Retinoids effecting gene expression in single cultured intrahepatic cholangiocyte organoids

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Layman summary

The liver can repair itself after it has been damaged. The repair is done by a subset of cells that are capable to multiply in numbers and become the cells of the tissue that has been damaged. These cells are what we call stem cells. There are different mechanisms of repair and one of these is called the ductular reaction (DR). During DR the liver stem cells can grow into the cells that will form the bile ducts, cholangiocytes. DR is often accompanied by another reaction called liver fibrosis, a reaction where scar tissue is produced as a sort of quick fix. The main player in the formation of this scar tissue, is the activated hepatic stellate cell (HSC).
This mechanism of self-repair, the DR accompanied by liver fibrosis, has its limits, especially when the injury becomes chronic. During chronic liver injury the development of scar tissue can result in bigger problems: liver cirrhosis that can eventually lead to liver cancer if left unbothered. The only available treatment for a damaged liver in this stage is a (partial) liver transplantation.
To gain deeper insights in the communication between the liver stem cells and the HSC, a model system has been set up to be studied in the laboratory. What has already been found is, when liver stem cells and HSC are grown together the stem cells grow faster and the HSC are activated.
During the activation of HSC, they lose their vitamin A storage and become a scare tissue secreting cell. The excreted vitamin A can than act as a transcription factor for many genes, having a wildly divers impact on neighbouring cells.

What is largely unknown, is how these cells interact with each other, and specifically who triggers who. When more is known about the interaction of liver stem cells and HSCs, perhaps early stages of a fibrotic liver, a liver where scar tissue is already doing damage, can be kept in check. This could drastically reduce the onset to liver cirrhosis and liver cancer.
In this research, the effect of vitamin A and its metabolites on liver stem cells has been studied. There is no effect on cell division from these molecules, however, the expression of certain genes does seem to be influenced. The effects seen when the liver stem cells when they are grown together with HSCs, could be replicated only partially by vitamin A and is metabolites.

Abstract

During liver injury, a repair mechanism called the ductular reaction (DR) can be induced. During DR, hepatic progenitor cells (HPCs) proliferate and differentiate into cholangiocytes. DR is often accompanied by a response called liver fibrosis, where a build-up of extracellular matrix (ECM) leads to scaring of liver tissue. ECM is excreted by activated hepatic stellate cells (HSCs). During activation the HSC also lose their retinoid storage and secrete retinoic acid (RA).
An in vitro DR model, with intrahepatic cholangiocyte organoids (ICOs) representing HPCs and primary isolated HSCs, has been setup to investigate the communication between HPCs and HSCs. In this research the effect of retinoids on the organoids will be further investigated through measuring metabolic cell activity and changes in gene expression of *Plk1, Gpc3, Mmp7, and Lgr5*.
Culturing organoids with RA does not influence organoid proliferation. However, the gene expression of *Gpc3*, *Mmp7*, and *Lgr5* is influenced by retinoids. Expression of *Mmp7* and *Lgr5* seems to be increased in a dose dependent manner. Whereas the effects on *Gpc3* are not that overwhelming, research needs to be done on this matter.
Furthermore, a communicative model is presented, where after HSC activation by the organoids, the retinoids they secrete affect gene expression in the organoids.

Introduction

Ductular Reaction and Liver Fibrosis

Liver injury can have many causes, ranging from viral infections to abuse of alcohol or an unhealthy lifestyle, with little exercise and a high fat diet (Mayo Clinic, 2023). When the liver experiences injury it will react and try to fix it.

One such inflammatory response is the ductular reaction (DR). DR can be defined as biliary proliferation in which hepatic progenitor cells (HPCs) activate and differentiate into cholangiocytes. In parallel, cholangiocytes self-proliferate and hepatocytes can trans-differentiate into a biliary phenotype (Sato et al., 2019) (figure 1).

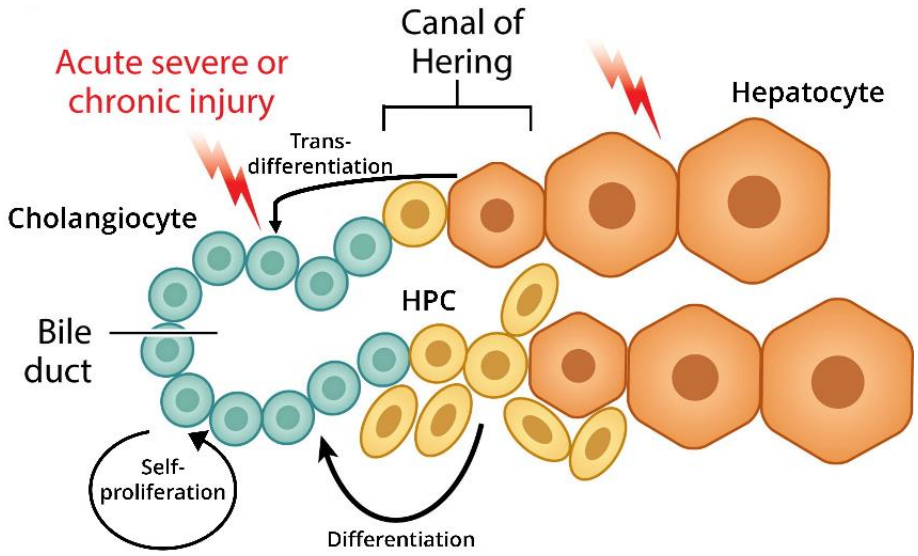

Figure 1: Schematic overview of cells involved in the ductular reaction (DR) and their actions. HPCs proliferate and differentiate into cholangiocytes, cholangiocytes self-proliferate, and hepatocytes trans-differentiate into cholangiocytes. HPC, hepatic progenitor cell. Adapted from: Ko, S., et. al (2020). Liver progenitors and adult cell plasticity in hepatic injury and repair: knowns and unknowns. Annual Review of Pathology: Mechanisms of Disease, 15, 23-50.

Another response of the liver after sustaining injury, is the onset of liver fibrosis. Initially, fibrosis is a natural response of the liver to heal itself after injury is sustained (Albanis & Friedman, 2001). The real problem occurs when the injury becomes chronic and the extracellular matrix (ECM) increasingly builds up, leaving scar tissue behind (Friedman, 1993). The cell responsible for the excretion of ECM is the activated hepatic stellate cell (HSC) (Acharya et al., 2021). In its quiescent state, the HSC functions as a vitamin-A storing cell (Friedman, 1993). During activation HSCs undergo a trans-differentiation into a myofibroblast-like cell, where it loses its vitamin-A storage and starts to excrete ECM (Friedman, 1993).

DR can be accompanied by liver fibrosis, and it has been described that HPCs and HSCs are found to be near each other during DR (Carpino et al., 2016). However, their exact interactions and the effect they have on each other during DR remain unclear.

In Vitro Ductular Reaction Model

To fill the knowledge gap of the communication between HPCs and HSCs, an in vitro DR model has been developed by Haaker et al. (Unpublished). In this model, HPCs are represented by intrahepatic cholangiocyte organoids (ICOs) and the HSCs are represented by primary isolated HSC from fresh liver tissue. Both ICOs and primary HSCs originate from mice (*Mus Musculus*).

ICOs have been first described by Huch et al. (2015) to be cultured for a longer period (>5 months). The ICOs were cultured with expansion medium in hydrogel, where differentiation is inhibited, and the cells stay in a proliferative state (Huch et al., 2015). The ICOs originated from bile duct cells, cholangiocytes, which were extracted from liver tissue (Huch et al., 2015). Cholangiocytes form cyst-like organoids with a lumen in the middle, different from, for example, hepatocyte organoids which form more of a cell lump (Marsee et al., 2021). Huch et al. (2015) also showed that, with the right effector molecules, ICOs can be differentiated into cells expressing mature hepatocyte markers. This shows that the ICOs resemble HPCs. In the DR model used for this research, the ICOs are cultured in the expansion medium to prevent differentiation and keep them in a proliferative state, resembling the HPCS. ICOs will be further referred to as 'organoids' in this thesis.

Primary HSCs stay in a quiescent state when cultured in hydrogel (Haaker et al., Unpublished). It becomes interesting when the primary HSC and organoids are cultured together in a co-culture in Matrigel. In the co-culture, the primary HSCs become activated, and the organoids proliferate more than in single culture (figure 2) (Haaker et al., Unpublished data). The expected co-culture activity, the black bar in figure 2, is generated by adding half of the activity from HSCs to half of the activity of the organoids. This is done because the number of cells seeded in the co-culture are half of the amount in the single cultures, for both HSCs and organoids. The actual measured cell activity is higher than that of the expected co-culture, meaning that the cells present in the co-culture are proliferating at a higher rate, since the single and co-culture are grown for the same amount of time. It has also been established that the increase seen in the cell activity is mostly from the organoids (Haaker et al., Unpublished).

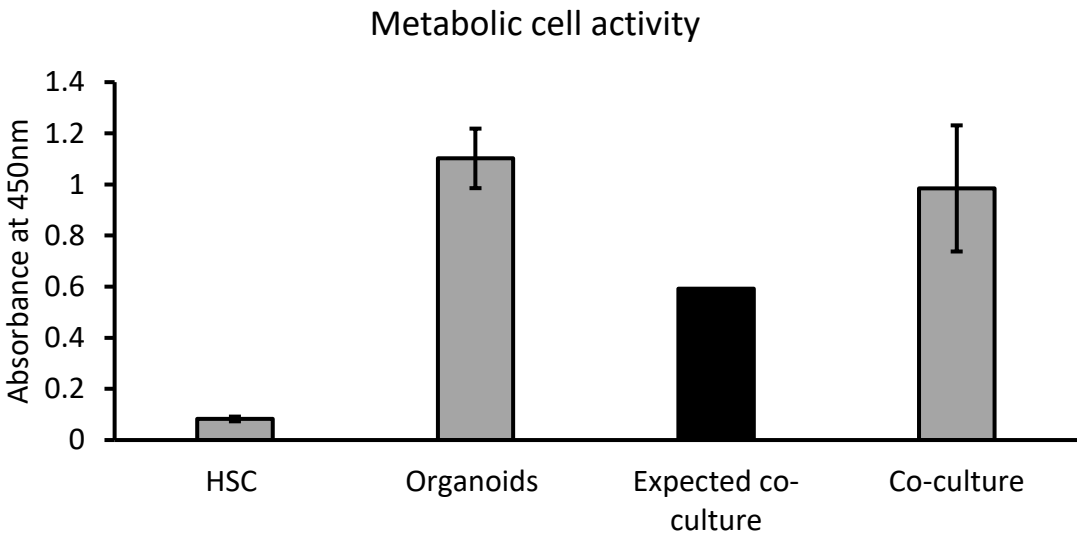

Figure 2: Metabolic cell activity of HSCs and organoids in single and co-culture, all grown in Matrigel. The expected co-culture bar, in black, is formed by adding half of the absorbance from the HSC bar to half of the absorbance from the organoid bar. HSC, hepatic stellate cell. Adapted from Haaker et al., Unpublished.

Furthermore, the gene expression of the organoids is altered in the co-culture when compared to the gene expression of single cultured organoids (Haaker et al., Unpublished). The organoids that were present in the co-culture have been separated from the HSCs through Fluorescence-activated cell sorting (FACS) prior to analysing gene expression. Noticeably, quite a few of the upregulated genes are associated with cell division and proliferation. For the simplicity of this thesis a few of these genes have picked out to investigate further: Glypican 3 (*Gpc3)*, Matrix metalloproteinase 7 (M*mp7)*, Leucine-rich repeat-containing G-protein coupled receptor 5 (*Lgr5)*, and Polo like kinase 1 (*Plk1)*.

Gpc3 has been described in literature to be a useful marker for proliferation in hepatocellular carcinoma (HCC) (Ho & Kim, 2011; Shirakawa et al., 2009). Loss of function of Gpc3 can lead to abnormal organ growth in mice (Liu et al., 2009), while overexpression leads to the inhibition of both liver regeneration and hepatocyte proliferation (Liu et al., 2010).

MMP7 is a secreted zinc- and calcium-dependent endopeptidase. It degrades various ECM substrates and cleaves non-ECM proteins which stick outside the cell (Liao et al., 2021).

Lgr5 is a marker for dividing cells present in self-renewing tissue, it binds Rspondin and enhances Wnt signalling. Single cells that are positive for Lgr5 can be grown in a 3D in vitro environment into organoids (Huch et al., 2013).

Plk1 is part of the Plk family, which are kinases involved in the regulation of cell division (Golsteyn et al., 1994). Plk1 has also been found effective as a marker for cancer, where it is upregulated in dividing tissue (Yuan et al., 1997).

Role of Retinoic Acid and Its Metabolism In The Hepatic Stellate Cell

As stated earlier, HSCs lose their retinoid storage during activation. The retinyl esters present in the lipid droplets are hydrolysed to retinol, retinol is then made into retinal and eventually converted into retinoic acid (RA), which is then excreted out of the cell (Haaker et al., 2020) (figure 3A). The conversion from retinal to RA is done by retinal dehydrogenase (RALDH) (O'Byrne & Blaner, 2013) (figure 3B). There are three cytosolic RALDH enzymes (RALDH1, RALDH2, & RALDH3) that are differentially expressed during mouse development and further organogenesis (Niederreither et al., 1999, 2002; Yoshida et al., 1998; Zhao et al., 1996).

The primary HSCs grown in co-culture with organoids activate, losing their retinoid storage and transdifferentiating into a myofibroblast-like cell. These activated HSCs also have a different gene expression pattern than the quiescent HSCs (Haaker et al., Unpublished). From this data it was observed that Raldh2 is upregulated in the activated HSC.
From these findings, the loss of retinoid storage and the upregulation of the enzyme responsible for RA synthesis, it can be speculated that the activated HSC loses its retinoids storage in the form of excreted RA.
When looking closer at the molecule RA, it is described as a highly reactive compound and it can act as a transcription factor for many genes in different tissues (reviewed in Di Masi et al., 2015; Ghyselinck & Duester, 2019; Napoli, 1996). RA is especially important in embryological development (Maden, 2000).

RA can bind specific and non-specific receptors, of which I will highlight two groups. Retinoic Acid Receptor α, β, and γ (RARα,β,γ) and Retinoid X receptor α, β, and γ (RXRα,β,γ). RAR and RXR can form heterodimers, homodimers or stay as monomers to bind RA (Germain et al., 2021). When bound to RA these heterodimers form a co-activator complex and can initiate transcription of target genes (Germain et al., 2021).

The aim of this research is to get a better understanding if retinoids are responsible for the effects on organoids that are seen in the co-culture, namely higher proliferation, and an upregulation of *Gpc3, Mmp7, Lgr5, and Plk1*. First, a study will be done on the RAR/RXR binding sites in the promoter regions of the target genes *Gpc3, Mmp7, Lgr5, and Plk1*. Second, organoids will be cultured in the presence or absence of the retinoids RA and Retinol. Third, Organoids will be cultured with a RALDH2 inhibitor in the presence or absence of retinoids.

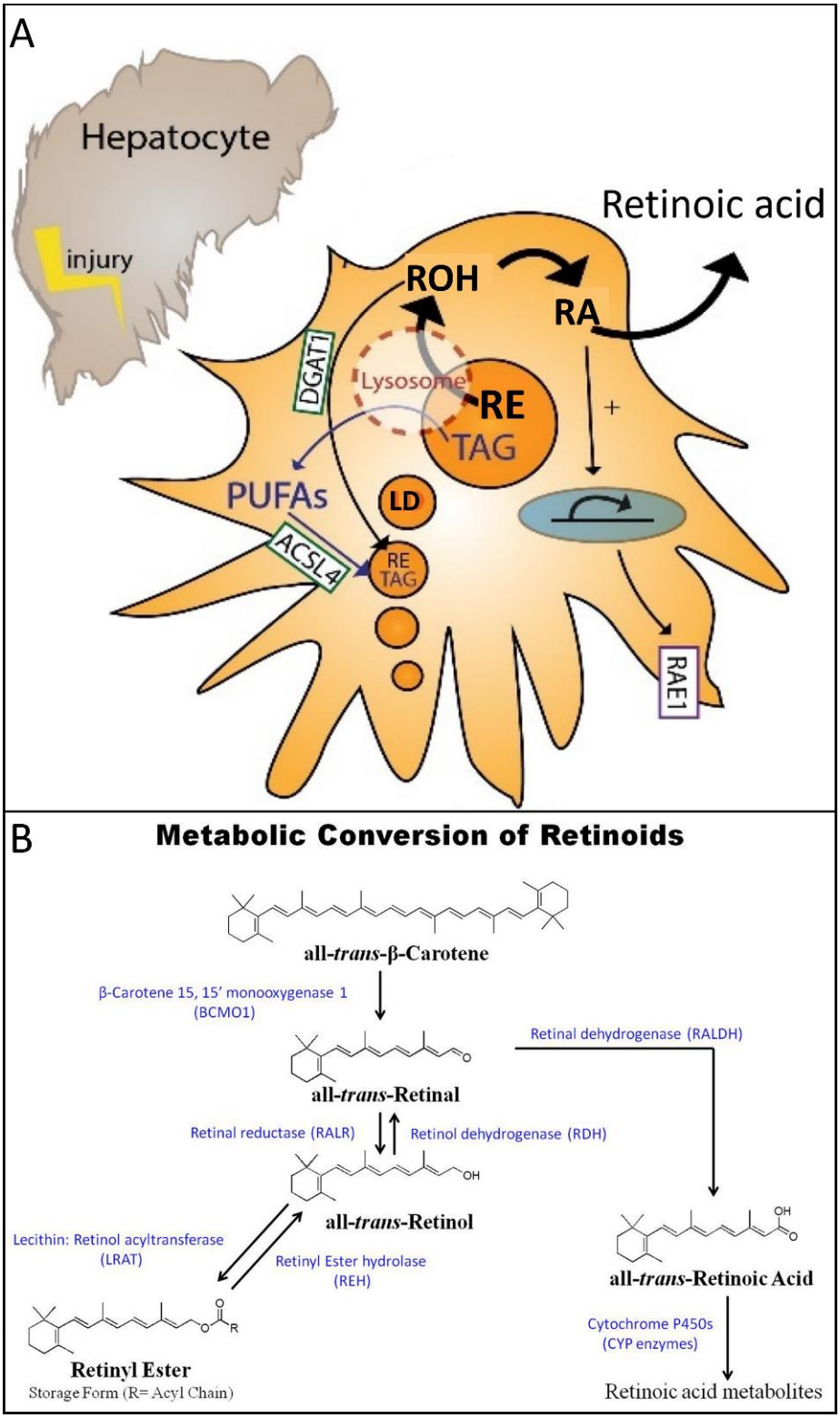

Figure 3: Schematic overview of retinoid metabolism during activation of the hepatic stellate cell (HSC). A, Overview of the pathway in which HSCs lose their retinoid storage. Retinyl ester are conversed to retinol, which in turn is converted int retinal and eventually becomes retinoic acid. Retinoic acid is then transported out of the cell. B, Overview of the metabolites and enzymes involved in the metabolism of retinoids. RE, Retinyl ester. ROH, Retinol, RA, Retinoic acid. A is adapted from Haaker, M. W., Vaandrager, A. B., & Helms, J. B. (2020). Retinoids in health and disease: A role for hepatic stellate cells in affecting retinoid levels. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1865(6), 158674. B is adapted from: O'Byrne, S. M., & Blaner, W. S. (2013). Retinol and retinyl esters: biochemistry and physiology: thematic review series: fat-soluble vitamins: vitamin A. Journal of lipid research, 54(7), 1731-1743.

Materials & Methods

Cell culture of intrahepatic cholangiocyte organoids

Organoids were cultured in Cultrex® RGF BME, Type 2 select (R&D systems, Minnesota, USA) or in Matrigel (BD Biosciences, USA) on a 24 well cell culture plate; flat bottom with lid; tissue culture treated; non-pyrogenic, polystyrene (Corning Incorporated, USA). Organoids were cultured in expansion medium as described by Huch et al. (2015). Medium was refreshed two times per week. Organoids were passaged every two weeks in a 1:4 dilution as followed:
Hydrogel was dissolved using cold PBS and cells were spun off at 500G for 5 minutes at 4°C. Cells were fragmented with a pipet point coated in ice cold FBS (Gibco, UK) and spun off at 500G for 5 minutes at 4°C. Cell were resuspended in BME or Matrigel and plated out on a new 24 well cell culture plate in 50µl droplets per well. 500µL expansion medium was added to each well containing cells in hydrogel. The wells around the cells were filled with 500µl PBS. The plate was incubated at 37°C with 5% CO_2.

Organoids were plated for experiments in a 48 well cell culture plate; flat bottom with lid; tissue culture treated; non-pyrogenic, polystyrene (Corning Incorporated, Kennebunk, USA). The passaging protocol was followed the same up until the organoids were resuspended. For plating the organoids were resuspended in 25µL BME per well, plated out in the 48 well cell culture plate. 250µl of co-culture medium, in the absence or presence of retinoids, was added to the cells. Co-culture medium was made by mixing expansion medium and passage medium, DMEM + GlutaMAX (Gibco, UK) with 10% FBS (Gibco, UK), in a 1:1 ratio. The wells around the cells were filled with 250µl PBS. The plate was incubated at 37°C with 5% CO_2. Organoids were cultured for two days, in the presence or absence of retinol in the form of all-trans retinol (Sigma-Aldrich, Germany), retinoic acid in the form of all-trans retinoic acid (Merck, Germany), and the Raldh2 inhibitor WIN 18,446 (SanBio bv, Netherlands). Retinoids were handled with care in the dark.

Metabolic cell activity/CCK8 assay

Metabolic cell activity was measured using a tetrazolium salt-based assay, namely CCK-8 (cell counting kit 8) (Sigma-Aldrich, Germany). Cells were incubated for 1 hour with a 1:10 dilution of the CCK8 liquid, diluted in passage medium. Absorption was measured at 450nm using the microplate reader SPECTROstar® nano (BMG Labtech, Germany).

Quantitative PCR

RNA was isolated from samples using the RNeasy Micro Kit (Qaigen, Germany), with a 1% beta-mercaptoethanol (Sigma-Aldrich, Germany) added to the lysis buffer. RNA concentrations were measured using the Nanodrop 2000 (Thermo Fisher) cDNA was made with the iScript cDNA Synthesis Kit (Bio-Rad, USA) in a thermocycler (Bio-Rad, USA). Amplification cycles were performed on a C1000 Touch thermocycler (Bio-Rad, USA) with an iQ SYBR Green Supermix (Bio-Rad, USA) following a 2-step assay protocol: denaturing was performed at 95°C for 10 seconds, annealing and elongation were performed at 60°C for 30 seconds for all primers, in a total of 40 cycles. The retrieved data was analysed with Bio-Rad CFX Maestro version 2. *Hprt*, *Hmbs*, and *Gapdh* were used as reference genes.

Table 1: Primers used for quantitative PCR. All primers are targets for mouse genes.

Gene	Primer sequence	Tm
Glypican 3 *(Gpc3)*	Forward – GATCTATATTGGCGTTGCTG Reverse – GTGGTCATGCAAGGCTGTA	60°C
Polo like kinase 1 *(Plk1)*	Forward – CCAAGCACATCAACCCAGTG Reverse – TGAGGCAGGTAATAGGGAGACG	60°C
Matrix metalloproteinase 7 *(Mmp7)*	Forward – ACTTCAGACTTACCTCGGATCG Reverse – TCCCCCAACTAACCCTCTTGA	60°C
Leucine-rich repeat-containing G-protein coupled receptor 5 *(Lgr5)*	Forward – TGCCCATCACACTGTCACTGT Reverse – CACCCTGAGCAGCATCCTG	60°C
Hypoxanthine-guanine phosphoribosyltransferase *(Hprt)*	Forward – GTGATTAGCGATGATGAACCA Reverse – CAAGTCTTTCAGTCCTGTCCA	55 – 63,5°C
Hydroxymethylbilane Synthase *(Hmbs)*	Forward – CTGTTCAGCAAGAAGATGGTC Reverse – TGATGCCCAGGTTCTCAG	57,5 – 63,5°C
Glyceraldehyde 3-phosphate dehydrogenase *(Gapdh)*	Forward – GAAGGTCGGTGTGAACGG Reverse – TGAAGGGGTCGTTGATGG	61°C

Promoter region study

Promoter regions were analysed with Eukaryotic Promoter Database (https://epd.expasy.org/epd/). The genes of interest (*Gpc3*, *Mmp7*, *Lgr5*, and *Plk1*) were searched for in the species *Mus* Musculus. Within the 'Search Motif Tool' the library was set to 'Transcription Factor Motifs (JASPAR CORE 2018 vertebrates)' and the motifs chosen were the following: for monomers of RAR and RXR the subtypes RAR-α(& var.2),-β(& var.2), & -γ(& var.2) and RXR-α,-β, & -γ; for the heterodimers there was a cutoff made when either RAR or RXR subtypes were missing. The range of base pairs (bp) was set from -1000 to 100 bp relative to the transcription start site (TSS) and the cut-off (p-value) was set to 0,001. The binding sites for each of the available subtypes of monomers or heterodimers were counted by hand, see table 2.

Statistics

For statistical analysis in figures 4 and 6 a one tailed paired t-Test with a Gaussian distribution was performed. For the statistical analysis in figure 8 a paired one-way ANOVA using a Ŝídák test was performed.

Results

Study of promoter regions of upregulated genes in the organoids in co-culture

As stated earlier, RA has the capability to function as a transcription factor when bound to RAR and/or RXR, forming co-activator complex. In the process of HSC activation, it loses its retinoid storage in the form of secreted RA. In the co-culture we see HSC activation and an increase in gene expression in the organoids. With this knowledge the question arises if the upregulated genes in the organoids could be due to the activation of transcription through RA.

In order to answer this, the promoter regions of the target genes *Gpc3*, *Mmp7*, *Lgr5*, and *Plk1* have been studied for biding sites of the monomers RAR and RXR, or the heterodimer RAR:RXR and their variants, see Table 2. Here we can see that the promoter region of *Gpc3* has the highest count of total retinoid receptor binding sites and Lgr5 has the lowest count. Theoretically, this could mean that the expression of *Gpc3* is more responsive to retinoids than the expression of *Lgr5*.

From this study, it can be hypothesised that the expression of all four genes can be increased by the aid of RA, since all four genes contain binding sites for retinoid receptors.

Table 2: Table the amount of retinoic acid receptor (RAR) and retinoid X receptor (RXR) binding cites in the genes Gpc3, Mmp7, Lgr5, and Plk1.

Gene	RAR subtypes binding sites	RXR subtypes binding sites	Dimer binding sites	Total binding sites
Gpc3	13	7	6	26
Mmp7	5	7	0	12
Lgr5	7	2	0	9
Plk1	8	3	6	17

Effect of retinoids on organoids

Effects of retinoic acid on organoids

The promoter region study revealed binding sites for the retinoid receptors. So, the next step was to see if the upregulation of *Gpc3*, *Mmp7*, *Lgr5*, and *Plk1* as seen in the co-culture can be mimicked by supplementing RA directly to the organoids.

When organoids are cultured with 1µM of RA in the medium, they do not seem to alter their proliferation. Both metabolic cell activity (figure 4A) and cell division, depicted as *Plk1* expression, (Figure 4B) are unchanged.
Gpc3 expression seems to be influenced by supplementation of 1µM RA. However, it cannot be said with certainty that there is an increase in expression due to the large error bars (Figure 4C). *Mmp7* and *Lgr5* expression does increase when organoids are cultured with 1µM RA (figure 4D & 4E, respectively).

After seeing the effects on gene expression of 1µM RA, we were curious to find out what would happen at different concentrations of RA. Figure 5 shows a dose response curve with a range of 100pM – 10µM RA in steps of 10 (Figure 5). Culturing organoids with RA has no effect on cell proliferation (Figure 5A). However, the increase in RA concentration does seem to have a negative effect on *Plk1* expression in the organoids (Figure5B).
Although, it seems as if there is an increase in *Gpc3* expression, it cannot be said with certainty that *Gpc3* expression has a dose dependency pf RA (Figure 5C). For *Mmp7,* there is a more distinct dose

dependent increase of gene expression (Figure 5D). Figure 5E shows that Lgr5 expression seems to increase as the concentration of RA increases. However, it is hard to interpret this effect due the dip and subsequent rise of Lgr5 expression after 10nM RA.

From this data it can be concluded that organoid proliferation is not increased when organoids are cultured with RA in the medium. RA might influence the expression of *Gpc3* and *Lgr5* in a dose dependent manner. For *Mmp7* expression this effect is more noticeable, where expression increases with the increasing RA concentration.

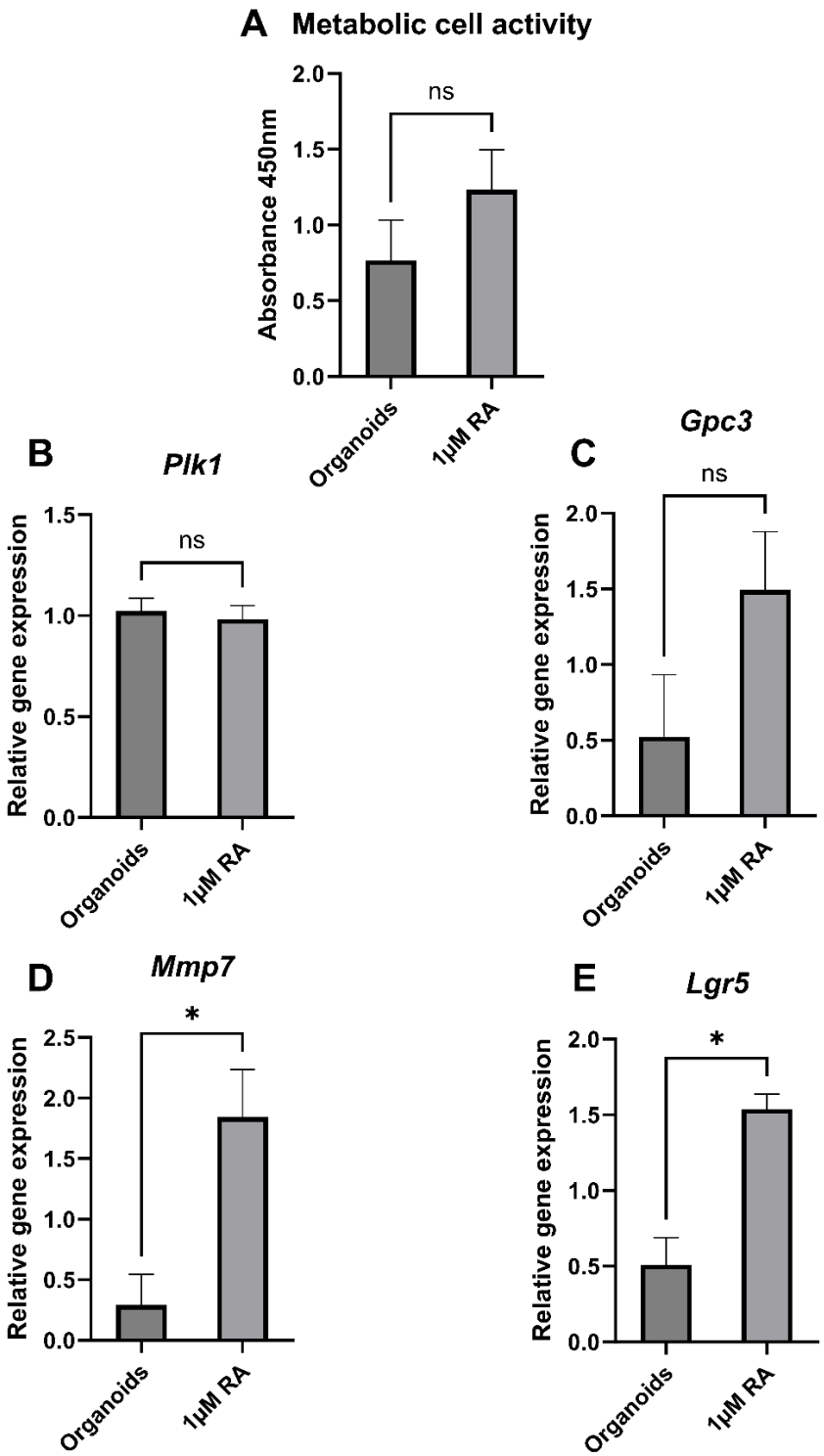

*Figure 4: Effects on proliferation and gene expression in organoids cultured alone (control) or with 1µM RA present in the medium (N=3). A, Metabolic cell activity is plotted as absorbance at 450nm performed on medium samples. B, Gene expression of Plk1. C, Gene expression of Gpc3, D, Gene expression of Mmp7. E, Gene expression of Lgr5. RA, retinoic acid. ns = p > 0.05; * = p < 0.05. In all graphs of this figure the data is normalised over the total signal within each experiment.*

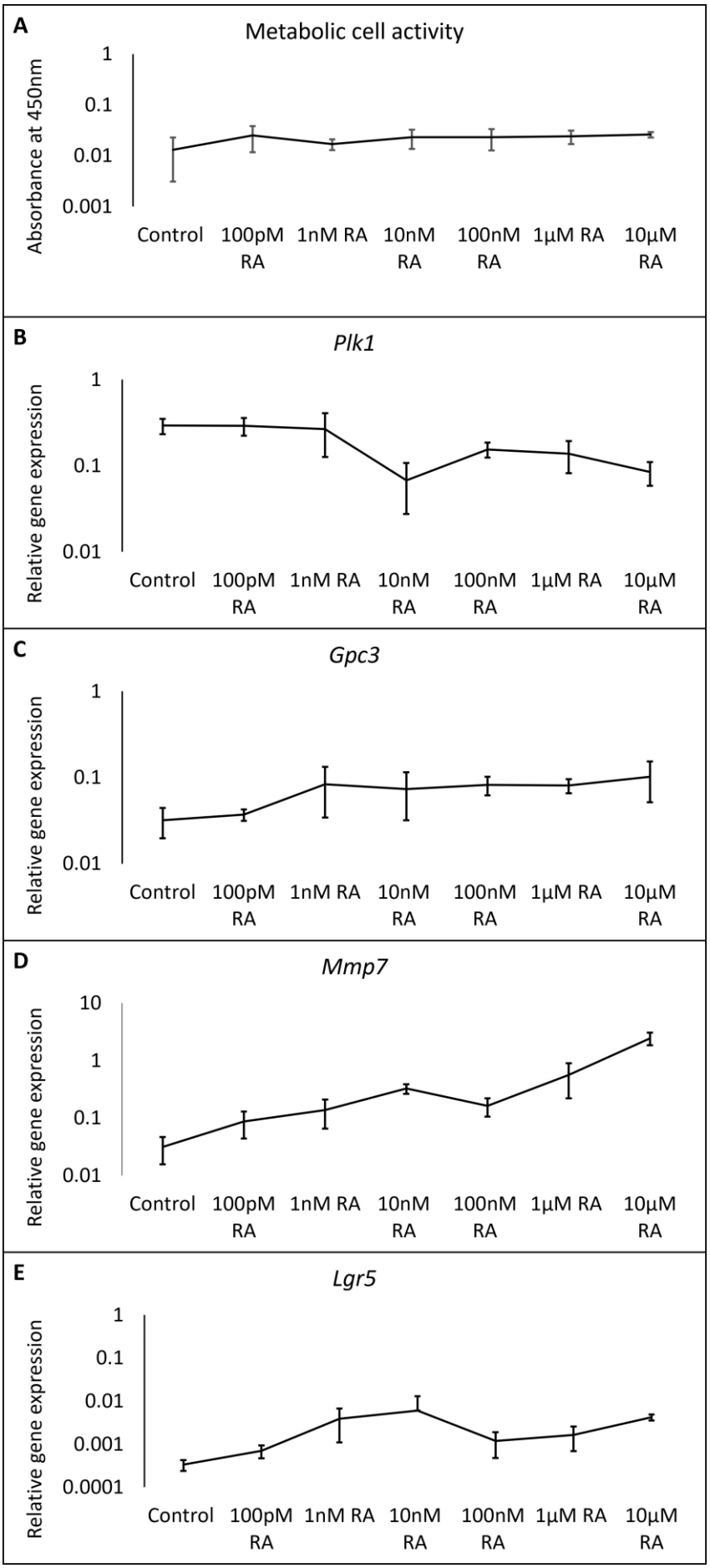

Figure 5: Effects on organoid proliferation and gene expression of RA. RA is added to the medium in different concentrations ranging from 100pM to 10µM in steps of the order of 10. All graphs are plotted with the y-axis in a log10 scale. All samples were plated out in triplicate, except for the control which is in duplicate. The experiment was done once. A, Metabolic cell activity is plotted as absorbance at 450nm performed on medium samples. B, Gene expression of Plk1. C, Gene expression of Gpc3, D, Gene expression of Mmp7. E, Gene expression of Lgr5. RA, retinoic acid.

Effects of retinol supplementation on organoids

During activation, HSC lose their retinoid storage first in the form of retinol, which is eventually converted into RA (Figure 3A) (Haaker et al., 2020). So, as retinol is a precursor of RA, the question came forward if retinol would also affect gene expression of the target genes *Gpc3*, *Mmp7*, *Lgr5*, and *Plk1*.

First, organoids were cultured in the presence of 10µM retinol. This had little to no effect on their proliferation. Both metabolic cell activity (Figure 6A) and cell division, depicted as *Plk1* expression (Figure 6B), were unchanged.
Gpc3 expression seems to be affected by 10µM retinol, although, it cannot be said with certainty if the increase is valid (Figure 6C). On the other hand, *Mmp7* and *Lgr5* expression are both increased in the presence of 10µM retinol (Figure 6D & 6E, respectively).

As supplementation of 10µM retinol shows similar effect on metabolic cell activity and gene expression as 1µM RA, we wanted to know if a range of retinol concentrations show the same trend as seen with the range RA concentrations. Figure 7 shows the results from this approach. Here organoid proliferation is unaffected by retinol. Both metabolic cell activity (Figure 7A) and cell division, depicted as *Plk1* expression (Figure 7B), are unchanged.

There seems to be a dose dependency of *Gpc3* expression, although this cannot be said with certainty due to the large error bars (Figure 7C). *Mmp7* and *Lgr5* expression does seem to be dose dependent on retinol (Figure 7D & 7E, respectively).

The data from organoids cultured with retinol has comparable trends as those of the organoids cultured in RA. Where there is no effect on organoid proliferation. *Gpc3* expression seems to have a dose dependency, and *Mmp7* and *Lgr5* are dose dependent of both retinol and RA.

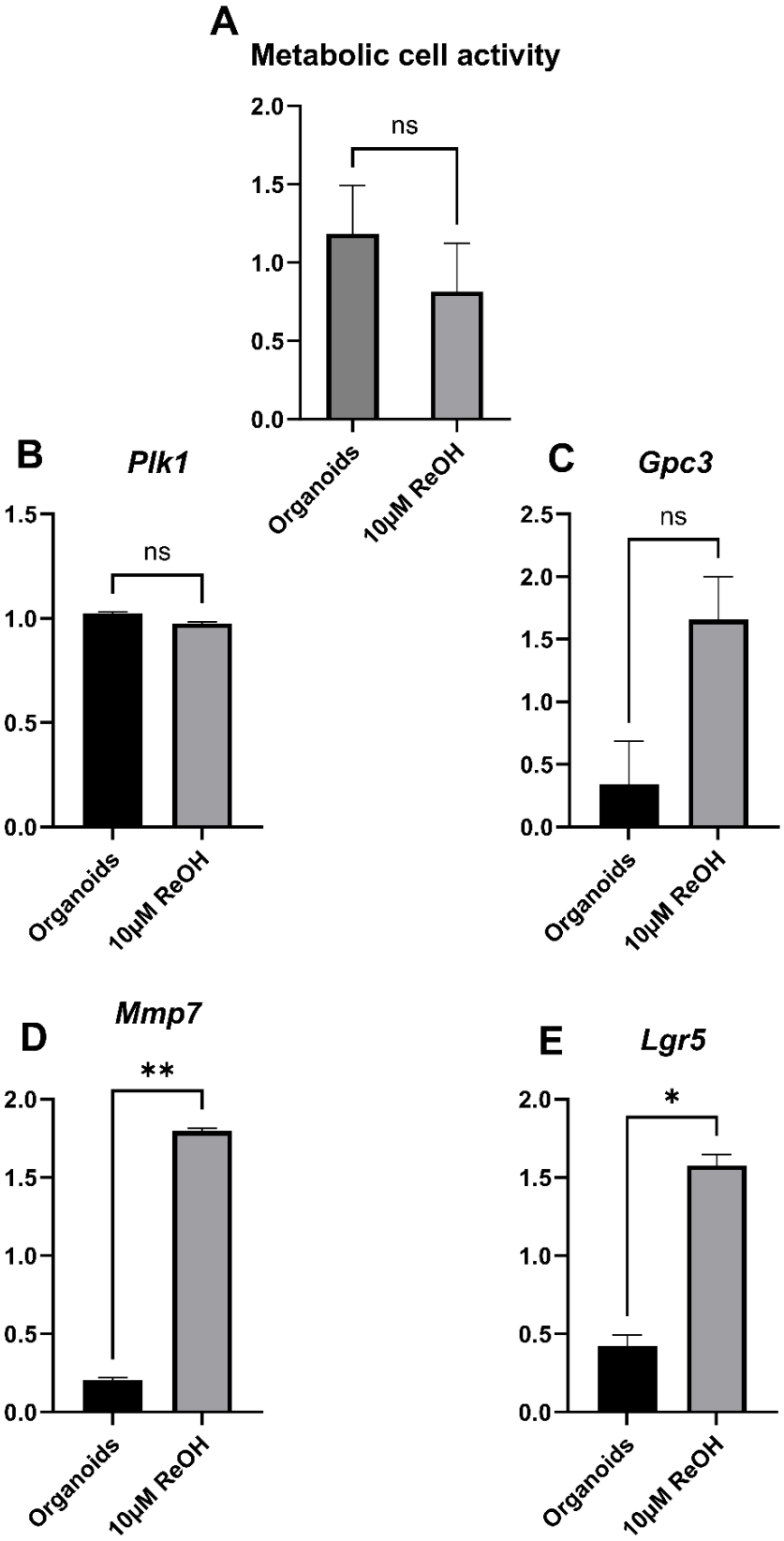

*Figure 6: Effects on proliferation and gene expression in organoids cultured alone (control) or with 10µM Retinol in the medium (N=2). A, Metabolic cell activity is plotted as absorbance at 450nm performed on medium samples. B, Gene expression of Plk1. C, Gene expression of Gpc3, D, Gene expression of Mmp7. E, Gene expression of Lgr5. ReOH, retinol. ns = p > 0.05; * = p < 0.05; ** p < 0.01. In all graphs of this figure the data is normalised over the total signal within each experiment.*

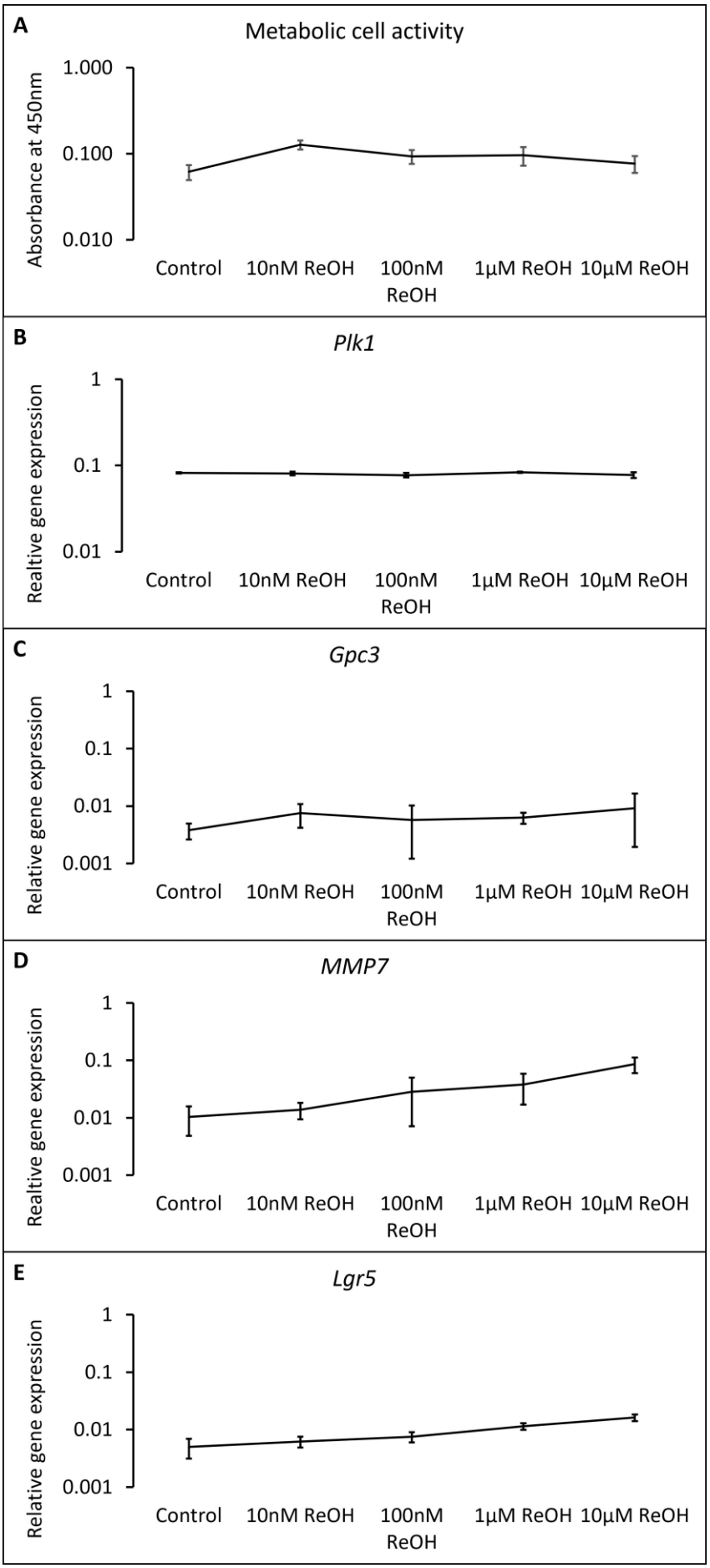

Figure 7: Effects on organoid proliferation and gene expression of retinol. Organoids are cultured with different concentrations of retinol, ranging from 10nM to 10µM in steps of the order of 10. All graphs are plotted with the y-axis in a log10 scale.A, Metabolic cell activity is plotted as absorbance at 450nm performed on medium samples. B, Gene expression of Plk1. C, Gene expression of Gpc3, D, Gene expression of Mmp7. E, Gene expression of Lgr5. ReOH, retinol.

Effects of inhibiting Raldh2 on organoids

Raldh2 is present in both HSCs and organoids, although during co-culture *Raldh2* expression is significantly upregulated in the HSCs. Inhibiting Raldh2 in the co-culture seemed to prevent the increase in *Gpc3* expression that was observed in the organoids (Haaker et al., Unpublished). This could imply that the expression of *Gpc3* is regulated by RA. We wanted to test if this effect of Raldh2 inhibition could be mimicked in the single cultured organoid in the presence of retinol, which is downstream in RA synthesis. Organoids were cultured with the Raldh2 inhibitor, with retinoids, or with retinoids in the presence of the Raldh2 (Figure 8).

The addition of the Raldh2 inhibitor had no effect on organoids proliferation (Figure 8A). Plk1 expression seemed to be lowered when cultured with the Raldh2 inhibitor, regardless of if there were retinoids present or not (Figure 8B).
It seems that *Gpc3* expression is already influenced by addition of the Raldh2 inhibitor. In the presence of 10µM retinol, *Gpc3* expression seems to increase. Ther is no change when Raldh is inhibited in the presence of 10µM retinol (Figure 8C). TThe expression of both Mmp7 and Lgr5 are unaffected when the Raldh2 inhibitor is added to the culture (Figure 8D & 8E, respectively). In the presence of retinoids, the Raldh2 inhibitor does not influence the expression of *Mmp7* and *Lgr5* (Figure 8D & 8E, respectively).

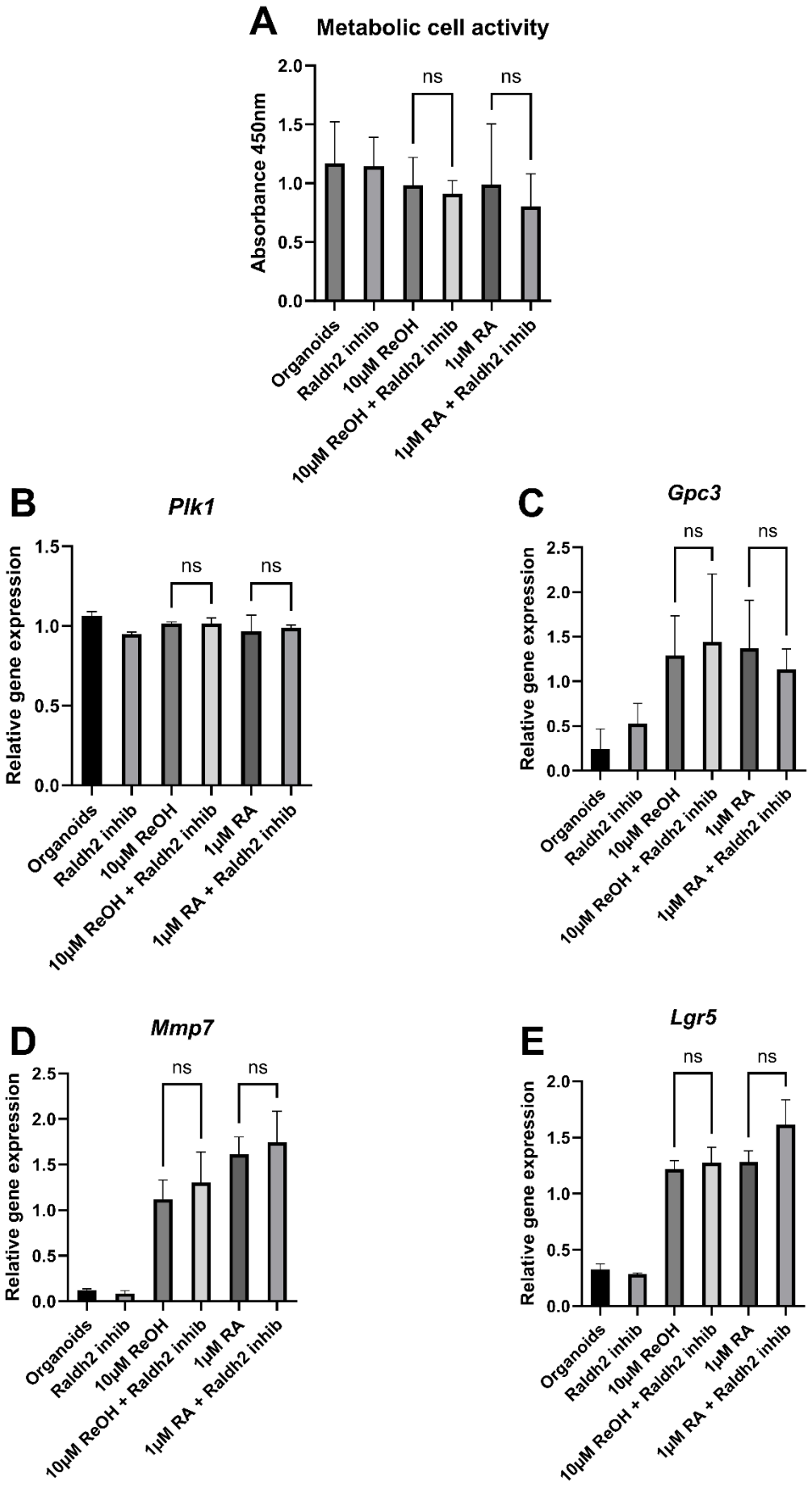

Figure 8: *Effects on proliferation and gene expression in organoids cultured alone (control) or cultured with 10µM retinol, 1µM retinoic acid, Raldh2 inhibitor or a combination in the medium. Raldh2 is inhibited by 2µM WIN-18,446 (N=2). A, Metabolic cell activity is plotted as absorbance at 450nm performed on medium samples. B, Gene expression of Plk1. C, Gene expression of Gpc3, D, Gene expression of Mmp7. E, Gene expression of Lgr5. ReOH, retinol. RA, retinoic acid. In all graphs of this figure the data is normalised over the total signal within each experiment. ns = p > 0.05.*

Proposed Communicative Model

Organoid proliferation is a process that acts independently from the RA pathway. Both cellular activity and cell division are unbothered when retinoids are present in the medium.

Gpc3 expression is altered in the presence of retinoids, both retinol and RA seem to increase the expression (Figure 4C, 5C, 6C & 7C). Interestingly, Raldh2 inhibition in the presence of retinol seems to prevent the increase in *Gpc3* expression in organoids that is seen when organoids are cultured with retinol alone. This can be interpreted as that the expression of *Gpc3* is dependent on the activity of Raldh2 synthesizing RA. Whereas *Mmp7* and *Lgr5* expression can be altered by either retinol itself, or perhaps other retinoids, or by RA that is synthesized through Raldh1 or Raldh3.

From the data obtained in this thesis, a communicative model has been established and is depicted in Figure 9. Organoids activate HSCs through different signals, where a close interaction is needed (Haaker et al., Unpublished). Upon activation, HSCs begin to lose their retinoid storage and start to produce retinol. Retinol can then act either on its own or as RA through a Raldh2 independent manner, to stimulate *Mmp7* and *Lgr5* expression in the organoids.

From earlier data (Haaker et al., Unpublished) it seemed as if the rise in *Gpc3* expression, in co-culture with HSCs, could be prevented when Raldh2 was inhibited. This effect could not be replicated by culturing organoids with the RA precursor retinol in the presence of Raldh2.

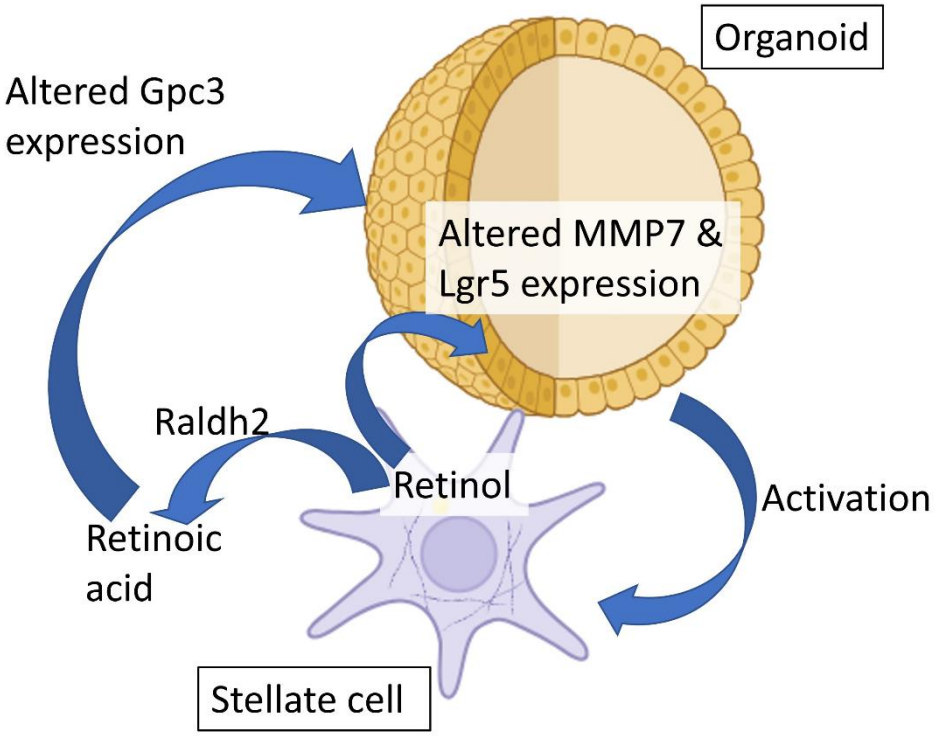

Figure 9: Schematic overview of the communication between organoids and HSCs. The organoid is depicted as the large sphere and the HSC as the myofibroblast-like cell. The organoid stimulates the HSC to activate, where it loses its retinoid storage. Retinol can act on Mmp7 and Lgr5 independent of Raldh2. Whereas Gpc3 is dependent on RA synthesized through RAldh2.

Discussion

Promoter region study

Looked only into RAR, RXR, or RAR:RXR binding sites in the promoter regions of Gpc3, Mmp7, Lgr5, and Plk1. There are indeed other receptors that can form a complex with either RAR or RXR that were not considered. This approach came forward from the culture conditions that the organoids would be exposed to, namely culture with RA or Retinol. RA can either bind RAR/RXR, and retinol can bind RXR proteins. For the simplicity of this study, the other possibilities were not investigated further.

Retinoid supplementation

All experiments where organoids were cultured in the presence or absence of retinoids were done over 2 days. This was done to give the organoids time to settle after plating. When the organoids are plated out, they are fragmented into clumps of cells, after which they need some time to accumulate in the new gel. During this time the retinoids could be broken down since they are unstable molecules. In future studies the organoids could first be seeded, and after 2 days the retinoid treatment can be started and done over a shorter period.

Raldh2 inhibitor WIN-18,446

Upon Raldh2 inhibition the increase in *Gpc3* expression specifically is prevented in the co-culture of organoids and HSCs. This effect has not been reproducible during this research. However, it would be interesting to look further into the effect of inhibiting Raldh2 in *Gpc3* expression.
This disturbance in *Gpc3* expression could be an interesting way of tackling both early onset of liver fibrosis and perhaps also in the progression of HCC. On this subject, one sidenote must be made regarding the RAldh2 inhibitor WIN 18,446. Namely, WIN 18,466 described to have testicular toxicity in mice (Singh & Dominic, 1995) and has also been used as a male contraceptive in clinical studies (Beyler et al., 1961; Coulston et al., 1960). These clinical trials were stopped soon after they had begun due to one side effect of the drug: an increased toxicity due to alcohol consumption. WIN-18,446 inhibits aldehyde dehydrogenase that is also involved in the breakdown of alcohol, where the toxic substance acetaldehyde is broken down by aldehyde dehydrogenases. Inhibiting this enzyme leads to a build-up of acetaldehyde, resulting in the worsening of the aftereffects of alcohol. It would thus be quite dangerous to use WIN-18,446 as a drug to put a brake on liver fibrosis due to its broad working mechanism on different members of the aldehyde dehydrogenase family.

Communicative model

The proposed communicative model only considers the data obtained from single cultured organoids supplemented with either retinol or RA. In the co-culture situation, there are a lot more factors that could play a role in the interaction between the HSCs and the organoids that do not involve retinoids. Also, the data shown in this research has its limits and no hard conclusions can be drawn from the graphs where there is no significance. This is especially the case for *Gpc3* expression. Where there seems to be an effect of retinoids, is could also be a coincidence. To validate the hypothesis of *Gpc3* being dependent on RA that is synthesized through Raldh2 solely, more experiments need to be done both in single cultured organoids as well as in the co-culture of organoids and HSC.
What can be concluded from the data shown in figure 8, is that inhibiting Raldh2 in the organoids has little effect on the gene expression and metabolic cell activity.

The main message that I want to give with this research is that the communication between HPCs and HSCs during DR is complicated. Retinoids do play a role in the stimulation of organoids, although, the effects are subtle. Perhaps in the in vivo situation, the retinoids excreted by HSCs are in a higher concentration then those that have been tested here.

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