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Investigating non-small cell lung cancer epithelial cell immune properties *ex vivo*

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Tekijä(t) Otsikko Sivumäärä Aika	Una Ojanen Ei pienisoluisen keuhkosyövän epiteelisolujen immunologiset ominaisuudet ex vivo- olosuhteissa 38 sivua + 2 liitettä 20.4.2017
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Ohjaaja(t)	Lehtori Hannele Pihlaja Tutkimuksen johtaja Emmy Verschuren Post doc tutkija Jennifer Devlin Tohtoriopiskelija Ashwini Nagaraj

Tämä projekti toteutettiin Suomen molekyylilääketieteen instituutissa (FIMM) Emmy Verschurenin tutkimusryhmässä. Ryhmä tutkii keuhkosyöpää ja keskittyy keuhkosyöpään liittyvien tuumorisuppressorien kemiallisten ja biologisten ominaisuuksien tutkimiseen. Ryhmä käyttää tätä tietoa luodakseen parempia keuhkosyöpämalleja, jotka mahdollistavat uusien sairauksien ehkäisyyn tähtäävien strategioiden kehityksen. (Verschuren group.)

Tämän projektin tarkoituksena oli selvittää, vaihteleeko immunologisten geenien ilmentyminen hiirien *Kras;Lkb1*-geenien muutosten aikaan saamissa ei-pienisoluisen keuhkosyövän kasvaimissa. Kasvainsoluviljelmät olivat luotu ja viljelty uudelleenohjelmoituina epiteelisoluina (conditionally reprogrammed cell) perustuen kasvaimien solualkuperään (CC10+ ja SPC+). Projektissa vertaillut geenit olivat *Interleukiini 1β (Il-1β), Arginaasi 1 (Arg1), Interleukiini 6 (Il-6,) Cxcl5* ja *Ccl2*. Tarkastelimme projektia kolmesta eri koeasetelmasta: (1) eri kasvaimien geenien ilmentymisen vertailu, (2) kasvaimien ja niistä luotujen uudelleenohjelmoitujen solujen geenien ilmentymisen vertailu sekä (3) kasvatusliuoksen vaikutus uudelleenohjelmoitujen solujen geenien ilmentymiseen.

Ensimmäisessä koeasetelmassa *Il-1β, Arg1* ja *Cxcl5* geenit ilmentyivät KL-CC10-kasvaimissa enemmän verrattuna KL-SPC-kasvaimiin, kun taas *Il-6* geenin ilmentyminen oli päinvastaista ja *Ccl2* geenin ilmentymisessä ei näkynyt eroa.

Toisen koeasetelman tulokset viittaavat siihen, että solulinjat ilmentävät mainittuja geenejä vähemmän kuin niiden vastaavat kasvaimet. Tämä oli nähtävissä erityisesti *Il-1β* geenin kohdalla. *Il-1β* geenin ilmeneminen solulinjoissa oli niin vähäistä, että se oli hankala todentaa qRT-PCR:llä. Poikkeuksia oli kuitenkin havaittavissa, KN85A-solulinja ilmensi *Il-6* ja *Ccl2* geenejä suuremmissa määrin kuin vastaava kasvain.

Kolmannessa koeasetelmassa selvisi, että kasvatusliuoksella ei ollut vaikutusta geenien ilmentymiseen ¾ solulinjassa, mutta *Il-6* ja *Ccl2* geenien ilmentyminen oli suurempaa KN85A solujen maljalla, johon oli vaihdettu stroomasoluviljelmästä eristetty kasvatusliuos. Tämä tulos viittaa siihen, että kasvatusliuos joka on eristetty stroomasoluviljelmästä, vaikuttaa vain tiettyjen solulinjojen geenien ilmentymiseen.

Avainsanat	CRC, Ei pienisoluinen keuhkosyöpä, NSCLC, qPCR, geenien ilmentyminen

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This project was carried out at the Institute for Molecular Medicine Finland (FIMM) in Emmy Verschuren's research group. The group is investigating lung cancer and the main focus is on the chemical and biological properties of tumor suppressors related to lung cancer. They use this information to create better lung cancer model systems to investigate new strategies for disease prevention and therapeutic intervention. (Verschuren group.)

The purpose of this project was to determine whether there are any differences in the expression of immune-related genes between murine *Kras*-mutant; *Lkb1*-loss of function driven non-small cell lung cancer tumors. These tumors were established *ex vivo* as epithelial conditionally reprogrammed cells (CRCs) based on their tumor cell-of origin (KL-CC10+ and KL-SPC+). The genes which were compared in this project were *Interleukin-1β (Il-1β,) Arginase-1 (Arg1)*, *Interleukin-6 (Il-6)*, *C-X-C motif chemokine 5* (*Cxcl5*) and *C-C motif chemokine ligand 2* (*Ccl2*). We had three different experimental conditions: (1) CC10+ tumor versus SPC+ tumor, (2) tumor versus CRCs and (3) normal media versus conditioned media from stromal cell culture.

In the tumor versus tumor experiment *Il-1β*, *Arg1* and *Cxcl5* gene expression was higher in KL-CC10+ positive tumors than in KL-SPC+ tumors while *Il-6* expression had an opposite trend and *Ccl2* showed no difference.

Tumor versus CRC results indicated that CRCs have lower expression of selected genes, especially in *Il-1β*. In CRCs the expression of *Il-1β* was so low that it was almost not detectable by qRT-PCR. However, there were exceptions. KN85A-cell line which is a KL-CC10 tumor, had higher *Il-6* and *Ccl2* expression in CRCs when compared to the matching tumor.

Conditioned media had no effect on selected gene expression in 3 of 4 CRCs, but increased *Il-6* and *Ccl2* gene expression in KN85A CRCs compared to the normal media. The KN85A result in conditioned media experiment suggests that there may be a cell-line specific effect of conditioned media from stromal cells on immune-gene expression.

Keywords	CRC, non-small cell lung cancer, NSCLC, qPCR, gene expression

Contents

Appendices

Appendix 1. Culture of Murine NSCLC Conditionally Reprogrammed Cells (CRC's). Talwelkar, Nagaraj and Devlin. Verschuren group

Appendix 2. Gene comparison protocol. Verschuren Group.

Abbreviations

AC	Adenocarcinoma
ASC	Adenosquamous carcinoma
AT2	Alveolar Type II
CAF	cancer-associated fibroblast
CC	Club cell
CC10	club cell antigen 10
cDNA	Complementary Deoxyribonucleic acid
Ct	Threshold cycle
Cq	Quantification cycle
CRC	Conditionally Reprogrammed Cell
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded DNA
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
Il	Interleukin
NSCLC	Non-small cell lung cancer
NTC	No Template Control
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RT	Reverse Transcription
SCC	Squamous cell carcinoma
SPC	Surfactant protein C
TAM	Tumor-associated macrophages
TME	Tumor Microenvironment

1 Introduction

This project was carried out at the Institute for Molecular Medicine Finland (FIMM) in Emmy Verschuren's research group. The group is investigating lung cancer and the main focus is on the chemical and biological properties of tumor suppressors related to lung cancer. They use this information to create better lung cancer model systems to investigate new strategies for disease prevention and therapeutic intervention. (Verschuren group.)

The purpose of this project was to determine whether there are any differences in the expression of immune-related genes between murine *Kras*-mutant; *Lkb1*-loss of function driven non-small cell lung cancer tumors. These tumors were previously established *ex vivo* as epithelial conditionally reprogrammed cells (CRCs) based on tumor cell-of origin. The genes which were compared in this project were *Interleukin-1β (Il-1β), Arginase 1 (Arg1), Interleukin-6 (Il-6), C-X-C motif chemokine 5* (*Cxcl5*) and *C-C motif chemokine ligand 2* (*Ccl2*). This project was inspired by study where there were found histotype-specific immune gene signatures for certain subtypes of non-small cell lung cancers (Nagaraj et al. 2017).

2 Non-small cell lung cancer

The majority of the lung cancer cases (up to 85%) are non-small cell lung cancers (NSCLC). Non-small cell lung cancer can be divided into subtypes which are based on the different types of lung cancer cells and morphological features. These subtypes include adenocarcinoma (AC, 40%), squamous cell carcinoma (SCC, 30%) and adenosquamous carcinoma (ASC) which is less common than the other subtypes. (American cancer society 2016.) Human lung adenocarcinomas have been found to be more common in the distal part of the lung rather than in the proximal airways whereas the squamous cell carcinomas (SCC) more typically initiate from proximal airways (Asselin-Labat – Filby 2012: 2).

There are two types of gene families which have a big role in cancer formation; oncogenes and tumor suppressor genes. Normally the human genome contains proto-

oncogenes which play an essential role in the regulation of cell growth. If these genes mutate they can be permanently activated and, as a result, the cell growth becomes uncontrolled and this can lead to cancer initiation. Tumor suppressor gene products repair DNA, slow down cell division and guide cells to apoptosis. When these tumor suppressor genes do not function as intended, cell growth can also become uncontrolled. (American Cancer Society 2014.)

In NSCLC the most commonly mutated oncogene, *Kras* is detected in around 25 % of all tumors in adenocarcinomas (de Castro Carpeño — Belda-Iniesta 2013). From 144 analyzed samples, 19% of squamous cell carcinomas and 34% of adenocarcinomas were found to have an inactivating mutation of tumor suppressor gene *Lkb1* (Ji 2007: 807). Other studies showed that in about 30% of non-small cell lung cancer cases the tumor suppressors gene *Lkb1* is inactivated, thus making it the most commonly mutated tumor suppressor gene in NSCLC (Mahoney et al. 2009; Koyama et al. 2016: 999).

2.1 Cell-of origin

Normal cells with genetic mutations can go through tumorigenic transformation, starting tumor initiation. These cells are referred as cell-of origin of the tumor. (Rycaj – Tang 2015: 4003) NSCLC may originate from many different progenitor cell types, 2 of which will be introduced here.

One of the many cell types located in bronchial epithelium are club cells (i.e. CCs, Clara cells or bronchiolar exocrine cells) which do not secrete mucus or have a cilium. It is estimated that 11-12% of all CCs are located in terminal bronchioles and these comprise approximately 15-44% of proliferating cells in bronchial tree. The main secretory protein of CCs is club cell protein 10 (CC10), also known as uteroglobin. (Rokicki – Rokicki – Wojtacha – Dżeljijli 2016: 26–27.)

Alveolar epithelium is mainly comprised by type I and type II cells, with the majority (60%) constituted by type II cells. Alveolar type II (AT2) cells express surfactant protein C (SPC) (Mao et al. 2015:1–2). AT2 cells and CCs have potential to proliferate under different conditions and they could potentially acquire mutations and thus initiate tumor development (Lin et al. 2012: 2).

2.2 Mouse model

The mouse genome is well characterized and correlates highly with the human genome, especially with respect to the genes shown to be important for cancer. The similarities in gene organization between human and murine are paralleled by similarities in the phenotypes which are engendered by homologous gene mutations. Even though characterization of mouse mutants obtained through mutagenesis or breeding has been very beneficial, an improved technology was developed using recombinant DNA technology. Creating transgenic breeding lines by injecting DNA into mouse oocytes has increased the capability to generate mice with developmental abnormalities and genetic diseases. (Wilson 2000: 201–203.) Originally the transgenic approach was compromised by multiple or exceptional insertion of genes and so homologous recombination techniques were developed instead to accurately remove or replace an endogenous gene segment. (Murray – Maga 2005: 307, 309; Wilson 2000: 201–203.) The advantage from the angle of developmental genetics is the capability to use transgenic mice to evaluate the effects of new genes on mammalian development. (Wilson 2000: 201–203.)

3 Background

3.1 Tumor cell of origin and immune related gene expression

The idea for this project stemmed from a recently published study where a histotype-specific immune gene expression signature was found in NSCLC tumors (Nagaraj et al. 2017). The model used in Nagaraj et. al. (2017: 673–674) study were transgenic mouse model with loxP-conditional oncogenic *Kras* mutant (G12D) and *Lkb1* loss-of-function alleles. The mutations were knocked in after the mice were infected with adenoviruses that express Cre-recombinase. Mutations of lung progenitor cell (either CC10+ or SPC+ cells) was achieved by using CC10+ and SPC+ specific adenoviruses and this was delivered to the mice intranasally, causing NSCLC development. (Nagaraj et. al. 2017: 674.) Later in this thesis this model introduced above will be referred as the *Kras;Lkb1* mutant mouse model.

CC10+ and SPC+ progenitors with mutant *Kras* (G12D oncogenic mutation) and loss of *Lkb1* were both able to generate different lung cancer subtypes (invasive AC, AC in situ,

papillary AC and ASC), but it was shown that aggressive ASCs were predominantly initiated from CC10 positive progenitors. Acinar ACs (AAC) and pure mucinous ACs (MAC) were also initiated from CC10 positive cells, but not from SPC positive cells. (Nagaraj et al. 2017: 680–681.)

The histotype-specific expression of immune related- genes was identified by a gene expression microarray using RNA from KL-CC10 ASC and KL-SPC AC tumors. Gene expression microarray results exhibited that genes functioning in immune suppression and chemotaxis, such as *Cxcl5*, *Il-1β* and *Arg1* were more highly expressed in KL-CC10 ASCs than in KL-SPC papillary ACs. *Il-1β* and *Arg1* expression levels were also validated by quantitative real time polymerase chain reaction (qRT-PCR), while validation of the other immune-related genes was not performed. The study showed that ASCs specifically had higher expression of pro-inflammatory genes such as *Il-1β* and *Arg1* and *S100 calcium-protein A8* and *A9*. (Nagaraj et al. 2017: 673, 677–678.)

3.2 Conditionally reprogrammed cells (CRC)

Reproduction of adult epithelial cells demands special medium for culturing and it is limited by the early aging of cells, also known as senescence. It is possible to avoid this aging by expressing viral oncogenes in cells, but it has been shown that these modified cell lines can have abnormal regulatory pathways. (Liu et al. 2012: 599.) Primary human adult cells can also be immortalized by using exogenous human Telomerase Reverse Transcriptase (hTERT) and cellular genes (e.g. *cdk4 gene*). However, Liu et al. showed (2012: 599–600) that using only hTERT to immortalize breast cells led to disrupted differentiation in Matrigel, a commercial basement membrane matrix used for a number of different kind of cell culture applications (BD Biosciences 2008).

Liu et al. (2012: 606), showed in the same study that epithelial cells derived from different tissues (tumor and normal tissue) can proliferate indefinitely using a combination of fibroblast feeder cells and Rho kinase inhibitor, without transferring any genetic material. Conditionally reprogrammed cells (CRCs) (from normal tissue) remained non-tumorigenic and maintained a normal karyotype, unlike in the other methods described above. Co-cultivation of irradiated feeder cells (fibroblasts) and Rho kinase inhibitor in the CRC medium are the main critical factors in the process of CRC culture. (Liu et al. 2012: 606.) Fibroblast feeder cells work as supporters to the target cells and help them

grow and expand by providing nutrients, growth factors and active signals (Llames – García-Pérez – Meana – Larcher – del Río 2015).

In Verschuren group, the CRC method is being used to culture epithelial NSCLC tumor cells from *Kras;Lkb1* mutant mouse tumors for *ex vivo* investigation (Nagaraj 2016).

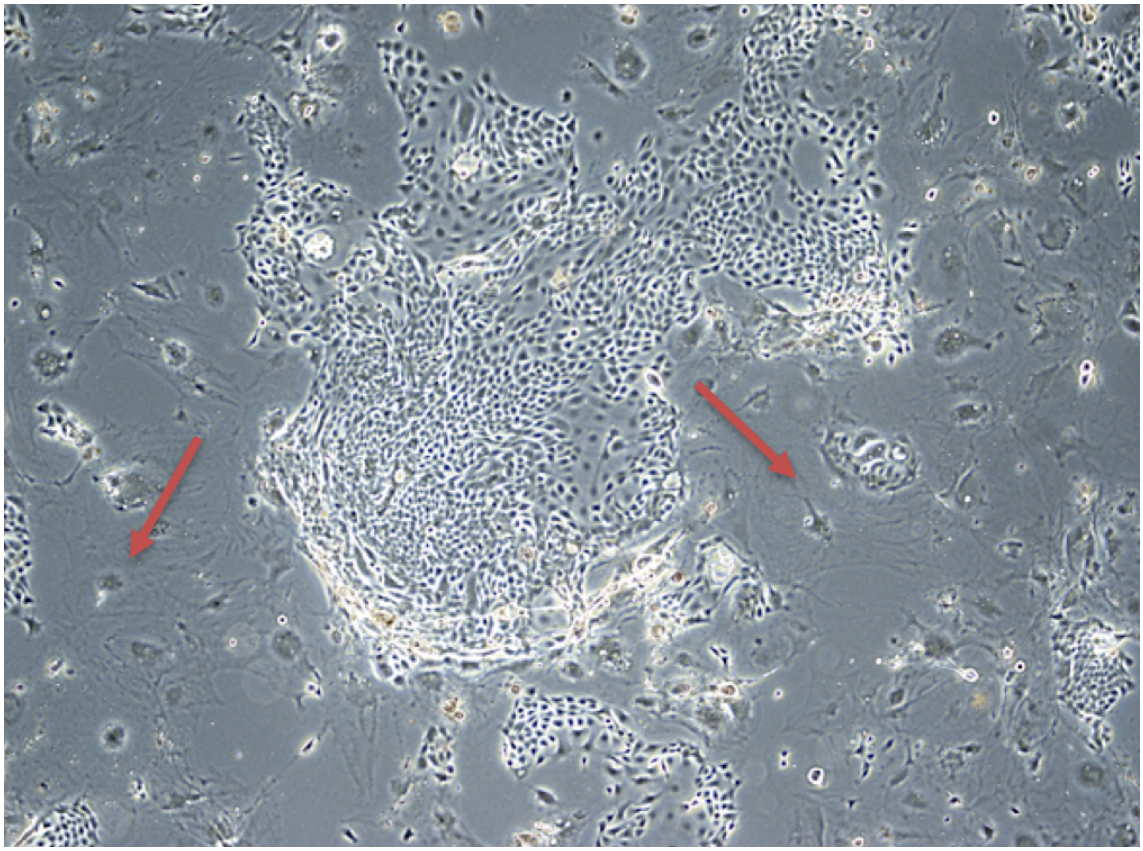

Figure 1. Example of CRC culture. KN13B CRCs. Red arrows points to fibroblasts. The big cluster in the middle is a CRC colony. 10x magnification, Zeiss Axio. (Ojanen 2016).

3.3 Tumor microenvironment

Tumors are infiltrated and surrounded by blood vessels, stromal and immune cells, extracellular matrix (ECM) and other supporting structures which form human organs. Together these factors comprise a microenvironment of a tumor. (Betof – Dewhirst 2011: 7.) Tumor microenvironment (TME) is identified to have a crucial role in malignant progression, neoplastic cell initiation and in the metastasis of tumor cells. The cell types which have been recognized in TME are inflammatory cells, vascular cells, fibroblasts, immunocytes, hematopoietic-derived cells and epithelial cells. TME isn't just a physical

space, it is also a venue for different factors to interact. (Siemann 2011; Betof – Dewhirst 2011: 7, 11.)

Cancer cells and stromal cells (e.g. vascular endothelial cells, fibroblasts and immune cells) constitute tumors. Non-malignant (benign) stromal cells surround non-differentiating malignant cancer cells. A major element of the cancer stroma are cancer-associated fibroblasts (CAFs), which secrete numerous growth factors (chemokines and cytokines) and have a role in regulating tumor proliferation, invasion and metastasis. (Shiga et al. 2015: 2443, 2447.)

The immune system is a critical part of the TME. Mast cells are the first cells to react to the tumor growth factors. Macrophages normally are the next inflammatory cells to react and infiltrate the growing tumor microenvironment. These tumor-associated macrophages (TAMs) start to secrete interleukin-1, interleukin-6, interleukin-8, tumor necrosis factor alpha, (TNF-α) and/or basic fibroblast growth factor (bFGF) once they are activated by tumor-derived transforming growth factor beta 1 (TGF- β1). TAMs are thought to have an essential role in signaling angiogenesis, that is necessary for tumor growth. Cytokines, growth factors and chemokines secreted by inflammatory cells, provokes epithelial proliferation and produce reactive oxygen species (ROS) that damage DNA, thus boosting tumor progression. (Betof – Dewhirst 2011: 10–11.) Neutrophils are another important immune cell type which are part of the tumor microenvironment (Gabrilovich – Nagaraj 2009: 20).

Several types of cells and secreted proteins compose an extracellular matrix which helps to maintain the organization of cellular structures. The matrix is filled with proteins such as laminins, plasminogens, proteases, collagens, fibronectin, hyoluronan and many other, and this together creates an inflexible system to which cells attach. (Parker – Siemann 2011: 3.) Cancer cells have ability to upregulate and release proangiogenic factors which can induce vascular outgrowth from regular blood vessels and make endothelial cells unstable. In order to form a new capillary net for the tumor mass, the endothelial cells proliferate in the direction of the chemoattractant angiogenic factor source. Normal vasculature is highly organized, whereas newly constructed tumor neovasculature lacks organization, vessel integrity and has abnormal structure. It is supposed that vascular endothelial growth factor (VEGH) plays a critical role in tumor angiogenesis. (Parker – Siemann 2011: 3.)

3.4 Project specific genes of interest

In this study the main focus was in the immune-related genes such *as Interleukin 1β (Il-1β), Interleukin 6 (Il-6), C-X-C motif chemokine ligand 5 (Cxcl5), C-C motif chemokine ligand 2 (Ccl2)* and *Arginase 1 (Arg1).* These genes were chosen for this project since there have been interesting results in gene expression comparisons in a previous studies. In Nagaraj et al. (2017) study, *Il-1β, Arg1* and *Cxcl5* were identified to be differentially expressed between ASCs and ACs. In one study (not published yet), gene expression was compared by RNA sequencing between CRCs and the results showed that *Ccl2* gene is more highly expressed in ASC CRCs compared to AC CRCs (Nagaraj 2016). Another study identified *Il-6* gene as important for *Kras;Lkb1* driven lung tumors, making it one of the genes-of interest in this project (Koyama et al. 2016: 999).

Interleukin 1β (Il-1β) is a gene that encodes protein from the interleukin 1 cytokine family. Activated macrophages and other cell types produces this cytokine, that has an important role in inflammatory responses and it is involved in numerous cellular activities such as cell proliferation, and differentiation and apoptosis. (Interleukin 1 beta 2017.)

It has been recorded that there are at least two forms of a mammalian arginase – type 1 and type 2 which are encoded by different genes. These isoforms differ in their subcellular localization, physiologic function, tissue scattering and immunologic cross reactivity. The type 1 form is encoded by this gene mentioned previously and it is an enzyme involved in urea cycle. *Arginase 1* is mainly expressed in the liver and it works as a catalyst in a reaction where arginine hydrolyses to urea and ornithine. (Arginase 1 2017.) *Arginase 1* is commonly expressed by neutrophils which have been stimulated by *Il-1β* and other factors in the tumor microenvironment (Gabrilovich – Nagaraj 2009: 21).

Chemokines have a role in activation and recruitment of a leukocytes and they are grouped by structure or by function. *Cxcl5* gene encodes a protein which is a member of C-X-C motif chemokine ligand subfamily. In cancer, it is suggested that this protein is involved in cancer cell migration, invasion and proliferation. (C-X-C motif chemokine ligand 5 2017.)

Interleukin 6 (Il-6) gene encodes a cytokine which is involved in maturation of B cells and in inflammation. It has been shown that this protein also has a capability to induce fever in patients with infections or autoimmune diseases. Primarily the protein is produced in

acute and chronic inflammation. In response to inflammation the encoded protein induces transcriptional inflammatory response via Il-6 receptor alpha, right after it has been secreted in the serum. (Interleukin 6 2017.) It has been showed that CAFs are one source of an Il-6 (Shiga et. al. 2015: 2443).

As been mentioned previously, chemokines have a role in inflammatory processes. Ccl2 chemokine belongs to a CC chemokine subfamily and it exhibits chemotactic activity for basophils and monocytes but not for eosinophils nor neutrophils. (C-C motif chemokine ligand 2 2017.)

3.5 Hypothesis and aims of the project

Based on the previous studies (Nagaraj et al. 2017), it was expected that there will be differences in the immune gene expression between different tumors (KL-CC10 and KL-SPC), and also in tumor versus CRC comparison. In addition, as stromal cells are an important part of the tumor microenvironment, they may have an effect on tumor cell immune gene expression. However, there is no previous publications about stromal-cell conditioned media effects on CRC immune gene expression in these same experimental conditions so it was hard to predict the outcome.

The main aims for this project were to compare gene expression between different tumors and CRCs and to investigate if there are any effects of stromal cell conditioned media to gene expression in CRCs.

4 Materials and methods

4.1 Cell culture

Many of the cells derived from animals or plants can survive and proliferate in a favorable artificial environment (Alberts et al. 2012; Life technologies: 2). This environment often needs a suitable medium enriched with specific protein growth factors and nutrients (Alberts et al. 2012). Cell cultures can be derived from the tissue directly by enzymatic or mechanical methods or they can also be derived from cell strains or cell lines which have been established before (Life technologies: 2).

The most important requirement of a cell culture laboratory is an aseptic work area. The cell culture hood is the most economical and simplest way to maintain aseptic conditions and it decreases the risk of contamination from aerosols. (Life technologies: 7, 11.) The key concepts for aseptic technique is a sterile handling, good personal hygiene, sterile reagents and media, and sterile work area. It is crucial to keep the cells free from contamination by micro-organisms. Sources of biological contamination can be dirty work surfaces, non-sterile supplies or reagents and the airborne particles. (Life technologies: 11.)

4.1.1 CRC culture

In this project, tumors used for CRC culture were previously collected from *Kras;Lkb1* (KL)-CC10 or KL-SPC mice and cut into pieces (Table 1). One of the pieces were used to generate CRCs and the rest of the tumor pieces were snap frozen in liquid nitrogen and stored. Later, these frozen sections were used for RNA isolation (see section 4.2).

Table 1. Tumor and the CRC details.

CRC / Tumor ID	***Genotype /Cell of origin***	***Histotype***
KN6B-T1	KL-SPC	AC
EV1093-T4	KL-SPC	AC
KN85A-T2	KL-CC10	ASC
KN13B-T1	KL-CC10	ASC

Epithelial CRCs which had been previously created in Verschuren lab from murine NSCLC tumors were co-cultured with murine NIH3T3 fibroblasts (ATCC® CRL-1658™). First these NIH3T3 fibroblasts were cultured on 10 cm tissue culture dishes and incubated overnight at 37°C in the presence of 5% carbon dioxide (CO_2). The NIH3T3 is a cell line established from mouse embryos (ATCC 2016).

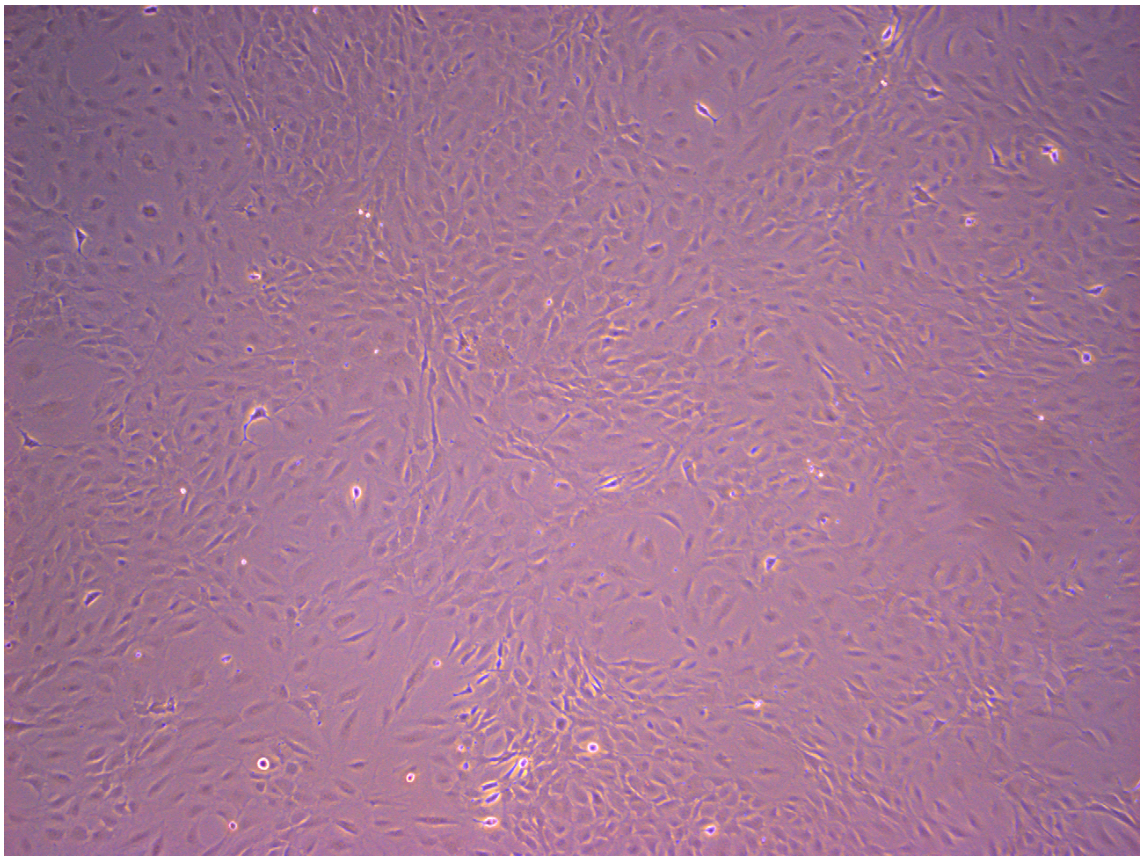

Figure 2. Newly thawed NIH3T3 fibroblasts with 10x magnification, Zeiss Axio (Ojanen 2016).

The medium (Dulbecco's Modified Eagle Medium) which is used to culture fibroblasts contained 10% heat-inactivated fetal bovine serum (HI FBS), L-glutamine and antibiotics (penicillin/streptomycin). Before use the medium was prepared by adding components listed above. After incubation NIH3T3 fibroblasts were irradiated by gamma-irradiation. Gamma-irradiation is one of the methods used to stop the cells from proliferating (Llames – García-Pérez – Meana – Larcher – del Río 2015).

CRCs were seeded onto irradiated NIH3T3 cells with F-12/DMEM (medium) which was enriched by numerous components. First 152 ml of media was removed from F12 media bottle. Into 348ml of F12 media (Invitrogen #11765-054) was added 116ml of DMEM with high glucose (Invitrogen #11965-084), 25 ml heat inactivated FBS (Gibco), 5 ml of PenStrep (antibiotics) and other components listed below (Table 2). For tumor versus CRC experiment, the cells which have been already seeded onto NIH3T3 cells were cultured 48 hours before used for RNA isolation (see section 4.2).

Table 2. Concentrations of stock solutions.

Chemical	*Stock concentration*	*Working concentration/ml*	*For 500ml of media*	*Storage*
rhEGF *(from stock)*	0,1 mg/ml	10 ng/ml	50 µl	-20°C
Insulin *(from stock)*	4 mg/ml	5 µg	625 µl	-20°C
Cholera toxin *(from stock)*	0,5 mg/ml	10 ng	10 µl	+4°C
Adenine *(from stock)*	2,4 mg/ml	24 µg/ml	5 ml	-20°C
ROCK inhibitor *(Y-26732) (from stock)*	5 mg/1,5ml 10mM	10 µM/ml	500 µl	-20°C
Hydrocortisone *(from stock)*	1 mg/ml	0,4 µg/ml	200 µl	-20°C

In conditioned media experiment the key was to replace the regular CRC-media with conditioned media from stromal cell cultures to see if there are any effect. This conditioned media was obtained from mouse bone marrow stromal cell (Ingo Ringshausen, University of Cambridge) culture. These stromal cells were first cultured into 10 cm plate with stromal cell media MEM Alpha + GlutaMAX (Invitrogen), with additional components, 10% horse serum (StemCell Technologies #06850), 10% heat inactivated FBS, 10uM B-mercaptoethanol (Sigma Aldrich #M6250) and 1% penicillin/streptomycin (Gibco #15140148). The stromal cells were cultured until they reached 50% confluency and afterwards a fresh F-12/DMEM media was put on the cells for 48 hours. This ‘conditioned’ media from the plates were collected and added to the CRCs for 24 hours.

For the conditioned media experiments (Figure 3), all of the CRCs, generated from different mouse NSCLC tumors, were cultured in two 10 cm plates at the same density. In one plate per each CRC line, the CRC media was replaced with conditioned CRC media after 24 hours. Thus we had two different experimental conditions; exponentially growing cells in CRC-media and exponentially growing cells in conditioned media from

stromal cell culture. These cultures were used for RNA isolation after additional 24- hour culture (see section 4.2).

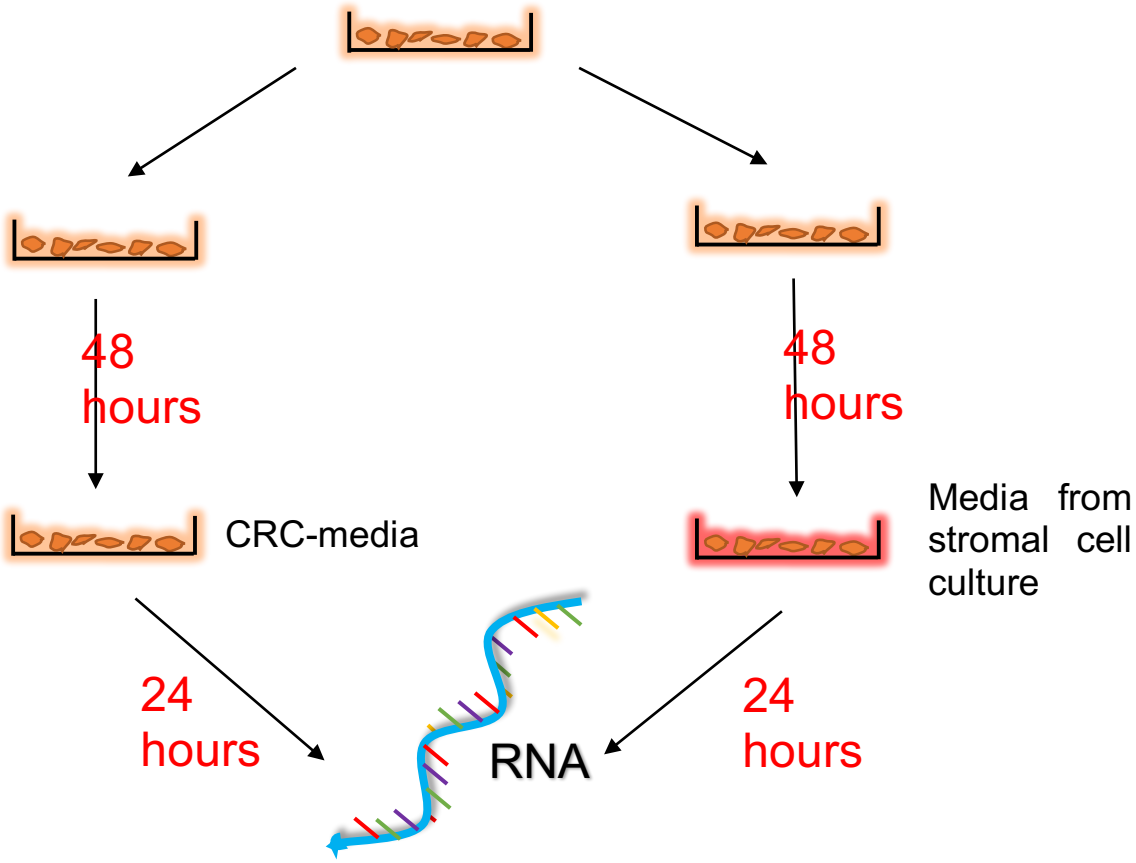

Figure 3. Workflow for conditioned media experiment.

4.2 RNA isolation

RNA isolation methods are comparable to DNA isolation methods, but it is known that RNA molecules are shorter and thus not so easily damaged by shearing. It means that the cells can be disrupted more vigorously. RNases which can be found on fingers or endogenously in a certain cell types, have ability to digest RNA. To avoid this digestion from happening, gloves and strong detergent included in the isolation medium should be used. (Rapley 2005: 3.)

For the isolation of RNA from cells, the cells must first be lysed. As a result, there is total RNA, mixture of messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA)

and other forms of non-coding RNA. Only about 2% of the total RNA mixture is mRNA (relevant for this project). (Mulhardt 2010: 96.)

First, the CRCs had to be separated from the NIH3T3 cells in the plate. To segregate CRCs from NIH3T3 fibroblasts, a double trypsin incubation method was a method used in Verschuren lab. In the first incubation trypsin detached the NIH3T3 cells allowing them to be discarded from the plate. During the second incubation the CRCs were detached from the plate by trypsin, washed with phosphate buffered saline (PBS) and freezed at -80°C (Appendix 1). To isolate cells from snap frozen tumor pieces matching the cultured CRCs (section 4.1), solid tumor tissue was homogenized by using Precellys® homogenizer from Bertin instruments.

In this project we used commercial RNA isolation kit (Nucleospin® RNA kit) to isolate RNA from frozen tumor tissues and CRCs. The kit includes a lysis buffer which also inactivates RNases, columns used to isolate clean RNA by binding to RNA molecule and DNase that eliminates genomic DNA in the column (Machereney-Nagel 2016). We obeyed the protocol made by manufacturer, with a slight difference in the final steps, where we did one extra centrifugation step in order to dry the column entirely and eluted the RNA in 50 µl of RNase-free H_2O instead of 60 µl (Machereney-Nagel 2015: 18–20). Quantitation of isolated RNA was performed by using spectrophotometer (NanoDrop).

In order to remove DNA, it is recommended that the RNA samples are treated with DNase (RNase-free) before reverse transcription, especially when using Sybr Green 1 detection as it requires that a single amplicon is produced (Sugden 2005: 337). In this project this step was included in RNA isolation, as the Nucleospin® RNA kit contained RNase-free DNase.

4.3 qRT-PCR

qRT-PCR is a quantitative reverse transcription polymerase chain reaction. This method is used when the material to be analyzed is RNA. First total RNA or messenger RNA must be transcribed into a complementary DNA by a reverse transcriptase. cDNA generated by the reverse transcription is then used as a template for the quantitative PCR (qPCR). (Thermo Fisher Scientific 2015a.)

There are some guidelines for aseptic technique in PCR working in order to avoid contamination. It is important to use tips that contain a filter (aerosol barrier), to wear proper laboratory clothing (gloves etc.) and to decontaminate surfaces where the pre-PCR is carried out with appropriate solution. Premixing the reagents before aliquoting reduces the pipetting steps and consequently reduces the chance of a contamination. It is suggested to perform PCR in a separate area e.g. in laminar hood with UV-light, where contamination measurements can be taken. The temperature cycler and area where post-PCR products are handled should be separated from previously mentioned area. (Hartley – Rashtchian 1993: S10.)

A No Template Control (NTC) should be analyzed at the same time as other samples, to rule out any contamination caused by reagents or surfaces. NTC comprises all the reagents used in RT-PCR but not a RNA template, which is normally replaced with nuclease-free water. There should be no synthetization in NTC, however if there is any fluorescence, it can mean that one or more of the reagents used are contaminated. (Thermo Fisher Scientific 2015c.) In this project, for each primer pair on each qRT-PCR plate, triplicate reactions using RNase free H_2O instead of the template cDNA were included to check for contamination of reaction reagents.

4.3.1 Synthesis of complementary DNA (cDNA)

Viruses have ability to generate DNA from RNA, as they contain an enzyme called reverse transcriptase (Mulhardt 2010: 99). Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and Avian myeloblastosis virus reverse transcriptase (AMV-RT) are generally used enzymes for the transcription (Thermo Fisher Scientific 2015a). For the synthesis of complementary DNA (cDNA), components needed are isolated RNA template, primer, buffer, RNase inhibitors, nucleotides and reverse transcriptase (Mulhardt 2010: 99).

The synthetization of cDNA from isolated RNA were performed by using a BioRad thermocycler and High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The kit contains MultiScribe® Reverse Transcriptase (RT), 100 mM dNTP Mix (nucleotides), RT Random Primers and RT Buffer (Thermo Fisher Scientific 2015b). Protocol we used for synthetization was High Capacity cDNA Reverse Transcription Kit protocol provided by Applied Biosystems. (Applied Biosystems 2010: 7–9). Before performing the procedure, isolated total RNA was first diluted to either 930 ng or 1000

ng, and the dilutions were made based on the concentrations measured by NanoDrop. Reverse transcription program is presented in the Table 2. After the reverse transcription, the cDNA was diluted using RNase-free H_2O. The amount of synthetized cDNA was measured using the Nanodrop and the cDNA was diluted to 80ng/µl stock solutions.

Table 3. Reverse transcription program on BioRad thermocycler.

Step	***Temperature (°C)***	***Time***
1	25°C	10 min
2	37°C	120 min
3	85°C	5 sec
4	10°C	Infinity

4.3.2 qRT-PCR with validated primers

The design of two oligonucleotides primers is the key for sensitive and specific real-time assay. In gene expression measurements, primers should only anneal to distinct exons in order to avoid amplification of genomic DNA. (Bimal 2005: 65; Sugden 2005: 337.) The primers have to be complementary for the target sequence, but they can't be self-complementary or form dimers when binding to each other. They also have to have matching GC-content and have same temperature for annealing. The use of different softwares when designing primers have made the design and choosing reaction conditions simpler and less time-consuming. All necessary information, including GC-content, product size and primer length can be input and the resources (softwares) provide an option of matching sequences for primers. (Bimal 2005: 65.)

Primers used in Verschuren lab were designed previously based on relevant publications and using the NCBI PrimerBlast (Ye et al. 2012). These primers were validated prior to use in experiments. First, to determine if primers amplified a single, correct sized specific product, a terminal PCR with KAPA HiFi DNA polymerase was performed. For the primers which produced a single product by agarose gel electrophoresis, the efficiency was also assessed by generating standard curves by qRT-PCR.

In this project the qRT-PCR was performed by using BioRAD CFX96 qRT-PCR machine and SybrGreen technology via BioRad iQ™ Sybr® Green Supermix. This preblended

supermix contains SybrGreen dye, iTaq™ DNA Polymerase, buffer, stabilizers for SybrGreen and dNTPs (BioRad 2016). The reaction involves the primers validated previously (reverse and forward, Table 4), SybrGreen mix and the cDNA template. If the cDNA template contains the gene of interest, the PCR detection system detects template amplification via changes in the Sybrgreen fluorescent signal. This signal is produced by the binding of the SybrGreen dye to double stranded DNA (dsDNA) as it is synthetized (Dharmaraj 2017). The fluorescence is still low in the unbound state, but upon binding to dsDNA the fluorescence intensifies. In the reaction there can be non-specific binding since SybrGreen dye will bind to any dsDNA (Nolte – Hill 2011: 1274). If a gene of interest is highly expressed, the cDNA template will be amplified more quickly and the Sybrgreen fluorescent signal will be detected at an earlier cycle in the PCR protocol (Life technologies 2012). The ΔΔCT method was used to analyze the raw qRT-PCR data and the differences in gene expression between the samples were compared.

Table 4. Reverse and forward primer details.

Gene Name	***Forward (5'→3')***	***Reverse(5'→ 3')***	***Expected Product Length (bp)***	***Reference***
Il-6	CTCTGGGAAA TCGTGGAAAT	TCCAGTTTGGT AGCATCCATC	135	Primer BLAST
Il-1β	TGCCACCTTTT GACAGTGATG AGA	CCTGGAAGGT CCACGGGAA	225	Verschuren lab stocks
Rpl19 (+control)	CGGGAATCCA AGAAGATTGA	TTCAGCTTGTG GATGTGCTC	110	Verschuren lab stocks
Arginase 1	TCGTGTACATT GGCTTGCGA	GCCAATCCCC AGCTTGTCTA		Primer BLAST
Ccl2	AGGTCCCTGT CATGCTTCTG	TCTGGACCCA TTCCTTCTTG	249	Lu and Kang, *J. Biol. Chem.* 2009, 284(2): 29087
Cxcl5	GCATTTCTGTT GCTGTTCACG CTG	CCTCCTTCTG GTTTTTCAGTT TAGC	205	Duchene et al., *J. Immunol* 2007, 179; 4849-4856

The desired concentrations for qRT-PCR reactions were 20 ng/µl cDNA, 300 nM forward primer, 300 nM reverse primer and 1X Sybgreen mix and the reaction volume was set to

10 µl with RNase free H_2O. Each reaction was performed in triplicate, meaning that 3 wells per reaction were used (Appendix 2). The qRT-PCR was performed in 3-step program with initial denaturing. This 3-step program (Table 3) comprised denaturing, annealing and extension phase which were repeated 40 cycles. The annealing phase with *Arginase 1* primers was performed at 58 degrees as primer validation experiments identified this as the preferable temperature for *Arginase 1* primers to anneal. Melt curves were generated by increasing the temperature by 0.5°C increments from 55°C to 95°C, 10 second each.

Table 5. qRT-PCR program.

Step	***Temperature (°C)***	***Time***
Initial denaturation	95°C	3 minutes
Denaturing	95°C	10 seconds
Annealing	60°C	15 seconds
Extension	72°C	30 seconds
Melt curve	55 – 95°C	10 seconds (0.5 degree increments)

4.4 ΔΔCT method

There are different approaches for qPCR data analysis. A commonly used method is where concentrations of a known cDNA are used to quantify the absolute copy number by creating a standard curve. Another approach for data analysis is ΔΔCT method (also known as Comparative CT method) which utilizes a reference gene to gain relative gene expression results. In this method the relative gene expression differences are calculated by normalizing the results against a reference gene (e.g. housekeeping gene). (Dharmaraj 2017.) Quantitative PCR data normalization is necessary since there can be variability between samples in starting material as well as in mRNA isolation, and differences at efficiency in reverse-transcription. Internal reference gene expression is quantitated in the same samples at the same PCR plate, as the target gene expression. Reference gene should be expressed at a constant level in the same tissue type between different samples. (Sugden 2005: 337.)

For example, in the Figure 4, average target gene *Cxcl5* Cq was normalized by using the housekeeping reference gene Cq (*Rpl19).* Therefore, ΔCq = Cq $_{\text{target gene}}$ – Cq $_{\text{reference gene}}$. In this case it was decided that the CRCs work as a calibrator, so ΔCq $_{\text{sample}}$ - ΔCq $_{\text{calibrator}}$ = ΔΔCq. Fold expression (2^(- ΔΔCq)) shows how many times more the sample is expressing the gene of interest.

Sample	RPL19 Cq	Average RPL19C	Cxcl5 Cq	Average Cxcl5 Cq	Delta Cq	Delta Delta Cq	Fold Expression (2^(-deltadeltaCq)
KN13B-CRC	19,09	19,16	23,52	23,51	4,35	0,00	1
	19,20		23,52				
	19,18		23,49				
KN13B-T1	18,13	18,03	18,39	18,51	0,48	-3,87	14,63769973
	17,96		18,50				
	18,00		18,65				

Figure 4. Example of the ΔΔCT method.

5 Results

These relative results of each experiment are merged into graphs in order to make it clearer. The results from different experiments are presented in different graphs and are not comparable between each other. In the tumor versus tumor comparison, the gene expression was folded over KL-SPC- tumors and in the tumor versus CRC comparison, the gene expression was folded over CRCs. In conditioned media experiment, the gene expression was folded over normal media samples.

5.1 Tumor versus tumor comparison

Tumors in this project were from *Kras*;*Lkb1* mutant mouse model with either CC10+ or SPC+ cell-of origin (see section 3.1). Comparison were made between two different tumors for each cell of origin (n=2). KL-SPC tumors – namely KN6B and EV1093 displays orange bars in the graphs and KL-CC10 tumors (KN85A and KN13B) displays the blue bars in the graphs. As shown in Figure 5, there can be variety between the different tumors with the same cell-of origin. Therefore, the results for each tumor are exhibited individually instead of showing only the average for the same cell-of origin.

qRT-PCR analysis showed that the expression of *Il-1β* mRNA was higher in KL-CC10 tumors compared to KL-SPC tumors (Figure 5). *Il-1β* is an important immune gene which

was identified to be expressed more highly in ASC compared to AC tumors in the tumor gene expression microarray (Nagaraj et. al. 2017, see section 3.3).

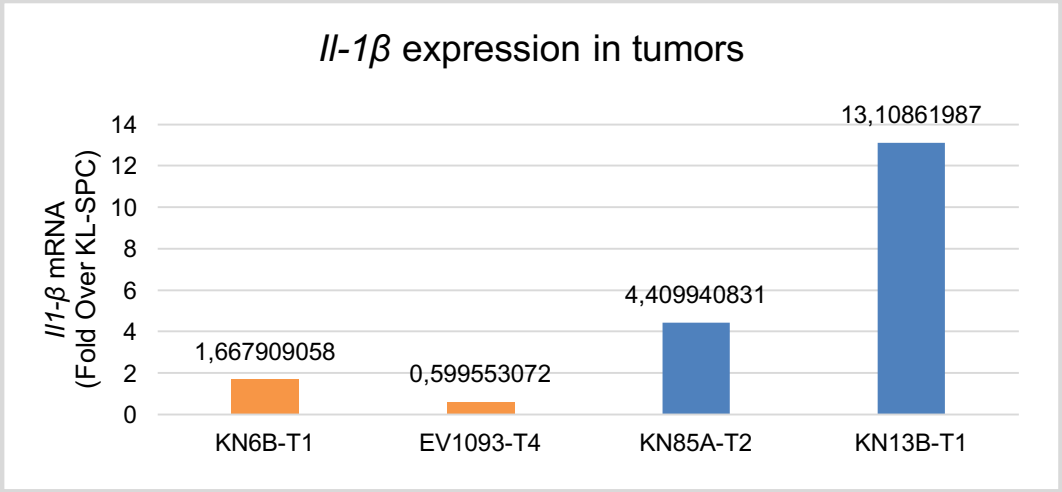

Figure 5. *Il-1β* mRNA expression, fold over KL-SPCs average.

Compared to the KL-SPC-tumors (Figure 6), KL-CC10 tumors were expressing *Arginase 1* mRNA at higher levels. Specially, KN85A expressed *Arginase 1* expressed 17 fold more when comparing to the KL-SPC-tumors. Also KN13B had higher expression level than KL-SPC-tumors (orange bars).

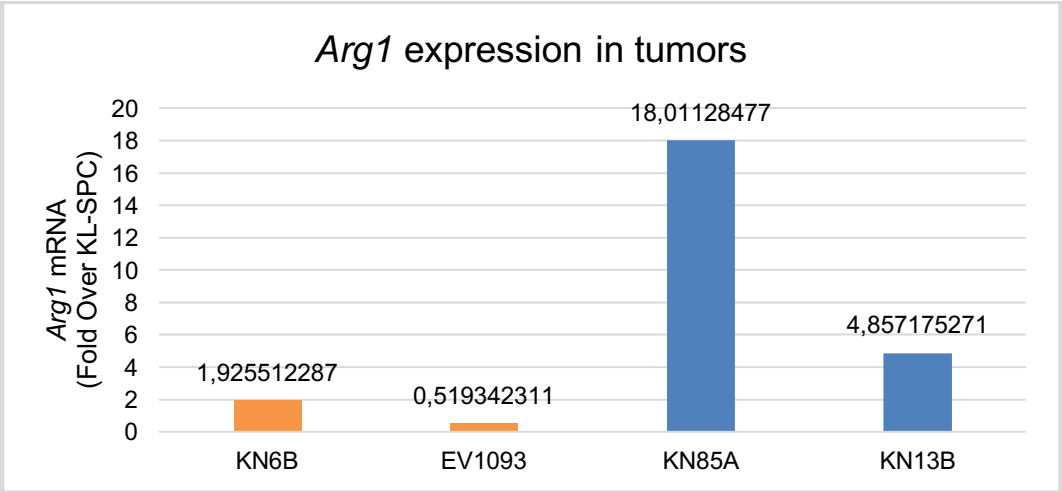

Figure 6. Expression level comparison of *Arginase 1* mRNA.

Cxcl5 was also identified in the tumor gene expression microarray, but the difference in gene expression between KL-CC10 and KL-SPC tumors was not confirmed by qRT-PCR, until now. In Figure 7, it is clear that *Cxcl5* was expressed more in KL-CC10 tumors

compared to KL-SPC tumors, consistent with the tumor gene expression microarray results (see section 3.3).

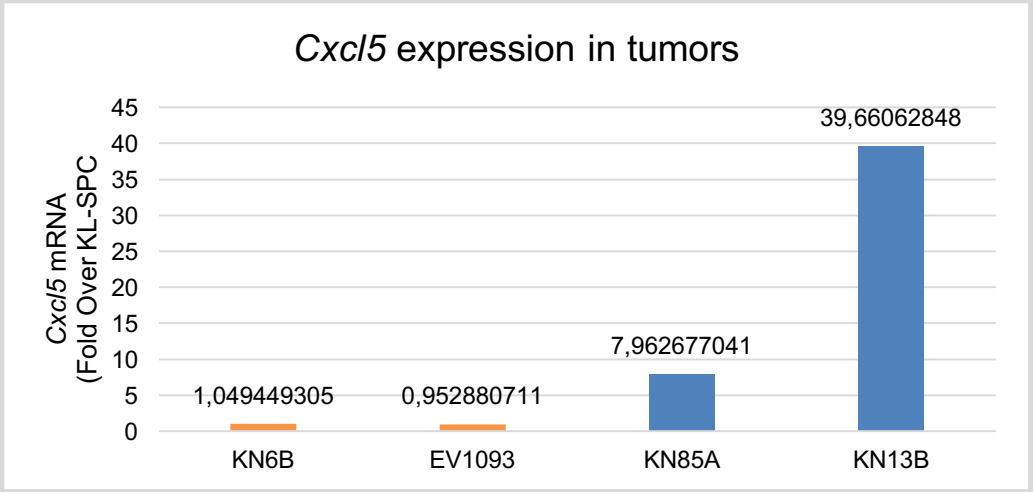

Figure 7. Expression comparison of *Cxcl5* gene in tumors.

Figure 8 shown below, reveals that there is an opposite trend to the other genes of interest. Comparison of *Il-6* gene expression between KL-CC10 and KL-SPC tumors indicates that there is no clear difference between the two tumor types. There is variation between individual tumors with the same cell-of origin, however, when the average of both tumor types is calculated, there is no difference.

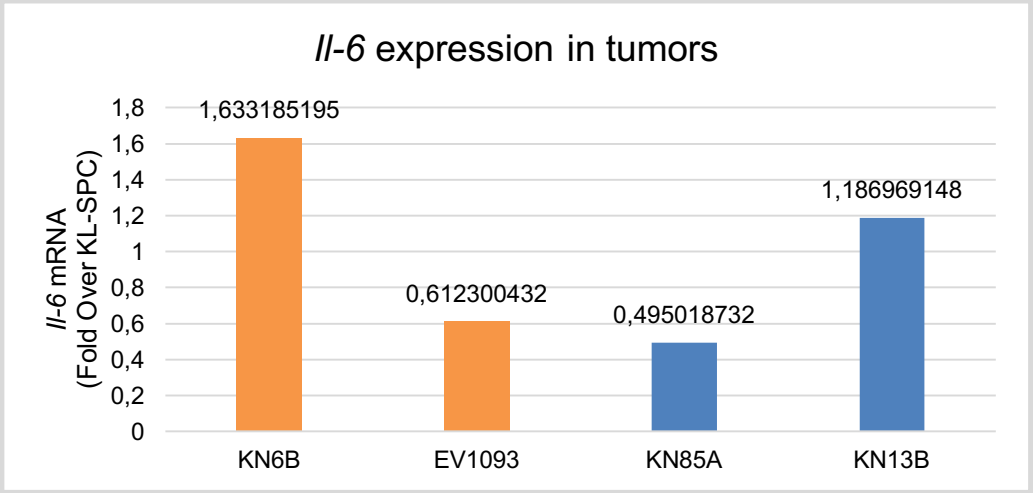

Figure 8. Expression level comparison of *Il-6* mRNA in tumors.

Similar to *Il-6*, *Ccl2* gene expression comparison in Figure 9 shows that there is no difference between KL-CC10 and the KL-SPC tumors.

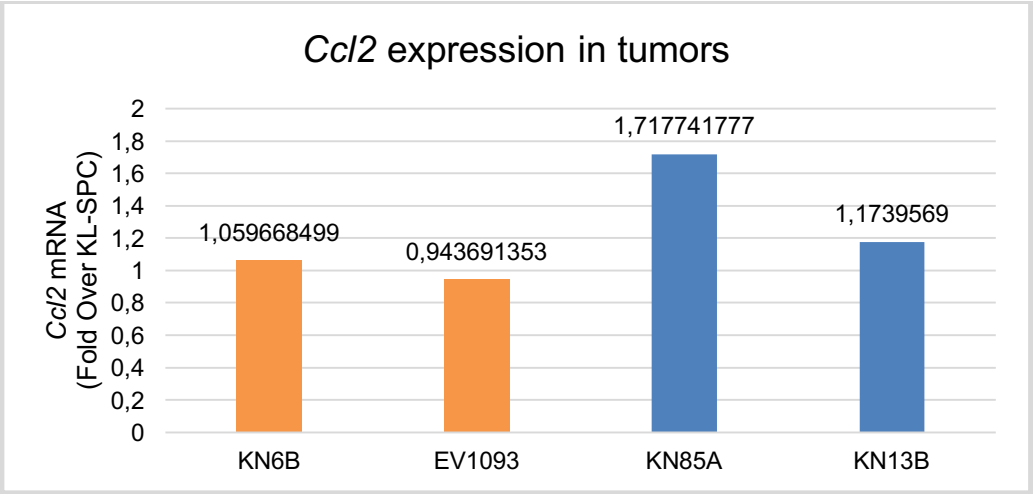

Figure 9. Expression comparison of *Ccl2* gene in tumors.

In tumor versus tumor comparison we have confirmed that in additional independent tumors the expression of *Il-1β* is higher in the KL-CC10 ASC tumors compared to the KL-SPC AC tumors. Also *Arginase 1* and *Cxcl5* comparison had the same trend as *Il-1β* with a big difference in gene expression levels. *Il-6* had an opposite trend to the other genes, but it was similar to *Ccl2* expression comparison which revealed that there was no difference between different cell-of origin tumors.

5.2 Tumor versus CRCs

Isolated RNA from CRCs was compared to the RNA isolated from the matching tumor tissue, to see if there are any differences in the expression of immune-related genes of interest. In all graphs the results can be compared only between CRC and its matching tumor (same colored bars). CRCs had lower expression of immune genes compared to matching tumor tissue, however there were some exceptions that will be discussed.

In all tumors tested, the *Il-1β* expression was higher when compared to their matching CRCs. However, the Figure 10 shows that there is a big difference between the samples, which is consistent with variability in *Il-1β* expression shown in Figure 5.

Expression of *Il-1β* in KN6B CRCs was not detected by qPCR at all so it was excluded from Figure 10. KN13B has the greatest difference in the expression of *Il-1β* between the matching tumor and the CRC according the qPCR analysis.

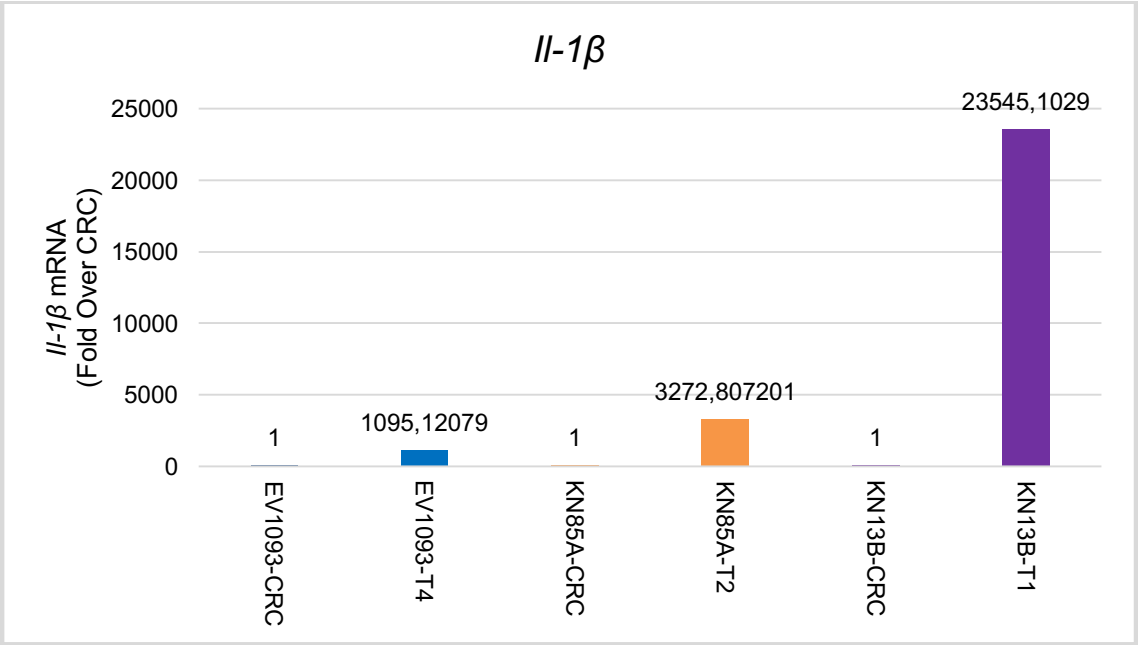

Figure 10. *Il-1β* comparison between tumor and CRCs.

Results shown in the Figure 10 were confirmed and visualized by running the qPCR reaction products in an agarose gel. KN6B, EV1093, KN85A and KN13B in Figure 11, are names of the tumors and the CRCs, whereas KL-SPC and KL-CC10 (vertically), stands for the genotype and cell of origin. *Il-1β* is visualized in the upper box and *Rpl19* in the box below. Tumor samples are placed on the left in the boxes and CRCs on the right.

In Figure 11, we can see that for the tumor samples there is a strong *Il-1β* band. This also shows that there is very low or no *Il-1β* expression in CRCs compared to matching tumors. *Rpl19* the housekeeping gene has a strong band which means that there was a similar amount of RNA present in the CRCs compared to the tumors.

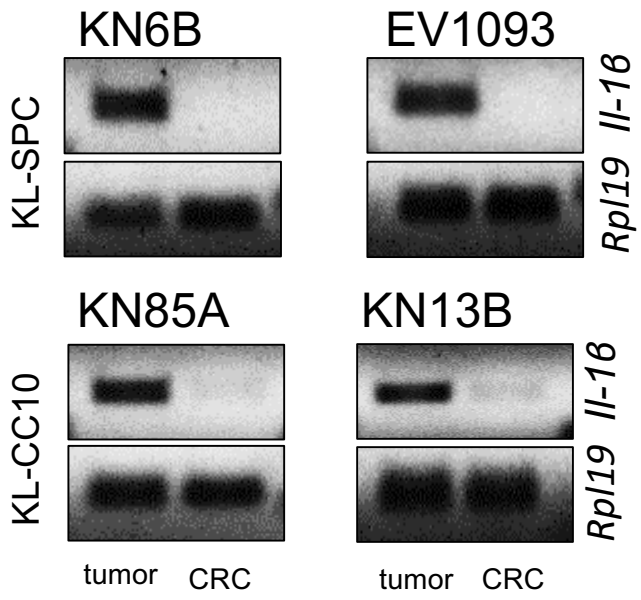

Figure 11. *Il-1β* qPCR products visualized in agarose gel.

Comparison of *Arginase 1* expression (Figure 12) shows that there is a very clear difference when comparing CRCs to the matching tumors; *Arginase 1* expression is dramatically lower in CRCs than in the tumors. This result is similar to *Il-1β* expression comparison above, however, the *Arginase 1* expression could be detected for all the CRCs by qRT-PCR.

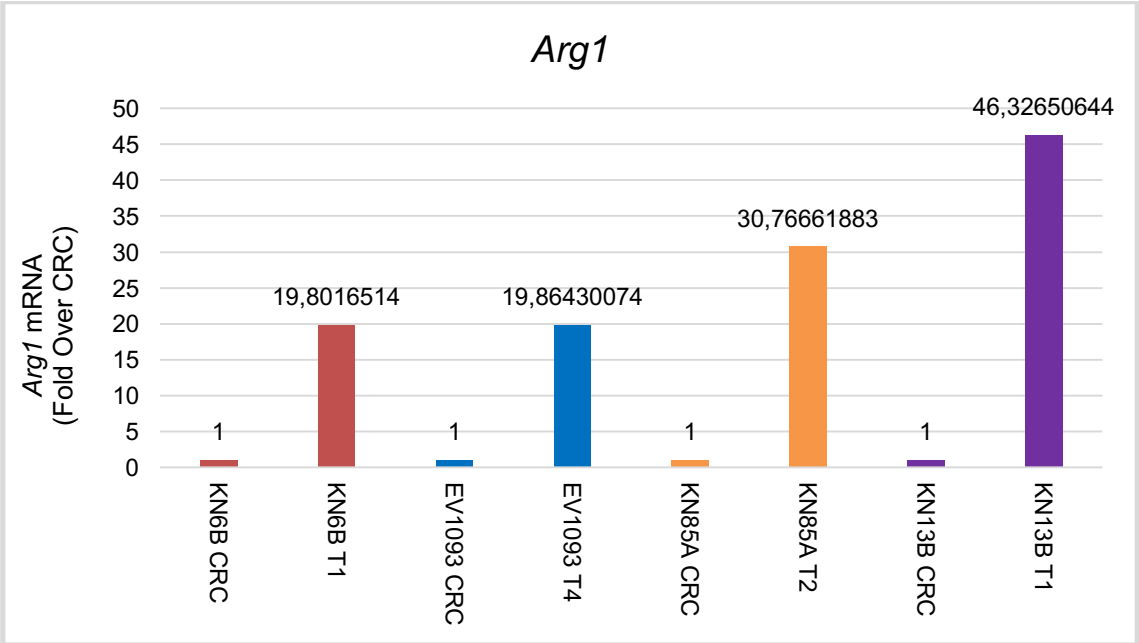

Figure 12. *Arginase 1* expression between tumor and CRCs.

For all samples there was higher expression of *Cxcl5* in the tumors compared to the CRCs (Figure 13). The difference was quite large for KN6B, EV10903 and KN13B, however for KN85A the difference was less than 2 fold.

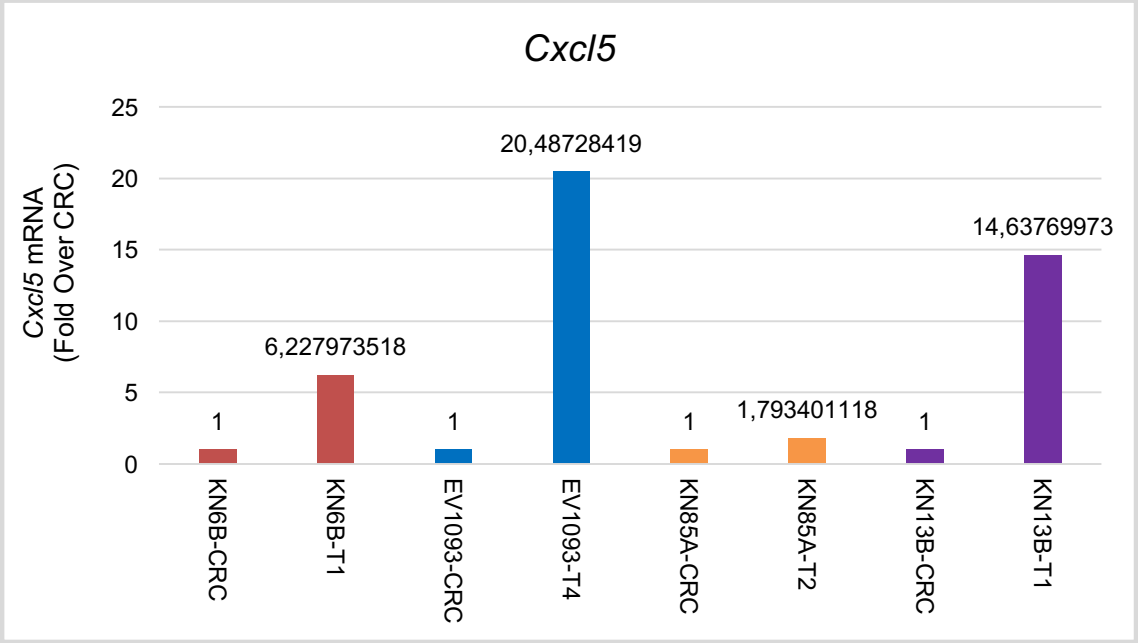

Figure 13. The *Cxcl5* expression comparison between tumor and CRCs.

In figure shown below (Figure 14), we can see higher *Il-6* expression in the tumors compared to the CRCs for 3/4 samples, however the largest difference is seen for the 2 KL-SPC tumors (KN16B and EV1093). KN85A has an opposite trend to the others, with twice as much *Il-6* gene expression in the CRC compared to the tumor. KN13B has quite small difference.

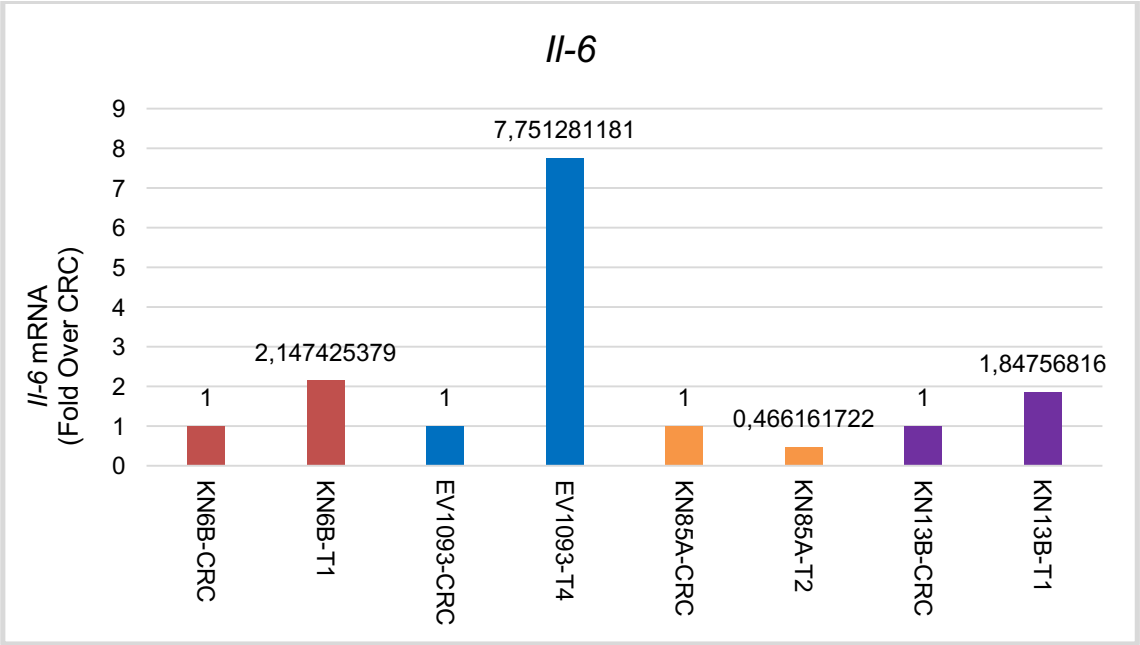

Figure 14. *Il-6* expression comparison between tumor and CRCs.

There is higher expression of *Ccl2* in the tumors compared to the CRCs for the KL-SPC samples while for 1 KL-CC10, KN13B, there is no difference between the CRC and the tumor for this gene. Similar to *Il-6*, KN85A has an opposite profile to the other tumors, with greater than 2 fold higher *Ccl2* gene expression in the CRC compared to the tumor tissue (Figure 15).

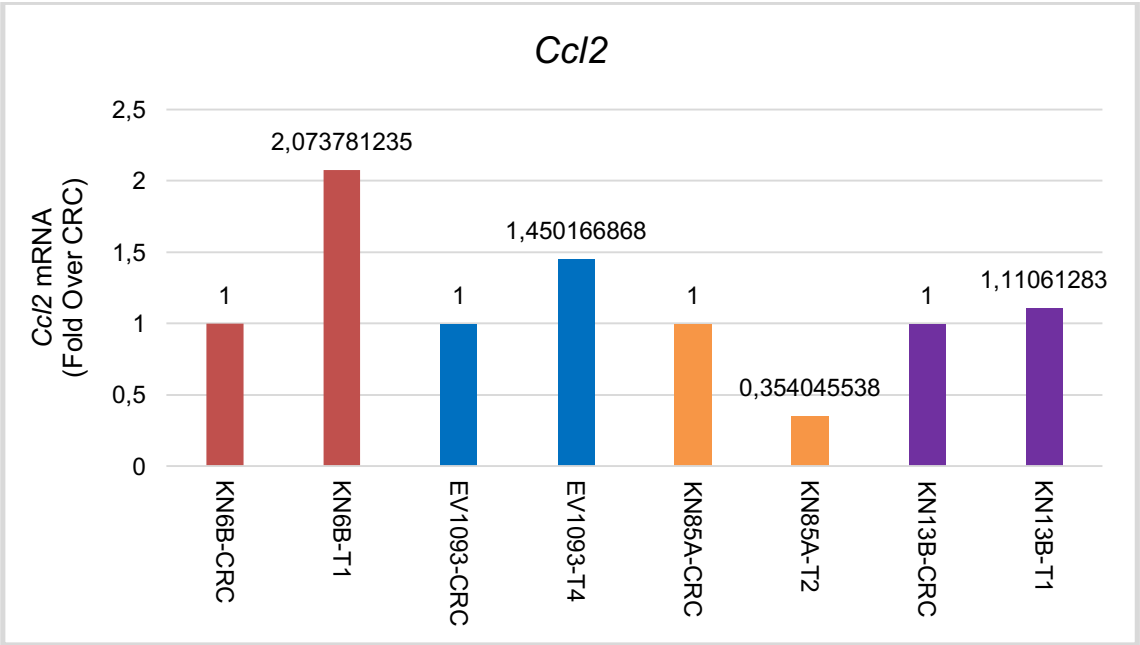

Figure 15. *Ccl2* gene expression comparison between tumor and CRCs.

Generally, CRCs have lower expression of immune genes than tumors. *Il-1β* and *Arginase 1* expression was dramatically lower in CRCs than in tumors. Nevertheless, there were a few exceptions: KN85A had higher expression of *Il-6* and *Ccl2* in CRCs and KN13B showed no difference in *Ccl2* expression.

5.3 Conditioned media experiment

RNA from CRCs cultured in normal and stromal cell culture conditioned media was isolated and the gene expression of different immune genes of interest was compared in order to find out if there were any effect of stromal cells. *Il-1β* was excluded from this experiment since the previous experiment showed that the *Il-1β* gene was not expressed in the CRCs.

There were no major differences in *Arginase 1* expression with normal media when compared to the conditioned media (Figure 16).

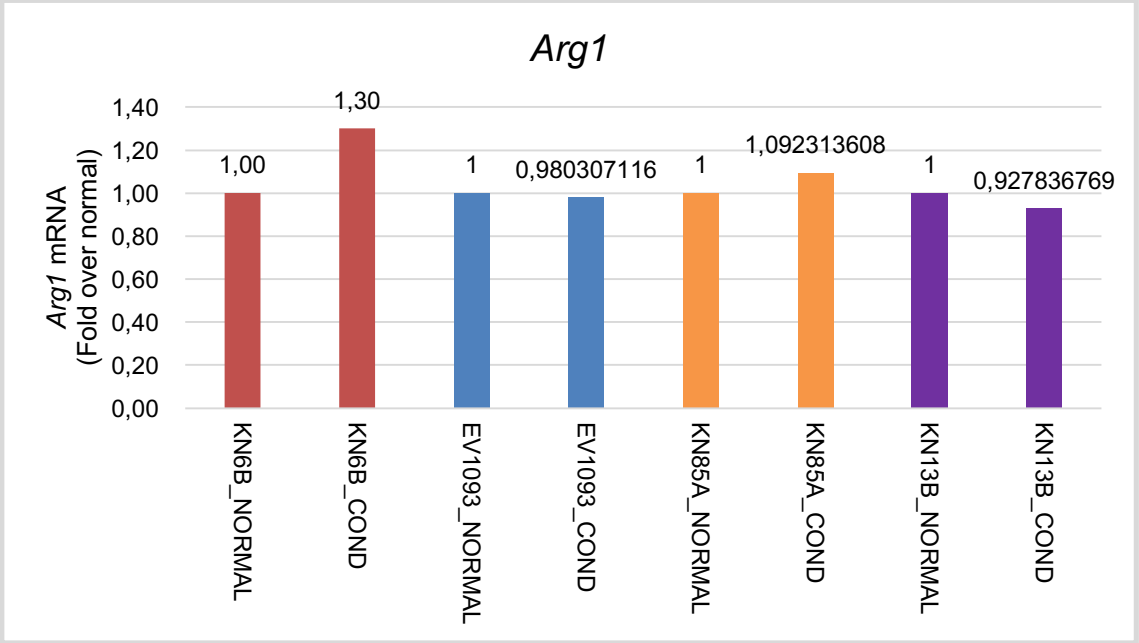

Figure 16. *Arginase 1* expression between normal media and conditioned media.

There was no major difference in *Cxcl5* gene expression between different media conditions (Figure 17). *Cxcl5* expression is slightly increased in KN85A cells in conditioned media but this difference is not as large as seen for other genes of interest.

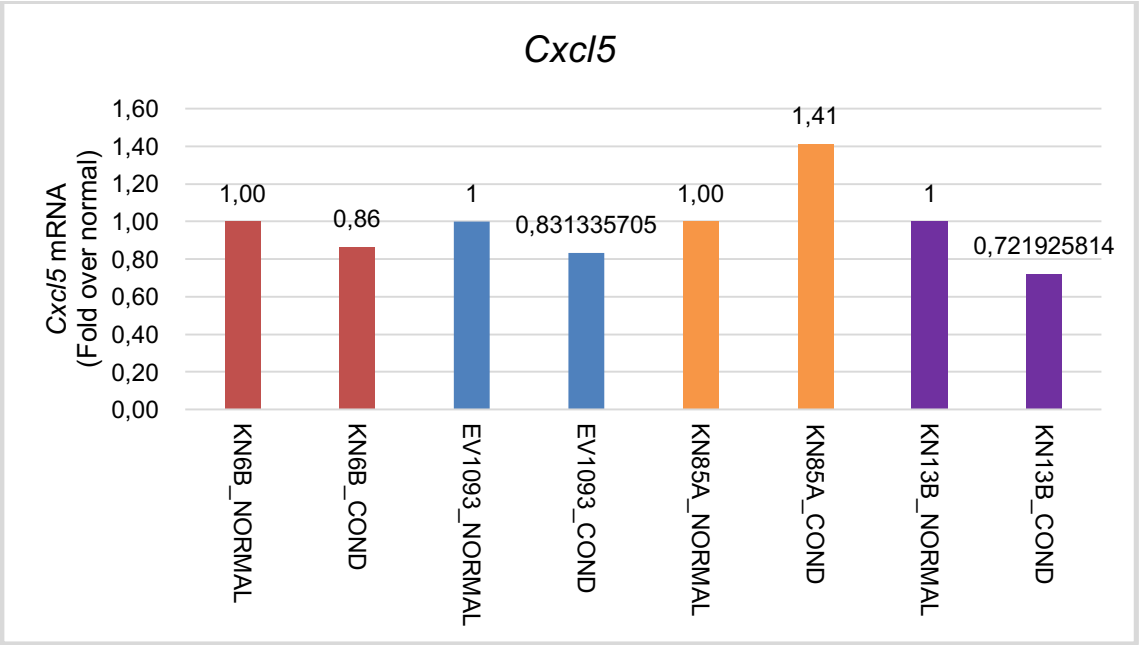

Figure 17. *Cxcl5* gene expression between normal media and conditioned media.

There was no difference in the *Il-6* gene expression between normal and conditioned media, except in KN85A, where there is almost 2-fold higher expression in *Il-6* gene expression when CRCs are cultured with conditioned media (Figure 18).

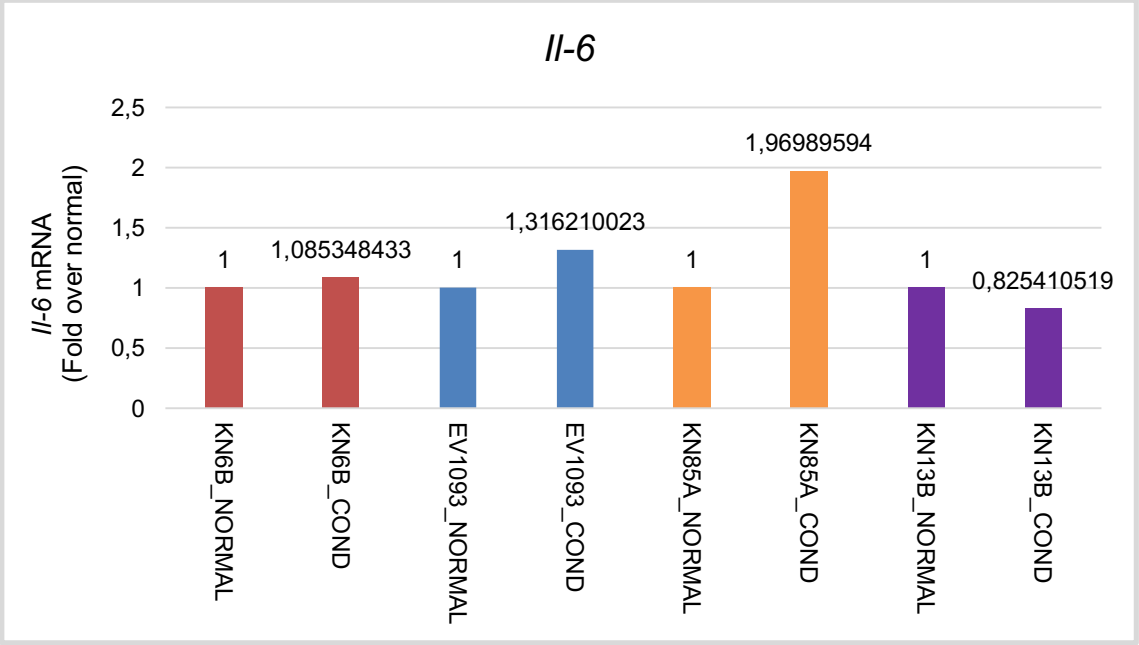

Figure 18. *Il-6* expression comparison between normal media and conditioned media.

KN85A with conditioned media has over 2-fold higher expression of a *Ccl2* gene than with a normal media (Figure 19). KN13B has also a major difference, although it is opposite to KN85A, 2-fold decrease in the expression of *Ccl2* in the conditioned media compared to the normal media. Also, KN6B shows a 40% decrease in the expression of conditioned media. In different CRCs, the conditioned media had variable effect on the *Ccl2* expression, independent of their cell-of origin.

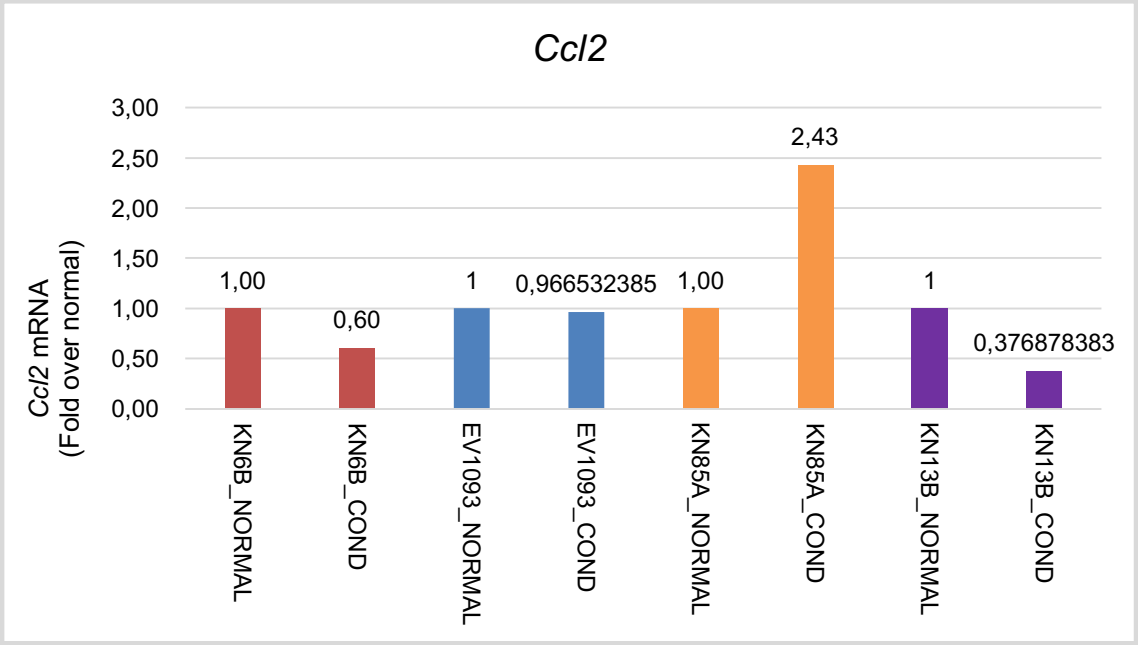

Figure 19. *Ccl2* gene expression comparison between normal media and conditioned media.

It was clear that there was no difference for ¾ cell lines (Figure 16-19), but as in tumor versus CRC experiment, there were exceptions. KN85A with conditioned media had higher expression of *Ccl2* and *Il-6* than with regular CRC-media (Figure 16 and 18). In *Cxcl5* and *Arginase 1* comparison there was no difference.

6 Discussion

In this project we used tumors and CRCs from *Kras;Lkb1* mutant mouse model to investigate differences in immune gene expression. The three different conditions we had for the gene expression comparison, were tumor versus tumor, tumor versus CRC and CRCs with normal media versus CRCs with conditioned media from stromal cell culture.

6.1 Expression of immune-related genes in *Kras;Lkb1* mutant NSCLC tumors and CRCs

In this project we confirmed that expression of *Il-1β* and *Arginase 1* is higher in the KL-CC10+ tumors compared to KL-SPC+ tumors, which means that these tumor samples are comparable to the ones used in the microarray in Nagaraj et al (2017) study. Also *Cxcl5* expression is higher in KL-CC10+ tumors than in KL-SPC+ tumors. This was expected from the microarray (Nagaraj et al. 2017) but the differential expression had not been confirmed before this. Other immune-related genes of interest for this project had different results, with *Il-6* expression having an opposite trend and *Ccl2* showing no large difference between KL-CC10 and KL-SPC tumors.

As we can see in the graphs in section 5.2, CRCs generally had lower expression of selected genes compared to matched tumors, especially for *Il-1β*. When evaluating the raw data, we saw that in KN85A-CRC and EV1093-CRC only 1/3 replicates gave fluorescence (Cq >34) and in KN6B-CRC there was no fluorescence in a single well indicating no or very low expression of *Il-1β*. The reason for this may be that in tumors *Il-1β* may be expressed only by immune cells (e.g. macrophages) or that *Il-1β* might only be expressed by tumor cells *in vivo,* making its expression immune microenvironment dependent. We can also make a suggestion that *Il-1β* is not expressed in CRCs independent of cell-of origin.

Also, *Arginase 1* expression was dramatically lower in CRCs than in the tumors. We can speculate is this due the absence of *Il-1β,* since these two genes are both linked to the activity of neutrophils in cancer.

So why is immune gene expression lower in CRCs? The reason for this might be found in the presence of immune cells in the tumor microenvironment and in the signaling between tumor and immune cells *in vivo.* As opposed to tumor tissue samples, which contain immune cells as well as tumor cells, there is no immune microenvironment present CRC epithelial cultures and no signaling between the tumor cells and immune cells. The results suggest that the interaction between tumor and immune cells is important for the expression of immune related genes, particularly to *Il-1β, Arginase 1* and *Cxcl5.*

The tumor microenvironment is composed by different cell types including stromal cells and this can affect expression of genes by the tumor cells. This was the main reason why the effect of conditioned media from stromal cell cultures on CRC expression of immune-related genes was investigated.

Conditioned media had no effect in 3 of 4 CRCs, but increased *Il-6* and *Ccl2* gene expression in KN85A CRCs compared to normal media. This KN85A result in conditioned media experiment suggests that there may be a cell-line specific effect of conditioned media from stromal cells on immune-gene expression in *Kras;Lkb1* mutant CRCs. This means that more experiments with more cells lines would need to be done to confirm this result.

Results from these experiments presents the importance of the tumor immune microenvironment and the tumor cell-of origin for the expression of immune-related genes. Variation seen in the results between different tumors and between CRCs in gene expression is consistent with NSCLC being a complex disease.

6.2 Quality control

As we can see in the Table 6, I performed six different qPCR runs in total. No Template Controls were included on each plate for each primer set. In H_2O controls there should not be any fluorescence as the sample used is just pure water rather than cDNA. In each run, there were fluorescence detected in *Rpl19* (housekeeping gene) H_2O controls. It might be that the primers used for *Rpl19* master mixes has been contaminated or the water itself and that caused the higher Cq values. However, when evaluating the raw qPCR data, we saw that the Cq value for *Rpl19* is normally between 17-19, so we concluded that the result from *Rpl19* H_2O controls are not relevant and that the results for the samples of interest could be analyzed.

On the 02.12 performed qPCR run, shows that there was fluorescence in every H_2O controls for each primer set. We checked the other results, and we saw that the Cq values for all primer sets in samples was lower than 30, whereas in H_2O controls the Cq value was over 35. We therefore concluded that these values over 35 were not relevant to the analysis of the gene expression

Table 6. NTC contaminations in qPCR runs.

Date	***Run***	***Contaminations***	***Cq value***
01.12	*Il-6*	*Rpl19* H2O control 3/3 wells	>30
	Il-1β		
	Rpl19		
02.12	*Ccl2*	*Ccl2* H2O control 2/3 wells	>35
	Cxcl5	*Cxcl5* H2O control 3/3 wells	>35
	Rpl19	*Rpl19* H2O control 1/3 wells	>35
08.12	*Arginase1*	*Rpl19* H2O control 1/3 wells	>30
	Rpl19		
13.12	*Ccl2*	*Ccl2* H2O control 2/3 wells	>40
	Cxcl5	*Rpl19* H2O control 3/3 wells	>30
	Rpl19		
13.12	*Il-6*	*Rpl19* H2O control 3/3 wells	>30
	Rpl19		
14.12	*Arginase1*	*Rpl19* H2O control 2/3 wells	>30
	Rpl19		

6.3 Reliability of the results

Procedures were performed by obeying the protocols and everything was written down in a personal lab book so that possible errors can be tracked down and procedures can be redone. A No Template Control reactions using RNase free H_2O instead of cDNA template were used to identify contaminations in the qPCR experiment. A number of NTC wells were contaminated, however, the Cq value for these wells was significantly higher than the correct Cq values for the primer pairs of interest (Table 6). While it would have been best to repeat the qPCR plates if there was sufficient time, I believe that these incidents did not affect the outcome of the results.

Prior to this project I did not have much experience with the procedures performed. My lack of experience was reflected for example in the qPCR procedure as the technical replicates varied from each other, meaning that there could have been pipetting errors. With practice I became more confident and also my pipetting skills improved. Reason for such a major difference (Figure 5) in gene expression could be also found in humane errors. After cDNA synthetization, the concentrations were measured by Nanodrop.

Errors in measuring could have led to a wrong calculation before dilution and therefore the amount of cDNA could have been greater in a certain sample.

In the original plan it was envisaged that analysis of other immune-related genes would have been included but these had to be excluded as the primer validation was not successful (results not shown). The primer pairs either did not produce single specific product band following the terminal PCR experiment or their efficacy in a standard curve qPCR was not at a desired level. Also comparisons between tumors and normal lung were excluded because of the lack of time. For further experiments, I believe it would be convenient to choose larger sample size, since the sample size exhibited in this project (n=2 each for KL-CC10 ASC and KL-SPC AC) was relatively small to argue that the results are reliable representation of the immune gene expression patterns in mouse *Kras;Lkb1* mutant NSCLC.

6.4 Ethics

This project obeyed good scientific practices. Working was based on the honesty, accuracy and diligence. All the information is shown in its entirety in this study, without leaving any details out. All the achievements and publications of other authors were respected by marking the references properly. (Finnish Advisory Board on Research Integrity 2014.)

When it comes to animal experimentation, it is estimated that each year up to 50 million animals are used for research worldwide. Majority of the cases use mice and rats in the procedures (Flossos 2005). Verschuren lab has approval to work with mouse models from the Experimental Animal Committee of the University of Helsinki and the State Provincial Office of Southern Finland (ESAVI/9752/04.10.07/2015).

The government of Finland has set an act to regulate animal experimentation; Act on the Protection of Animals Used for Scientific or Educational Purposes. The purpose of the act is to make sure that animals are used for experimentation only when it is necessary, and when there is no other scientifically reliable method to achieve the desired result. In addition, the act is intended to ensure that the experimentations cause the least possible amount of suffering, distress, pain or lasting harm to the animals used. Experimental animal establishments must have adequate and appropriate equipment, instruments and

facilities, including organized veterinary services. (Act on the Protection of Animals Used for Scientific or Educational Purposes 497/2013.)

There are alternative methods to animal experimentations such as computer model simulations, artificial skin which has been developed for toxicity testing and also human volunteers are an option. It is also possible to reduce animal suffering by improving the experiments themselves, treating pain with medication, and by use of less invasive methods for experimentation. (Flossos 2005).

Although there are alternative methods for animal experimentation, some of the experiments still require living organism. If any substitutive methods are developed, the law will obligate scientist to use it as it stands in the Act on the Protection of Animals Used for Scientific or Educational Purposes 497/2013. But until that they have no other choice.

6.5 Future

For the future it would be interesting to find out what causes the *Il-1β* expression in tumors and why the expression is higher in KL-CC10 ASCs compared to KL-SPC ACs. Separation of immune cells and tumor cells from fresh tumors by flow cytometry comparison of immune gene expression in the different cell populations is an option to answer this question. It may also reveal new information about tumor immune microenvironment and the immune related gene expression in *Kras;Lkb1* mutant NSCLC.

As discussed in section 6.2 the small sample size in this project is also a valid reason to repeat these experiment with extra tumors and CRCs in the future so that the results can be confirmed. For now, the results will work as a precursor, and they can't be presented as facts. However, it is positive that the results gained from this project are in line with the results exhibited in Nagaraj et. al. (2017) study.

I believe that I have grown professionally and improved my knowledge in this field of science. As been mentioned previously, I did not have much of a hands-on experience when I first started this project. I became familiar with the methods used in this project and learned to perform them independently. I am very grateful for everything I have

learned during this project and for the support and guiding I have received from Verschuren group.

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Culture of Murine NSCLC Conditionally Reprogrammed Cells (CRC's)

Materials: sterile

Irradiated NIH3T3 feeder cells

- 10cm plate cultured in DMEM + 10% HI FBS + Pen/Strep + L-Glut

CRC media: store at 4°C protected from light when not in use

F12 Media (Invitrogen-11765-054)	348ml
DMEM (Invitrogen-11965-084)	116ml
HI FBS (Gibco-10270-106)	25ml
Pen-strep solution (Gibco-15140-122)	5ml
Adenine (2.4mg/ml; Sigma A2786)	5ml
Insulin (Sigma-I2643)	625µl
Y-27632 (Enzo Life sciences ALX-270-M0055)	500µl
EGF (BD-354052)	50µl
Hydrocortisone (Sigma-H4001)	200µl
Cholera Toxin (List Biological Labs- 1000B)	10µl

1. Adenine (Sigma A2786)
Stock solution: 2.4 mg/ml
Dissolve 120 mg of powder in 45 ml of 0.5N HCl on a magnetic stirrer by adding couple of drops of concentrated HCl. The solution becomes clear only after adding HCl. Filter sterilize and store 10 ml aliquots in 15 ml falcon tubes. Store at -20°C.

2. Insulin (Sigma-I2643)
Stock solution: 4 mg/ml
Dissolve 50 mg of insulin in 2.5 ml of 0.0005N HCl by keeping it on a magnetic stirrer for 1 hr at room temperature (solution looks turbid). Add 10 ml of sterile MQ water; now the solution becomes clear. Filter through 0.2 uM low protein binding filter. Aliquot in sterile eppendorf tubes.
Store at -20°C.

(Note: Insulin dissolves when the pH of solution is around 2.5 to 3, if the solution does not become clear, lower the pH of the solution)

3. Rho kinase inhibitor/ Y27632 (Enzo Life sciences ALX-270-M0055)
Stock solution: 10 mM
Dissolve 5 mg of the compound in 1.5 ml of sterile MQ water. Aliquot in sterile eppendorf tubes, 100-200 ul/tube.
Store at -20°C.

4. Human recombinant (**hr) EGF (BD-354052)**
Stock solution: 0.1 mg/ml
Pipet 1 ml of sterile MQ into the vial, dissolve by pipetting up and down. Aliquot in sterile eppendorf tubes, 250ul/tube.
Store at -20°C for up to 3 months.

5. Cholera toxin - Azide free (List Biological Labs- 1000B)
Stock solution: 0.5 mg/ml
Add 0.5 ml of sterile MQ water into the tube mix well by pipetting. Aliquot in sterile eppendorf tubes as 100 ul/tube.
Store at 4°C.

Trypsin

PBS

DMEM + 10% HI FBS (for inactivation of trypsin)

Cell lifter (Corning-3008)

Protocol: for passaging, collection of cell pellet and freezing of cell cultures

1. Ensure there are sufficient plates with recently irradiated NIH3T3 feeder cells

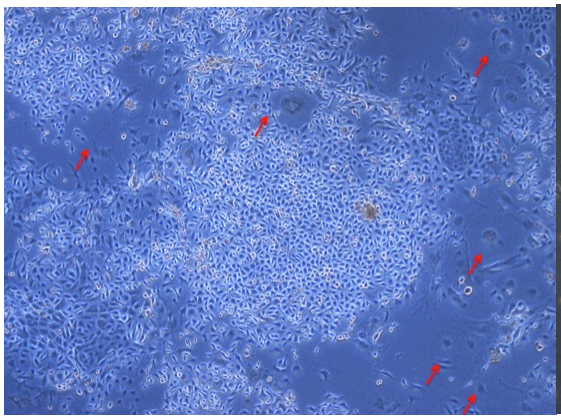
2. Ensure you had sufficient media and other reagents – warm media and trypsin in the water bath (37°C)

3. Check CRC culture plates under microscope and decide what to do

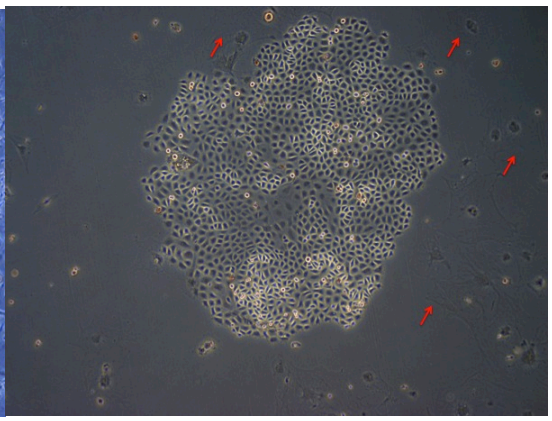
4. In laminar:
    a. Remove old CRC media with vacuum and Pasteur pipette
    b. Add 3-4mL PBS to the side of the plate and tilt to cover all cells
    c. Remove PBS with vacuum and Pasteur pipette
    d. Add 1mL pre-warmed trypsin to each plate – cover all cells and incubate at 37°C for 4-5 minutes to detach irradiated 3T3 cells
        i. During incubation time prepare 15mL tubes – if passaging and collecting pellet / freezing cells then prepare 2 tubes per CRC culture
    e. Check plate under microscope to see if i) 3T3 cells have detached from plate and ii) CRCs are still attached
    f. Remove trypsin + detached 3T3 cells with vacuum and Pasteur pipette
    g. Add 3-4mL PBS to the side of the plate and tilt to cover all cells
    h. Remove PBS with vacuum and Pasteur pipette
    i. Add 1mL pre-warmed trypsin to each plate – cover all cells and incubate at 37°C for 4-5 minutes to detach CRCs (as much as possible)
    j. Add 5-9mL DMEM + HI FBS to each plate to inactivate trypsin
        i. Note; volume added depends on confluency of cells – more confluent = more media to prevent clumping
    k. Use cell lifter to gently detach any CRCs still adhered to the plate – check under microscope to see if all cells are in suspension
    l. Transfer cell suspension to 15mL tube(s) as necessary for passaging / collecting pellet / freezing pellet
    m. Centrifuge cell suspension to pellet cells: 1000rpm, 5min, RT
        i. During centrifugation:
            a. For passaging of cells; label fresh irradiated 3T3 plates with CRC details (ID, passage number, date) and replace 3T3 culture medium with fresh CRC media
            b. For collection of cell pellets: label 1.5mL tubes with CRC details (ID, passage number, date)
            c. For freezing of cells: label 2mL cryovials with CRC details (ID, passage number, date) and prepare freezing solution (90% HI FBS + 10% DMSO) – get ice
    n. Remove media with vacuum and Pasteur pipette but do not suck up cell pellet

o. **For passaging cells:** resuspend cell pellet in fresh CRC media (1mL for each plate to be split) and transfer resuspended cells to 3T3 plates from step m (1mL per plate) → 37°C / 5% CO_2 incubator
p. **For cell pellet collection:** resuspend cell pellet in 1mL PBS and transfer to 1.5mL tube from step m → centrifuge to pellet cells at 10000rpm, 1min, RT → remove PBS with vacuum → freeze cell pellet at -80°C
q. **For freezing of cells:** resuspend cells in freezing solution (1mL for each cryovial to be frozen) and transfer resuspended cells to 2mL cyrovials from step m (1mL per cryovial) → ice → isopropanol freezing container → -80°C

~70% confluent CRC's



CRC colony



Red arrows indicate irradiated flat fibroblast cells.

Gene comparison protocol

1. Dilute primers from 100uM stock to 10uM
2. Prepare cDNA stock (80ng/μL) with nuclease-free water (see example below)

cDNA dilutions 1.12.2016						
	cDNA (ng/ul)	Desired concentration 80ng/ul	cDNA required		H2O required	Total
KN6B-T1	123,79		64,63	ul	35,37	100
EV1093-T4	119,31		67,05	ul	32,95	100
KN85A-T2	125,07		63,96	ul	36,04	100
KN13B-T1	122,97		65,06	ul	34,94	100
KN13B-CRC	124,15		64,44	ul	35,56	100
KN85A-CRC	122,53		65,29	ul	34,71	100
KN6B-CRC	241,75		33,09	ul	66,91	100
EV1093-CRC	143,21		55,86	ul	44,14	100
3T3-IRR	120,42		66,43	ul	33,57	100

3. Design how to organize the primers and samples on a 96 well plate
    a. (8 samples + 1 H2O) x 3 primer sets x 3 replicates = 81 wells
    b. total 27 + 3 error per one primer pair/gene
    c. Prepare Sybrgreen and primer master mixes – one per primer pair

Reagent	***Concentration***	**1X**	**30X**
2X Sybrgreen	***1X***	5μL	150μL
10μM Forward Primer	***300nM***	0,3μL	9 μL
10μM Reverse Primer	***300nM***	0,3μL	9 μL
Nuclease-free H20	-	1,9 μL	57 μL
Total Volume		7,5 μL	225 μL

96-well layout

	1	2	3	4	5	6	7	8	9	10	11	12
A: 1-9										*H2O*	*H2O*	*H2O*
B: 1-9										*H2O*	*H2O*	*H2O*
C: 1-9										*H2O*	*H2O*	*H2O*
D: 1-9												
E: 1-9												
F: 1-9												
G: 1-9												
H: 1-9												

BLANK

4. Aliquot master mixes into assigned wells on 96 well qPCR plate – 7,5μL per well

5. Aliquot 2,5μL of cDNA dilutions to assigned wells. For H20 control wells aliquot 2,5μL of nuclease free H2O
    a. Add cDNA to the side of the wells avoiding the tip from touching the sybr/primer mastermix
    b. Change tip for each well to increase chance of accurate pipetting

6. Apply coverslip and spin plate briefly (2min) at low speed (200g) to remove bubbles

7. Run plate – or put at 4 degrees for short time

BioRAD recommended cycling protocols:

3-step protocol: JD_PCR_MELTC

Check reaction volume: set to 10μL

Initial denaturation	95 degrees	3 minutes
40 cycles:		
Denaturing	95 degrees	10 seconds
Annealing	60 degrees	15 seconds
(*Arginase 1* 58 degrees)		
Extension	72 degrees	30 seconds
Melt curve	55 – 95 degrees (0.5 degree increments)	10 seconds