The effect of YKL-40 on cell migration

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Líf og umhverfisvísindadeild
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12 eininga ritgerð sem er hluti af
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Leiðbeinandi
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Útdráttur

YKL-40 er utanfrumuprótein sem er m.a. seytt við vefjaskemmdir og á meðan á bólgusvari stendur hjá spendýrum. Því er einnig seytt af nokkrum gerðum æxla. YKL-40 er kítínasalíkt prótein og getur bundist kítini, YKL-40 getur hvatt til frumuskiptingar. Það hefur verið verið sýnt *in vitro* að próteinið hefur áhrif á frumufar VSMC fruma (vascular smooth muscle cells) og HUVEC fruma (human umbilical vein endothelial cells). Mesenchymal stofnfrumur geta sérhæfts yfir í mismunandi miðlagsfrumur s.s. VSMC frumur. Þessar frumur sýna frumufar og berast til vefja þar sem vefjaskemmdir eða annað slíkt hefur átt sér stað. Þessu frumufari er stýrt af efnatogum (e. chemokine). Þar sem að YKL-40 er seytt af vefjum í svipuðum aðstæðum er möguleiki á að það hafi hlutverk við að stýra frumufari mesenchymal stofnfruma.

Markmið þessa verkefnis er að athuga hvort YKL-40 hefur áhrif á frumufar mesenchymal stofnfruma og MCF-7 brjóstakrabbameinsfruma. YKL-40 próteinið sem var notað í þessari rannsókn var safnað frá HEK293T frumum sem höfðu verið ummyndaðar til að tjá manna YKL-40. Ætinu sem frumurnar voru í var síðan safnað 1 og 4 dögum eftir að ummyndunin átti sér stað og magn þess mælt. Áhrif YKL-40 á frumufar Mesenchymal stofnfruma og MCF-7 fruma var síðan mælt í "modified Boyden chamber". Þau reyndust vera óveruleg.

Abstract:

YKL-40 is a extracellular protein that is secreted in response to tissue damage in mammals and during the inflammatory response, it is also secreted by a number of solid tumors. YKL-40 is a chitinase like protein and can bind chitin. It has the ability to induce proliferation. It has been shown *in vitro* to function as a chemo attractant for cells such as vascular smooth muscle cells (VSMC) and human umbilical vein endothelial cells (HUVEC). Mesenchymal stem cells have the ability to differentiate into cells of the mesodermal lineage such as VSMC. They have the ability to migrate, their migration is mostly steered towards tissues under pathological conditions. Chemokines play a role in their recruitment. Since YKL-40 is secreted from tissues in pathological conditions it is possible that it has a role in mesenchymal stem cell recruitment.

The goal of this project was to determine the effect YKL-40 has on the migration of Mesenchymal stem cells (MSC) and MCF-7 breast cancer cells. The YKL-40 used in this research was collected from cultured HEK293T cells which had been transfected with the human YKL-40 gene, the cell was collected after 1 and 4 days after transfection. A migration assay was performed in a modified Boyden chamber to determine the effect YKL-40 has on migration of mesenchymal stem cells and MCF-7 breast cancer cells. No difference in cell migration was observed in either cell type.

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1. Introduction

1.1 YKL-40

Human YKL40 is an extracellular 40 kDa protein composed of a single polypeptide chain of 383 amino acids. The name is derived from its molecular weight and the three amino acids on its NH_2-terminal, tyrosine (Y), lysine (K) and leucine (L) (Johansen J.S. *et. al*. 2006).

YKL-40 is a part of the glycosyl hydrolase family 18 which is split into two groups, chitinases and chi-lectins. The chi-lectins *e.g.* YKL-40, are chitinase-like lectins which lack the ability to hydrolyze chitin. The ability of chitinases to hydrolyze chitin is dependent on the presence of glutamic (E) and aspartic acid (D) at the catalytic site. YKL-40's lack of enzymic activity is most likely due to the lack of glutamic acid at the catalytic site which has been replaced with leucine (Bussink A.P. *et.al.* 2007 and Renkema G.H *et. al.* 1997).

YKL 40 is secreted in response to tissue damage and during the acute inflammatory response. It stimulates can cell proliferation and has the ability to bind chitin and heparin (Fusetti F. *et.al.* 2003). It is secreted by macrophages, chondrocytes, differentiated vascular smooth muscle cells (VSMC) and fibroblast-like synovial cells as well as being secreted by a number of solid tumors such as breast, colon, lung, kidney, ovary, prostate, pancreas and osteosarcoma (Johansen J.S *et. al.* 2006). Elevated serum YKL-40 levels are therefore often found present in many patients suffering from cancer. A number of clinical studies have shown that a high serum level of YKL-40 is a biomarker of poor prognosis (Renkema G.H. *et.al.* 1998 and Jensen B.V. *et.al.* 2003). A likely reason for this correlation with poor prognosis is that YKL-40 has been shown to play a role in cancer angiogenesis, which is prerequisite for cancer growth. Shao *et.al.* showed that YKL-40 promotes the growth and development of tumors (Shao R. *et.al.* 2009) . YKL-40 is also expressed during the acute inflammatory response caused by the tumor.

1.1.2 The role of YKL-40 in cell migration

YKL-40 can function as a chemo attractant and has been shown *in vitro* to enhance migration, attachment and spreading of VSMC's (Nishikawa K.C. *et.al.* 2003). The effect of YKL-40 is not limited to VSMC's, it has also been shown to

have an effect on the migration of human umbilical vein endothelial cells (HUVEC) *in vitro*. YKL-40 is secreted when VSMC's are undergoing morphological changes; affecting cell organization and enhancing tube formation of VSMC's leading to possible angiogenesis (Malinda K.M. *et.al.* 1999).

Angiogenesis is a biological process where new blood vessels are formed. This is an essential event for biological processes such as embryonic development and wound healing (Otrock Z.K. *et.al.* 2007). Angiogenesis is also an important step in tumor growth as tumor development requires increased neovascularization. Avascular tumors do not have the ability to grow larger than 1 to 2 mm in diameter. By secreting angiogenesis factors such as YKL-40 the tumor supports capillary proliferation and enhances angiogenesis (Folkman J. 1974).

1.2 T-ChOS

YKL-40 has the ability to bind chitin. Chitin, ((1-4)-poly-N-acetyl D-glucosamine) is one of the most abundant polysaccharides found in nature, second only to cellulose. It is found in bacterial and fungal cell walls and in the exoskeletons of various animals such as beetles, ants, crabs, shrimp and lobsters (Lee C.G. 2009). The company Genís ehf. has been producing biologically active chitooligosaccharides from shrimp shells for the past few years. These chitooligosaccharides are formed by deacetylating chitin, resulting in a water soluble end product. A well defined mixture of chitooligosaccharides called T-ChOS (therapeutical chitosan oligosaccharide) is one of Genís' main products. This product cannot be degraded by chitin-degrading enzymes (personal communication with Jón M. Einarson). T-ChOS is a mixture of chitooligosaccharides, it can therefore potentially inhibit enzymes which bind to chitin (chitinase-like enzymes), such as YKL-40 (Renkema G.H. *et.al.* 1998 and Shackelton L.M *et.al.* 1995). Hence, T-ChOS could be able to inhibit the effect YKL-40 has on cell migration or angiogenesis. It was the goal of this project to determine whether the effect of YKL-40 could be determined, in order to late measure potential inhibition of YKL-40 by T-ChOS.

1.3 Mesenchymal stem cells

Mesenchymal stem cells (MSC) are multipotent stem cells that have the ability to differentiate into cells of the mesodermal lineages except hematopoietic cells. MSC reside in the bone marrow where they represent about 1/10.000 of the nucleated

cells in the marrow. They are also found in other tissues because of their ability to migrate, their migration is especially steered towards injured tissues or tissues in pathological conditions. Chemokines are thought to play a role in steering MSC migration to sites of injury, though the mechanism behind this recruitment of MSC is not completely understood (Chamberlain G. *et.al.* 2007). Migration of MSC can be induced *in vitro* by specific cytokines such as basic fibroblast growth factor (bFGF). The effect of bFGF is different depending on circumstances, for example in a medium with high levels of FCS (fetal calf serum), high levels of bFGF increases migration where as in low levels of FCS low bFGF concentration increases migration but high concentration decrease migration (Schmidt A. *et. al* 2006).

1.4 MCF-7 cell line

MCF-7 is a stable breast cancer cell line, the most commonly used cell line in breast cancer models (Burdall S.E. *et.al.* 2003). The breast cancer cell line was established in June 1973 by Dr. Soule. It was derived from the pleural effusion taken from a 69 year old patient suffering from metastatic breast cancer (Levenson A.S. and Jordan V.C. 1997). The cell line was then developed in the Michigan Cancer Foundation which is what the name MCF stands for. (Burdall S.E. *et. al.* 2003).

1.5 Boyden chamber migration assay

A commonly used method to analyze cell migration is the Boyden chamber migration assay. The method is based on two chambers that are separated by a microporous membrane. Cells in medium containing low serum levels are placed in the upper compartment and can migrate to the lower compartment which usually contains higher serum levels as well as chemo attractants. The cells are incubated for an appropriate period of time which differs between cell types. The membrane is fixed and stained, at last the number of cells that migrated through the membrane are determined (Chen H.C. 2004).

2. Materials and methods

2.1 Cell Culture:

All cells were grown at 37°C in an incubator (5% CO_2 atmosphere, humidified). Mesenchymal stem cells were obtained from Dr. Ólafur Eysteinsson at the Blood Bank. The Mensenchymal stem cells were isolated from bone marrow of three donors. The donors were all healthy and at the age of 18-45 years old. MCF-7 cells were obtained from the Cancer Research Laboratory at UI. Unfortunately, the identity of this cell line, had not been determined and is currently being examined. It is therefore possible that the cell line used in this study is different, possibly a different cancer line used by the Cancer Research Laboratory.

2.1.1 Materials

- PBS (Phosphate buffered saline) pH 7.4:
  - -137 mM NaCl
  - -2.7 mM KCl
  - -10 mM Na_2HPO_4
  - - 1.76 mM KH_2PO_4
- DMEM + Glutamax (GIBCO)
- FBS (fetal bovine serum)
- 100 mm culture dishes
- 25 cm^2 Falcon culture flasks

2.1.2 HEK293T cells

- Frozen HEK293T cells provided by Alexander Schepsky were thawed
- 5 ml PBS were added and the cells centrifuged at 2000 rpm for 3 min
- The supernatant is removed
- 1 ml of DMEM+ GlutaMax with 10% FBS is added
- Cells placed in a 25 cm^2 Falcon culture flask
- 4 ml of DMEM+ GlutaMAX with 10 % FBS is added
- Every 2nd-3rd days the cells were split 1:5 and placed on a 100 mm culture dish.

2.1.3 MCF-7

- Frozen MCF cells were thawed
- 5 ml PBS were added and the cells centrifuged at 2000 rpm for 3 min.
- The supernatant is removed
- 1 ml of DMEM+ GlutaMax with 10% FBS is added
- Cells placed in a 25 cm^2 Falcon culture flask
- 4 ml of DMEM+ GlutaMAX with 10 % FBS is added
- Every 4th-5th days the cells were split 1:8 and placed in a 25 cm^2 Falcon culture flask

2.2 Transfection of HEK293T cells with YKL-40 plasmid

2.2.1 Materials:

- *Competent E-coli*
- YKL-40 plasmid (pCMV6-XL5 origene SC125328)
- pUC19 plasmid (50 pg/μL) (New England Biolabs)
- LB medium
- Agar plates
- QIAGEN plasmid midi-kit
- 6 well plates
- GFP plasmid (pEGFP-N1)
- HEK293T cells
- DMEM (10% FBS) + Glutamax (GIBCO)
- Trans pass D1 (New England Biolabs)

2.2.2 Transformation of *E-coli*

To prepare for transfection of HEK293T cells with a YKL-40 plasmid, amplification of an YKL-40 plasmid was performed by transformation of *E-coli* and midiprep.

- Bacteria thawed on ice (had been stored at -80 °C)
- 3 solutions were prepared :

- Solution 1: 1μL of YKL-40 plasmid put in 30-50 μL of bacteria
- Solution 2: As a positive control 1 μL of pUC19 plasmid (50 pg/μL) put in 30-50 μL of bacteria
- Solution 3: As a negative control 30-50 μL of bacteria

- The bacteria was left to stand on ice for a few minutes
- 42°C heat shock for 35 sec
- Left to stand for a moment to cool on ice
- 300 μL LB medium added
- Grown at 37°C for 5 min
- 5 LB plates were used
    - 1. Plate : 100 μL of solution 1
    - 2. Plate: 100 μL of solution 1 (diluted 1:10)
    - 3. Plate: 10 μL of solution 1 (diluted 1:10)
    - 4. Plate: 100 μL of solution 2
    - 5. Plate: 100 μL of solution 3
- Grown overnight at 37°C

2.2.3 YKL-40 plasmid purification

YKL-40 plasmids were purified from the transformed *E-coli* mentioned earlier by using QIAGEN Plasmid midi kit. The purification was preformed according to the manufacturer's instructions catalog nr. 12143

2.2.4 Transfection of HEK293T cells

YKL- 40 plasmids were used to transfect HEK293T cells. As a control HEK293T cells were transfected with a GFP-plasmid.

- HEK293T cells were grown in two 6 well plate and had reached up to 70% confluence.
- For each well the following solution was prepared:
    - 250 μL DMEM with 10% FBS
    - 3 μg YKL-40 plasmid/ GFP plasmid
    - 9 μL trans pass D1
- The solution was left to stand for 20 min

- Medium removed
- 1 mL of DMEM with 10 % FBS was put in each well
- In 7 wells the solution containing YKL-40 plasmid was added
- In 2 wells the solution containing GFP plasmid was added

2.2.5 Collection of conditioned medium from transfected HEK293T cells

The transfected HEK293T cells were grown in DMEM medium containing 10% FBS. The conditioned medium (CM) was collected after 1 and 4 days and stored at -20°C.

2.3 Elisa

Elisa (Enzyme-linked immunosorbent assay) was used to see how much YKL-40 the transfected HEK293T cells had secreted during the 4 days of culture after transfection. MicroVue YKL-40 EIA kit was used to determine this.

- Microassay plates that had been coated with streptavidin and biotinylated murine YKL-40 monoclonal antibody were used
- In wells A-H was added:
    - A: 5 μL CM from HEK293T cells transfected with YKL-40 (1 day in culture) + 35 μL water
    - B: 5 μL CM from HEK293T cells transfected with YKL-40 (2 days in culture) + 35 μL water
    - C: 5 μL CM from HEK293T cells transfected with YKL-40 (3 days in culture) + 35 μL water
    - D: 5 μL CM from HEK293T cells transfected with YKL-40 (4 days in culture) + 35 μL water
    - E: 10 μL CM from HEK293T cells transfected with GFP (4 days in culture) + 10 μL water
    - F: 0.5 μL CM from HEK293T cells transfected with YKL-40 (1 days in culture) + 20 μL water
    - G: 0.5 μL CM from HEK293T cells transfected with YKL-40 (3 days in culture) + 20 μL water
    - H: 0.5 μL CM from HEK293T cells transfected with YKL-40 (4 days in culture) + 20 μL water

- I: 20 μL YKL-40 standard 0 ng/ml
- J: 20 μL YKL-40 standard 300 ng/ml

- 100 μL of capture solution was put into each well, mixed well and left to stand for 60 min
- each well was washed 4 times with a wash buffer
- 100 μL of substrate solution was added to each well and left to stand for 60 min, after that each well was washed 4x with a wash buffer
- 100 μL of stop solution was added
- The optical density was read at 405 nm

2.4 Migration assay

2.4.1. Materials

- Falcon Multiwell ^{TM} 24 wells
-Cell culture Insert with 8 μm pore size
- PBS (Phosphate buffered saline) pH 7.4
- DMEM-F12 media (with 0.5% FBS)
- DMEM + Glutamax (with 10% FBS)
- conditioned medium(CM) containing GFP
- conditioned medium (CM) containing YKL-40 (7.7 μg/ml)
- T-CoHS (16.44mg/ml) (Genis ehf)
- Mesenchymal stem cells
- MCF-7 cells
- cotton swab
- 4 % formaldehyde
- 0.5 % crystal violet blue
- bright field microscope

2.4.2 Migration assay of MSC towards YKL-40 in a modified Boyden chamber

Migration assay was carried out in 24 transwells using membranes with 8μm pores. 100μL of MSC at adensity of 6 x 10^5/ mL in DMEM F12 media (with 0.5% FBS) were placed in the upper chamber of the transwell assembly. The lower chamber contained 600 μL of DMEM-F12 (10% FBS) media with the following combinations:

- 594 μL of DMEM media and 6 μl of conditioned medium (7.7μg YKL-40/ml) from HEK293T cells transfected with YKL-40 plasmids
- 570 μL of DMEM and 30 μl conditioned medium (7.7μg YKL-40/ml) from HEK293T cells transfected with YKL-40 plasmids
- 594 μL of DMEM media and 6 μl of conditioned medium from HEK293T cells transfected with GFP plasmids
- 570 μL of DMEM and 30 μl conditioned medium from HEK293T cells transfected with GFP plasmids
- 594 μL of DMEM and 6 μl unconditioned medium
- 570 μL of DMEM and 30 μl unconditioned medium

Cells were incubated for 6 hours, the upper surface of the membranes scraped gently (using a wet cotton swab) to remove non-migrating cells and washed with PBS. The membranes were then fixed for 15 minutes in 4% paraformaldehyde and stained with 0.5% crystal violet blue for 10 minutes and washed gently in PBS. The number of migrating cells was counted using a bright field microscopy. Values of MSC migration were estimated by the average number of migrated cells bound per microscopic filed over four field per assay. All experiments were run in duplicate from three MSC donors.

2.4.3 Migration assay of MCF-7 cells migration towards YKL-40 in a modified Boyden chamber

Migration assay was carried out in 24-well transwell using membranes with 8μm pores (Becton Dickinson, San Diego, CA, USA). 100 μL of MCF-7 at a density of 1 x 10^5 cells/ mL in DMEM media (with 1% FBS) were placed in the upper chamber of the transwell assembly. The lower chamber contained the following

combinations:

- 594 µL of DMEM media and 6 µl of conditioned medium (7.7µg YKL-40/ml) from HEK293T cells transfected with YKL-40 plasmids
- 570 µL of DMEM and 30 µl conditioned medium (7.7µg YKL-40/ml) from HEK293T cells transfected with YKL-40 plasmids
- 594 µL of DMEM media and 6 µl of conditioned medium from HEK293T cells transfected with GFP plasmids
- 570 µL of DMEM and 30 µl conditioned medium from HEK293T cells transfected with GFP plasmids
- 570 µL of DMEM and 30 µl unconditioned medium

Cells were be incubated for 10 hours, then the membranes of the modified Boyden Chamber were fixed, stained and the number of migrated cells estimated like described above in 2.4.2 Migration assay of MSC towards YKL-40 in a modified Boyden chamber.

2.4.4 Migration assay of MCF-7 cells towards YKL-40 and T-chos in a modified Boyden chamber

Migration assay was carried out in 24-well transwell using membranes with 8 µm pores (Becton Dickinson, San Diego, CA, USA). 100 µL of MCF-7 at a density of 1 x 10^6 cells/ mL in DMEM media (with 1% FCS) were placed in the upper chamber of the transwell assembly. The lower chamber contained the following combinations:

- 570 µL of DMEM and 30 µL unconditioned medium
- 591 µL of DMEM media and 6 µL of conditioned medium (7.7 µg YKL-40/mL) from HEK293T cells transfected with YKL-40 plasmids and 3 µL of T-chos (16.44 mg/mL)
- 555 µL of DMEM and 30 µl conditioned medium (7.7 µg YKL-40/mL) from HEK293T cells transfected with YKL-40 plasmids and 15 µL of T-chos (16,44 mg/mL)
- 594 µL of DMEM media and 6 µl of conditioned medium (7,7µg YKL-40/mL) from HEK293T cells transfected with YKL-40 plasmids
- 570 µL of DMEM and 30 µL conditioned medium (7.7 µg YKL-40/mL) from

HEK293T cells transfected with YKL-40 plasmids

- 15 μL of T-chos (16.4 mg/mL)

Cells were be incubated for 10 hours, then the membranes of the modified Boyden Chamber were fixed, stained and the number of migrated cells estimated like described above in 2.4.2 Migration assay of MSC towards YKL-40 in a modified Boyden chamber.

3. Results

3.1. *E-coli* transformation and plasmid purification

Transformation of *E-coli* with the YKL-40 plasmid was successful. As expected the negative control where no plasmid had been mixed in with the bacteria had no colonies. The positive control where 1 μL of pUC19 (50 pg/μL) was mixed in with the bacteria gave one colony. 1μL of YKL-40 plasmid mixed with the bacteria gave 1 colony when it was diluted 10x and 4 colonies when it was not diluted. The YKL-40 plasmid was subsequently isolated from two of these colonies using a QIAGEN plasmid midi-kit. The purification of the plasmids was measured with a Nanodrop. The concentration of the first purification was 809.64 ng/μL, the concentration of the second was only 55.22 ng/μL.

3.2. Transfection of HEK293T

HEK293T cells were transfected with the YKL-40 plasmid, as a control HEK293T cells were transfected with a eGFP plasmid. The day after transfection the success of the transfection was evaluated by eGFP-expression in the cells (see Figure 1, left panel). It was estimated that under 30% of the cells were transfected. The cells were cultured in the same medium for 4 days and their conditioned medium collected after 1 and 4 days. To estimate the survival of the cells after 4 days in the same medium the eGFP expression on day 4 was compared with the eGFP expression the day after the transfection (see Figure 1). The GFP expression decreases substantially after 4 days which is probably due to the fact that cells had died during this period, due to low concentration of nutrients and accumulation of waste since the medium was not changed during this period.

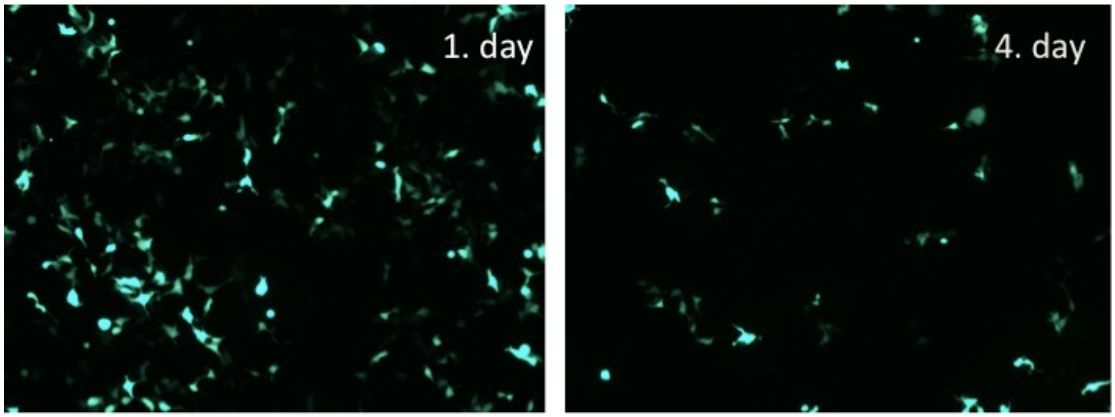

Figure 1: eGFP expression of transfected HEK293T cells. The picture on the left shows eGFP expression one day after transfection and the picture on the right shows eGFP expression four days after transfection.

3.3 HEK293T production of YKL-40

Transfection of HEK293T cells with the YKL-40 plasmid significantly increased their YKL-40 production. In figure 2 is shown the measurements on their YKL-40 production after 1, 3 and 4 days which was measured with Elisa.

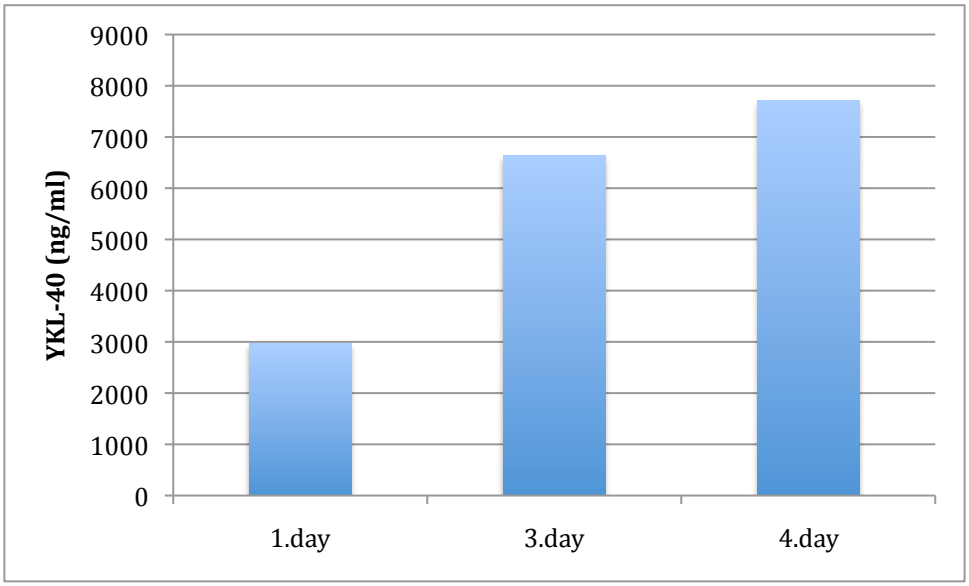

Figure 2: Measurements of YKL-40 concentration in conditioned medium from HEK293T cells that were transfected with a YKL-40 plasmids. YKL-40 concentration was measured with Elisa on day 1 day 3 and day 4.

3.4 The effect of YKL-40 on Mesenchymal stem cells migration

The effect of YKL-40 on the migration of Mesenchymal stem cells was measured with a modified Boyden chamber. The migration assay was performed on Mesenchymal stem cells from three donors, where difference in migration towards conditoned medium containing different concentration of YKL-40 was compared to migration towards conditioned medium from HEK293T cells that had been transfected with a GFP plasmid as well as migration towards unconditioned medium. All the conditioned medium were collected from 4 days old HEK293T cell cultures which had been transfected with an YKL-40 plasmid or an GFP plasmid.

To estimate whether YKL-40 had an enhancing effect on MSC migration, the average number of migrated cells bound per microscopic filed over four fields per assay were counted, the image was taken in a bright field microscope (see Figure 3).

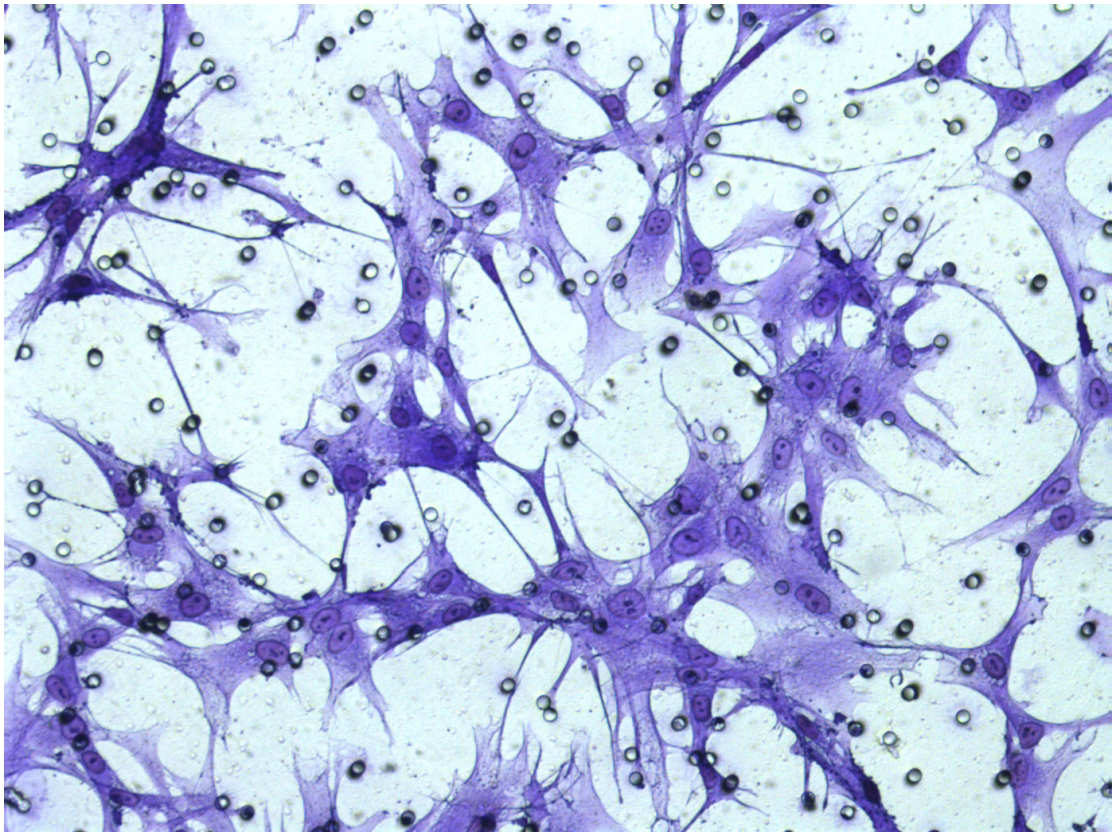

Figure 3: Mesenchymal stem cells that have migrated through a membrane with 8µm pores in a modified Boyden chamber. The image was taken in a bright field microscope.

MSC from the first donor shows increased migration towards higher concentration of YKL-40 in the medium (see Figure 4). Migration towards medium from HEK293T transfected with a GFP plasmid appears random and in some cases

higher than towards medium containing high levels of YKL-40. Statistical analyses revealed that there is a significant difference between groups (Table 1). A closer look at the difference between individual groups shows that there is a significant change in migration between Mesenchymal stem cells migrating towards 1% GFP medium compared to migration towards 5% GFP medium (p value = 0.0044). There is also a significant difference in migration towards 10% YKL-40 compared with both 1 % GFP (p value = 0.0021) and 1% YKL-40 (p value= 0.01). Between the other groups there seems to be no significant difference.

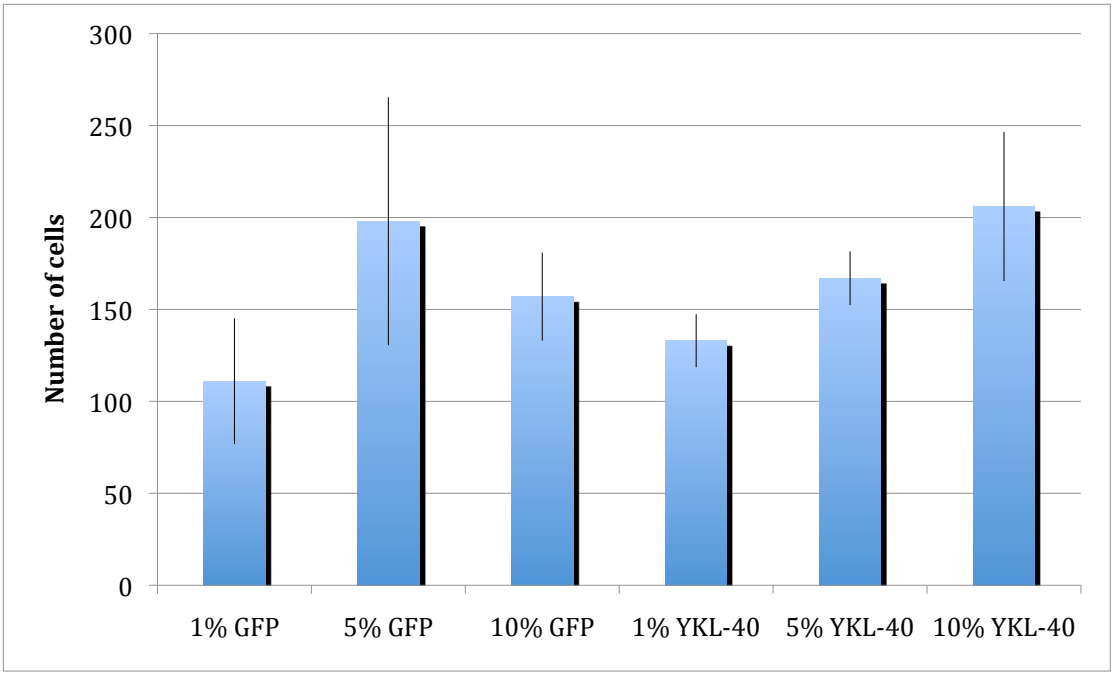

Figure 4: Results from a migration assay of MSC from donor 1 in a modified Boyden chamber. It shows the difference in MSC migration towards conditioned medium containing different concentration of YKL 40 and towards conditioned medium from HEK293T cells transfected with a GFP plasmids.

Table 1: ANOVA for data from donor 1

Degrees of freedom	Sum of Sq.	Mean of Sq.	F-value	Pr(<F)
5	20110,4	4022,1	8,2186	0,00416

As shown in figure 5 the second donor showed increased migration of MSC towards conditioned medium compared with un-conditioned medium. MSC showed increased migration towards both medium from HEK293T cells transfected with GFP

and YKL-40 plasmid and the migration increased from 1% concentration to 5% concentration in both YKL-40 conditioned medium and GFP. Statistic analyses revealed that there is a significant difference between groups (see Table 2). A closer look on difference between individual groups shows that there is a significant change between migration towards 5% YKL-40 concentrated medium compared with migration towards the other mediums (p value between 0.003-0.03) except for the 5% GFP medium (p value= 0.44). There is not a significant difference in migration between the other groups.

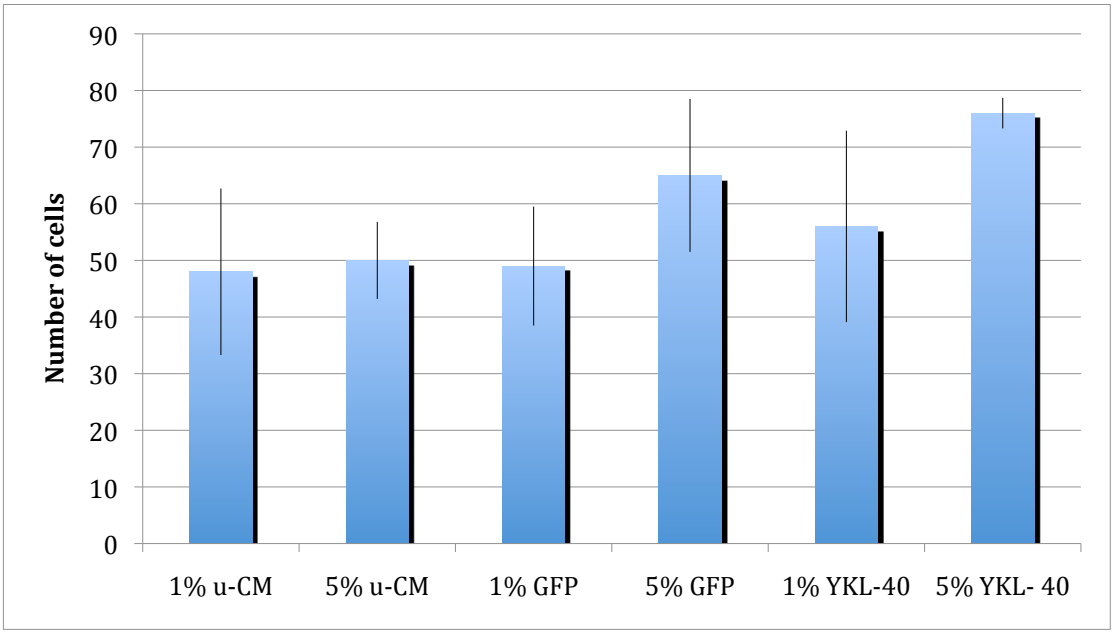

Figure 5: Results from a migration assay of MSC from donor 2 in a modified Boyden chamber. It shows the difference in MSC migration towards conditioned medium containing different concentration of YKL 40 and medium from HEK293T cells transfected with a GFP plasmids and towards un-conditioned medium.

Table 2: ANOVA for data from donor 2

Degrees of freedom	Sum of Sq.	Mean of Sq.	F-value	Pr(<F)
5	1918,94	383,79	7,71	0,00186

The third donor shows no correlation between migration towards increased YKL-40 concentration in the medium, in fact the migration diminishes with increased YKL- 40 concentration and seems random. Statistical analysis showed that there is no

significant change in migration towards any of the different mediums (see Table 3). There is a slight increase in migration of MSC towards conditioned medium compared with un-conditioned medium.

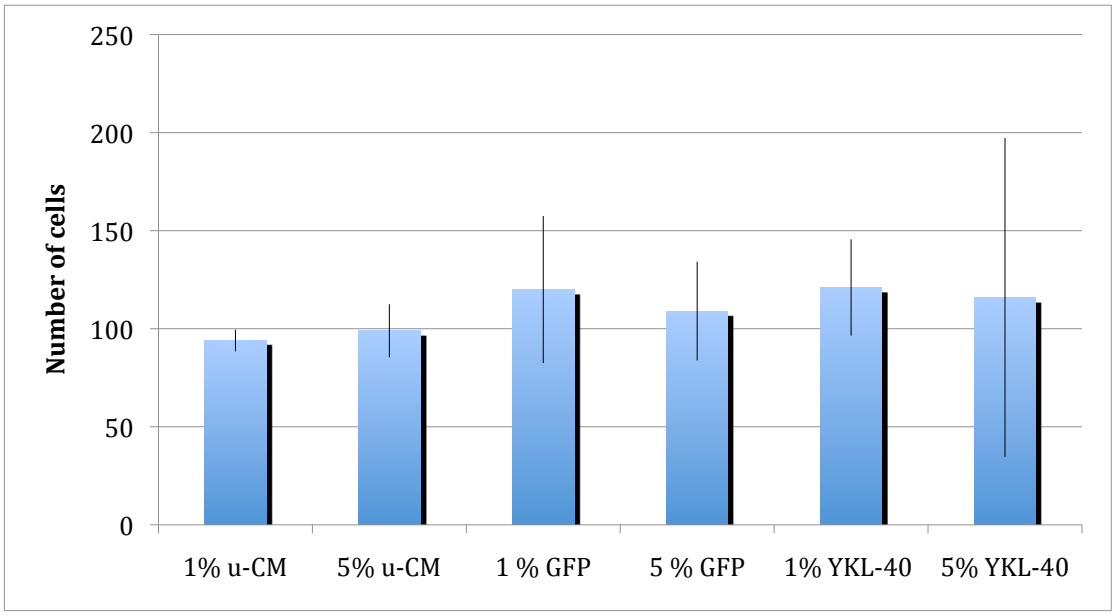

Figure 6: Results from migration assay of MSC from donor 3 in a modified Boyden chamber. It shows the difference in MSC migration towards conditioned medium containing different concentration of YKL 40 and conditioned medium from HEK293T cells transfected with a GFP plasmids and unconditioned medium

Table 3: ANOVA for data from donor 3

Degrees of freedom	Sum of Sq.	Mean of Sq.	F-value	Pr(<F)
5	1861,2	372,23	1,9276	0,1631

By comparing the effect of YKL-40 on MSC migration between donors (Figure 7) one can see that there is no obvious connection between increased YKL-40 concentration and increased migration. It is interesting to note the difference in migration between donors, it is obvious that the migration ability differs greatly between these three donors for unknown reasons.

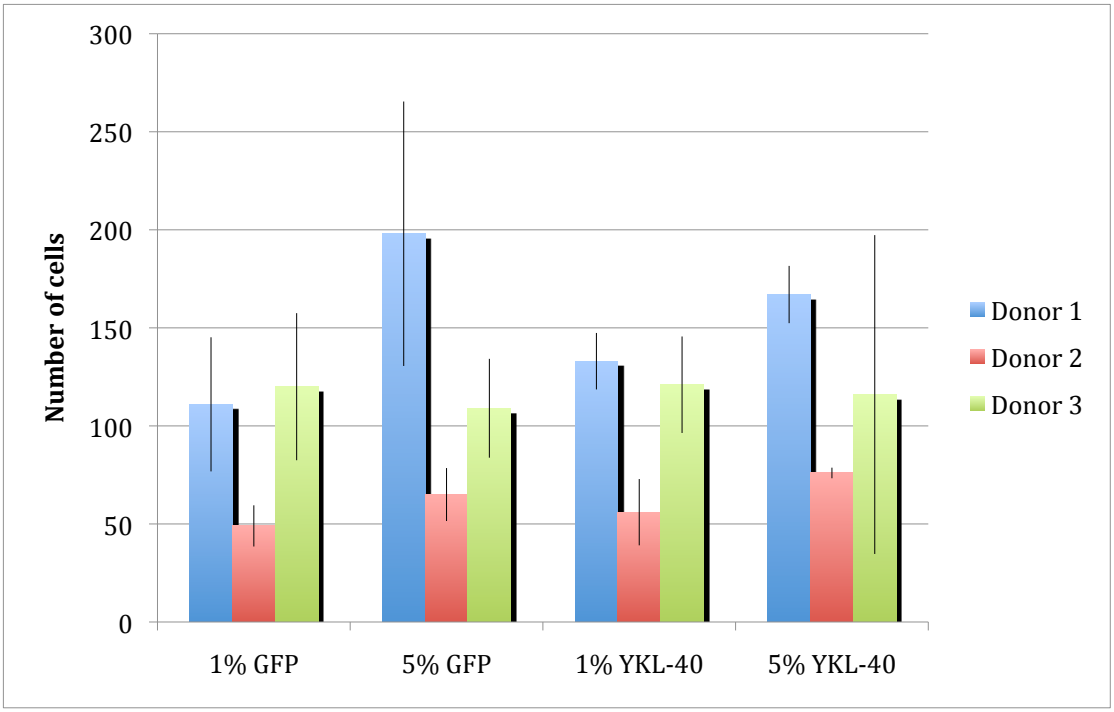

Figure 7: A comparison of the difference in migration of MSC between the three donors both towards different concentrations of YKL-40 and different concentrations of GFP.

3.5 The effect of YKL-40 on MCF-7 cell migration

The effect of YKL-40 on the migration of MCF-7 breast cancer cells was also measured with a modified Boyden chamber. The cell migration towards conditioned medium containing different amounts of YKL-40 was compared with conditioned medium containing GFP. All the conditioned medium were collected from 4 days old HEK293T cell cultures which had been transfected with an YKL-40 plasmid or an GFP plasmid.

To estimate whether YKL-40 has a migration enhancing effect on MCF-7 cells the sum of migrated cells bound per microscopic filed over four fields per assay was counted, images were taken in a bright field microscope (see Figure 8).

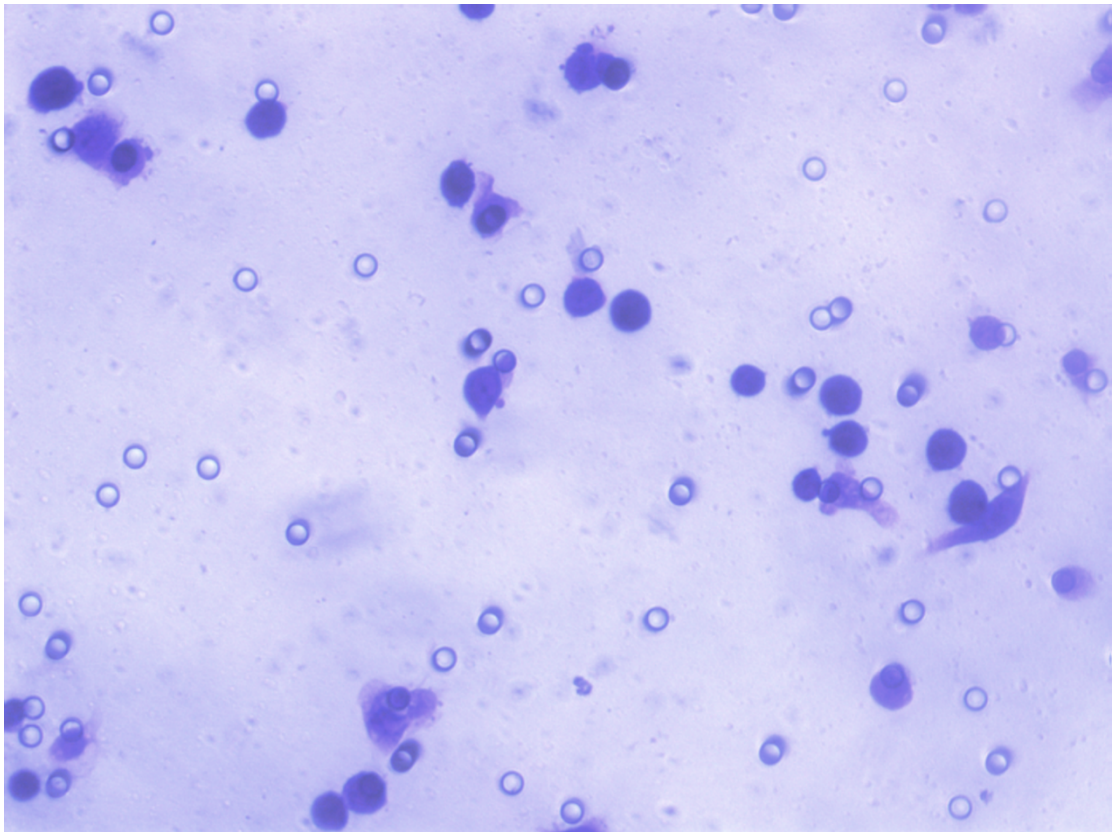

Figure 8: MCF-7 cells that have migrated through a membrane with 8μm holes in a modified Boyden chamber. The image was taken in a bright field microscope.

3.6 The effect of YKL-40 and T-ChOS on MCF-7 cell migration

The effect YKL-40 had on the migration of MCF-7 cells is shown in figure 9. There is a slight increase in cell migration towards higher concentration of YKL-40, though the variation in migration levels within each group is quite high and statistic analysis revealed that there is not a significant change between the groups (see Table 4).

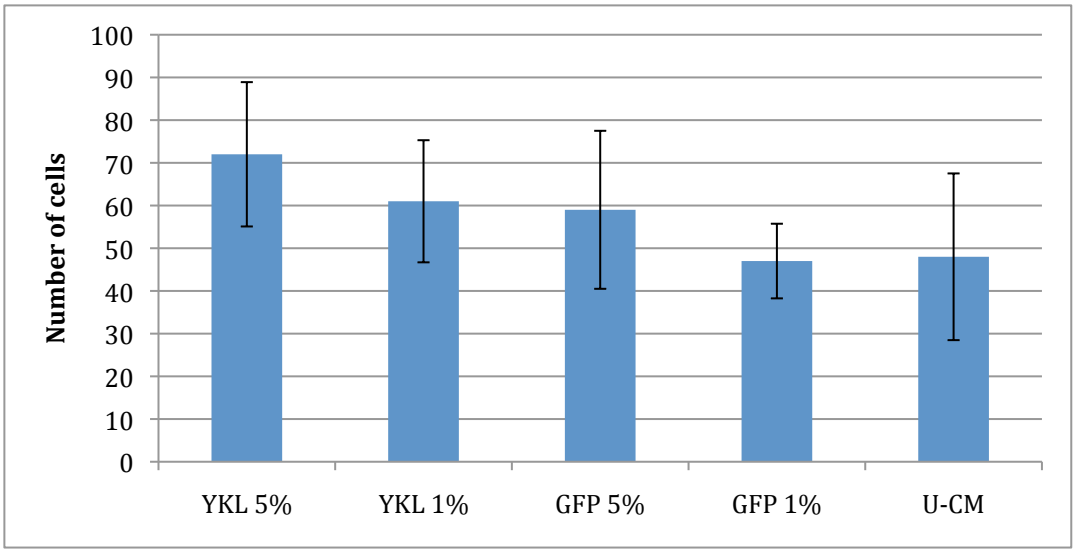

Figure 9: Results from a migration assay of MCF-7 cells performed in a modified Boyden chamber. It shows the difference in MCF-7 migration towards conditioned medium containing different concentrations of YKL 40 compared with migration towards conditioned medium from HEK293T cells transfected with a GFP plasmid and unconditioned medium (U-CM).

Table 4: ANOVA for data on migration of MCF-7 cells shown in Figure 9

Degrees of freedom	Sum of Sq.	Mean of Sq.	F-value	Pr(<F)
4	1734,5	433,63	1,8759	0,1671

YKL-40 has the ability to bind to chitin. T-ChOS is a chitosan made by Genis ehf. T-ChOS was added to the mixture to see if YKL-40 potentially bound by T-ChOS had a different effect on MCF-7 cell migration. As shown in figure 10 there is a slight decrease in migration towards 5% YKL-40 when it also contains T-ChOS, there is no difference in 1% YKL-40. There is a slight increase in migration towards medium containing YKL-40, though the difference between groups is not statistically significant for the variation within each group is too high (see Table 5).

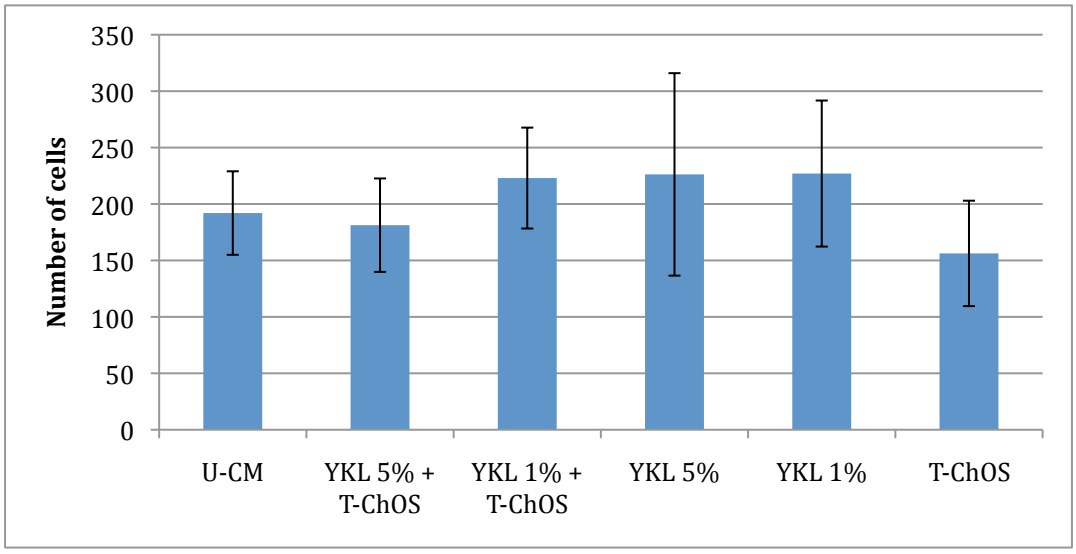

Figure 10: Result from a migration assay of MCF-7 cells performed in a modified Boyden chamber. It shows the difference in MCF-7 cell migration towards conditioned medium containing different concentrations of YKL 40 with and without T-ChOS. Migration towards unconditioned medium was used as a control.

Table 5: shows ANOVA for data on migration of MCF-7 cells shown in Figure 10.

Degrees of freedom	Sum of Sq.	Mean of Sq.	F-value	Pr(<F)
5	17085	3416,9	1,0535	0,4176

4. Discussion:

As shown in figure 1 the transfection of HEK293T cells was successful and by comparing GFP expression from day 1 to the expression on the 4th day we see that the expression has diminished on the 4th day. This is due to the increased cell death due to the diminishing levels of nutrients in medium and the accumulation of waste. By keeping the cells for so long in the medium we get higher levels of YKL-40 (see Figure 2) than if we would have used medium from 1 or 2 day old cultures. This comes at a price, the conditioned medium now contains increased levels of dead cells which might have burst and leaked some of their content into the medium.

Figures 4, 5 and 6 show the effect of YKL-40 on migration of Mesenchymal stem cells (MSC) from three different donors. As shown in figure 4 the first donor showed increase in MSC migration with an increased concentration of YKL-40 in the medium. However MSC migration towards medium from HEK293T cells transfected

with a GFP- plasmid which contained low levels of YKL- 40 seemed random and in some cases higher than towards medium containing higher levels of YKL-40. MSC from donor 2 showed increased migration towards 5% YKL-40 medium compared to 1% YKL-40 medium. The same difference was visible in migration towards GFP containing medium. The migration was higher towards 5% GFP containing medium compared with 1% YKL-40 medium. The third donor shows no correlation between migration towards increased YKL-40 concentration in the medium, in fact the migration diminishes with increased YKL- 40 concentration and seems random.

Migration increases towards higher levels of YKL-40 for both donor 1 and donor 2 but in both those cases the migration is higher towards the control medium containing high levels of GFP containing medium. There is an increase in migration towards higher levels of conditioned medium compared with un-conditioned medium. The increase in migration towards higher levels of YKL-40 containing medium for donor 1 and donor 2 might therefore be due to the increased levels of conditioned medium in the samples and not due to the increase in YKL-40 levels. That raises the question whether there is something else beside YKL-40 that the HEK293T cells are secreting that attracts the MSC. As shown in figure 7 there is a difference in migration between donors which indicates that the migration ability of MSC differs between individuals.

It seems that YKL-40 does not have an effect on MSC migration in our experiments. This is interesting because MSC has the ability to differentiate into cells such as VSMC and YKL-40 has been shown *in vitro* to have migration enhancing effect on these cells (Nishikawa K.C. and Millis A.J.T. 2003). It is possible that changes in MSC during differentiation causes them to develop sensitivity to YKL-40. It is also possible that changing our experimental approach, such as using isolated YKL-40 might show that MSC are sensitive towards YKL-40 levels

YKL-40 can functions as a chemo attractant and it has been shown *in vitro* that it enhances VSMC (Nishikawa K.C. *et.al* 2003) and HUVEC migration (Malinda K.M. *et.al.* 1999). The fact that YKL-40 is secreted by a number of solid tumors raises the question if it has any effect on migration of cancer cells which could lead to metastasis. I therefore examined the effect of YKL-40 on migration of the breast cancer cell line MCF-7 in a modified Boyden chamber. The data did not show a connection between YKL-40 and increased migration of MCF-7 cells. Though it is possible that it has an effect, the MCF-7 cells show a tendency to increase migration

towards higher concentration of YKL-40 compared with controls (see figures 9 and 10). Even though the cells show a tendency to migrate towards higher levels of YKL-40 the variation within each group is too high, so it is not possible to draw the conclusion from these results that YKL-40 has an effect. YKL-40 is not secreted by all types of cancers and as a matter of fact it is not secreted by MCF-7 cells (Shao R. *et.al* 2009). Since YKL-40 is not secreted by MCF-7 cells one can speculate that YKL-40 probably did not play a role in the spreading and angiogenesis of the cancer these cells are derived from. The question whether YKL-40 has an effect on cancer cell migration still remains. The next step could be to take a cancer cell line known to secrete YKL-40 and see if YKL-40 has an effect on their migration.

It is not possible to conclude from these results that YKL-40 has an effect on the migration of either Mesenchymal stem cells nor MCF-7 breast cancer cells. The identity of the MCF-7 cell line used in this research has not been confirmed and is currently under examination, it is therefore possible that the cell line used in this study is different, possibly a different cancer line used by the Cancer Research Laboratory at UI. It is therefore possible that the results from this research don't apply to the MCF-7 breast cancer cell line. There is a possibility that the results for the MSC might be flawed because of few measurements per sample or large variation between donors. Possibly using differentiated MSC might show that during differentiation these cells become more sensitive towards YKL-40, as has been shown for VSMC.

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