of 1:4-1:8 depending on the cell density in the droplets.

EM keeps cells in the culture medium at the definitive endoderm stage, these cells need to be differentiated into hepatocytes for functional studies. For this purpose, cells are treated with 25 ng/ml BMP7 for 3-5 days and then 1% B27, EGF (50 ng/mL), gastrin (10 nM), HGF (25 ng/mL), FGF19 (Incubate for 8-10 days in differentiation medium (DM) containing 100 ng/mL), A8301 (500 nM), DAPT (10 μ M), BMP7 (25 ng/mL), and dexamethasone (30 μ M). Following day 5, cells appear to differentiate into hepatocytes.

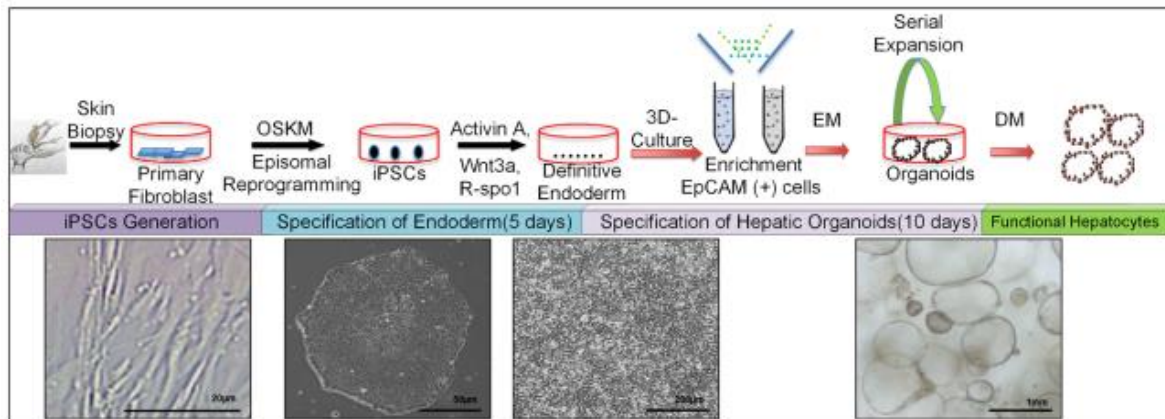


Figure 16. Organoid culture from IPSCs

3.6.2.5 Primer Design

The gRNA used for p53 knockout was taken from the reference article (Senturk et al., 2017). Accordingly, the gRNA sequence targeting exon 4 is as follows.

TP53-F 5'- CACCGCCATTGTTCAATATCGTCCG -3'

TP53 -R 5'- AAACCGGACGATATTGAACAATGGc -3'

Two designs were made for the NF1 primer gRNA using the CRISPOR primer design tool.

NF1_3F 5' CACCGCTCGTCGAAGCGGCTGACCA 3'

NF1_3R 5' AAAGTGGTCAGCCGCTTCGACGAGC 3'

NF1_4F 5' CACCGCGCGCACAGGCCGGTGAAT 3'

NF1_4R 5' AAACATTCCACCGGCCTGTGCGCGC 3'

Primer design for the APC gene:

Reference primers from the article:

APC-EXON-16F 5' - CACCgGTTTGAGCTGTTTGAGGAGG- 3'

APC-EXON-16R 5'- AAACCCTCCTCAAACAGCTCAAACc - 3'

APC-EXON-6F 5' - CACCgAAGGCAAATCAGAGTTGCGA- 3'

APC-EXON-6R 5' – AAACTCGCAACTCTGATTTGCCTTc - 3'

APC-EXON-7F 5' – CACCgGTCCCAAGCAACAGAAGCAG - 3'

APC-EXON-7R 5' – AAACCTGCTTCTGTTGCTTGGGACc - 3'

Primers designed with the CRISPOR tool

APC#E-5F 5' CACCGCCCCGGCTTCCATAAGAACGG 3'

APC#E-5R 5' AAACCCGTTCTTATGGAAGCCGGGC 3'

APC E1 #F6 5' CACCGTGCTGCCGGACTCGGAAAT 3'

APC E1 #R6 5' AAACATTTCCGAGTCCGGCAGCAC 3'

APC E10 F#7 5' CACCGTGATATCCATGCGACAGTC 3'

APC E10 R#7 5' AAACGACTGTCGCATGGATATACAC 3'

3.6.2.6 Validation of Primers

In order to carry out primer validations, the primer oligos should be double stranded (1.3.1) and cloned into linearized (1.3.2) the empty Px458 (Figure 17) plasmid carrying the GFP sequence and Cas9 sequence (1.3.3 - 1.3.4).

3.6.2.6.1 Annealing of primers

Primer pairs prepared in the PCR tube as given below are incubated for 5 minutes at 25°C for 5 minutes, starting from 95°C for 30 minutes at 37°C and decreasing 5 degrees each to 25°C.

1 ul oligo 1 (100mM)

1 ul oligo 2 (100mM)

1 ul 10X T4 Ligation Buffer (NEB)

6.5 ul ddH₂O

0.5 ul T4 PNK (NEB)

10 ul Total

3.6.2.6.2 Cut and linearization of px458 empty plasmid with BbsI plasmid

1ug pX458 empty plasmid is cut with BbsI enzyme and incubated for 30 minutes at 37C.

1 ug pX458

1 ul Fast Digest BbsI (Fermentas)

1 ul Fast AP (Fermentas)

2 ul 10X Fast Digest Buffer

X ul ddH2O

20 ul total

The product obtained after cutting is loaded with agarose gel and the linear plasmid observed to be cut is isolated from the gel and made ready for ligation with the cleanup kit.

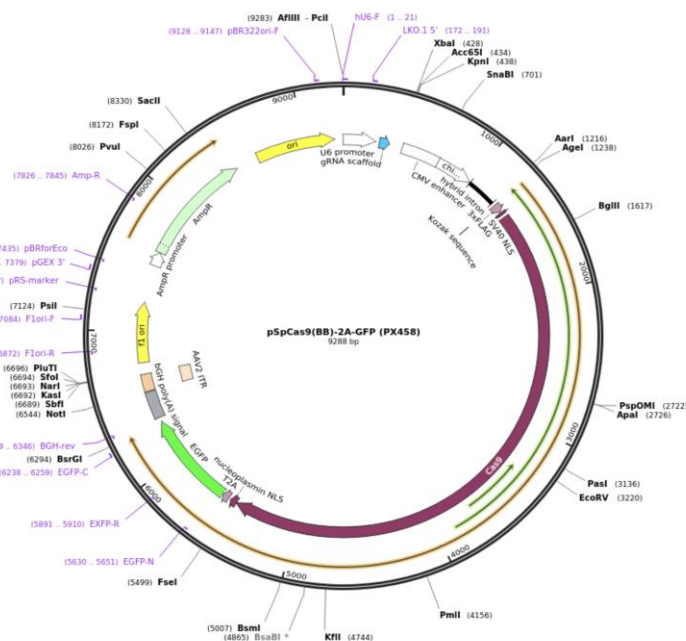


Figure 17. Empty px458 plasmid

3.6.2.6.3 Ligation of linearized empty plasmid with annealed primer

X ul (50 ng) pX458 vector with BbsI cut in step 1.3.2 and cleaned with clean up kit

1 ul (1:200 diluted) phosphorylated and coupled oligo duplex in step 1.3.1

1 ul 10x T4 DNA ligase buffer

0.5 ul T4 DNA Ligase

X ul ddH2O

10 ul Total

Ligation reaction is established and 3-16 h ligation is left and transformation is started.

3.6.2.6.4 Transformation of ligated NF1-px458 plasmids with Stbl3

1. A 100µL bacterial stock, previously rendered competent and stored at -80°C, is kept on ice for 10 minutes. 10 µl of plasmid obtained from the ligation reaction obtained from 1.3.3 is added and incubated on ice for 30 minutes.
2. Bacteria are removed from ice and incubated for 90 seconds in a 42°C heat block or water bath.
3. Bacteria taken from 42°C are kept on ice for 2-5 minutes.
4. Then, 900µL of LB (or SOC) broth pre-incubated at 37°C is added onto the bacteria and the bacteria are dispersed in the broth by gentle pipetting.
5. Cells are incubated for 1 hour on a shaker at 37°C.
6. Cells taken from the shaker are centrifuged at 2500 rpm for 3 minutes, the supernatant is removed.
7. 900µL of supernatant is drawn over the collapsed cells with a micropipette and discarded. Cells that have settled as pellets are resuspended in 100µL of medium remaining in the tube.
8. Bacteria are seeded on LB agar plates containing the antibiotic in which the plasmid carries the resistance gene. (Bacteria without transformation as a control group, only groups without insert, without insert and ligase are used for ligation.) and incubated at 37°C overnight.
9. Selected from single clones, incubated in 5 ml of LB medium containing antibiotics, 12-16 hours at 37°C with a shaker, controlled with cutting enzymes by miniprep, and midiprep performed.

3.6.2.6.5 Selection of suitable colonies and isolation of plasmids with miniprep

It was made in accordance with the protocol of Miniprep MN's Nucleospin Plasmid kit. Briefly, 1-5 ml of bacterial stock was dissolved in 250 ul Buffer A1, 250 ul of A2 buffer was added and gently inverted 6-8 times. Then, 300 µl of A3 buffer was added on it and the lysate was centrifuged and the plasmid DNAs in the supernatant were attached to the columns and washed with Buffer AW and A4, and the DNA was eluted.

3.6.2.6.6 Control of isolated plasmids.

Firstly, to check whether the isolated plasmid is correct, EcoRI-HF enzyme cut was performed by cutting our px458 plasmid DNA from two regions and forming an 800 bp band. A standard PCR reaction was established using the universal U6 forward primer of the plasmid DNAs we validated and the reverse primer of the relevant gene. The aim here is to understand whether the cloned plasmid successfully contains the gRNA sequence of our interest. Here, if the cloning of the relevant gRNA sequence into the region is unsuccessful, no product will be seen in the PCR result, and if it is successful, a 250 bp product is expected to be obtained.

10X Taq Buffer	2
MgCl ₂	1
10mM dNTPs	0,5
Forward primer (U6 promoter primer) (10μM) (5'-GAGGGCCTATTTCCCATGATT-3')	0,8
Reverse primer (Reverse oligo) (10μM)	0,8
Taq Polymerase	0,2
ddH ₂ O	X
Plasmid DNA (500ng)	X
Total	20

Step	Temperature	Time
Initial Denaturation	95°C	5 minutes
25-35 Cycles	95°C	30 seconds
	60°C	30 seconds
	72°C	45 seconds
Final Extension	72°C	5 minutes
Hold	4°C	

Clones that were successful in this step were sent to the Sanger sequence control and their accuracy was confirmed. In order to reduce the cost in organoid, the clones that were confirmed to be accurate were first examined how successfully they could silence the target gene in Hek293T cells by the WB method, and the most successful plasmid clone was selected for Organoid studies.

3.6.2.6.7 Transfection protocol in Hek293T cells

For the transfection of Hek293T cells, the cells are seeded into a 6-well plate at 70% density for the next day (350-400 thousand cells/well). The next day, it is incubated for 6 h with the prepared transfection mix with 2.5 μg plasmid at a 1:5 μg DNA/PEI ratio. At the end of the incubation, the medium is withdrawn and the cells are allowed to grow in fresh medium. The next day, the GFP⁺ content is examined under the microscope, and on the 3rd day, the cells with GFP⁺ are separated by FACS method and inoculated on a new plate with a density of 45-50% according to the number of cells removed. The seeded cells are grown in culture for 3-5 days and then the lysate is prepared for western blotting.

3.6.2.6.8 Confirmation of gene silencing by Western in Hek293T cells

Total cell lysate method was used to obtain lysate from Hek293T cells. For this, the cells were incubated with RIPA buffer for 30 minutes on ice and then sonicated for 30 seconds. Afterwards, it was centrifuged at maximum speed for 20 minutes at 4°C and the supernatant was transferred to a new tube kept on ice. Protein concentrations were determined by BCA method. 60 µg protein for each gene and clone was loaded on gels. After the protein was transferred at 400 mA for 4 h, it was incubated with 5% BSA for 1 h blocking and overnight NF1, Bioss(bs-4140R), APC, Abcam(ab154906) antibodies were 1:250 and 1:1000, respectively. After incubation, washings were made with TBST containing 0.1% TritonX-100 and incubated with 1/10,000 secondary antibodies. The results were viewed on the Licor instrument.

3.6.2.7 Transfection in Organoids

1. Y-compound (10 µM) and CHIR (10 µM) were added to the cell medium 2 days before transfection of early passage organoids.
2. After 24 hours, this medium was withdrawn and replaced with medium containing Y-compound + CHIR + 1.25% DMSO.
3. After 24 h, for transfection, the cells were lysed with trypsin for 7-10 min, and the nucleofactor was electroporated with CD150 and EX147, which we saw working well in the nucleofactor.
4. For each transfection 5 µg DNA 100 µl transfection reagent was used and a total of 5x10⁵ cells were transfected.
5. After transfection, 400 µl of Y-compound and CHIR-containing medium was added to the cells, and the cells were waited for 4-8 h for self-assembly and transfection to occur, and then inoculated into Matrigel.
6. Cells 24-48h after transfection were visualized under a fluorescent microscope.
7. Transfection success rates were analyzed by Flow analysis. (With the optimizations the efficiency has been increased from 1-7% to 45%.

3.6.2.8 Flow Cytometry

For flow experiments, 40-50 dots (50 µl) of cells were collected with cold Advanced DMEMF12 and precipitated at 400g for 5 minutes by centrifugation pre-set to +4 degrees. After removing the medium containing the matrigel residues, it was turned into a single cell by trypsinification at 37°C for 8-10 minutes. Cells were then thawed in 1x FACS (2% FBS, 0.5% EDTA, 2.5% HEPES) buffer and treated with EpCAM-FITC (Miltenyi Biotech, Cat#130-110-

998) (1:50), CD133-APC (Miltenyi) Biotech, Cat#130-113-106) (1:50), CD90-APC (Miltenyi Biotech, Cat#130-114-903), CD44-FITC (Biolegend, Cat#103022) and CD24-APC (Miltenyi Biotech, Dyes were made using Cat#: 130-112-657) (1:50). Mouse IgG1-FITC (Miltenyi Biotech, Cat# was used as isotype control. The staining protocol was kept on ice for 15 minutes as recommended, then washed to remove the remaining dye residues. DAPI was added to the sample samples at a final rate of 0.5 ug/ml and the cells were BD LSR Analyzed with Fortessa instrument Results analyzed in FlowJo interface.

3.6.2.9 Reporter assay

For the TCF/LEF and pSBE4luc/pRL-TK luciferase method, cells were harvested, trypsinified and single celled on day 12 as described previously. For each experimental condition, 100 ng of TCF/LEF, 40 ng of Renilla and 200 ng of pSBE4luc 30 ng of pRL-TK plasmids were transfected 1:3 using lipofectamin 3000. Luminometric measurements were taken 24 hours after transfection and the results were edited via GraphPad Prism.

3.6.2.10 Immunohistochemical stainings (IHC)

All organoid samples used for immunohistochemical (IHC) staining were fixed with formalin and embedded in paraffin. 4-5 µm thick sections were prepared from paraffin-embedded tissues and placed on electrostatically charged slides (Xtra™, Surgipath Medical Industries, Richmond, Illinois, USA). It was then deparaffinized and dehydrated using a graded ethanol series using routine protocols. After dehydration, it was stained with primary antibodies. Briefly for this procedure, sections were incubated at 50 °C overnight, dewaxed in xylol and rehydrated with graduated alcohol rinses. For antigen retrieval, all slides prepared for each antibody were heated in a microwave oven (650 W for 5 min plus 450 W for 20 min) in 0.1 mmol/L EDTA buffer (pH 8) or immersed in citrate buffer (10 mmol) in a vacuum. heated for 2.5 minutes and cooled for 20 minutes. Peroxidase activity was blocked by treatment with 30 mL/L H₂O₂ for 5 minutes. The incubation time with monoclonal antibodies was optimized as 30 minutes. Immunostaining antibodies were performed by the streptavidin-peroxidase method (Labvision, TM-125-HL) and alkaline phosphate-based visualization system. 3,3'-diaminobenzidine (Dako, TA-125 HD) was used as the chromogen. Slides were read under a light microscope after staining with hematoxylin. Slides were examined with an Olympus BX61 microscope (Olympus Corporation, Tokyo, Japan) according to the positivity rate method.

Organoids used for periodic acid-Schiff (Caligiuri, Gentilini, Pastore, Gitto, & Marra) staining were similarly fixed with formalin and embedded in parafilm, and 4-5 µm thick sections were

placed on electrostatically charged slides (Xtra™, Surgipath Medical Industries, Richmond, Illinois, USA). Then it was deparaffinized and dehydrated by passing through an ethanol series. The slides prepared for PAS staining were placed in Periodic Acid Solution at room temperature (18–26°C) for 5 minutes and washed several times with distilled water. Then, slides kept in Schiff Reagent at room temperature (18–26°C) for 15 minutes were washed under running tap water. Then, the slides stained with Hemotoxylin for 90 seconds were covered with a xylene-based mounting medium.

Similarly, for Mason Trichome staining, the preparations that were passed through alcohol series and deparaffinized were stained with the help of the kit. Briefly, cells fixed in Bouin's solution for 1 h were stained with Weigert's iron hematoxylin for 10 min. Afterwards, they were stained with Biebrich scarlet-acid fuchsin solution for 15 minutes and then kept in aniline blue solution for 15 minutes. After being rinsed under water and kept in 1% acetic acid solution for 5 minutes, it was dehydrated and covered with mounting medium.

For OCT staining, on Day 12, differentiated organoids (approximately 10 dots/30 µl for each condition) were collected by incubating in cold PBS for 5 min on ice. Afterwards, the organoids were collected without fragmentation and centrifuged at 200 g for 5 minutes and the matrigel was removed. After this process, it was incubated overnight at 4°C in 10% formalin buffer and fixed. It was then incubated in 30% sucrose for 2 or 3 days. At the end of the period, organoids were collected, embedded in OCT and stored at -80°C. Then they were cut at 8 µm thickness and transferred to slides and left for about 1 h to dry at RT. At the end of the time, the OCT was removed by immersing it in distilled water for 5 minutes. Afterwards, the organoids that were drawn around with a pakpen were incubated for 15 minutes with 0.5% permeabilization buffer. After the incubation, it was incubated in the blocking buffer (1% BSA, 10% goat serum, 0.1% Tween 20 0.225g glycine for 10ml) prepared for 1 h and incubated overnight with primary antibodies in the same blocking buffer. The antibodies used in the study are as in Table 3. At the end of the period, it was incubated with secondary antibodies for 1 h and closed with a mounting medium. The images were taken with the C-Apochromate 40x/1.2 W Korr FCS M27 lens on the LSM 880 AxioObserver confocal device and edited with the imageJ program.

Table 3. Antibody details.

Antibody	Cat No	Dilution
Ki67	9449S	1:800

COL1A1	ab34710	1:100
COL4A1	ab6586	1:100
CK19	sc-6278	1:100
CK18	sc-51982	1:100
ALB	sc-51515	1:100
Cleaved-caspase 3	9664S	1:400
H2A.X (Ser139)	9718S	1:400
SERPINA1	ab166610	1:100
ZO-1	40-2200	1:50
PDGFRB	3169S	1:100

3.6.2.11 Albumin Elisa

Albumin Elisa analyzes were performed as specified in the protocol. Briefly, the solutions to be prepared such as coating buffer, wash buffer, blocking buffer, sample diluent/conjugate buffer and stop buffer were prepared as specified and pH adjustments were made. Plates incubated overnight with coating buffer were washed 5 times with wash buffer, 200 µl of blocking buffer was added to each well and incubated for 30 minutes at room temperature. Then, it was washed 5 times with wash buffer, samples and standards were added and incubated for 1 hour. After incubation, it was washed 5 times and incubated with HRP antibodies for 1 hour for detection. Then, it was washed 5 times, 100 µl of TMB solution was added, and it was kept in the dark for 15 minutes. At the last stage, 100 µl of stop buffer was added and the signal was collected at 450 nm. The results obtained were normalized according to cell numbers and the amount of albumin secreted by 1×10^6 cells in 24 hours was presented graphically.

3.6.2.12 Cell titer glo-3D assay

The relevant method was performed as recommended by the manufacturer's kit protocol. Briefly, Cell-titer Glo-3D reagent was added to the Organoid medium at a ratio of 1:1 and centrifuged for 5 minutes at room temperature. Then, it was waited for 25 minutes for the normalization of the signal at room temperature and the luminometric signal was read for 1 second with a Berthold luminometer device and the signal was collected. Obtained values were arranged using GraphPad prism.

3.6.2.13 Pro-carcinogenic TME secretom production

CAF secretome: To produce activated cell secretome from LX-2 cells, cells were stimulated for 24h with 10 ng/ml TGFB-1(in RPMI-2) for myofibroblast formation. It was then replaced with fresh drug-free RPMI-2. After waiting 48h for the activated myofibroblasts to secrete their secretomes during this process, they were filtered to remove cell residues and stored at -20 for adding to the organoid culture. LX2-2 activation was controlled with COL1A1 and PDGFRB (Wei et al., 2019; Yu et al., 2018).

TAM (M2 macrophage) secretome: To prepare the secretome to be obtained from THP1 monocyte cells, the cells were first induced with 200ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 hours to ensure macrophage differentiation (25×10^4 cell in 12 well plate). Then, they were kept in PMA-free environment for 48 hours for the cells to self-recover and incubated with 20ng/ml IL4 for 48 hours to obtain M2 polarization. At the end of the incubation, the relevant secretome was collected by filtration and stored at -20 to add to the organoid medium. Polarization was confirmed by the CD206(MRC1), TGM2 and CCL22 marker expressions (Genin, Clement, Fattaccioli, Raes, & Michiels, 2015; C. Li, Levin, & Kaplan, 2016; Surdziel et al., 2017; Zhao et al., 2015).

3.7. Work Plan and Timeline

Table 4. Work plan and Timeline

Work	Timeline
gRNA design and validation for p53 APC and NF1 genes in HEK293T cells	09.2017-09.2018
First transfection trials in eHEPO using lipofectamine, PEI, X-treame reagent	09.2018-06.2019
First transfection trials in eHEPO using nucleofector	06.2019-08.2019
Optimization of nucleofector method	08.2018-12.2019
Covid-19 pandemic	12.2019-05.2020
Further optimization and obtaining p53 KO organoids	05.2020-09.2020
The validation of p53 KO organoids with IF, Western blot, Sanger seq., and immunohistochemical staining	09.2020-04.2021
Selection medium trials for APC and NF1 KO organoids	04.2021-06.2021

To create a fibrosis model by searching the literature and decide the way and content of the pro-carcinogenic tumor microenvironment	06.2021-07.2021
To obtain a pro-carcinogenic tumor microenvironment produce activated stellate and M2 polarise macrophage medium	07.2021-09.2021
Immunohistochemical staining's in the pro-carcinogenic tumor microenvironment	09.2021-11.2021
Flow analysis and repeats	11.21-12.2021
Albumin ELISA and repeats	12.2021-01.2022
qPCR and repeats	01.2022-02.2022
Reporter assays and repeats	02.2022-03.2022
OCT staining for Coll, CollIV, ZO1, PDGFRB, A1AT	03.2022-04.2022
Check results for other p53 KO clons with immunohistochemical staining	04.2022-05.2022

3.8. Data Assessment

All statistical analyzes were performed using GraphPad Prism 7 (GraphPad Software, Inc., California, United States). Two-tailed unpaired student t-test or two-way anova was used to determine statistical changes according to the relationship of the groups with each other. For statistical evaluation, the process “> 0.05” (n.s.), “≤ 0.05” (*), “≤ 0.01” (**), “≤ 0.001” (***), “≤ 0.0001” (****) was used. Image J program was used to analyze OCT images. The Flowjo program was used to analyze and graph the Flow results.

3.9. Limitations of Study

Due to insufficient budget, detailed molecular analyzes for all three genes originally planned in the research had to be continued with a single gene. At the same time, GFP+ cells, which is one of the most important steps of the study, could not be separated via FACS correctly and effectively due to the problems caused by the device and personnel used in the IBG Flow unit. For this reason, studies with APC and NF1 genes were stopped and focused on early fibrosis mechanisms that developed after p53 mutation. Although our study showed that p53KO organoids can be used as an effective fibrosis model, the possible molecular mechanisms of p53 triggering the fibrotic process could not be examined in detail due to insufficient budget.

3.10. Ethics Committee Approval

The related project was found suitable for the execution of the studies by IBG-HAYDEK with the meeting numbered 18/2017 on 06/09/2017.

 T.C. DOKUZ EYLÜL ÜNİVERSİTESİ İZMİR ULUSLARARASI BİYOTİP VE GENOM ENSTİTÜSÜ HAYVAN DENEYLERİ YEREL ETİK KURULU (İBG-HADYEK) KARARI 			
TOPLANTI TARİHİ	06/09/2017	TOPLANTI GÜNÜ	Çarşamba
TOPLANTI SAYISI	13	TOPLANTI SAATİ	15:00

Sayın Prof. Dr. Esra ERDAL,

18/2017 protokol no'lu "Karaciğer Kanseri'nin modellenmesinde İndüklenebilir Pluripotent Kök Hücre (iPSC) kaynaklı Hepatik Organoidler" isimli projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.
Bilgilerinizi ve gereğini rica ederiz.

 Prof. Dr. Esra ERDAL Başkan	 Prof. Dr. H. Alper BAĞRIYANIK Başkan Yardımcısı
 Prof. Dr. Belgin UNAL Üye	 Doç. Dr. Ralph Meuwissen Üye
 Doç. Dr. Devrim PESEN OKVUR Üye	(Katılmamıştır) Doç. Dr. H. Güneş ÖZHAN Üye
 Uzm. Kerem ESMEN Üye	 Uzm. Umur KELEŞ Üye
(Katılmamıştır) Ecz. Ferdane KAHRAMAN Üye	

4. RESULTS

4.1 SASP mediated microenvironment modulates the hepatocytes senescence morphology and stemness

After a certain proliferation step of cells enter the senescence. Senescent cells have structural alterations, from enlarged and more flattened morphology, altered composition of the plasma membrane (PM), accumulation of lysosomes, mitochondrial changes and nuclear changes are observed. Also, senescent cells secrete same cytokine and chemokines to modulate their microenvironment. This is also called as senescence associated secretory phenotype (SASP) and have role on near tissue proliferation, stemness, cell cycle arrest and also immune cells activation or inhibition in the damaged area. SASP potently fuels the chronic, proinflammatory systemic state that characterizes aging, impairs the regenerative ability of stem cells, and increases the risk of developing multiple pathologies including cancer.

Within the scope of the thesis, it has been shown that cancer stem cells create different senescence and stemness responses depending on the microenvironment, and these responses vary depending on the expression of p53 (Karabicici, 2021) In this context, condition medium (secretome) collected from cells with and without cancer stem cell characteristics was placed on mature hepatocyte differentiated cells produced from iPSCs and examined for 5 days. At the end of this period, it was observed that the conditioning medium collected from the Doxorubicine-induced population, especially without stem cell characteristics, caused a more pronounced senescence morphology as well as an increased stemness in hepatocytes (Figure 18).

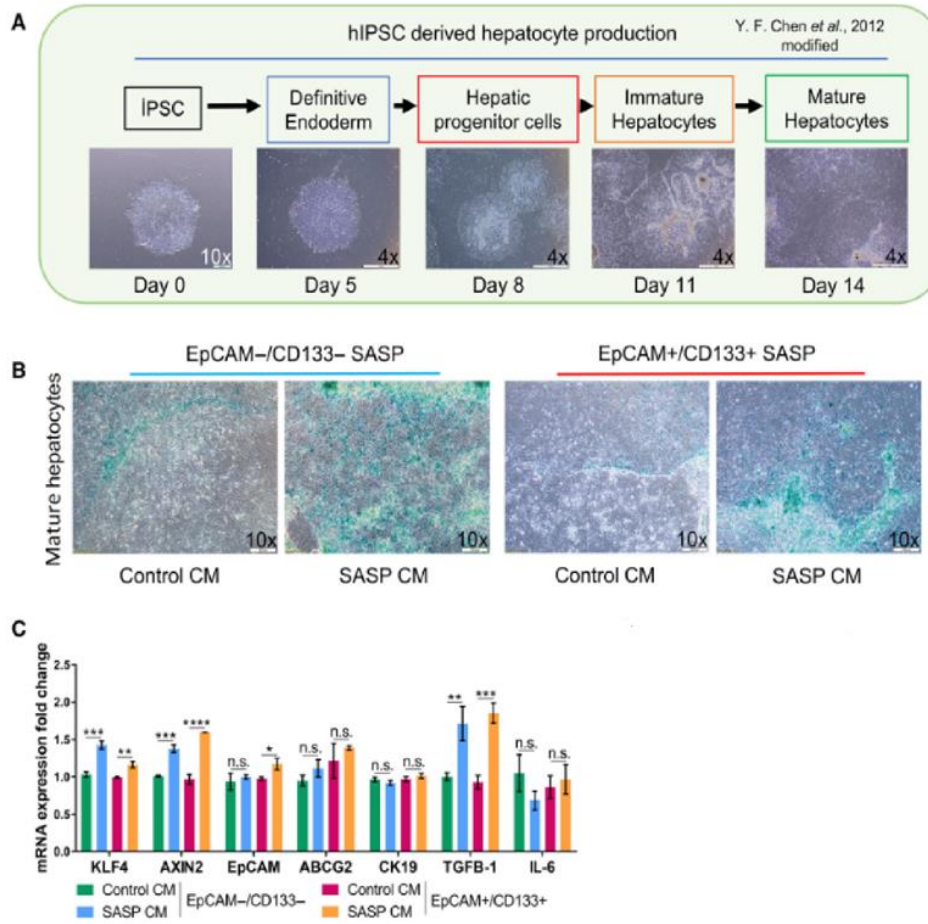


Figure 18. SASP mediated microenvironment trigger senescence morphology and stemness in the iPSCs derived mature Hepatocytes. (SASP: Senescent associated secretory phenotype) A. iPSC derived mature hepatocytes steps. B. SASP medium treated mature hepatocytes after 5-day treatment. C. SASP mediated changes in stemness marker gene expressions.

Similarly, an increase in senescence morphology was observed after induction with SASP in HuH7 parental cell lines (Figure 19).

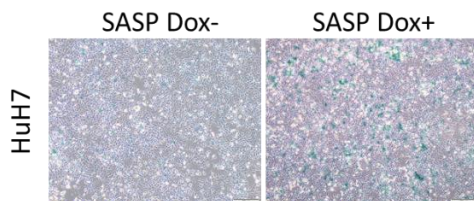


Figure 19. SASP induced senescence in HuH7

In our ongoing studies, when the senescence responses in different cell lines were examined, it was observed that the senescence responses changed depending on the expression of p53 in the cell (Figure 20).

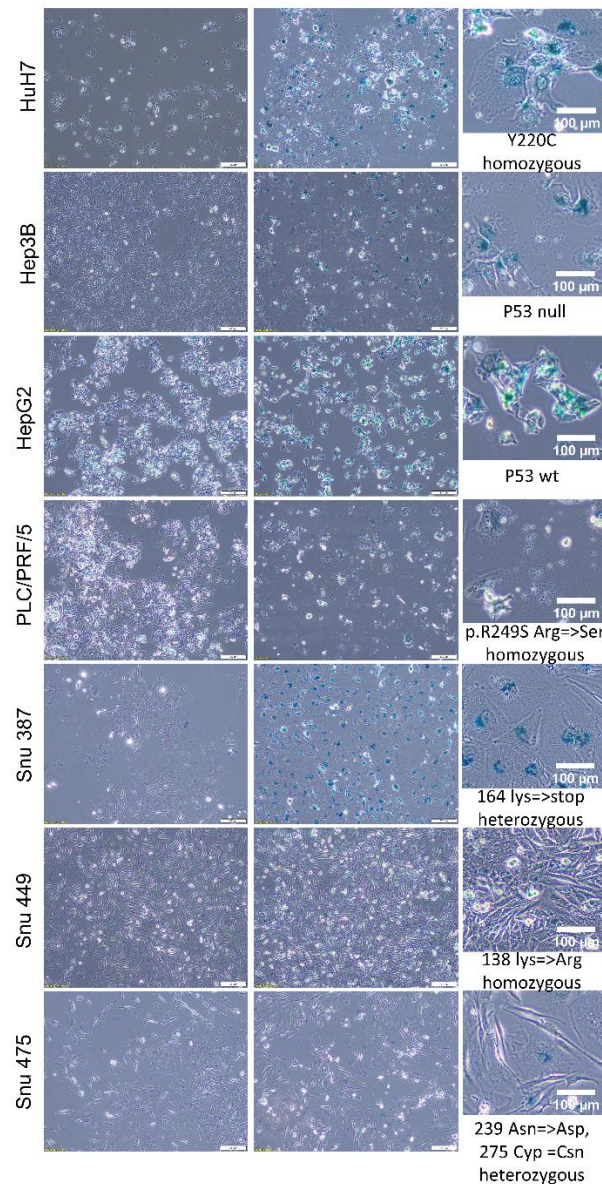


Figure 20. Senescence morphology in different HCC cell lines.

As a result, it has been shown in our previous study that the microenvironment modulates stemness, which causes different responses in cancer cells depending on p53, and in the continuation of our studies, it is aimed to establish a fibrosis model in eHEPO organoid culture in order to elucidate the relationships of the senescence-related fibrotic processes of the microenvironment with p53. In this context, p53 KO-eHEPOs were obtained by CRISPR method and cultured with pro-carcinogenic microenvironment. To mimic the pro-carcinogenic microenvironment, secretomes collected from LX2 and macrophage cells were added to the culture medium of WT and P53-KO eHEPOs mutant with the CRISP method, the optimization steps of which are described below.

4.2 Increasing transfection efficiency in eHEPOs

Candidate genes based on a one-step knockout strategy are planned to be silenced in eHEPOs. For this reason, attempts were made to transfect the p53 gene into organoids, but the success rate could be increased up to a maximum of 4% with chemical methods such as lipofectamine 2000, lipofectamine 3000, X-treameGENE reagent, Fusion, and PEI tested in organoid culture. In addition, it is reported in the literature that the CRISPR/Cas9 system works with 5-10% efficiency even in cell experiments where transfection is very good (Hendriks, Artegiani, Hu, Chuva de Sousa Lopes, & Clevers, 2021).

For this reason, the low transfection efficiency we encountered in organoid culture in the ongoing processes has emerged as an obstacle to be overcome. At this point, different optimizations were tried in chemical-based methods such as lipofectamine, PEI and X-treameGENE, but no significant increase in yield rates could be obtained. For this reason, instead of chemical methods, nucleofector technology, which is a physical transfection-based method that makes the cell membrane permeable by applying a sudden electric current in a certain range and moves the molecules to the cell through the electric field, has been used.

In this context, many programs, each of which is characterized by different electrical pulses and durations, suggested by the device for the relevant cells and cell groups, have been tried to transfer the P53 plasmid to WT organoids. Although the efficiency was lower than we expected in the first trials, it was observed that some programs provided transfection efficiency around 7-8%. In this context, as a result of several different trials we conducted, the most efficient (high transfection rate and low cell death) programs EX147, CD118 and DS150 were selected for further studies and electroporation-based transfection protocols made in different organoid types in the literature were tried for optimizations in the nucleofector protocol (Figure 21-22).

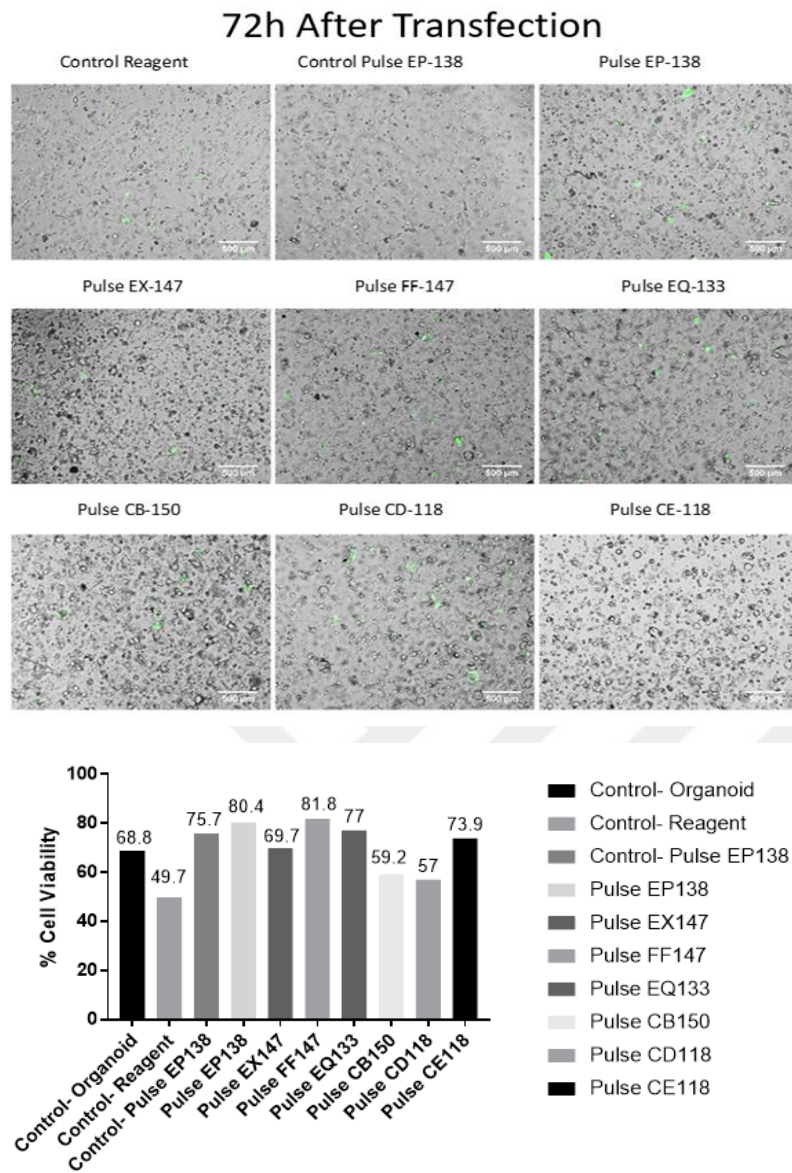


Figure 21. Effect of different nucleofector programs on cell viability

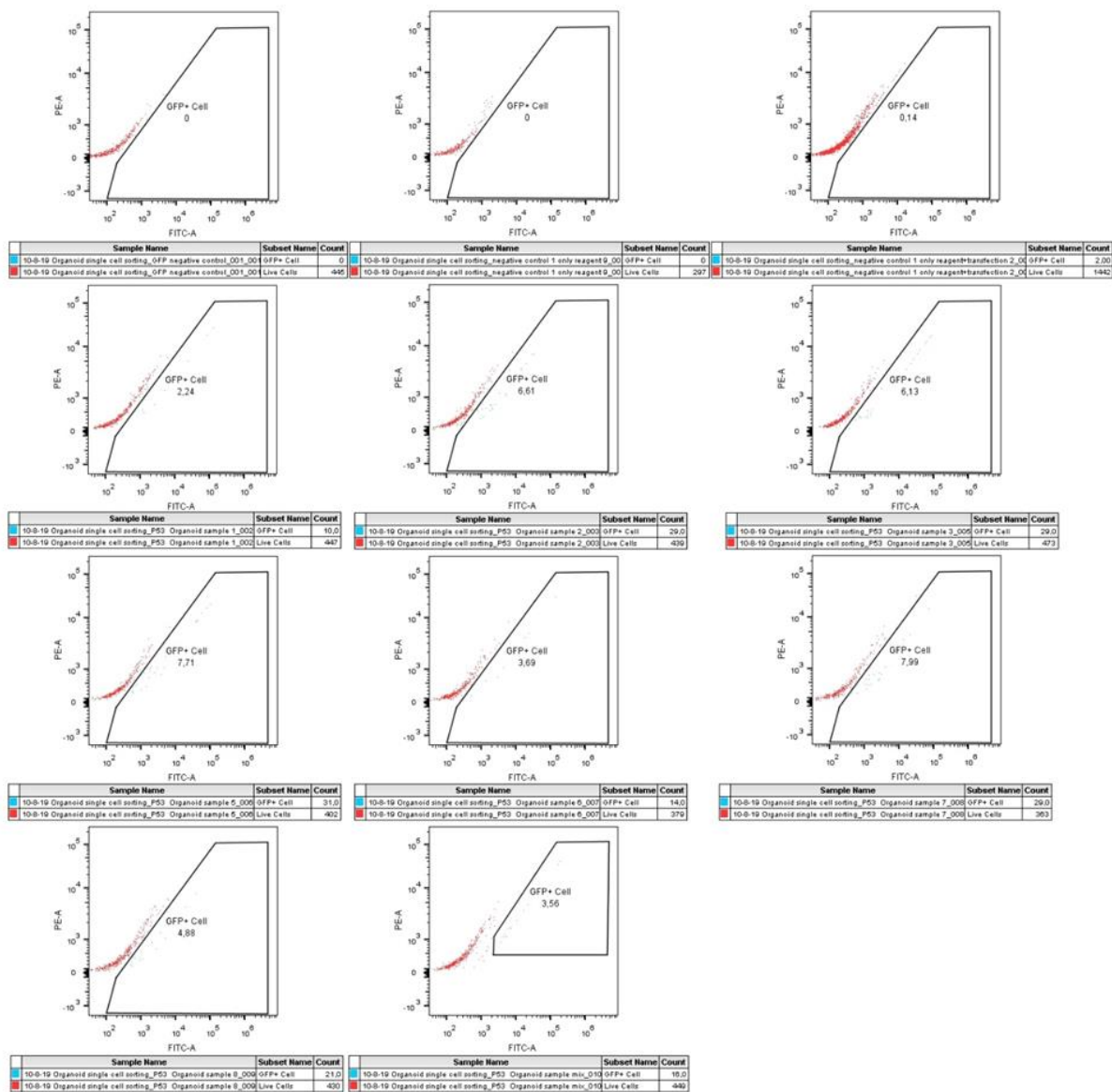


Figure 22. Determination of transfection success of different nucleofector programs.

One of these studies, were able to successfully perform gene editing in intestinal organoids using the electroporation method and shared their detailed protocols (Fujii, Matano, Nanki, & Sato, 2015). In this study, it was stated that in order for the cells to have high pre-transfection efficiency, they should be in early passage and passaged 2-3 days before transfection. After the cells were passaged, additional Rock inhibitor Y-compound (10 μ M final concentration) and GSK3B inhibitor CHIR (5 μ M final concentration) were added to the cell medium. It was stated that after 24 h, when an extra 1.25% DMSO was added to the medium, the transfection efficiency increased up to 8 times. After transfection, extra Y-compound CHIR and DMSO were added to the medium and incubated for 24 h. Afterwards, DMSO was withdrawn from the

medium and after 2 days of supplementation with CHIR and Y-compound only, puromycin-mediated selection was performed.

In another study, it was reported that removing antibiotics from the medium increases the yield, and nicotinamide can be added to the medium to increase survival after transfection (Merenda et al., 2017). In another study conducted in 2020, optimization studies were carried out in different organoids with the plasmid px458, which we used in our study, and then GFP positive cells were selected by FACS method. It has been reported that the efficiency can reach 40% in this process (Gaebler et al., 2020).

Based on these protocols, we added extra Y-compound (10 μ M) and CHIR (10 μ M) to the cell medium 2 days before the transfection as seen in Figure 23 in the first step. After 24 hours we replaced it with media containing Y-compound + CHIR + 1.25% DMSO.

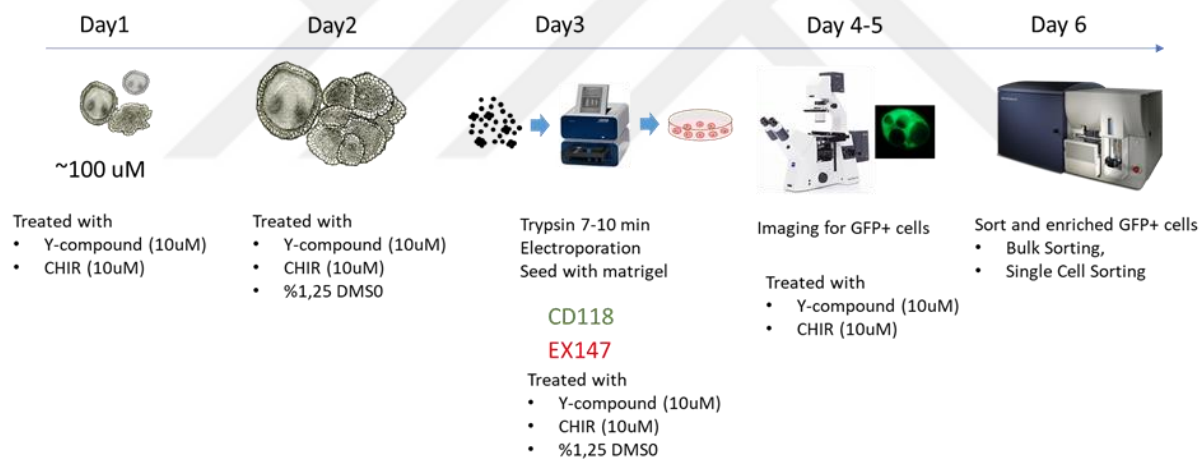


Figure 23. Summary of transfection optimization experiment with p53-KO plasmid in eHEPO

Cells were lysed with trypsin for 7-10 min without being single cell and electroporated with CD118, EX147 and DS150, which we saw working well in the nucleofector. 5 μ g DNA 100 μ l transfection reagent was used for each transfection and a total of 5x10⁵ cells were transfected. After transfection, 400 μ l of Y-compound and CHIR-containing medium was added to the cells and the cells were waited for 30 minutes and then inoculated into matrigel. Organoids were tracked and photographed for 2 days after transfection. Of the pulses used, the EX147 was observed to have a much more successful GFP+ rate (Figure 24).

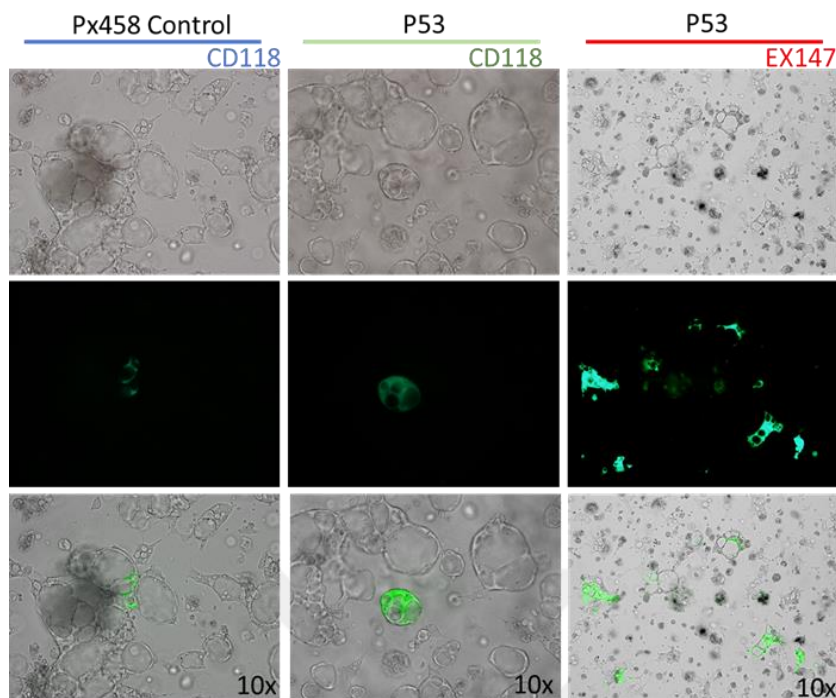


Figure 24. Light and fluorescent images of cells after transfection.

At the end of the sixth day, GFP+ cells were tried to be separated by sort. A parallel increase in the proportions of GFP+ of cells, similar to that seen under the microscope, was also obtained in flow analysis. Accordingly, the transfection efficiency was increased from around 5-7% to around 12% (Figure 25).

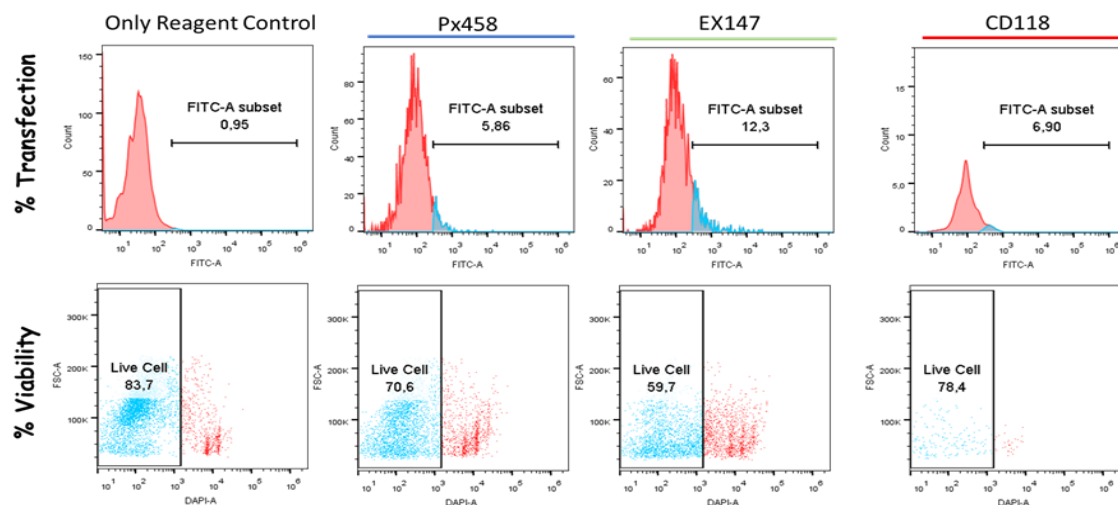


Figure 25. GFP+ and viable cell ratios of cells after transfection.

However, due to the fact that these rates were far from the rates we wanted to reach and the survival was poor in the ongoing process, different optimizations continued to be tried.

In the optimization we made in the second stage, after transfection, organoids were incubated on ultra-low attachment plates for 4-8 h without matrigel, as suggested by the protocol of the Nucleofector kit (Figure 26).

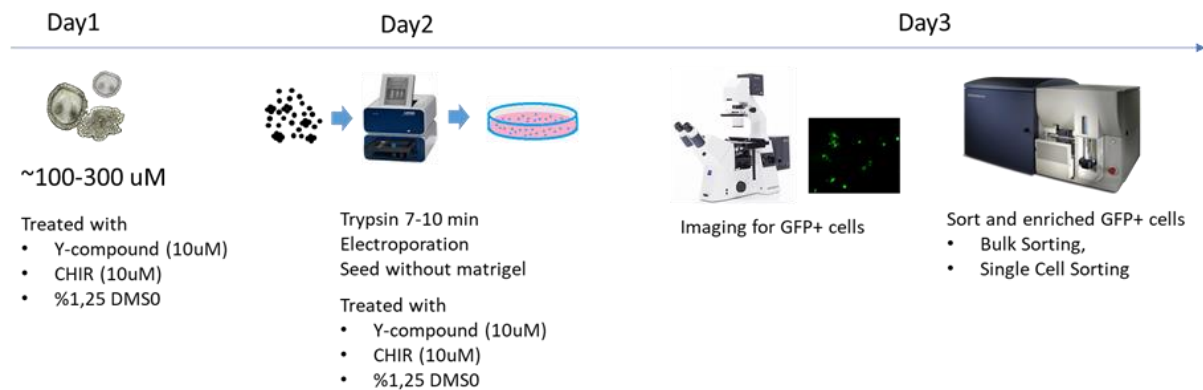


Figure 26. Summary of the new transfection optimization experiment with plasmid p53-KO in eHEPOs. Post-transfection cells were visualized under fluorescence microscopy and GFP+ cell ratios were observed to reach much higher levels (Figure 27).

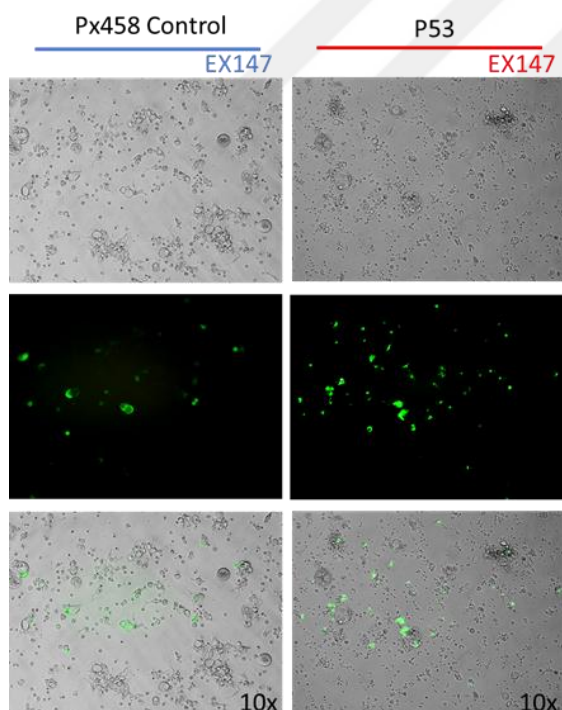


Figure 27. Light and fluorescent images of cells after transfection.

When we analyzed the rates with flow analysis, it was observed that the transfection efficiency increased from 5% to 45% after electroporation with EX147 and the survival was quite high (Figure 28).

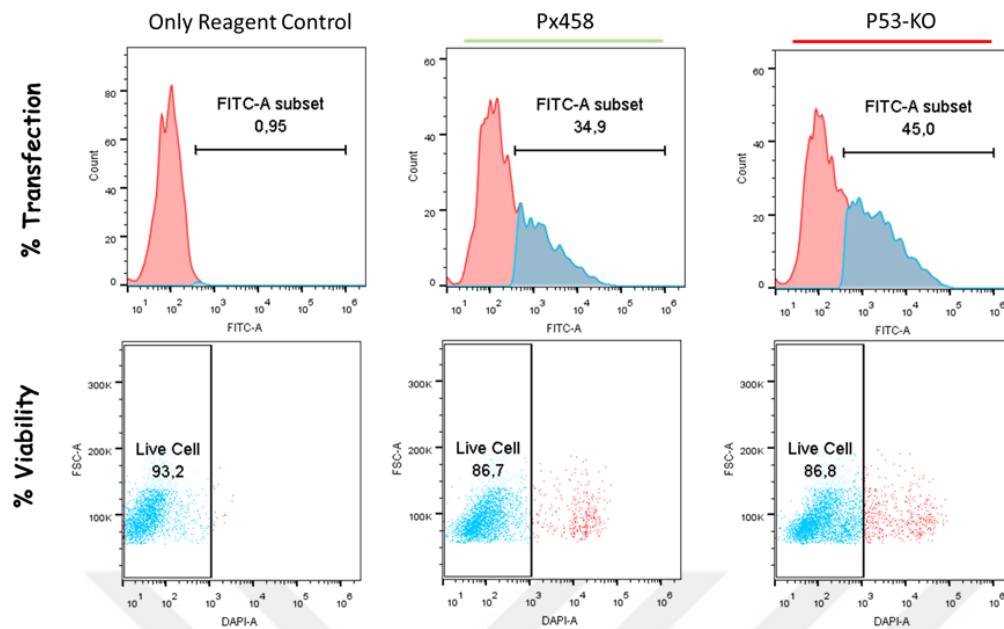


Figure 28. GFP+ and viable cell ratios of cells after transfection.

In further studies, higher GFP+ cell ratios were observed in early passage organoids, but quantitative flow analyzes were not performed again because the protocol was optimized (Figure 29).

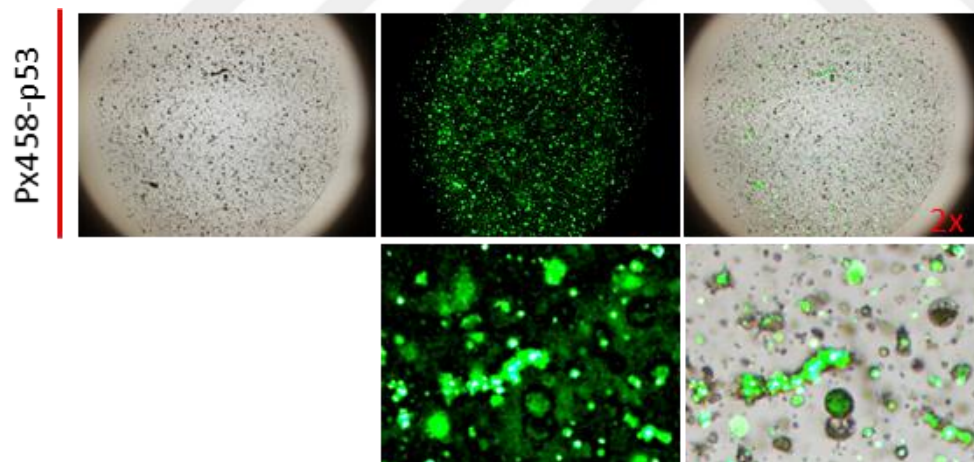


Figure 29. Light and fluorescent images of early passage cells after transfection.

As a result, the problem of low transfection efficiency, one of the biggest obstacles in front of the project, was overcome to a certain extent and progress was made.

4.3 CRISPR/Cas9-mediated P53, APC and NF1 gene silencing in HEK293T cells

4.3.1 For p53: generation of gRNAs, cloning into plasmid px458 and confirmation of clones

In our study, guide RNAs (gRNA) targeting the 4th exon designed by Şentürk et al. within the scope of the study published in 2017 on the <http://crispr.mit.edu> site was used. Transformed versions of these gRNAs into the DD-Cas9 plasmid system have been reported to target multiple genes that control various biological processes such as oncogenic transformation (X. Liu et al., 2018; Senturk et al., 2017). In this study, a lentiviral backbone-mediated p53 was shown to be significantly silenced in the A549 cell line.

In our project, with the same strategy, we first cloned the p53 gene in HEK293T and then eHEPOs into px458, which does not contain lentiviral particles in the gRNAs designed in the article, but instead contains a GFP tag to be used in selection. For this, gRNAs were oligomerized and incubated for 3 hours at room temperature with plasmid px458 which linearized with BbsI enzyme, respectively. Then, the obtained px458 plasmids were transformed into Stbl3 bacterial strain and allowed to grow overnight. The next day, 3-5 of the growing colonies were selected and plasmid verification by EcoRI enzyme digestion, and PCR method using reverse p53 primer sequence confirmed whether the px458 plasmid contained the target gRNA or not (Figure 30).

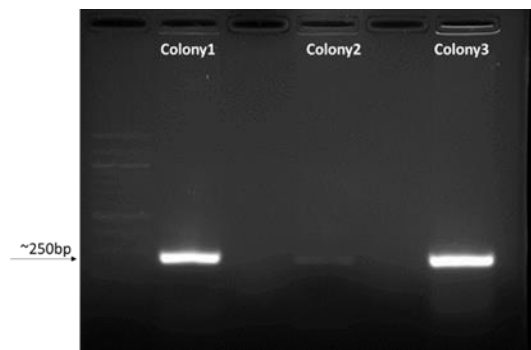


Figure 30. PCR results of selected colonies

As a result, the selected colonies were sent for sequencing and it was confirmed that they contained the target gRNA and that there were no mutational changes. After validation, plasmids px458-p53 were used for transfection into HEK293T cells (Figure 31).

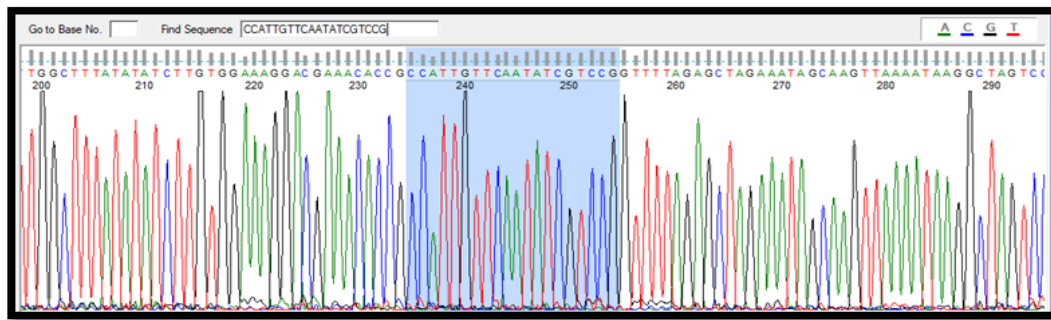


Figure 31. Sequencing result. The blue area shows the Tp53 gRNA sequence.

4.3.2 To target NF1 gene: generation of gRNAs, cloning into plasmid px458 and confirmation of clones

CRISPOR, the CRSPR-Cas9 tool, was used in the gRNA design for the NF1 gene. The reason why this tool is preferred is that it gives possible off target numbers, SNP status on the primer and presents the primers that are expected to work most effectively, respectively. Although the cloning procedures are the same as in p53, briefly annealing the primers, cutting and linearizing the px458 empty plasmid with BbsI plasmid, ligation of the annealed primer and linearized empty plasmid, transformation of the ligated NF1-px458 plasmids with Stbl3, selection of suitable colonies and finally selected Includes miniprep isolation of plasmids from colonies (Figure 32). In total, 2 primer designs were made (see method). px458-NF1 plasmids, whose accuracy was checked by sequencing after isolation, were prepared for control by transfection in HEK293T cells.

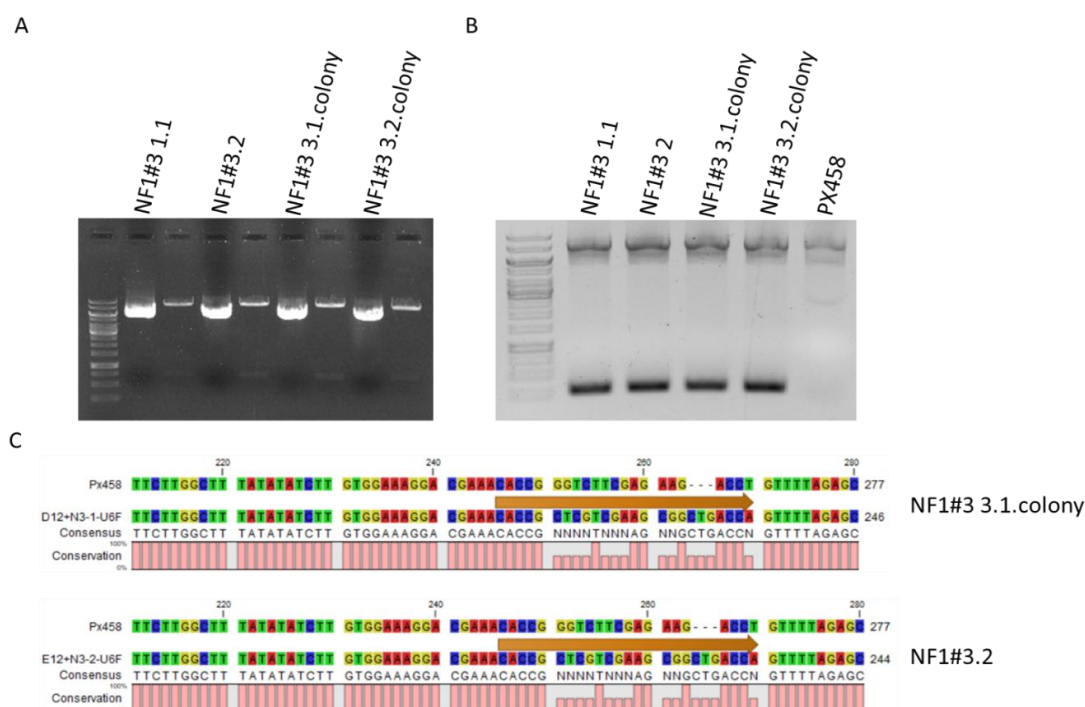


Figure 32. Production and control of NF1 gRNAs. A. EcoR1 cut. Candidates for uncut and EcoRI cut gRNA, respectively. B. Checking the success of cloning by standard PCR. C. Sanger sequence control. The portion marked with the arrow is the designed gRNA, showing successful cloning of each candidate.

4.3.3 To target APC gene: generation of gRNAs, cloning into plasmid px458 and confirmation of clones

Primers specific to the APC gene are referenced from publications in which sequential cancer mutations are made in intestinal stem cells (Drost et al., 2015) and CRISPR-Cas9-mediated genome editing in human Barrett epithelial organoids (X. Liu et al., 2018), the relevant sequences are referenced in a CRISPR publication before ordering. Confirmed via CHOPCHOP, the Cas9 editing tool. In addition to these, specific sequence-specific primers were designed and tested using the CRISPOR tool. The respective primers were cloned into the empty px458 plasmid containing the 2x GFP and Cas9 sequences. In this process, PCR controls were similarly performed with EcoRI cut and reverse APC primers, and the selected primers were confirmed by sequence analysis (Figure 33) (see method for primer sequences).

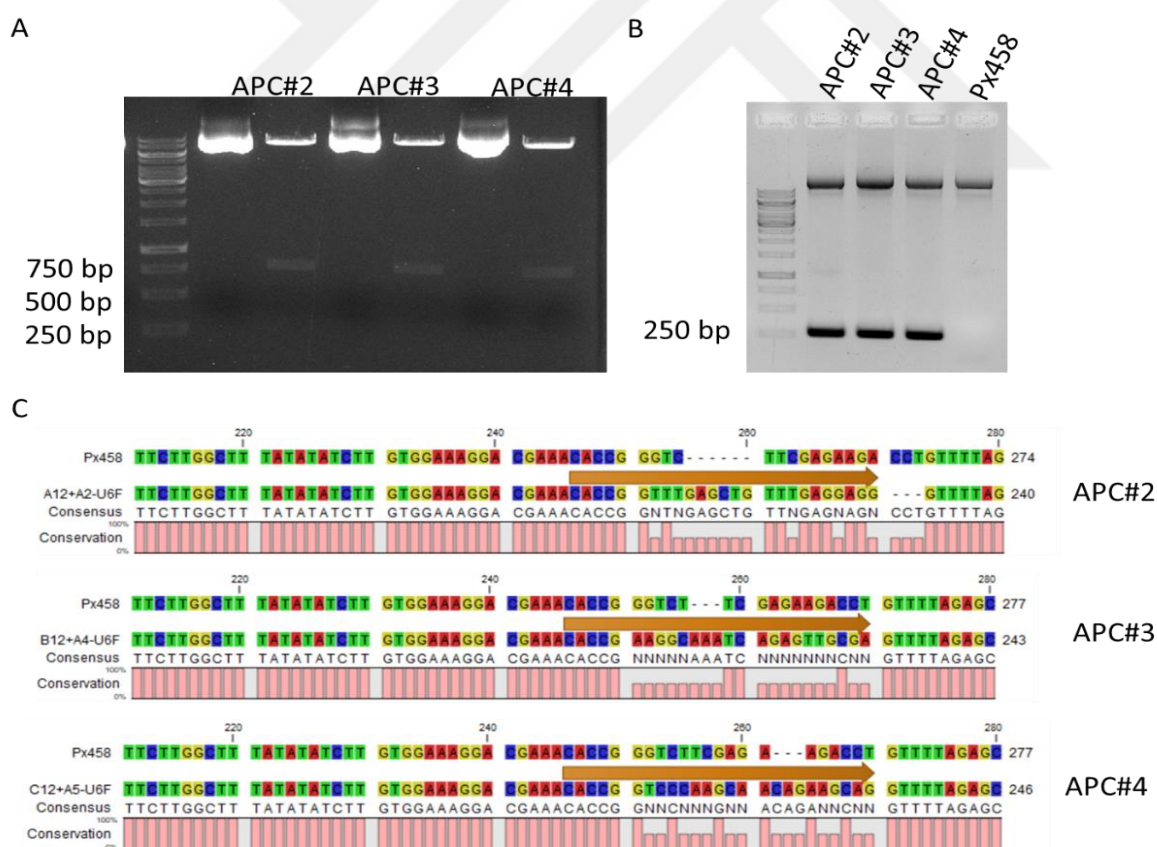


Figure 33. Production and control of APC gRNAs. A. EcoR1 cut. Candidates for uncut and EcoRI cut gRNA, respectively. B. Checking the success of cloning by standard PCR. C. Sanger sequence control. The portion marked with the arrow is the designed gRNA, showing successful cloning of each candidate.

4.4 Transfection of px458-gRNA plasmids into HEK293T cell line and investigation of their silencing efficiency.

p53: Using plasmids confirmed by sequencing, Hek293T cells were transfected with the PEI method as given in the material method section, and the lysates obtained on day 6 were examined by western blot for the altered activity of the gene of interest (Figure 34).

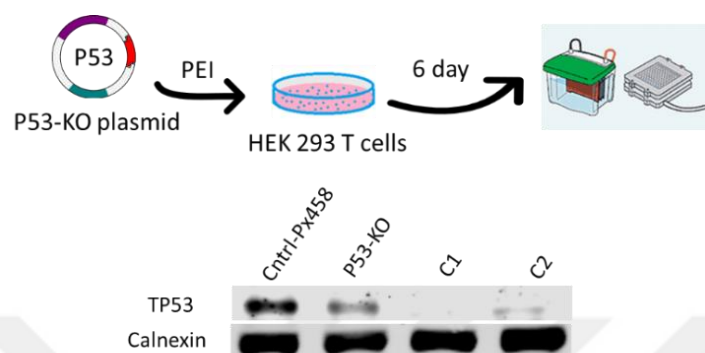


Figure 34. Activity validation of gRNA targeting p53 in the HEK293T cell line.

Empty plasmid, lysate obtained after transfection into Hek293T, C1-C2 p53 KO control clones, respectively.

On day 6 after transfection, cells were harvested from the culture medium and the resulting lysate was loaded at 50 μ g. Also, clones that were clonally confirmed to be p53 KO as controls were loaded in the same amount. As a result, it was shown that the gRNA-p53 plasmid successfully reduced the activity. After this stage, work has started on eHEPO.

APC: All of the plasmids confirmed by sequencing for the APC gene were transfected into HEK293T cells by the PEI method, and at the end of the 6th day, the cells were harvested as lysate. The lysates obtained were calculated and loaded at 50 μ g in each well (Figure 35).

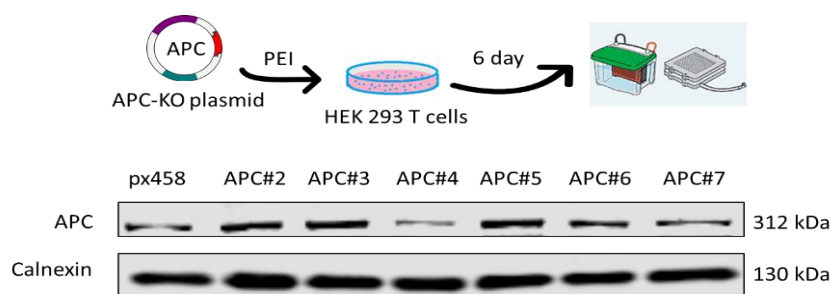


Figure 35. Activity validation of gRNA targeting APC in the HEK293T cell line

As a result, it was determined that gRNA number 4 decreased the protein level by 50% compared to the control group, px458 plasmid, and could be used in further studies.

Candidate gRNA APC#4:

APC-EXON-7F 5'CACCgGTCCCAAGCAACAGAAGCAG-3'

APC-EXON-7R5' – AAACCTGCTTCTGTTGCTTGGGACC- 3'

NF1: Of the plasmids confirmed by sequencing for the NF1 gene, one was produced in sufficient quantity for transfection due to lack of budget. This produced px458-NF1 plasmid was similarly transfected into HEK293T cells by PEI method and at the end of the 6th day, the cells were harvested as lysate. The lysates obtained were calculated and loaded at 50 µg in each well (Figure 36).

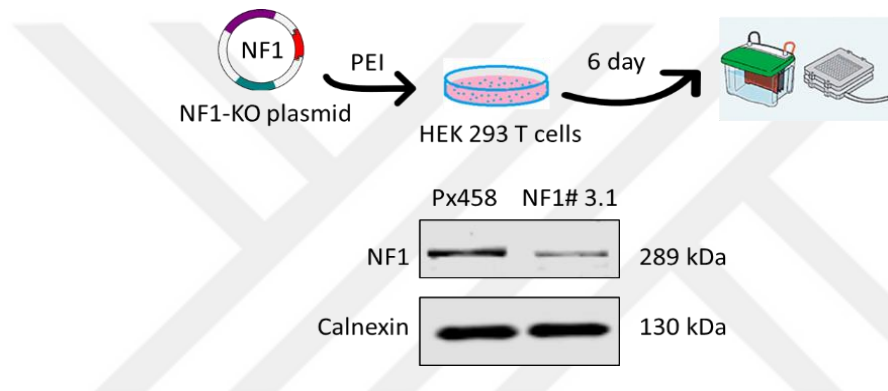


Figure 36. Activity validation of gRNA targeting NF1 in the HEK293T cell line

After transfection with the selected px458-NF1 plasmid set, up to 64% reduction in protein level was observed.

Candidate gRNA NF1# 3.1:

NF1_3F 5' CACCGCTCGTCGAAGCGGCTGACCA 3'

NF1_3R 5' AAAGTGGTCAGCCGCTTCGACGAGC 3'

4.5 Crispr/Cas9-mediated silencing of the p53 gene in the eHEPO model

WT organoids transfected with the protocol optimized in our study were grown in supportive medium containing Y-compound and CHIR for 2 days to increase cell survival after transfection, protect cells from apoptosis, and strengthen their proliferative signal. Then the cells were made single cell using trypsin. During this process, care was taken to ensure that the cells kept in trypsin for 7-10 minutes are not completely single cells, but in a suspension containing trypsin in a community of 3-5 cells in order to preserve the eHEPO-forming capacity

of the cells. After this stage, the cells, which were kept without matrigel for 4 hours, were mixed with matrigel at the end of the time and prepared for sorting within the following 3 days. However, at this stage, due to the device failures we experienced in the FACS sort unit and the lack of access to the unit during the Covid-19 pandemic process, selection was made with the Nutlin3a method following the bulk sorting. Nutlin3a, in principle, cuts the connection of MDM2, the E3 ubiquitin ligase, which is the negative regulator of TP53, with p53 and ensures the accumulation of p53 in the cell. If the cell is WT, death mechanisms work depending on p53 and the cells die. On the other hand, p53 knockout cell groups can proliferate independently of Nutlin-3a treatment (Bartfeld et al., 2015; Matano et al., 2015). Nutlin3 was added to the medium for the selection of P53 KO organoids when the cells were at a certain health and density. Then, the medium was refreshed every 3 days and the cells were left to grow in selective medium for about 20 days. In this process, it was observed that some of the cells that were left to grow in natural selection environments containing Nutlin3 (20 μ M) died (the cells in which CRISPR-cas9 did not work as expected), and some of them proliferated excessively and formed quite large organoids (our target cells in which the CRISPR system worked). These overgrown organoids were separated with 200 μ l pipettes under the microscope and grown as single organoid clones in selective medium for a while, and then functional experiments were started (Figure 37).

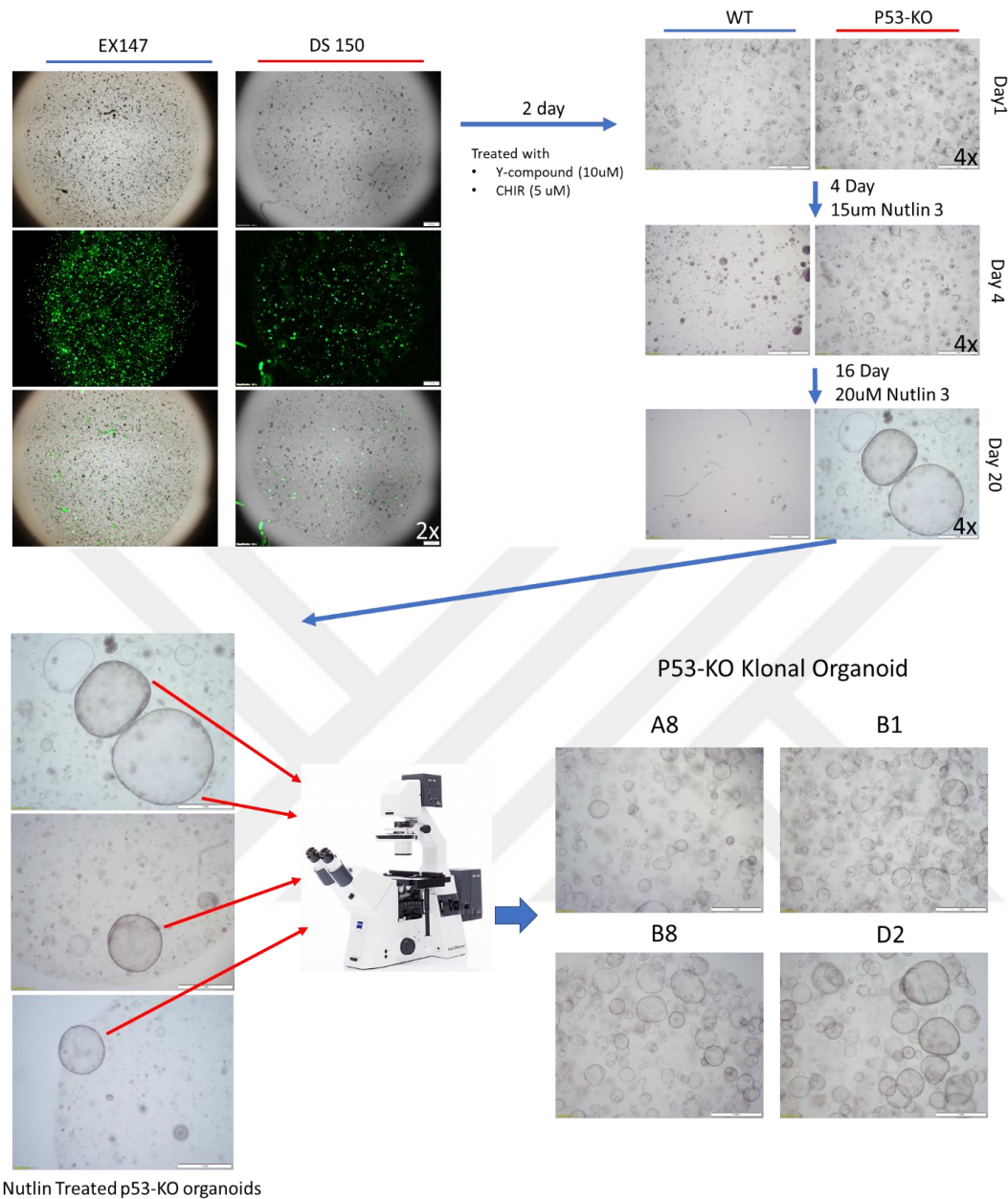


Figure 37. Obtaining P53 KO eHEPOs

In the clonal organoids obtained, first immunofluorescence staining approach, which is possible with few cells, was performed and it was shown that p53 activity was lost in the selected D2 clone. Subsequently, western blot analysis was performed with abundantly amplified clonal organoids. In this system, WT organoids treated with Doxorubicin or Nutlin3a were used as positive controls. As a result, it has been shown that p53 can be successfully KO in all clones. Clones 5 and 6 were also excluded from the candidate clones for further studies due to contamination. In the following process, the selected clonal organoids were sent to Sanger sequencing, and it was shown that an Adenine(A) nucleotide was added after the 3rd nucleotide

following the PAM sequence in all clones in the target sequence, and Frameshift mutation occurred accordingly (Figure 38).

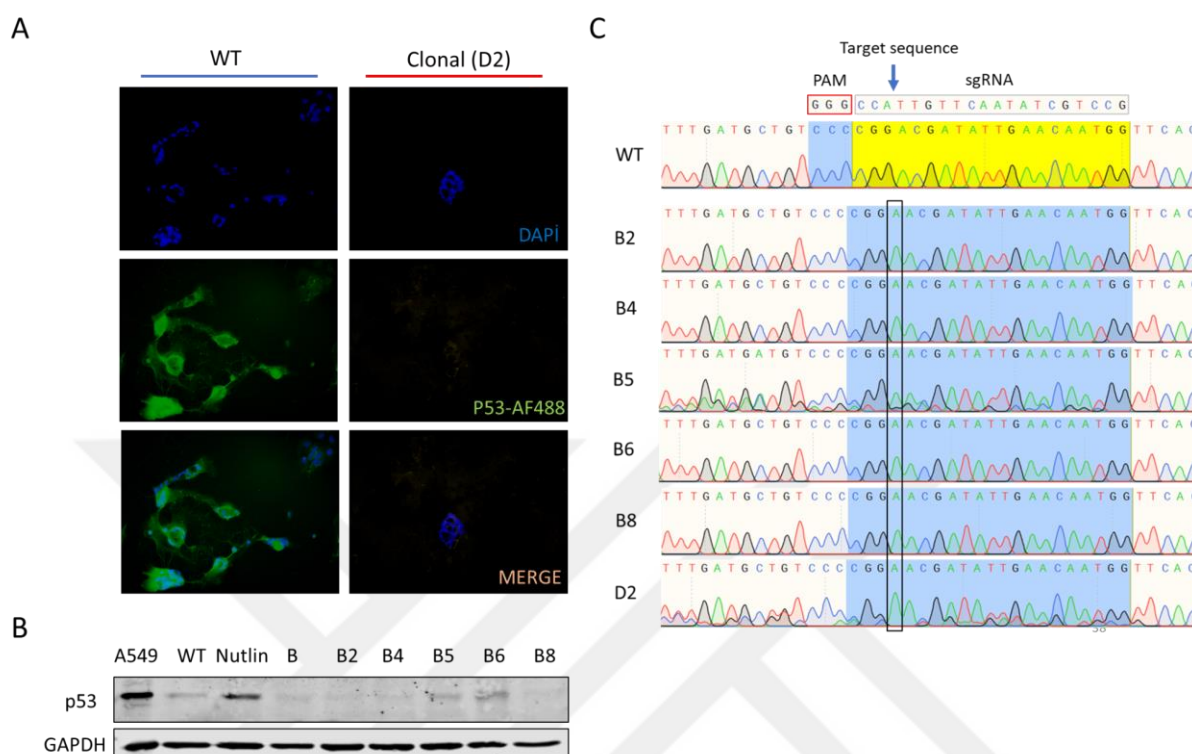


Figure 38. Confirmation of P53 protein loss in p53 KO-eHEPOs. A.p53 Immunofluorescent staining. B. Western blot C. Sanger sequencing

4.6. Determination of selective media content for use in APC and NF1 gene silencing

4.6.1 NF1 selection medium; Blocking the EGFR signaling pathway

Although Ras, Raf mutations are not frequently encountered in HCC, it has been shown that Neurofibromin (NF1) inhibition plays a major role in keeping the Ras/MAPK signaling pathway active (Calvisi et al., 2011). Again, at the genome level, CRISPR-Cas9 library scans have shown that NF1 is an important tumor suppressor gene in liver cancer and has an important role in the regulation of liver bipotent cell population in the process of carcinogenesis (Song et al., 2017; Zender et al., 2008). Therefore, silencing of NF1 is thought to have a very important place in the tumorigenesis process (Oprita et al., 2021).

In order for cells to grow in a healthy way, there must be sufficient growth factors and basic supplements in the cell environment. In order to ensure a controlled growth in the organoid culture medium, the supplements needed to the reduced serum medium are added one by one. For this reason, not adding a specific supplement and/or growth factor to the medium may affect

the proliferation and survival of cells. Similar selective media have been prepared for APC and NF1 KO models in the literature (Drost et al., 2015). NF1 KO organoids are expected to be able to grow in cell medium without EGF and supplemented with Gefitinib, the EGF receptor inhibitor.

Accordingly, in our preliminary studies with ancestral organoids, it has been shown that the KO clone selection strategy will be successful by changing the content of this medium in our preliminary studies with ancestral organoids, following the withdrawal of EGF from the medium and the administration of EGFR inhibitor to the medium to obtain NF1-KO eHEPO (Figure 39). As a result, we think that in clones with NF1 KO, Ras will remain bound to GTP and with the constitutive activation of the activation-independent pathway via EGF, the organoids will proliferate without the need for EGF in the medium, and growing organoids can be selected.

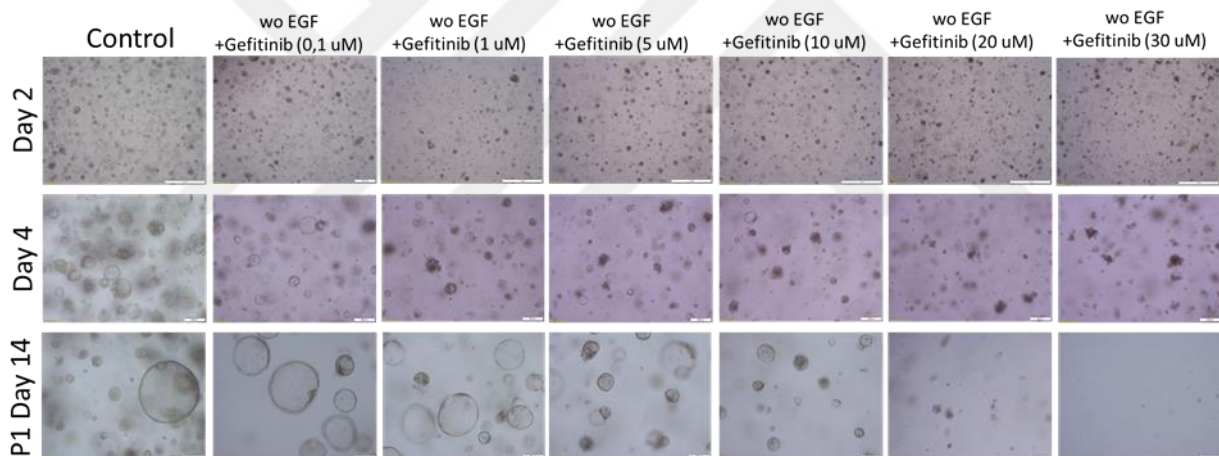


Figure 39. Growth states of WT organoids in selective medium without EGF and supplemented with Gefitinib

In our project, the production of organoids containing single gene knockout was abandoned due to lack of budget. Instead, it was planned to silence the NF1 gene in the p53-KO organoids we obtained. For this reason, the same preliminary study performed in WT organoids was also tested in organoids with P53 KO, and it was observed that proliferation slowed down (Figure 40). Thus, it was predicted that successful selection of NF1 KO to be made on P53 KO organoids could be achieved.

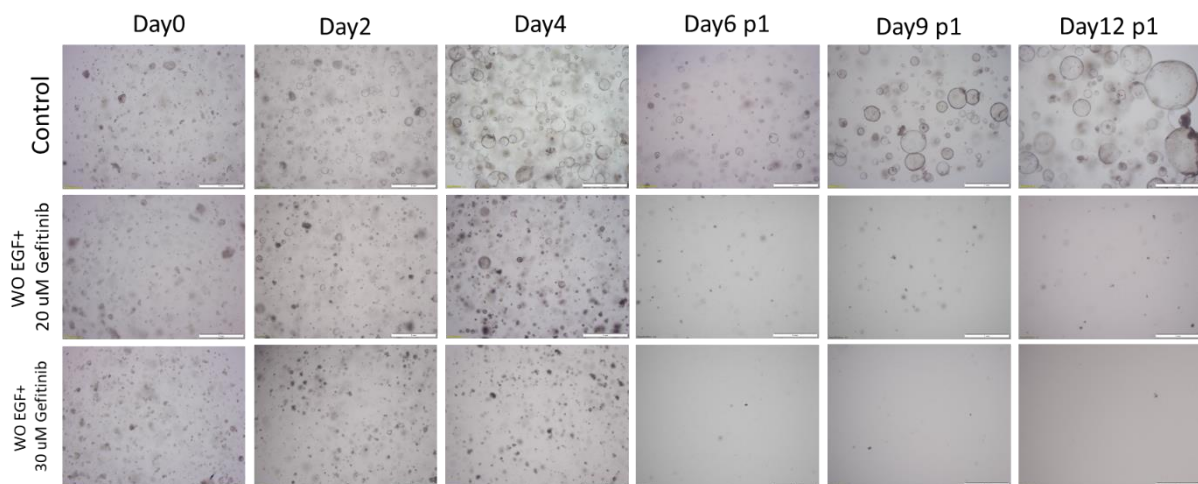


Figure 40. Growth status of p53KO organoids in selective medium without EGF and supplemented with Gefitinib prepared for NF1.

The medium of the cells was changed every 3 days. After the cells were photographed on the 4th day, they were passaged at 1:5 in all three conditions.

4.6.2. APC Selection medium; Modulation of the Wnt/R-spo1 signaling pathway

In the literature, Drost et al. 2015 reported that the removal of Wnt and RSPO from the medium caused a decrease in cell proliferation and the death of organoids. On the other hand, it was observed that the cells in the APC-KO group were not affected by this situation and continued to proliferate. From this point of view, it was aimed to transfect the px458-APC plasmids we designed into organoids and to examine their growth status by removing RSPO from the medium. Sort by FACS will be attempted in the first preliminary trials of the selected APC and NF1 plasmids. However, if GFP⁺ cells cannot be obtained during or after the sorting process, for the positive selection of cells where CRISPR-Cas9 works successfully without sorting, long-term R-spondin was removed from the environment of healthy organoids and how the organoids were affected was investigated. As a result, it was observed that healthy organoids could not proliferate and even died in the absence of R-spo over time (Figure 41). In other words, hepatic organoids are dependent on R-spo, which activates the canonical Wnt signaling pathway, to proliferate, and since constitutive Wnt activation will be achieved when APC is KO, organoids can proliferate and be selected without the need for this dependency.

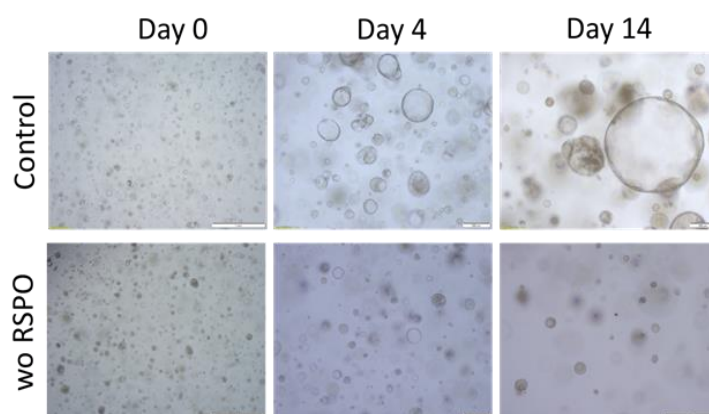


Figure 41. Growth states of WT organoids in RSPO-free selective media prepared for APC

In our project, the production of organoids containing single gene knockout was abandoned due to lack of budget. Similarly, APC gene silencing was planned to be performed on p53-KO organoids. For this reason, the same preliminary study performed in WT organoids was also tested in organoids with P53 KO, and it was observed that proliferation slowed down (Figure 42). Thus, it was predicted that successful selection of APC KO to be made on P53 KO organoids could be achieved.

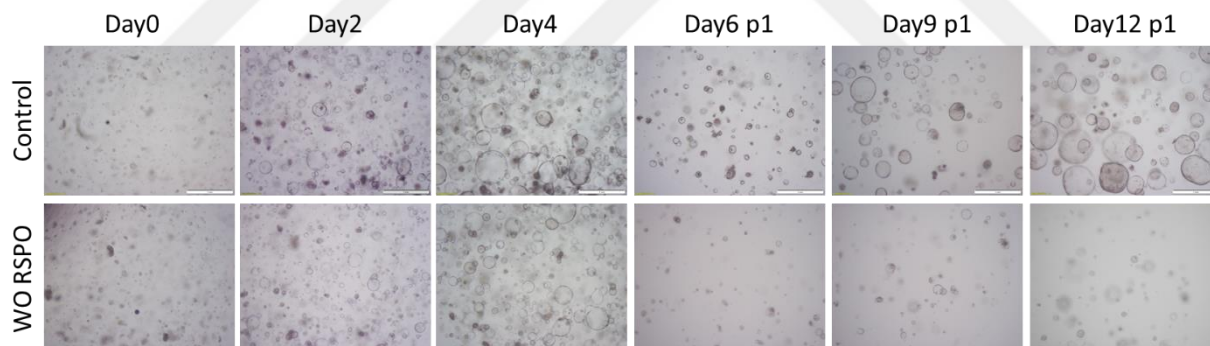


Figure 42. Growth states of p53KO organoids in selective medium without RSPO prepared for APC.

The medium of the cells was changed every 3 days. After the cells were photographed on day 4, they were passaged at 1:5 in both conditions.

4.7 Generation of cells that mimic the pro-carcinogenic tumor microenvironment

CAFs and TAMs, which have a role in the initiation and development of liver cancer, are activated stellate (myofibroblast) and macrophages polarized into M2 form, respectively. In our study, LX2 stellate cells were transformed into myofibroblasts by induction of 24h 10ng/ml TGF β . It was observed that the activated stellate cells changed morphologically and there was a significant increase in the myofibroblast marker Col1A1 and PDGFRB genes (Figure 43).

After induction with TGFB, it was incubated in fresh RPMI 2 medium for 48 h and the medium was collected to be added to the Organoid culture in further studies.

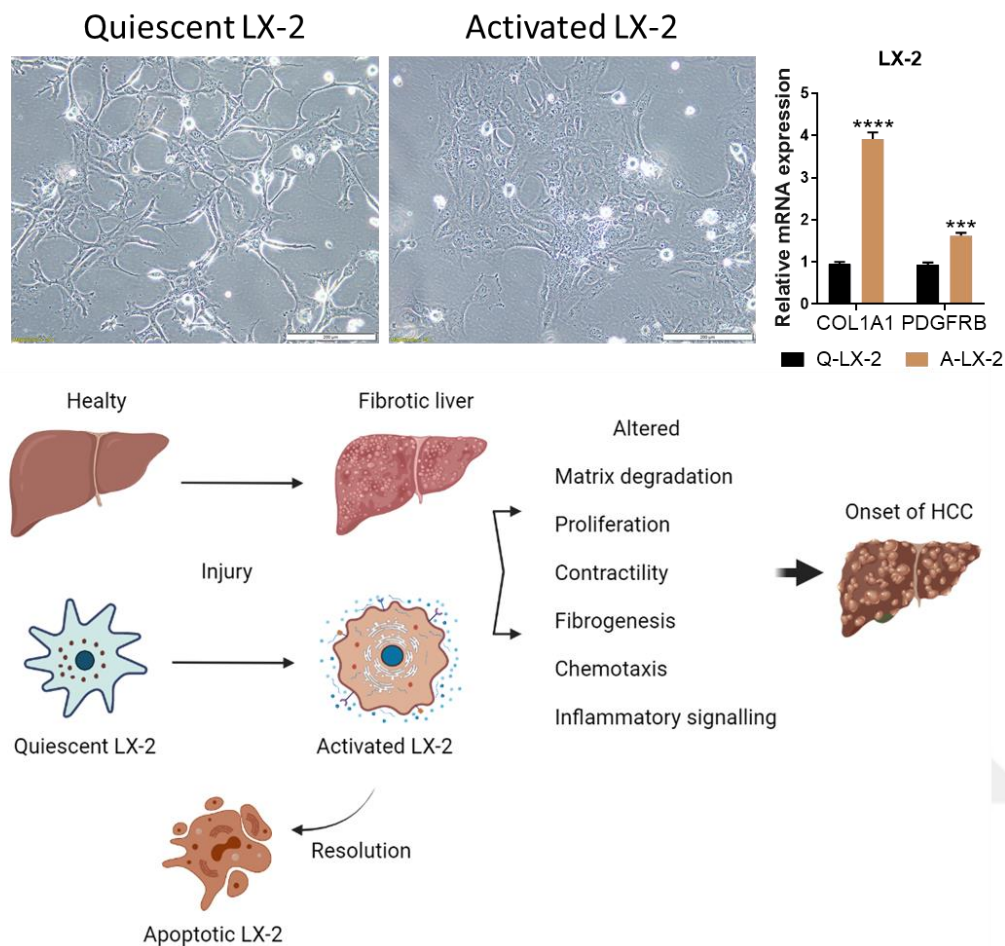


Figure 43. Stellate cell activation and validation

Similarly, THP1 cells, a human monocyte cell line, were induced with PMA for 2 days and differentiated into macrophage cell type. Then, it was waited for 2 days for the cells to self-recover and all macrophages were polarized to M2 type by inducing IL-4, which is widely known and accepted in the literature, for 2 days (Fig. 44).

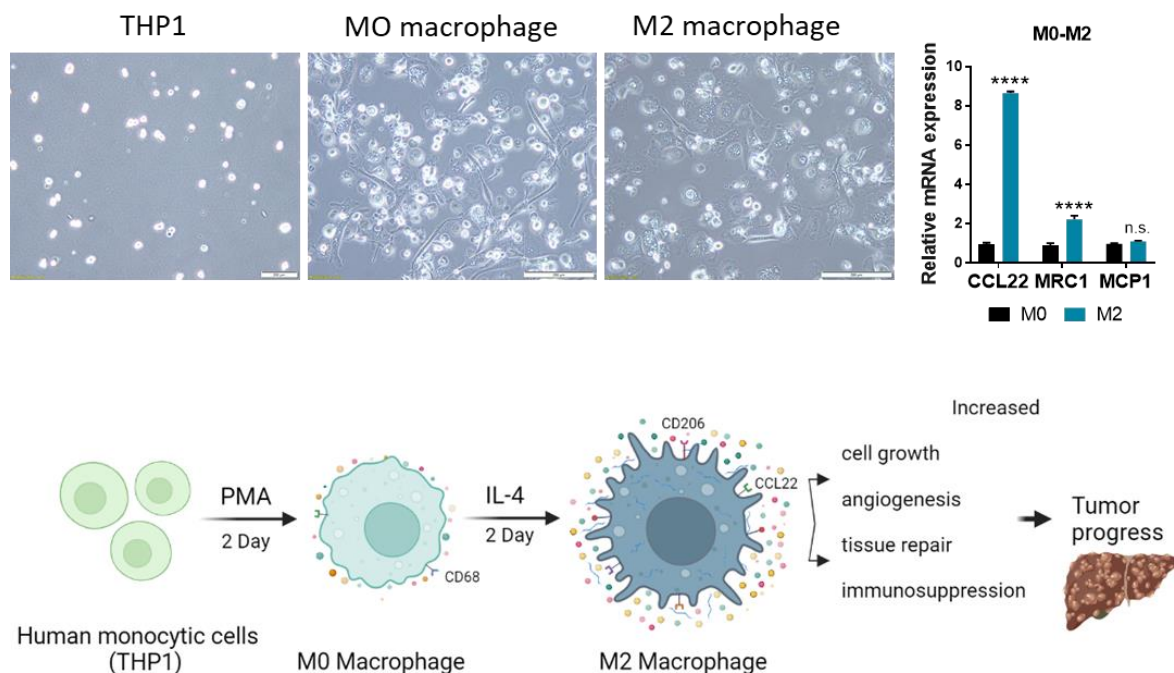


Figure 44. Extraction and characterization of M2 polarized macrophages

In addition to morphological changes, an increase in the M2 markers CCL22 and CD206 gene expressions was observed in cells differentiated into M2 macrophage, while no change was observed in M1 marker gene MCP1. Similarly, the medium of M2 polarized macrophages was changed with fresh medium and after 48h collected for inclusion in organoid culture.

4.8 Characterization of p53 KO eHEPOs

4.8.1. Investigation of the effects of silencing the p53 gene on proliferation in eHEPOs.

It has been shown that the CRISPR-Cas9 system works successfully in all of the p53 KO clonal organoids we have obtained. In the ongoing process, both western blot and immunohistochemical staining were performed on all clones in order to select one or two of these clones for further studies (Figure 45).

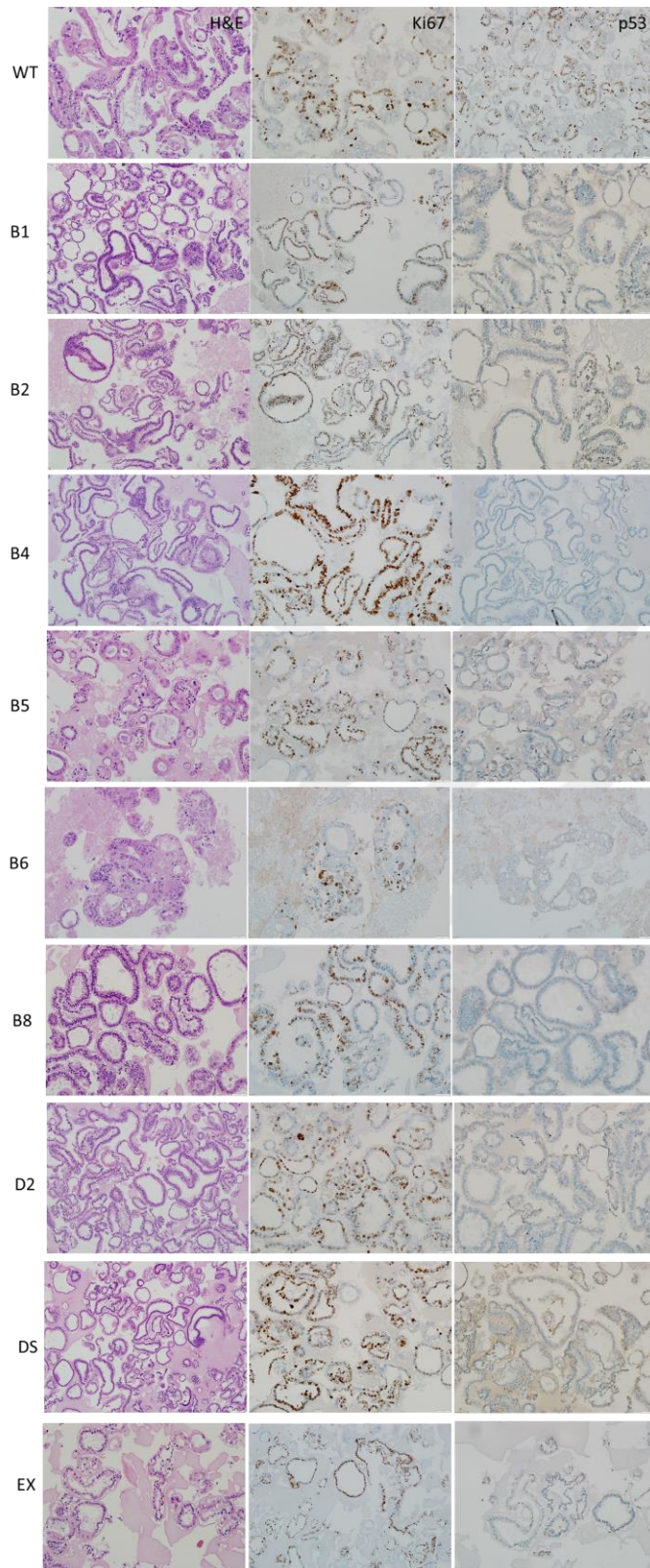


Figure 45. Ki67 and p53 staining of all clones obtained

With these staining's, B4 and B8, the two cleanest clones with the highest proliferative index (Ki67 positivity) and without p53 positive staining, were selected (Figure 46). Because of the

slower proliferation of B8 from these two clones compared to B4, the B4 clone was used in the continues of the studies.

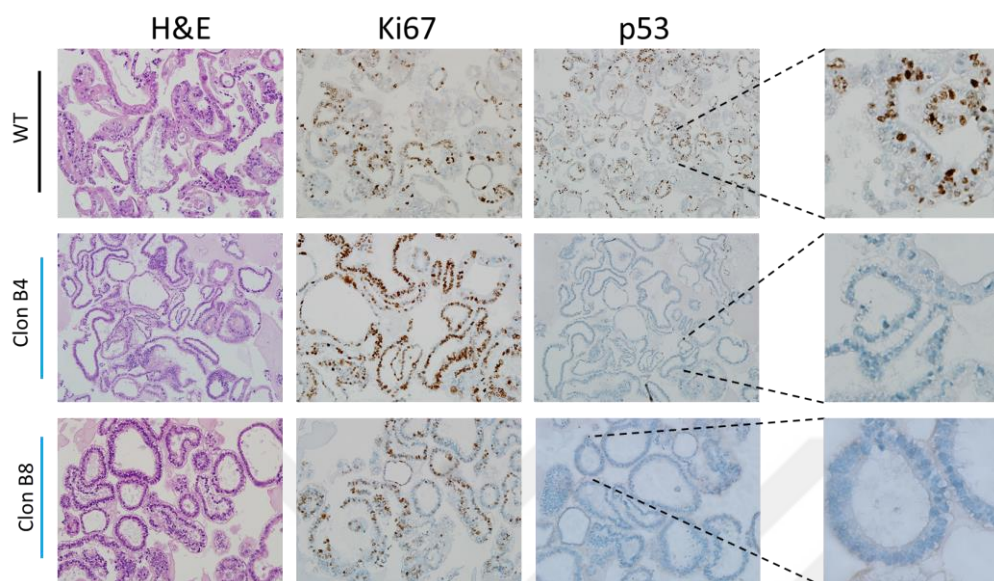


Figure 46. Immunohistochemical staining of selected P53 KO eHEPOs

In addition to the Ki67 staining results of the B4 clone, measurement was taken with cell titer glo 3D to quantitatively examine the proliferative changes in the culture medium. As a result of these measurements, it was shown that the proliferative responses of p53 KO eHEPOs were significantly higher, especially after the 3rd day, compared to WT. Similarly, the cells photographed on the days of the experiment were shown to form larger structures (Figure 47)

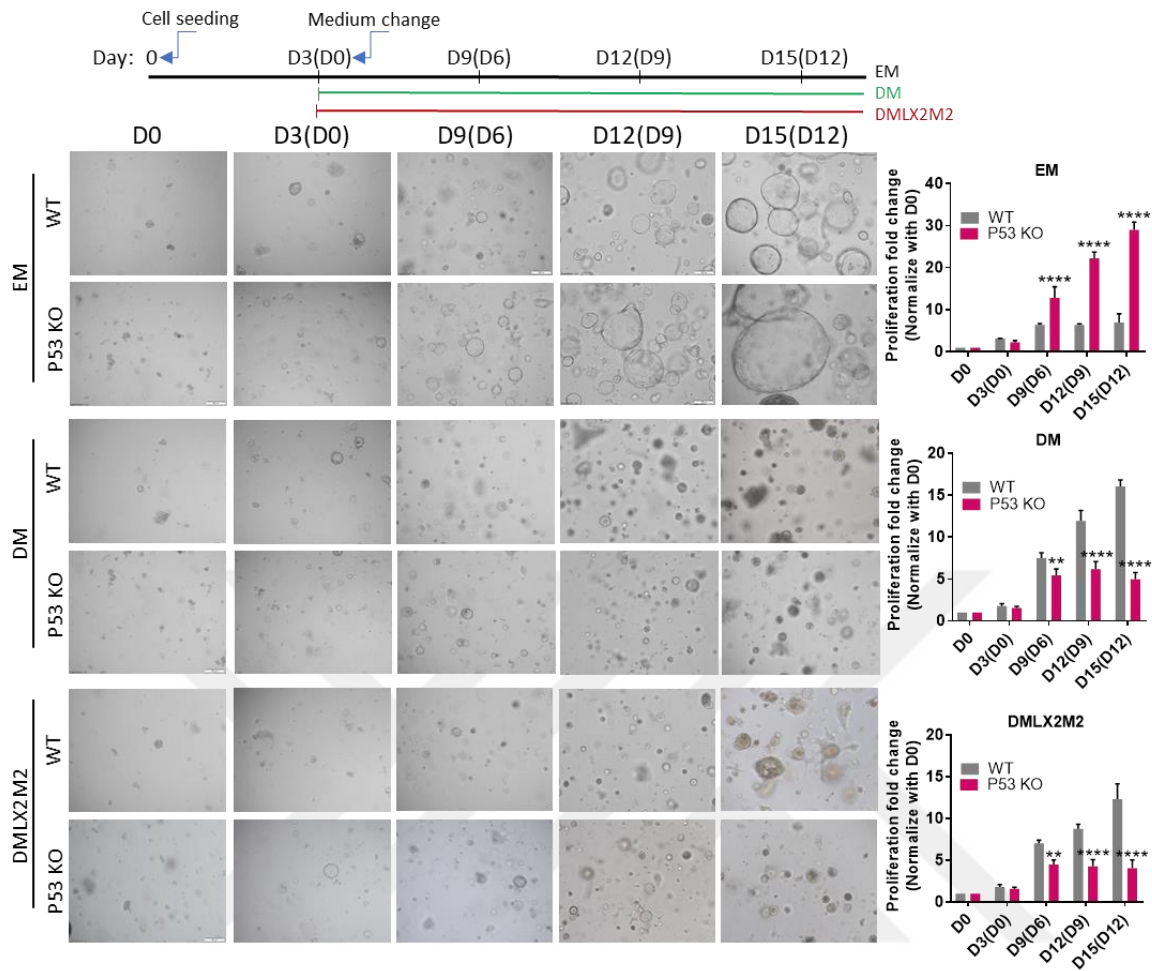


Figure 47. Investigation of changes in the proliferative processes of WT and p53 KO organoids in EM DM and DMLX2 culture medium. left. Microscope image. 10x zoom Right. Monitoring of quantitative growth rates obtained with Cell titer glo-3D.

4.8.2. Examination of the histopathological effects of p53 KO in eHEPOs

Although p53 and Ki67 staining was performed at the beginning of our study, hematoxylin-eosin staining was repeated and histopathological examinations were carried out in order to examine the morphological changes in these cell groups in detail in the future. In HE staining, p53 KO organoids have been shown to have increased atypical and inflammatory-like cell types related to the onset of HCC (Jiang, Al-Diffhala, & Centeno, 2018; Schlageter, Terracciano, D'Angelo, & Sorrentino, 2014) and have a tubular and pseudo-glandular pattern (Figure 48).

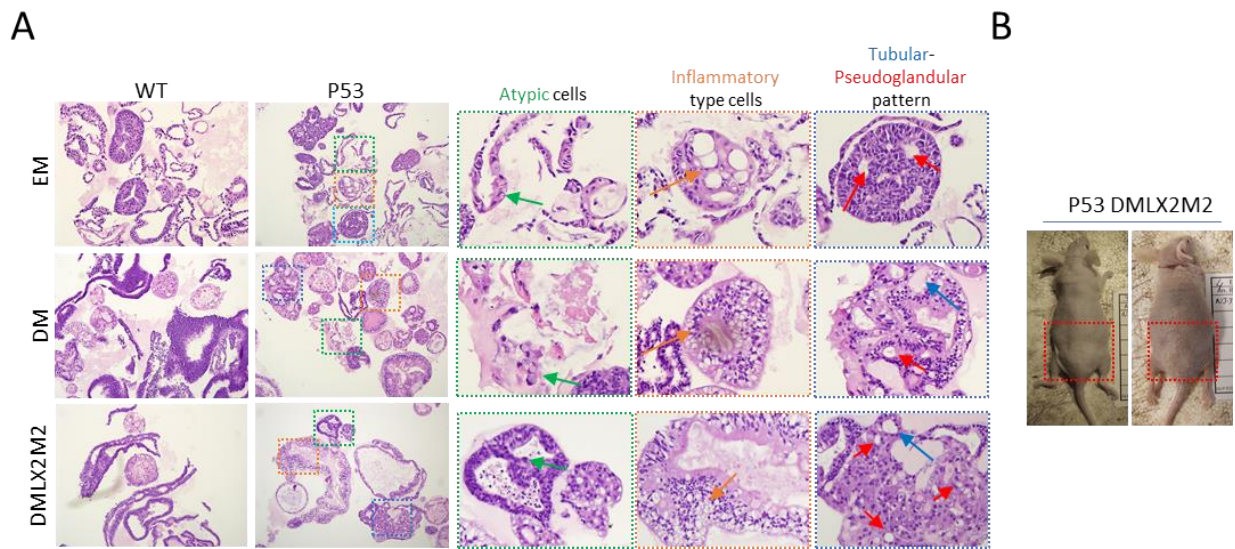


Figure 48. Immunohistopathological examination of WT and p53 KO organoids.

Again, in order to examine the changes in the maturation process of p53 KO organoids, their glycogen storage capacities were examined by PAS staining. Similarly, the amounts of GPC3 and MUC1 (Bozkaya et al., 2012; Suzuki et al., 2010) tumor markers in HCC in p53 KO organoids were investigated. Although the glycogen storage capacity was higher in P53 KO organoids compared to WT, it was observed that the amount of GPC3 and MUC1 staining was also higher (Figure 49).

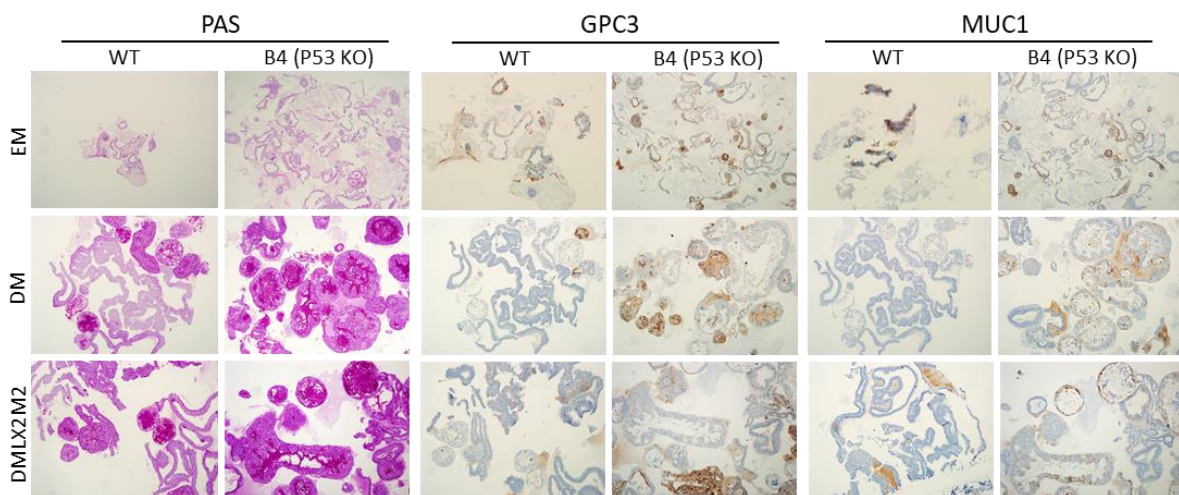


Figure 49. Histopathological examination of PAS, GPC3 and MUC1 levels in WT and P53 KO organoids

4.8.3. Investigation of the changes of p53 KO in cancer stem cell surface marker expressions in eHEPOs.

In recent studies in the literature, it has been shown that cell groups with positive or negative expression of cancer stem cell markers, especially at tumor initiation, have some decisive roles. In one of these studies, it was reported that CD44 could differentiate into tumorigenic hepatic precursor cells via the AKT pathway by controlling the nuclear translocation of p53 (Dhar et al., 2018). For this reason, the changes of these markers in WT and p53 KO organoids were investigated by the flow method (Figure 50). In these analyses, flow analysis was performed on the 12th day of differentiation. In the analyzes performed, it was observed that especially CD24 and EpCAM positive cell groups exhibited major changes, in addition, the pro-carcinogenic microenvironment increased the expression of all surface markers at a certain rate.

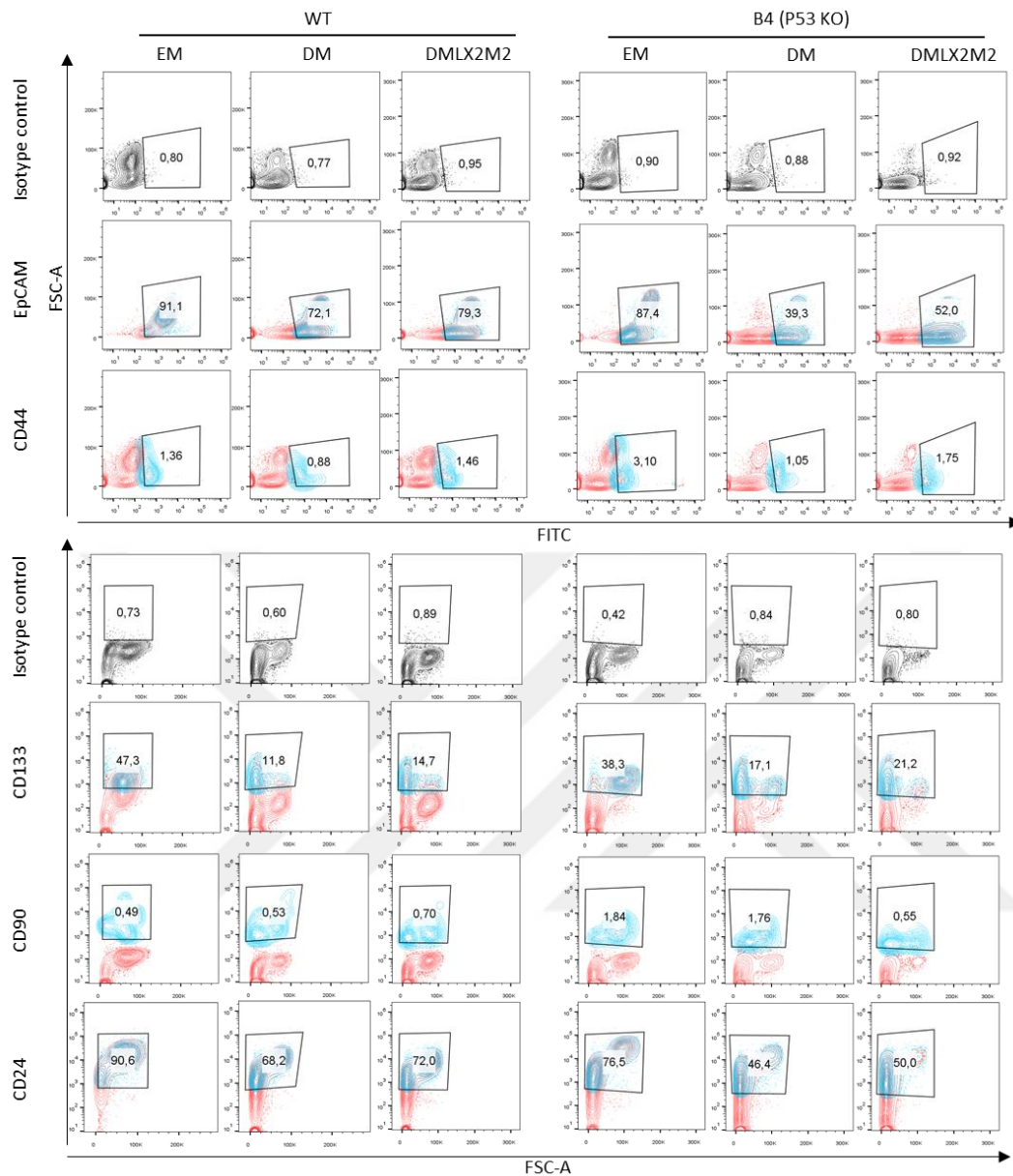


Figure 50. Investigation of changes in cancer stem cell surface marker levels of WT and p53 KO organoids.

Cells were collected and analyzed on day 12 after differentiation for the experiment. Analyzes were made on the Fortessa device and the results were arranged with the Flowjo program.

4.8.4. Investigation of the effects of p53 KO on Wnt-TGFB pathway activity in eHEPOs

Significant changes in EPCAM and CD24 were observed in the changes in the flow analysis. At the same time, the interactions of p53 with Wnt and TGFB pathways have been emphasized in the literature and it has been reported that the pathway activities may change depending on the mutation status of p53. Regarding this issue, it has been shown in the literature that the loss of function in the p53 gene activates the Wnt pathway, whereas the TGFB pathway activity is

dependent on the presence of p53 (Cordenonsi et al., 2003; Donehower, Godley, et al., 1995; Kalo et al., 2007; Wellenstein et al., 2019). In our study, we examined the Wnt and TGF β pathway activities of WT and P53 KO organoids with reporter assays (Figure 51).

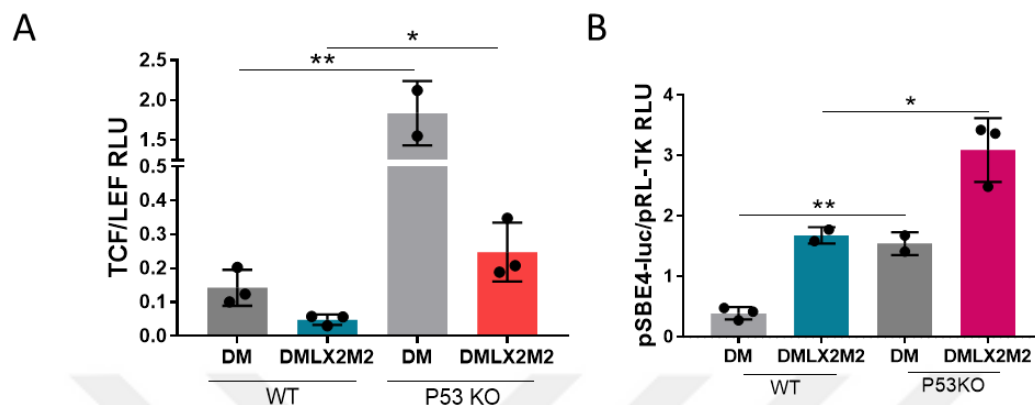


Figure 51. Investigation of Wnt and TGF β pathway activities of WT and P53 KO organoids, respectively, by TCF/LEF and pSBE4-luc/pRL-TK relative luciferase assay

In the analyzes performed, a significant increase was observed in the Wnt and TGF β pathways in P53 KO organoids in DM medium. On the other hand, while the pro-carcinogenic microenvironment decreases Wnt activity, the activity of TGF β pathway increases.

4.8.5. Investigation of the effects of p53 KO on hepatic maturation in eHEPOs

It is reported that p53 acts as a barrier in the dedifferentiation of normal cells and is a guarantor for the preservation of their current state (Krizhanovsky & Lowe, 2009; Onder et al., 2012). From this point of view, it was examined whether the differentiation capacities of the P53 KO organoids we obtained were different in the DM medium compared to WT, and the level of Albumin, a maturation marker, was measured by the Albumin ELISA method (Figure 52).

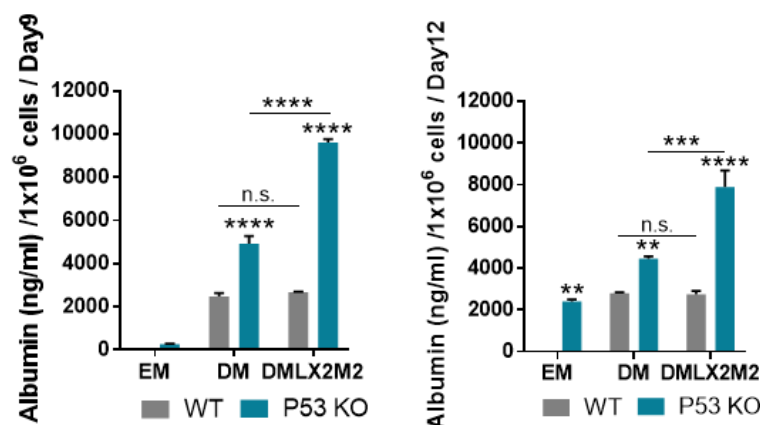


Figure 52. Investigation of changes in albumin levels of WT and P53 KO organoids

For the experiment, the albumin production capacity of the organoids in the unit amount of which the medium was collected and the cell numbers were determined on the 9th and 12th days of differentiation was shown. It was observed that the P53 KO group secreted Albumin in the EM phase, unlike WT, and this amount increased in DM. As a result, it showed us that hepatocyte differentiation of cells was induced depending on the content of the EM medium by breaking the resistance barrier in front of the cells. Based on this result, we think that it may be beneficial to temporarily inhibit p53 to create a more mature hepatic organoid model.

4.8.6. Changes in fibrosis maturation and apoptosis markers in p53 KO eHEPOs

Changes of the most prominent markers of early stage fibrotic tissue due to silencing of the p53 gene in a pro-carcinogenic microenvironment were investigated by OCT method. Accordingly, it was observed that the collagen markers COL1A1 and COL4A1, which are known to increase in fibrotic tissue, were significantly increased in p53KO-eHEPO. Yet another marker, PDGFRB, was observed to be expressed only in p53 KO-eHEPOs. In addition, it was observed that differentiated p53 KO-eHEPOs had decreased Ki67+ proliferative index compared to WT, confirming our previous data (Figure 53). In our ongoing experiments, it was observed that p53KO-eHEPO with different passage numbers further increased collagen accumulation in DMLX2M2, which mimics the pro-carcinogenic tumor microenvironment. (Figure 54) Relevant fibrosis marker gene expressions were also controlled by qPCR method, and it was observed that especially COL1A1 and COL3A1 increased significantly in p53-KO eHEPO. In addition, significant changes were observed in the expression levels of fibrotic markers PDGFRB, TNF α , MMP9, TGFB-R1 (Figure 55)

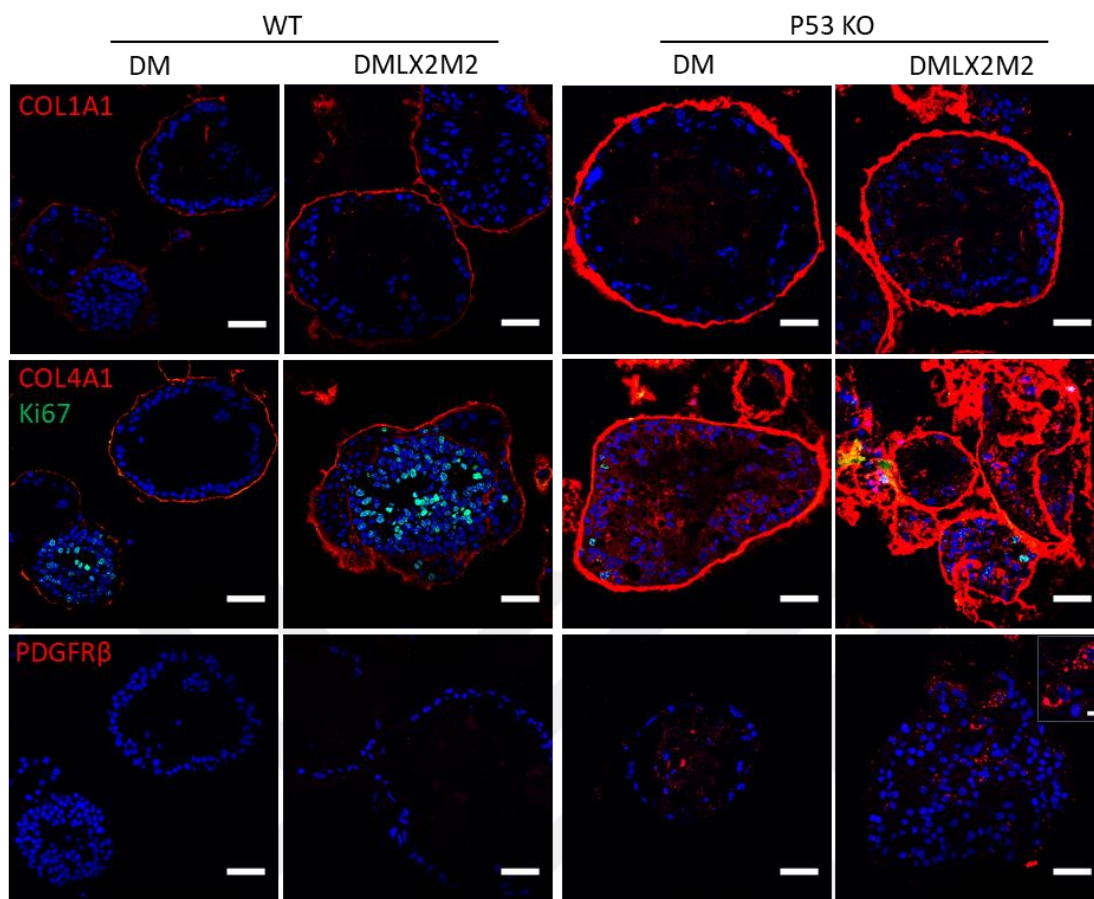


Figure 53. Liver fibrosis marker gene expressions increase in p53 KO eHEPOs

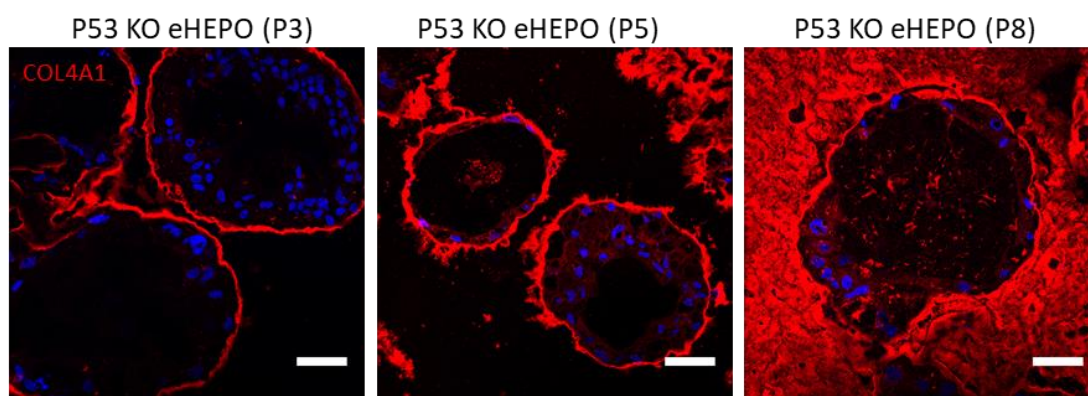


Figure 54. COL4A1 expression levels in P3, P5 and P8 p53 KO-eHEPOs in DMLX2M2 pro-carcinogenic medium. (P3, P5, P8: passage number 3, 5, 8)

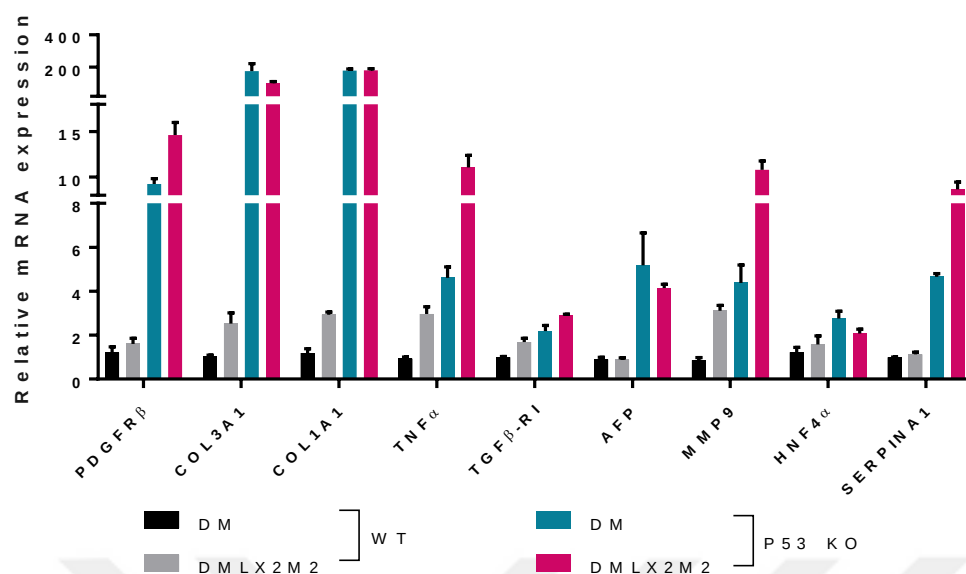


Figure 55. Fibrosis and maturation marker gene expressions in WT and p53-KO eHEPOs.

In addition, this, mason trichome staining was performed in WT and p53KO-eHEPO under similar conditions to confirm these results, and as expected, a highly significant collagen deposition was observed in p53 KO-eHEPO (Figure 56)

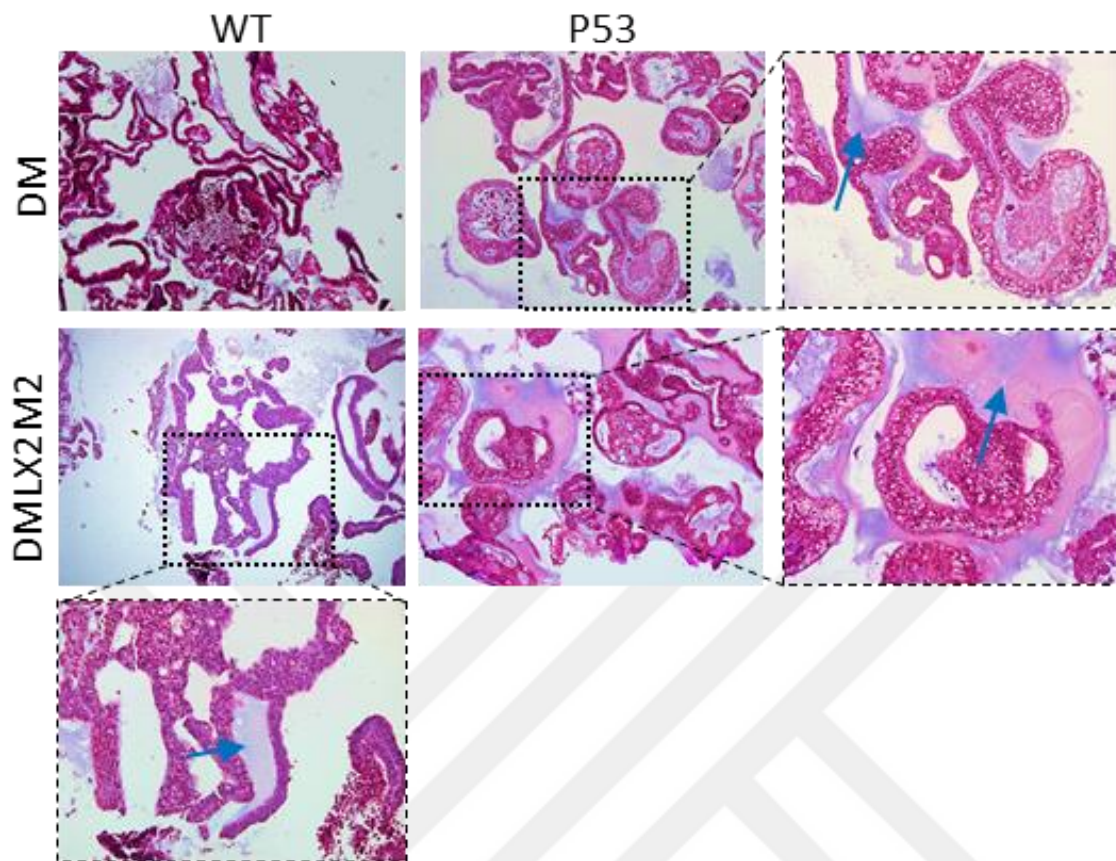


Figure 56. Mason trichome staining in WT and p53KO-eHEPOs. Blue arrows show mason trichome positive areas.

In the continuation of our study, as we observed before, significant increases were observed in the changes of mature liver markers in parallel with the decrease in proliferation. Among these markers, CK19 is a colongocyte marker whose expression is lost in mature hepatocytes. In studies in the literature, it has been reported that hepatocyte-mimicking organoid cultures express CK19 to a certain extent. Similarly, in our method, it is observed that WT organoids express CK19 significantly. Interestingly, CK19 expression amounts of p53 KO-eHEPOs seem to be decreased. This decrease was also observed indistinctly in the pro-carcinogenic setting. In support of this, it was observed that CK18, which was expressed only in mature hepatocytes, was significantly increased in p53KO-eHEPO. Interestingly, the pro-carcinogenic microenvironment highly expresses both markers together in p53 KO organoids. In addition, two other maturation markers, SERPINA1 (A1AT) and ALB positivity, were observed to increase significantly in p53 KO-eHEPOs. In conclusion, the specific expression pattern observed in these markers indicates the emergence of a characteristic bipotent feature similar to that in tumor-initiating hepatic progenitor cells. In addition, when the gene expressions

showing hepatic maturation were examined, it was seen that AFP, HNF4a and SERPINA1 were significantly higher in p53-KO eHEPOs (Figure 57)

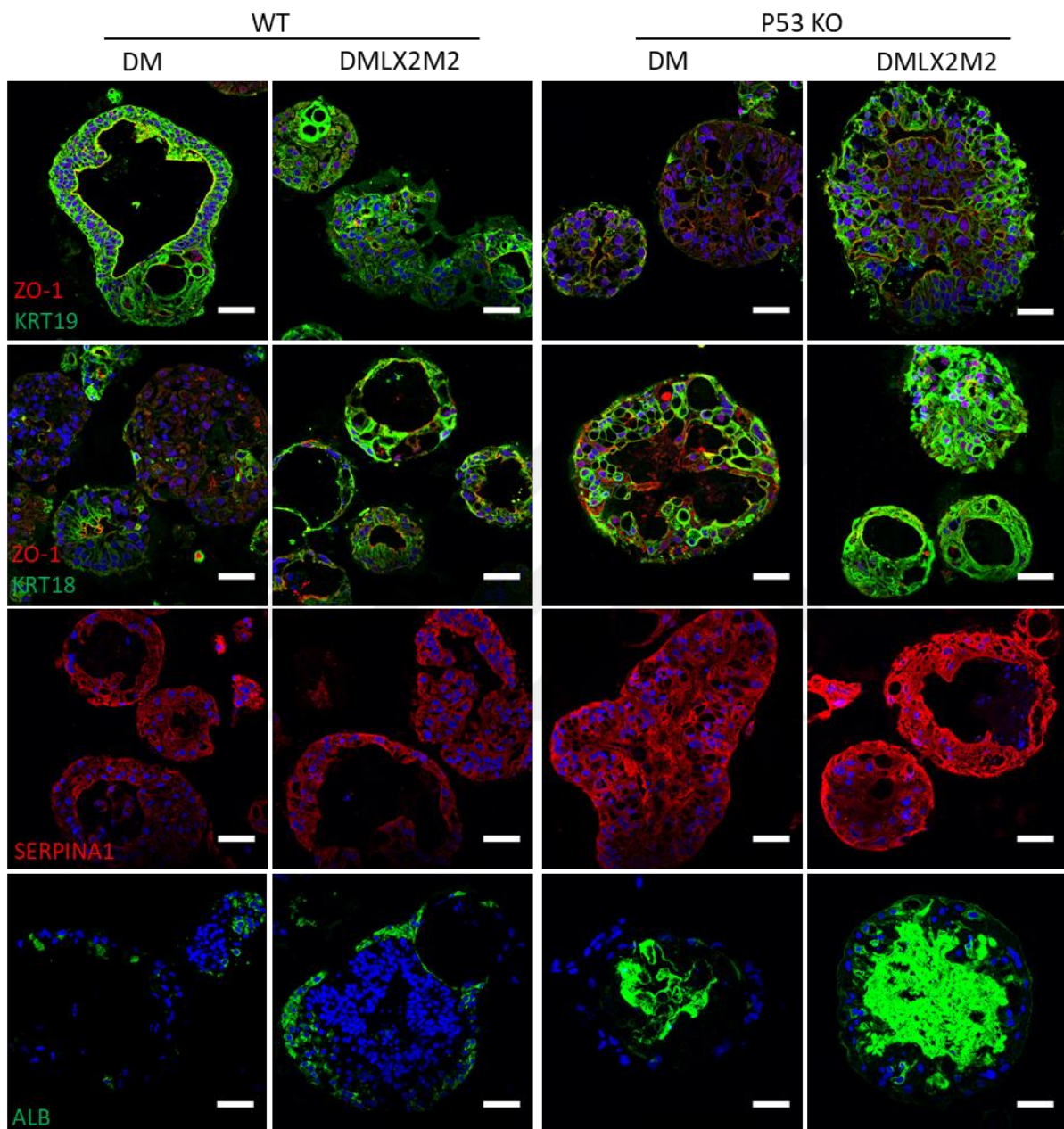


Figure 57. Hepatic marker gene expressions strengthen in p53 KO-eHEPOs.

Finally, cleaved-caspase3 and H2A.X staining's were performed to examine the extent and distribution of DNA damage accumulated due to silencing of the p53 gene and the extent to which the cells died during the differentiation process. Accordingly, it was observed that apoptotic bodies increased in the pro-carcinogenic inflammatory microenvironment, as expected, and this increase was generally on the inner surface of organoids. It was observed

that the increase in DNA damage increased significantly in p53 KO-eHEPOs and similarly, a higher positivity was observed in the cell groups located in the lumen part of the organoids. (Figure 58)

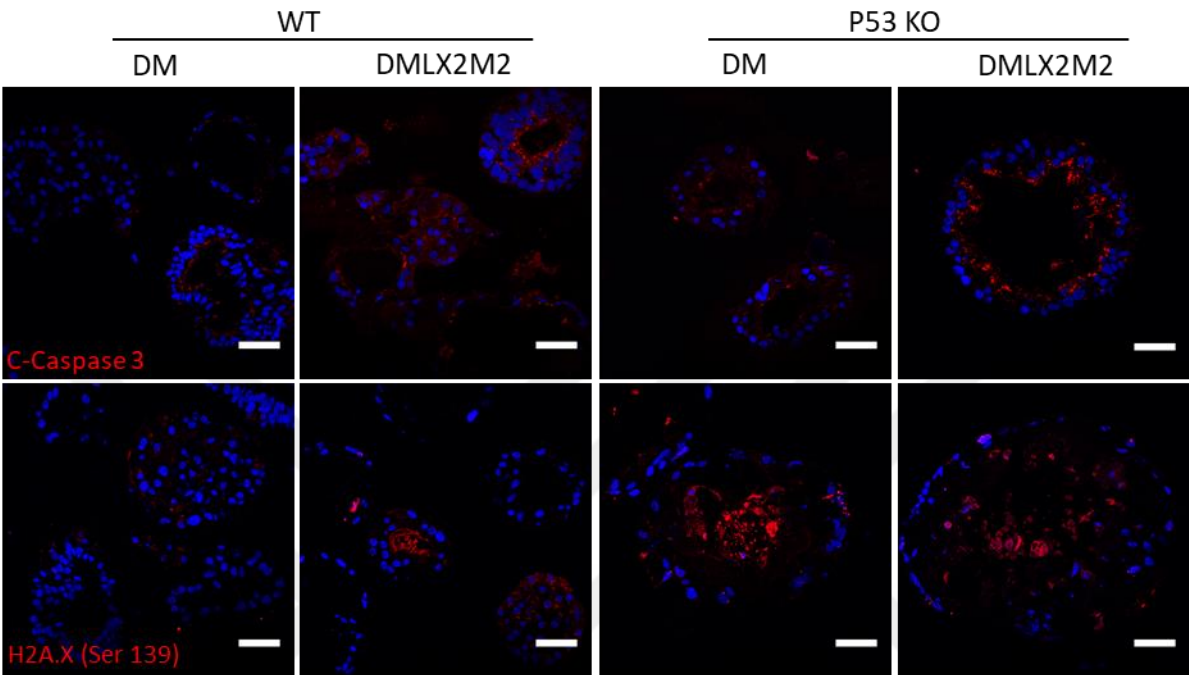


Figure 58. Apoptotic cell and DNA damage ratio increase in p53KO-eHEPOs.

5. DISCUSSION

Liver cancer is the 6th most common cancer in the world and ranks 4th in terms of mortality rates. It is a type of cancer characterized by its late prognosis and drug resistance. There are very limited treatment options in advanced cancer, and current drugs have short-term effects and side effects that reduce the patient's life standards considerably. At this point, it is very important to understand cancer at an early stage and to develop therapeutic approaches for the mechanisms in the tumorigenesis process (Cheng et al., 2009). A cirrhotic process is involved in 90% of liver cancer. Increased regeneration and fibrosis in the stage before cirrhosis can be tolerated and the development of cirrhosis can be prevented if appropriate conditions and signals are provided. P53, one of the genes involved in this process, plays a central role in the development of cirrhosis of a fibrotic tissue. Activated stellate (myofibroblast) cells located in the inflammatory region, which push the cells to proliferate, enter senescence with the activation of the P53 gene and are recognized and removed from the environment by immune cells. Thus, regeneration nodules dissolve and liver homeostasis are restored (Caligiuri et al., 2021; Kwon et al., 2015; Sakurai et al., 2013). Recent studies have focused on examining the role of the p53 gene in more detail in the early stages of cancer. In a related study, the relationship of p53 with CD44 molecule, which is one of the cancer stem cell surface markers and whose expression has been shown to increase at tumor initiation, was shown. In this study, it is stated that CD44 inhibits p53 by inducing nuclear translocation of MDM2 in tumor-initiating hepatic progenitor cells, and then proliferation increases with Akt pathway activation (Dhar et al., 2018). Similarly, it is reported that p53 plays an active role in tumor initiation processes of widely known cancer stem cell surface markers (F. Liu & Qian, 2021; Tang et al., 2012; L. Wang et al., 2015). In addition, it has been reported that hepatic damage and tumors are observed in p53 KO mice within 4-6 months (Donehower, 1996; Yahagi et al., 2004; Yamamoto et al., 2000). Again, it is reported that p53 plays a role in the initiation and progression of liver inflammation, fibrosis and tumorigenesis by supporting the stem cell population and by modifying the TME, controlling the entry and exit of immunosuppressive cells and tumor infiltrating cells into the region (Boutelle & Attardi, 2021; Ghatak et al., 2020). From this point of view, the organoids we obtained in our study and which we showed to be knockout, constitute a very valuable tool to evaluate the role of p53 in the onset of cancer.

In this thesis, it is also planned to carry out KO studies for APC and NF1 gene on p53 KO organoids. For this, gRNAs have been designed and shown to work successfully in HEK293T

cells. The APC gene is a tumor suppressor gene involved in the Wnt/b-catenin pathway, which is mutated in approximately 30% of patients with HCC. Hypermethylation related to APC gene inactivation has been reported in the promoter region of 81% of patients with tumors (Csepregi et al., 2008). Likewise, it has been reported that mice with double KO with the cre/lox system in the APC gene have a 67% more tendency to develop HCC and the tumor frequency increases in the following processes (Colnot et al., 2004). Again, in the colon organoid model, APC mutants on p53 were successfully made on p53, and increased aneuploidy, chromosomal instability and delays in chromosomal segregation, which are widely accepted cancer markers, were observed in these mutants (Drost et al., 2015). In our study, a similar approach was tried for HCC, and it was aimed to create a model that mimics the human tumorigenesis process, which is closer to in vivo models compared to other in vitro and animal models.

It has been reported that the NF1 gene, another tumor suppressor, is expressed in healthy tissue, but its expression is lost in tumor tissue. In parallel, increased expression of p21-RAS was associated with increased proliferation in the region. It has also been reported that HCC formation was triggered in a patient with NF1 mutation (Kimura, Tanaka, Umemura, & Tanaka, 2015). Although there are not many case studies on the mutation of the NF1 gene, in a study conducted in mice in 2017, it was shown that mutation insertions and deletions mediated by CRISPRCas9 in the NF1 gene significantly triggered the formation of HCC. In this study, p53 $-/-$; myc; In Cas9 cells, NF1 inactivation with various NF1 gRNAs showed increased levels of phosphor ERK, which is the downstream biomarker of the RAS-MAPK signaling cascade. It has been reported that NF1-RAS pathway disorders can be detected in patients through HMGA2 and Sox9, which is another marker, and the success of treatment with specific inhibitors can be increased. However, in this study, both the results obtained in an animal model and the use of a lentiviral Cas9 system are disadvantageous (Song et al., 2017). In our study, it has been shown that the NF1 gene can successfully silence gene activity in Hek293T cells. It was also shown that WT and p53 KO cells could not grow in the next step, NF1 specific selective medium (no EGF+ 20 μ M gefitinib). Therefore, in the organoid model, KO of the NF1 gene with the CRISPR/Cas9 method and selective growth of successful cells and obtaining clonal organoids will be ensured.

Thus, a basic tumor model will be created in the literature, and the fact that this model has been developed in organoids, which are mini-organs that can be stably cultured for a long time,

preserving the changes in tumorigenesis processes and their function and physiology in real tissue, is a reference for many future studies.

At the same time, further validation and characterization of the obtained P53 KO organoids were performed and a fibrosis model induced by the pro-carcinogenic tumor microenvironment was developed. In our studies, it has been shown that p53 KO organoids are more proliferative and have atypical inflammatory-like cell morphology as seen in tumorigenic histological sections. Similarly, it was observed under the microscope that P53 KO organoids formed tubular-pseudo-glandular structures (Schlageter et al., 2014; Suzuki et al., 2010). Similar morphological changes were also seen in the article that tried to produce colon cancer with a similar approach (Drost et al., 2015). In addition, immunohistochemical staining showed that the expression of GPC3 and MUC1 levels, which were shown to be associated with the cancerization process in the literature, increased in our p53 KO model (Bozkaya et al., 2012; Suzuki et al., 2010). Based on these results, it is observed that our model works successfully.

It has been reported in the literature that p53 plays a role in controlling the activation of many signaling pathways. It has been reported that p53, together with Smads, modifies the TGFB pathway. It has been reported that the TGFB signal is significantly reduced in conditions where p53 is double knocked out or null, and the activation of the TGFB pathway increases synergistically due to p53 activation (E et al., 2014; Elston & Inman, 2012). On the other hand, there are studies that say that silencing p53 suppresses TGFB-mediated fibrotic responses and migration (Higgins et al., 2018; N. Li & Zhang, 2020). In our reporter assay, an increase in TGFB gene expression was observed.

It has been reported that DKK1 protein levels decrease due to the loss of function of the Wnt pathway in p53, and the pathway is activated and systemic inflammation is induced. Similarly, it has been reported that when p53 is silenced, proliferation and differentiation rates of mesenchymal progenitor cells are increased in a Wnt pathway-dependent manner (Cordenonsi et al., 2003; Donehower, Godley, et al., 1995; Donehower, Harvey, et al., 1995; E et al., 2014; Kalo et al., 2007; Wellenstein et al., 2019). Obtaining similar results with the reporter assay in our study is another indicator that our model reflects the real physiology exactly.

In addition to these results, it has been reported in the literature that P53 acts as a barrier in the conversion of normal cells to induced pluripotent stem cells, allowing them to remain in a stable state (Krizhanovsky & Lowe, 2009; Onder et al., 2012). In our study, it was observed that the

PAS positivity (glycogen storage capacity), which is a mature hepatocyte marker, increased in p53KO-eHEPOs, and when the cells were cultured for a long time without differentiation, they produced Albumin in the EM medium, so the differentiation barrier was broken. On the other hand, the analysis with Cell titer glo-3D shows that the cells with high proliferation observed in EM are both proliferative and have a more plastic structure. Similarly, in a study in the literature, it was reported that the cells in the p53 KO mouse model were in a blast-shaped morphology and secreted Albumin. These cell groups were evaluated as hyperproliferative, albumin secreting and maintaining stem cell status (Dumble, Knight, Quail, & Yeoh, 2001). The Organoid model we obtained reflects this physiology.

Compared to WT, a significant increase in fibrotic marker gene expression was observed in p53 KO organoids induced by the pro-carcinogenic tumor microenvironment, which we created to mimic the initial stage of liver cancer. In parallel with this increase, collagen deposition in P53 KO organoids was demonstrated in Mason trichome staining. Similarly, increased fibrotic marker gene expressions in OCT staining showed that p53 KO organoids are more prone to fibrosis in the pro-carcinogenic microenvironment and can be used as a fibrosis model. Significant changes in EPCAM and CD24 seen in flow analyzes suggested that these surface markers may have a role in forming cancer-initiating stem/progenitor cells depending on p53. However, detailed molecular analyzes could not be performed within the scope of this thesis.

As a result, at this point, understanding the mechanisms of tumor formation in a carcinogenic microenvironment by using p53 KO organoids, which is one of the driver genes, will make significant contributions to the literature. Similarly, with this approach, it becomes possible to mimic different stages of tumorigenesis. Thus, it is to create a very valuable model to understand the molecular mechanisms of cancer, one of the most basic health problems that humanity has been dealing with for many years, with step-by-step and real physiology.

6. CONCLUSION

Within the scope of the thesis, the targeted result was achieved and a pro-carcinogenic tumor microenvironment inducible P53 KO-eHEPO fibrotic organoid model was created. However, the lack of qualified personnel for the use of the devices in the institute, their change and the appointment of untrained people caused the limitation of our work in terms of budget, which is the loss of materials and experiments. In addition, the protocol has been tried for the first time by us in the literature for liver organoids, and the transfection efficiency is very low in organoids compared to normal cells, and the optimizations to increase this require time and money, which has led to this process getting longer. Again, the desired success rate could not be reached in the desired time due to reasons such as the fact that the total budget of the study was depleted very quickly due to the changing exchange rate differences, and our work was interrupted in a period of about 1 year due to the Covid-19 pandemic.

Despite these disadvantages, p53 KO organoids were successfully obtained in this study and it was shown that APC and NF1 KO organoids could also be obtained. At this point, the APC and NF1 KO organoid modeling studies planned for these two genes were canceled due to the additional budget requirement. In future studies, in addition to P53 in the advanced stages of the cancerization process, cumulative KO models are created in other genes that are observed to have KO, and examining the transcriptomic changes in all these organoids compared to WT will provide an understanding of the molecular mechanisms of the tumorigenesis process. Thus, candidate molecules that can be used in the development of new treatment strategies will be possible by targeting the molecular pathways that vary according to each cancer-mutation type.

Investigation of transcriptomes of WT and P53 KO organoids obtained within the scope of this study in DM and DMLX2M2 media is a very important cornerstone for the study. Thus, the molecular mechanisms that change depending on the p53 gene will be discovered during the development of fibrosis. For this reason, it is planned to perform single cell RNA-seq (scRNA-seq) in the relevant groups to be presented in the paper to be published for the future of our study. Mechanistic studies targeting candidate signaling pathways or ECM changes for the treatment to be found with the results to be obtained from here should definitely be done in the future.

Finally, I would like to thank TÜBİTAK for funding the 1001 project numbered 117Z858 carried out within the scope of this thesis, and the YÖK100/2000 program and BİDEB 2211-C program that supported me during my thesis work.

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8. SUPPLEMENTARY DATA

Paper 1: Karabicici, M., Alptekin, S., Karagonlar, Z. F., & Erdal, E. (2021). Doxorubicin-induced senescence promotes stemness and tumorigenicity in EpCAM[−]/CD133[−] nonstem cell population in hepatocellular carcinoma cell line, HuH-7. *Molecular oncology*, 15(8), 2185.

Paper 2: Genetic ablation of p53 in a pro-carcinogenic microenvironment triggers at the onset of HCC in liver organoid (eHEPO) model. (under process for Cell Death and Disease)

