INVESTIGATION OF THE EFFECTS OF NICOTINE ON THE EXPRESSION PROFILE OF SW620 COLON ADENOCARCINOMA CELLS USING A FUNCTIONAL GENOMICS APPROACH

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MASTER OF SCIENCE

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AUGUST 2009

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TO MY PARENTS SELVİ -KEMAL KAYA; MY BROTHER OKAN;

and TO SEVGİ ŞAHİN…

ABSTRACT

INVESTIGATION OF THE EFFECTS OF NICOTINE ON THE EXPRESSION PROFILE OF SW620 COLON ADENOCARCINOMA CELLS USING A FUNCTIONAL GENOMICS APPROACH

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Colon cancer is the third most common form of cancer with approximately 655,000 deaths worldwide annually and the second principal cause of cancer-related death in the Western world. Studies focusing on genomic instability and cell culture in recent years have shown that there is a statistically significant link between tobacco smoking and colorectal cancer. Although nicotine is one of the most potent chemical in tobacco, it was not studied extensively in colorectal cancers. Nicotine works as an agonist of nicotinic acetylcholine receptors and modulates the intracellular calcium concentrations hence deregulating multiple signal transduction pathways (e.g., PI3K/AKT, MAPK, mTOR). It has been shown that nicotine accelerates cell proliferation while it increases cell migration, metastasis and angiogenesis, and inhibits apoptosis in lung and gastric cancers. The aim of this study was to give more insight into the association between nicotine and colon cancer by investigating the gene expression profiles of SW620 colon adenocarcinoma cells under 48h 1μM nicotine treatment at different serum levels to reflect molecular response to growth factor-induced and –depleted conditions (10% FBS or 0.1% FBS). We used multiple approaches including cell culture techniques, microarray technology, and gene-network analysis to assess the effects of nicotine on cell proliferation and transcriptome profile. Furthermore, the

selected genes that are involved in cell cycle and apoptosis were used to confirm and evaluate the transcriptome analysis results with real time qRT-PCR and Western Blot techniques. In this project, our findings indicated that serum starvation of SW620 colon adenocarcinoma cell line resulted in decreased cell proliferation, which could be rescued by 1μM nicotine via deregulation of multiple pathways including cell cycle, apoptosis, Ca^{2+} signaling, and ribosomal protein expression. This study implicated that nicotine-, thus acetylcholine-mediated signaling may have an important role in tumor development and metastasis.

Key words: Nicotine, nicotinic acetylcholine receptors, SW620, colon cancer, microarray, serum starvation

ÖZET

NİKOTİNİN SW620 KOLON ADENOKARSİNOM HÜCRELERİNİN GEN İFADE PROFİLİ ÜZERİNE OLAN ETKİSİNİN İŞLEVSEL GENOMİK YAKLAŞIMLARLA ARAŞIRILMASI

Onur KAYA

Moleküler Biyoloji ve Genetik Yüksek Lisansı

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Kolon kanseri her yıl dünyada 655.000'e yakın kişinin ölümüne sebep olmakta dünyada en üçüncü, Batı dünyasında ise kanserle ilgili ölümlere sebep olmakta ikinci sıra bulunan kanser tipidir. Son yıllarda genomik kararsızlıklarla ilgili yapılan çalışmalar sigara kullanmakla kolorektal kanser arasında istatistiksel olarak kayda değer bir ilişki olduğunu göstermiştir. Sigarada bulunan kimyasallar içinde nikotin, sigaranın etkisini belirlemede çok önemli bir yer teşkil etmesine rağmen, kolorektal kanser araştırmalarında çok fazla kullanılmamıştır. Nikotinik kolinerjik reseptörlerine agonist olarak bağlanan nikotin, hücre içi kalsiyum konsantrasyonu üzerine etki ederek bir çok sinyal yolağının (PI3K/ AKT, MAPK, mTOR yolakları gibi) düzensizleşmesine, aktif ya da inaktif olmasına neden olur. Nikotin üzerine yapılan çalışmalar göstermiştir ki, nikotin hücre çoğalmasını hızlandırmakla beraber hücre göçü, hücre metaztazı, ve anjiyogenez (damar gelişmesini) olaylarını da arttırmakta, ayrıca akciğer ve gastrik kanserlerde apoptozu engellemektedir. Bu çalışmamızda, SW620 kolon adenokarsinom hücrelerini farklı serum şartlarında, 48 saat boyunca 1μM nikotine maruz bırakarak nikotin-kolon kanseri ilişkisini aydınlatmayı amaçladık. Bu amacımız doğrultusunda, hücre kültürü teknikleri,

mikrodizin teknolojisi ve gen-ağ analizleri içeren yaklaşımlar kullanarak nikotinin hücre çoğalması ve gen ifadesi profiline olan etkisini araştırdık. Bunların yanında, apoptozda veya hücre döngüsünde görev alan ve mikrodizinde etkili bir biçimde ifade olan genleri belirleyerek bu genleri nicel gerçek zamanlı polimeraz zincir reaksiyonu ve "Western blot" tekniklerinde kullanarak mikrodizin çalışmasının doğrulamasını yaptık. Bu çalışmada, serumdan mahrum bırakılan SW620 kolon adenokarsinom hücrelerinin çoğalmasında belirgin bir azalma olmasına rağmen, aynı serum şartlarında 1μM konsantrasyondaki nikotin sayesinde hücreler hücre döngüsü, apoptoz, kalsiyum iyonu ve ribosomal protein yolakları gibi çeşitli sinyal yolaklarının aktivitelerini değiştirerek çoğalma özelliklerini belirli bir derecede geri kazanmıştır. Bu çalışma göstermiştir ki nikotin tarafından, bundan hareketle asetilkolin tarafında düzenlenen hücre içi sinyaller tümor gelişimi ve metaztaz mekanizmalarında önemli bir etken teşkil etmektedir.

Anahtar sözcükler: Nikotin, nikotinik asetilkolin reseptörleri, SW620, kolon kanseri, mikrodizin

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ABBREVIATIONS

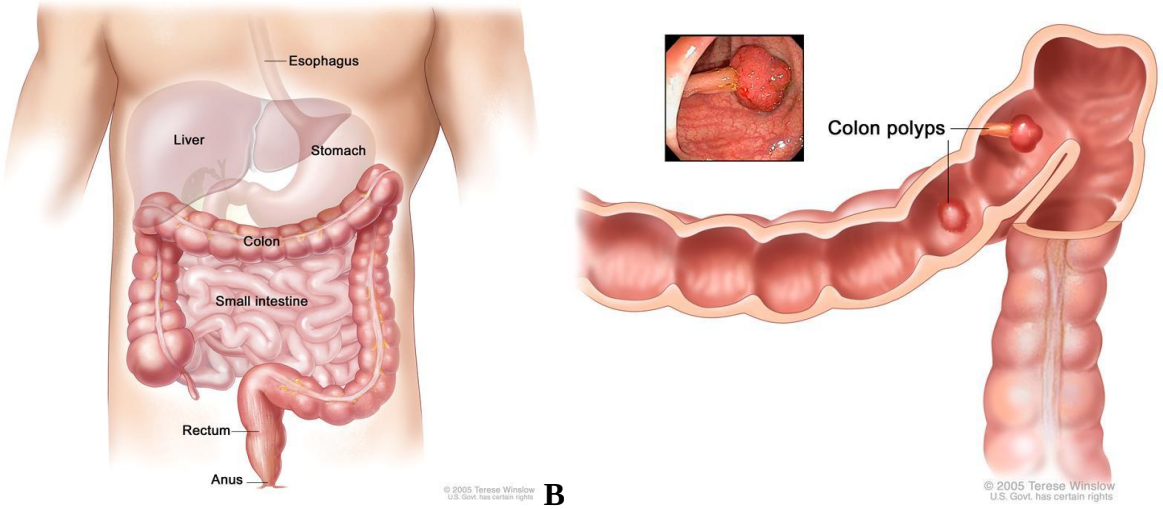
HNPCC	Hereditary non-polyposis colon cancer
TNM	Tumors/nodes/metastases
AJCC	American Joint Committee on Cancer
IARC	International Agency for Research on Cancer
IUPAC	International Union of Pure and Applied Chemistry
NNO	Nicotine N'-oxide
CNO	cotinine N-oxide
XIAP	X-linked Inhibitor of Apoptosis Protein
BIRC4	Baculoviral inhibitor of apoptosis repeat-containing 4
BIRC5	baculoviral inhibitor of apoptosis repeat-containing 5
NSCLC	non-small cell lung cancer
GSK-3β	Glycogen synthase kinase 3 beta
nAChR	Nicotinic Acetylcholine Receptor
COX-2	Cyclooxygenase-2
ERK	Extracellular signal-regulated kinases
ATCC	American Type Culture Collection
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
KEGG	Kyoto Encyclopedia of Genes and Genomes
CDKN2A	Cyclin dependent kinase inhibitor 2A
ATR	Ataxia telangiectasia and Rad3 related
RP	Ribosomal Protein
UGT	Uridine 5'-diphospho-glucuronosyltransferase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MDM	Murine double minute

CHAPTER 1. INTRODUCTION

1.1 Colon Cancer

1.1.1 Colorectal Cancer

Colorectal cancers consist of cancers of the digestive system, namely colon and rectal cancers; the former includes cancers of the lower part of the digestive system (larger intestine-colon) while rectal cancers are characterized as originating from the end part of colon (last 6 inches) (***Figure 1.1A***) [1]. With approximately 655,000 deaths worldwide annually, it is the third most common form of cancer and the second principal cause of cancer-related death in the Western world [2]. About 112,000 people are diagnosed with colon cancer per year, and approximately 41,000 new cases of rectal cancer are diagnosed annually, according to the American Cancer Society [1]. According to the 2009 statistics, among these cases, nearly 50,000 people have died because of the colorectal cancer [3]. Most of the colon cancer cases begin as small-benign clumps of cells called **adenomatous polyps (*Figure 1.1B*)**. Thereafter, some of these polyps progress to become colon cancers.



A **B**

Figure 1.1: Human colon physiology **A)** Illustration of normal colon physiology in human body. **B)** Imaging of colon polyps inside of the large intestine tissue [3].

1.1.2 Risk Factors

Although exact causes of colorectal cancer are not well known, there are some predicted reasons that may trigger the formation of cancer according to National Cancer Institute. Heritability and age are among the main risk factors for colorectal cancers. More than 90% of people with colorectal cancer are diagnosed after age 50 as colorectal polyps increase after that age and the average age for the disease is 72 [3]. Although colorectal cancer is not contagious, familial history takes important role in development of cancer. Close relatives of a person with a history of colorectal cancer are somewhat more likely to develop this disease [1]. Moreover, mutations in some key-genes increase the risk of colorectal cancer. For instance, "Hereditary Non-Polyposis Colon Cancer (HNPCC)" is the most common type of inherited colorectal cancer. It accounts for approximately 2% of all colorectal cancers [4]. Most people with an altered HNPCC gene develop cancer at an early age [3]. Recurrence is a common theme in colorectal cancers [5]. In addition, women with a history of ovary, uterus or breast cancer have higher risks to develop colorectal cancers [3, 6]. Long-term *Ulcerative colitis* and *Crohn's disease* history and diets high in fat and low in calcium foliate may also increase the risk of colorectal cancer [7, 8]. Another very important risk factor for development of colorectal cancer is tobacco smoking. A person who smokes cigarettes may have increased risk of developing adenomatous polyps. According to Strate et al., women and men who smoked have increased risk of dying from the disease, more than 40% and 30% respectively, when compared with those who never smoked [9].

1.1.3. Early Detection, Diagnosis and Staging

Weakness, fatigue, change in bowel habits, diarrhea or constipation, red or dark blood in stool, weight loss, gas, abdominal pain, cramps, or bloating are some of the symptoms of colorectal cancer. Some other diseases also mimic these symptoms such as ulcerative colitis, diverticulitis, and peptic ulcer disease [3]. Colorectal cancer may develop for many years before the symptoms,

which vary according to the place of the tumor in the intestine [2]. Therefore, early detection of colorectal cancer gives a very important chance for treatment and better prognosis. The National Cancer Policy Board of the Institute of Medicine predicted in 2003 that efforts for developing and applying additional colorectal cancer screening methods would result in a 29% decrease in colorectal cancer deaths in 20 years [10]. There are many screening methods to detect colorectal cancer formation such as digital rectal exam (DRE), fecal occult blood test (FOBT), endoscopy, sigmoidoscopy, and colonoscopy**.** A colonoscopy has the advantage that if polyps are found during the procedure they can be immediately removed and tissue can also be taken for biopsy [11, 12].

Colon cancer staging is an approximation of the quantity of diffusion of a particular cancer [13, 14]. It is performed for both diagnostic and research purposes, and in order to decide the best way for cure. The systems for staging colorectal cancers depend on the extent of local invasion, the degree of lymph node participation and whether there is outlying metastasis [14]. Staging of metastasis frequently include Abdominal Ultrasound, CT, PET scanning, and other imaging studies. The most common staging system is the **TNM** (for tumors/nodes/metastases) system, from the **American Joint Committee on Cancer (AJCC)**. The TNM system assigns a number based on three classes: "T" represents the grade of invasion of the intestinal wall, "N" denotes the degree of lymphatic node participation, and "M" symbolizes the degree of metastasis. Stages I to IV progressively indicate a more advanced cancer and worse prognosis (***Table 1.1***) [15].

Table 1.1: The stages of the colorectal cancer [15].

AJCC stage	**TNM stage criteria for colorectal cancer**
Stage 0	Tis: Tumor confined to mucosa; cancer-in-situ
Stage I	T1: Tumor invades submucosa
Stage I	T2: Tumor invades muscularis propria
Stage II-A	T3: Tumor invades subserosa or beyond (without other organs involved)
Stage II-B	T4: Tumor invades adjacent organs or perforates the visceral peritoneum
Stage III-A	N1: Metastasis to 1 to 3 regional lymph nodes. T1 or T2.
Stage III-B	N1: Metastasis to 1 to 3 regional lymph nodes. T3 or T4.
Stage III-C	N2: Metastasis to 4 or more regional lymph nodes. Any T.
Stage IV	M1: Distant metastases present. Any T, any N.

1.1.4 SW620 Human Colorectal Adenocarcinoma Cells

The SW620 is a metastatic colon cancer cell line, which was established from a lymph node of a 51-year-old Caucasian male (blood group A, Rh+) patient with an undifferentiated adenocarcinoma of the ascending colon [16]. It consists mainly of individual small spherical and bipolar cells lacking microvilli that are highly tumorigenic [17]. In this cell line, p53 is mutated (Arg → His substitution at codon 273) [18] while several oncogenes also are expressed (c-myc, K-ras, H-ras, N-ras, Myb, sis and fos; www.atcc.org). Moreover, APC, which is key regulator in β-catenin signaling pathway, is mutated in SW620 cells [19].

1.2 Nicotine

1.2.1 Smoking and Cancer

Smoking is one of the worldwide reasons for cancer–related death. Research demonstrates that 69% of all lung cancers in women and 91% in men are related to smoking [20]. Moreover, smoking is also one of the major reasons for the oral cavity, larynx, esophagus and stomach cancers for both smoking males and females [21]. Cigarettes consist of more than 4000 diverse chemicals and of these chemicals more than 60 are identified as carcinogens according to the research of International Agency for Research on Cancer (IARC). These carcinogens in cigarette smoke react with DNA leading to DNA modifications and mutations and finally causing genetic alterations that might have a role in cancer [22]. Nicotine is one of the carcinogens in the tobacco smoke. Its absorption may arise through the oral cavity, skin, lung, urinary bladder, and gastrointestinal tract [22]. Dissimilarly to the stomach, nicotine is well absorbed in the small intestine due to the higher pH and a large surface area [23]. Nicotine is known as a highly addictive chemical in tobacco taking the main role in addiction that leads to ongoing consumption of tobacco products. On the other hand, nicotine has an important role in

therapeutic applications for neurodegenerative diseases, such as Alzheimer's and Parkinson's disease [24, 25].

1.2.2 History of the Nicotine

Nicotine is an active alkaloid named after the tobacco plant *Nicotiana tabacum (Solanaceae)* [22].The systematic International Union of Pure and Applied Chemistry (IUPAC) name is (S)-3-(1- methyl-2-pyrrolidinyl) pyridine ***(Figure 1.2)***. Pure nicotine is a clear liquid and it has a characteristic odor, while the color changes into brown on exposure to air. Nicotine is soluble in water and can be mixed with an equal amount of water. However, it prefers organic solvents [22].

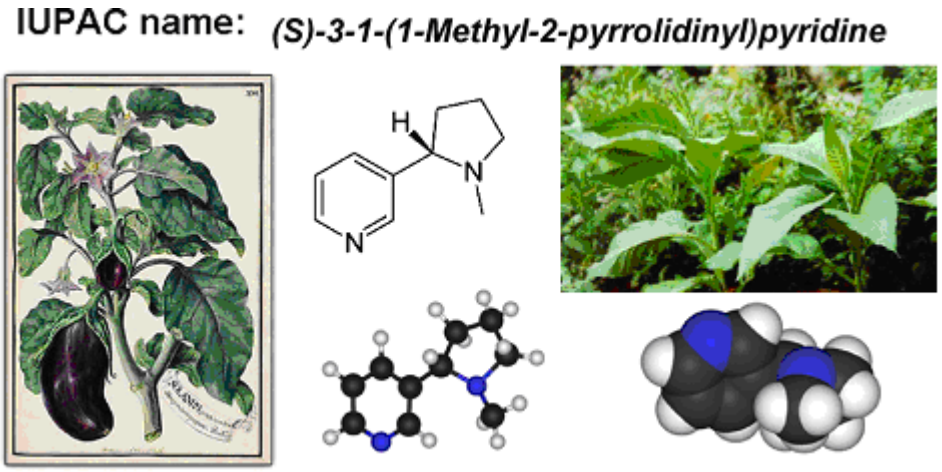


Figure 1.2: IUPAC name and chemical structure of nicotine; tobacco plants images (left); illustration for the correlation of smoking and lung cancer. The illustration modified from Catassi et. al., 2008 [22].

1.2.3 Nicotine Metabolism

Absorption of nicotine throughout the cell membrane relies on the pH property. If the pH is acidic, nicotine is ionized and does not easily pass through membranes [23]. In the lungs, nicotine is

rapidly captivated by the systemic circulation. The active form of nicotine is a cation, which is very similar to acetylcholine. It has been demonstrated that nicotine might compete and/or interferes with acetylcholine, which is the major neurotransmitter of the brain [26]. The binding of nicotine opens the ionic channel in a very few milliseconds and opening of these channels leads to a brief depolarization (Na^+ through the cell) [27]. Nicotine also activates the dopamine system within the brain. An expanded exposure of these receptors to nicotine decreases the effectiveness of dopamine by repressing the number of suitable receptors [27]. When nicotine concentration decreases in the brain, the receptors turn into active state from desensitized state. The re-activation of receptors increases neurotransmission to an unusual rate [28].

Nicotine is chiefly metabolized in the liver as well as in the lungs and the kidneys into many different metabolites (***Figure 1.3***). Quantitatively, cotinine (70-80% of nicotine converted to cotinine) and nicotine N'-oxide (NNO) (4-7% of nicotine converted to NNO) are the most important and primary metabolites of nicotine. They are the products hepatic oxidation of nicotine via cytochrome P-450 [29]. Cotinine is further metabolized to a number of metabolites including 3'-Hydroxycotinine (3HC), 3HC-glucuronide conjugate (3HC-Gluc), and cotinine N-oxide (CNO) [30]. Indeed, it appears that most of the reported urinary metabolites of nicotine are derived from cotinine [30]. NNO can also be reduced back to nicotine that will lead to recycling of nicotine in the body. Other than these metabolites of nicotine, about 3-5% of nicotine is converted to nicotine-glucuronide (Nicotine-Gluc) and excreted in urine [29]. Nornicotine is also shown as a metabolite of nicotine in human. It has been detected in smokers' urine and about 1% of nicotine is converted to nornicotine.

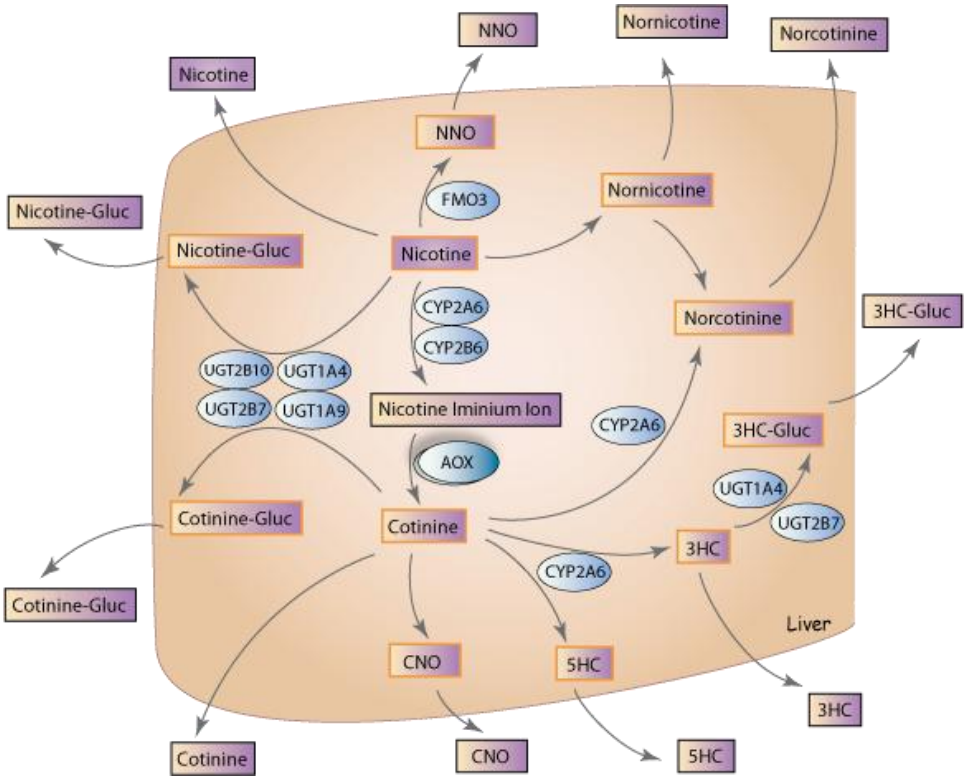


Figure 1.3: Illustration of nicotine metabolism and its primary metabolites in the human liver (www.pharmgkb.org).

1.2.4 Nicotine and Apoptosis

Nicotine has a very important role in deregulation of key and regulator genes in apoptosis events. It is shown by Mai *et al*. that 1μM nicotine may prevent cisplatin-induced apoptosis via phosphorylation of Bcl2 in human lung cancer cells [31]. Another similar study performed with oral cancer cells has shown parallel results [32]. Banerjee *et al*. also have shown that nicotine inhibits apoptosis triggered by nitric oxide induction in oral epithelial cells and they have proposed that inhibition of apoptosis may lead tobacco- induced oral cancer formation [33]. Nicotine may also deregulate apoptotic pathways via inducing phosphorylation of Bax through activation of AKT [34]. Besides Bax phosphorylation, it is shown that phosphorylation of Bad may inactivate its pro-apoptotic activity via 1μM nicotine exposure in A549 lung cancer cells thus increasing cell survival [35, 36]. NF-κB signaling pathway and nicotine may also have

important associations in terms of apoptosis inactivation. Zhang *et al*. has shown that nicotine prevents menadion-induced apoptosis in A549 lung cancer cells via activation of NF-κB [37]. Nicotine also suppresses opioid-induced apoptosis in human lung cancer cells [38]. According to another study nicotine alleviates oxidative stress, activates NF-kB and sensitizes cells to genotoxic/ xenobiotic stresses in HCT116 colon cancer cell line [39]. Furthermore, nicotine prevents apoptosis triggered by some apoptotic drugs like taxol and gemcitabin via increasing gene expression of apoptosis inhibitor XIAP (i.e. BIRC4) and Survivin (i.e. BIRC5) genes in non-small cell lung cancer (NSCLC) in a dose and time dependent manner [40]. Nicotine also weakens and decreases apoptosis rate triggered by serum starvation [41]. Besides these findings, nicotine unfavorably affects the cytotoxicity of DNA-damaging agents like cisplatin, UV, and gamma radiation in head and neck cancer cells. According to studies of Onoda *et al*. in these cells, nicotine does not affect the DNA damage repair mechanism but directly interferes with the signaling of the death pathway, reducing the signaling of the JNK1 pathway [42].

1.2.5 Nicotine and Cell Proliferation

Nicotine has an effective role in stimulation of cell proliferation and growth via many diverse mechanisms and signaling pathways. In one study, Ye *et al*. have shown that nicotine promotes cell proliferation in SW1116 colon cancer cell lines via phosphorylation of (epidermal growth factor receptor) EGFR and c-Src followed by an increased in 5-LOX (lipoxygenase) expression [43]. Besides, nicotine suppresses the growth inhibitory effects of trans-retinoic acid (RA) by inhibiting RA receptor-β (RARβ) expression throughout its induction of TR3 expression in lung cancer cells according to the results of Chen *et al* [44]. Shin *et al*. has also shown the promoting activity of nicotine on gastric cancer cell proliferation and vascularization through sequential gene activation of ERK/COX-2/VEGF signaling pathway [45]. Another interesting

study was performed with mouse epithelial cells. Long-term exposure of these cells to nicotine disorders cell cycle restriction machinery and cell cycle check points via targeting Ras pathway and induces Cyclin D1 [46]. Nicotine has also a role in AKT pathway. Nicotine and NNK activates Akt via α3/α4 or α7 nAChRs thus lessens etoposite-, UV- or H_2O_2- induced apoptosis through phosphorylation of GSK-3β, p70S6K, 4EBP-1, and FKHR [47]. Nicotine increases the proliferation of human small cell lung carcinoma SHP77 cells, indirectly increasing the expression levels of bombesin-like peptides [48]. Nicotine and its nitrosated carcinogenic derivative NNK actively bind to α7-nicotinic acetylcholine receptor (nAChRα7), and this activates MAPK through PKC/Raf-1, leading to the cell proliferation and finally development of lung cancer [49]. Nicotine also promotes gastric cancer via cyclooxygenase-2 (COX-2) /c-myc/ODC sequential activation [50]. Besides these, nicotine persuades cell proliferation of pancreatic cancer cell line AR42J via activating p-ERK1/2 in independent manner of its secretory response [51]. Also, rapid activation of ERK1/2 and Stat-3 by nicotine stimulates cell proliferation in bladder cancer [52]. Moreover, nicotine increases Rb-Raf1 complexes in human lung cancer cells lines via interaction with β-arrestin via nAChR α7 and depends on Src activity [53]. In one of the recent studies of Wong *et al*., it has been shown that β-adrenergic activation plays a regulatory role in the proliferation of colon cancer via promotion of nicotine [54]. They also imply that nicotine stimulates cell proliferation via nAChRα7 in human colon adenocarcinoma HT-29 cells [55]. Another recent study suggests that nicotine increases proliferation of lung cancer cells through induction of fibronectin and α5β1 integrins and these events refereed by nAChR-mediated signals that consist of ERK and PI3-K/mTOR pathways [56].

1.2.6 Nicotine and Microarray Studies

Saito *et al*. have shown that multiple MAPK signaling components, GABA receptors and protein phosphatases have been involved in nicotine-induced changes in brain [57]. Konu *et al*, have suggested that the genes belong to PKC, MAPK, NF-κB and ubiquitin/ proteasome signaling pathways are affected by nicotine via their microarray studies in rat brains [58]. Konu *et al*. also suggest via microarray studies with PC12 cells (derived from a pheochromocytoma of the rat adrenal medulla) that nicotine might lead to regulation of ribosomal and proteosomal protein subunits, many growth factors, and heat-shock proteins [59]. It is also worth noting that although there are such microarray studies performed with nicotine in neuronal cells, no microarray study exists for understanding the effects of nicotine on colon cancer cells.

1.3 Nicotinic Acetylcholine Receptors

1.3.1 General Information

Nicotinic acetylcholine receptors, also known as nAChRs or CHRNs, are cholinergic receptors that form ionotropic (i.e. ligand-gated) ion channels in the plasma membranes of definite neuronal cells. nAChRs are directly associated to an ion channel and do not activate a secondary messenger as some other receptor types [60]. nAChRs take their names from nicotine, agonist of cholinergic receptors as they can also be triggered by nicotine besides acetylcholine ligand [61]. Although nAChRs are mainly found in the central nervous system and the peripheral nervous system [60], current research show the expression of nAChRs in non-neuronal tissues [62].

1.3.2 Structure of nAChRs

nAChRs, with a molecular mass of 290 kDa, form hetero-pentamers on the cell surface and they settle symmetrically around a central pore [60, 61, 63] ***(Figure 1.4)***. These receptors are clustered into two subtypes (muscle and neuronal type) according to their primary sites of expression. The muscle types are found in neuromuscular connections and they are either the embryonic form, composed of α1, β1, δ, and γ subunits in a 2:1:1:1 ratio, or the adult form composed of α1, β1, δ, and ε subunits in a 2:1:1:1 ratio [60, 61, 64]. The neuronal subtypes have diverse homomeric or heteromeric pentamers combinations of twelve different nicotinic receptor subunits: α2 -α10 and β2 -β4 like $(\alpha4)_3(\beta2)_2$ [60]. In both of the subtypes, the subunits are similar to each other in terms of hydrophobic regions. The neuronal subunits differ from the muscle ones in one way they are not responsive to α-bungarotoxin [60].

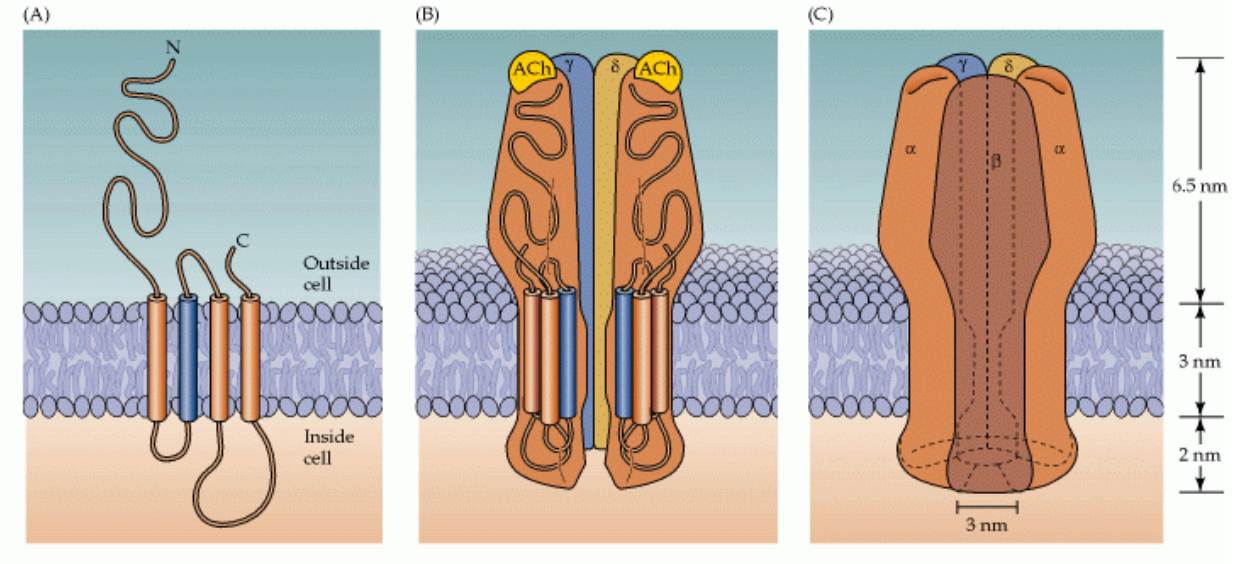


Figure 1.4: The structure of the nAChR receptor/channel. (A) Each receptor subunit crosses the membrane four times. The membrane-spanning domain that shapes the pore is shown in blue. (B) Five such subunits come together to create a pentamer structure containing twenty trans-membrane domains that surround a central pore. (C) The openings at either end of the channel are very large - ca. 3 nm in diameter; yet the narrowest area of the pore is approximately 0.6 nm in diameter (in comparison, the diameter of Na^+ or K^+ is less than 0.3 nm.) [60].

1.3.3 Function of nAChRs

1.3.3.1 General Functions of Nicotinic Acetylcholine Receptors

The existence of the loops on the extracellular regions of nAChRs preserves the binding site for the neuro-transmitter acetylcholine (ACh) which is the physiologically agonist of all nAChRs subunits [65, 66]. Long-exposure to acetylcholine might lead to stabilization of the receptor causing desensitization of the receptor [67, 68]. Aside from the agonistic or antagonistic binding site, diverse interaction sites are present on nAChRs for both non-competitive inhibitors and allosteric modulators [69].

1.3.3.2 Nicotine and Nicotinic Acetylcholine Receptors

Similar to ACh interactions with the receptor complex, nicotine binds as an agonist to the α subunits of nAChRs [70] ***(Figure 1.5)***. Nicotine interacts with nAChRα4β2 in higher affinity than it does with the nAChRα7 [71]. Interestingly, chronic nicotine exposure causes desensitization of nAChRα4β2, but not of nAChRα7 [72]. Desensitization is short-lived and removal of nicotine exposure re-establishes receptor affinity [73].

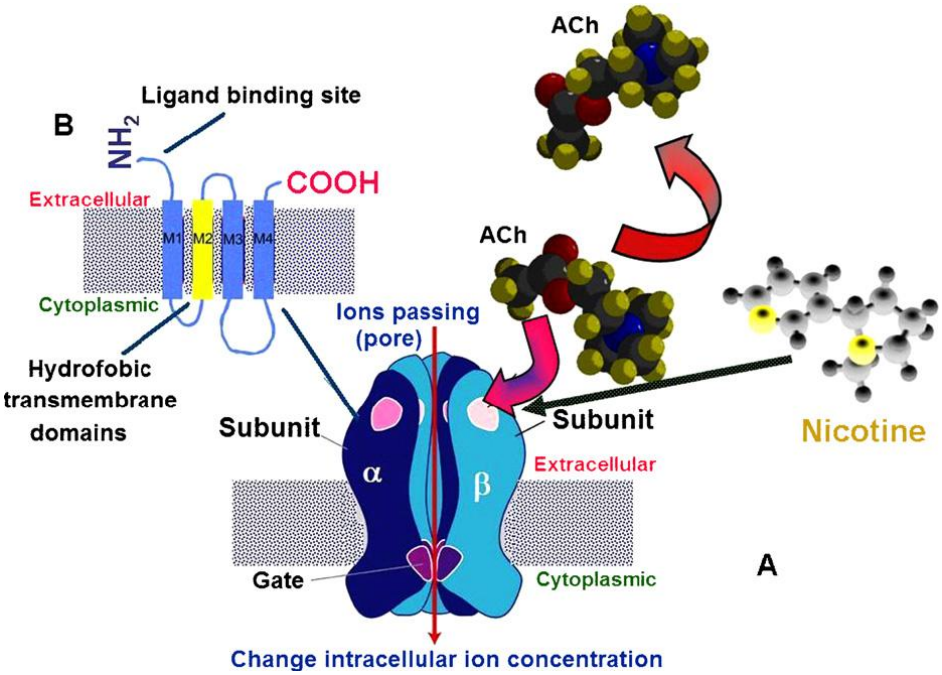


Figure 1.5: Nicotine and acetylcholine interaction with nAChRs. A) Depiction of nicotine and acetylcholine interaction with nicotinic receptor subunits (nAChR). B) Schematic representation of nAChRs structure [22].

Nicotine-receptor interaction guides a conformational change in the receptor and releases the gate on the intracellular side of the ion channel in the plasma membrane. Ion influx into the cell causes membrane depolarization [74, 75]. Following this initial depolarization voltage-activated Ca^{2+} channels gates open, resulting in an extra Ca^{2+} influx to the cells, which in turn leading to deregulation of Ca^{2+} activated signaling cascades , and release of neurotransmitters. Accordingly, activation of nACHRs might have a very critical role in cell division, self-renewal, differentiation, proliferation, senescence, apoptosis, and migration [76, 77].

CHAPTER 2. OBJECTIVES AND RATIONALE

Colon cancer is one of the leading causes of death in the world and early diagnosis provides better treatment opportunities. Nicotine, an addictive and modulatory molecule in tobacco has been associated with lung cancer initiation and progress yet the impact of nicotine-driven signaling on colorectal cancer cells have not been studied in detail and/or at the transcriptional profile level. Furthermore, it is apparent that serum levels in cell culture systems reflect changes in growth factors, and serum starvation models help us understand the cellular proliferation and apoptosis mechanisms in cancer cells. Previous studies are focused on nicotine's effects in systems with serum *ad libitum*, however, there is indications that serum levels modify nicotine's effects [46]. Moreover recent studies have shown that nicotine has a critical role in cell cycle proliferation, in apoptosis and cell invasion, yet mostly in lung cancers [40, 53, 78]. But there is no microarray study, which may help understanding the nicotinic signaling pathways in cancer cells of colon. Therefore, the aim of this study is to understand serum-dependent and –independent as well as dose- and time-specific actions of nicotine on colon cancer using molecular and biochemical methods as well as whole genome transcriptome profiling.

The following questions were asked in this study:

1) At what dose and time period does nicotine show its effect for cell proliferation? This question was addressed primarily by using MTT cell viability assay.
2) Do proliferative or apoptotic effects of nicotine differ with respect to levels of serum in cell growth media? This question was addressed by the following approaches a) MTT assays for cell proliferation b) Determination of cell cycle and apoptotic genes expression at protein levels c) Comparing the expression profiles of the nicotine treated cells with or without serum.

3) What cellular signaling pathways are involved in nicotine's serum-dependent effects? This question was addressed by using gene lists obtained by gene set-enrichment analysis and network visualization methods.

CHAPTER 3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 General chemicals

All laboratory chemicals were supplied by Sigma-Aldrich (St. Louis, MO, U.S.A), Farmitalia Carlo Erba (Milano, Italy) and Merck (Schucdarf, Germany), except Ethanol and methanol were from Riedel-de Haën (Germany). Agarose was obtained from Gibco (Carlsbad, CA, USA).

3.1.2 RNA Isolation, PCR and cDNA synthesis kits

The RNA isolation was performed using Promega RNA isolation kit, (Madison, USA; Z3100). RevertAid first strand cDNA synthesis kit (MBI Fermentas) and qRT-PCR kit, SYBR-Green (Finnzymes), were used to obtain cDNA and to amplify cDNA, respectively.

3.1.3 Oligonucleotides: The oligonucleotides used in quantitative Reverse Transcriptase-polymerase chain reaction (qRT-PCR) were obtained from Iontek Inc. (Istanbul, Turkey). The oligo sequences of the primers were shown on ***Table 3.1***.

Table 3.1: The sequences of primers used for microarray verification experiment via qRT-PCR (F: Forward primer, R: Reverse primer)

Primer ID	**Sequence (5' → 3')**	**Product Length (bp)**	**Temperature(^{o}C)**
XIAP (F):	TCACTTGAGGTTCTGGTTGC	113	60
XIAP (R)	CGCCTTAGCTGCTCTTCAGT		
SURVIVIN (F)	GTTGCGCTTTCCTTTCTGTC	141	60
SURVIVIN (R)	TCTCCGCAGTTTCCTCAAAT		

3.1.4 Western Blotting materials

3.1.4.1 Kits and reagents: ECL plus western blotting reagent was supplied from Amersham (UK; RPN2132) and SuperSignal West Femto reagents was obtained from Perbio Perbio (UK; 34095). Protein size marker was obtained from MBI Fermantas (PageRuler Prestained, Cat No: SM0671).

3.1.4.2 Antibodies: Calnexin primary antibody (CL731, Sigma) and anti-mouse HRP conjugated secondary antibody (A0168, Sigma) were kindly provided by Mehmet Ozturk group. Pro-apoptotic gene Bax primary antibody (SC-529, Santa Cruz) and cell-cycle protein antibody (Cyclin E (SC-481, Santa Cruz) were kindly provided by K. Can Akcali Group. Anti-rabbit HRP conjugated secondary antibody was obtained from Santa Cruz (USA). The primary antibodies were diluted into 1:200 to 1:5000 concentrations, while the secondary antibodies were diluted in blocking solution (1:5000, as recommended by the supplier).

3.1.4.3 Protein transfer materials: Immobilen P transfer (PVDF; Roche; Germany), and 3mm Whatman filter paper (Whatman International Ltd.; Madison, USA) were used for protein transfer.

3.1.5 Electrophoresis, photography, spectrophotometer and autoradiography: The films (KODAK; Rochester, USA) were developed using Hyperprocessor (Amsderdam, UK). The power supply Power-PAC300 and Power-PAC200 were from Bio Rad Laboratories (CA, USA). Beckman Spectrophotometer Du640 (CA, USA) and Nanodrop ND-1000 Full-spectrum UV/Vis Spectrophotometer purchased from Thermo Fisher Scientific (Wilmington, DE, USA) were used for protein visualization, gel electrophoresis and nucleic acid measurements, respectively.

3.1.6 Tissue culture reagents and cell lines: Dulbecco's modified Eagle's Medium (DMEM), and trypsin were obtained from Biochrom (UK); fetal bovine serum and penicillin/streptomycin were supplied from HyClone (Logan, USA). SW620 Colon Adenocarcinoma cells (ATCC; CCL-227) were kindly provided by Assist. Prof. Dr. Sreeparna Banerjee, METU.

3.1.7 Microarrays: HG-U133 plus 2 microarray chips and reagents were supplied from Affymetrix (USA).

3.1.8 Nicotine: Liquid nicotine was supplied from Sigma (USA; 54-11-5).

3.1.9 Cell proliferation experiment kits and reagents: Vybrant MTT cell proliferation kit was obtained from Invitrogen (V13154, USA)**.**

3.2 SOLUTIONS and MEDIA

3.2.1 General solutions

50X Tris-acetic acid-EDTA (TAE): 2 M Tris-acetate, 50 mM EDTA pH 8.5. Diluted to 1X for working solution.

10X PBS: 80 g NaCl, 2 g KCl, 14.4 g Na2HPO4 and 2.4 g KH2PO4 were dissolved in 800 ml dH2O and adjusted the pH 7.4 with HCl. ddH2O was added to complete the final volume 1 liter.

2 M NaCl: 58.44 gr NaCl in 500 ml ddH2O

10% SDS: 100gr SDS was dissolved n 900 ml ddH2O and heated to 68 0C to assist dissolution. Then, pH was adjusted to 7.2 and final volume was completed to 1lt by adding ddH2O.

3.2.2 Cell culture solutions:

Complete media: DMEM was supplemented with 10% fetal bovine serum and 1% penicillin /streptomycin mixture and stored at 4^{o}C.

Serum starvation media: DMEM was supplemented with 0.1% fetal bovine serum, 1% penicillin /streptomycin mixture and stored at 4^{o}C.

Cell freezing solution: 5% DMSO and 95% complete media were mixed.

1XPBS: 10X PBS was diluted to 1X PBS solution with dd-water and adjusted pH at 7.4. Then, it was autoclaved and filtered before use.

Media with nicotine: 2 µl Nicotine was dissolved in 1248 µl serum starved tissue culture medium to make 10mM Nicotine stock solution, prepared freshly. 100nM, 1µM, and 10 µM nicotine

solutions were prepared via serial dilution from 10mM Nicotine stock solution. All work was performed in dark and in a fume cupboard.

3.2.3 SDS (Sodium Deodecyl Sulfate)-PAGE (Polyacrylamide Gel Electrophoresis) solutions:

30% Acrylamide mix (1:29): *Per 100 ml*: 29 g acrylamide, 1 g bisacrylamide in double-distilled water, filtered, degassed, and stored at 4°C (stock solution). 5X SDS gel-loading buffer 3.8 ml double-distilled water, 1 ml of 0.5 M Tris-HCl, 0.8 ml glycerol, 1.6 ml of 10% SDS, 0.4 ml of 0.05% bromophenol-blue. Before use, ß-mercaptoethanol was freshly added to a final concentration of 5% to reach 1% when mixed with protein samples.

10% Ammonium persulfate (APS): 0.1 g/ml solution in double distilled water (Prepared freshly).

1.5 M Tris-HCl, pH 8.8: 54.45 g Tris base (18.15 g/100 ml) ~150 ml distilled water. Adjust to pH 8.8 with 1 N HCl. Completed to 300 ml with distilled water and stored at 4° C.

1 M Tris-HCl, pH 6.8: 12.14 g Tris base ~ 60 ml distilled water. Adjust to pH 6.8 with 1 N HCl. Completed to 100 ml with distilled water and store at 4° C.

3.2.4 Western-blotting solutions:

Semi-dry transfer buffer *per liter*: 2.5 gr Glycine, 5.8 gr Tris base, 3.7 ml 10% SDS, 200 ml MeOH were dissolved in ddH2O to a final volume of 1 lt.

10X Tris-buffer saline (TBS) *Per liter:* 100 mM Tris-base, 1.5 M NaCl, pH 7.6 in double distilled water.

TBS-Tween (TBS-T): 0.5% Tween-20 solution in TBS. (Prepared freshly)

Blocking solution: 5% (w/v) non-fat milk, 0.5% Tween-20 in TBS. (Prepared freshly).

NP40 lysis buffer: 150 mM NaCl, 50 mM Tris.Cl at pH 8.0, 1% NP40 and 1X protease inhibitor mix wered mixed in dd H2O.

Bradford working solution: 10 mg Coomassie brilliant blue was dissolved in 5 ml 95% ethanol and 10 ml 85% phosphoric acid completed to a final volume of 1 lt. with ddH_2O. Then, the solutions were filtered by using whatman paper and stored at +4 ^oC in dark.

2X protein loading buffer: 50 Mm Tris. HCl at pH 6.8, 2 Mm EDTA at pH 6.8, 1% SDS, 10% glycerol and 0.02% Bromophenol blue solutions.

5X Running Buffer: 15 gr Tris.base, 72 gr Glycine and 5 gr SDS were dissolved in ddH2O to a final volume of 1 lt.

Resolving Gel Solution: 12% polyacrylamide gel (15 ml) was prepared:

4.9 ml of dH_2O, 6.0 ml of 30% Acrylamide mix, 3.8 ml of 1.5 M Tris, pH=8.8, 150 µl of 10% SDS, 150 µl of 10% APS, and 6 µl of TEMED.

Stacking Gel Solution: 5% polyacrylamide gel (8 ml) was prepared:

5.5ml of dH_20, 1.3 ml of 30% Acrylamide mix, 1 ml of 1.0 M Tris, pH=6.8, 80 µl of 10% SDS, 80 µl of 10% APS, and 8 ml of TEMED.

3.3 METHODS

3.3.1 Cell culture techniques:

3.3.1.1 Cell lines: SW620 Colon Adenocarcinoma cell line (ATCC; CCL-227) is a lymph node derived metastatic colon adenocarcinoma. They were originally isolated from the tissue of a 51-year-old Caucasian male (blood group A, Rh+) (www.lgcstandards-atcc.org). They are epithelial cells and strongly adherent to the plate.

3.3.1.2 Growth conditions of the cell lines: SW620 cells were grown up in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin mixture. The cells were incubated in the 37^oC incubator with 5% CO_2. The cells were seeded to new flasks (or plates) in 1:3- 1:5 ratio, every 2-4 days before reaching 90% confluence. The complete media were aspirated and cells were washed with 1X PBS for two times. Trypsin/EDTA solution was added to the flask to force cells to detach from the surfaces. The cells were dispersed by pipetting with the fresh media. Then, the cells were transferred to new flasks after counting with a hematocytometer. One day later, the media were replaced with fresh media. Media and 1X PBS were kept at +4^oC,

trypsin/EDTA was kept at -20^{o}C. All cell culture solutions were heated to 37^{o}C and equipment was cleaned with 70% ethanol before use.

3.3.1.3 Cryopreservation of cell lines: Cells were harvested by using trypsin/EDTA and neutralized by adding fresh growth medium. The cells were precipitated by centrifuge at 1200 rpm for 3 minutes. The media/trypsin mixture was aspirated and the cells were resuspended in freezing media containing 5% DMSO in complete medium; resuspended cells in the freezing media were transferred into cryotubes; stored at -80^{o}C overnight and then transferred to the liquid nitrogen tank for long-term storage.

3.3.1.4 Thawing of the frozen cell lines: The cells were thawed rapidly at 37^{o}C and mixed with 3 ml complete growth medium; centrifuged at 1200 rpm for 3 minutes and precipitated. After removal of supernatant via aspiration, the cells were resuspended with fresh growth medium and transferred in 75-t flasks to 37^{o}C.

3.3.2 Nicotine treatment of the cells

3.3.2.1 Time and dose-dependent nicotine treatments: Cells were harvested as described in section *3.3.1.2.* Harvested cells were counted under the light microscope with hematocytometers. For 48 hours treatment, 10,000 cells/ well; for 72 hours 5000 cells/well; for 5 and 7 days 3000 cells/well; for 15 days 1000 cells/well were seeded into the 96 well-plates. The cells were incubated for 24 h to attach the surface of the plate. After 24 h, the medium was replaced with the starvation medium to induce quiescence. After 24 h quiescence, the cells were treated with different levels of serum with or without different nicotine concentrations (cells Seeded → 24h → 0.1% FBS treatment for quiescence → 24h → cells treated with different conditions for 48h or 7 days). To determine the optimum dose, a nicotine dose curve was applied using 100ηM, 1μM and 10μM concentrations of freshly prepared nicotine (in the media). Nicotine concentrations were prepared in 10% FBS containing complete media or 0.1% FBS containing serum-deprived media.

After obtaining the quiescent cells, freshly prepared nicotine including media was applied to the cells for 48 and 72 hours experiments. For 5, 7 and 15 days experiments; first cells were incubated in the nicotine media for 3 days and then the media were changed with freshly prepared media with nicotine. The media were changed in between every 3 days.

3.3.2.2 Nicotine treatment to the cells for RNA and protein extraction: The exponentially growing cells were harvested and counted. 1x 10^6 cells were seeded to the 75 cm^2 flasks in complete media and were incubated for 24 h for attachment to the plate. 24 hours later, complete media was replaced with the 0.1% FBS containing media for inducing quiescence. After another 24h, the media was replaced with the nicotine containing media (four conditions: complete media-control, 1 μM nicotine in complete media, 0.1% FBS media, and 1μM nicotine in 0.1% FBS containing media) and cells were incubated in the treatment medium for 48 h. Then, the cells were harvested via scrapper in the ice-cold 1X PBS. Later, cells were centrifuged at 1200 rpm for 3 min at 4 ^oC and the cell pellets were subjected to liquid nitrogen for snap-frozen. Then the pellets were stored at -80^oC for further steps. Each condition had three biological replicas. The protein assays were performed only for two biological replicas.

3.3.3 MTT cell viability assay: Vybrant MTT cell proliferation kit was used to see the cell proliferation rates of SW620 cells under different nicotine concentration and the results were obtained by ELISA reader. Firstly, MTT-powder was suspended in ice-cold 1 ml of 1X PBS and solution was kept in the dark. Later, the media including nicotine (or control media) were removed. 100 μl of medium without serum was added to cells in each well and two wells were kept for empty (for blank reading). Next, 10 μl of MTT solution was added to the each well. The cells were incubated for 4 h at 37^oC. Then, the media with MTT solution was removed and 100 μl ice-cold DMSO was added to the wells and mixed by pipetting. After 10 min incubation, the plates were read in Elisa-reader at 540 nm wavelength, with 450 nm reference wavelength. During analysis, blank wells were used for background reading and calculation. In MTT cell proliferation assay, the

significance of each condition's effect was compared with the control using ANOVA followed by Fisher's multiple comparison tests.

3.3.4 Microarray analyses:

3.3.4.1 Obtaining raw data: Four different conditions (Complete media control (1 replicate), 1μM nicotine-treated complete media (1 replicate), 0.1% FBS containing media control (2 replicates, and 1μM nicotine-treated 0.1% FBS containing media (2 replicates) were used for microarray experiments. The samples were prepared and hybridized on Affymetrix U133 plus 2 GeneChips. 5μg RNA sample was used for each experiment (amplification/labeling/hybridization). Amplification, labeling and hybridizations were performed at the Genomics Core Facility of Bilkent University by the facility technician under the supervision Assoc. Prof. Dr. Işık Yuluğ according to manufacturer's protocols. Affymetrix analysis software GCOS was used to perform the preliminary probe-level quantification of the microarray data.

3.3.4.2 Data normalization: The data were further normalized using the justRMA normalization method by using the BrbArray Tools, 3.7 Version (http://linus.nci.nih.gov/BRB-ArrayTools.html). The default option of RMA (with background correction, quantile normalization, and log transformation) was used to generate the normalized intensity for each probeset. The quality of microarray sets were tested via BRBArray Tools, Affy Data Quality Control utility (***Figure 4.3***).

3.3.4.3 Determination of differentially expressed genes: Identification of differentially expressed genes was determined by BRB-Array-Tools using Class Comparison utility. The Geneset enrichment analysis with KEGG option (BrbArrayTools > Class Comparison > Between Group of Arrays) was performed with a threshold p-value <= 0.05. The differentially expressed genes were selected for the following conditions: Effects of nicotine on SW620 cells grown in 0.1% FBS supplemented media in the presence or absence of nicotine. Effects of serum starvation

on SW620 cells was identified by subtracting 0.1% FBS treated cell expression profile from 10% FBS-treated cell expression profile (single replicate fold change).

3.3.4.4 Gene Ontology (GO) analyses: After determination of the differentially expressed genes, the GO ontology analysis was performed in order to see the effect of nicotine on the biological functions and determine which biological functions were altered via nicotine and what kind of genes were differentially expressed under nicotine exposure conditions. To perform the GO ontology analysis, Webgstalt (WEB-based GEne SeT AnaLysis Toolkit) were used (bioinfo.vanderbilt.edu/webgestalt). When performing the gene ontology analyses, hyper-geometric test were used with a p-value<0.05 and two genes were selected as a minimum number of genes. Level 4 and 5 were chosen for the biological level and molecular functions' graphics. If the groups were significant according to selected criteria, the functions were written in red color. The graphs are shown in the ***Appendix A***.

3.3.4.5 Cluster analysis: Cluster 3.0 software together with Java TreeView software were used to hierarchically cluster the targeted genes according to different conditions of our microarray analyses. Upon median gene centering average linkage method of hierarchical clustering process was performed. During this process, both gene and array clustering were done, uncentered correlation was calculated to find the similarity.

3.3.4.6 Pathway Miner analysis: It is important to observe the interactions between genes and/or proteins to understand the biological system in a holistic manner. Pathway Miner is one of the freeware programs that allow for visualization of genes that have roles in the same pathway thus each node represents a gene and each edge represents a co-occurrence within a cellular signaling or metabolic pathway (http://www.biorag.org). Differentially expressed gene lists obtained from different comparisons were filtered based on fold-change and consistency between experiments criteria and visualized using Pathway Miner.

3.3.5 Microarray Verification- RNA experiments

3.3.5.1 Total RNA isolation: Total RNA isolation was performed directly by using Promega SV Total RNA isolation kit according to the manufacturer's instructions. The RNA was eluted in a total volume of 50 µl RNAse free-H_2O. The concentration of the isolated RNA and the ratio of absorbance at 260 nm/280 nm were measured with the Nanodrop ND-1000 spectrophotometer. Isolated RNA samples were stored at -80^oC. For microarray analyses, 5µg/8µl concentration of RNAs were prepared. The integrity of isolated RNA samples for microarray chips was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The RNA integrity number (RIN) values and RNA concentrations were shown on ***Table 4.1.***

3.3.5.2 cDNA synthesis: For qRT-PCR experiments 2µg RNA used to get cDNAs. RevertAid first strand cDNA synthesis kit was used for cDNA production according to the manufacturer's instructions.

3.3.5.3 Quantitative real time RT-PCR experiments: For qRT-PCR mastermix, 10µl SYBR-Green qRT-PCR kit, 8 µl dd-H_2O, 0.5 µl 20 pmol forward primer, 0.5 µl 20 pmol reverse primer and 1 µl cDNA sample were put into RT-PCR plates. 10µl of mineral-oil used to cover the surface of the mixture. Then the plate was sealed with the plastic wrap and was put into the iCycler analyzer.

3.3.5.4. Quantification of relative expression with respect to GAPDH: Threshold cycles were determined using iCycler visualization tools for target and GAPDH gene expression curves. Unmodified $\Delta\Delta C_T$ method was used by using 2 as the efficiency. The formula of $\Delta\Delta C_T$ method is: $\Delta\Delta C_T$= (Avg. $\Delta C_{T,\,target}$ - Avg. $\Delta C_{T,GAPDH}$), where ΔC_T = (Avg. $C_{T,target}$ - Avg. $C_{T,GAPDH}$) [79].

3.3.6 Protein preparation and Western Blot experiments:

3.3.6.1 Protein extraction: The cell pellet was lysated in the NP40 lysis buffer (explained in *3.2.4*) and vortexed at every 5 min for half an hour and kept on ice during this period. Later lysates

were centrifuged at 13000 rpm for 30 min at +4^{o}C, the supernatant, which contains the proteins, was collected and stored at -80^{o}C for quantification by Bradford assay and Western blot experiments.

3.3.6.2 Quantification of protein concentrations: The concentration of cell lysed was detected by Bradford assay. 2 µl of protein samples were mixed with 98 µl of ddH2O and then 900 µl of Bradford working solution was added to the sample to finalize the volume to 1 ml. For the blank, 2 µl of NP40-lysis buffer was added to the 98 µl of ddH2O and then 900 µl of Bradford working solution was added to this mix. After 5 min of incubation, the protein concentrations were measured at OD_{595} λm versus blank solution. In order to calculate the protein concentrations, a standard curve was generated by using different concentrations of BSA protein. 1 mg BSA was dissolved in 1 ml of ddH2O to make 1mg/ml BSA concentration. Then, 0, 1, 2, 4, 8, 16, and 32 µl of BSA added to 100, 99, 98, 96, 92, 84 and 68 µl of ddH2O, respectively and 900 µl of Bradford working solution was put into all of these samples. After 5 min of incubation, the OD_{595} λm values of each samples were measured and the standard curve was prepared with these values. The unknown concentrations of the samples were calculated by using this from the standard curve. The concentration data of the protein samples are given in ***Table 4.3.***

3.3.6.3 Western blotting:

3.3.6.3.1 SDS polyacrylamide gel electrophoresis:

The gel apparatus was set and resolving and stacking gel solutions (explained at *3.2.4 part*) were loaded. Equal amounts (50 µg/well) of proteins were mixed with 6X loading dye containing 1% β-mercaptoethanol and boiled in the water for 5 min to denature the proteins and break S-S bonds. Then, samples and Fermentas PageRuler Protein size marker were loaded on gel. Gels were run at 80 V during until the samples leave the stacking gel and 120 V when the samples were run at resolving gel in the 1X running buffer. The run was stopped just after loading dye leaves the gel.

3.3.6.3.2 Protein transfer to PVDF Membrane: Since all of our targeted proteins were smaller than 120 kDa, semi-dry transfer was performed. Whatman papers and PVDF membranes were cut in suitable sizes with gels. The membranes were incubated into 100% methanol for 30 seconds and then, immediately merged into ddH_2O for 2 min. Later, whatman papers and membrane were incubated into the semi-dry transfer buffer for 5 min. Then 2 whatman papers, PVDF membrane, gel, and another 2 whatman papers were put on top of each other into the tank. Then the system was set to 160mA and the proteins were run for 30 min.

3.3.6.3.3 Immunological detection of membrane transferred proteins: The membrane was incubated with 5% blocking solution for an hour on a slowly rotating platform to get rid of non-specific bindings. After blocking, the membrane was washed with 0.1% TBS-T three times for 5 min. The membrane was incubated overnight with primary antibody at $+4^{o}C$ on a slowly rotating platform, or alternatively incubated at room temperature for 1 hour. After, the membrane was washed with 0.1% TBS-T three times for 5 min, the membrane was incubated with HRP-conjugated secondary antibody together with the primary for an hour at room temperature on a slowly rotating platform. Then, the membrane was washed three times with 0.1% TBS-T for 5 min. Then, the membrane was treated with ECL plus or FEMTO reagents for 5 min, according to the manufacturer's instructions, and was wrapped with stretch film. The autoradiography was performed for various exposure times to obtain the different signal intensities of the labeled proteins.

CHAPTER 4. RESULTS

4.1 Determination of optimum time and dose of nicotine treatment

4.1.1 Nicotine treatment of the SW620 cells and its effect on cell proliferation

The effect of different nicotine concentrations (10ηM, 100ηM, 1μM, and 10μM) on SW620 cell proliferation activity was tested to determine the dose of nicotine and suitable time (48h and 7 days) course of the treatment while cells the cells were grown in 10% FBS or 0.1% FBS containing culture media. MTT cell proliferation assay was used to test for cell proliferation activity. Our findings indicated that SW620 cells were responsive to 0.1% FBS serum-starvation at 48 hours and 7 days by a reduction of 67% and ca. 99.9%, respectively when compared with physiological serum level (***Figure 4.1*** and ***Figure 4.2***). Nicotine showed a different effect at 0.1% and 10% FBS serum levels (Two way ANOVA; P-value $_{(serum)}$ <0.001; P-value $_{(dose)}$ = 0.105). One-Way ANOVA results demonstrated that nicotine at concentrations starting from 10ηM to 10μM consistently rescued the loss of cell proliferative activity while at 1μM concentration nicotine exhibited the highest rescue power (***Figure 4.1B***) under serum-starved conditions at 48 h. No effect of nicotine was observed under physiological serum levels (10% FBS) (***Figure 4.1A***). Similarly, cell proliferation activity differed significantly between the cells grown in 0.1% FBS and 10% FBS at day 7 (Two way ANOVA; P-value $_{(serum)}$ <0.001; P-value $_{(dose)}$ = 0.922). Interestingly, nicotine at 1μM significantly inhibited the cell proliferative activity when compared with control at 10% FBS-treated cells, while other doses of nicotine did not affect the cell proliferation ratio extensively at day 7 (***Figure 4.2A***). On the other hand, One-Way ANOVA implicated significant induction of cell proliferation in response to all doses of nicotine while

100ηM nicotine treated cells exhibited a more significant response (***Figure 4.2B***) under serum starved conditions.

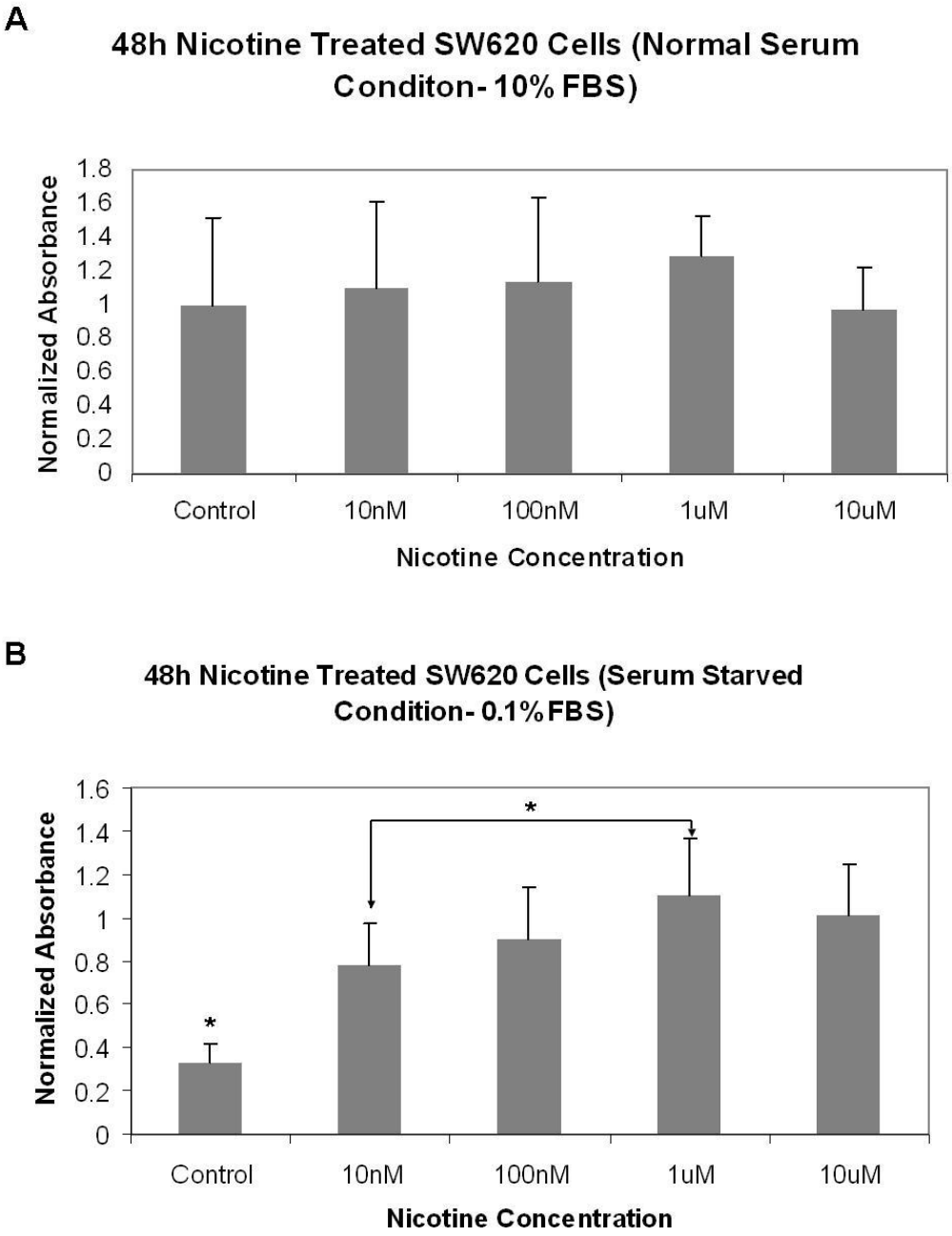
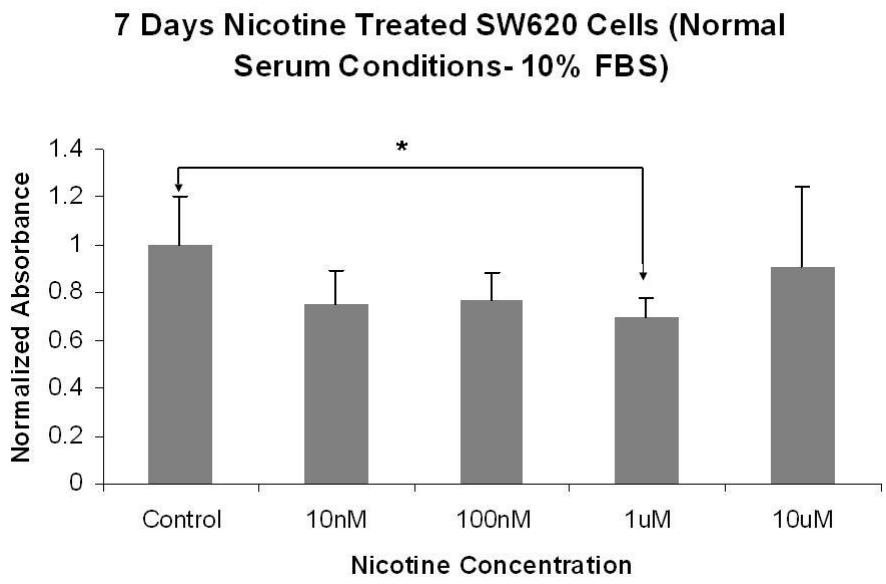


Figure 4.1: MTT cell proliferation results for 48h nicotine treated quiescent SW620 colon adenocarcinoma cells. **A)** MTT cell proliferation results for 48h nicotine treated quiescent SW620 colon adenocarcinoma cells under normal (10% FBS in media) growth conditions. One-Way ANOVA were performed to indicate the significance level (F-value = 0.36, DF = 4, P-value =

0.835). **B)** MTT cell proliferation results for 48 h nicotine-treated quiescent SW620 colon adenocarcinoma cells under serum deprived (0.1% FBS in media) conditions. One-Way ANOVA findings followed by multiple comparisons were indicated with asterisks (F-value = 8.05, DF = 4, P-value = 0.001). Asterisk represents the significantly different sample from other samples. (Asterisk with arrows indicates that only samples that were shown by arrows are significantly different from each other.)

A



B

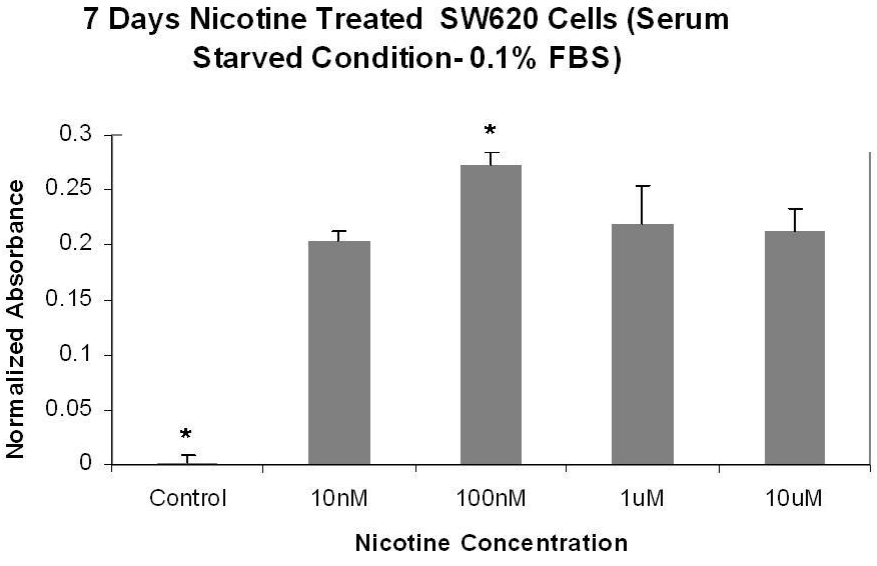


Figure 4.2: MTT cell proliferation results for 7 days nicotine treated quiescent SW620 colon adenocarcinoma cells. **A)** MTT cell proliferation results for 7 days nicotine treated quiescent SW620 colon adenocarcinoma cells under normal (10% FBS in media) serum conditions. (One-Way ANOVA; F-value =1.58, DF = 4, P-value = 0.230). **B)** MTT cell proliferation results for 7 days nicotine treated quiescent SW620 colon adenocarcinoma cells under serum deprived (0.1% FBS in media) conditions (One-Way ANOVA; F-value = 89.20, DF = 4, P-value < 0.001). (Asterisk represents the significantly different sample from other samples. Asterisk with arrows indicates that only samples that were shown by arrows are significantly different from each other.)

4.2 Microarray Experiments

4.2.1 Quality Control of RNA samples and microarray chips

4.2.1.1 Pre-hybridization quality control of the RNA samples

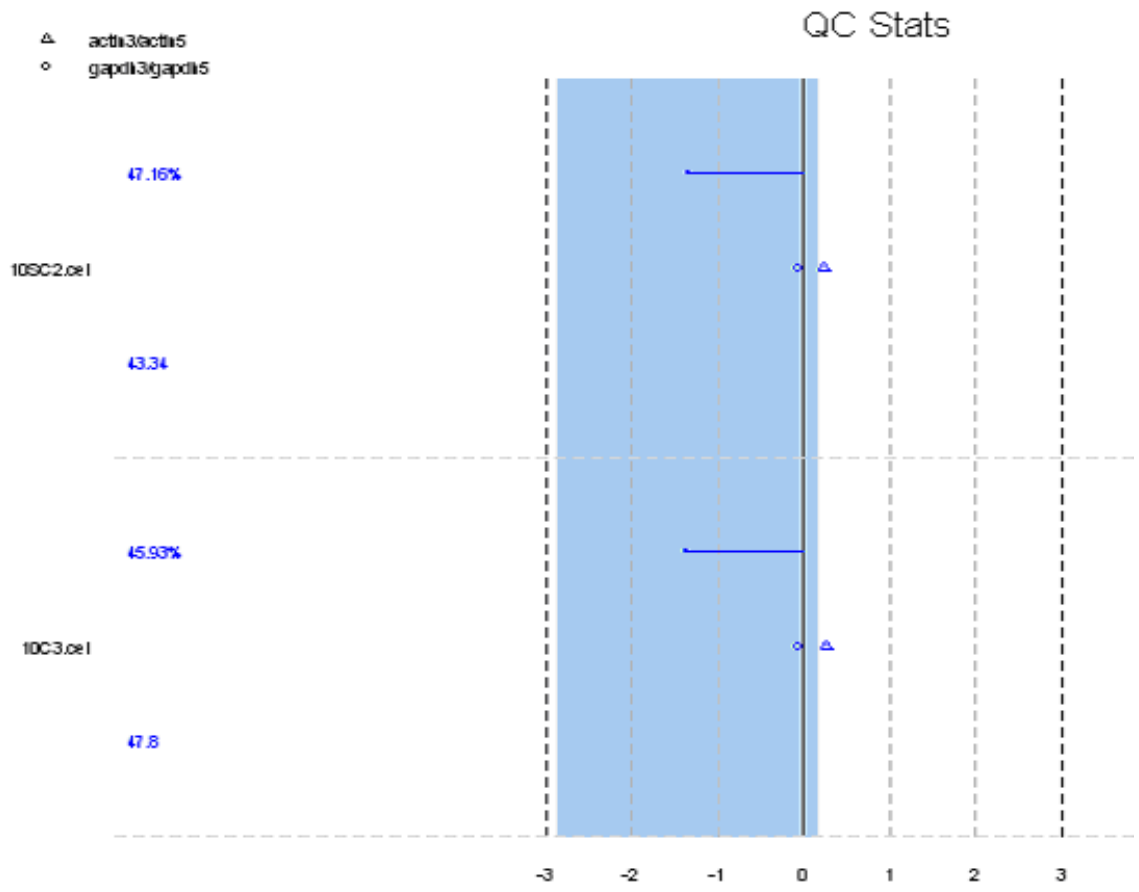
To investigate the acute effect of nicotine on the cells, 48 hour treatment time and 1μM nicotine dose were used for the microarray experiments to determine the gene expression profile of the cells. Accordingly, the RNA samples from the cells grown in 0.1% and 10% FBS containing media with or without 1μM nicotine addition were extracted as described in the Materials and Methods section (***Table 4.1***). Initially, RNA sample quality and quantity analyses were performed with Agilent 2000 Bioanalyzer and Nanodrop. The results showed that we have high quality RNA samples (***Table 4.1***).

4.2.1.2 Post-hybridization quality control of microarray results

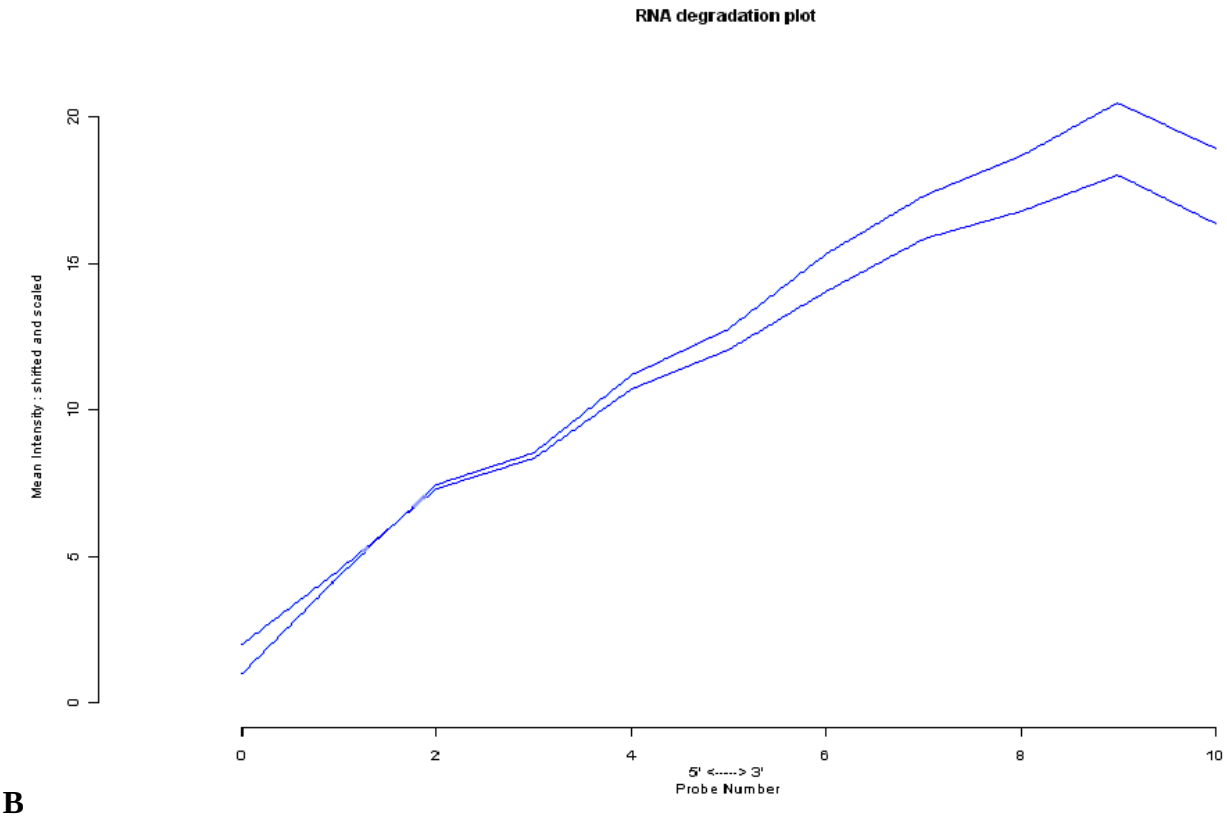
Post-hybridization quality control analyses also showed that all samples had similar degradation slopes (***Figure 4.3***) and comparable background, 5'3'-GAPDH and ACTIN values as well as scaling factors (***Table 4.2***).

Table 4.1: The list of experimental conditions of the SW620 cells and the concentration and RIN values of the RNA samples that were prepared for microarray experiments.

Time Course	Dosage	Serum	Biological Replicate	Concentration (ηg/μl)	260/280 value	RIN value
48h	1μM Nicotine	0.1% FBS	Set 2	1913.39	2.12	8
48h	No Treatment	0.1% FBS	Set 2	1039.20	2.07	8.2
48h	1μM Nicotine	0.1% FBS	Set 3	1274.42	2.1	8.4
48h	1μM Nicotine	10% FBS	Set 3	1168.4	2.09	9.6
48h	No Treatment	0.1% FBS	Set 3	668.34	2.11	8.8
48h	No Treatment	10% FBS	Set 3	727.7	2.11	9.7



A



B

Figure 4.3: Representative figures for microarray hybridization quality control results for 10% FBS treated control samples (For the whole list, ***Appendix C***). **A)** Quality Control (QC) plot. The QC plot represents the QC metrics for arrays within each class. Within each class, each array is represented by a segment of the image. The scale factor for each array is plotted as a horizontal line from the vertical zero-fold line to the solid point that represents the scale factor. Scale factors in blue are within the three-fold range and those in red are out of range. The circle and triangle points represent GAPDH and B-ACTIN ratios, respectively. **B)** RNA degradation plot. The RNA degradation plot represents the scaled mean intensity for 5' to 3' probes. Each line represents an array within a class. The consistent slopes of arrays within a class indicate good quality (http://linus.nci.nih.gov/BRB-ArrayTools.html).

Table 4.2: Representative data for microarray post-hybridization quality control analysis. The background found to be comparable for 5'3'-GAPDH and ACTIN values as well as scale factors.

Samples	%Present	Scale Factor	b.Actin3.5	GapDH3.5	b.Actin3.M	GapDG3.M	Avg. Background
0.1% FBS Control Set3	47.36	0.38	0.57	0.33	0.03	0.16	59.42
0.1% FBS Control Set2	47.09	0.57	1.25	0.67	0.10	0.22	44.82
0.1% FBS 1uM Nicotine Set3	49.38	0.42	1.15	0.54	0.36	0.30	45.60
0.1% FBS 1uM Nicotine Set2	49.19	0.41	0.57	0.25	0.07	0.22	43.16
10% FBS Control Set3	45.93	0.39	0.25	0.16	-0.06	0.08	47.80
10% FBS 1uM Nicotine Set3	46.27	0.45	0.30	0.19	-0.02	0.11	47.57

All analyses indicated that the six arrays performed in this study were comparable.

4.2.2 Scatter plot analysis

We performed scatter plots of the microarray data to see the extent of dispersion between groups of treatments at fold-change level (i.e., 2). Accordingly, we also aimed to see the distribution of these genes in scatter plots to compare the effects (upregulation or downregulation, respectively) of normal serum and nicotine exposure under different serum levels (***Figure 4.4*** and ***Figure 4.5***).

According to the results of scatter plots, there are more genes upregulated by nicotine than those downregulated in response to nicotine treatment at 0.1% FBS level (***Figure 4.4A*** and ***Figure 4.5A***). Moreover, these genes were also upregulated or downregulated in response to addition of serum (10% FBS) in a fashion mimicking the effects of nicotine under serum starvation (***Figure 4.4B*** and ***Figure 4.5B***). Although these genes similarly acted in normal serum condition in comparison to the nicotine treatment under serum deprivation, when we compared the 10% FBS treatment with nicotine treatment under serum starvation, we saw that these genes although much less in number were greatly downregulated at 10% FBS (***Figure 4.4C***). A similar distribution was

also apparent in the nicotine-mediated downregulation profiles (***Figure 4.5C***). We also compared the effects of nicotine at 10% FBS levels; our results indicated the extent of modulation by nicotine was relatively small when compared with that observed under serum starvation, however there were a number of genes that were located closely at the fold change threshold indicating that a part of the nicotine-response under serum starvation might still exist at 10% FBS levels, although mildly (***Figure 4.4D*** and ***Figure 4.5D***).

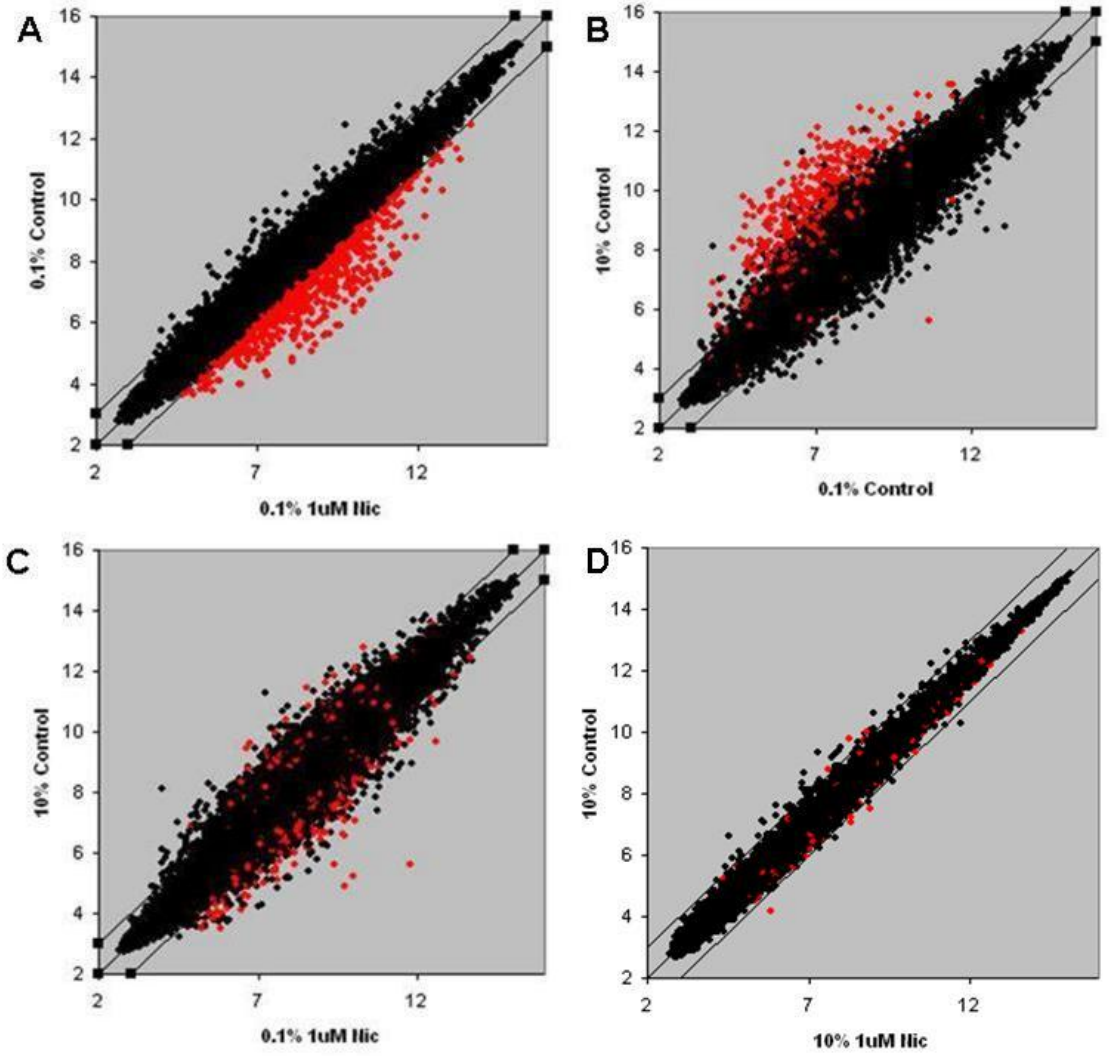


Figure 4.4: Different Scatter plot illustrations of 1µM nicotine exposed samples' downregulated genes in 0.1% FBS control in comparison with 1µM nicotine under same serum conditions. **A)** Red dots indicate downregulated genes in 0.1% FBS treatment in comparison with 1µM nicotine under

same serum conditions. **B)** Box-B represents the locations of significant genes in box A under 10% FBS control compared to 0.1% FBS control. **C)** Box-C represents the locations of significant genes in box A under 10% FBS control compared to 0.1% FBS 1μM Nicotine treated sample. **D)** Box-D represents the locations of significant genes in box A under 10% FBS control compared to 10% FBS 1μM nicotine treated sample.

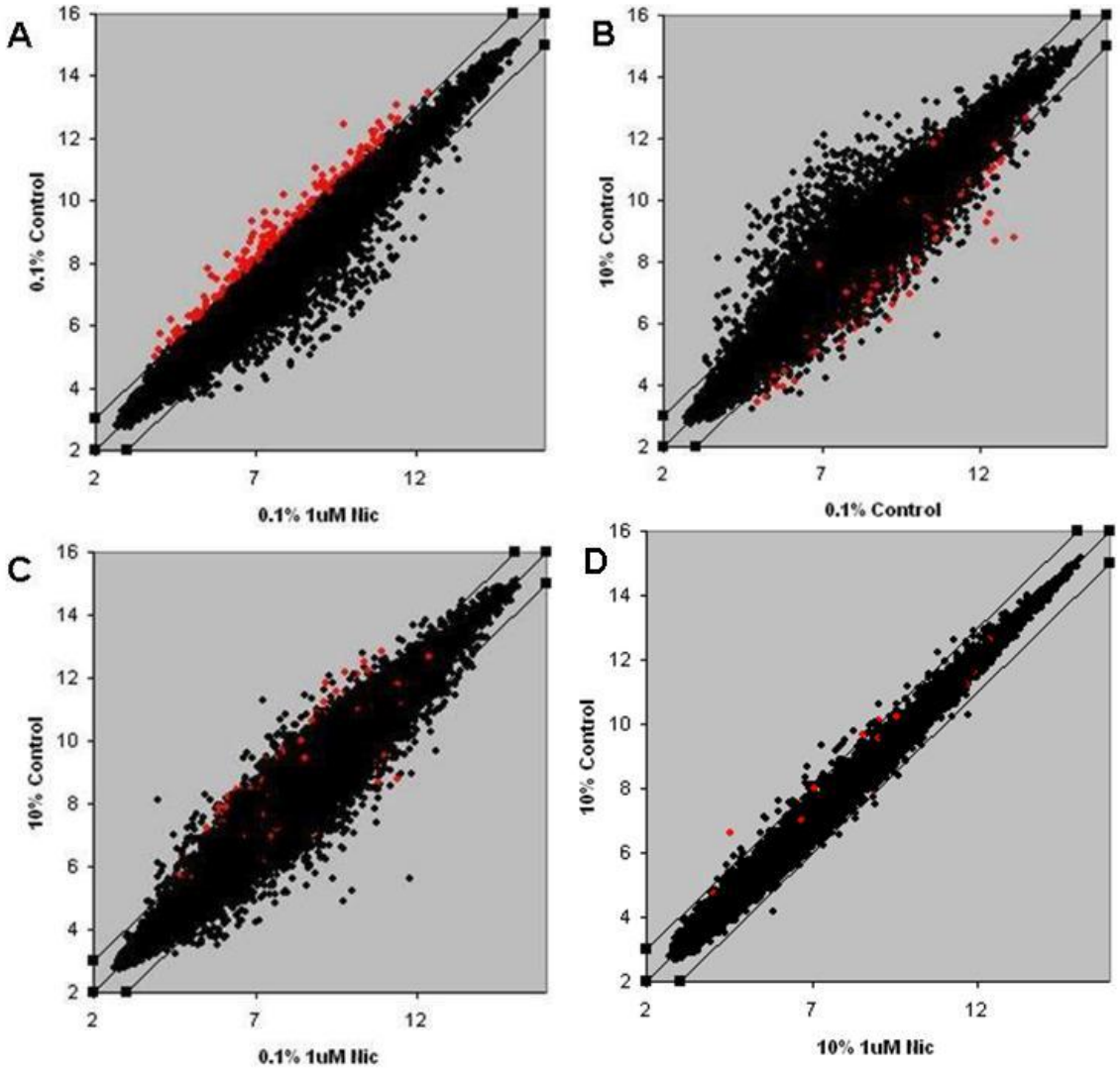


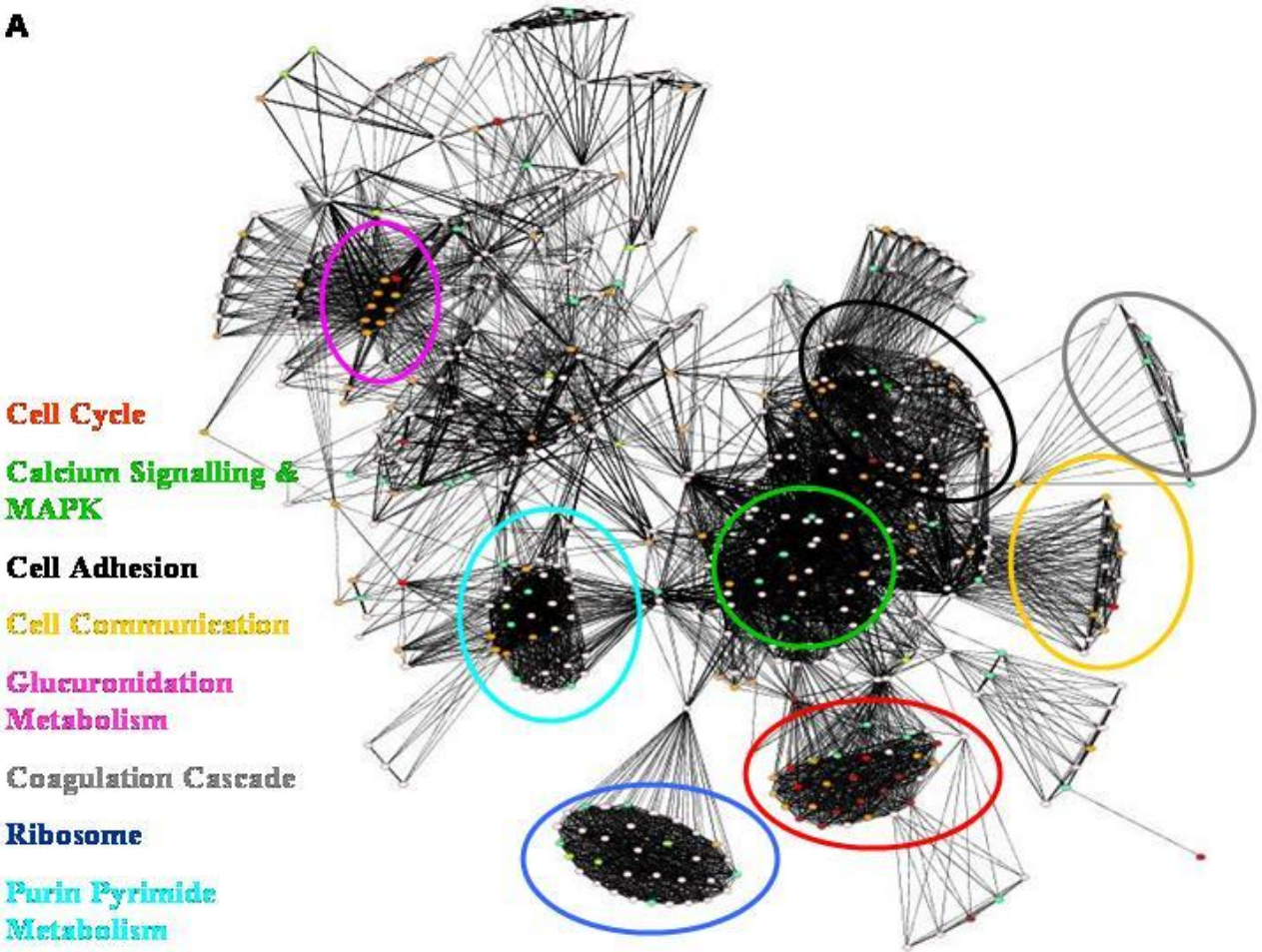
Figure 4.5: Different Scatter plot illustrations of 1μM nicotine exposed samples' upregulated genes in 0.1% FBS control in comparison with 1μM nicotine under same serum conditions. **A)** Red dots indicate upregulated genes in 0.1% FBS treatment in comparison with 1μM nicotine under same serum conditions. **B)** Box-B represents the locations of significant genes in box A under 10% FBS Control compared to 0.1% FBS control. **C)** Box-C represents the

locations of significant genes in box A under 10% FBS Control compared to 0.1% FBS 1μM Nicotine treated sample. **D)** Box-D represents the locations of significant genes in box A under 10% FBS Control compared to 10% FBS 1μM Nicotine treated sample.

4.3 Determination of differentially expressed gene profiles upon nicotine exposure under different serum conditions after microarray analyses

4.3.1 Statistical and functional analysis of nicotine response

In order to see the effects of nicotine under serum starvation in comparison with the effects of 10% FBS addition, we performed a gene set enrichment analysis regarding the significant KEGG pathways (***Appendix B***); thus we filtered out the significant genes at $p<0.05$ (over 800) that were up- or down-regulated under nicotine treatment to illustrate the interactions of the genes in terms of cellular regulatory and metabolism pathways with the help of Pathway Miner. In the following graphs, upregulated and downregulated genes (with greater or less than 1.5 fold change, respectively) were represented by red and green coloring. The gene clusters involved in both cellular and metabolic pathways were shown in ***Figure 4.4***. The most striking interactions belonged to cell cycle, calcium signaling, purine/ pyrimidine metabolism, cell adhesion, cell communication, estrogen metabolism, coagulation and ribosomal proteins interactions (***Figure 4.6; Appendix B***).


A
Cell Cycle
Calcium Signalling & MAPK
Cell Adhesion
Cell Communication
Glucuronidation Metabolism
Coagulation Cascade
Ribosome
Purin Pyrimide Metabolism

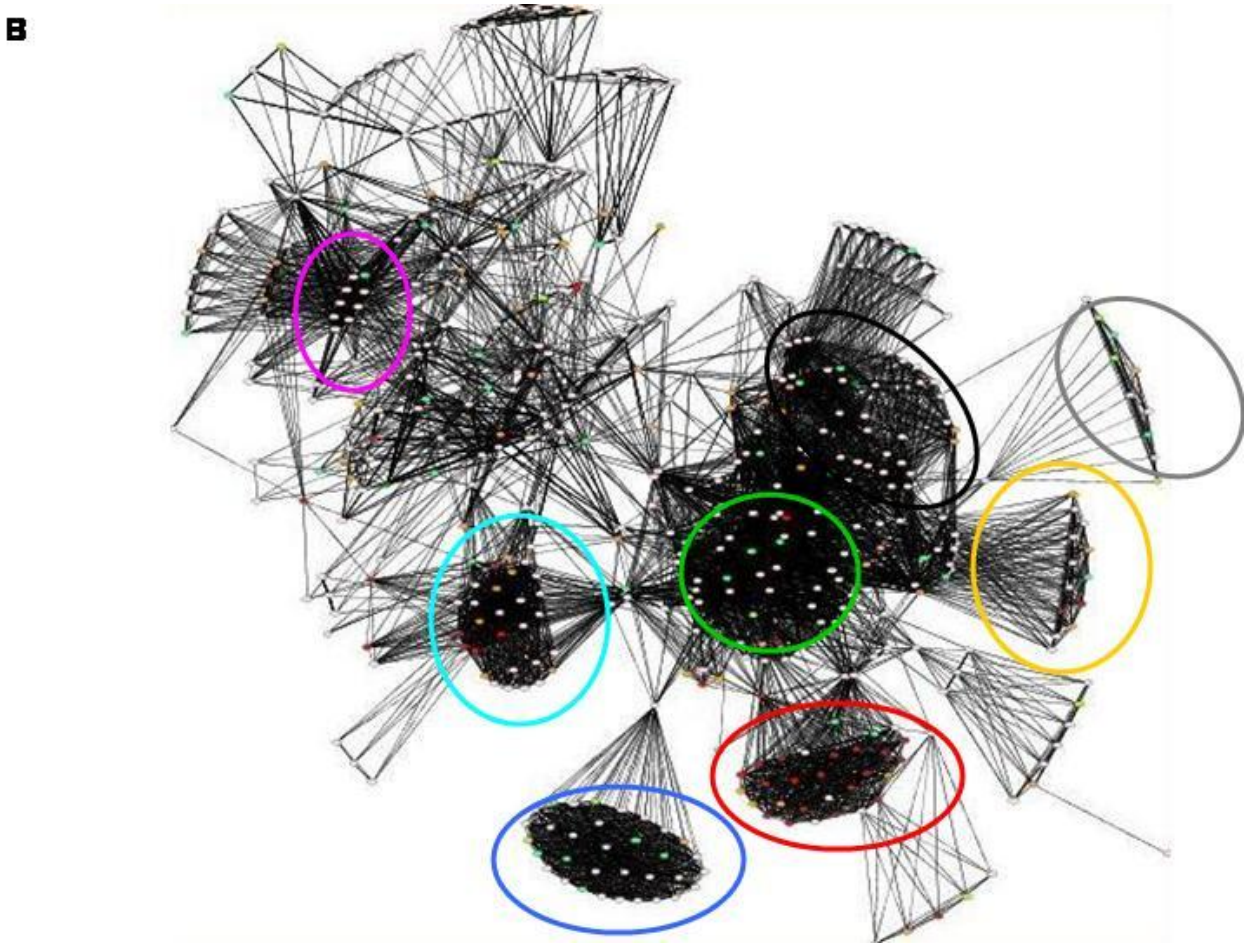
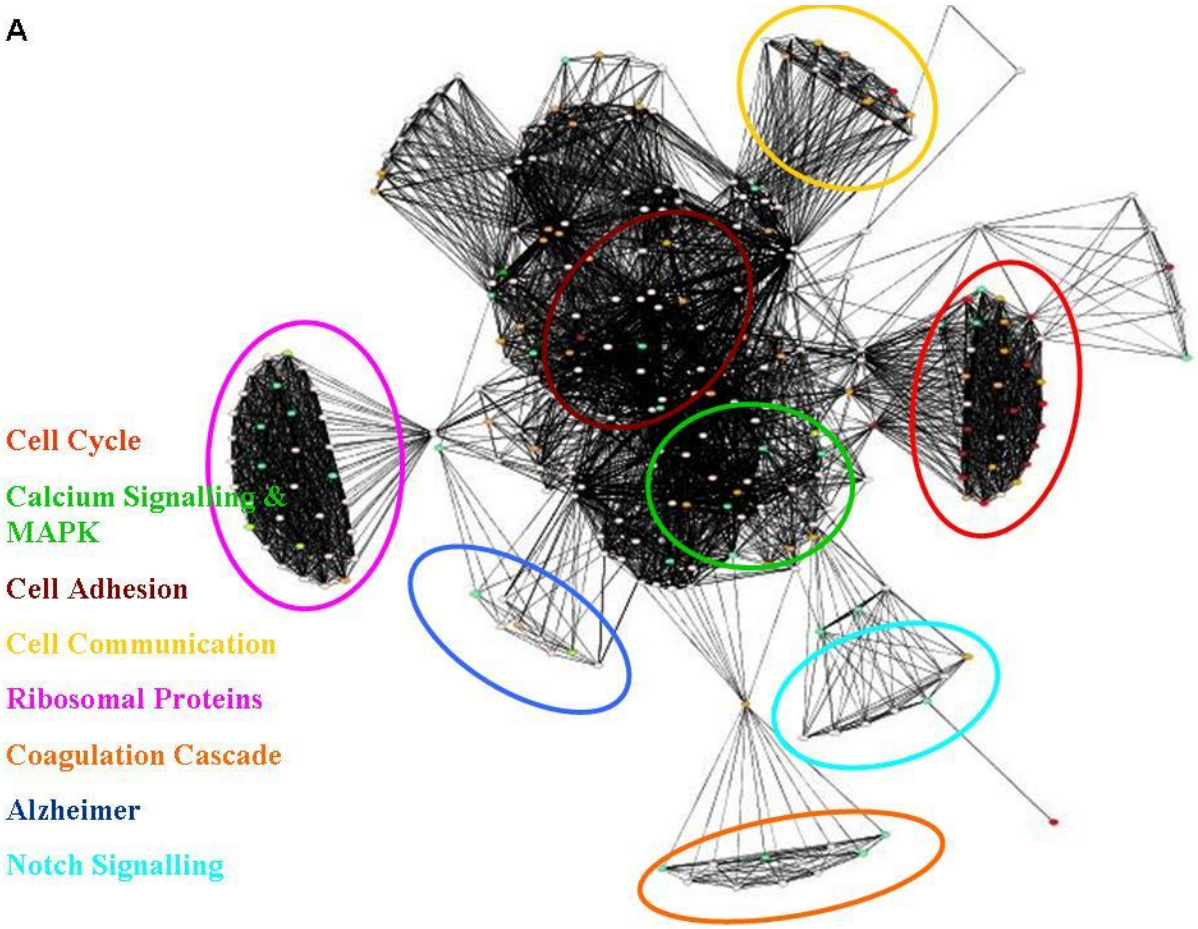


Figure 4.6: KEGG Pathway illustrations sketched via significant genes of 10% FBS serum conditions and 1μM Nicotine treatment in comparison with 0.1% FBS serum treatments. **A)** The genes affected by 1μM nicotine treatment under starvation conditions in comparison with starvation controls. **B)** The genes affected by normal serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of downregulation and upregulation of the normal serum or 1μM Nicotine genes in comparison with serum starvation, respectively.

We also specifically focused on cellular regulatory process pathways (***Figure 4.7***) and metabolic pathways (***Figure 4.8***). The cell cycle, calcium and MAPK signaling, coagulation, cell communication, cell adhesion, ribosomal machinery proteins, Alzheimer related genes, and Notch

signaling genes were extensively regulated by nicotine under serum starvation conditions in cellular regulatory pathways, which also mimicked the response to 10% FBS in terms of these pathways (***Figure 4.7***). Similarly, estrogen metabolism, amino acid metabolism and biosynthesis, glycan biosynthesis, glycolipid metabolism and purine/ pyrimide metabolism were regulated by nicotine in terms of KEGG metabolic pathways (***Figure 4.8; Appendix B***).



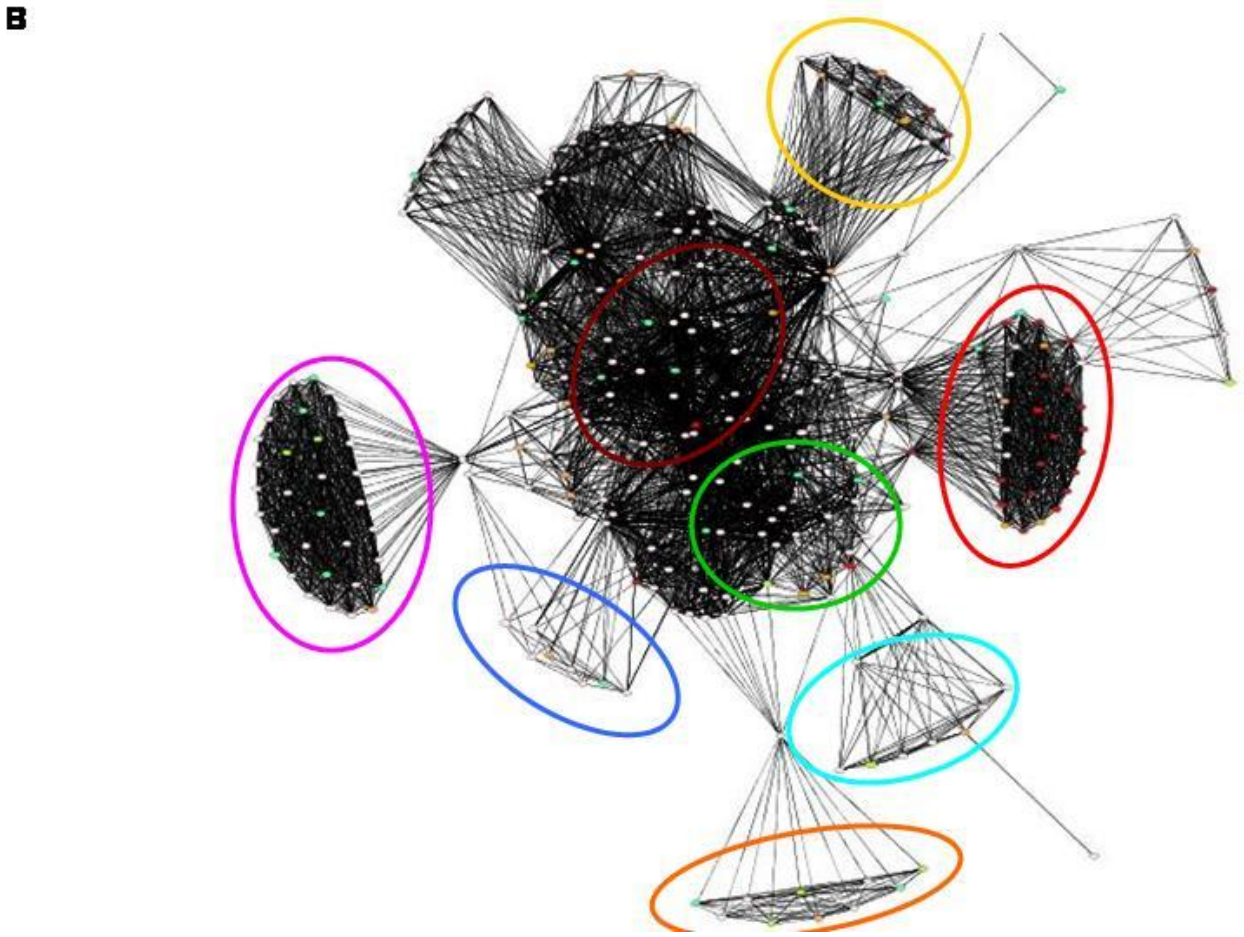


Figure 4.7: The Cellular and Regulatory Process Pathways were illustrated via KEGG Pathway representing significant genes of 10% FBS serum conditions or 1µM Nicotine treatment in comparison with 0.1% FBS serum treatments. **A)** The genes affected by 1µM nicotine treatment under starvation conditions in comparison with starvation controls. **B)** The genes affected by normal serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of downregulation and upregulation of the normal serum or 1µM Nicotine genes in comparison with serum starvation, respectively.

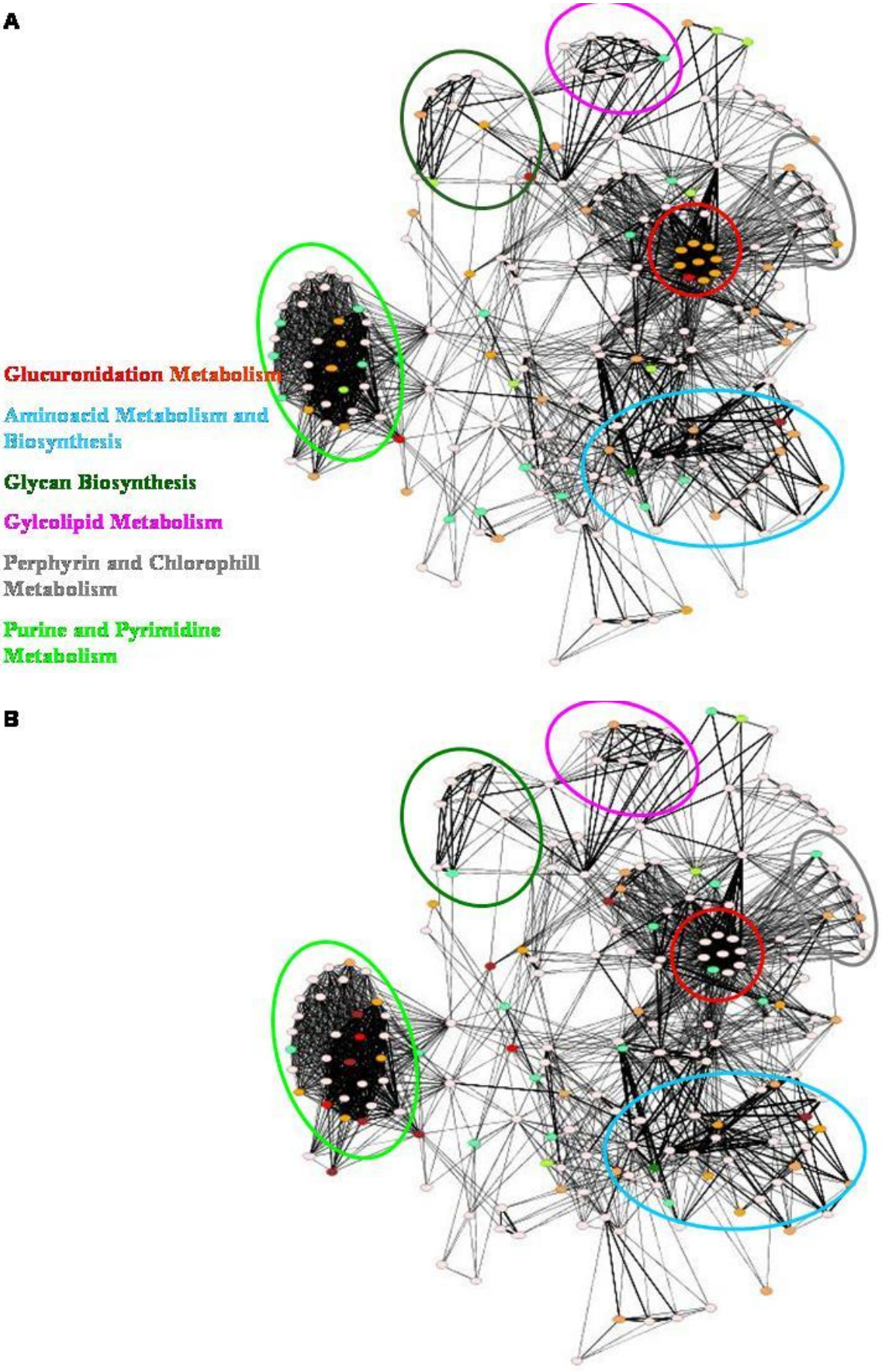
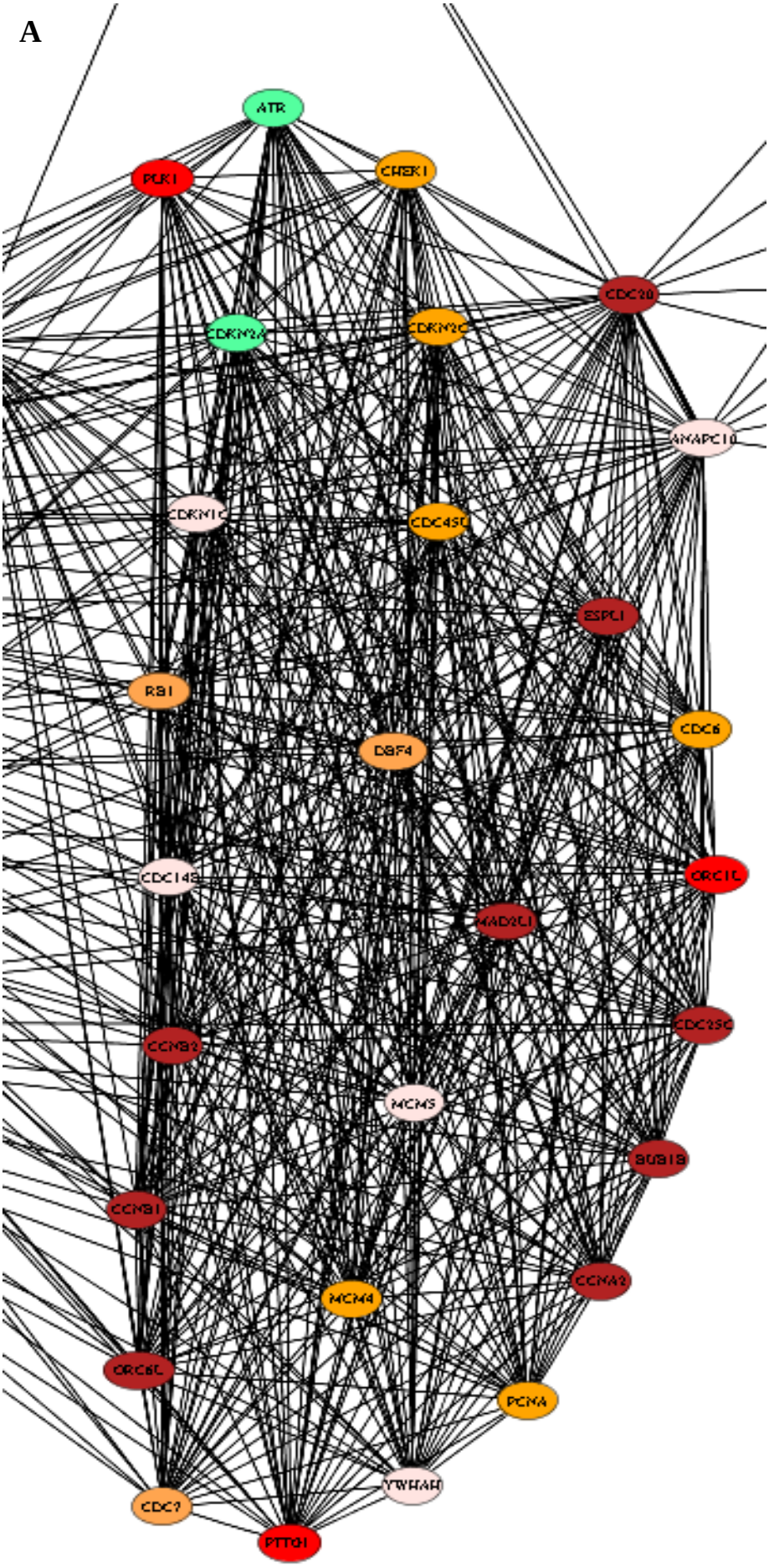

A
Glucuronidation Metabolism
Aminoacid Metabolism and Biosynthesis
Glycan Biosynthesis
Gylcolipid Metabolism
Perphyrin and Chlorophill Metabolism
Purine and Pyrimidine Metabolism
B

Figure 4.8: The Metabolic Pathways were illustrated via Pathway Miner using KEGG Pathway genes representing significant genes of 10% FBS serum conditions or 1μM Nicotine treatment in comparison with 0.1% FBS serum treatments. **A)** The genes affected by 1μM nicotine treatment under starvation conditions in comparison with starvation controls. **B)** The genes affected by normal serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of downregulation and upregulation of the normal serum or 1μM Nicotine genes in comparison with serum starvation, respectively.

Among the pathways mentioned above, we decided to focus on some of them separately to identify candidate genes affected by nicotine. Cell cycle pathway genes were one of the preferences regarding the nicotine effect. The class comparison analyses as visualized in Pathway Miner indicated that nicotine upregulated the genes included processes like DNA replication and cell division such as cell cycle genes (e.g., **Cyclin E1, A1, B1**), **CDCs** (cell division cycle genes), **PCNA** (Proliferating cell nuclear antigen), **MCM5** (DNA helicase) (***Figure 4.9, Appendix A1***). Furthermore, **ATR** (Ataxia telangiectasia and Rad3 related) and **CDKN2A** (Cyclin dependent kinase inhibitor 2A) that results in cell cycle arrest, were strikingly downregulated by nicotine treatment under serum starvation conditions (Figure 4.9A).


A
ATR
PLK1
CHEK1
CDC20
CDKN2A
CDKN2C
ANAPC10
CDKN1C
CDC45L
ESPL1
RB1
CDC6
DBF4
CDC14B
ORC1L
MAD2L1
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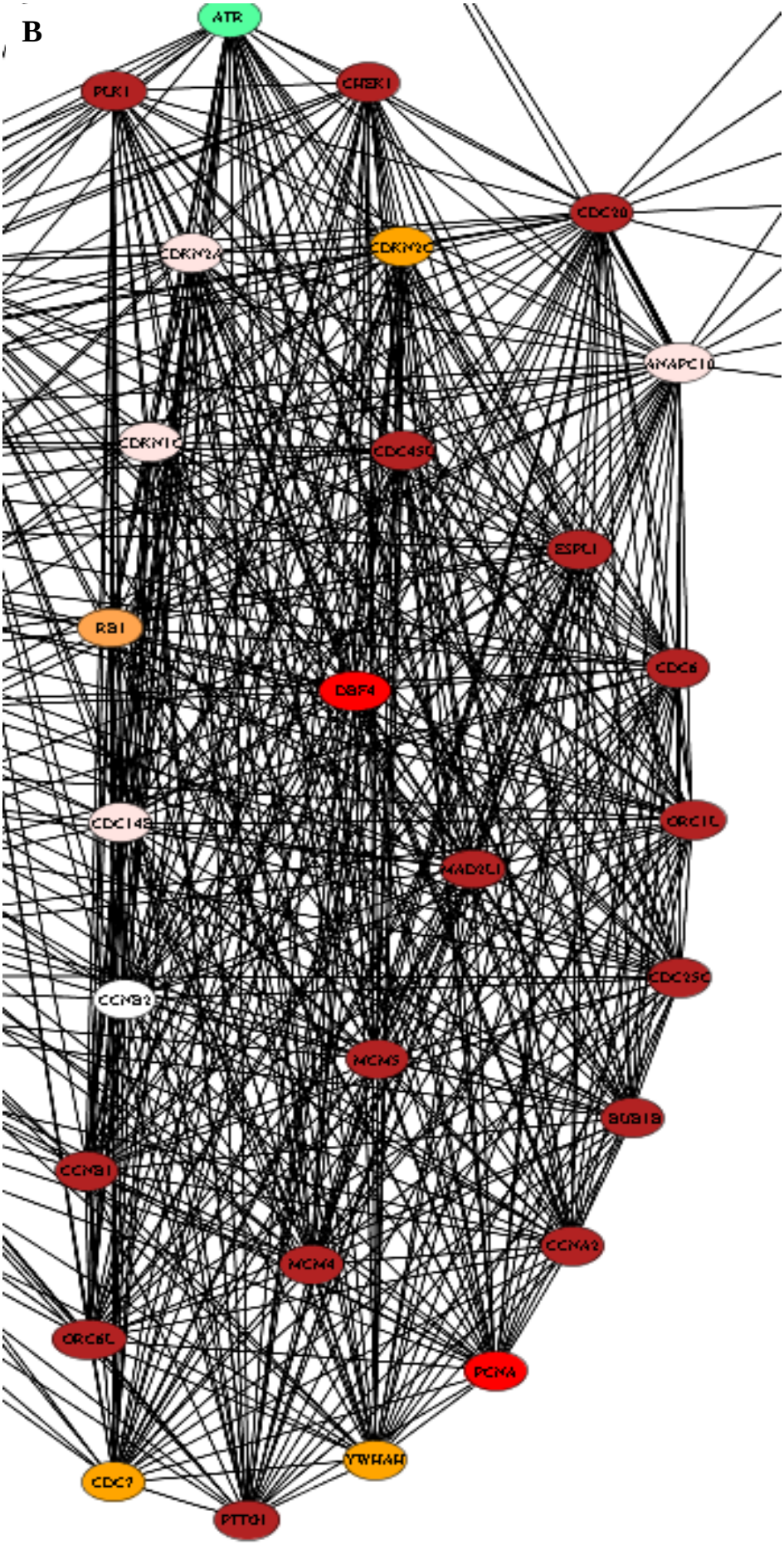
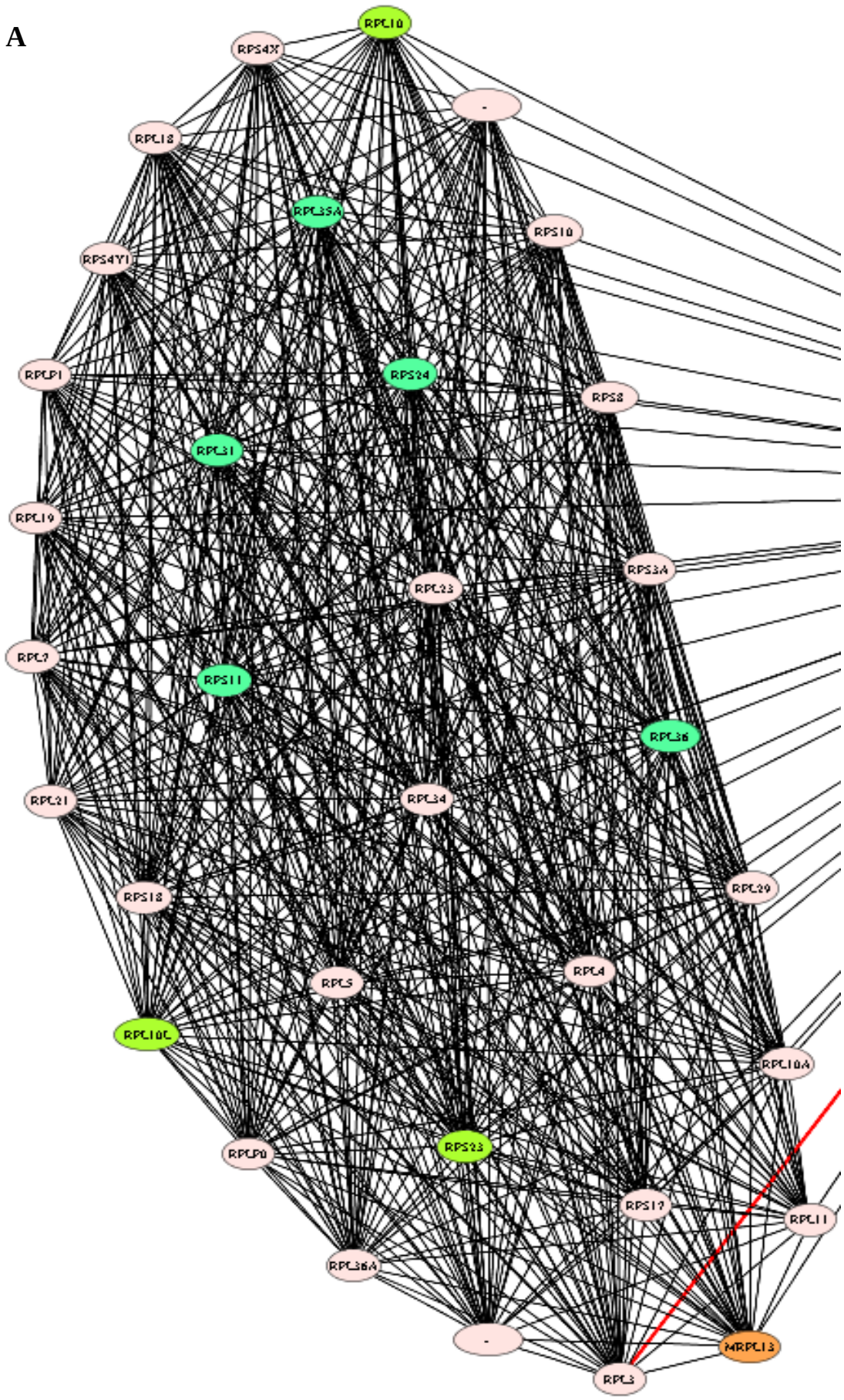
CCNB1
CCNA2
MCM4
ORC6L
PCNA
YWHAH
CDC7
PTTG1


B
ATR
PLK1
CHEK1
CDC20
CDKN2A
CDKN2C
ANAPC10
CDKN1C
CDC45L
ESPL1
RB1
CDC6
DBF4
CDC14B
ORC1L
MAD2L1
CDC25C
CCNB2
MCM3
BUB1B
CCNB1
CCNA2
MCM4
ORC6L
PCNA
YWHAH
CDC7
PTTG1

B

Figure 4.9: The cell cycle pathway genes illustrated via Pathway Miner with KEGG pathway genes. **A)** The genes affected by 1μM nicotine treatment under starvation conditions in comparison with starvation controls. **B)** The genes affected by 10% serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of downregulation and upregulation of the normal serum or 1μM Nicotine genes in comparison with serum starvation, respectively.

Ribosomal protein genes were also detected as deregulated genes via nicotine treatment. Many of the ribosomal (**RPLs, RPSs**) protein genes had smaller fold-changes (under 1.5), but the rest of the ribosomal proteins were significantly downregulated in both nicotine treatment and normal conditions (***Figure 4.10***). Only one gene, **MRPL13**, which is mitochondrial, was slightly upregulated (***Figure 4.10***).


A
RPL10
RPS4X
-
RPL18
RPL35A
RPS10
RPS4Y1
RPLP1
RPS24
RPS8
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RPL19
RPS3A
RPL23
RPL7
RPS11
RPL36
RPL21
RPL34
RPS18
RPL29
RPL5
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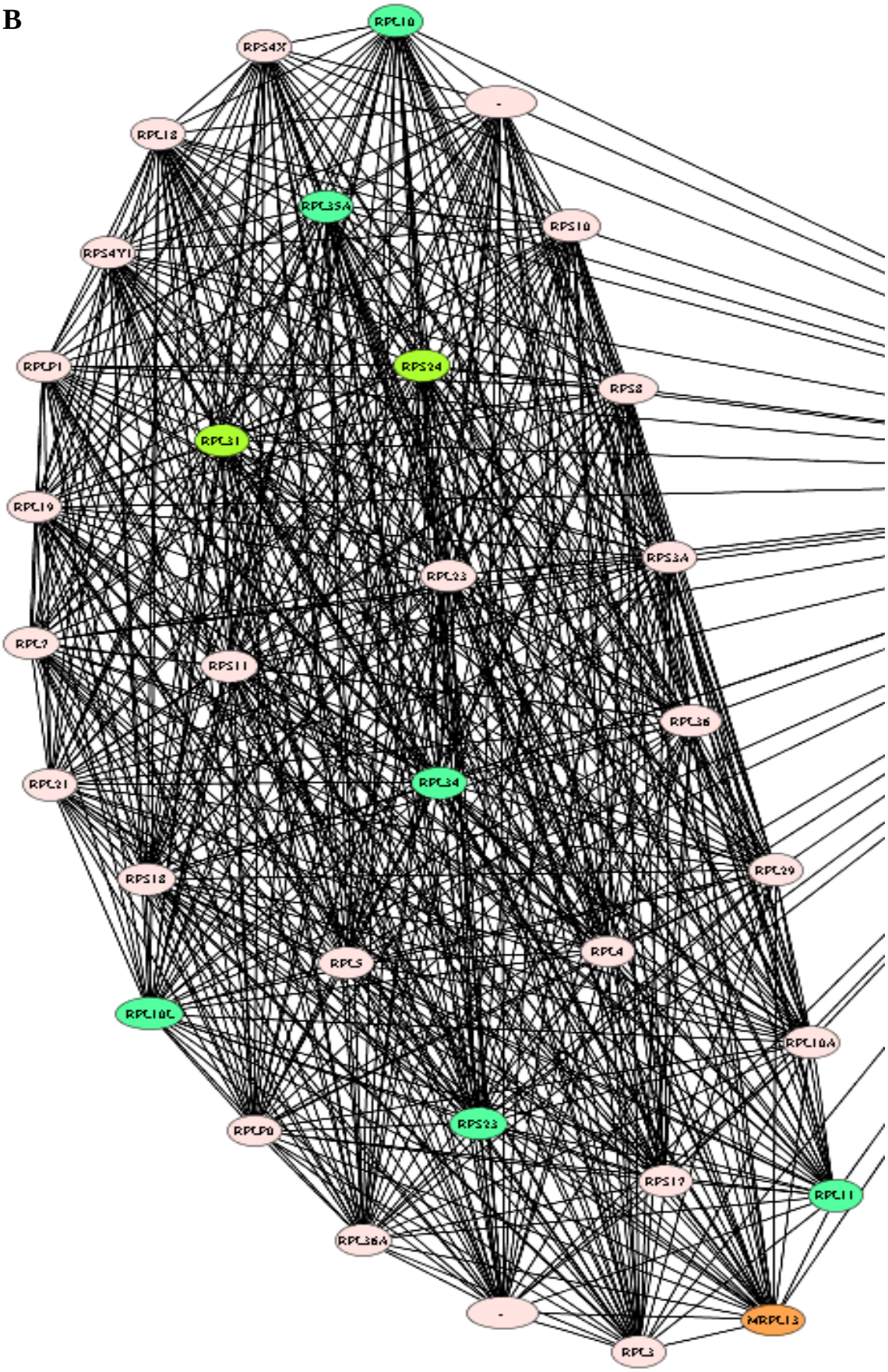
RPS17
RPL11
RPL36A
-
MRPL13
RPL3

B


RPL10
RPS4X
-
RPL18
RPL35A
RPS10
RPS4Y1
RPLP1
RPS24
RPS8
RPL31
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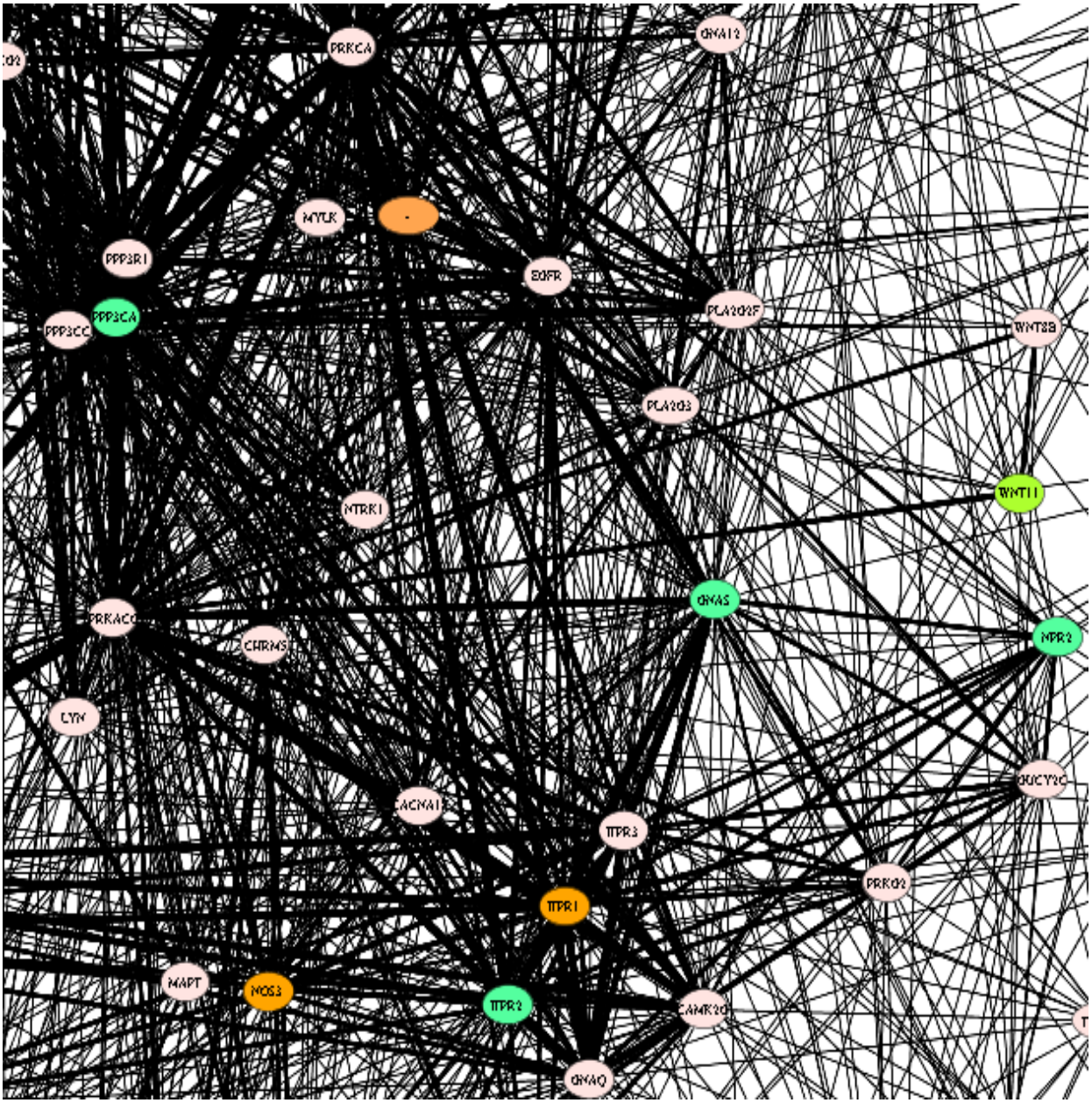
RPS3A
RPL23
RPL7
RPS11
RPL36
RPL21
RPL34
RPS18
RPL29
RPL5
RPL4
RPL10L
RPL10A
RPLP0
RPS23
RPS17
RPL11
RPL36A
-
MRPL13
RPL3

Figure 4.10: The ribosomal protein interactions illustrated via Pathway Miner with KEGG pathway genes. **A)** The genes affected by 1μM nicotine treatment under starvation conditions in comparison with starvation controls. **B)** The genes affected by 10% serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of downregulation and upregulation of the normal serum or 1μM Nicotine genes in comparison with serum starvation, respectively.

One of the most interesting pathways was Ca^{2+} signaling pathway for the differential role of nicotine exposed to serum deprived cells in comparison with normal conditions. Although other pathways had the significant expression differences on the same genes, the differentially expressed Ca^{2+} signaling pathway genes were different under nicotine treated serum starvation condition in comparison with physiologic serum conditions (***Figure 4.11***).While **NOS3** and **TTPR1** were upregulated and **GNAS, NPR2, TTPR2** and **PPP3CA** genes were downregulated under nicotine treatment (***Figure 4.11A***), **MAPT, GNAS, NPR2** were downregulated and **PPP3R1** gene was upregulated in response to 10% serum (***Figure 4.11B***).

Accordingly, purine/ pyrimidine metabolism was another very affected pathway due to nicotine treatment in serum deprivation conditions and normal physiological conditions of the SW620 cells. Similar genes were upregulated or downregulated in both conditions (***Figure 4.12***).

A


PRKCA
GNA12
MYLK
-
PPP3R1
EGFR
PPP3CA
PPP3CC
PLA2G2F
WNT2B
PLA2G3
WNT11
NTRK1
GNAS
PRKACG
CHRM3
NPR2
LYN
GUCY2C
ACNA1S
ITPR3
PRKG2
ITPR1
MAPT
NOS3
ITPR2
CAMK2G
GNAQ

B

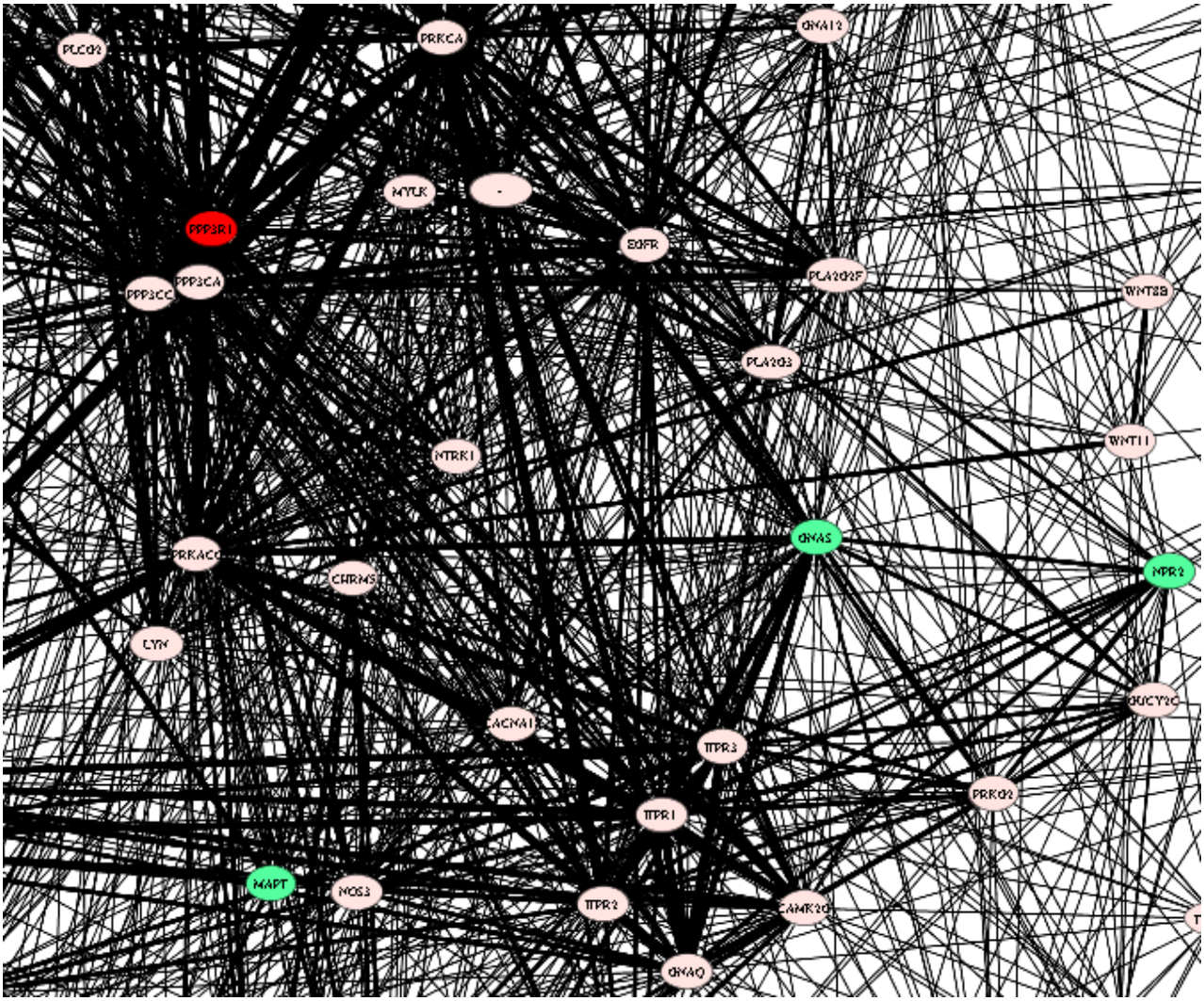
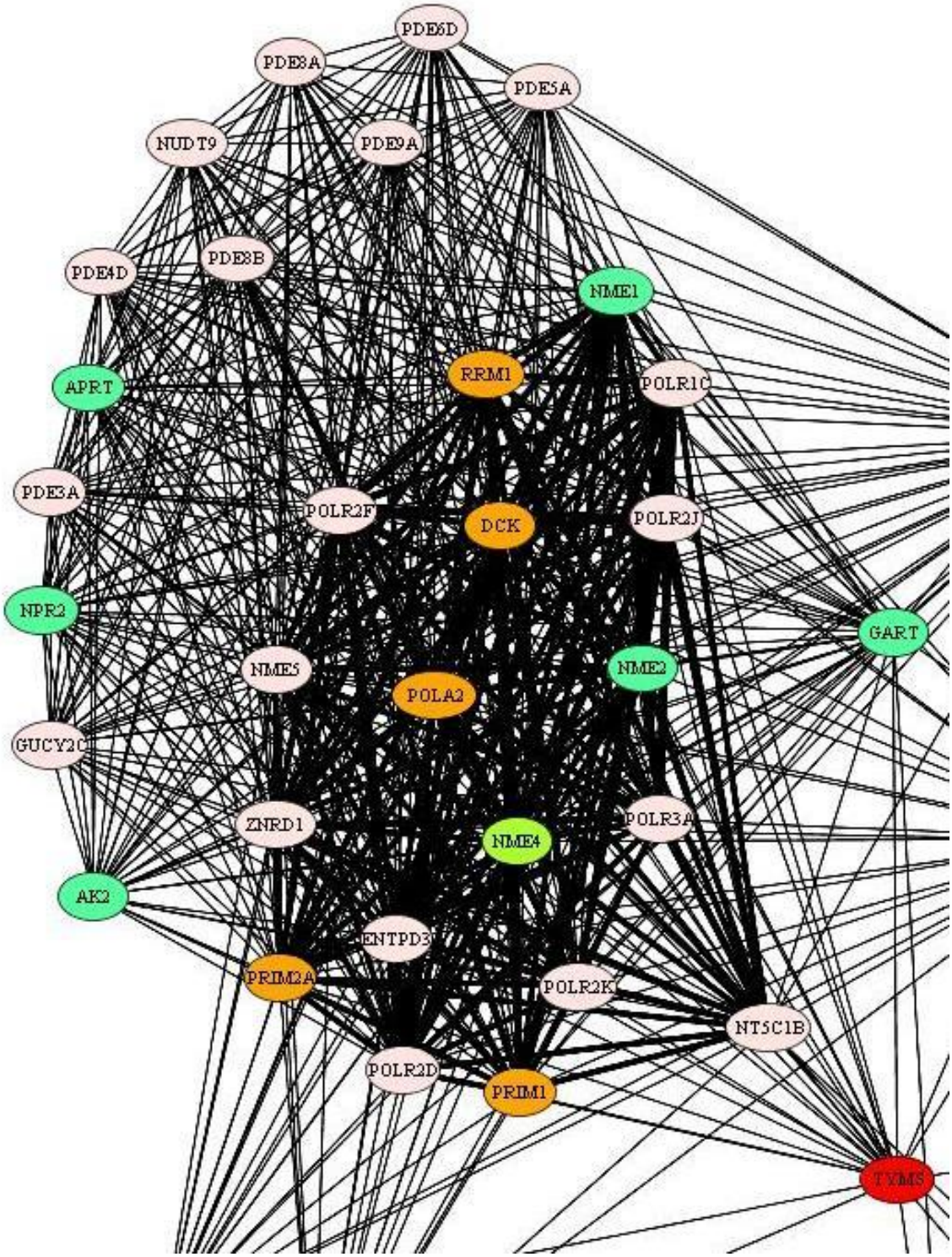
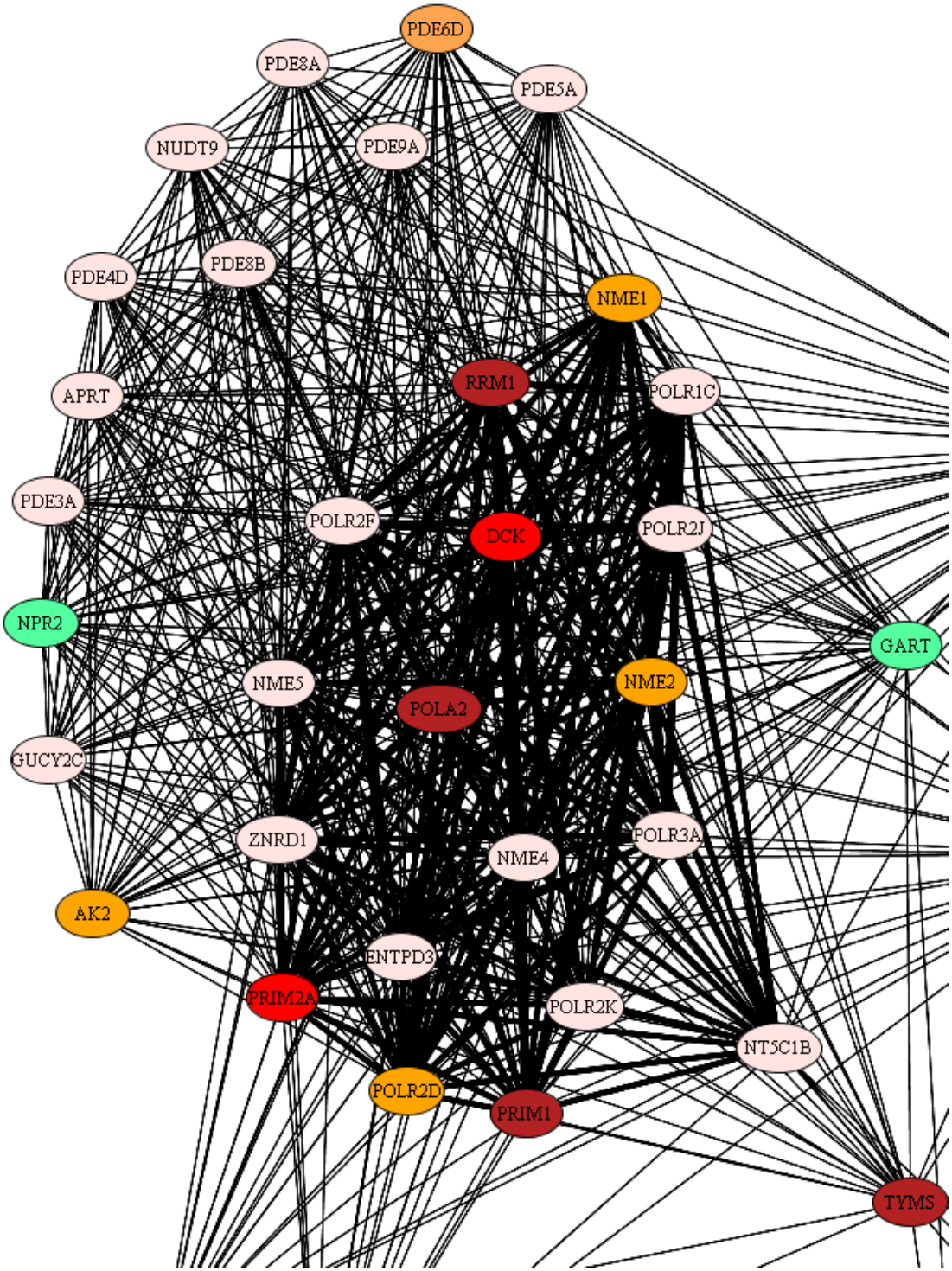


Figure 4.11: The Calcium Signaling genes illustrated via Pathway Miner with KEGG pathway genes. **A)** The genes affected by 1µM nicotine treatment under starvation conditions in comparison with starvation controls. **B)** The genes affected by 10% serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of downregulation and upregulation of the normal serum or 1µM Nicotine genes in comparison with serum starvation, respectively.


PDE6D
PDE8A
PDE5A
NUDT9
PDE9A
PDE4D
PDE8B
NME1
APRT
RRM1
POLR1C
PDE3A
POLR2F
DCK
POLR2J
NPR2
GART
NME5
NME2
POLA2
GUCY2C
ZNRD1
POLR3A
NME4
AK2
ENTPD3
PRIM2A
POLR2K
NT5C1B
POLR2D
PRIM1
TYMS

A



B

Figure 4.12: The Purine/ Pyrimidine metabolism illustrated via Pathway Miner with KEGG pathway genes. **A)** The genes affected by 1µM nicotine treatment under starvation conditions in comparison with starvation controls. **B)** The genes affected by 10% serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of

downregulation and upregulation of the normal serum or 1μM Nicotine genes in comparison with serum starvation, respectively.

Another interesting metabolism pathway was glucuronidation pathway in which the genes were significantly upregulated under nicotine treatment in comparison with normal conditions (***Figure 4.13***). The genes (Uridine 5'-diphospho-glucuronosyltransferases 1A- **UGT1As**) have been shown to be very important in the body's elimination of foreign substances such as drugs and medications, as well as endogenous substances including endogenous toxins [80]. One of the genes of this family, **UGT1A6**, was very interesting such that it was highly upregulated in nicotine treated serum but was detectably downregulated under physiological serum conditions (***Figure 4.13***).

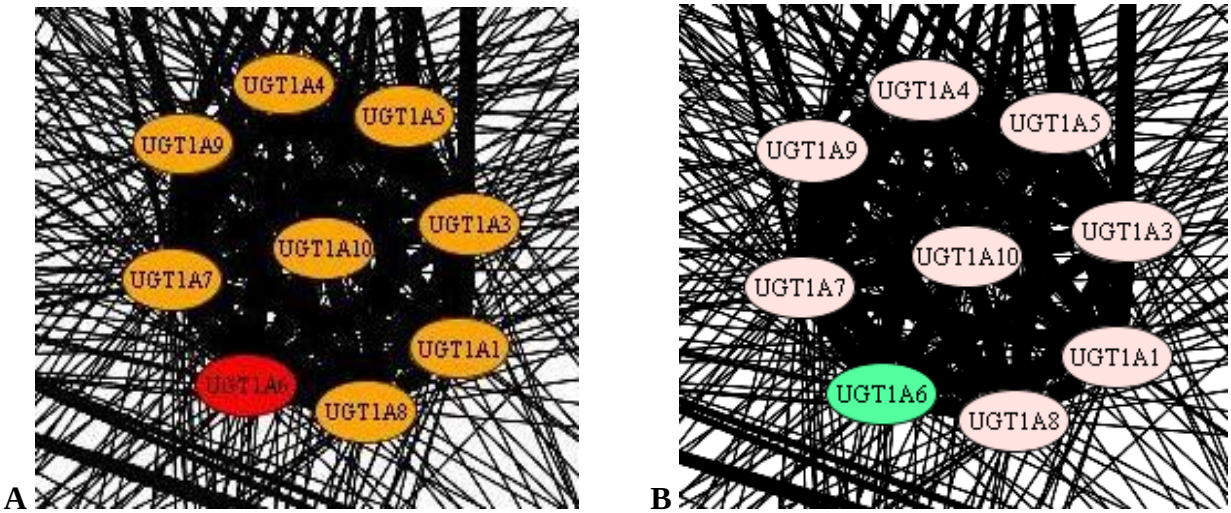
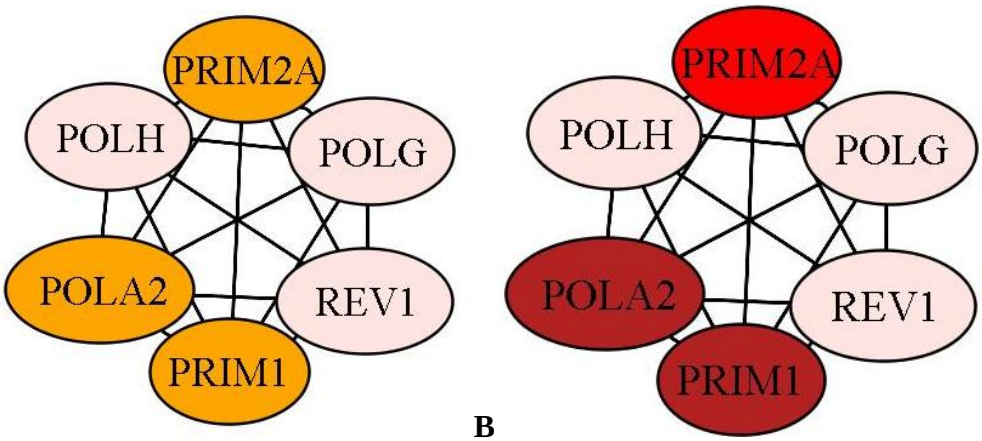


Figure 4.13: The Glucuronidation metabolism illustrated via Pathway Miner with KEGG pathway. A) The genes affected by 1μM nicotine treatment under starvation conditions in comparison with starvation controls. B) The genes affected by10% serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of downregulation and upregulation of the normal serum or 1μM Nicotine genes in comparison with serum starvation, respectively.

DNA polymerase activity was also observable in nicotine treated serum starvation conditions; however, the upregulation of DNA polymerase genes was much higher in response to addition of serum, expectedly (***Figure 4.14***).



A **B**

Figure 4.14: DNA polymerase genes illustrated via Pathway Miner with KEGG pathway genes. **A)** The genes affected by 1μM nicotine treatment under starvation conditions in comparison with starvation controls. **B)** The genes affected by 10% serum conditions compared to serum starvation. The different intensities of green and red colors represent the strength of downregulation and upregulation of the normal serum or 1μM Nicotine genes in comparison with serum starvation, respectively.

4.3.2 Effects of serum and of nicotine on cholinergic receptor expression

Nicotine did not have significant effects on cholinergic receptor expression except that CHRNA1 expression was greater under nicotine treatment when cells were serum-starved while the same increase was not observed under physiological serum concentrations (p-value <0.05, $CHRNA1_{mean}$= 5.98 (10% FBS control), 5.88 (10%FBS 1μM Nic), 6.30 (0.1% FBS Control), 7.31 (0.1% FBS 1μM Nic)) (***Figure 4.15***). On the other hand, CHRNA5 was the most

significantly affected receptor among other followed by CHRNE in response to serum (p-value < 0.05, $CHRNA5_{geoMean}$= 8.58 (10% FBS) and 7.02 (0.1% FBS); $CHRNE_{geoMean}$= 7.10 (10% FBS) and 6.66 (0.1% FBS)) (***Figure 4.15***).

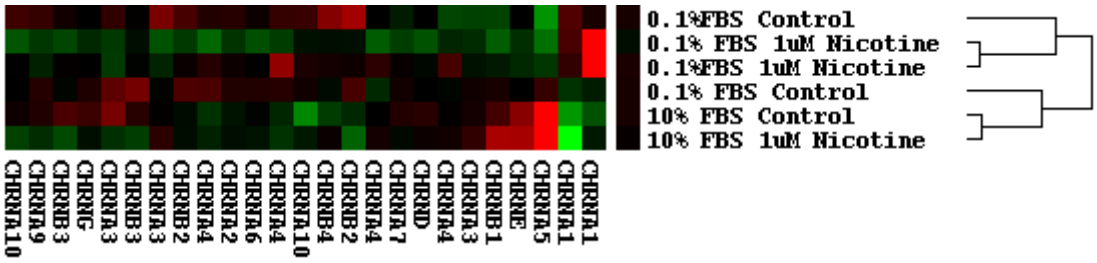


Figure 4.15: Treeview image of nicotinic cholinergic receptor genes' expression profiles. Nicotine has significant effect on the cluster of CHRNs but has not very noteworthy effect on the receptors individually. Red and green colors indicate a reduction and induction of expression when each gene is normalized to its median.

4.3.3 Microarray verification experiments of selected differentially expressed genes using real-time RT-PCR and Western Blot analyses

Western blot experiments were performed to see whether we can observe the similar expression pattern we obtained using the microarray profiling also at the protein level. For that purpose, we used Bax protein (a pro-apoptotic gene) levels; under normal serum conditions the protein levels decreased via nicotine treatment. When cells were deprived of serum the protein level increased drastically as expected yet but BAX protein expression was totally lost when cells were treated with nicotine together with serum deprived media (***Figure 4.16***). In the case of p53, the protein levels considerably decreased under serum starved condition when nicotine was present in media in comparison with the control samples. The level of this protein as expected did not change very momentously under normal serum conditions (***Figure 4.16***). The cell cycle regulator CYCLIN E

protein levels on the other hand, did not change drastically yet it is possible that under serum starvation the level of this protein might have been slightly induced by nicotine.

Table 4.3: 48 hours with or without 1 μM nicotine exposed SW620 cells' total protein concentration values calculated via Bradford technique after isolation.

Protein Sample ID	Concentration (μg/μl)
48h 10% FBS Control Set2	16.3
48h 10% FBS 1μM Nicotine Set2	14.3
48h 10% FBS Control Set3	7.6
48h 10% FBS 1μM Nicotine Set3	7.4
48h 0.1% FBS Control Set2	3.7
48h 0.1% FBS 1μM Nicotine Set2	5.5
48h 0.1% FBS Control Set3	6.1
48h 0.1% FBS 1μM Nicotine Set3	5.7

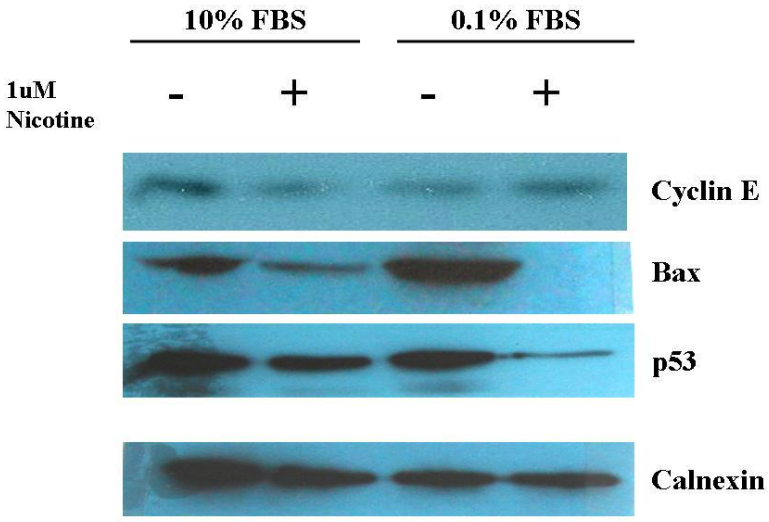


Figure 4.16: Western blotting results of 48 hour 1μM nicotine treated SW620 cells under two different serum conditions.

To confirm microarray experiment results, we first identified candidate genes by using Class comparison method. XIAP and Survivin were two significant candidates for our purpose and

we used GAPDH as a control (house-keeping gene) according to our microarray results (***Figure 4.17***). Therefore, we performed a qRT-PCR experiment with XIAP and Survivin, anti-apoptotic genes, together with the control gene GAPDH (***Figure 4.18*** and ***4.19***, respectively). According to these qRT-PCR results, under normal serum conditions neither XIAP nor Survivin have any noteworthy differential expression in comparison with what was observed with nicotine exposure under normal serum treated controls (preliminary data). However, under serum deprivation, nicotine treatment drastically increased the expressions of both of the genes. The expression difference was much stronger for the SURVIVIN gene. When these cells were deprived of serum, SURVIVIN expression was almost totally lost, but when nicotine was provided to the cells, the expression level of the SURVIVIN gene increased dramatically, even when compared to the levels obtained under normal serum conditions (***Figure 4.19***).

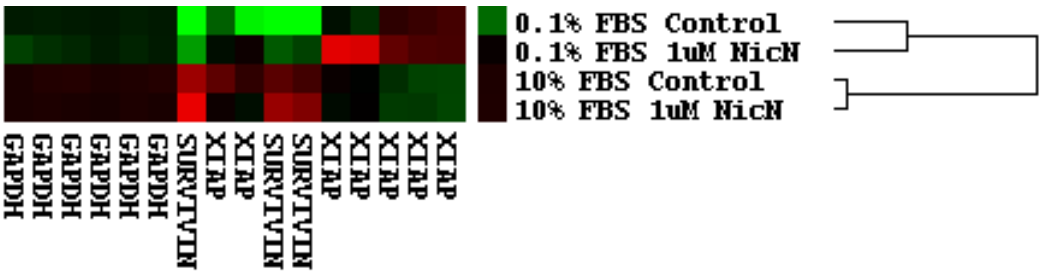


Figure 4.17: Treeview imaging of SURVIVIN and XIAP gene expression values from microarray analyses together with GAPDH. The red and green color intensity expresses the upregulation and downregulation of gene expression values, respectively.

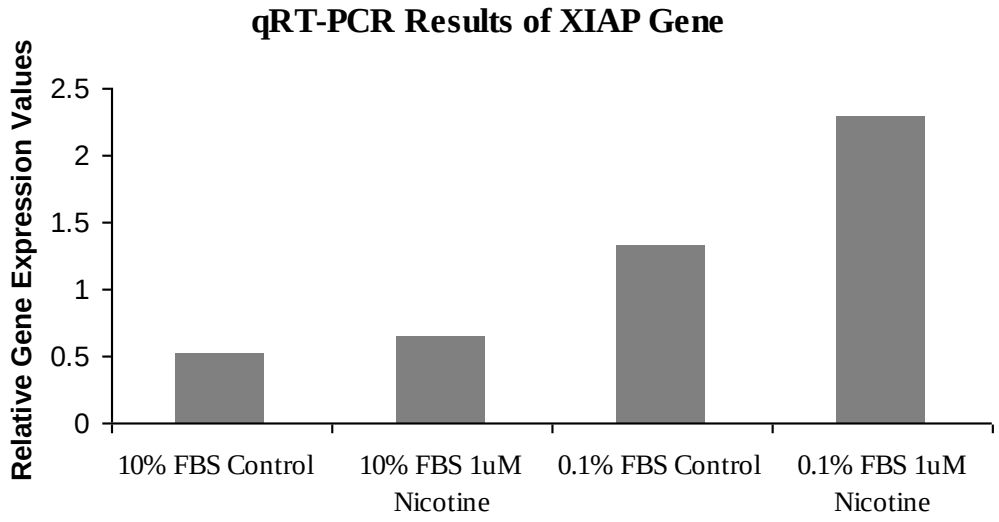


Figure 4.18: qRT-PCR results of XIAP (anti-apoptotic gene) under different conditions. GAPDH used as an internal control for qRT-PCR.

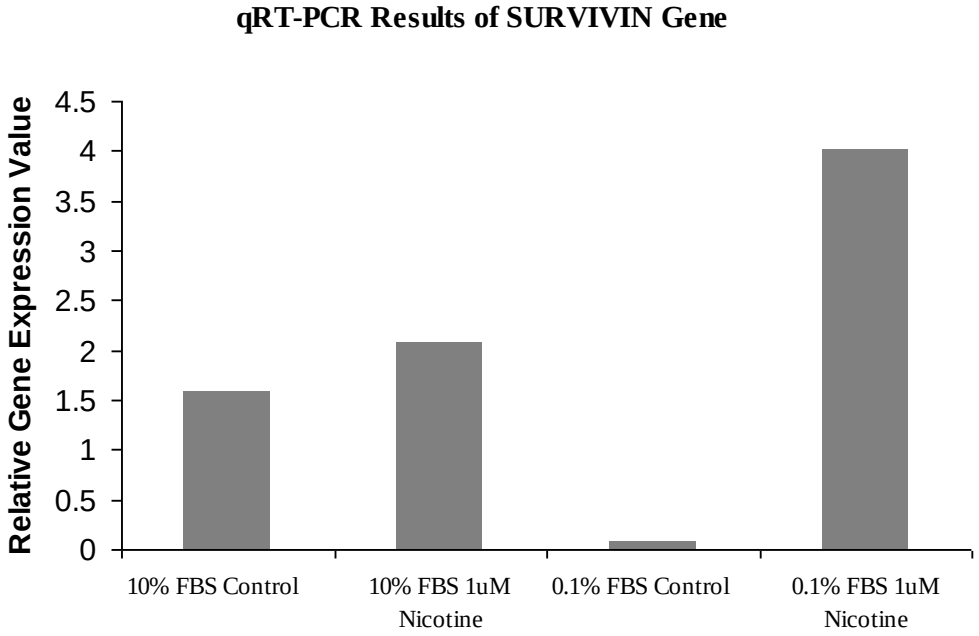


Figure 4.19: qRT-PCR results of SURVIVIN (anti-apoptotic gene) under different conditions. GAPDH used as an internal control for qRT-PCR.

CHAPTER 5. DISCUSSION and CONCLUSION

In the USA, colorectal cancer is the third most common cite of new cases and deaths in both men and women [13]. An individual's lifetime risk of developing colorectal cancer in the US is approximately 6%, with 90% of these occurring after age 50 [81]. Nearly 90% of all colorectal cancers and deaths are estimated to be preventable based on symptoms and early detection of polyps. As the symptoms of the colorectal cancer are not rapidly seen but very slowly developing, thus cancerous tissue cannot be diagnosed at the very early stages of the tumor. Therefore, it is important to investigate candidate genes and signaling pathways for their diagnostic potential with further implications on prognosis, as well. There are many risk factors for colorectal cancer. Genetic reasons (family history), physical inactiveness, red meat consumption, obesity, *ulcerative colitis, Crohn's disease,* alcohol and smoking are significant risk factors for this disease. Family history and physical inactivity have the highest relative risk ratios with 1.8 and 1.7, respectively [81]. Smoking has the next highest ratio with 1.5, meaning that smoking is one of the very crucial leading factors for colorectal cancer.

Tobacco does not only associate with colorectal cancer, but also 30% incidences of all cancer types, in which oral and lung cancer rates are higher than this rate, are directly related to smoking. Consequently, there are a lot of studies regarding tobacco and its ingredients. Nicotine is an active-alkaloid in tobacco and it is the strongest addictive chemical by which the dopaminergic system is deregulated in brain. Although there are many studies with nicotine's effect on cancer formation in lung, larynx, and oral cancer, only few studies are present regarding colorectal cancer cells and the action of nicotine through key genes modulated by nicotine exposure. Hence, these reasons led us to study the activity of nicotine upon colon cancer cells.

In this study, the cell proliferative and apoptotic effects of nicotine were studied using MTT assays, Western Blots, microarray transcription profiling and real-time RT-PCR studies. Our findings indicated that nicotine exhibited drastic proliferative effects under serum starvation

conditions but did not have a significant role in cell proliferation under normal growth conditions at different time courses (48 hour, 7 days, 15 days (data not shown)).

In terms of the design of the microarray and confirmatory experiments, we have used a 1μM as the optimum dose. Although all small concentrations of nicotine significantly play a role in cell proliferation, nicotine at 1μM closely resembles the higher limit of the concentration (0.025- 0.444 μM/ ml) of nicotine found in the blood of women and men (3-18 year-smokers) [82, 83]. Moreyra *et al*. also have shown that after the first two cigarette smoked, the nicotine concentration increases to 45 ± 8 ηg/ml (ca. 400 μM/ml) in the blood [84]. Therefore, 1μM nicotine concentration for 48 hour exposure to SW620 cells seems to be an optimum condition to assess the acute and early gene expression regulation of nicotine.

5.1 Nicotine Regulated Cell Proliferation and Cell Cycle

Since unstoppable cell proliferation and division is the most important feature of tumor development serum starvation does lead to cell cycle arrest and ceases the cell proliferation. When we examined the effects of nicotine under serum starvation conditions, nicotine seemed to have a definite role in rescuing from loss of cellular proliferation. Alongside with our MTT experiment data (***Figures 4.1 and 4.2***), microarray pathway analyses and further verification with Cyclin E RNA and protein levels coming from the class comparison we could propose that nicotine at 1μM systematically modifies the cell cycle machinery at both the RNA and protein levels. In contrary to the significant increase of cell proliferative genes under serum starvation conditions upon nicotine treatment, under normal conditions nicotine has not significant effect on the expression of these gene families (***Appendix A1, Figure 4.9***). This may be due to the competition of nicotine with other growth factors and subsequent proliferative effects. Indeed, the recurrent exposure to nicotine enhances the activity of EGFR in bronchial epithelial cells which are finally differentiated [85]. Moreover, nicotine also activates VEGF-C gene alongside the EGFR level in cervical cancer cells [86]. These results can be interpreted as nicotine might exhibit a growth factor like behavior

and nicotine exposure deregulates intracellular signaling pathways as if they have got stimuli from a growth factor. When there is enough growth factor in the media, nicotine may not have a significant acute activity (48 h) in terms of cell growth; however, it does have a very striking act on serum starved cells in order to force them to proliferate and grow. Another possibility, and not necessarily mutually exclusive, would be that nicotine initiates receptor activity through binding to its own receptors and recruits Rb-Raf-1 and c-src pathways via β-arrestin [53]. Although this has been shown in lung cancer cells colon cancer cells have not been studied in this context.

5.2 Nicotine Mediated Apoptosis

Besides cell proliferation, apoptosis is another key mechanism in cancer development. Normally, cancerous tissues lose their feature of entering apoptosis and they start to proliferate *ad infinitum*. This is sometimes because of a repressive mutation of a tumor suppressor- apoptosis triggering gene (as in the case of p53 mutations), or an over expression of a proto-oncogene (as c-myc). It is worth noting that tumorigenesis due to non-apoptotic features does generally not depend on a single gene mutation, but it requires multiple hits along with some epigenetic deregulations [87, 88]. In addition, as apoptosis is triggered by serum starvation [89], it is essential to investigate whether the nicotine activity under different serum conditions result in changes in the RNA and protein levels of apoptotic and/or anti-apoptotic genes. Although XIAP expression significantly increased under serum starvation conditions by nicotine treatment, one important difference from Survivin's behavior grasped the attention. Survivin's expression was lost under serum starvation while that of XIAP was relatively higher when compared with XIAP expression at 10% FBS. However, nicotine increased expression of both genes, especially under serum starvation. This unexpected increase observed with XIAP expression under serum starvation may be due to the fact that XIAP could stop apoptosis induced either by viral infection or overexpression of primary cell death genes, caspases [90] via binding caspase 3, 7 and 9 [91]. Wilkinson *et al*. showed that deregulation of XIAP results in autoimmunity, neurodegenerative disorders and cancer [92]. This

result may be one of the explanations for heightened expression of XIAP under serum deprivation. Although deprived of serum, cancer cells might inherently increase XIAP which may have such a function to prevent cells from apoptosis at any cost. Moreover, in the development of lung cancer, the overexpression of XIAP not only inhibits caspase, but also stops the activity of cytochrome c (caspase activator) [90]. Therefore, it means that it interrupts the activity of mitochondria which is the energy supplier organelle of the cells. Taken together, XIAP can be resembled a blind-Samurai that fights for its rights at any cost by forcing cells not to enter the apoptosis when normal cells would. It would be interesting to perform similar experiments with normal colon cells under serum deprivation with or without nicotine exposure to see whether normal colon cells could escape this force.

In addition to real-time analyses of XIAP and SURVIVIN expression, changes of protein levels of Bax and p53 confirmed our findings obtained from the MTT results, the two apoptotic markers of cellular response were drastically downregulated in the presence of nicotine. It is known that nicotine induces cell proliferation in association with p53 down-regulation [93]. Moreover, according to a study regarding lung cancer and smoking relation in terms of p53 mutation it was shown that in lung cancers, the p53 mutational patterns differ between smokers and nonsmokers. such that smokers have frequent G to T transversions [94]. Besides p53, proapoptotic protein Bax expression was shown as downregulated by nicotine via inducing Bax phosphorylation and nicotine blocked stress-induced translocation of Bax from cytosol to mitochondria in lung cancer cells [34]. Although these studies suggests novel mechanisms regarding nicotine and apoptosis triggering proteins in different cell lines via using different approaches like triggering apoptosis via cisplatin, our study is the first showing nicotine also regulates the protein levels of these two proteins in SW620 cells.

5.3 Nicotine Effects upon Nicotinic Acetylcholine Receptors in SW620

As it has been mentioned in the introduction part, nicotine exerts its activity via nAChR [61]. nAChR forms hetero-pentamers on the cell membrane, although there are some preferred complexes such as nAChRα7 homo-pentamer or nAChRα4β2 pentamer in neuronal cells [52]. The regulation of nAChR subunit expressions is important for nicotine activity. To clarify the molecular mechanisms that are affected by nicotine, nAChRs should be well identified and discussed. As cholinergic receptors behave similar to each other in terms of receptor activity, they may have similar gene expression patterns and may have common regulatory elements (promoters, enhancers). To see their gene expression profile in response to serum deprivation and nicotine exposure, we filtered the nAChR probe sets from normalized microarray data and we obtained the gene expression values before clustering these genes for determination of coexpression tendency in terms of serum and nicotine conditions. As a result, nAChR genes did not change drastically with regard to serum addition and nicotine activity, except nAChRα5 (***Figure 4.17***). nAChRα5 significantly was affected by serum starvation and under poor serum conditions, nAChRα5 expression decreased independent of nicotine treatment in the SW620 cells. If we look at the heat map carefully, we can interpret that nAChRα5 has a very strong role in classifying normal serum treated samples versus serum deprived samples. On the other hand, nACHRα1 responded to nicotine under serum starvation. Previous studies classified the expression of lung cancer cell lines in terms of expression of nACHRs. No studies exist for the expression of nACHRs under serum starvation or nicotine exposure in colon cancer. Moreover, this is the first study that implicates nACHRα5 and α1 as differentially modulated genes by nicotine/ serum. Another interesting feature of the cluster analysis was that although nAChR genes have not strong expression values except nAChRα5, they can separate and cluster nicotine exposed samples and control samples under serum deprivation conditions. This suggested that although nicotine has not significant effect upon nAChR genes individually under serum depletion conditions, it actually may modulate the

cluster as a whole as if they were one. This suggestion also strengthens that nAChR genes may have common regulators at the transcriptional level.

5.4 Nicotine and Signaling Pathways

According to our Gene Set Enrichment Analysis and Pathway Miner studies of microarray experiments, many different signaling pathways including cell cycle, calcium signaling, cell adhesion, cell communication, ribosomal protein activity, purine/ pyrimidine metabolism, Alzheimer disease genes, and glucuronidation pathway genes were found to be modulated (***Appendix B***). Effect of nicotine on cell cycle regulation has been demonstrated in many previous studies [53, 78]. Accordingly, our data shows that in the nicotine-treated serum starved SW620 cells, the genes (like cyclins and CDCs) that support cell division were upregulated but the inhibitors (eg. ATR and CDKN2A) were downregulated and the expression profiles of these genes were significantly similar to the profile of normal serum treated cells. This may mean that nicotine may trick the starved cells as if they were not starving and force them to divide. The commitment to mitosis by nicotine exposure may result in tumor formation in normal cells and may result in malignancy and aggressiveness of benign tumors.

Other than cell cycle deregulation, nicotine also acts on ribosomal proteins' expression and regulation. According to our results of Pathway Miner, nicotine exposed serum starved cells and 10% serum treated cells act in parallel in terms of ribosomal protein expression profiles meaning both of these two conditions result in the down regulation of ribosomal protein encoding genes. Ribosomal proteins (RPs) are fundamental components, which assemble with four rRNA throughout a complex mechanism that takes place at different sites of the cell (nucleolus, nucleoplasm, cytoplasm, respectively), of ribosomes [95]. For a long time it was thought that RP regulation were controlled by growth factors, but recently different studies indicated that there are also some key signaling pathways that take place in RP mRNA

expression and translational regulation as PI3K, NK-κB, c-Myc and mTOR [96-99]. Although ribosome deficiencies due to RP gene mutations have been known for many years in *Drosophila* and *Xenopus* [100, 101], the scientists did not pay attention to these small proteins which were only considered as non-crucial subunits that play roles in formation of ribosome. But recent studies show that RP deregulation and mutations may result in very important defects. For instance, RPS6 conditional knock out in the liver of the mouse causes cell cycle block [102]. Besides, according to a very recent study RPL11 knock out experiments in zebrafish show that this gene is very important for embryonic development and its absence results in abnormalities in the brain and finally causes death through p53 dependent apoptotic response [103]. This protein also negatively regulates oncoprotein MDM2 via p53-dependent pathway [104]. Moreover, RPL11, RPL5, RPL23 and RPS7 were shown as MDM2 regulators via inhibiting its E3 ligase activity toward p53 tumor suppressor protein [105, 106]. Another RP protein, RPS3, was shown that its mRNA level is elevated in colorectal tumors [107], besides RPL15 and RPL19 in gastric cancer, and RPL7a and RPL37 in prostate cancer [108]. Nicotine has reduced RP expression to closer to the levels attained by the addition of 10% serum suggesting that nicotine and serum both might rescue from apoptosis/growth arrest induced by RP over-expression. The ribosomal protein genes such RPL10, RPS23, RPS11 and RPL36 are greatly downregulated via nicotine and they can be very important agents in terms of anti-cancer drug treatments.

In addition, nicotine treated serum starvation cells had upregulated genes in DNA polymerase activity and purine/ pyrimidine metabolism. The upregulation of these genes again supports that when cells under serum starvation, meaning poor growth factors in the media, nicotine mediates growth factor-less cell proliferation which may be possible via cell division without cell growth. This can be tested by enzymatic activities of growth and cell division regulating enzymes.

Another interesting data came up with the "Glucuronidation metabolism**"**, in which UDP-glucuronosyltransferase (UGT), a glycosyl transferase catalyzes the reaction [109]. This reaction basically involves addition of the sugars to lipids and to the other apolar xenobiotics and is an important step in the organism's elimination of foreign substances such as xenobiotic metabolism of substances such as drugs, pollutants, bilirubin, androgens, estrogens, mineralocorticoids, glucocorticoids, fatty acid derivatives, retinoids, and bile acids as well as endogenous substances including endogenous toxins [110]. The genes belong to this metabolic pathway were significantly upregulated under nicotine treated serum starvation circumstances, in comparison with the addition of 10% serum. Although in the previously mentioned pathways, the gene expression profiles of nicotine treated serum starvation and of normal serum treated cells were acting in parallel, the genes of glucuronidation were totally acting in the opposite direction. The most significant gene, UGT1A6, was strongly upregulated under nicotine treated serum deprivation, but drastically downregulated under physiological serum conditions. Although there are not much study regarding UGT1A6 and colorectal cancers, it was shown that UGT1A6 variants influence colorectal carcinogenesis neoplasia recurrence and decreases the risk of recurrence [111]. This data may be important in terms of showing that UGT family (especially UGT1A6) genes have important role in tumorigenesis prevention. According to our data UGT1A6 upregulation under nicotine exposed serum starvation condition may show that UGT1A6 may specifically target nicotinic effect in the cell. Therefore, UGT1A6 may be a very important for repressing nicotine deregulation of the cell signaling.

CHAPTER 6. FUTURE PERSPECTIVES

In the present study, we showed that 1μM nicotine regulates cell proliferation under serum starvation conditions via deregulating many different families of genes (Section 4.1.2). The future studies regarding the results of this project will focus on the molecular mechanisms of the significantly regulated genes by nicotine.

One of the future perspectives will be the understanding the nicotine and nAChRs relationship in the colon cancer cell lines. Although we showed that nicotine has a proliferative effect on SW620 cell lines under serum starvation conditions, we do not know that which subunits of nAChRs are important for nicotine activity. Even, we do not know surely that whether nicotine act only through these receptors. Therefore, experiments regarding the inactivation of nAChRs should be done. The common ligand binding domains of these receptors can be blocked by chemical drugs and this may prevent nicotine binding. Another approach may be using siRNA technique to silence specific nAChRs and see whether nicotine activity differs. nAChRα1 and nAChRα5 are two good candidates for siRNA experiments. On the contrary, also these candidate receptors can be overexpressed in the colon cancer cells and nicotine effect can be compared to the receptor silenced or normal cell behavior.

Another future approach may be using different cancer cell lines in order to screen nicotine's effect on different cancer types. In addition, normal cell lines should be exposed to nicotine and compared to cancer cells in terms of cellular activity and cell proliferation targeting specifically regulated genes by nicotine according to this study. As we studied with only one type of colon cancer cell, some part of our results may be specific to SW620 cell line and in order to get a more reliable data about nicotine and activity on colon cancer/tissue, using many different types of cells can be a good idea.

As it was mentioned before, nicotine regulates apoptosis and may downregulate important proapototic genes (such as Bax). Although we showed that nicotine has a obvious effect on these

kinds of genes via real time RT-PCR and western blot (although these experiments are preliminary) with SW620 colon cancer cells, we did not test nicotine's activity against other apoptotic conditions such as UV and drug treatment. Therefore, it would be a very good approach to trigger apoptosis and then test the nicotine effect in terms of rescuing cells from apoptosis via different experimental setups like TUNEL assay, FACS analysis, caspase assays or using different apoptosis detection kits.

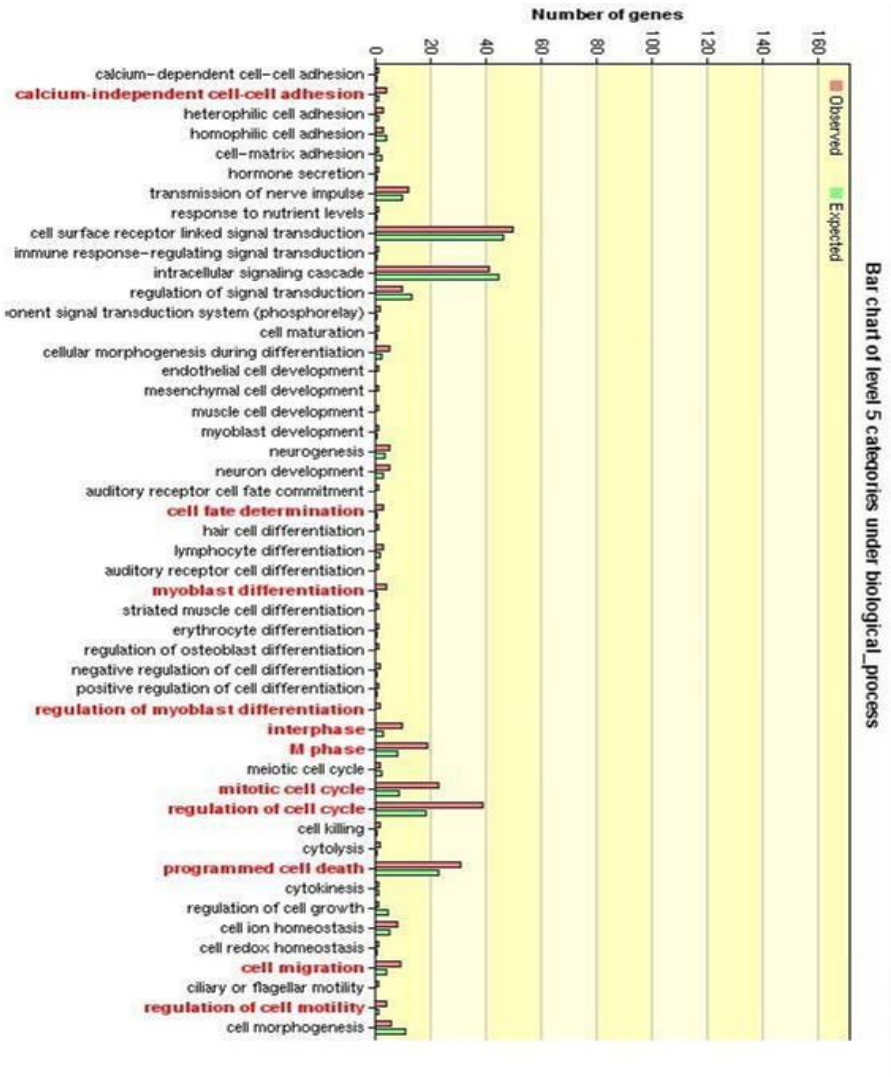
Throughout our microarray analyses, we showed that there are thousands of genes affected by nicotine activity under serum starvation conditions, more replicates of each condition may be necessary for conclusive results. Furthermore, there many interesting gene families arose as targets of nicotine under serum starvation; among these genes are proteasomes, ribosomal proteins, specific miRNA targets, cell cycle genes, MAPK signaling genes. Candidate genes will be selected among this rich informative data and they will be used in order to catch some key features to enlighten the mechanisms that are regulated by nicotine.

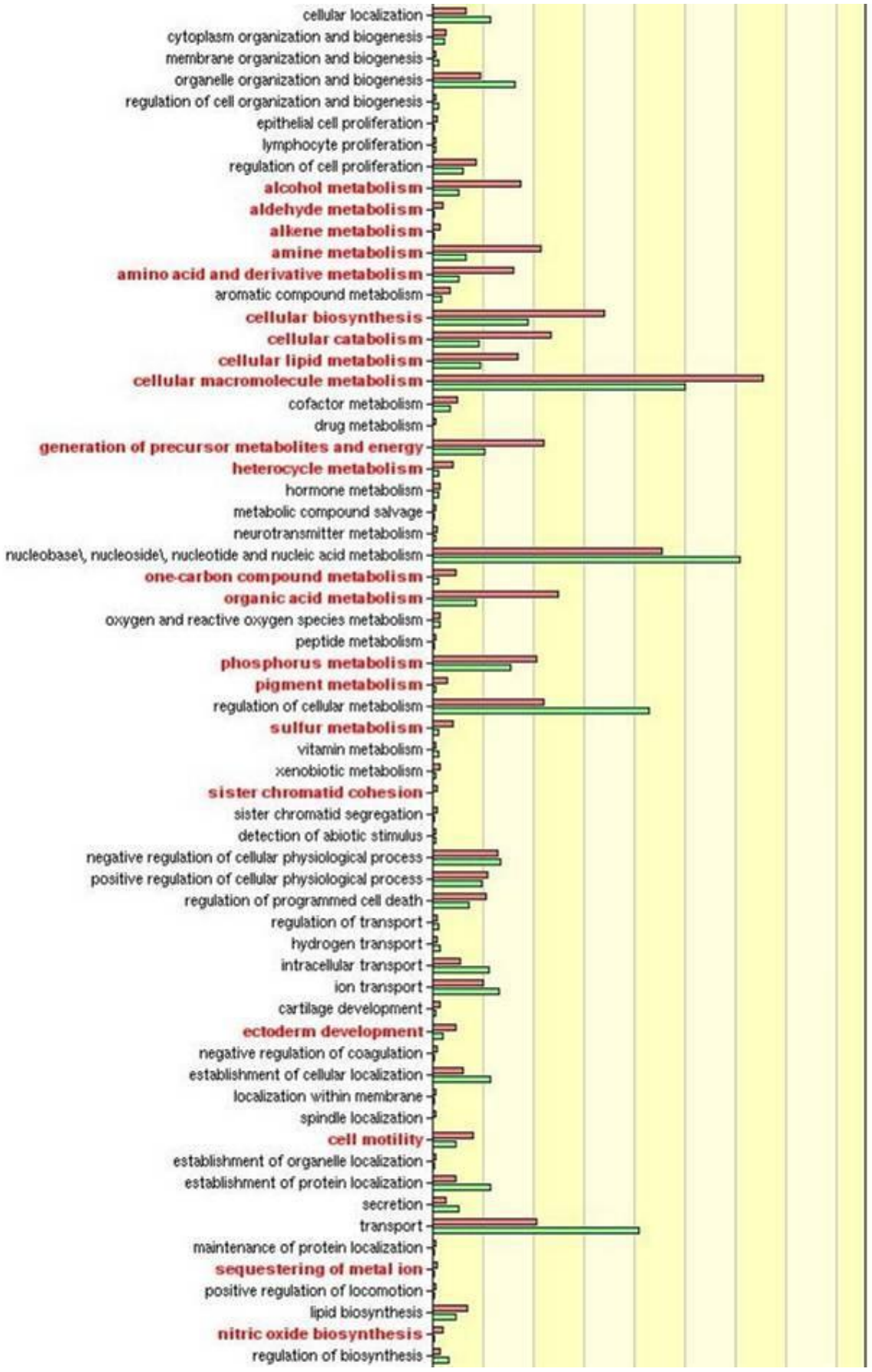
In addition, different time courses for nicotine exposure may help to understand the behavior of nicotine under short and long times. As cancers do not develop rapidly but it takes years to develop a tumor on a tissue, treating the cells with small concentrations of nicotine for very long periods may give better ideas about nicotine role in development of colon cancer. For a starting experiment, nicotine treatment for 7 days microarray experiments can be considered as a further step to the 48 h microarray experiments as we have very significant results at 7 days under nicotine treatment in serum starved cells.

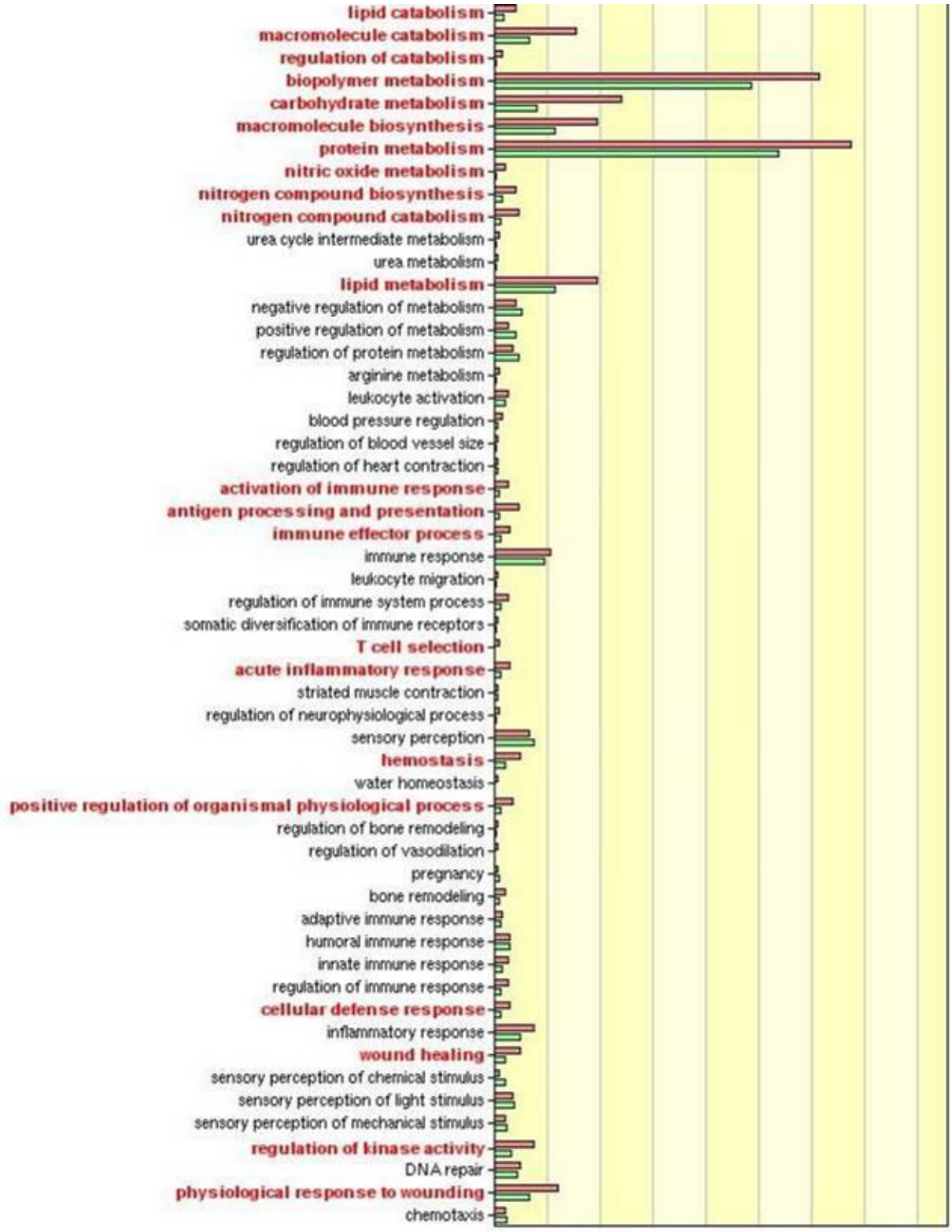
APPENDICES

APPENDIX A: The results of the gene set enrichment analysis of the significant gene lists that generated by comparing the expression profiles of the nicotine treated serum starved cells to the cells grown under physiological serum level

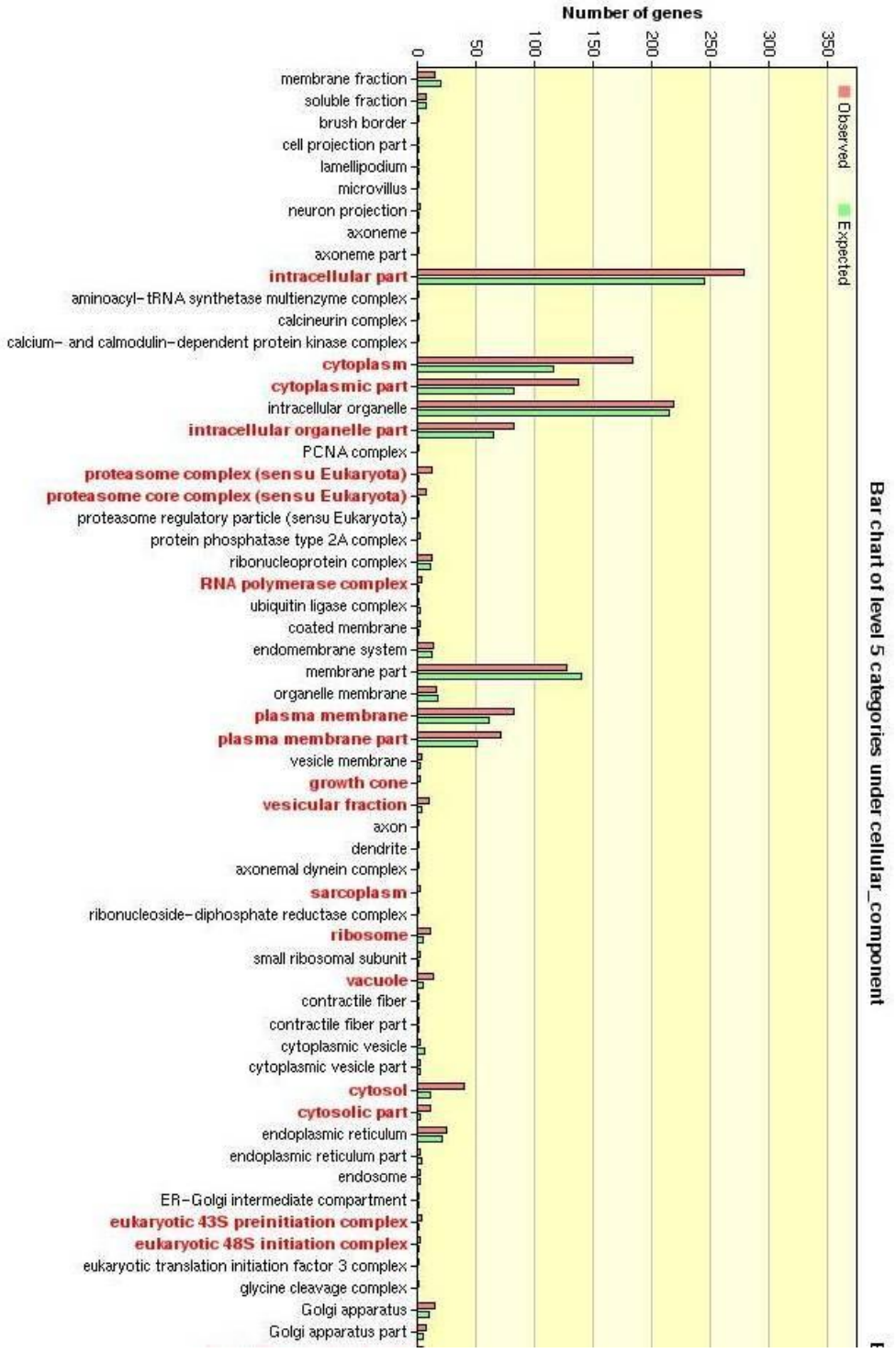
Appendix A1: The significant genes act in biological process pathways. The significant genes, of nicotine effect in comparison with serum starvation, act in biological process, listed according to the results of gene set enrichment analysis of comparison of nicotine treated samples with serum starvation conditions. The figure generated via "Webgestalt" tool. The pathways shown in red represent significance statue (p-value<0.05).

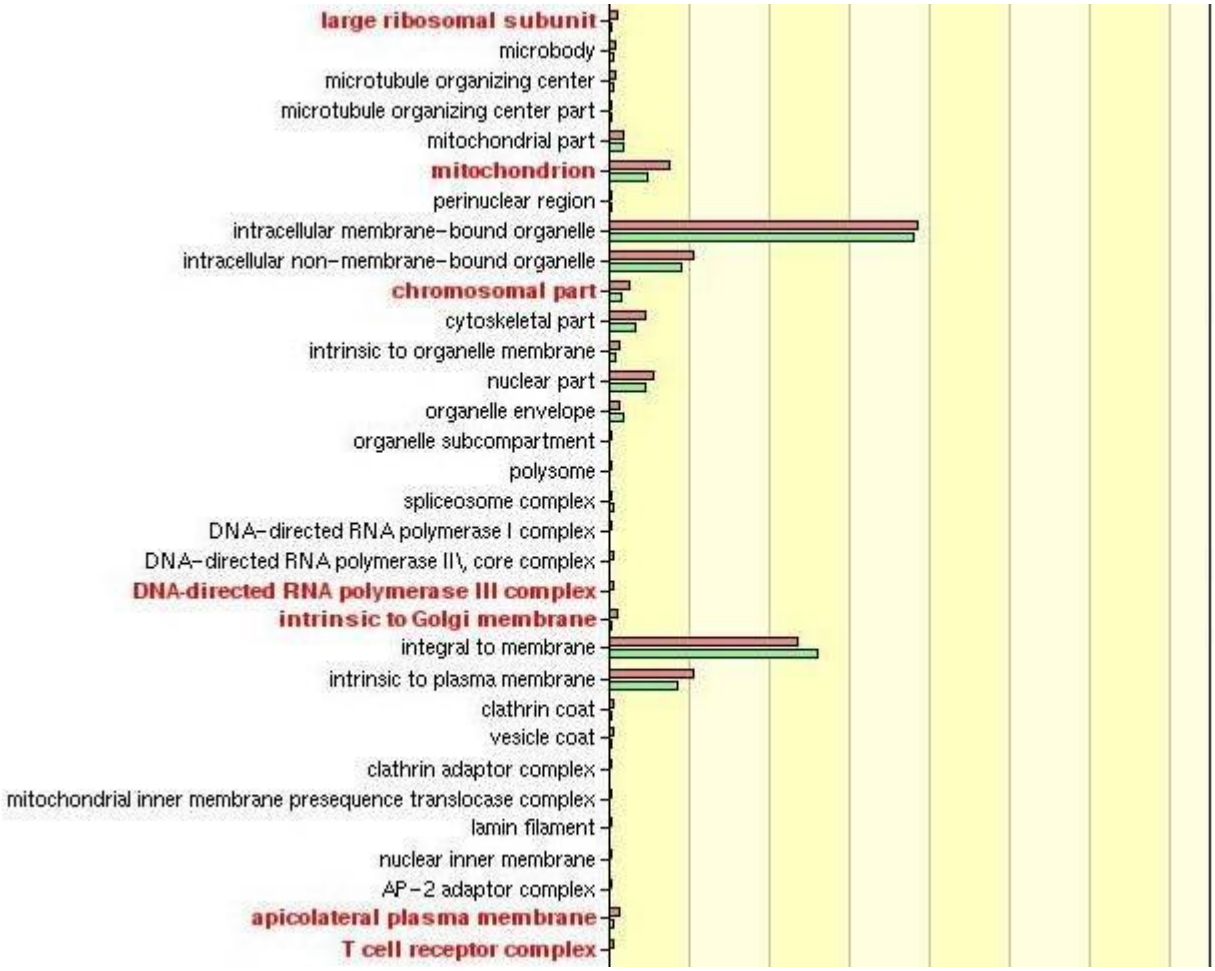


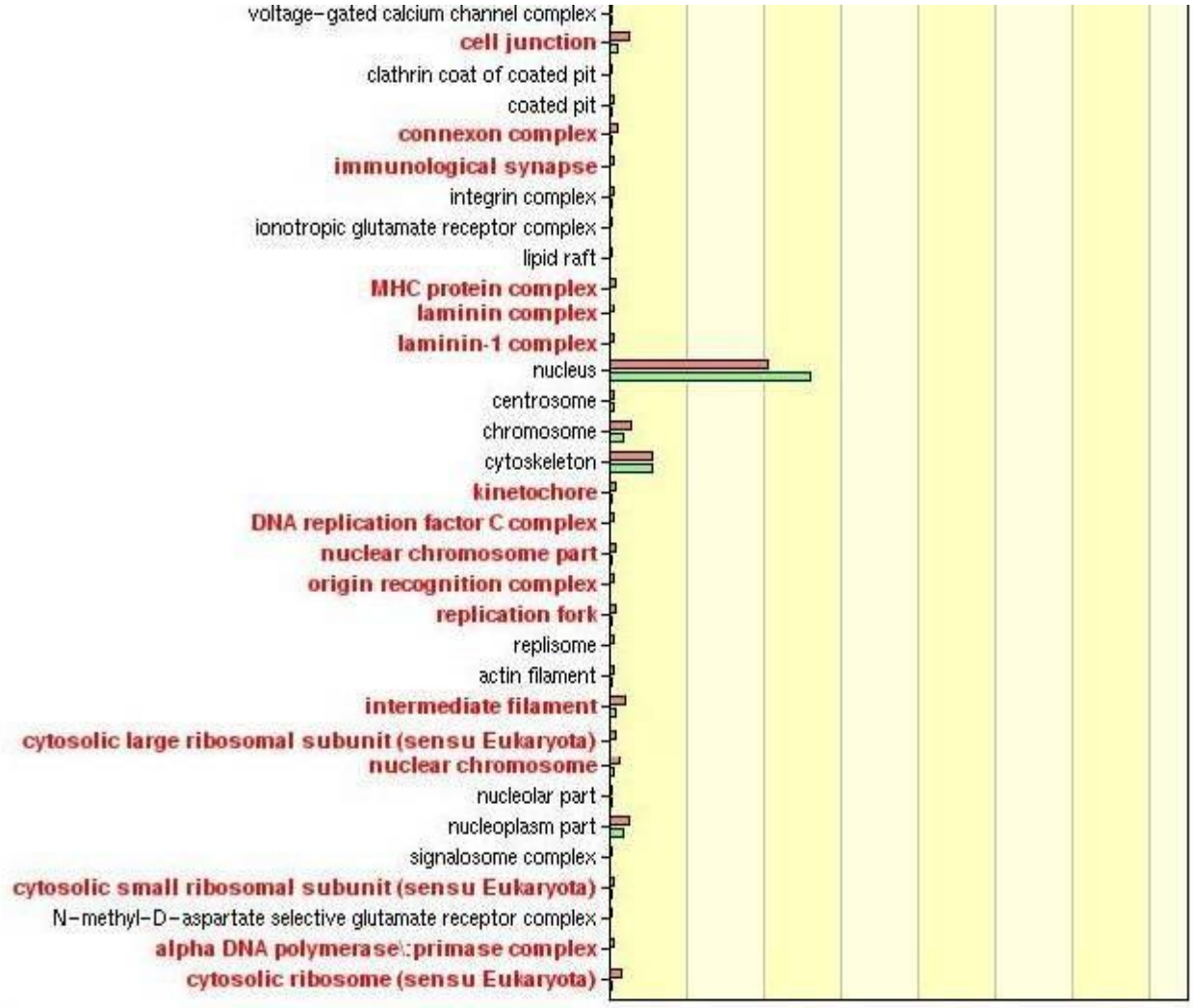

cellular localization
cytoplasm organization and biogenesis
membrane organization and biogenesis
organelle organization and biogenesis
regulation of cell organization and biogenesis
epithelial cell proliferation
lymphocyte proliferation
regulation of cell proliferation
alcohol metabolism
aldehyde metabolism
alkene metabolism
amine metabolism
amino acid and derivative metabolism
aromatic compound metabolism
cellular biosynthesis
cellular catabolism
cellular lipid metabolism
cellular macromolecule metabolism
cofactor metabolism
drug metabolism
generation of precursor metabolites and energy
heterocycle metabolism
hormone metabolism
metabolic compound salvage
neurotransmitter metabolism
nucleobase\, nucleoside\, nucleotide and nucleic acid metabolism
one-carbon compound metabolism
organic acid metabolism
oxygen and reactive oxygen species metabolism
peptide metabolism
phosphorus metabolism
pigment metabolism
regulation of cellular metabolism
sulfur metabolism
vitamin metabolism
xenobiotic metabolism
sister chromatid cohesion
sister chromatid segregation
detection of abiotic stimulus
negative regulation of cellular physiological process
positive regulation of cellular physiological process
regulation of programmed cell death
regulation of transport
hydrogen transport
intracellular transport
ion transport
cartilage development
ectoderm development
negative regulation of coagulation
establishment of cellular localization
localization within membrane
spindle localization
cell motility
establishment of organelle localization
establishment of protein localization
secretion
transport
maintenance of protein localization
sequestering of metal ion
positive regulation of locomotion
lipid biosynthesis
nitric oxide biosynthesis
regulation of biosynthesis


lipid catabolism
macromolecule catabolism
regulation of catabolism
biopolymer metabolism
carbohydrate metabolism
macromolecule biosynthesis
protein metabolism
nitric oxide metabolism
nitrogen compound biosynthesis
nitrogen compound catabolism
urea cycle intermediate metabolism
urea metabolism
lipid metabolism
negative regulation of metabolism
positive regulation of metabolism
regulation of protein metabolism
arginine metabolism
leukocyte activation
blood pressure regulation
regulation of blood vessel size
regulation of heart contraction
activation of immune response
antigen processing and presentation
immune effector process
immune response
leukocyte migration
regulation of immune system process
somatic diversification of immune receptors
T cell selection
acute inflammatory response
striated muscle contraction
regulation of neurophysiological process
sensory perception
hemostasis
water homeostasis
positive regulation of organismal physiological process
regulation of bone remodeling
regulation of vasodilation
pregnancy
bone remodeling
adaptive immune response
humoral immune response
innate immune response
regulation of immune response
cellular defense response
inflammatory response
wound healing
sensory perception of chemical stimulus
sensory perception of light stimulus
sensory perception of mechanical stimulus
regulation of kinase activity
DNA repair
physiological response to wounding
chemotaxis

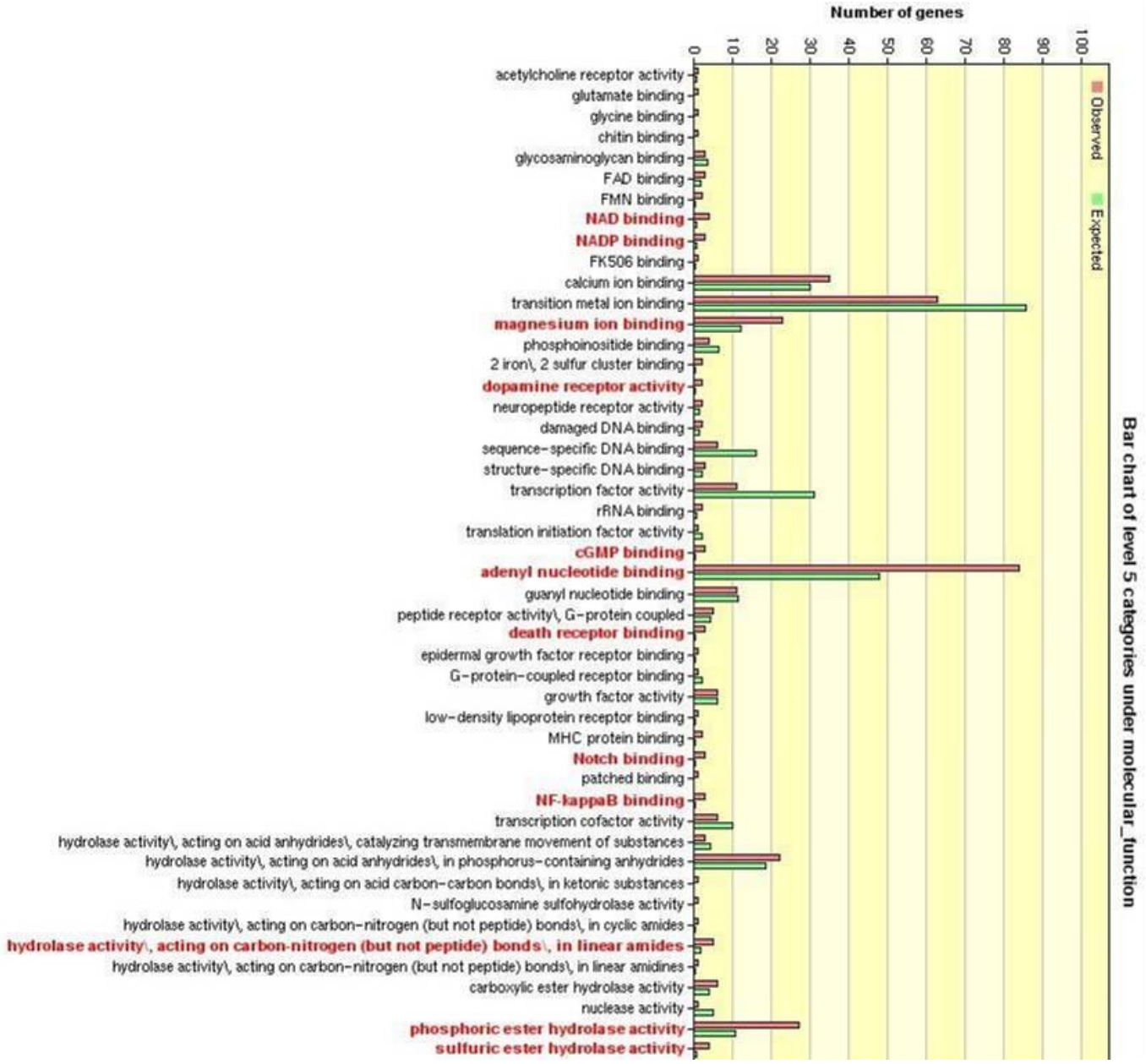
Appendix A2: The significant genes act in cellular component pathways. The significant genes, of nicotine effect in comparison with serum starvation, act in cellular component, listed according to the results of gene set enrichment analysis of comparison of nicotine treated samples with serum starvation conditions. The figure generated via "Webgestalt" tool. The pathways shown in red represent significance statue (p-value<0.05).

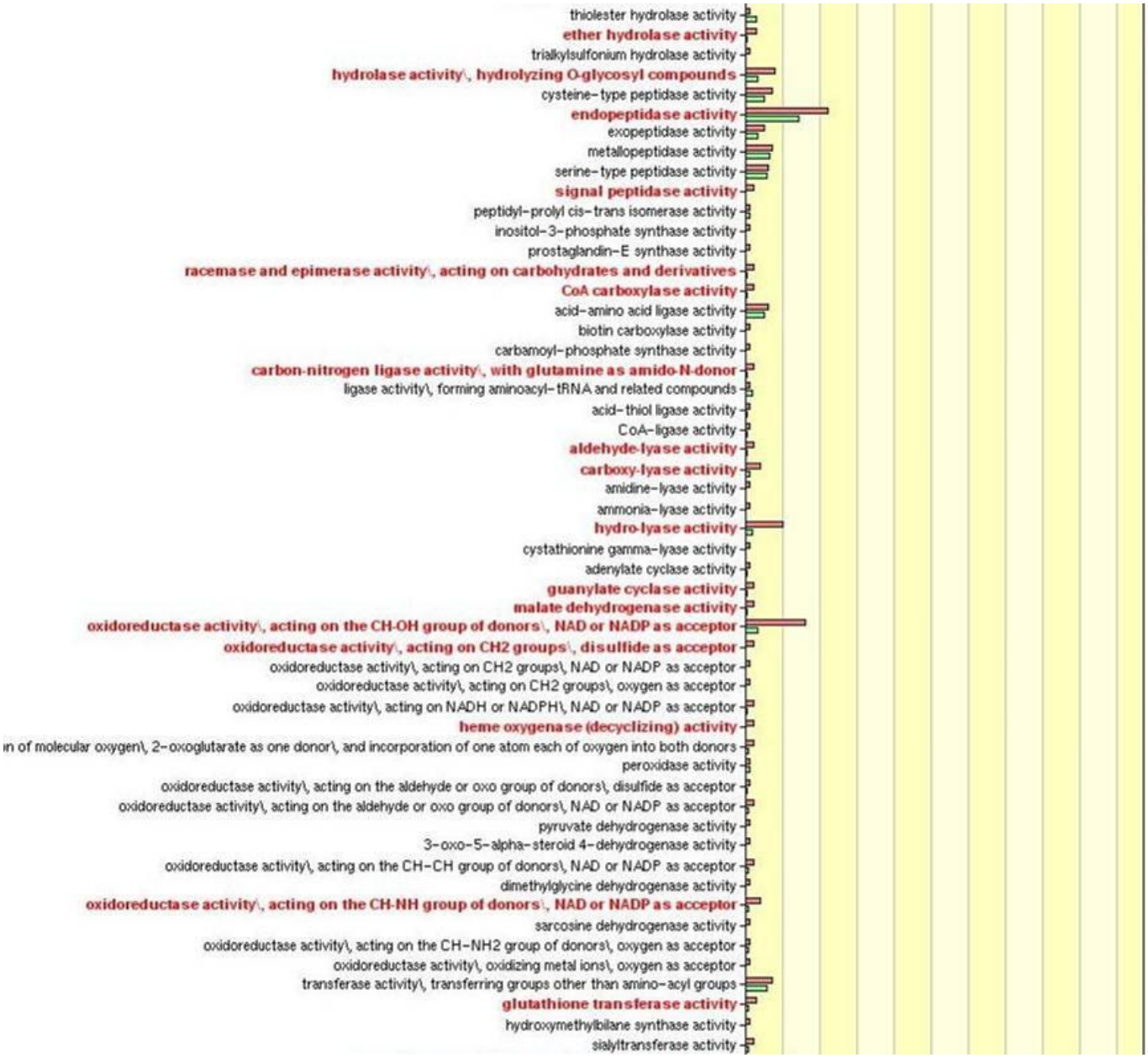


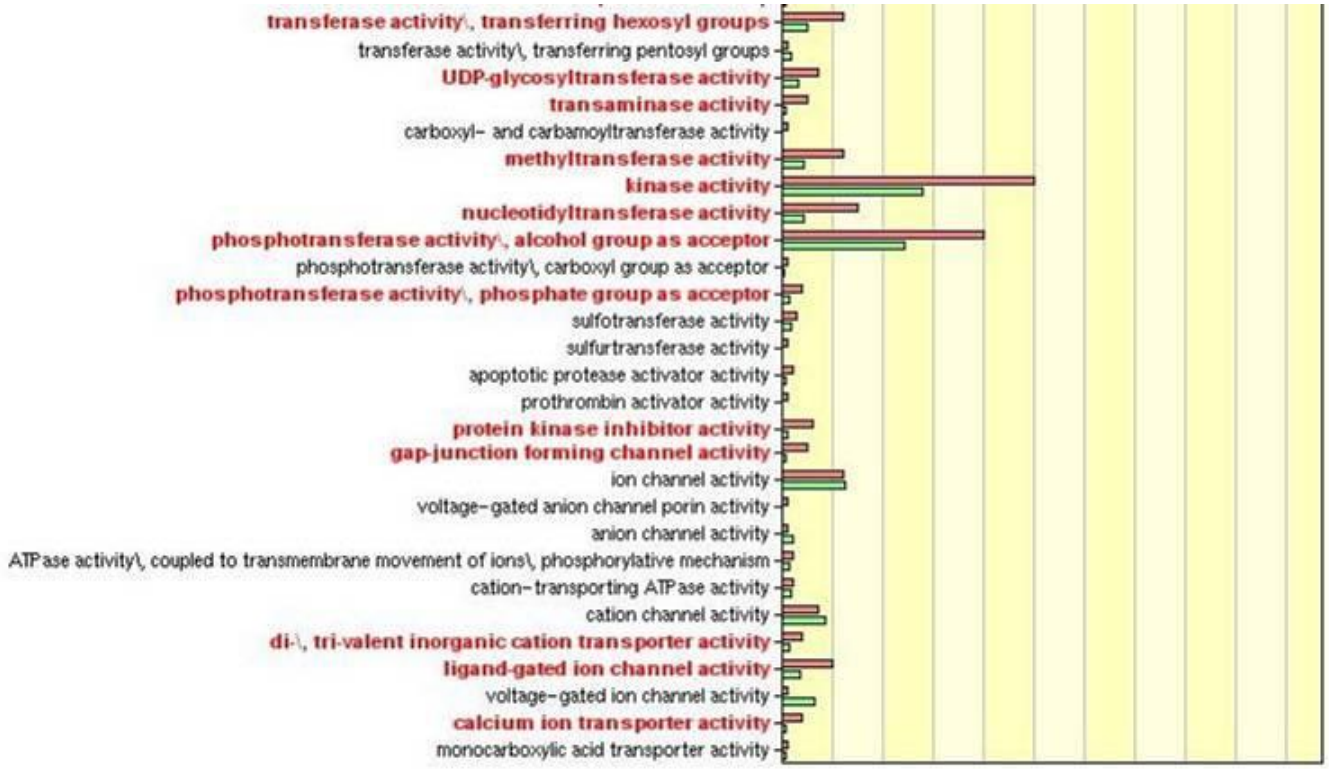

large ribosomal subunit
microbody
microtubule organizing center
microtubule organizing center part
mitochondrial part
mitochondrion
perinuclear region
intracellular membrane-bound organelle
intracellular non-membrane-bound organelle
chromosomal part
cytoskeletal part
intrinsic to organelle membrane
nuclear part
organelle envelope
organelle subcompartment
polysome
spliceosome complex
DNA-directed RNA polymerase I complex
DNA-directed RNA polymerase II\, core complex
DNA-directed RNA polymerase III complex
intrinsic to Golgi membrane
integral to membrane
intrinsic to plasma membrane
clathrin coat
vesicle coat
clathrin adaptor complex
mitochondrial inner membrane presequence translocase complex
lamin filament
nuclear inner membrane
AP-2 adaptor complex
apicolateral plasma membrane
T cell receptor complex


voltage-gated calcium channel complex
cell junction
clathrin coat of coated pit
coated pit
connexon complex
immunological synapse
integrin complex
ionotropic glutamate receptor complex
lipid raft
MHC protein complex
laminin complex
laminin-1 complex
nucleus
centrosome
chromosome
cytoskeleton
kinetochore
DNA replication factor C complex
nuclear chromosome part
origin recognition complex
replication fork
replisome
actin filament
intermediate filament
cytosolic large ribosomal subunit (sensu Eukaryota)
nuclear chromosome
nucleolar part
nucleoplasm part
signalosome complex
cytosolic small ribosomal subunit (sensu Eukaryota)
N-methyl-D-aspartate selective glutamate receptor complex
alpha DNA polymerase\:primase complex
cytosolic ribosome (sensu Eukaryota)

Appendix A3: The significant genes act in molecular function pathways. The significant genes, of nicotine effect in comparison with serum starvation, act in molecular process, listed according to the results of gene set enrichment analysis of comparison of nicotine treated samples with serum starvation conditions. The figure generated via "Webgestalt" tool. The pathways shown in red represent significance statue (p-value<0.05).




thiolester hydrolase activity
ether hydrolase activity
trialkylsulfonium hydrolase activity
hydrolase activity\, hydrolyzing O-glycosyl compounds
cysteine-type peptidase activity
endopeptidase activity
exopeptidase activity
metallopeptidase activity
serine-type peptidase activity
signal peptidase activity
peptidyl-prolyl cis-trans isomerase activity
inositol-3-phosphate synthase activity
prostaglandin-E synthase activity
racemase and epimerase activity\, acting on carbohydrates and derivatives
CoA carboxylase activity
acid-amino acid ligase activity
biotin carboxylase activity
carbamoyl-phosphate synthase activity
carbon-nitrogen ligase activity\, with glutamine as amido-N-donor
ligase activity\, forming aminoacyl-tRNA and related compounds
acid-thiol ligase activity
CoA-ligase activity
aldehyde-lyase activity
carboxy-lyase activity
amidine-lyase activity
ammonia-lyase activity
hydro-lyase activity
cystathionine gamma-lyase activity
adenylate cyclase activity
guanylate cyclase activity
malate dehydrogenase activity
oxidoreductase activity\, acting on the CH-OH group of donors\, NAD or NADP as acceptor
oxidoreductase activity\, acting on CH2 groups\, disulfide as acceptor
oxidoreductase activity\, acting on CH2 groups\, NAD or NADP as acceptor
oxidoreductase activity\, acting on CH2 groups\, oxygen as acceptor
oxidoreductase activity\, acting on NADH or NADPH\, NAD or NADP as acceptor
heme oxygenase (decyclizing) activity
n of molecular oxygen\, 2-oxoglutarate as one donor\, and incorporation of one atom each of oxygen into both donors
peroxidase activity
oxidoreductase activity\, acting on the aldehyde or oxo group of donors\, disulfide as acceptor
oxidoreductase activity\, acting on the aldehyde or oxo group of donors\, NAD or NADP as acceptor
pyruvate dehydrogenase activity
3-oxo-5-alpha-steroid 4-dehydrogenase activity
oxidoreductase activity\, acting on the CH-CH group of donors\, NAD or NADP as acceptor
dimethylglycine dehydrogenase activity
oxidoreductase activity\, acting on the CH-NH group of donors\, NAD or NADP as acceptor
sarcosine dehydrogenase activity
oxidoreductase activity\, acting on the CH-NH2 group of donors\, oxygen as acceptor
oxidoreductase activity\, oxidizing metal ions\, oxygen as acceptor
transferase activity\, transferring groups other than amino-acyl groups
glutathione transferase activity
hydroxymethylbilane synthase activity
sialyltransferase activity


transferase activity\, transferring hexosyl groups
transferase activity\, transferring pentosyl groups
UDP-glycosyltransferase activity
transaminase activity
carboxyl- and carbamoyltransferase activity
methyltransferase activity
kinase activity
nucleotidyltransferase activity
phosphotransferase activity\, alcohol group as acceptor
phosphotransferase activity\, carboxyl group as acceptor
phosphotransferase activity\, phosphate group as acceptor
sulfotransferase activity
sulfurtransferase activity
apoptotic protease activator activity
prothrombin activator activity
protein kinase inhibitor activity
gap-junction forming channel activity
ion channel activity
voltage-gated anion channel porin activity
anion channel activity
ATPase activity\, coupled to transmembrane movement of ions\, phosphorylative mechanism
cation-transporting ATPase activity
cation channel activity
di-\, tri-valent inorganic cation transporter activity
ligand-gated ion channel activity
voltage-gated ion channel activity
calcium ion transporter activity
monocarboxylic acid transporter activity

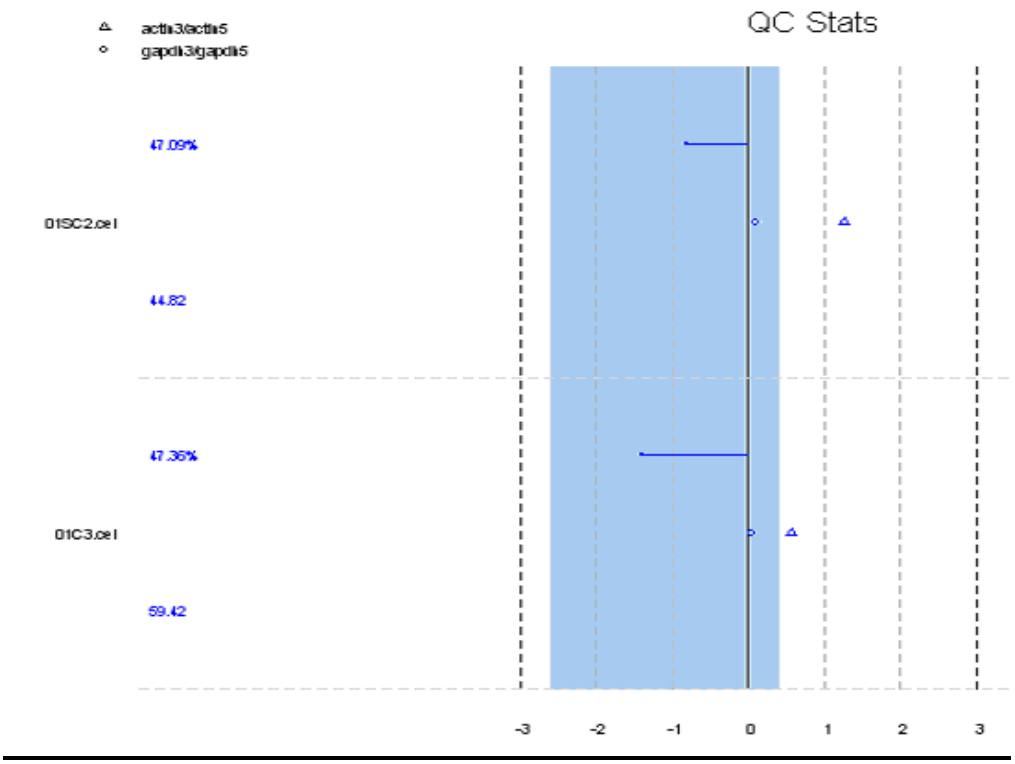
APPENDIX B: The KEGG pathway list of the significant genes that are generated by comparing the expression profiles of the nicotine treated serum starved cells to the untreated serum starved cells by the gene set enrichment analysis

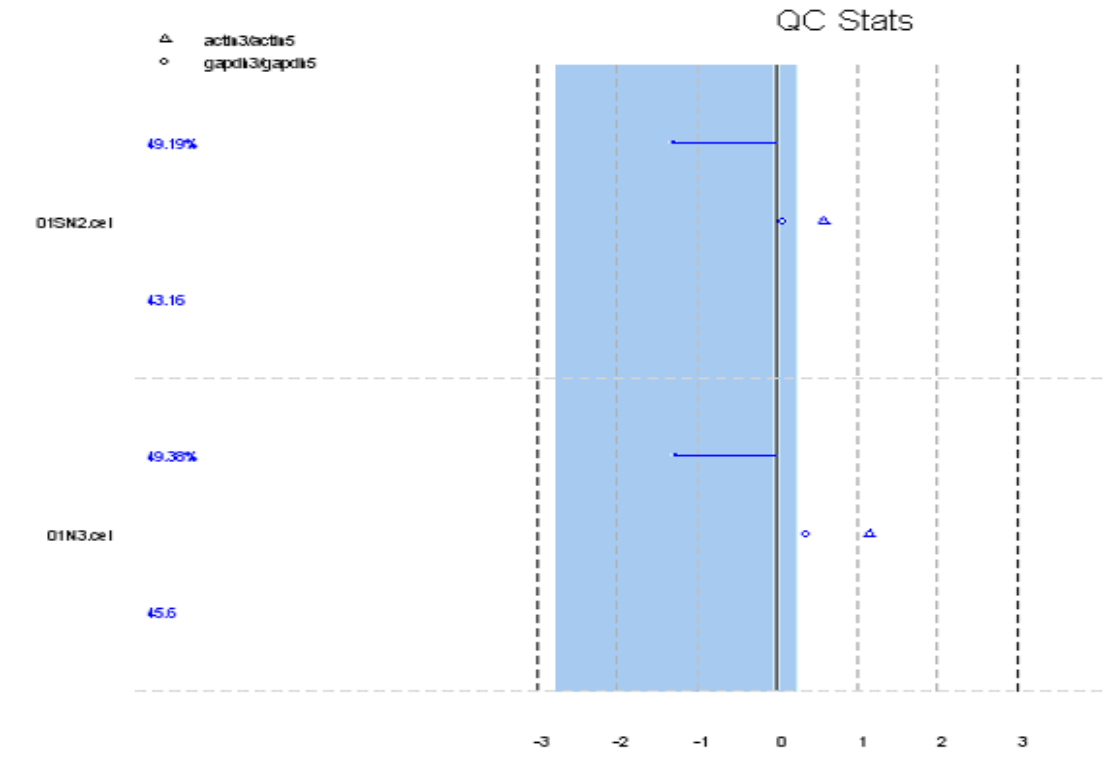
	Kegg Pathway	Pathway description	Number of genes	LS permutation p-value	KS permutation p-value
1	hsa03010	Ribosome	219	0.00001	0.00001
2	hsa04110	Cell cycle	274	0.00001	0.00076
3	hsa04940	Type I diabetes mellitus	123	0.00001	0.00019
4	hsa03050	Proteasome	56	0.00007	0.00003
5	hsa04540	Gap junction	281	0.00048	0.02811
6	hsa00860	Porphyrin and chlorophyll metabolism	65	0.00178	0.00127
7	hsa00460	Cyanoamino acid metabolism	25	0.00437	0.03776
8	hsa00500	Starch and sucrose metabolism	128	0.005	0.01821
9	hsa00362	Benzoate degradation via hydroxylation	7	0.00582	0.03093
10	hsa01032	Glycan structures - degradation	71	0.00785	0.00578
11	hsa04612	Antigen processing and presentation	210	0.01045	0.19397
12	hsa00240	Pyrimidine metabolism	182	0.01081	0.1912
13	hsa04210	Apoptosis	218	0.01127	0.05984
14	hsa00600	Sphingolipid metabolism	89	0.01363	0.00272
15	hsa00040	Pentose and glucuronate interconversions	33	0.01494	0.01643
16	hsa00670	One carbon pool by folate	45	0.01886	0.15329
17	hsa00511	N-Glycan degradation	26	0.02655	0.01597
18	hsa00980	Metabolism of xenobiotics by cytochrome P450	125	0.02695	0.00114
19	hsa01430	Cell Communication	257	0.0271	0.02781
20	hsa00643	Styrene degradation	7	0.03211	0.17557
21	hsa00430	Taurine and hypotaurine metabolism	24	0.0335	0.06485
22	hsa03030	DNA polymerase	48	0.03395	0.04967
23	hsa00271	Methionine metabolism	31	0.03497	0.1594
24	hsa00480	Glutathione metabolism	72	0.03771	0.05048
25	hsa00532	Chondroitin sulfate biosynthesis	38	0.03911	0.0051
26	hsa04514	Cell adhesion molecules (CAMs)	358	0.03963	0.31504
27	hsa00620	Pyruvate metabolism	82	0.04311	0.01934
28	hsa00530	Aminosugars metabolism	54	0.04367	0.17379
29	hsa00790	Folate biosynthesis	81	0.04544	0.06403
30	hsa03060	Protein export	22	0.04562	0.06916
31	hsa00930	Caprolactam degradation	37	0.046	0.02581
32	hsa04650	Natural killer cell mediated cytotoxicity	319	0.04881	0.41028
33	hsa00150	Androgen and estrogen metabolism	87	0.0499	0.40821
34	hsa00230	Purine metabolism	329	0.04991	0.22131
35	hsa00904	Diterpenoid biosynthesis	7	0.05539	0.13764
36	hsa00062	Fatty acid elongation in mitochondria	16	0.0633	0.32756
37	hsa00220	Urea cycle and metabolism of amino groups	50	0.06802	0.08224
38	hsa00521	Streptomycin biosynthesis	18	0.08982	0.17166
39	hsa00531	Glycosaminoglycan degradation	45	0.09137	0.12173
40	hsa00052	Galactose metabolism	59	0.09848	0.35361
41	hsa00950	Alkaloid biosynthesis I	10	0.11526	0.09158

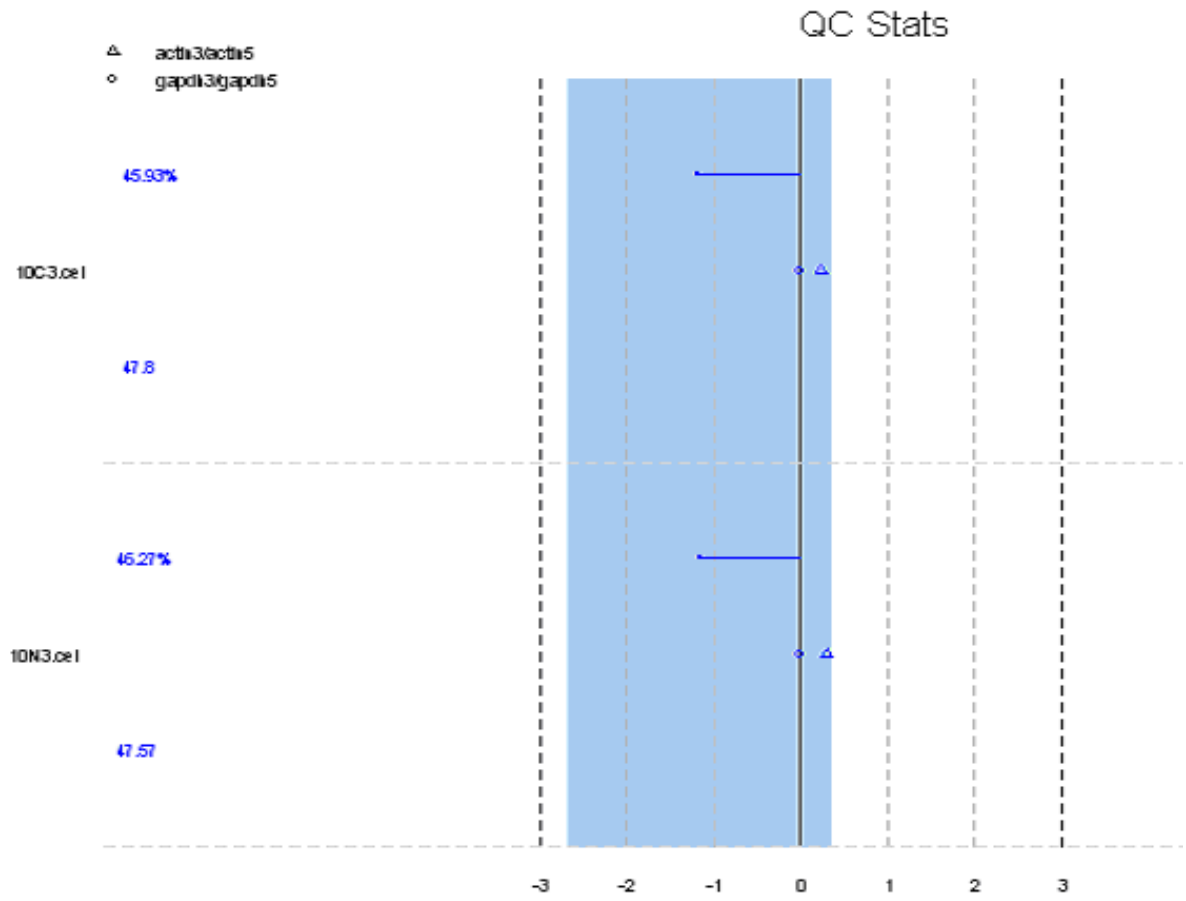
	Kegg Pathway	Pathway description	Number of genes	LS permutation p-value	KS permutation p-value
42	hsa00940	Stilbene, coumarine and lignin biosynthesis	18	0.11827	0.33383
43	hsa00920	Sulfur metabolism	31	0.12827	0.06269
44	hsa04730	Long-term depression	211	0.13289	0.15454
45	hsa00260	Glycine, serine and threonine metabolism	98	0.13673	0.21129
46	hsa00640	Propanoate metabolism	78	0.14639	0.00829
47	hsa00590	Arachidonic acid metabolism	107	0.16531	0.11577
48	hsa00602	Glycosphingolipid biosynthesis - neo-lactoseries	50	0.17407	0.26578
49	hsa00360	Phenylalanine metabolism	61	0.17846	0.09649
50	hsa00310	Lysine degradation	114	0.18368	0.20991
51	hsa00830	Retinol metabolism	6	0.18508	0.13715
52	hsa00410	beta-Alanine metabolism	52	0.19474	0.0146
53	hsa00903	Limonene and pinene degradation	76	0.19822	0.04336
54	hsa00051	Fructose and mannose metabolism	115	0.19901	0.16073
55	hsa00340	Histidine metabolism	86	0.21398	0.15742
56	hsa04720	Long-term potentiation	186	0.22095	0.13386
57	hsa05040	Huntington	94	0.22645	0.09444
58	hsa00791	Atrazine degradation	11	0.23033	0.51708
59	hsa04950	Maturity onset diabetes of the young	46	0.2693	0.32074
60	hsa00450	Selenoamino acid metabolism	76	0.3249	0.28904
61	hsa04330	Notch signaling pathway	118	0.32723	0.43381
62	hsa04120	Ubiquitin mediated proteolysis	116	0.33481	0.49485
63	hsa05130	NA	66	0.38437	0.54156
64	hsa05131	NA	66	0.38437	0.54156
65	hsa03022	Basal transcription factors	67	0.45139	0.25404
66	hsa00363	Bisphenol A degradation	35	0.45805	0.75343
67	hsa05010	Alzheimer	66	0.4855	0.3627
68	hsa04020	Calcium signaling pathway	478	0.49788	0.43331
69	hsa01031	Glycan structures - biosynthesis 2	164	0.52345	0.17911
70	hsa00910	Nitrogen metabolism	55	0.52558	0.73335
71	hsa00533	Keratan sulfate biosynthesis	37	0.53564	0.09225
72	hsa04610	Complement and coagulation cascades	135	0.55709	0.15736
73	hsa00710	Carbon fixation	58	0.67427	0.53794
74	hsa04340	Hedgehog signaling pathway	130	0.72643	0.55913
75	hsa00561	Glycerolipid metabolism	128	0.84407	0.48472

APPENDIX C: Microarray post-hybrizdization quality control results

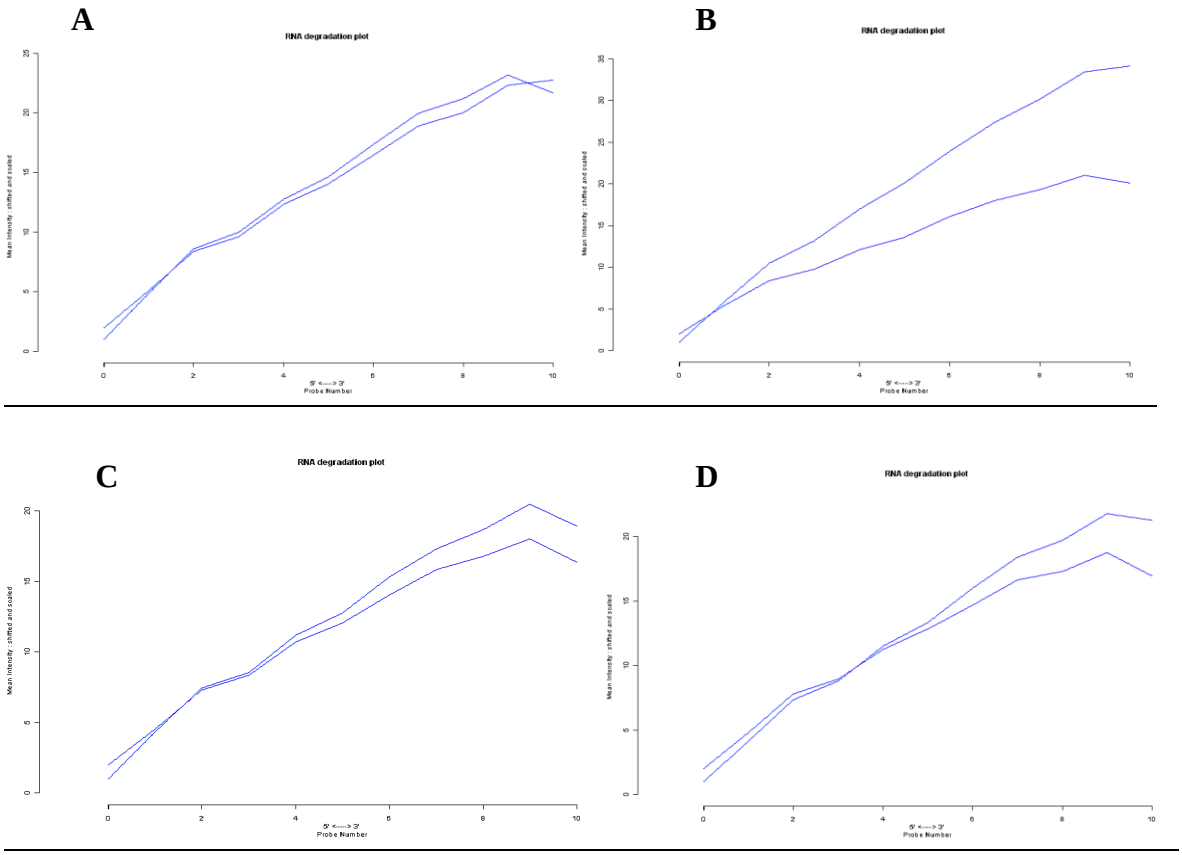
Appendix C1: Quality Control Plots






QC Stats
actin3/actin5
gapdh3/gapdh5
45.93%
10C3.cel
47.8
45.27%
10N3.cel
47.57
-3
-2
-1
0
1
2
3

Appendix C2.a: RNA Degradation Plots



Appendix C2.b: The data associated with the RNA degradation plot indicating significance levels

	0.1% FBS Control Set3	0.1% FBS Control Set2	0.1% FBS 1uM Nicotine Set3	0.1% FBS 1uM Nicotine Set2	10% FBS Control Set3	10% FBS 1uM Nicotine Set3
slope	2.17	2.09	3.36	1.87	1.57	2.11
pvalue	0	0	0	0	1.00E-06	0

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