

THE FUNCTIONAL EFFECTS OF AKR1B10 OVEREXPRESSION IN  
COLORECTAL CANCER CELL LINES

A THESIS SUBMITTED TO  
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES  
OF  
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ESİN GÜLCE SEZA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
FOR  
THE DEGREE OF MASTER OF SCIENCE  
IN  
MOLECULAR BIOLOGY AND GENETICS

SEPTEMBER 2017



Approval of the thesis:

**THE FUNCTIONAL EFFECTS OF AKR1B10 OVEREXPRESSION IN  
COLORECTAL CANCER CELL LINES**

submitted by **ESİN GÜLCE SEZA** in partial fulfillment of the requirements  
for the degree of **Master of Science in Molecular Biology and Genetics in  
Biology Department, Middle East Technical University** by,

Prof. Dr. Gülbin DURAL ÜNVER  
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Orhan ADALI  
Head of Department, **Biology**

Assoc. Prof. Dr. Sreeparna BANERJEE  
Supervisor, **Biology Dept., METU**

**Examining Committee Members:**

Prof. Dr. Mayda GÜRSEL  
Biology Dept., METU


Assoc. Prof. Dr. Sreeparna BANERJEE  
Biology Dept., METU

Assoc. Prof. Dr. Can ÖZEN  
Biotechnology Dept., METU

Assoc. Prof. Dr. Çağdaş Devrim SON  
Biology Dept., METU

Assoc. Prof. Dr. Ali Osmay GÜRE  
Molecular Biology and Genetics Dept., Bilkent University

**Date:** 05/09/2017



**I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.**

Name, Last name: Esin Gülce SEZA

Signature :

## **ABSTRACT**

### **THE FUNCTIONAL EFFECTS OF AKR1B10 OVEREXPRESSION IN COLORECTAL CANCER CELL LINES**

Seza, Esin Gülce

M.Sc., Department of Molecular Biology and Genetics

Supervisor: Assoc. Prof. Dr. Sreeparna Banerjee

September 2017, 73 pages

Aldo-keto reductases (AKRs) are nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) dependent oxidoreductases that are involved in many anabolic and catabolic reactions. When they are over-activated, the polyol pathway is activated that results in oxidative stress. AKRs are implicated in many inflammation-associated diseases including diabetes mellitus, asthma, uveitis, sepsis, atherosclerosis, periodontitis, and many cancers. AKR1B10, a member of the AKR family that is also known as small intestine like aldose reductase is highly expressed in the small intestine and colon.

Analysis of publicly available microarray datasets indicated that colorectal cancer (CRC) patients showed lower AKR1B10 expression compared to normal tissues, although AKR1B10 was overexpressed in other cancers such as liver cancer. Gene set enrichment analyses indicated significant enrichment of metabolism related genes in tumors that expressed high amounts of AKR1B10. In order to understand the functional effects of AKR1B10, we overexpressed AKR1B10 in CRC cell lines that do not express the protein. We observed no alterations in cellular proliferation or cell cycle; however, cellular motility was reduced, along with a decrease in the nuclear translocation, DNA binding and transcriptional activity of NF- $\kappa$ B, which is an important transcription factor that is necessary for cell survival and inflammation.

The work carried out in this thesis suggests that the expression of AKR1B10 in colon cancer cells may not directly affect cancer progression by affecting cell proliferation or cell cycle; rather, the protein has a more indirect effect, perhaps through the activation of metabolism related pathways.

**Keywords:** Aldo-keto reductase, colorectal cancer, AKR1B10, motility, NF- $\kappa$ B

## ÖZ

### AKR1B10 AŞIRI İFADELENMESİNİN KOLOREKTAL KANSER HATLARI ÜZERİNDEKİ FONKSİYONEL ETKİLERİ

Seza, Esin Gülce

Yüksek Lisans, Moleküler Biyoloji ve Genetik Bölümü

Tez Yöneticisi: Doç. Dr. Sreeparna Banerjee

Eylül 2017, 73 sayfa

Aldo-keto redüktazlar (AKRler) birçok anabolik ve katabolik reaksiyoniyelerde rol oynayan nikotinamid adenin dinükleotit (NAD(P)H) bağımlı oksidoredüktazlardır. Fazla aktif edildikleri zaman polyol yolağını aktive ederek oksidatif strese sebep olurlar. Aldo-keto redüktazlar diyabet, astım, üveit, sepsis, aterosklerosis, periyodontit ve çeşitli kanserler gibi enflamasyona bağlı hastalıklarda yer almaktadır. AKR ailesinin üyelerinden olan AKR1B10, ince bağırsak aldoz redüktazı olarak bilinir ve özellikle ince bağırsak ve kolonda aşırı ifadelenir.

Karaciğer kanseri gibi birçok kanser türünde aşırı ifalenmesine rağmen, mikrodizin dalarına göre, kolorektal kanser hastaları normal kolorektal dokuları ile kıyaslandığında az miktarda AKR1B10 ifade eder. Gen

zenginleştirme analizleri, bu hastalarda tümörün daha çok metabolizma genleri ile alakalı olduğunu göstermiştir. AKR1B10 aşırı ifadenmesinin etkilerini daha iyi anlayabilmek için, kolorektal kanser hücre hatlarından protein ifade etmeyenleri seçilmiştir. Hücre çoğalmasında ve hücre döngüsünde değişim görmememize rağmen, hücre hareketliliğinde ve NF-κB adı verilen hücre yaşaması ve enflamasyonda önemli olan transkripsiyon faktörünün hücre çekirdeğine geçişinde , DNA'ya bağlanmasında ve transkripsiyonel aktivitesinde önemli miktarda azalma gözlemlenmiştir.

Bu tez çalışmasında, kolon kanseri hücrelerinde AKR1B10 ifadenmesinin, doğrudan hücre çoğalması ile kanser ilerleyişini etkilemesinden ziyade, daha dolaylı bir şekilde metabolizma ile ilgili yolları aktifleştirerek etkilediği öngörülmektedir.

**Anahtar kelimeler:** Aldo-keto redüktaz, kolorektal kanser, AKR1B10, hücre hareketliliği, NF-κB



*To my family*

## ACKNOWLEDGMENTS

First, I would like to express my sincere gratitude to my supervisor Assoc. Prof. Dr. Sreeparna Banerjee for the guidance, comments, support and encouragement throughout my research. I would like thank her for patience even with a very tiny problem. I always believe that she choose us with an algorithm that let the lab make us feel like cozy as home.

I would like to thank Mustafa Kemal Atatürk for his idealism and contributions for this country.

I would like to thank all my jury members; Prof. Dr. Mayda Gürsel, Assoc. Prof. Dr. Can Özen, Assoc. Prof. Dr. Çağdaş Devrim Son and Assoc. Prof. Dr. Ali Osmay Güre for their contributions.

I would like to thank once more to Assoc. Prof. Dr. Ali Osmay Güre and his student Seçil Demirkol for their invaluable GSEA analysis and comments.

I am deeply grateful and thankful to former and present Lab B59 family. Many thanks go to Dr. Sinem Tunçer, Melis Çolakoğlu, Betül Taşkoparan, Sinem Ulusan, Burak Kızıl, Ege Solel, Güniz Sırt, Aydan Torun, İsmail Güderer, Çağdaş Ermiş, Müge Sak and Rezwan Siddiquee for their support and help in most challenging problems.

I would like to thank semi-lab mate Hlyya poęlu for her support and invaluable friendship throughout my undergraduate and graduate studies.

I am thankful to my dearest friends Glay İlgen, Atiye Merve Karayazılı, Erkan Durmuş, Berfin Şahiner and Glistan Yeşilmen for their endless support, friendship and encouragements.

I would like to thank my dear love Efe Boyacıgiller for his support in good times and funny jokes in hard times.

Lastly, many thanks to my parents Sevda and Hasan Seza for their endless love, spiritual support and eternal understanding.

This study has been supported by TBİTAK through Bilateral Cooperation Project with Slovak Academy of Science (Project No: 113S006).

## TABLE OF CONTENTS

ABSTRACT.....	v
ÖZ.....	vii
ACKNOWLEDGMENTS .....	x
TABLE OF CONTENTS.....	xii
LIST OF FIGURES .....	xv
LIST OF TABLES .....	xvii
LIST OF ABBREVIATIONS .....	xviii
INTRODUCTION .....	1
1.1    Cancer Cell Metabolism.....	1
1.2    Warburg effect.....	2
1.3    PI3K Pathway.....	3
1.4    AMPK Pathway.....	4
1.5    AKR Superfamily.....	6
1.5.1    AKR1B1 .....	9
1.5.2    AKR1B10 .....	11
1.5.3    AKRs and cancer .....	11
1.6    Aims of the study .....	13
1.6.1    Aim of the thesis .....	14
MATERIALS AND METHODS.....	15
2.1    Cell Culture .....	15
2.2    Transfection.....	16

2.3	Protein Isolation and quantification .....	16
2.4	Western Blot.....	17
2.5	RNA Isolation .....	20
2.6	cDNA Synthesis .....	20
2.7	RT-PCR and qRT-PCR .....	20
2.8	Proliferation Assays .....	22
2.9	Cell Cycle Assay .....	22
2.10	Luciferase Assay.....	23
2.11	Transwell Migration Assay .....	24
2.12	Statistical Analysis .....	24
RESULTS .....		25
3.1	Expression of AKR1B10 in CRC Cell lines .....	27
3.2	Overexpression of AKRB10 in HCT-116, SW 480 and LoVo.....	30
3.3	Effects of AKR1B10 overexpression on cellular proliferation.....	31
3.4	Effects of AKR1B10 on the Mitogen Activated Protein Kinase (MAPK) pathway.....	32
3.5	Effects of AKR1B10 overexpression on cell cycle progression.....	34
3.6	Effects of AKR1B10 overexpression on nuclear localization of NF- $\kappa$ B	39
3.7	Effects of AKR1B10 overexpression on NF- $\kappa$ B transcriptional activity.....	41
3.8	Effects of AKR1B10 overexpression on cell migration and EMT markers.....	43
DISCUSSION .....		47

CONCLUSIONS AND FUTURE STUDIES .....	55
REFERENCES .....	59
APPENDIX A.....	69
APPENDIX B .....	71



## LIST OF FIGURES

<b>Figure 1.1</b> Downstream pathways of phosphorylated AMPK. ....	5
<b>Figure 1.2</b> Polyol Pathway. ....	10
<b>Figure 3.1</b> Gene Set Enrichment Analysis for AKR1B1. ....	26
<b>Figure 3.2</b> Gene Set Enrichment Analysis for AKR1B10. ....	27
<b>Figure 3.3</b> Transcript levels of AKR1B10 and AKR1B1 in CRC cell lines. ....	28
<b>Figure 3.4</b> Protein levels of AKR1B10 and AKR1B1 in CRC cell lines. ....	29
<b>Figure 3.5</b> Confirmation of overexpression of HCT-116, SW 480 and LoVo with respect to empty vector (EV). ....	30
<b>Figure 3.6</b> Effect of AKR1B10 overexpression on cell proliferation. ....	31
<b>Figure 3.7</b> Effects of AKR1B10 transfection on cell proliferation by using MTT assay in HCT-116 cells. ....	32
<b>Figure 3.8</b> Effects of AKR1B10 overexpression on JNK1 and p-SAPK/JNK MAPK pathway. ....	33
<b>Figure 3.9</b> Effects of AKR1B10 overexpression on Erk1/2 phosphorylation with respect to empty vector control (EV) in HCT-116, SW 480 and LoVo. ....	34
<b>Figure 3.10</b> Effects of overexpression of AKR1B10 on cell cycle progression. AKR1B10 overexpression did not change the cell cycle distribution of HCT-116 cells with respect to empty vector (EV). ....	36
<b>Figure 3.11</b> Cell cycle distribution in AKR1B10 overexpressing HCT-116 cells. ....	37
<b>Figure 3.12</b> Determination of markers of cell cycle progression in AKR1B10 or EV transfected HCT-116 cells. ....	39

<b>Figure 3.13</b> Effects of AKR1B10 overexpression on the translocation of NFκB to the nucleus. ....	40
<b>Figure 3.14</b> Effects of AKR1B10 overexpression in NF-κB transcriptional activity.....	42
<b>Figure 3.15</b> DNA binding activity of the NF-κB p65 subunit in the presence of AKR1B10 overexpression by a DNA binding ELISA assay.....	43
<b>Figure 3.16</b> The effect of AKR1B10 expression on cell migration. ....	44
<b>Figure 3.17</b> The expression of epithelial and mesenchymal markers in AKR1B10 or EV transfected cells.....	45

## LIST OF TABLES

<b>Table 1.1.</b> List of the human specific AKRs.....	7
<b>Table 2.1.</b> List of the antibodies used in this study.....	19
<b>Table 2.2.</b> List of the primers used in this study. ....	21



## LIST OF ABBREVIATIONS

<b>AKR</b>	Aldo-keto reductases
<b>CRC</b>	Colorectal cancer
<b>ROS</b>	Reactive oxygen species
<b>NAD(P)H</b>	Nicotinamide adenine dinucleotide phosphate
<b>PI3K</b>	Phosphatidylinositol 3-kinase
<b>mTOR</b>	Mammalian target of rapamycin
<b>AMPK</b>	AMP-activated kinase
<b>G1</b>	Gap 1 phase of cell cycle
<b>S</b>	Synthesis phase of cell cycle
<b>G2/M</b>	Gap 2 phase of cell cycle/Mitosis
<b>EMT</b>	Epithelial-Mesenchymal transition
<b>GSEA</b>	Gene set enrichment analysis
<b>PBS</b>	Phosphate buffer saline
<b>TBS-T</b>	Tris buffer saline-Tween 20
<b>GO</b>	Gene ontology
<b>MAPK</b>	Mitogen-activated protein kinase
<b>ERK</b>	Extracellular signal-regulated kinase
<b>ATP</b>	Adenosine triphosphate

# CHAPTER 1

## INTRODUCTION

### 1.1 Cancer Cell Metabolism

Cancer cells are capable of unlimited survival and growth. Numerous deletions, amplifications, insertions and substitutions resulting from defects in DNA repair machinery, replication errors, and/or environmental effects such as carcinogen exposure or aging may contribute to a cancer cell phenotype. As a result, mutations may accumulate that may alter cellular pathways. Cancer cells have the ability to escape from the immune system by altering signals in their microenvironment. If the environment is favorable, the cells start to proliferate and grow. At that point, the cells acquire some common characteristics such as continuous proliferation, angiogenesis, invasion and metastasis, evasion from cell death and growth suppressors (Hanahan & Weinberg, 2011). Moreover, other characteristics such as enabling replicative immortality, altering energy metabolism and escaping from immune system have attracted considerable attention in recent years and are considered as emerging hallmarks of cancer (Hanahan & Coussens, 2012).

Various intrinsic and extrinsic mechanisms may decide the fate of a precancerous cell. Of these, cellular metabolism (consisting of both anabolism and catabolism) is a key factor that is controlled tightly. ATP is an important metabolic molecule in anabolic or catabolic reactions; cancer cells carry out many more catabolic reactions compared to normal cells and the induction of catabolic pathways is often dependent on the cellular ATP levels. All four major macromolecules, namely, proteins, lipids, nucleic acids and carbohydrates, provide energy for proliferative cells. Cancer cells need even more energy to maintain their proliferative state. Therefore, cancer cells generally use glucose for aerobic glycolysis (please see below for further details) while normal cells use glucose in the oxidative phosphorylation pathway (Annibaldi & Widmann, 2010). This phenotype may give advantages to cancer cells. Energy metabolism of the cells may affect oncogenes, pH of microenvironment, pro-inflammatory signals, anti-proliferative mechanisms and redox management.

Glycolysis is the major energy supply for all living organisms. Glycolysis may often be enhanced in cancer cells under anaerobic conditions but cancer cells may prefer aerobic glycolysis even in the presence of abundant oxygen. This phenomenon is called the Warburg effect (Warburg, 1956).

## **1.2 Warburg effect**

Increased glycolysis despite the presence of oxygen is a characteristic of cancer cells and is called aerobic glycolysis or Warburg effect. In the cancer microenvironment, pH of the extracellular space is lower when compared to the microenvironment of normal cells (Annibaldi & Widmann, 2010). The

switch from oxidative phosphorylation to glycolysis causes accumulation of lactate by-products, thereby reducing the pH in the tumor microenvironment and this accumulation is one of the characteristics of tumor cell metabolism (Cairns, Harris, & Mak, 2011). Aerobic glycolysis is more helpful for cancer cells due to less production of ROS (Reactive Oxygen Species). It helps to adapt to hypoxia more easily in rapidly growing tumors that are poorly vascularized (Cairns et al., 2011).

Decreased pH and hypoxia may elevate ROS formation, which in turn causes cellular stress. Cellular stress may lead to the secretion of growth factors that can enhance proliferation; additionally, cell-to-cell interactions in the tumor environment may help the cells to induce angiogenesis and escape from immune cells. Signaling mechanisms of neighboring cells may be changed in the presence of lactate by-products. For example, endothelial cells activate the VEGF, PI3K, HIF-1 $\alpha$  and NF- $\kappa$ B pathways, which provide essential signaling molecules for the proliferation and continuing survival of cancer cells. Among changes in the immune cells, a decrease in the classical M1 macrophages and an increase in the alternate M2 macrophages are seen; this polarization contributes to the suppression of the immune system. Lastly, increased hyaluronic acid production from fibroblasts has been reported and it may contribute to tumor invasion (Pavlova & Thompson, 2016).

### **1.3 PI3K Pathway**

The PI3K (phosphatidylinositol 3-kinase) pathway is one of the key pathways that link cellular energy metabolism and proliferation, growth, survival and motility. Stimulated receptor tyrosine kinases (RTKs) and G protein coupled

receptors (GPCRs) transduce signals to PI3K, which phosphorylates inositol phospholipids and activates the serine/threonine kinase AKT (protein kinase B) (Liu, Cheng, Roberts, & Zhao, 2009 and Fresno Vara et al., 2004). PTEN (phosphatase and tensin homolog deleted from chromosome 10) is a tumor suppressor and a negative regulator of PI3K signaling pathway (Liu et al., 2009). If PTEN is mutated in tumor cells, the PI3K pathway is active continuously.

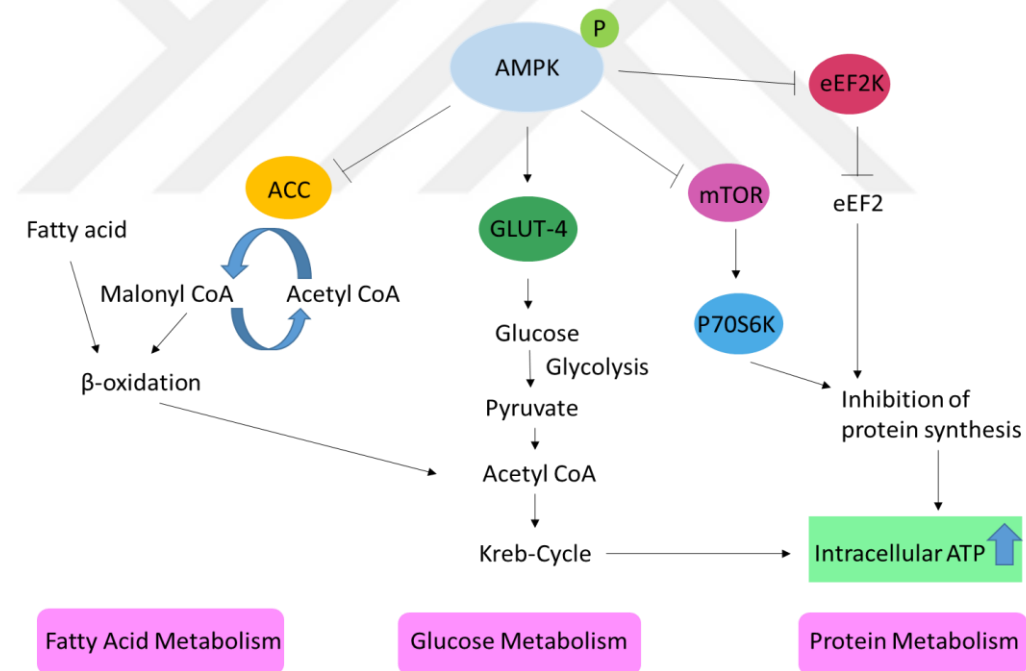
mTOR (mammalian target of rapamycin) is the downstream kinase of AKT and the phosphorylation of AKT activates mTOR in elevated glycolysis. Nutrient sensing and metabolic transformation by mTOR is well known (Cairns et al., 2011). mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2) constitute mTOR kinase, which regulates cellular growth and proliferation. mTORC1, the best characterized mTOR complex, regulates cellular responses to five major variables including stress, growth factor availability, oxygen level, energy status and amino acid availability (Laplante & Sabatini, 2012). AMPK (AMP-activated protein kinase) activation when AMP levels are high may suppress synthesis of energetically costly molecules by inhibiting mTOR (Palm & Thompson, 2017).

#### **1.4 AMPK Pathway**

AMPK (AMP-activated protein kinase) is another key molecule that regulates energy metabolism of cells by relying on the AMP:ATP ratio. When the ratio increases (i.e. there is less ATP in the cell), AMPK is phosphorylated and activated. p-AMPK activates glucose related biochemical pathways, enhances ATP production and restores homeostasis. AMPK is considered as a potential

therapeutic target in metabolic syndrome and cancer (Kim, Yang, Kim, Kim, & Ha, 2016).

AMPK contains three different subunits,  $\alpha$ ,  $\beta$  and  $\gamma$ . The protein can be activated directly by AMP or AMP mimics. Phosphorylation of the AMPK $\alpha$  subunit at Thr-172 activates further signaling. AMPK $\beta$  is the regulatory domain that decides whether anabolic (i.e lipogenesis, glycogenolysis, protein synthesis) or catabolic (i.e glucose uptake/glycolysis, fatty acid oxidation, autophagy) processes will be activated. AMPK $\gamma$  activates AMPK in the presence of the cellular adenosine nucleotides (AMP, ADP or ATP) (Kim et al., 2016). Activated AMPK may affect fatty acid, glucose and protein metabolism (**Figure 1.1**).



**Figure 1. 1** Downstream pathways of phosphorylated AMPK. Figure is adapted from Wong et al., 2009.

As shown in *Figure 1.1*, AMPK activation directly inhibits mTOR (mammalian target of rapamycin) and abrogates the anabolic process of protein synthesis. In fatty acid metabolism, ACC (acetyl-CoA carboxylase) is the key molecule for the activation of the  $\beta$ -oxidation through AMPK activation. Lastly, active AMPK increases expression of the glucose transporter GLUT-4 in order to enhance uptake of glucose inside the cells. More glucose intake elevates the level of ATP and AMPK is deactivated through its regulatory domain.

### **1.5 AKR Superfamily**

The aldo-keto reductases (AKRs) are a family of proteins that bind to nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) in order to activate various metabolism related pathways in both health and disease. Over 150 AKR enzymes belonging to 15 different families (classified according to sequence similarity) can catalyze the reduction of carbonyl groups of primary and secondary alcohols. These enzymes have conserved sequence identity, common protein fold and the  $(\alpha/\beta)_8$ -barrel structure although their functions may be very different from each other (Mindnich & Penning, 2009). Moreover, aliphatic and aromatic aldehydes, ketones, ketoprostaglandins, ketosteroids and xenobiotics are the substrates that can be reduced by AKRs.

Nomenclature system of AKRs starts with family name and continues with subfamily, unique protein sequence and multimers, which exist only in AKR6 and AKR7 families. A comprehensive overview about all types of AKRs is on a website (<https://www.med.upenn.edu/akr/>).

There are 14 known human AKRs as represented in Table 1.1.

**Table 1.1.** List of the human specific AKRs.

<b>Name</b>	<b>Enzyme</b>	<b>Specificity</b>
AKR1A1 (ALR)	Aldehyde reductase	
AKR1B1 (AR)	Aldose reductase	
AKR1B10 (ARL-I)	Small intestine like aldose reductase	Small intestine
AKR1B15	Aldose reductase	Mitochondrial
AKR1C1	Dihydrodiol dehydroxygenase 1	
AKR1C2	Dihydrodiol dehydroxygenase 2	
AKR1C3	3 $\alpha$ -Hydroxysteroid dehydrogenase, type II	
AKR1C4	Dihydrodiol dehydroxygenase 4	
AKR1D1	Ketosteroid-5 $\beta$ -reductase	Hepatic homeostasis
AKR1E2 (AKR1CL2)	Aldo-keto reductase family 1 member C-like 2	Testis specific
AKR6A3 (KCNAB1)	Potassium voltage-gated channel member 1	
AKR6A5 (KCNAB2)	Potassium voltage-gated channel member 2	
AKR6A9 (KCNAB3)	Potassium voltage-gated channel member 3	
AKR7A2 (AFAR1)	Aflatoxin aldehyde reductase	
AKR7A3 (AFAR2)	Aflatoxin aldehyde reductase	

AKR1B, AKR1C and AKR7A families seem to be specific to vertebrates. Duplications and diversification may have contributed to the formation of the vertebrate AKRs. There are potential pseudogenes or new isoforms in the vertebrate genome (Mindnich & Penning, 2009).

The AKRs are water-soluble enzymes with an average molecular weight of 37 kDa. Many of these proteins have been implicated in cancer and metabolic disorders. Aldose reductases (ARs), AKR1B subfamily, play a major role in the polyol pathway (*Figure 1.2*). Under normal conditions, less than 3% of the glucose is catalyzed through the polyol pathway, but under hyperglycemia, this pathway is highly activated (Ramana, 2011). Sorbitol dehydrogenase catalyzes sorbitol to fructose by utilizing NAD when there is excess sorbitol. The highly active polyol pathway causes the accumulation of reactive oxygen species (ROS) in tissues such as heart, vasculature, kidney, eyes, and neurons (W. H. Tang, Martin, & Hwa, 2012).

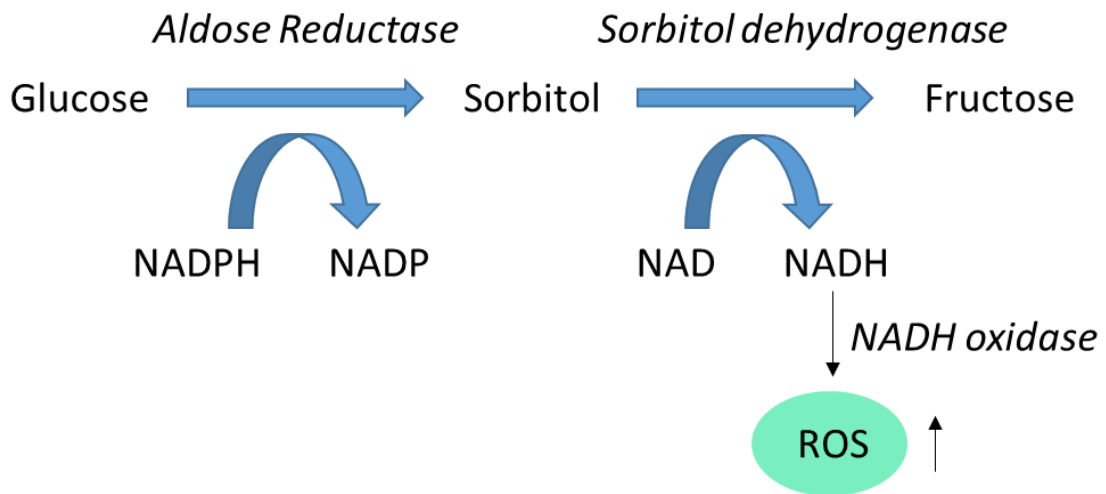
AKR1B1 inhibitors have been developed to diminish diabetic complications such as cataractogenesis, retinopathy, neuropathy, nephropathy and microangiopathy (Srivastava et al., 2011). . AKR inhibitors can be classified into three main groups based on chemical structures:

- Acetic acid derivatives: Tolrestat, Epalrestat, Ponalrestat, Zoporestat, Zenarestat
- Spirohydantoin: Sorbinil, Fidarestat
- Succinimide class ranirestat

To treat diabetic neuropathy, epalrestat is approved for use in the Japanese market. Ranirestat is one of the recently developed inhibitors that is currently at Phase III clinical trials in USA. Fidarestat failed at the Phase III clinical trial although it had less cytotoxic effects. In order to address potential cytotoxic effects of AKR inhibitors, efforts have been made to utilize natural compounds such as quercetin and make them more effective by altering their chemical structures and side chains (Milackova et al., 2014). CHNQ (3,7-Dihydroxy-2-[4-(2-chloro-1,4-naphthoquinone-3-yloxy)-3-hydroxyphenyl]-5-hydroxychromen-4-one) is a semi-synthetic derivative of quercetin that was designed as a potential AKR inhibitor. However, our group reported that cells incubated with low doses of CHNQ showed enhanced oxidative stress and cellular toxicity (Enayat et al., 2016).

### **1.5.1 AKR1B1**

AKR1B1 (also known as ALDR1) that plays an important a role in diabetic pathophysiology is the most studied mammalian AKR enzyme. The expression level of the enzyme can vary in different tissues such as immune system, neurons and muscle tissues. The expression of AKR1B1 is regulated by thyroid hormone, CREB (cAMP-responsive element-binding protein), NFAT5 (nuclear factor of activated T-cells), NRF2 (nuclear factor-erythroid 2 related factor 2), NO (nitric oxide), EGF (epidermal growth factor), TGF $\beta$ 1 (transforming growth factor- $\beta$ 1), hydrogen peroxide, methylglyoxal and atorvastatin (Chen & Zhang, 2012). AKR1B1 is a rate-limiting enzyme in the polyol pathway, which catalyzes the conversion of excess glucose to sorbitol (*Figure 1.2*).



**Figure 1. 2 Polyol Pathway.** Figure is adapted from Tang et al., 2012.

In normal cells, glucose is phosphorylated by hexokinase as soon as it enters the cell. In the presence of excess glucose (such as in diabetes) the polyol pathway gains importance in the removal of glucose if the hexokinase is saturated. In this case, glucose is converted to sorbitol, which cannot cross the cell membrane. Excess sorbitol is converted to fructose in the presence of sorbitol dehydrogenase enzyme, which catalyzes NAD to NADH. Excess NADH may lead to elevated levels of reactive oxygen species (ROS). Both increased oxidative stress and accumulation of the sorbitol disturb cellular mechanisms. The polyol pathway affects only 3% of glucose flux under normal conditions; on the other hand, it can increase up to 25-30% of the glucose flux in hyperglycemia (Ramana, 2011).

### **1.5.2 AKR1B10**

AKR1B10 is a member of aldo-keto reductase family 1 and subfamily B similar to AKR1B1. Although both proteins have a number of structural and functional similarities, there are also a few crucial differences. Unlike the ubiquitous expression of AKR1B1, AKR1B10 is mostly expressed in the colon, small intestine, adrenal glands and liver (Chung et al., 2012a). Interestingly, both AKR1B10 and AKR1B1 are located on chromosome 7 and in close proximity, therefore unsurprisingly; AKR1B1 and AKR1B10 have 70% similarity in amino acid sequence.

Although these two enzymes have very similar nucleotide sequences, they are able to catalyze different substrates. AKR1B1 reduces glucose in the polyol pathway but AKR1B10 is a poor reductant of the glucose, xylose and prostaglandin H<sub>2</sub>. However, AKR1B10 has a high capacity for the reduction of retinals, isoprenyl aldehydes (farnesal and geranylgeranial), and cytotoxic aldehydes (Matsunaga et al., 2012). AKR1B10 regulates retinoic acid pathways in addition, it is thought that AKR1B10 may contribute to carcinogenesis because retinoids play an important role in proliferation, differentiation and morphogenesis (Matsunaga et al., 2012).

### **1.5.3 AKRs and cancer**

In colon cancer, aldose reductase inhibition prevented oxidative stress induced signaling and activation of NF- $\kappa$ B (Ramana, 2011). Ramana et al. showed that inhibition of aldose reductase in SW480 human colon adenocarcinoma cells

prevented tumor growth in nude mice xenografts due to inhibition of G1-S transition phase (Ramana, Tammali, & Srivastava, 2010).

Analysis of data available in the Oncomine cancer gene database indicated that the expression of AKR1B1 and AKR1B10 was variable in different cancer types (Laffin & Petrash, 2012). AKR1B1 was reported to be overexpressed in bladder, brain, cervical, head and neck, esophageal, kidney, leukemia, lymphomas and melanomas when compared with corresponding normal tissue. On the other hand, AKR1B10 was found to be overexpressed in leukemia and pancreatic cancer. Interestingly, AKR1B10 was reported to be under-expressed in colon, gastric and head and neck cancers unlike AKR1B1 (Laffin & Petrash, 2012).

Various studies have indicated that AKR1B1 is overexpressed in cancers associated with inflammation and oxidative stress (Tammali, Srivastava, & Ramana, 2011). Highly metastatic and poorly prognostic basal-like breast cancer showed upregulation of Twist2, which in turn enhanced the expression of AKR1B1. Increased AKR1B1 transcription can activate NF- $\kappa$ B, which regulates Twist2 indicating the presence of a feedback loop. This positive feedback was shown to activate EMT (Epithelial- Mesenchymal Transition) meaning a stronger tumorigenic and metastatic phenotype (Wu et al., 2017).

In cancer, it is suggested that AKR1B10 may be involved in pancreatic cancer by enhancing cellular apoptosis and prenylation of proteins. Additionally, a role of AKR1B10 has been suggested in hepatocellular carcinoma, tobacco-related lung and bladder carcinoma where AKR1B10 was shown to be overexpressed may act as a tumor biomarker (Chung et al., 2012a).

AKR1B10 was shown as a direct target of p53, one of the most important tumor suppressor genes (Ohashi, Idogawa, Sasaki, Suzuki, & Tokino, 2013). Overexpression of AKR1B10 resulted in enhanced p53-induced apoptosis in a colorectal cancer nude mouse model. On the other hand, low expression of AKR1B10 led to poor prognosis and decreased survival due to inhibition of p53-mediated apoptosis (Ohashi et al., 2013).

### **1.6 Aims of the study**

The aldo-keto reductases AKR1B1 and AKR1B10 are major contributors towards metabolic homeostasis and are considered as a drug target due to their role in diabetic pathophysiology and inflammation. We have therefore designed a study where we have combined *in silico* and wet lab work to compare the effects of AKR1B1 and AKR1B10 expression in colorectal cancer.

Analyses of a publicly available colon cancer microarray dataset as well as a colon cancer cDNA panel indicated that the expression AKR1B1 was remained unchanged between tumor and normal samples while the expression of AKR1B10 was lower in tumor samples compared to normals (Taskoparan, Seza et al., 2017). GSEA (Gene Set Enrichment Analysis) showed that tumors in the high 30% AKR1B1 expressing patients were enriched in genes related to inflammation and migration while the samples from low 30% AKR1B1 expressing tumors were enriched in cell cycle related genes. *In vitro* data corroborated with the *in silico* analysis; AKR1B1 silencing resulted in slower progression through the cell cycle, reduced motility and reduced transcriptional

activity of NF- $\kappa$ B, a master regulator of inflammation. HCT-116 cells (Taskoparan, Seza et al., 2017). My thesis was designed as a part of the described study to examine the role of AKR1B10 in colon cancer.

### **1.6.1 Aim of the thesis**

In this thesis, we aimed to systematically uncover the role played by AKR1B10 in CRC with the following aims:

- To establish whether the *in silico* data (GSEA with high 30% and low 30% AKR1B10 expressing colon tumors) obtained by our group could be confirmed *in vitro* using cell lines
- To determine whether the exogenous overexpression of AKR1B10 resulted in alterations in the major hallmarks of cancer such as proliferation, cell cycle, motility and inflammation.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Cell Culture

The colon cancer cell lines SW480, LoVo, Caco-2, and RKO were purchased from ATCC (Middlesex, UK), HCT-116 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and HT-29 was purchased from ŞAP Enstitüsü (Ankara, Turkey). HCT-116, HT-29 and LoVo were grown in RPMI supplemented with 10% FBS, 2mM L-glutamine and 1% Penicillin-Streptomycin. SW480 cells were grown in DMEM supplemented with 10% FBS, 4mM L-glutamine and 1% Penicillin-Streptomycin. RKO cells were grown in EMEM supplemented with 10%FBS, 2mM L-glutamine, 1% Non-Essential Amino Acid, 1mM Sodium Pyruvate, 0.15% Sodium Bicarbonate and 1% Penicillin-Streptomycin. Caco-2 cells were grown in EMEM supplemented with 20% FBS, 2mM L-glutamine, 1% Non-Essential Amino Acid solution, 1mM Sodium Pyruvate, 0.15% Sodium Bicarbonate and 1% Penicillin-Streptomycin. All cell lines were grown in a humidified incubator with 5% CO<sub>2</sub> at 37°C. All cell culture consumables were purchased from Biochrom AG (Germany).

## 2.2 Transfection

AKR1B10 cDNA cloned into a pCOLD1 vector (a kind gift from Dr. Satoshi Endo, Gifu Pharmaceutical University, Japan) was digested with the restriction enzymes *NheI* and *EcoRI* and subcloned into a pcDNA3.1(-) mammalian expression vector (cloning was carried out by Betül Taşkoparan). The cloning was confirmed by sequencing (Refgen, Ankara). For the overexpression of AKR1B10, HCT-116, SW 480 and LoVo cells were seeded in tissue culture plates and allowed to attach for 24 h. When the cells were 70% confluent, they were transfected with AKR1B10 plasmid at 1:1 ratio of DNA:transfection reagent (X-tremeGENE HP DNA Transfection Reagent, Roche, Switzerland) according to the manufacturer's instructions. The cells were collected after 24 h and processed for RNA or protein isolation.

## 2.3 Protein Isolation and quantification

Total protein was isolated by using the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, USA) containing protease inhibitor cocktail and phosphatase inhibitor (Roche, Germany) according to the manufacturer's instructions. For the fractionation of proteins in nuclear and cytoplasmic fractions, the cells (HCT-116, SW480 and LoVo) were washed with PBS containing Phosphatase inhibitor (PhosStop) and re-suspended 240  $\mu$ l (HCT-116), 120  $\mu$ l (SW480) and 240  $\mu$ l (LoVo) of hypotonic buffer containing 100 mM HEPES at pH 7.5, 40 mM NaF, 1 mM EDTA, 100  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>, protease inhibitor cocktail, and phosphatase inhibitor. After 15 min of incubation on ice, 10% Nonident P-40 (60  $\mu$ l for HCT-116 and LoVo cells, and 30  $\mu$ l for SW 480 cells) was added. Centrifugation for 30 s at the highest speed separated cytoplasmic (supernatant) and nuclear (pellet) fractions. The

supernatant containing the cytoplasmic fraction was carefully collected to prevent cross contamination with the nuclear fraction and transferred to ice-chilled eppendorf tubes. Any remaining supernatant was discarded. The pellet was suspended in Nuclear Extraction Buffer containing 20 mM HEPES at pH 7.9, 3mM MgCl<sub>2</sub>, 840 mM NaCl, 20% glycerol along with protease inhibitor cocktail, phosphatase inhibitor and 1 μM DTT (Dithiothreitol). The re-suspended pellet was vortexed for 15 s at the highest speed and incubated in ice for 15 min. This step was repeated once more and the tubes were centrifuged at 14000 x g for 10 min at 4 °C. After centrifugation, the supernatant was transferred into ice-chilled eppendorf tubes and stored at -80 °C.

Protein content of the isolated proteins was measured by Bradford assay with Coomassie Protein Assay Reagent (Thermo Fisher Scientific, USA) with the help of a standard curve generated by bovine serum albumin.

## **2.4 Western Blot**

Western blotting was used to investigate the expression level of different proteins of interest. Total or nuclear/cytoplasmic proteins were separated by SDS-Polyacrylamide Gel Electrophoresis at 100 V by loading 15-50 μg proteins in 10% gels. PageRuler Plus Prestained protein ladder (Thermo Fisher Scientific, USA) was used as a marker in the 10-250 kDa range. After separation, the proteins in the gel were wet-transferred to a PVDF (Polyvinylidene Fluoride) membrane at 4°C and 115 Volts for 75 minutes. Blocking was conducted in TBS-T containing 5% skimmed milk or TBS-T containing 5% BSA (Bovine Serum Albumin). Primary antibody incubation was carried out overnight at 4°C. The membranes were washed extensively and

incubated with a secondary antibody for 1h at room temperature. Visualization of the proteins in the membranes was conducted using the Clarity ECL Substrate (BioRad, USA) and imaged on a Chemi-Doc MP (BioRad, USA). Where necessary, the membranes were stripped and reprobed with additional antibodies. Before reprobing the membranes, a mild stripping protocol was carried out. Mild stripping buffer was heated at 60°C and applied twice for 10 min, followed by extensive wash at least three times in TBS-T. Moreover, the membranes were also blotted with a  $\beta$ -actin or GAPDH antibody as a loading control to ensure equal protein loading.



**Table 2.1.** List of the antibodies used in this study.

<b>Description</b>	<b>Origin</b>	<b>Brand</b>	<b>Dilutions</b>	<b>Media</b>
GAPDH	Rabbit	Santa Cruz	1:4000	5% skimmed milk
NF- $\kappa$ B p65	Mouse	Santa Cruz	1:500	5% skimmed milk
NF- $\kappa$ B p50	Rabbit	Santa Cruz	1:500	5% skimmed milk
$\alpha$ -tubulin	Mouse	Protein Tech	1:5000	5% skimmed milk
AKR1B10	Rabbit	Thermo Fisher	1:500	5% skimmed milk
AKR1B1	Rabbit	Thermo Fisher	1:500	5% skimmed milk
TopoII $\beta$	Rabbit	Santa Cruz	1:500	5% skimmed milk
I $\kappa$ B- $\alpha$	Rabbit	Santa Cruz	1:500	5% BSA
E-cadherin	Mouse	Santa Cruz	1:200	5% skimmed milk
Vimentin	Goat	Santa Cruz	1:500	5% skimmed milk
JNK1	Mouse	Cell Signaling	1:1000	5% BSA
p-SAPK JNK	Rabbit	Cell Signaling	1:1000	5% BSA
p38	Rabbit	Cell Signaling	1:1000	5% BSA
p-p38	Rabbit	Santa Cruz	1:500	5% skimmed milk
p-ERK 1/2	Rabbit	Santa Cruz	1:500	5% BSA
HDAC1	Mouse	Santa Cruz	1:500	5% skimmed milk
Transgelin	Rabbit	Santa Cruz	1:200	5% skimmed milk
ZO-1	Goat	Santa Cruz	1:200	5% skimmed milk
$\beta$ -actin	Mouse	Santa Cruz	1:4000	5% skimmed milk

## **2.5 RNA Isolation**

Total RNA isolation was performed by using RNeasy RNA Extraction Kit (Qiagen, Germany) according to manufacturer's instructions. The RNA content was measured using a Nanodrop and stored at -80°C. A260/280 ratio near to 2.0 and A260/230 ratio between 2.0 and 2.2 were accepted.

## **2.6 cDNA Synthesis**

The total RNA was treated with DNase I (Thermo Fisher Scientific, USA) and cDNA synthesis was carried out with 1 µg of RNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) by using random hexamers according to manufacturer's instructions. The synthesized cDNA was stored at -20°C.

## **2.7 RT-PCR and qRT-PCR**

Reverse Transcriptase –Polymerase Chain Reaction (RT-PCR) was performed in a final volume of 20 µl containing 2 µl of cDNA and 18 µl reaction mix containing 0.5 µM forward and reverse primers, 0.2 mM dNTP mix, 1.5 mM MgCl<sub>2</sub>, proper buffer and 1 U of Taq Polymerase (Thermo Fisher Scientific, USA). The final volume was completed with PCR grade water. Reaction was performed in a thermal cycler (Applied Biosystems, USA) with initial denaturation (3 min) and denaturation (30 sec) at 95 °C, annealing (30 sec) at an optimized temperature, extension (30 sec) and final extension (7 min) at 72 °C. β-actin was used as an internal control for every PCR reaction along with other proper controls. For visualization, 20 µl of RT-PCR product was mixed

with 4  $\mu$ l of 6X loading dye were run on a 2% agarose gel at 100 V. The gels were incubated in 10 mg/ml ethidium bromide solution (Applichem, Germany) and imaged under UV light.

Quantitative RT-PCR (qRT-PCR) was performed in a final volume of 10  $\mu$ l containing 1  $\mu$ l of cDNA (diluted 1:10) and 9 $\mu$ l reaction mix containing 0.5  $\mu$ M forward and reverse primers, 5  $\mu$ l of BioRad Sso Advanced Universal SYBR Green Supermix (BioRad, USA). For every primer pair, a standard curve was generated.  $\beta$ -actin was used as an internal control for every PCR reaction with other proper controls. The reaction was performed in CFX Connect Real-Time PCR Detection System (BioRad, USA). Expression values of individual genes were calculated by Pfaffl method, which suggests relative quantification based on target genes and reference genes expression level difference reflective of physiological changes (Pfaffl, 2001).

**Table 2.2.** List of the primers used in this study.

<b>Gene Name</b>	<b>Forward (5'-3')</b>	<b>Reverse (5'-3')</b>	<b>Tm (°C)</b>
AKR1B1	AAGCCGTCTCCTGCTCA	TTGCTGACGATGAAGAGC	55
AKR1B10	CAGAATGAACATGAAG TGGGG	GCTTTTCCACCGATGGC	55
Beta-Actin	CAGCCATGTACGTTGCT ATCCAGG	AGGTCCAGACGCAGGATG GCATG	60

## **2.8 Proliferation Assays**

To determine whether overexpression of AKR1B10 in colon cancer cell lines affected their proliferation, two different methods were used. First, an MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was used as per the manufacturer's instructions (Thermo Fisher Scientific, USA). 10,000 cells were seeded per well in three different 96-well plates and allowed to attach for 24h. The cells were transfected with the AKR1B10 plasmid. 24h after transfection, the medium was changed and the cells were allowed to grow for a further 24, 48 or 72h. At the end of each time point, the medium was changed and MTT solution, dissolved in PBS, was added directly onto the cells. 4 h later, 1% SDS-0.01M HCl (hydrochloric acid) solution was added to each well. The plate was incubated for 16 h and the color formed was measured in a microplate reader (Thermo Fisher Scientific, USA) at 370 nm. As a second method, a Trypan blue exclusion assay was carried out followed by counting in a TC20™ Automated Cell Counter (BioRad, USA). After 24 h of transfection with the AKR1B10 plasmid, the cells were collected by trypsinization and washed. 1:1 ratio of cells and 0.4% Trypan blue solution (BioRad, USA) were mixed. 10 µl from each sample was added directly to the ready to use slides. According to given values (cells per milliliter) were analyzed.

## **2.9 Cell Cycle Assay**

Colon cancer cells transfected with the relevant plasmids were collected and washed once with PBS. For fixation of the cells, 1.0 ml of ice-cold 70% ethanol was added dropwise to each sample and vortexed gently. After 2 h of incubation

at -20°C, the samples were centrifuged at 400 x g for 5 min and all of the ethanol was carefully removed from the samples. The cells were washed with PBS and centrifuged at 400 x g for 5 min. Staining solution containing 0.1% Triton X-100, 2 mg/ml RNase A and 20 mg/ml Propidium Iodide (Sigma Aldrich, USA) was added to every sample and incubated for 30 min in the dark at room temperature. The cells were analyzed for cell cycle distribution using Accuri C6 Flow Cytometer (BD Biosciences, USA). Proper staining controls and unstained controls were included and analyzed using the SSC (side scatter), FSC (forward scatter) and FL-3 channels.

### **2.10 Luciferase Assay**

In order to determine whether AKR1B1 or AKR1B10 expression affected NF- $\kappa$ B transcriptional activity, a luciferase reporter assay using the Pathdetect reporter plasmid (Agilent Genomics, USA) containing 5 copies of the binding sites for NF- $\kappa$ B upstream of a firefly gene was used. pRL-TK Renilla plasmid (Promega, USA) was used as an internal control. 50,000 cells were seeded in 48 well-plates and transfection was carried out at 1:250-500 Firefly to Renilla ratio using X-tremeGENE HP DNA Transfection Reagent (Roche, Switzerland). After 24 h of transfection, the cells were lysed by using Passive Lysis Buffer (Promega, USA). The remaining steps were carried out according to manufacturer's instructions using the Dual-Glo Luciferase Assay Kit (Promega, USA). Luminescence was read in white opaque 96-well plates using a luminometer (Turner Biosystems, USA).

## **2.11 Transwell Migration Assay**

In order to observe the migration ability of AKR1B10 transfected cells, a Transwell Migration Assay was performed with 8.0 µm PET 24-well ThinCert Cell Culture Insert (Greiner Bio-One, Germany). 50,000 cells suspended in RPMI supplemented with 1% FBS was applied to the inserts and placed in the upper chamber. Complete medium supplemented with 10% FBS was placed in the lower chamber. 48 h after cell seeding, the inserts were removed and the upper chambers were cleaned with sterile cotton buds. The insert membranes were then fixed in 100% methanol for 10 min and stained with Giemsa solution (Merck Millipore, USA) for 2 min at room temperature. Excess dye was washed with sterile distilled water at least three times. The inserts were allowed to air dry in a fume hood for 15 min. Completely dried inserts were cut with a sterile bistoury and were placed on a sterile glass slide with a drop of immersion oil and covered with a cover slip. The cells were counted at 20X magnification under an inverted light microscope (Leica, Germany).

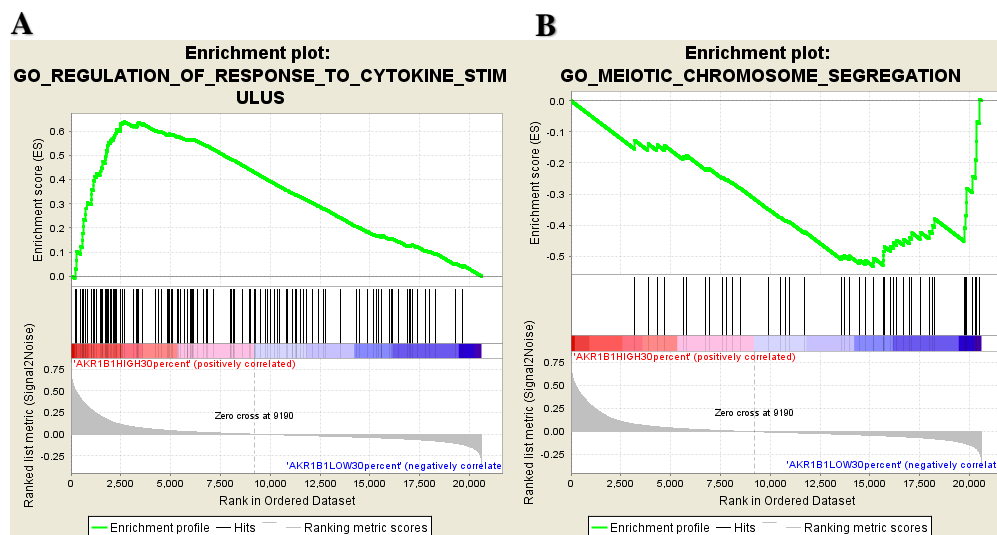
## **2.12 Statistical Analysis**

All experiments were carried out at least two or three times independently. All independent biological replicates were conducted with at least three technical replicates. Statistical analyses and graphing were carried out using GraphPad Prims 6.1 Software (GraphPad Software Inc., USA). Student's t Test, One-way ANOVA or Two-way ANOVA were employed to determine statistics according to experimental groups.  $p < 0.05$  was considered as statistically significant.

## CHAPTER 3

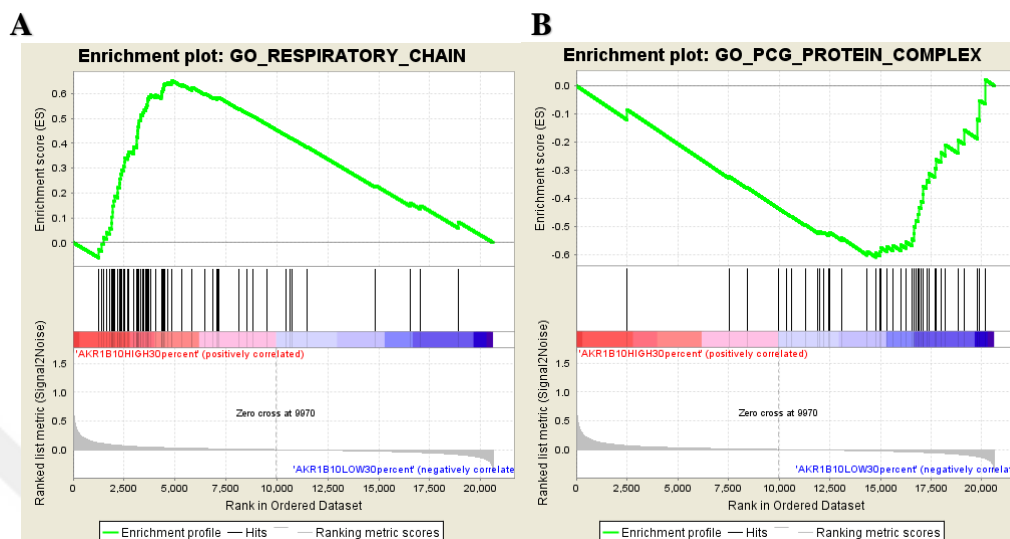
### RESULTS

AKR1B10 and AKR1B1 have 70% similarity in amino acid sequence and common enzymatic functions in the reduction of aldehydes. However, a comparison of their expression and activity in CRC is currently unknown. Using a publicly available microarray dataset (GSE 39582) of colon cancer patients, we carried out a stratification of the patients into high (30%) AKR1B10 or high (30%) AKR1B1 expressing and low (30%) AKR1B10 or low (30%) AKR1B1 expressing. Extraction of the microarray data, normalization and stratification was carried out by Seçil Demirkol and Dr. Ali Osmay Güre (Bilkent University). GSEA (Gene Set Enrichment Analysis) of the stratified data showed that high the 30% AKR1B1 group showed gene expression enrichment of genes related to inflammation (*Figure 3.1 A*). Top and highly significant GO (Gene Ontology) terms were REGULATION OF RESPONSE TO CYTOKINE STIMULUS, CELL SUBSTRATE JUNCTION and Fc RECEPTOR SIGNALING PATHWAY. On the other hand, gene enrichment analysis of the low 30% AKR1B1 expressing samples were associated with cell cycle regulation (*Figure 3.1 B*) and related to the GO terms: MEIOTIC CHROMOSOME SEGREGATION, NON-RECOMBINATIONAL REPAIR, MEIOSIS I and MEIOTIC CELL CYCLE.



**Figure 3. 1** Gene Set Enrichment Analysis for AKR1B1. A. Top GO term for high 30% AKR1B1. B. Top GO term for low 30% AKR1B1.

When we carried out GSEA with the high 30% AKR1B10 expressing group, metabolism related genes were enriched, represented by the GO terms: RESPIRATORY CHAIN, ELECTRON TRANSPORT CHAIN and OXIDATIVE PHOSPHORYLATION. The low 30% AKR1B10 expressing samples were enriched in epigenetic pathways such as POLYCOMB GROUP PROTEIN COMPLEX, HISTONE METHYLTRANSFERASE COMPLEX and NEGATIVE REGULATION OF CELL SUBSTRATE ADHESION.



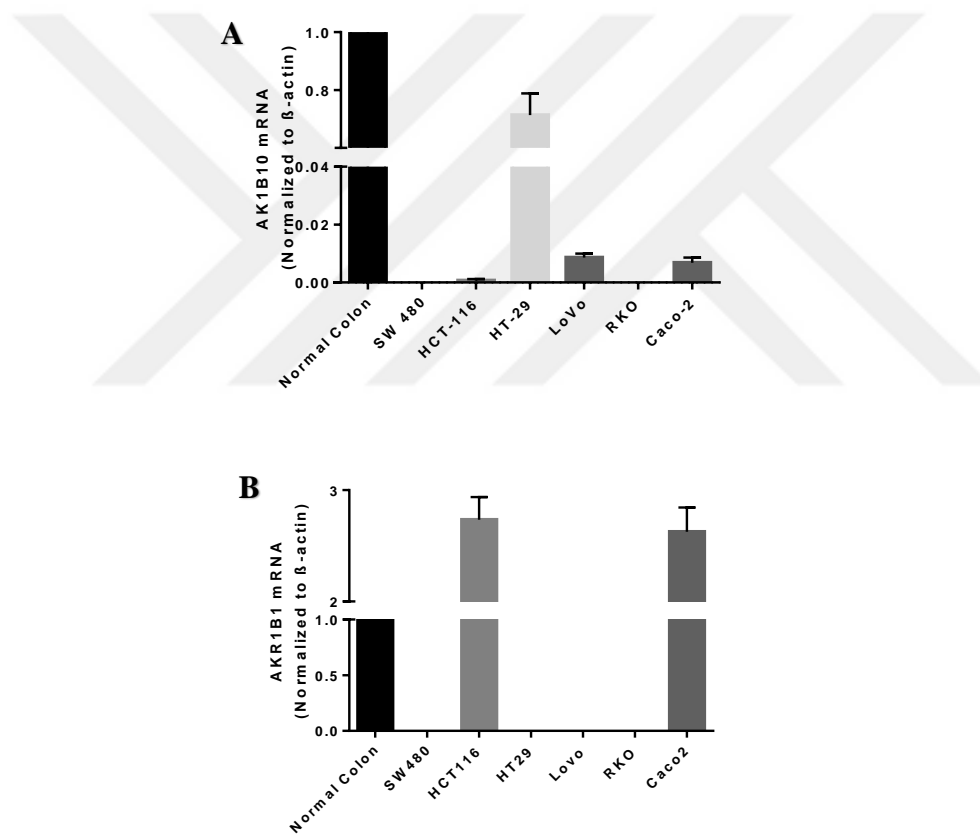
**Figure 3. 2** Gene Set Enrichment Analysis for AKR1B10. A. Top GO term for high 30% AKR1B10. B. Top GO term for low 30% AKR1B10.

Based on the data we obtained with GSEA, we hypothesized that the consequences of the expression of AKR1B1 and AKR1B10 in colon cancer were quite divergent. Using the enriched GO terms obtained from GSEA as a guide, we examined the effect of expression of AKR1B10 on cellular characteristics in the context of the hallmarks of cancer. Where indicated, comparison with an AKR1B1 expressing cell line was also carried out.

### 3.1 Expression of AKR1B10 in CRC Cell lines

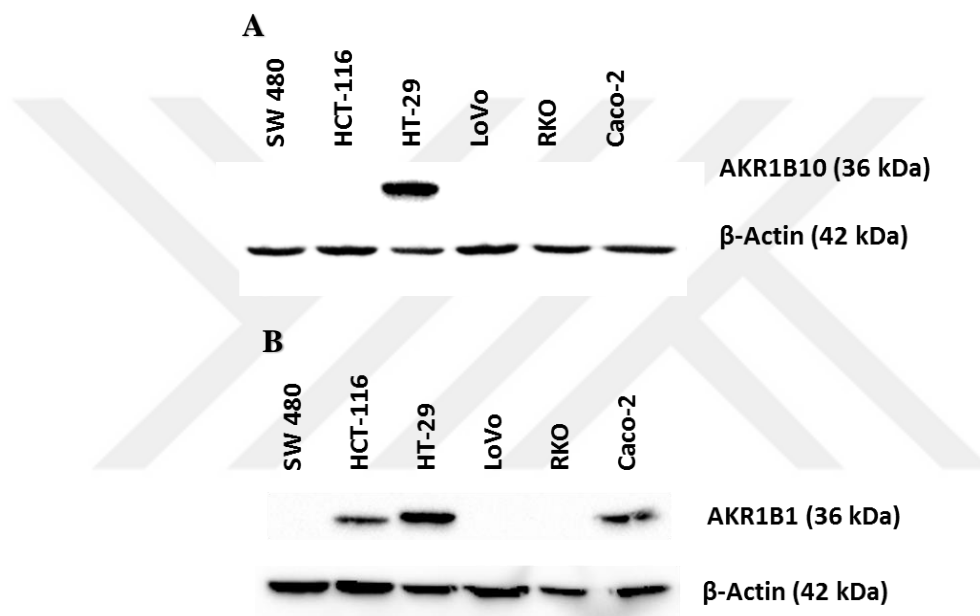
The expression of AKR1B10 was reported to be lost in some CRC cell lines (Ebert, Kisiela, Wsól, & Maser, 2011). To confirm this, the expression of AKR1B10 at the mRNA and protein levels were determined by qRT-PCR and

western blot, respectively, in six different CRC cell lines. qRT-PCR was normalized according to the expression of AKR1B10 normal colon tissue, using a commercially sourced normal RNA sample. Accordingly, we observed that the expression of AKR1B10 was lower in every cell line compared to the normal colon. The HT-29 cells showed comparatively higher AKR1B10 transcript level than other CRC cell lines but slightly lower than normal colon (**Figure 3.3A**). When we compared the AKR1B1 mRNA levels, HCT-116 and Caco-2 cell lines had almost more than 2-fold higher transcript levels than normal colon (**Figure 3.3B**).



**Figure 3.3** Transcript levels of AKR1B10 (A.) and AKR1B1 (B.) in CRC cell lines. 1 $\mu$ g RNA was reverse transcribed and normalization was done with two replicates according to  $\beta$ -actin, which is an internal control for qRT-PCR.

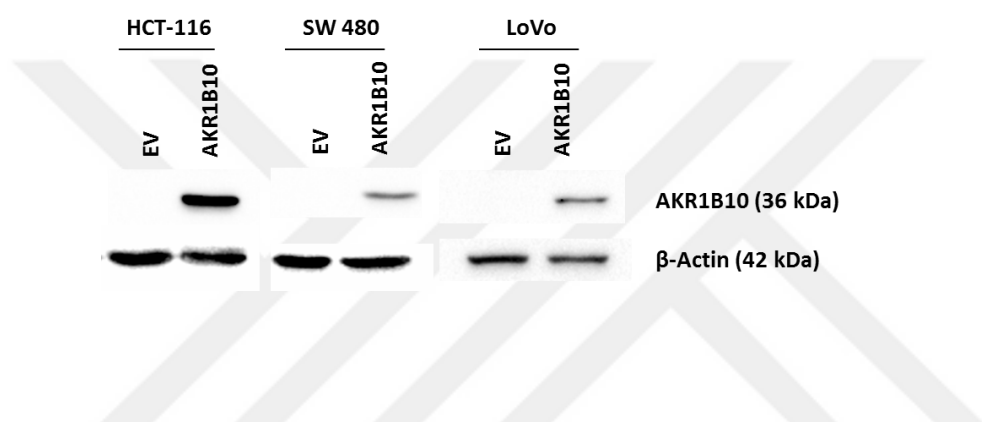
Although, LoVo and Caco-2 showed a very low level of AKR1B10 transcript, western blot demonstrated that only HT-29 expressed the AKR1B10 protein among these CRC cell lines (**Figure 3.4A**). However, HCT-116 and Caco-2 cell lines expressed AKR1B1, although there was a band in HT-29 line. This band was slightly upper than the AKR1B1 and we suspected that it might be one of the other AKRs recognized by same antibody.



**Figure 3.4** Protein levels of AKR1B10 (A) and AKR1B1 (B) in CRC cell lines. 30  $\mu$ g of protein from whole cell lysate was loaded and  $\beta$ -actin was used as a loading control for western blot. Anti-AKR1B10, Anti-AKR1B1 and anti- $\beta$ -actin were diluted 1:1000, 1:1000 and 1:4000 respectively.

### 3.2 Overexpression of AKRB10 in HCT-116, SW 480 and LoVo

As shown in **Figure 3.4A**, there was no expression of AKR1B10 in HCT-116, SW 480 and LoVo cells. Therefore, AKR1B10 was transiently overexpressed in those cell lines. Transient overexpression of AKR1B10 was confirmed by western blotting before each experiment (**Figure 3.5**).

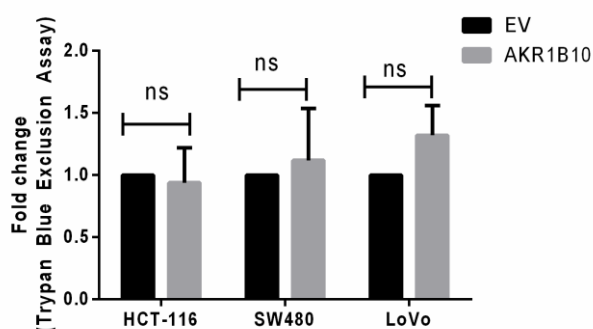


**Figure 3.5** Confirmation of overexpression of HCT-116, SW 480 and LoVo with respect to empty vector (EV). Expression was assayed using 30  $\mu$ g of protein from whole cell lysate and  $\beta$ -actin was used as a loading control for western blot. Anti-AKR1B10 and anti- $\beta$ -actin were diluted 1:1000 and 1:4000 respectively.

Since we observed loss of AKR1B10 from most of the CRC cell lines examined, we next tested whether the overexpression of AKR1B10 had any effects on the main hallmarks of cancer such as proliferation, motility and cell cycle progression.

### 3.3 Effects of AKR1B10 overexpression on cellular proliferation

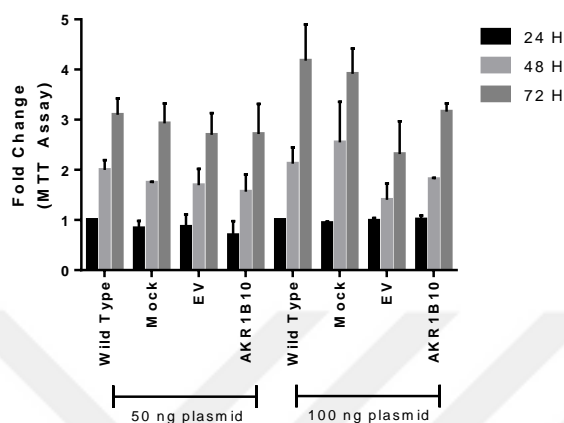
To determine whether AKR1B10 overexpression affected cellular proliferation, two different methods were used. Trypan blue exclusion assay followed by cell counting in an automated (TC 20 Bio-Rad) cell counter using HCT-116, SW 480 and LoVo cells transiently transfected with the AKR1B10 plasmid for 24h indicated that there was no statistically significance between the number of live cells between empty vector (EV) and AKR1B10 transfected cells in each cell line (*Figure 3.6*).



**Figure 3.6** Effect of AKR1B10 overexpression on cell proliferation. Trypan blue exclusion assay indicated that with respect to empty vector (EV) control, AKR1B10 expression in HCT-116, SW 480 and LoVo cells for 24 h did not lead to any change in the proportion of live or dead cells. Student's t-test was used for statistical analysis (ns: not significant). Three independent biological replicates were used for statistics.

To further confirm the Trypan blue exclusion data, an MTT assay was carried out. Transfection of cells with the AKR1B10 plasmid indicated no difference in cell proliferation between EV and AKR1B10 transfected cells for 24-72h after transfection. Wild type control and mock control were used to test the

effects of transfection reagent; however, there was no effects of transfection reagent alone (mock) on cell proliferation (**Figure 3.7**).



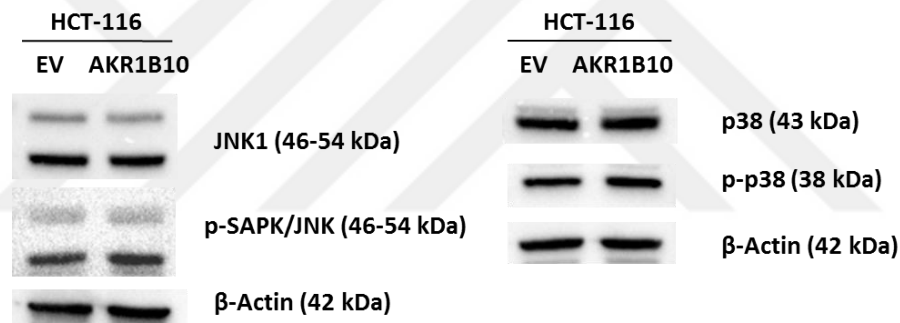
**Figure 3.7** Effects of AKR1B10 transfection on cell proliferation by using MTT assay in HCT-116 cells. HCT-116 cells were transfected with 50 ng or 100 ng of the AKR1B10 plasmid or the empty vector for 24h. The cells were washed and incubated for a further 24, 48 and 72 h after which they were processed for the MTT assay. No difference in proliferation was observed at any time point. Wild type and mock were used as controls. Three independent biological replicates were assessed.

### 3.4 Effects of AKR1B10 on the Mitogen Activated Protein Kinase (MAPK) pathway

The Mitogen Activated Protein Kinase (MAPK) pathway in mammalian cells is involved in many cellular functions such as proliferation, apoptosis, differentiation and migration (Dhillon, Hagan, Rath, & Kolch, 2007). We next wanted to establish whether the lack on any effect of AKR1B10 on cell proliferation was also reflected in the activation of the MAPK pathway. The MAPK pathway, particularly the JNK and p38 pathways, can also be activated

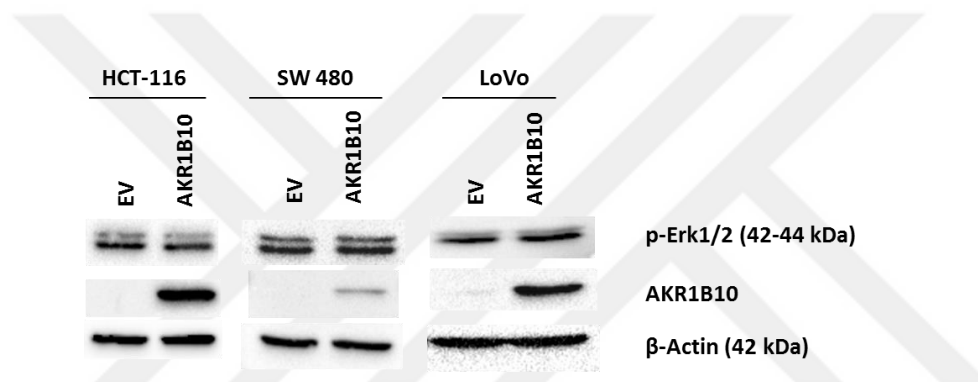
with stress such as UV irradiation, cytokines and other inflammatory molecules and growth factor deprivation (Dhillon et al., 2007). Previous work revealed that AKR1B10 silencing decreased the phosphorylation of the c-Raf, MEK and ERK in breast cancer (Huang, Verhulst, Shen, Bu, Cao, He, et al., 2016).

To determine if AKR1B10 expression affected signaling through the MAPK pathway, the expression and activation of the MAPK proteins including p-ERK1/2, p-38 and p-p38, and p-SAPK/JNK and JNK1 were confirmed by western blotting in HCT-116. We observed no differences in the activation of the stress related MAPK pathway (*Figures 3.8 and 3.9*).



**Figure 3.8** Effects of AKR1B10 overexpression on JNK1 and p-SAPK/JNK MAPK pathway. 30 µg of whole cell lysate from HCT-116 transiently transfected with AKR1B10 or the corresponding empty vector (EV) was blotted (First column) and the effects of AKR1B10 overexpression on p38 and p-p38 MAPK pathway molecules with respect to empty vector (EV) control (Second column). β-actin was used as a loading control. Anti-JNK1, anti-p-SAPK/JNK, anti-p38, anti-p-p38 and anti-β-actin were diluted 1:1000, 1:1000, 1:1000, 1:500 and 1:4000 respectively.

The ERK pathway is known to regulate cellular proliferation (Meloche & Pouyssegur, 2007). Growth factors and mitogens have an ability to activate upstream kinases of ERK1/2 such MEK (MEK1 and MEK2); therefore, the activation of ERK1/2 through phosphorylation is generally considered to be synonymous with cell proliferation (Dhillon et al., 2007). We did not observe any difference in the activation of ERK1/2 after the overexpression of AKR1B10, corroborating the lack of any effect of AKR1B10 overexpression on cellular proliferation in HCT-116, SW 480 and LoVo (**Figure 3.9**).

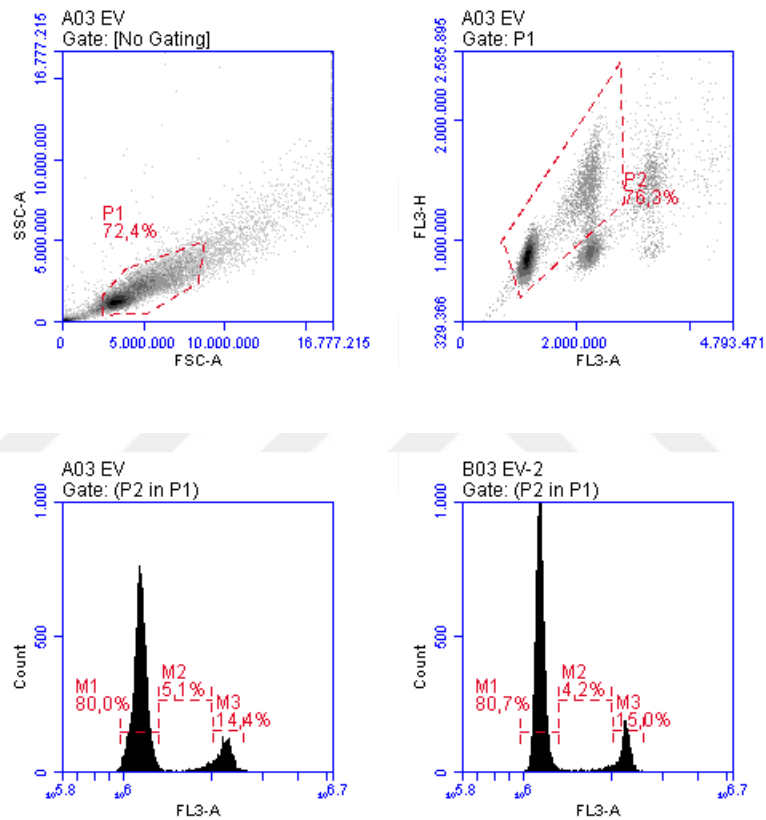


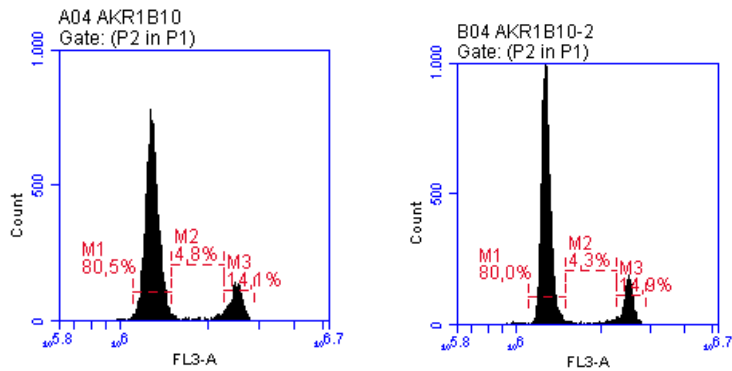
**Figure 3.9** Effects of AKR1B10 overexpression on Erk1/2 phosphorylation with respect to empty vector control (EV) in HCT-116, SW 480 and LoVo. 30 $\mu$ g of whole cell lysates was loaded and  $\beta$ -actin was used for equal loading. Anti-p-Erk1/2, anti-AKR1B10 and anti- $\beta$ -actin were diluted 1:500, 1:1000 and 1:4000 respectively.

### 3.5 Effects of AKR1B10 overexpression on cell cycle progression

The low 30% AKR1B1 expressing group was observed to be highly related with cell cycle mechanisms (**Figure 3.1**). We therefore examined whether the expression of AKR1B10 could also affect cell cycle progression. For this, HCT-116 cells were transfected for 24h with the AKR1B10 plasmid or the

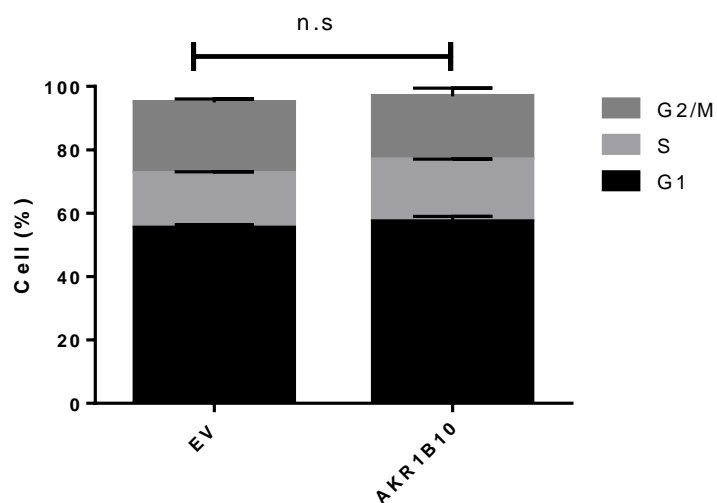
empty vector and then examined for cell cycle distribution after PI staining using flow cytometry. AKR1B10 expression showed no effect on cell cycle progression in any of the phases of the cells cycle. The histograms after proper gating were obtained using BD Acuri C6 software (BD Biosciences, USA) (*Figure 3.10*).





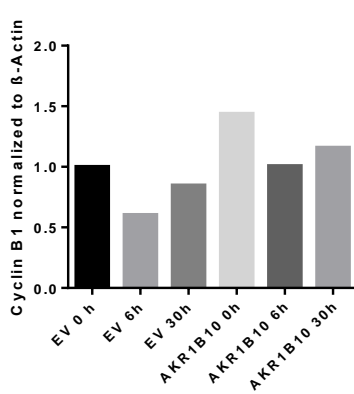
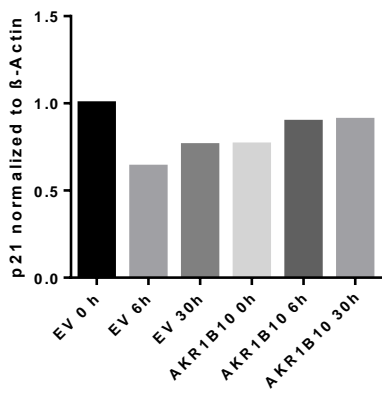
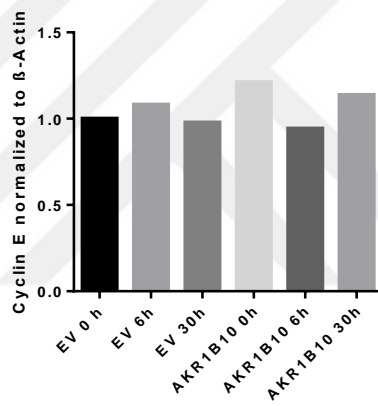
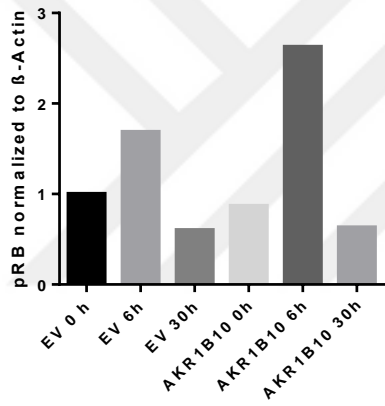
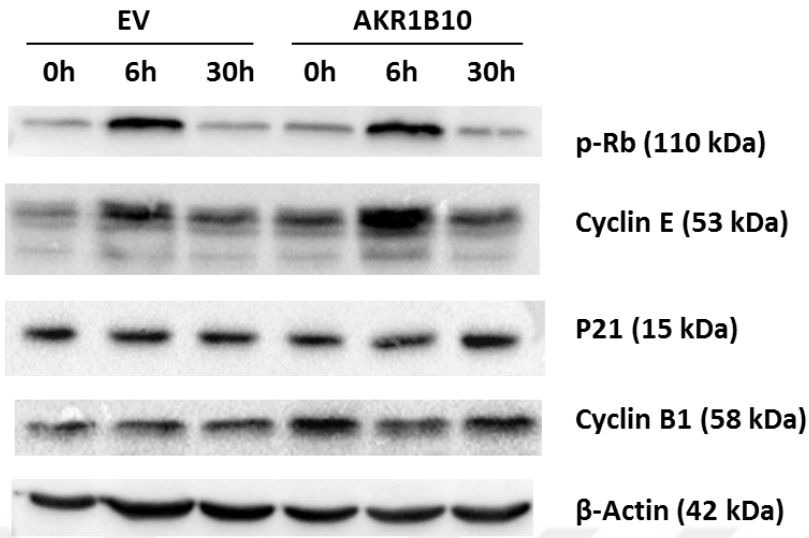
**Figure 3.10** Effects of overexpression of AKR1B10 on cell cycle progression. AKR1B10 overexpression did not change the cell cycle distribution of HCT-116 cells with respect to empty vector (EV). First left panel demonstrates a single population of cells that was gated to distinguish from cell debris. First right panel demonstrates the presence of cell singlets and doublets, which has to be discriminated from cell cycle. Rest of the figures demonstrates cell cycle distribution of EV or AKR1B10 transfected cells showing the percentage of cells at S (M1), G1 (M2) and G2/M (M3) phases, respectively.

The percentage of cells at G1, S and G2/M in AKR1B10 or EV transfected cells are shown as a line diagram below (**Figure 3.11**).



**Figure 3.11** Cell cycle distribution in AKR1B10 overexpressing HCT-116 cells. Statistical analysis was determined by two-way ANOVA. (n.s: not significant) Three independent biological replicates were carried out.

In order to further establish any role of AKR1B10 overexpression on cell cycle, HCT-116 cells were starved during transfection for 24 h. The starved cells were collected and referred to as 0 h cells. Then, the cells were released from the starvation by the addition of 10% FBS containing medium and collected at the 6<sup>th</sup> h and 30<sup>th</sup> h and examined for the expression of cell cycle related genes. The increase in phosphorylation of Rb and accumulation of Cyclin E indicate that the cells started cycling after release from starvation. However, there was no difference in the activation of Rb or cyclin accumulation between EV and AKR1B10 transfected cells (**Figure 3.12**).



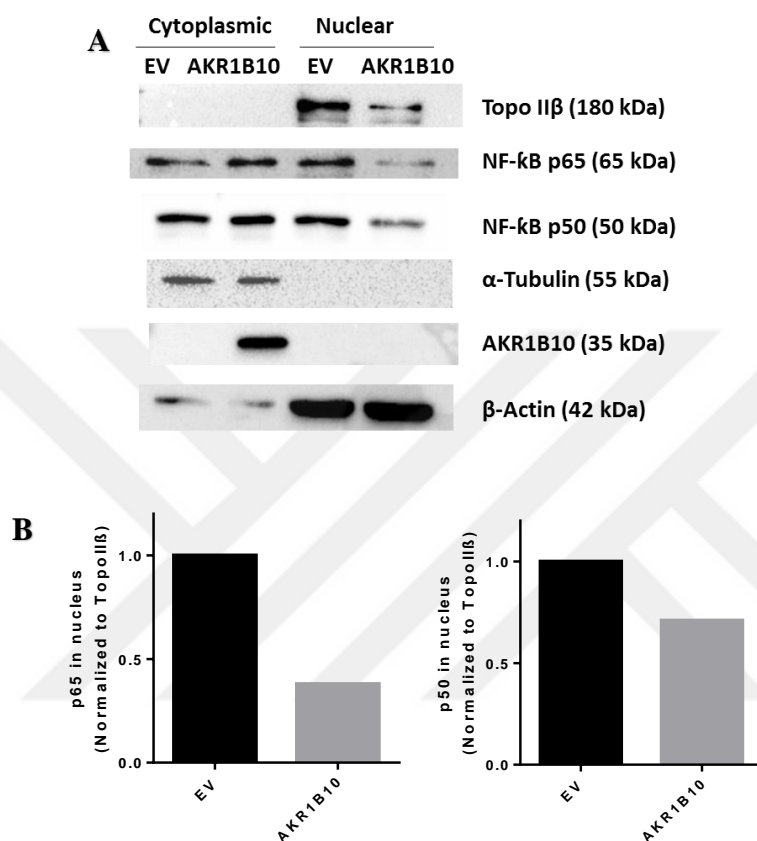
**Figure 3.12** Determination of markers of cell cycle progression in AKR1B10 or EV transfected HCT-116 cells. The cells were synchronized by starvation and then released and collected immediately (0h), or after 6h and 30h. The cells were processed for protein isolation and 50 µg of protein was separated by SDS-PAGE and blotted for p-Rb, cyclin E, p21 and cyclin B1. AKR1B10 overexpression did not alter the protein levels of any of these markers. β-actin was used as a loading control.

### **3.6 Effects of AKR1B10 overexpression on nuclear localization of NF-κB**

A GO term that was enriched in the 30% high AKR1B1 expressing samples was related to cytokine signaling. Therefore, we examined whether inflammation related signaling pathways were activated in the AKR1B10 expressing cells. Canonical NF-κB pathway is activated by microbial products and proinflammatory cytokines such as TNF-α (tumor necrosis factor alpha) and IL-1 (interleukin-1) (Lawrence, 2009). IKK (IκB kinase), IκB, RelA (p65) and p50 are the components of the canonical pathway. IKK activation leads to IκB degradation by ubiquitin-proteasome pathway. Free NF-κB starts to migrate through nucleus. In the nucleus, target inflammatory genes are regulated by NF-κB, which is composed of RelA and p50 (Wuerzberger-Davis, Nakamura, Seufzer, & Miyamoto, 2007).

In order to observe the nuclear localization (and thereby activation) of the canonical NF-κB subunits (p50 and p65), cytoplasmic and nuclear fractions were isolated from HCT-116 cells transfected with the AKR1B10 vector or the corresponding EV. Western blot showed that AKR1B10 overexpression led to a decrease in the translocation of NF-κB to the nucleus when compared with

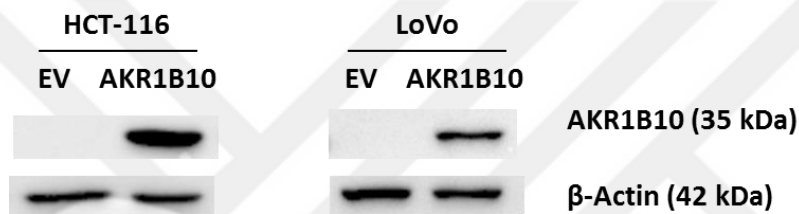
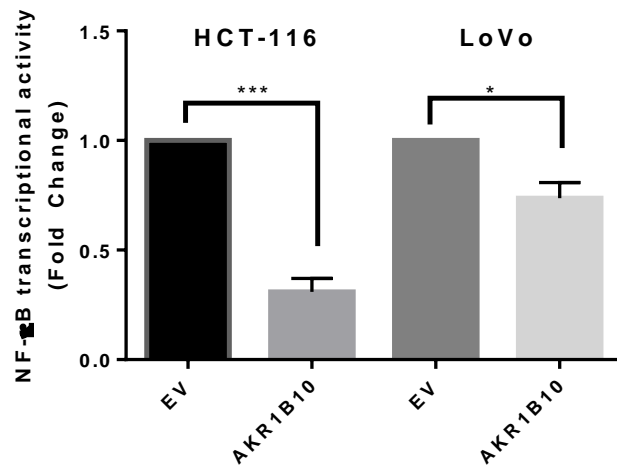
empty vector (EV). Topo II $\beta$  and  $\alpha$ -Tubulin were used as nuclear or cytoplasmic markers, respectively.



**Figure 3.13** (A) Effects of AKR1B10 overexpression on the translocation of NF $\kappa$ B to the nucleus. HCT-116 cells were transfected and the levels of p65 and p50 in cytoplasmic and nuclear fractions were assayed by western blot with respect to empty vector (EV). A decrease in the nuclear translocation of both p65 and p50 was observed. Topo II $\beta$  was used as a nuclear marker and  $\alpha$ -Tubulin was used as a cytoplasmic marker. 30  $\mu$ g of protein was loaded for every fraction. Five different independent replicates were used to confirm the nuclear translocation of the NF- $\kappa$ B. (B) Nuclear p65 and p50 normalization according to TopoII $\beta$  (nuclear marker) showed a decline with AKR1B10 overexpression. Normalization was based on densitometric analysis in Image Lab (BioRad, USA).

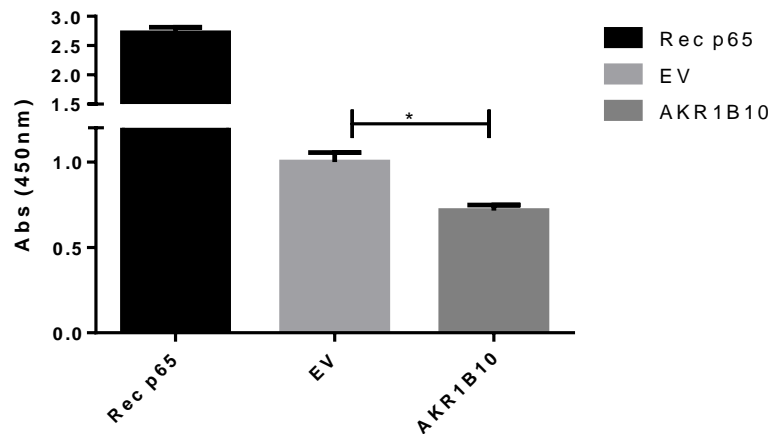
### **3.7 Effects of AKR1B10 overexpression on NF- $\kappa$ B transcriptional activity**

In order to determine whether the reduced nuclear translocation of the NF- $\kappa$ B subunits was accompanied by a reduction in the NF- $\kappa$ B transcriptional activity in AKR1B10 overexpressing cells, a luciferase reporter assay was carried out. HCT-116 and LoVo cells were transfected with the AKR1B10 plasmid along with the NF- $\kappa$ B Pathdetect plasmid (Agilent Genomics, USA) containing five tandemly repeated NF- $\kappa$ B responsive element in the upstream region of firefly luciferase gene. For normalization of the data in a dual luciferase assay, the cells were co-transfected with a pRL-TK Renilla plasmid (Promega, USA). Firefly luminescence levels were normalized to Renilla luciferase luminescence. Both HCT-116 and LoVo cells showed a decrease in the transcriptional activity of NF- $\kappa$ B when the cells expressed AKR1B10 (*Figure 3.14*).



**Figure 3.14** Effects of AKR1B10 overexpression in NF-κB transcriptional activity. Upper figure shows that AKR1B10 overexpression activity was normalized to empty vector (EV). Three independent biological replicates were used for statistical analysis. Significances were determined by Student's *t*-test. \* $p < 0.05$ , \*\*\* $p < 0.001$ . Lower figure shows the confirmation of overexpression of AKR1B10 by western blotting in both HCT-116 and LoVo. 30 μg of total lysates was loaded and normalized to β-actin.

We next investigated whether the reduced transcriptional activity of NF-κB (p65) in AKR1B10 expressing cells was related to the DNA binding ability of the p65 subunit, a DNA binding ELISA (enzyme-linked immunosorbent assay) was carried out. We observed reduced DNA binding ability in the nuclear extract of AKR1B10 transfected HCT-116 cells (**Figure 3.15**).

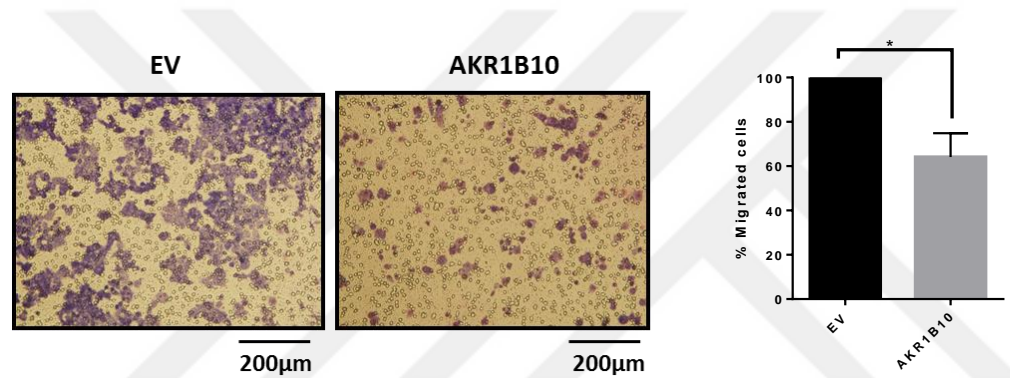


**Figure 3.15** DNA binding activity of the NF- $\kappa$ B p65 subunit in the presence of AKR1B10 overexpression by a DNA binding ELISA assay. A NF- $\kappa$ B response element specific dsDNA (double stranded DNA) is immobilized into the wells of a 96-well plate. The nuclear extract was added followed by an NF- $\kappa$ B p65 specific primary antibody for recognition. Secondary antibody with HRP conjugation provided for detection at 450 nm. Rec p65 is a recombinant p65 protein that was used as a positive control. One-way ANOVA was carried out with respect to empty vector (EV). \* $p < 0.05$

### 3.8 Effects of AKR1B10 overexpression on cell migration and EMT markers

Activating motility is one the hallmarks of cancer (Hanahan & Weinberg, 2011). Additionally, GSEA showed that the high 30% AKR1B1 samples were enriched for the GO term CELL SUBSTRATE JUNCTION. Therefore, to understand if AKR1B10 expression affected the motility of HCT-116 cells, an *in vitro* Transwell Migration assay was carried out. AKR1B10 transfection was carried out in serum free medium. After 24 h of transfection, cells were placed into Transwells with 1% FBS in the upper compartment. The lower compartment contained medium with 10% serum. The cells were allowed to

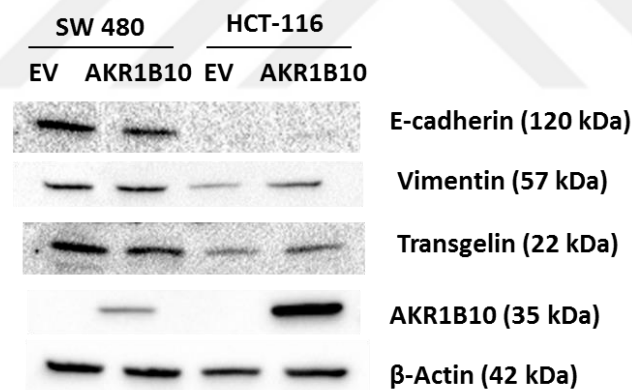
migrate through the pores of the Transwell for 48h. The non-migrated cells were removed by scrubbing with sterile cotton swabs. After which the inserts were fixed in methanol and stained with Giemsa solution (Merck Millipore, USA). The membranes were then cut out using a bistoury, mounted on a slide and covered with a coverslip. The stained cells representing migrated cells were counted at 20X magnification under inverted light microscope (Leica, Germany). HCT-116 cells overexpressing AKR1B10 were observed to migrate less compared to the empty vector (EV) control cells (**Figure 3.16**).



**Figure 3.16** The effect of AKR1B10 expression on cell migration. Left panel shows the representative images from the Transwells showing AKR1B10 transfected HCT-116 cells. The cells were stained purple and significantly fewer migrated cells were seen in the AKR1B10 transfected cells. Right panel shows quantitative analysis of percent of migrated cells. AKR1B10 transfected cell percent were calculated according to empty vector (EV). Unpaired t-test was carried with three independent biological replicates as a statistical test. \* $p < 0.05$ .

Epithelial-mesenchymal transition (EMT) is a multistep process of the transformation of epithelial cells to mesenchymal cells that help cells move during embryogenesis, wound healing and metastasis (Hanahan & Weinberg, 2011). When epithelial cells gain this property, they may start to invade and

migrate. Cancer cells may gain the EMT phenotype due to the accumulation of mutations and they may invade and metastasize to a distant tissue. A loss of epithelial markers such as E-cadherin or a gain of mesenchymal markers such as vimentin are the best characterized reasons of migration in carcinoma (Hanahan & Weinberg, 2011; Liu, Lin, Tang, & Wang, 2015). Since the AKR1B10 transfected cells showed reduced motility in the *in vitro* Transwell migration assay, we examined the expression of EMT markers in both HCT-116 and SW480 cells transiently transfected with AKR1B10. However, contrary to our expectations, none of the epithelial markers tested such as E-cadherin and transgelin (inhibitor of matrix metallo protease-9 (MMP-9), Assinder, Stanton, & Prasad, 2009) or the mesenchymal marker vimentin showed any change in expression when AKR1B10 was overexpressed in HCT-116 cells (**Figure 3.17**).



**Figure 3.17** The expression of epithelial and mesenchymal markers in AKR1B10 or EV transfected cells. E-cadherin, vimentin, transgelin, AKR1B10 and  $\beta$ -actin antibodies were diluted 1:200, 1:500, 1:200, 1:500, and 1:4000, respectively. 30  $\mu$ g of whole cell lysate were loaded and  $\beta$ -actin was used as a control. Three independent biological replicates were used but none of them showed any change in the expression of these markers.



## CHAPTER 4

### DISCUSSION

The Aldo Keto Reductase (AKR) family of enzymes that catalyze redox reactions involved in detoxification of xenobiotics, biosynthesis and metabolism (Barski, Tipparaju, & Bhatnagar, 2008). AKRs are best known for their ability to catalyze the reduction of aldehydes or ketones. These proteins do not require any cofactors such as metal ions or flavin; this makes these enzymes less efficient as alcohol dehydrogenases. AKRs preferably use NADPH over NADH as a source of protons. In cells that are metabolically active, NADP exists primarily the reduced form (Pollak, Dölle, & Ziegler, 2007) therefore, in such cells reduction is favored over oxidation. A high ratio of NADPH/NADP reflects anabolic pathways in cells, which are generally active in living cells (Barski et al., 2008). This ensures a constant supply of NADPH that allows AKRs to remain enzymatically active under a wide range of energetic states of the cell as seen during different rates of respiration, cell growth or starvation (Barski et al., 2008).

It is not surprising therefore that AKRs are involved in diseased states that are closely related to metabolism, such as diabetes and cancer. In diabetes, when intracellular glucose concentrations are high, the polyol pathway is commonly activated. This is a two-step metabolic pathway in which excess glucose is

reduced to sorbitol by AKRs using NADPH as a cofactor; sorbitol is then metabolized to fructose by sorbitol dehydrogenase using NAD<sup>+</sup> as a cofactor. These products may have a number of consequences for the cell including alteration of intracellular tonicity, generation of fructose-3-phosphate and 3-deoxyglucosone that can act as precursors of advanced glycation end products (AGE), and enhanced production of ROS, all of which can initiate and exacerbate cellular damage associated with diabetes (Lorenzi, 2007).

It has been appreciated greatly in recent years that cellular metabolic pathways and mitogenic signaling pathways closely work with each other in mediating cellular transformation (Boroughs & DeBerardinis, 2015). A number of studies have indicated that AKRs, particularly AKR1B1 and AKR1B10, were differentially expressed in normal and cancer tissues (Laffin & Petrash, 2012). In general, the expression of AKR1B1 was reported to be higher in solid tumors and leukemia. This triggered a number of studies by Ramana et al. who showed that treatment of colon cancer cells with AKR1B1 inhibitors resulted in the amelioration of cell proliferation, motility and inflammatory markers (Tammali, Srivastava, et al., 2011; Ramana, 2011; Srivastava et al., 2011).

The expression of AKR1B10 was reported to be tumor site specific with high expression reported in some tumors and low expression in others (Laffin & Petrash, 2012). The oncogenic function of AKR1B10 has been shown in different tissues such as pancreatic carcinoma (Chung et al., 2012b), breast cancer (Li et al., 2017), hepatocellular carcinoma (Liu et al., 2015). In addition, AKR1B10 was suggested as a tumor marker in human oral squamous cell carcinoma (Ko et al., 2017) and downregulated in high-grade endometrial cancer (Sinreih et al., 2017). Silencing of AKR1B10 in HCT-8 (colorectal

cancer) cells was reported to reduce cell proliferation, indicating that the enzyme may play a role in the pathology of colorectal cancer as well (Yan et al., 2007).

Data on the functional effects of AKR1B10 expression in colorectal cancer are scarce. Using publicly available expression data of AKR1B1 and AKR1B10 in colon cancer patient samples using microarray data as well as the TCGA portal, we observed that the expression of AKR1B10 was significantly lower in cancer samples compared to normal samples whereas the expression of AKR1B1 was similar between cancer and normal (Taskoparan, Seza et al., 2017).

Based on our *in silico* finding that the expression of AKR1B10 was lost in colon cancer samples, we compared colorectal cancer (CRC) cell lines in terms of the expression of AKR1B1 and AKR1B10. AKR1B10 transcript level was the highest in a commercially sourced normal colon tissue, while very low or no expression was observed in LoVo, Caco-2 and HCT-116. HT-29 cells expressed AKR1B10, but at levels lower than the normal colon. On the other hand, AKR1B1 mRNA expression in HCT-116 and Caco-2 cells were almost three fold more than normal colon transcript levels (**Figure 3.3 A and B**).

Next, we examined the AKR1B10 protein levels that was detectable only in the HT-29 cell line among the other CRC cell lines by western blotting. However, HCT-116 and Caco-2 showed the AKR1B1 protein expression in the western blotting. Interestingly, a band appeared in HT-29 at slightly higher level in terms of molecular weight. A protein BLAST showed that AKR1B1 has 55% similarity in amino acid sequence with AKR1CL2, 68% similarity with AKR1B15 and 71% similarity with AKR1B10 (E.G.S and S.B unpublished

data). As we did not observe any AKR1B1 mRNA transcript of AKR1B1 in qRT-PCR, we may suggest that the AKR1B1 antibody may recognize one of these mammalian AKRs (**Figure 3.4 A and B**).

We therefore overexpressed AKR1B10 in colon cancer cell lines (HCT-116, SW 480 or LoVo) and carried out a systematic analysis of the subsequent functional effects. In the larger study that this thesis is a part of, we have compared the effects of AKR1B10 overexpression with the silencing of AKR1B1 in the same cell lines.

Tumor, a heterogeneous mass, contains many different cell types: cells of hematopoietic origin, cells of mesenchymal origin as well as carcinoma cells. In addition to cell types, tumor microenvironment has different extracellular matrix (ECM), which consists of proteins, glycoproteins and proteoglycans (Pattabiraman & Weinberg, 2014). *In vitro* experiments using human colon cell lines would naturally have limited influence of a true tumor microenvironment. Thus, since the publicly available microarray data set was obtained from human tumors that contain both epithelial and non-epithelial cells, we naturally expected differences between the *in silico* and *in vitro*. Nonetheless, cell lines are easy to handle and manipulate, and recent studies indicate that they are good representatives of tumors in terms of their mutation profile and behavior (Barretina et al., 2012). Manipulation of cells *in vitro* is easy and provide excellent models for mechanistic studies that investigate cellular pathways.

GSEA (gene set enrichment analysis) carried out by Seçil Demirkol and Dr. Ali O. Güre (Bilkent University) showed that patient colon tumor samples from the high 30% AKR1B1 expressing patients was enriched in genes related to

inflammation while the low 30% AKR1B1 expressing group was enriched in cell cycle associated genes (**Figure 3.1**). On the other hand, high 30% AKR1B10 group was enriched in metabolism related genes while the low 30% AKR1B10 group was enriched in pathways related to epigenetic alterations such as Polycomb group proteins (**Figure 3.2**). These data indicate that the pathways affected by AKR1B1 and AKR1B10 are very diverse. Pathways that may directly contribute to neoplastic transformation such as inflammation and cell cycle progression were associated with AKR1B1. Conversely, metabolism related genes, that perhaps represent the enzymatic function of AKR1B10 was greatly represented in this analysis. Thus, while we observed changes in cell proliferation and cell cycle progression in CRC cell lines where AKR1B1 was silenced, no such alteration in cell proliferation, cell cycle progression or apoptosis were observed in the AKR1B10 overexpressing cells. We therefore hypothesized that rather than a direct driver of cellular transformation, AKR1B10 expression may affect cancer cell characteristics in a more indirect manner, perhaps through its effects on metabolic pathways.

Corroborating this idea, AKR1B10 overexpression showed no effect on cellular proliferation in HCT-116, SW 480 and LoVo cell lines. (**Figure 3.6 and 3.7**). We also observed no alteration in cell cycle progression or in the expression of cell cycle related genes. We also examined the activation of p38, JNK and ERK proteins that are members of the mitogenic MAPK pathway (Dhillon et al., 2007). The p38 or JNK proteins are generally activated under cellular stress (e.g. serum depletion) whereas the activation of ERK may associated with cellular proliferation (Meloche & Pouyssegur, 2007). Keeping with the lack of any functional effects on cell proliferation or apoptosis, AKR1B10 overexpression had no effects on the phosphorylation of any of the MAPK pathway proteins (**Figure 3.8 and 3.9**). Similar to our expectations

from the *in silico* analysis, silencing of AKR1B1 resulted in a loss of proliferation, along with slower progression through the cell cycle, although we did not observe any effects on apoptosis (Taskoparan, Seza et al., 2017).

Activation of the polyol pathway can lead to the development of oxidative stress, which in turn can enhance the activation of inflammatory transcription factors such as NF- $\kappa$ B and AP-1 (Srivastava et al., 2011). Inflammatory pathways were also enriched in GSEA obtained with high 30% AKR1B1 expressing colon cancer samples. We therefore examined whether the overexpression of AKR1B10 can affect inflammation related pathways. We observed that AKR1B10 overexpression led to reduced activation of NF- $\kappa$ B, a major transcription factor that can regulate proliferation, differentiation, apoptosis, and development (Oeckinghaus & Ghosh, 2009). On the other hand, we observed that AKR1B1 overexpression or silencing in CRC cell lines was directly correlated with NF- $\kappa$ B activation, most likely through enhanced ROS production (Taskoparan, Seza et al., 2017). No alteration in ROS production was observed in the AKR1B10 overexpressing cells.

When we examined the correlation of a set of inflammation related genes with the expression of AKR1B1 and AKR1B10 from TCGA CoadRead RNA-seq data, correlation was observed between distinct non-overlapping sets of inflammatory genes (Taskoparan, Seza et al., 2017). AKR1B1 was significantly positively correlated with several pro-inflammatory genes. Among the genes that were positively correlated with the expression of AKR1B10, the highest positive correlation was observed with IL1-R2, a negative regulator of Interleukin (IL)-1 signaling that binds to IL-1 $\beta$  and IL-1 $\alpha$  with high affinity, but does not induce any downstream signaling (Garlanda,

Riva, Bonavita, & Mantovani, 2013). Interestingly, the expression of IL-1 $\alpha$  was also significantly positively correlated with AKR1B10 indicating the possibility of negative feedback mechanisms. While it is premature to conclude the AKR1B10 has anti-inflammatory properties, it is likely that the type of inflammation induced by AKR1B1 and AKR1B10 serve different purposes.

Pharmaceutical inhibition of AKR1B1 in HT-29 cells was shown to reduce growth factor mediated adhesion to endothelial cells and expression of cell adhesion molecules (Tammali, Reddy, et al., 2011). These data were further supported by reduced hepatic metastasis of KM20 cells in nude mice that were given AKR inhibitors (Tammali, Reddy, et al., 2011). Additionally, AKR1B1 has recently been shown to be a target of the mesenchymal transcription factor Twist2 and a major inducer of an epithelial to mesenchymal transition in basal-like breast carcinomas (Wu et al., 2017). Our analysis of colon cancer microarray data (GSEA), as well as functional studies on colon cancer cell lines supported that high levels of AKR1B1 resulted in enhanced motility and migration (Taskoparan, Seza et al, 2017). On the contrary, overexpression of AKR1B10 in HCT-116 cells resulted in significantly slower cell motility. The role of AKR1B10 in metastasis is highly context dependent. In breast cancer, AKR1B10 has been reported to be significantly associated with metastasis (Ma et al., 2012) through the upregulation of integrin  $\alpha$ 5 and  $\delta$ -catenin (Huang, Verhulst, Shen, Bu, Cao, & He, 2016). On the other hand, similar to our data, AKR1B10 overexpression in a nasopharyngeal carcinoma cell line resulted in slower proliferation and slower motility in a scratch wound assay (Guo et al., 2016). These differences may be partly explained on the basis of the cell type examined. The colon cancer cell line HCT-8, with prolonged growth (20 days) on soft medium transitioned from an epithelial (E) phenotype to a rare more rounded (R) phenotype that were highly metastatic (X. Tang et al., 2014).

These ‘R’ cells expressed significantly higher amounts of AKR1B10 compared to the ‘E’ cells. Thus, it is possible that the expression of AKR1B10 is generally reduced in the epithelial (E) type CRC cells that predominate in most of the models used to date. Interestingly, the epithelial type MCF-7 breast cancer cells overexpressing AKR1B10 could not metastasize to lungs *in vivo*, whereas MDA-MB-231 cells that are more mesenchymal in nature (Lehmann et al., 2011), metastasized to the lungs when overexpressing AKR1B10 (Huang, Verhulst, Shen, Bu, Cao, & He, 2016). It remains to be seen whether primary colon cancer stem cells express higher amounts of AKR1B10 and whether this influences the metastatic capability of these cells.

## CHAPTER 5

### CONCLUSIONS AND FUTURE STUDIES

AKR1B1 and AKR1B10 are members of a vast family of enzymes called AKRs (Aldo-Keto Reductases) that are soluble, mostly monomeric, 37 kDa and NAD(P)H dependent oxidoreductases (Zu, Yan, Ma, Liao, & Cao, 2009). These enzymes can reduce aldehydes such as glucose into sorbitol and other downstream intermediates (Chung et al., 2012a). These intermediates are known to play a role in the production of reactive oxygen species and subsequently inflammation (Srivastava et al., 2011).

Colorectal cancer is frequently accompanied by the development of low grade chronic inflammation (Lucas, Barnich, & Nguyen, 2017). Therefore, we hypothesized that aberrant expression of the primary AKR family members, AKR1B1 and AKR1B10 may play a role in the development and progression of colorectal cancer.

In this study, we focused on the expression and functions of AKR1B10 in colorectal cancer. Our primary findings have been tabulated below.

1. Gene Set Enrichment Analysis using GSE 39582 (analysis carried out by Seçil Demirkol and Dr. Ali O. Güre, Bilkent University) indicated that

AKR1B10 expressing tumors were enriched in metabolism related genes in keeping with the function of AKR1B10 as an important metabolic enzyme. AKR1B1 expressing tumors, on the other hand enriched in genes associated with cancer (inflammation, cell cycle progression, motility etc.).

2. Corroborating the GSEA data, overexpression of AKR1B10 in different colon cancer cell lines did not lead to any major changes in the primary hallmarks of cancer including proliferation and cell cycle progression.

3. Overexpression of AKR1B10, however, did lead to a reduction in cellular motility in a Transwell assay. Nonetheless, the expression of the well-known EMT markers such as E-cadherin, vimentin and transgelin did not show any difference between the AKR1B10 expressing and control cells. It is currently unclear whether other markers of cellular motility may be functional in diminishing cellular motility.

4. A decrease in the nuclear translocation, DNA binding and transcriptional activity of NF- $\kappa$ B was observed in the AKR1B10 expressing cancer cells compared to the control cells.

There are several questions remaining to be answered in this study:

- Colon cancer patients' samples that showed high AKR1B10 expression were enriched in metabolism related genes according to GSEA. Future studies will show the relation between AKR1B10 and metabolism.
- According to GSEA, low AKR1B10 expressing colon cancer patient's samples were enriched in regulatory genes in epigenetics. The loss of AKR1B10 expression in colon cancer may result from epigenetic alterations. Preliminary data generated from our lab indicate that treatment of HCT-116 cells with the DNA methyltransferase inhibitor 5-azacytidine could restore expression of AKR1B10. Further studies are needed to better establish this.

- AKR1B10 overexpressing cells showed reduced in cellular motility. The mechanism for this event is currently unclear.

Based on the results obtained from this study, it may be suggested that the role played by AKR1B10 neoplastic transformation in the colon may be indirect, perhaps through the activation of metabolism related pathways. It has been greatly appreciated in recent years that nutrient sensing and growth factor mediated signaling pathways have significant cross-talk. The aberrant activation of the PI3K/AKT signaling pathway has been widely described in many different tumor types; activation of this pathway in cancer cells may ensure autonomy over uptake of nutrients and increasing the availability of precursors for macromolecular synthesis (Palm & Thompson, 2017). Our future studies will be aimed to better understand whether AKR1B10 expression leads to the activation of nutrient sensing pathways and how the activation of these pathways may alter cancer-associated characteristics such as proliferation, motility and sensitivity to cancer chemotherapy drugs.



## REFERENCES

- Annibaldi, A., & Widmann, C. (2010). Glucose metabolism in cancer cells. *Current Opinion in Clinical Nutrition and Metabolic Care*, 13(4), 466–470. <https://doi.org/10.1097/MCO.0b013e32833a5577>
- Assinder, S. J., Stanton, J. A. L., & Prasad, P. D. (2009). Transgelin: An actin-binding protein and tumour suppressor. *International Journal of Biochemistry and Cell Biology*. <https://doi.org/10.1016/j.biocel.2008.02.011>
- Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A. A., Kim, S., ... Garraway, L. A. (2012). The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*, 483(7391), 603–307. <https://doi.org/10.1038/nature11003>
- Barski, O. A., Tipparaju, S. M., & Bhatnagar, A. (2008). The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metabolism Reviews*, 40(4), 553–624. <https://doi.org/10.1080/03602530802431439>
- Boroughs, L. K., & DeBerardinis, R. J. (2015). Metabolic pathways promoting cancer cell survival and growth. *Nature Cell Biology*, 17(4), 351–9. <https://doi.org/10.1038/ncb3124>
- Cairns, R., Harris, I., & Mak, T. (2011). Regulation of cancer cell metabolism. *Nature Reviews Cancer*, 11(2), 85–95. <https://doi.org/10.1038/nrc2981>
- Chen, W. D., & Zhang, Y. (2012). Regulation of aldo-keto reductases in

human diseases. *Frontiers in Pharmacology*, 3 MAR.

<https://doi.org/10.3389/fphar.2012.00035>

Chung, Y. T., Matkowskyj, K. A., Li, H., Bai, H., Zhang, W., Tsao, M.-S., ...

Yang, G.-Y. (2012a). Overexpression and oncogenic function of aldo-keto reductase family 1B10 (AKR1B10) in pancreatic carcinoma.

*Modern Pathology*, 25(5), 758–766.

<https://doi.org/10.1038/modpathol.2011.191>

Chung, Y. T., Matkowskyj, K. A., Li, H., Bai, H., Zhang, W., Tsao, M.-S., ...

Yang, G.-Y. (2012b). Overexpression and oncogenic function of aldo-keto reductase family 1B10 (AKR1B10) in pancreatic carcinoma. *Mod Pathol*, 25(5), 758–766.

<https://doi.org/10.1038/modpathol.2011.191>

Dhillon, A., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene*, 26, 3279–3290.

<https://doi.org/10.1038/sj.onc.1210421>

Ebert, B., Kisiela, M., Wsól, V., & Maser, E. (2011). Proteasome inhibitors MG-132 and bortezomib induce AKR1C1, AKR1C3, AKR1B1, and AKR1B10 in human colon cancer cell lines SW-480 and HT-29.

*Chemico-Biological Interactions*, 191(1–3), 239–249.

<https://doi.org/10.1016/j.cbi.2010.12.026>

Enayat, S., Şeyma Ceyhan, M., Taşkoparan, B., Stefek, M., & Banerjee, S.

(2016). CHNQ, a novel 2-Chloro-1,4-naphthoquinone derivative of quercetin, induces oxidative stress and autophagy both in vitro and in vivo. *Archives of Biochemistry and Biophysics*, 596, 84–98.

<https://doi.org/10.1016/j.abb.2016.03.004>

Fresno Vara, J. a, Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., & González-Barón, M. (2004). PI3K/Akt signalling pathway and cancer.

*Cancer Treatment Reviews*, 30(2), 193–204.

<https://doi.org/10.1016/j.ctrv.2003.07.007>

- Garlanda, C., Riva, F., Bonavita, E., & Mantovani, A. (2013). Negative regulatory receptors of the IL-1 family. *Seminars in Immunology*, 25(6), 408–415. <https://doi.org/10.1016/j.smim.2013.10.019>
- Guo, Y., Luo, W., Hu, Z., Li, J., Li, X., Cao, H., ... Luo, D. (2016). Low expression of Aldo-keto reductase 1B10 is a novel independent prognostic indicator for nasopharyngeal carcinoma. *Cell & Bioscience*, 6(1), 18. <https://doi.org/10.1186/s13578-016-0082-x>
- Hanahan, D., & Coussens, L. M. (2012). Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. *Cancer Cell*. <https://doi.org/10.1016/j.ccr.2012.02.022>
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. Vol. 144, *Cell*. 2011. p. 646–74. Hallmarks of cancer: The next generation. *Cell*.
- Huang, C., Verhulst, S., Shen, Y., Bu, Y., Cao, Y., & He, Y. (2016). AKR1B10 promotes breast cancer metastasis through integrin  $\alpha 5 / \delta$ -catenin mediated FAK / Src / Rac1 signaling pathway, 7(28).
- Huang, C., Verhulst, S., Shen, Y., Bu, Y., Cao, Y., He, Y., ... Cao, D. (2016). AKR1B10 promotes breast cancer metastasis through integrin  $\alpha 5 / \delta$ -catenin mediated FAK/Src/Rac1 signaling pathway. *Oncotarget*, 7(28), 43779–43791. <https://doi.org/10.18632/oncotarget.9672>
- Kim, J., Yang, G., Kim, Y., Kim, J., & Ha, J. (2016). AMPK activators: mechanisms of action and physiological activities. *Experimental & Molecular Medicine*, 48(4), e224. <https://doi.org/10.1038/emm.2016.16>
- Ko, H.-H., Cheng, S.-L., Lee, J.-J., Chen, H.-M., Kuo, M. Y.-P., & Cheng, S.-J. (2017). Expression of AKR1B10 as an independent marker for poor

- prognosis in human oral squamous cell carcinoma. *Head & Neck*, 39(7), 1327–1332. <https://doi.org/10.1002/hed.24759>
- Laffin, B., & Petrash, J. M. (2012). Expression of the aldo-ketoreductases AKR1B1 and AKR1B10 in human cancers. *Frontiers in Pharmacology*, 3 JUN(June), 1–7. <https://doi.org/10.3389/fphar.2012.00104>
- Laplante, M., & Sabatini, D. M. (2012). MTOR signaling in growth control and disease. *Cell*. <https://doi.org/10.1016/j.cell.2012.03.017>
- Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor Perspectives in Biology*. <https://doi.org/10.1101/cshperspect.a001651>
- Lehmann, B. D. B., Bauer, J. a J., Chen, X., Sanders, M. E., Chakravarthy, a B., Shyr, Y., & Pietenpol, J. a. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *Journal of Clinical Investigation*, 121(7), 2750–2767. <https://doi.org/10.1172/JCI45014DS1>
- Li, J., Guo, Y., Duan, L., Hu, X., Zhang, X., Hu, J., ... Luo, D.-X. (2017). AKR1B10 promotes breast cancer cell migration and invasion via activation of ERK signaling. *Oncotarget*, 8(20), 33694–33703. <https://doi.org/10.18632/oncotarget.16624>
- Liu, C.-Y., Lin, H.-H., Tang, M.-J., & Wang, Y.-K. (2015). Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. *Oncotarget*, 6(18), 15966–83. <https://doi.org/10.18632/oncotarget.3862>
- Liu, P., Cheng, H., Roberts, T. M., & Zhao, J. J. (2009). Targeting the phosphoinositide 3-kinase (PI3K) pathway in cancer. *Nat. Rev. Drug. Discov.*, 8(8), 627–644. <https://doi.org/10.1038/nrd2926>. Targeting

- Liu, T.-A., Jan, Y.-J., Ko, B.-S., Wu, Y.-J., Lu, Y.-J., Liang, S.-M., ... Liou, J.-Y. (2015). Regulation of aldo-keto-reductase family 1 B10 by 14-3-3ε and their prognostic impact of hepatocellular carcinoma. *Oncotarget*, 6(36), 38967–82. <https://doi.org/10.18632/oncotarget.5734>
- Lorenzi, M. (2007). The polyol pathway as a mechanism for diabetic retinopathy: attractive, elusive, and resilient. *Experimental Diabetes Research*, 2007, 61038. <https://doi.org/10.1155/2007/61038>
- Lucas, C., Barnich, N., & Nguyen, H. T. T. (2017). Microbiota, Inflammation and Colorectal Cancer. *International Journal of Molecular Sciences*, 18(6). <https://doi.org/10.3390/ijms18061310>
- Ma, J., Luo, D.-X., Huang, C., Shen, Y., Bu, Y., Markwell, S., ... Cao, D. (2012). AKR1B10 overexpression in breast cancer: association with tumor size, lymph node metastasis and patient survival and its potential as a novel serum marker. *International Journal of Cancer. Journal International Du Cancer*, 131(6), E862-71. <https://doi.org/10.1002/ijc.27618>
- Matsunaga, T., Wada, Y., Endo, S., Soda, M., El-Kabbani, O., & Hara, A. (2012). Aldo-keto reductase 1B10 and its role in proliferation capacity of drug-resistant cancers. *Frontiers in Pharmacology*, 3 JAN. <https://doi.org/10.3389/fphar.2012.00005>
- Meloche, S., & Pouyssegur, J. (2007). The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene*, 26(22), 3227–39. <https://doi.org/10.1038/sj.onc.1210414>
- Milackova, I., Prnova, M. S., Majekova, M., Sotnikova, R., Stasko, M., Kovacikova, L., ... Stefek, M. (2014). 2-Chloro-1,4-naphthoquinone derivative of quercetin as an inhibitor of aldose reductase and anti-inflammatory agent. *Journal of Enzyme Inhibition and Medicinal*

- Chemistry*, 6366, 1–7. <https://doi.org/10.3109/14756366.2014.892935>
- Mindnich, R. D., & Penning, T. M. (2009). Aldo-keto reductase (AKR) superfamily: genomics and annotation. *Human Genomics*, 3(4), 362–70. <https://doi.org/10.1186/1479-7364-3-4-362>
- Oeckinghaus, A., & Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology*, 1(4), a000034. <https://doi.org/10.1101/cshperspect.a000034>
- Ohashi, T., Idogawa, M., Sasaki, Y., Suzuki, H., & Tokino, T. (2013). AKR1B10, a Transcriptional Target of p53, Is Downregulated in Colorectal Cancers Associated with Poor Prognosis. <https://doi.org/10.1158/1541-7786.MCR-13-0330-T>
- Palm, W., & Thompson, C. B. (2017). Nutrient acquisition strategies of mammalian cells. *Nature*, 546(7657), 234–242. <https://doi.org/10.1038/nature22379>
- Pattabiraman, D. R., & Weinberg, R. A. (2014). Tackling the cancer stem cells — what challenges do they pose? *Nature Reviews Drug Discovery*, 13(7), 497–512. <https://doi.org/10.1038/nrd4253>
- Pavlova, N. N., & Thompson, C. B. (2016). The Emerging Hallmarks of Cancer Metabolism. *Cell Metabolism*, 23(1), 27–47. <https://doi.org/10.1016/j.cmet.2015.12.006>
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9), e45. <https://doi.org/10.1093/nar/29.9.e45>
- Pollak, N., Dölle, C., & Ziegler, M. (2007). The power to reduce: pyridine nucleotides – small molecules with a multitude of functions. *Biochemical Journal*, 402(2), 205–218.

<https://doi.org/10.1042/BJ20061638>

Ramana, K. V. (2011). Aldose reductase: New insights for an old enzyme. *Biomolecular Concepts*. <https://doi.org/10.1515/bmc.2011.002>

Ramana, K. V., Tammali, R., & Srivastava, S. K. (2010). Inhibition of Aldose Reductase Prevents Growth Factor–Induced G1-S Phase Transition through the AKT/Phosphoinositide 3-Kinase/E2F-1 Pathway in Human Colon Cancer Cells. *Molecular Cancer Therapeutics*, 9(4). Retrieved from <http://mct.aacrjournals.org/content/9/4/813.long>

Sinreih, M., Štupar, S., Čemažar, L., Verdenik, I., Frković Grazio, S., Smrkolj, Š., & Rižner, T. L. (2017). STAR and AKR1B10 are down-regulated in high-grade endometrial cancer. *The Journal of Steroid Biochemistry and Molecular Biology*, 171, 43–53. <https://doi.org/10.1016/j.jsbmb.2017.02.015>

Srivastava, S. K., Yadav, U. C. S., Reddy, A. B. M., Saxena, A., Tammali, R., Shoeb, M., ... Ramana, K. V. (2011). Aldose reductase inhibition suppresses oxidative stress-induced inflammatory disorders. In *Chemico-Biological Interactions* (Vol. 191, pp. 330–338). <https://doi.org/10.1016/j.cbi.2011.02.023>

Tammali, R., Reddy, A. B. M., Saxena, A., Rychahou, P. G., Evers, B. M., Qiu, S., ... Srivastava, S. K. (2011). Inhibition of aldose reductase prevents colon cancer metastasis. *Carcinogenesis*, 32(8), 1259–1267. <https://doi.org/10.1093/carcin/bgr102>

Tammali, R., Srivastava, S. K., & Ramana, K. V. (2011). Targeting aldose reductase for the treatment of cancer. *Current Cancer Drug Targets*, 11(5), 560–571. Retrieved from <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=21486217&retmode=ref&cmd=prlinks%5Cnpapers2://publication/uu>

d/6C06683C-3083-4DDD-BC90-A4A90D8EE4D3

- Tang, W. H., Martin, K. A., & Hwa, J. (2012). Aldose Reductase, Oxidative Stress, and Diabetic Mellitus. *Frontiers in Pharmacology*, 3, 87.  
<https://doi.org/10.3389/fphar.2012.00087>
- Tang, X., Kuhlenschmidt, T. B., Li, Q., Ali, S., Lezmi, S., Chen, H., ... Kuhlenschmidt, M. S. (2014). A mechanically-induced colon cancer cell population shows increased metastatic potential. *Molecular Cancer*, 13(1), 131. <https://doi.org/10.1186/1476-4598-13-131>
- Taskoparan, B., Seza, E.G., Demirkol, S., Tuncer, S., Stefek, M., Gure, A.O., Banerjee, S. (2017). Opposing roles of the aldo-keto reductases AKR1B1 and AKR1B10 in colorectal cancer. *Cellular Oncology*.  
<https://doi.org/10.1007/s13402-017-0351-7>
- Warburg, O. (1956). Injuring of Respiration the Origin of Cancer Cells. *Science*, 123(3191), 309–14.  
<https://doi.org/10.1126/science.123.3191.309>
- Wu, X., Li, X., Fu, Q., Cao, Q., Chen, X., Wang, M., ... Dong, C. (2017). AKR1B1 promotes basal-like breast cancer progression by a positive feedback loop that activates the EMT program. *The Journal of Experimental Medicine*, 214(4), 1065–1079.  
<https://doi.org/10.1084/jem.20160903>
- Wuerzberger-Davis, S. M., Nakamura, Y., Seufzer, B. J., & Miyamoto, S. (2007). NF-kappaB activation by combinations of NEMO SUMOylation and ATM activation stresses in the absence of DNA damage. *Oncogene*, 26, 641–651. <https://doi.org/10.1038/sj.onc.1209815>
- Yan, R., Zu, X., Ma, J., Liu, Z., Adeyanju, M., & Cao, D. (2007). Aldo-keto reductase family 1 B10 gene silencing results in growth inhibition of

colorectal cancer cells: Implication for cancer intervention. *International Journal of Cancer*, 121(10), 2301–2306.

<https://doi.org/10.1002/ijc.22933>

Zu, X., Yan, R., Ma, J., Liao, D.-F., & Cao, D. (2009). AKR1B10: A potential target for cancer therapy. *Bioscience Hypotheses*, 2(1), 31–33.

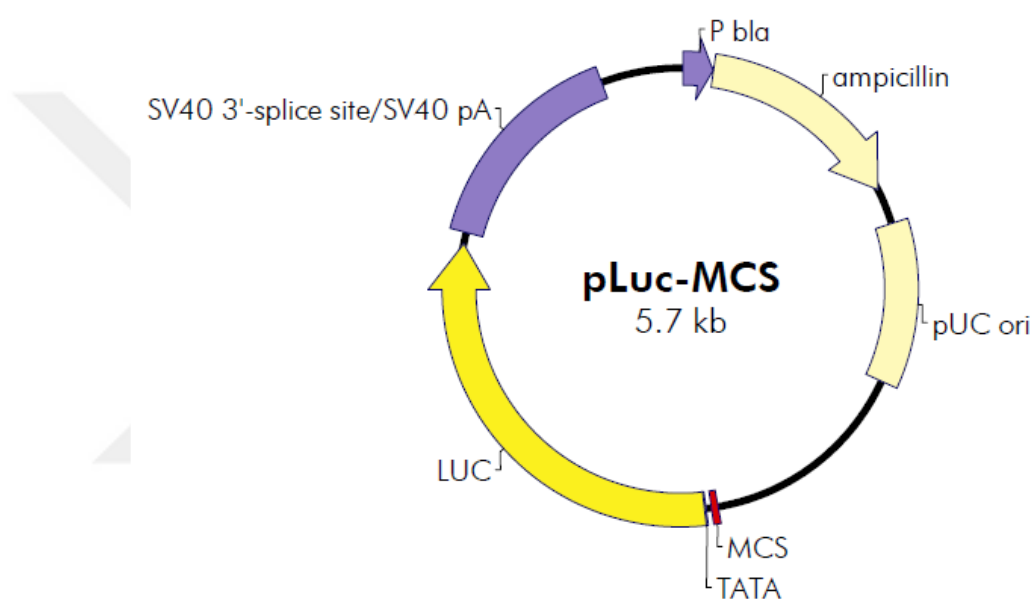
<https://doi.org/10.1016/j.bihy.2008.07.011>



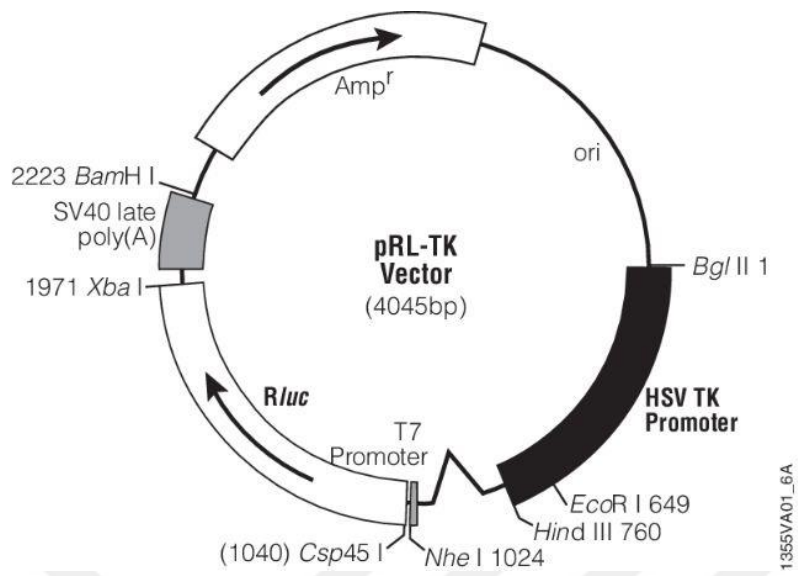


## APPENDIX A

### MAPS OF VECTORS USED IN THIS STUDY



**Figure A.1** The vector map for Pathdetect cis-Reporter plasmid (Agilent Technologies, USA). The NF- $\kappa$ B Pathdetect plasmid used in this study contains five tandem repeats of NF- $\kappa$ B response element, which is TGGGGACCTTTCCGC cloned of MCV of this vector.



**Figure A.2** The vector map for pRL-TK Renilla Luciferase vector (Promega, USA).

## APPENDIX B

### CONTENTS OF BUFFERS USED FOR WESTERN BLOT EXPERIMENTS

#### **6X SAMPLE LOADING DYE**

12% SDS  
30%  $\beta$ -mercaptoethanol  
30% Glycerol  
0.012% Bromophenol Blue  
0.375 M Tris-HCl pH 6.8

#### **10X BLOTTING BUFFER**

0.25 M Tris  
1.92 M Glycine  
Adjusted pH 8.3 in 1 L dH<sub>2</sub>O

#### **TRANSFER BUFFER (1L)**

200 mL Methanol  
100 mL 10X Blotting Buffer  
700 mL dH<sub>2</sub>O

#### **SDS-PAGE RUNNING BUFFER**

25 mM Tris  
190 mM Glycine  
0.1% SDS

### **10% SEPERATING GEL MIX**

5.4 mL dH<sub>2</sub>O

3.8 mL 10% SDS + 1.5 M Tris-HCl pH 8.8

5.6 mL 30% Acrylamide + Bisacrylamide solution

150 µl 10% Ammonium persulfate (APS)

20 µl TEMED

### **4% SEPERATING GEL MIX**

4.7 mL dH<sub>2</sub>O

2 mL 10% SDS + 1.5 M Tris-HCl pH 6.8

1.2 mL 30% Acrylamide + Bisacrylamide solution

50 µl 10% Ammonium persulfate (APS)

10 µl TEMED

### **TBS-T**

50 mM Tris-HCl pH 7.4

150 mM NaCl

Autoclaved, and 0.1% Tween-20 added before use.

### **PBS-T**

8 g NaCl

0.27 g KH<sub>2</sub>PO<sub>4</sub>

3.58 g Na<sub>2</sub>HPO<sub>4</sub>.12 dH<sub>2</sub>O

Adjusted pH 7.4

Autoclaved, and 0.1% Tween-20 added before use.

**MILD STRIPPING BUFFER**

15 g Glycine

1 g SDS

10 mL Tween-20

Adjusted pH 2.2 in 1 L dH<sub>2</sub>O

