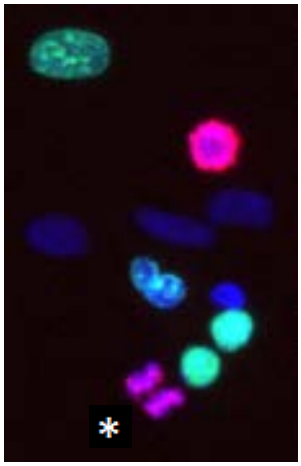

Activating Hepatic Progenitor Cells

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Honours Programme September 2012 – August 2013
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Final report Honours Programme project

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Cover photo: *Proliferating HepaRG cells stained for DAPI (blue), EdU (green) and pH3 (red), with mitotic figure (*) – Source: Screen data, H.S. Kruitwagen*

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Chapter 1.

General introduction

Summary
This general introduction describes the general organization of research at the Faculty of Veterinary Medicine, including the embedding of this research project. In order to formulate the research questions for this Honours Programme project some background information is required. Therefore this general introduction also includes information about the healthy liver and how Regenerative Medicine can contribute to recovery of the damaged or diseased liver.

Research at the Faculty of Veterinary Medicine

At the Faculty of Veterinary Medicine research is divided over five thematic and interdisciplinary programmes:

- Biology of Reproductive Cells
- Emotion and Cognition
- Risk Assessment of Toxic and Immunomodulatory Agents
- Strategic Infection Biology
- Tissue Repair

The research program Advances in Veterinary Medicine conducts additional research, including research training for residents with the specific goal to maintain top-level veterinary specialists. This comprises fields outside the focus of the thematic programmes mentioned above. This division has multiple advantages. It contributes to the integration and scientific innovation of different disciplines, thus creating an attractive scientific environment. Furthermore, it facilitates economic use of resources and enhances the chances of acquiring external research funds.

The Tissue Repair (TR) programme focusses on the pathophysiology of tissue dysfunction and on the potential repair mechanisms adult stem cells could provide. Adult stem cells include tissue specific stem cells (progenitor cells), induced Pluripotent Stem cells (iPS cells) or multi-potent Mesenchymal Stem Cells (MSCs). The aim of the TR programme is to use these stem cells to develop new methods of regeneration, in order to cure diseases that are presently incurable. This is achieved by finding signals such as cytokines, growth factors and hormones to manipulate *in vivo* or *ex vivo* proliferation, differentiation and migration of stem cells.

As there is great resemblance between certain inherited and spontaneous diseases in animals and human patients, animals can be used as a model for comparative studies of pathogenesis and therapeutic intervention. This type of research, called translational medicine, is also an important aim of the TR programme.

The TR programme comprises three research lines. The first research line investigates the musculoskeletal system, with a special focus on osteoarthritis and intervertebral disc disease. The second research line focusses on diseases of internal organs, especially the liver. The third research line investigates tumorigenesis in relation to stem cell transformation and stromal interactions.

This project is part of the second research line, focussing on diseases of the liver and potential therapies. The research groups involved in this research are the Department of Pathobiology (DP), the Department of Biochemistry and Cell Biology (DBC) and the Department of Clinical Sciences of Companion Animals (DCSCA). These groups investigate a variety of subjects such as angiogenesis as part of regenerative capacity (DP), lipid metabolism as a regulator of ‘stemness’ (DBC) and canine inherited copper storage disease as a model for human Wilson’s disease (DCSCA).

The DCSCA aims to unravel the molecular processes that regulate proliferation, differentiation and migration of adult liver stem cells, which are then analysed to translate from mouse to dog to man. As it is a clinical department, spontaneously occurring liver disease in dogs and cats can be investigated and translated to human clinical hepatology (1).

The healthy liver

The liver is located in the most cranial part of the abdomen, where it is positioned between the diaphragm cranially and the gastrointestinal mass caudally. Due to its important role in body homeostasis and its many other functions the liver is a vital organ for all animals (2). Its many functions comprise production of bile, excretion of waste products, storage of for example vitamins, synthesis of plasma proteins such as coagulation factors and albumin, detoxification and metabolism of proteins, carbohydrates and lipids (3). The latter function is facilitated by the part of the blood supply that comes directly from the portal vein, which is a combination of blood vessels draining the digestive tract, pancreas and spleen. Blood from the intestines is transported through the portal vein, thus entering the liver before reaching the general circulation. This results in the presentation of all products of digestion to the hepatic cells before passing to the rest of the body. The other part of the blood supply comes from the hepatic artery, which is a branch of the celiac artery. Between species there is great variation in the relative importance of these two supplies. In humans the hepatic artery provides the liver with one fifth of its total blood supply, whilst it is also responsible for three-fifth of the oxygen supply. All blood received by the liver is collected in a single set of veins, forming the few large hepatic veins opening into the caudal vena cava (2).

Several fissures divide the liver into lobes, resulting in a lobar pattern that differs between species (2). The liver is covered by the visceral peritoneum, overlying a thin connective tissue capsule. This capsule also extends into the lobes, surrounding and supporting individual lobules. This results in a lobular pattern that is highly conserved between species. Each lobe consists of numerous lobules; cords of hepatocytes that are arranged more or less radially around a central vein. Between these hepatic cords the sinusoids can be found. These are endothelial lined spaces and are analogous to capillaries in other organs. At the edge of each lobule is the portal triad, consisting of a portal vein, a hepatic artery and a bile duct. Blood in both the hepatic artery and –vein drain centrally, whereas bile drains peripherally to the bile ducts in the portal triad (4).

The cells in the liver are divided in a parenchymal and a non-parenchymal fraction. The parenchymal fraction accounts for 70% of all cells in the liver and consists of hepatocytes, which carry out most of the liver's metabolic and synthetic functions (3, 5). In acute liver disease or after a partial hepatectomy the hepatocytes are activated to regenerate the liver. This does not only include proliferation (compensatory hyperplasia), the hepatocytes also increase in size (5). The non-parenchymal fraction of cells is located in the sinusoidal compartment of the liver and consists primarily of three cell types: sinusoidal endothelial cells, hepatic stellate cells and Kuppfer cells. Sinusoidal endothelial cells line the walls hepatic sinusoids, functioning as a filter between the blood and the hepatocyte surface due to the presence of small fenestrations. Hepatic stellate cells are characterised by their intracytoplasmic fat droplets. The cells can be found in the perisinusoidal space, where they are responsible for storage of vitamin A and turnover of extracellular matrix. The transdifferentiation of stellate cells into contractile myofibroblasts is activated by acute hepatocyte damage and is noted as a major event in hepatic fibrogenesis (5, 6). The formed myofibroblasts play an important role in the inflammatory response, thus making the stellate cell vital to liver function and in the liver's response to injury. Kupffer cells are liver specific macrophages and are known for their phagocytic capacity. Their function includes the secretion of mediators of the inflammatory response such as cytokines. This process controls the early phase of liver inflammation, but can ultimately lead to damage to the liver. Additionally Kupffer cells play a role in the clearance of damaged erythrocytes and they modulate immune responses through several mechanisms (6).

Regenerative Medicine in the liver

Regenerative medicine (RM) aims to accomplish functional recovery of damaged tissue by stimulating and imitating the body's natural ability to repair damaged tissues and organs. RM comprises several

basic scientific disciplines such as cell biology and chemistry, as well as more application-oriented disciplines as implantation technology (7). The liver is well known for its ability to regenerate, so it appears that there is no need for regenerative medicine in the liver. Even with a partial hepatectomy of 70% of the total liver mass, the remaining tissue can completely recover, both in mass and function (8). The experimental setup of a partial hepatectomy is used to study the reaction of the liver to acute injury. Regeneration is strictly regulated, in such a way that proliferation stops when the appropriate liver to body weight ratio is achieved (8).

The regenerative capacity of the liver consists of two (patho)physiological mechanisms. The first line of defence against liver injury is the compensatory hyperplasia of mature, normally quiescent adult hepatocytes and non-parenchymal cells (e.g. endothelium). This type of regeneration is the main response of the liver to injuries caused by drugs, toxins, resection or acute viral diseases. This fraction of cells is also responsible for regeneration after partial hepatectomy. If regeneration by hepatocytes is insufficient the second line of defence against liver failure is activated. This is the proliferation and differentiation of resident adult stem cells of the liver, the so-called Hepatic Progenitor Cells (HPCs).

The presence of a hepatic progenitor cell population was first described in 1952, when Farber described the 'oval cells' in rats, named after their characteristically shaped nucleus (9). Further research in animal models has shown that these cells are involved in regeneration of the damaged liver, with a bipotential ability to differentiate into either bile duct cells or hepatocytes (10). In the human liver an equivalent to the oval cells has been found in the terminal bile ductules, the canals of Hering (11). HPCs reside in a specified niche of neighbouring cells and matrix components and can be found close to the portal area in a histological section. Upon activation, HPCs will enter the cell cycle and start to proliferate. They have the ability to migrate through the parenchyma to the site of injury and can differentiate into hepatocytes or cholangiocytes depending on the type of damage and the existing microenvironment (12-14). The regenerative process can compensate for the loss of healthy hepatocytes, thus delaying the onset of liver insufficiency (8).

HPCs are activated when adult hepatocytes fail to repair the damaged liver. This is the case in damage to the liver that is so acute and severe that the surviving hepatocytes cannot regenerate. Katoonizadeh et al. found in human patients that extensive hepatic progenitor cell activation only occurred after crossing a threshold of approximately loss of 50% of hepatocytes. In this case, reduced proliferation of the remaining hepatocytes was essential for HPC activation (15). In addition, the degree of HPC activation was negatively correlated with clinical outcome. This is probably due to the fact that HPCs are only activated when the damage to the liver is severe. In these cases the activation is often too little and mostly too late. Additionally, HPCs are activated when a patient suffers from chronic damage to the liver, thus exhausting hepatocyte proliferation (8).

Because HPCs have the potential to repopulate the liver with both hepatocytes and cholangiocytes it seems to be an ideal population of cells to target therapeutically. However, the signalling pathways involved in activating the progenitor cells are mostly unknown, making it is difficult to intervene in this process.

<u>High throughput screen</u>

In order to find signals that are involved in the stimulation or inhibition of HPC proliferation a High throughput screen (HTS) was performed. In this screen the effect of kinase knockdown on the HepaRG cell line, a human HPC like cell line, was studied. The effect on proliferation was measured by assessing the percentage of cells duplicating their DNA. For this the thymidine analogue EdU (5-ethynyl-2'-deoxyuridine) was used (16). The terminal alkyne group of this molecule replaces the methyl group in the 5 position during the replication of cellular DNA, thus EdU is incorporated in the DNA. The phenotype was determined by quantifying the percentage of EdU positive cells. Additionally the cells were stained

with DAPI (a nuclear counterstain) and a phospho-histone H3 antibody (M-phase detector) in a triple immunofluorescent staining procedure.

The primary screen included a library of 716 individual kinases, all knocked down individually by siRNA mediated gene silencing. This resulted in 100 hit kinases that gave a proliferation-related phenotype after knockdown. The experiment was repeated (primary rescreen), using a different plate setup to prevent false positives due to edge effect. This resulted in 41 hit kinases. The rescreen also included a negative selector, in the form of a second cell line (LX2, a human stellate cell line). Kinases that gave a phenotype in the LX2 were excluded, to ensure that only HPC specific signals were found. After this selection step 36 kinases remained. The last step of the screen was the secondary screen, or deconvolution, in which the 36 kinases were technically validated (ruling out off-target effects). This selection procedure resulted in a final list of 10 hit kinases. The knockdown of 9 of these kinases resulted in a decrease in percentage of EdU positive cells, suggesting that they are essential factors for HPC proliferation. One kinase (DYRK1A) showed an increase in percentage of EdU positive cells after knockdown, suggesting that this kinase normally has an inhibitory effect on the proliferation of HPCs.

DYRK1A

As a follow up to the performed screen the research proposal for this Honours Program project was written. The focus of this project is on the single kinase that gave an increase in EdU incorporation after knockdown. There were several reasons to select this kinase for further study. The first reason to choose Dual-Specificity Yak Related Kinase 1A (human: DYRK1A, mouse: Dyrk1A) was the fact that it was the only kinase in the screen that showed an increase in EdU incorporation after knockdown, suggesting an inhibitory role on HPC proliferation. This is interesting, because compared to activation of a kinase, inhibition is more easily accomplished. The second reason to investigate DYRK1A is because inhibition can not only be achieved *in vitro*, it could also be accomplished *in vivo* in patients. Inhibition of DYRK1A can be established by gene silencing (e.g. using siRNA mediated knockdown) or by chemical inhibition. For *in vivo* (clinical) use, chemical inhibition is most advantageous as it does not require genetic modification. It can also be used for *in vitro* (functional) studies, but might be less specific than siRNA mediated knockdown. For this purpose a commercially available chemical inhibitor can be used.

DYRK1A has, as its name indicates, dual specificity. It can phosphorylate both serine/threonine and tyrosine residues. Activation of DYRK1A is achieved by autophosphorylation of a conserved tyrosine residue. This is most likely not a regulatory process but a maturation step, as all proteins isolated from tissue are in their phosphorylated form. If autophosphorylation is not a regulatory step, the regulation of DYRK1A must have another mechanism (17). The first option is that regulation takes place on the level of gene expression (transcriptional regulation), as the mRNA levels of DYRK1A have been shown to be highly variable (18). The second option for regulation is proteasome-mediated degradation of the protein. Though this theory has been proposed as an option, there are currently no publications available confirming it (17). A third option is regulation of activity by the interaction with other proteins. Several proteins have been suggested to interact with DYRK1A (17). A phenomenon described, that could also influence DYRK1A activity, is the nuclear versus cytoplasmic localization in the cell (19, 20). Though there are multiple hypotheses about this, it is unclear what mechanism is responsible for DYRK1A regulation *in vivo*.

The DYRK1A gene is located on chromosome 21 in the so-called Down syndrome critical region (21). This region is associated with most of the phenotypic features in human Down syndrome. DYRK1A is thought to be involved in the signalling pathway regulating neural proliferation and development (17). Strict regulation of DYRK1A activity is required, as both an extra copy and DYRK1A deficiency result in neurodegenerative and cognitive disorders. In human patients with trisomy 21 DYRK1A overexpression is implicated to play a role in the pathogenesis of Down Syndrome (22). Conversely, haploinsufficiency of DYRK1A has been shown to cause a distinctive clinical syndrome, including mental retardation, primary

microcephaly, intrauterine growth retardation, facial dysmorphism, impaired motor functioning, and behavioural problems (23, 24).

The effects of Dyrk1A over- and under expression have also been studied in mouse models. Transgenic mice overexpressing the full length cDNA of DYRK1A presented a delayed craniocaudal maturation of the brain, which had functional consequences for neuromotor development. The mice displayed altered motor skill acquisition and hyperactivity. These characteristics were maintained through adulthood. Furthermore, the mice showed hippocampal and prefrontal cortex dysfunction (25). Both homozygous and heterozygous Dyrk1A-null mice have been created by gene targeting. The homozygous mice showed a general growth delay, resulting in death during midgestation. The neonatal viability of heterozygous mice was reduced, as was the body size from birth to adulthood. Specific areas of the brain of heterozygous mice were decreased in size (26).

It has been suggested that DYRK1A has an influence on the switch from proliferation to differentiation in neural progenitor cells (19, 27). As most of the research results published concern neural tissue and brain development, it is not known whether this function of DYRK1A also extends to other cell types and different kinds of tissue-specific stem cells. If DYRK1A induces the same effects in HPCs, it can perhaps be utilised to stimulate HPC proliferation *in vivo* or *in vitro*. Recent HTS data indicate that knockdown of DYRK1A in a hepatic progenitor-like cell line resulted in a proliferation-related phenotype. However, the exact nature of this proliferative response remains to be determined. In addition, it is not known whether this effect can also be observed in primary cells as it could be specific for the cell line that was studied.

<u>Research question</u>

The aim of this research project was:

- In part A - to find the effect of DYRK1A (knockdown) on HPC proliferation *in vitro,* and
- In part B - to validate the effect of Dyrk1A on proliferation in a primary HPC cell culture.

If DYRK1A has an effect on the proliferation of HPCs, it is a possibility that it could serve as a therapeutic target in severe liver disease.

The hypothesis is that DYRK1A has an inhibitory effect on the proliferation of HPCs, and that it is possible to enhance the rate of proliferation of HPCs by inhibiting DYRK1A. To answer this research question the results found in the screen are validated. This is done by performing siRNA mediated gene silencing in the HepaRG cell line. Chemical inhibition will also be investigated in this project. The readout for these experiments will be proliferation (measured by cell counts, fluorometric analysis and quantification of cell number by automated microscopy), EdU incorporation and pH3 phosphorylation. Additionally cell cycle analysis will be performed by performing flow cytometry and automated microscopy.

As a cell line differs from *in vivo* cells, the results found in the screen and in the follow-up on HepaRGs cannot be directly translated to primary cells or the *in vivo* situation. Based on the results found in the HepaRG experiments the phenotype also has to be followed up in primary cell culture. The preferred species for the primary cells is human, as this would fit with the human HepaRG cell line. However, for this project human primary cells were not available. Therefore, we decided to study mouse primary liver cells, more specifically mouse liver organoids. This is a new and promising three dimensional culture model of liver stem cells and the optimal cell type to use as a model for primary progenitor cells. So far, culture of liver organoids has only been described for mouse and not for dog or man. Therefore, results found in a different species would ultimately need to be confirmed in canine and human cells or tissue. However, major regulatory pathways tend to be conserved across species.

Organoids

Even though the liver has an enormous potential to replicate, it is currently not possible to sustain hepatocyte culture *in vitro* for longer than a few days. Other *in vitro* systems, such as iPS cells and embryonic stem cells, have been described as a potential source of functional hepatocytes. However, these culture systems are not yet capable of expanding to meet the required amount of cells for therapeutic cell transplantation. Also, the cells in these culture systems require genetic modifications or introduction of reprogramming factors in order to prevent senescence (28).

A new system of culture that does have this potential is the *in vitro* expansion of 'liver organoids'. These are hepatic progenitor cells that grow in the absence of a mesenchymal niche. The most important feature of the organoid culture is that it can be sustained over longer periods of time (over 12 months), while maintaining their differentiation potential (29). Liver organoid cultures are established by embedding biliary duct fragments into Matrigel (a semi-solid biological matrix containing laminin) and culturing them in expansion medium containing a specific set of growth factors (29). These primary cells then form three dimensional spherical structures in the Matrigel. The organoids grow in size and can be subcultured by fractioning the spheres and seeding them into new wells with Matrigel. The structures reform their spherical shape within a matter of hours.

Organoid cultures maintain their genomic integrity after long-term culturing and show no malignant transformation after transplantation. These features highlight the advantage organoid culture has over the currently used *in vitro* culturing systems that suffer from genetic stability problems. The combined advantages of organoid culture do not only make it suitable for the evaluation of stem cell transplantation, it can also be used a model for the *in vitro* study of diseases (28).

ODPCs

Organoid Derived Progenitor Cells (ODPCs) are cells obtained from liver organoids. They grow two dimensional, attached to a plastic culture plate. This is accomplished by incubating organoid fragments with trypsin, and thus digesting them to single cell level. These cells are plated out in expansion medium, either to be used directly in an experiment or to be subcultured.

The advantage of ODPCs over organoids is that it overcomes the hurdles of the 3D structure that are encountered with some techniques. Although the spherical shape is a key feature of organoids by definition, a transfection can be difficult to accomplish in a 3D structure. Also, scoring of cells after immunocytochemical or –fluorescent staining can be more challenging when compared to a monolayer of cells. Considering these practical objections to the use of Organoids, the ODPCs were chosen to be used as a model for primary HPCs. The ODPCs were not subcultured as such, but were created *de novo* from three dimensional organoid cultures for each experiment.

Chapter 2.

The effect of DYRK1A knockdown and inhibition on proliferation and cell cycle in HepaRG cells

Abstract

Background and aims - High throughput screening has proposed Dual-Specificity Yak Related Kinase 1A (DYRK1A) as a kinase that can influence the proliferation of Hepatic Progenitor Cells (HPCs). This kinase has also been suggested to influence the switch between proliferation and differentiation in neural progenitor cells. The effect expected based on the screen results is an increase in proliferation after knockdown of DYRK1A. If DYRK1A inhibition could influence HPC proliferation, it might function as a new therapeutic strategy to target a patient's own *in vivo* HPC population. *In vivo* inhibition of DYRK1A could be performed using harmine, a plant derived chemical and potent inhibitor of DYRK1A. Therefore, our aim was to use harmine for *in vitro* inhibition of DYRK1A and to find its effect on proliferation.

Materials and methods - The effect of DYRK1A knockdown and inhibition on the human HepaRG cell line was measured. Knockdown was established through siRNA mediated gene silencing. The cells were treated with either siDYRK or a negative control, a non targeting (NT) siRNA. Harmine was used for the chemical inhibition of DYRK1A. After knockdown or inhibition of DYRK1A for 48 and 72 hours the cells were pulsed with EdU (5-ethynyl-2'-deoxyuridine) for 3 hours. They were then fixed on the plate for automated microscopy using the Arrayscan VTI (600 series), version 6.6.1.3-1.00x. The effect of DYRK1A knockdown/inhibition on proliferation was assessed by measuring EdU incorporation, which is an S-phase detector, and pH3 staining, which is an M-phase indicator. In addition, the total number of cells was quantified and compared to the negative controls. Cell counts were performed using a Bürker-Türk counting chamber for manual counting, a Biorad Automated Counter and a CyQuant assay. Additionally, the amount of valid objects as counted by automated microscopy of DAPU was used as an indicator for the amount of cells per well. The cell cycle was studied by measurement of DNA content by automated microscopy of DAPI stained cells and analysis of this data by FlowJo software.

Results - Automated microscopy of EdU incorporation shows an increased percentage of EdU positive cells upon DYRK1A knockdown and in harmine treated conditions, suggesting increased proliferation. However, total cell number did not increase accordingly. Cell cycle analysis shows a different cell cycle distribution after DYRK1A knockdown and inhibition. The $G_0/_1$ population decreased, whereas the population in S phase and G_2/M increased.

Conclusion - Both cell counting experiments and analysis of the valid object count by automated microscopy by the Arrayscan show that there is no increase in cell number after DYRK1A knockdown or inhibition. Therefore the increased percentage of EdU positive cells after DYRK1A knockdown and inhibition is not the result of an increased proliferation. The increased percentage of EdU positive cells is the result of a shift in the cell cycle distribution, which occurs after DYRK1A knockdown. This effect is also visible after chemical inhibition of DYRK1A with harmine, though it is less pronounced than with siRNA mediated gene silencing. DYRK1A knockdown or inhibition results in a decrease of the $G_0/_1$ population and an increase in the population in S phase and G_2/M phase. This phenotype has not yet been described in hepatic cells, so a follow-up using primary liver cells is necessary. The most suitable model that is currently available is that of the liver organoids. The second part of this research project focusses on the effect of DYRK1A knockdown and inhibition in liver organoid-derived progenitor cells.

Introduction

It is necessary to find new therapies for severe liver disease, in both human and canine patients. The HPC is a potential therapeutic target cell population that can be used to regenerate the liver. Therefore new signalling pathways need to be unravelled in order to influence the proliferation of HPCs. This research project will focus on protein kinase DYRK1A. The effects of DYRK1A have mainly been studied in neural tissue, with a focus on the neural progenitor cell. In this tissue-specific stem cell it has been found that DYRK1A may play a role in the switch from proliferation to differentiation. A previously performed high throughput screen has shown that knockdown of this kinase gives a phenotype of an increased percentage of EdU positive cells in HepaRG cells, a human hepatoma-derived cell line that has HPC characteristics, suggesting increased proliferation.

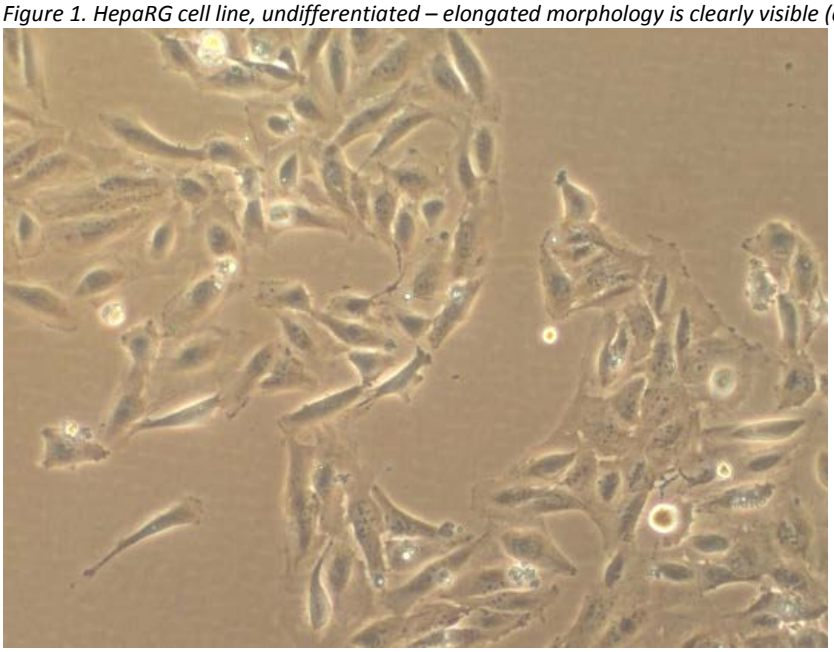
In the screen the only effect of DYRK1A knockdown evaluated was the percentage of EdU positive cells. To discover what the mechanism behind this phenotype is, further study is required. This follow up consists of proliferation assays and cell cycle distribution analyses. The knockdown established in the screen will be accomplished in the same manner, through siRNA mediate gene silencing. The percentage of mRNA knockdown will be established via quantitative Polymerase Chain Reaction (qPCR) analysis of mRNA expression levels. As an alternative for gene silencing, chemical inhibition is used. This method is less complex than siRNA mediated gene silencing. In contrast to gene silencing, harmine has the potential to be performed *in vivo* in patients suffering from liver disease. There are several substances available for chemical inhibition of DYRK1A. In this project the plant-derived alkaloid harmine was used to inhibit DYRK1A. This chemical substance has been shown to be the most potent inhibitor of DYRK1A, as a competitive inhibitor against ATP (the donor of phosphate required for autophosphorylation and thus activation) (30).

A readout included in the screen as well as in this research project is the percentage of phospho-histone-H3 (pH3) positive cells, used as an M-phase (mitosis) marker. The antibody against pH3 only detects histone H3 when phosphorylated at serine 28. Cells in metaphase contain chromosomes that have histone H3 that is highly phosphorylated at serine 10 and serine 28. Thus these cells stain intensely and are easily recognisable as mitotic cells (31).

HepaRG cell line

In this part of the project the human HepaRG cell line is used as a model for HPCs. This cell line is derived from a human hepatoma (32), HepaRG cells are bipotent progenitor-like cells, capable of differentiating into either biliary-like cells or hepatocyte-like cells (33). When seeded at low density the cells acquire an elongated and undifferentiated morphology, expressing markers of hepatic progenitor cells (figure 2). They divide actively, and after having reached confluency the cells differentiate into typical hepatocyte-like colonies that are surrounded by biliary epithelial-like cells (32), a process that can be enhanced by adding DMSO to the culture medium. HepaRG cells can be used as an alternative to *ex vivo* cultured primary human hepatocytes, as they also display features and properties of adult hepatocytes. In the screen and follow up HepaRG cells in their undifferentiated state are used to study proliferation, but they are most often used in their differentiated state as a tool to study drug metabolism (as many detoxifying enzymes are functionally expressed), carcinogenesis and infection by pathogens (33, 34).

Figure 1. HepaRG cell line, undifferentiated – elongated morphology is clearly visible (courtesy of B.A. Schotanus)



HepaRGs are cultured in William's medium E containing several additives, including Fetal Calf Serum (FCS). The standard percentage of FCS in HepaRG culture medium is 10%, ensuring maximum growth (cell division every 24h). As these cells are dividing at their maximum speed, it is near impossible to see an increase in proliferation due to treatment. For that purpose special 2% FCS HepaRG cell lines have been created by weaning down cells normally cultured on 10% FCS. These cells grow slower with an estimated division rate of once every 48 hours, thus enabling an increase in growth rate.

The cell cycle

Cells in the body go through the cell cycle to regenerate lost tissue. Its most basic function is therefore to duplicate its DNA in the chromosomes and consequently distribute these chromosomes over two genetically identical daughter cells. The amount of time it takes to go through the cell cycle differs greatly per cell type. A mammalian liver cell, in healthy liver tissue, divides not more than once a year. The cell cycle consists of four phases: G_0/G_1 phase, S phase, G_2 phase and M phase. In each of these phases the cell has a specific DNA content. In G_0 and G_1 the amount of DNA per cell is the lowest (2n). In S phase the cell is duplicating its DNA, thus going from 2n to 4n. In G_2 the cell has a DNA content of 4n. After mitosis both daughter cells end up with again a 2n nucleus. Additionally, polyploidy occurs in the liver, a phenomenon described in the liver for over 100 years (35). Hepatocyte ploidy depends two factors, both the DNA content per nucleus and the number of nuclei per cell are of influence (36).

The DNA content per cell can be determined by incubation with a hypotonic solution of Propidium Iodide (PI), resulting in staining of nuclear chromatin. Advantages of this method over earlier described methods are the rapid staining (5 minutes), the minimal amount of required material and the absence of clump formation (37). Flow cytometry can be used to measure the relative distribution of DNA content in cells stained with PI. A fluorochrome such as PI makes it possible to measure cellular DNA content by flow cytometric analysis, as it binds and labels DNA (38). This makes it an accurate method for cell cycle distribution analysis of cultured cell populations, as well as for monitoring for example cells of cancer patients.

Materials and methods

HepaRG culture

HepaRG progenitor cells were obtained from BioPredic International (Rennes, France) and were subcultured every 3 to 4 days. The cells were cultured on maintenance medium consisting of William's Medium E containing penicillin/streptomycin, 50 µM hydrocortisone hemisuccinate (Sigma-Aldrich Company Ltd., Zwijndrecht, The Netherlands), 5 µg/mL insulin (Sigma-Aldrich) and 2% or 10% Fetal Calf Serum. Cells were incubated in a humidified atmosphere with 5% CO_2, at 37°C.

Harmine

Viability pilot

Harmine was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO). To determine the toxicity of harmine a viability pilot was performed. In this pilot the effect of different concentrations of harmine on cell morphology and viability was tested. Cells were seeded 350,000 cells/well in 6 wells plates (Greiner Bio-one B.V., Alphen aan de Rijn, The Netherlands) and cultured on maintenance medium. After 24 hours the medium was replaced with medium containing harmine in concentrations of 0, 0.015625, 0.125, 0.5, 1, 2, 8, 32 and 64 µM. DMSO was used as vehicle control in the same concentration as with 1 and 16 µM harmine concentrations. After 48 hours of harmine incubation the cells were microscopically evaluated for changes in morphology. Cells were then incubated with trypsin to harvest them from the plate, after which total cell number and viability were determined using a trypane blue viability assay (TC20™ Automated Cell Counter; Biorad, Hercules, CA, USA). The principle of this test is that live cells possess an intact cell membrane that prevents the uptake of certain dyes, such as trypane blue, ensuring a clear cytoplasm. The cell membrane of dead cells is not intact, enabling the dye to enter the cell and stain the cytoplasm blue (39).

Concentration optimisation

To determine the concentration of harmine with the highest effect on proliferation of HepaRGs, cells were incubated with harmine for several days. The cell type used in this experiment is HepaRG, cultured on maintenance medium containing either 2% or 10% FCS.

On day 0 HepaRGs were seeded 100.000 cells/well in a 6 wells culture plate and kept on maintenance medium with 2% or 10% FCS for 24h. After these 24 hours the medium was supplemented with harmine in different concentrations: 0 (vehicle control/VC), 0.05, 0.25, 0.5, 1, 2, 4, 8 and 16 µM for both the 2% and 10% FCS conditions. The vehicle control consisted of an equal concentration of DMSO as the 8 µM harmine concentration. At 96 hours after seeding (10% FCS) and 120 hours after seeding (2% FCS) the first wells were 100% confluent. At this point cells were harvested and counted in combination with a trypane blue viability assay. Based on the results the experiment was redone only for the 10% FCS condition, with a concentration range VC, 0.05, 0.25, 0.5, 1, 2, 4, 8 and 16 µM harmine. The cells were harvested after 96 hours.

CyQuant assay

HepaRG cells were seeded 5000 cells/well in a 96 wells culture plate. After 6 hours harmine was added in different concentrations (VC, 0.05, 0.25, 0.5, 1, 2, 4, 8 and 16 µM). There were two vehicle controls, containing the same DMSO concentration as either the 1 µM or the 8 µM harmine condition. After 24, 48, 72 and 96 hours the plates were washed with Hank's Buffered Saline Solution (HBSS; PAA, Cölbe, Germany) and stored in -70°C. All plates were processed simultaneously, by incubating each well with 100 µL CyQuant GR dye/cell-lysis buffer (Invitrogen, Breda, The Netherlands) for 2-5 minutes. The lysate was transferred to a new (white) plate and the fluorescence was determined using a fluorescence microplate reader with a fluorescent filter with ~480 nm excitation and ~520 nm emission maxima. Measured fluorescence was translated to a cell number by comparing it to the standard, a dilution series

of which the number of cells/well is known. The standard was on the plate containing 2% HepaRGs harvested on 72 hours. Because background fluorescence is different for all plates, the formula used to calculate the amount of cells per well is different for all the plates. The trend line of the standard dilution can be described with the formula
y=ax – b. The slope (a) of each formula is the same, the only difference is in the intersection with the y-axis (b). This difference is based on the variation in background fluorescence between plates. The total number of cells per well (=x) is calculated from measured fluorescence (=y) by using the fixed (a) and plate-specific (b) component of the formula.

siRNA mediated gene silencing and chemical inhibition

Transfection

At a confluency of 30-50%, HepaRG cells were incubated in transfection medium containing 5 nM siRNA (Non Targeting or siDYRK, Thermo Scientific, Rochester, NY, USA) and 6 nM lipofectamin RNAiMAX transfection reagent (Invitrogen). After 24 hours the transfection medium was removed and replaced with maintenance medium. At 48 and 72 hours after transfection the cells were pulsed with 1 µM EdU (Invitrogen) for 3 hours (EdU omitted in the negative control). Cells were washed with phosphate buffered saline (PBS) with 0.1% Tween (PBST) and fixed and permeabilised using a 3.7% PFA and 0.5% Triton fixating solution for 10 minutes at room temperature. Plates were stored at 4°C until further use.

Chemical inhibition

HepaRG cells were incubated maintenance medium containing either 1 or 2 µM harmine or corresponding vehicle controls. The harmine medium was replaced after 48 hours (for the 72h time point). After 48 and 72 hours the cells were pulsed with 1 µM EdU for 3 hours (EdU omitted in the negative control). Cells were washed with PBST and fixed and permeabilised using a 3.7% PFA and 0.5% Triton fixating solution for 10 minutes at room temperature. Plates were stored at 4°C until further use.

DAPI-EdU-pH3 Triple stain

Both the transfected and the chemically inhibited plates were stained. The cells were stained using EdU staining buffer containing 100 mM Tris, 1 mM $CuSO_4$, 5 µM Alexa fluor 488-azide (Invitrogen) and 100 mM ascorbic acid. For the phospho-histone-3 stain a serum block was performed using 5% normal goat serum (NGS; Sigma-Aldrich). After this the cells are incubated for 1 hour at room temperature with Rabbit anti-phospho-Histone-3 primary antibody (1:500; Millipore, Billerica, MA, USA), followed by the goat anti-rabbit-AF568 secondary antibody (1:200; Dako, Glostrup, Denmark) under the same conditions. DAPI (CCI) was used as a nuclear counter stain in a concentration of 1:4000 for 30 minutes.

Automated microscopy (Arrayscan)

The Arrayscan VTI (600 series) is an automated fluorescence microscope capable of measuring fluorescence in three different channels. As image acquisition, analysis and storage are automated the system is capable of handling large numbers of samples, all the while being less prone to errors than manually possible. The microscope scans the well by taking pictures of the well which are simultaneously analysed by the software. Based on an algorithm developed specifically for each cell type the Arrayscan decides whether or not something is an object (nucleus). This algorithm is created using the target activation module of vHCS view software and is based on for example shape, size and staining intensity of the nucleus. Objects are further separated based on their average intensity, and whether or not this intensity exceeds the threshold value (positive versus negative nuclei).

The measurements in three channels enable a triple stain such as an EdU-pH3-DAPI stain. Each cells individual response in all three channels is recorded, generating an enormous amount of data. The Arrayscan was used to measure the percentage of EdU positive cells, the percentage of pH3 positive cells

and the average intensity of each nucleus of EdU, pH3 and DAPI. Because all wells were entirely scanned the number of nuclei per well was also determined. Average DAPI intensity per nucleus as measured by the arrayscan was used to analyse the cell cycle distribution with the aid of FlowJo analysis software. With this software a histogram of DNA content (based on DAPI intensity) was made, and the different populations of cells were quantified. The DNA content was also compared to the average EdU intensity.

FlowJo analysis

DAPI intensity was used as a parameter for DNA content of a nucleus. The data on DNA content acquired by automated microscopy were analysed using FlowJo analysis software. The intended input for this program is data acquired by flow cytometry and/or Fluorescence Activated Cell Sorting (FACS). This data is based on nuclear staining and the intensity of fluorescence this results in. As the arrayscan also produces data on the fluorescent intensity (of DAPI) of each nucleus, it is possible to convert the data on DAPI intensity to a format suitable for FlowJo. This software was used to make histograms of DNA content per nucleus, as well as to calculate the distribution of the nuclei over the phases of the cell cycle.

Statistical analysis

As there were not enough measurements to prove that the data were normally distributed, it was assumed that they were not normally distributed. Therefore statistical analysis was performed using a Mann-Whitney U test. All data were analysed using R software library (version2.11.1).

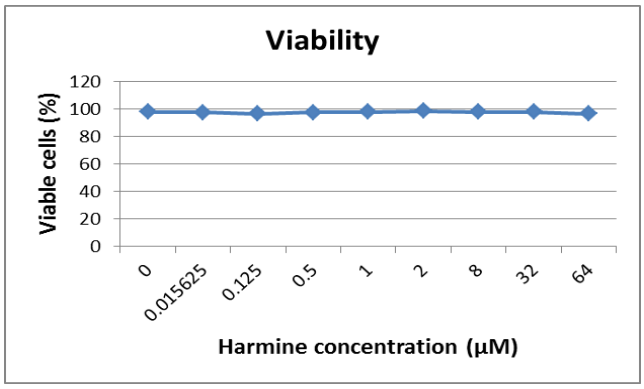
Results

Effect of harmine on HepaRGs

Effect on toxicity

Microscopic assessment of cell morphology in the viability pilot showed more toxic effect in the 8, 32 and 64 µM harmine treated wells. The cells were more rounded instead of flattened and there were more floating (dead) cells. The higher the concentration of harmine, the higher the amount of rounded and dead cells. In the lower concentrations and vehicle control the cells were outstretched and there were little to no floating cells. Though microscopically more dead cells were observed in the highest concentrations there was no difference in viability between the conditions (figure 2). Absolute cell numbers showed that there were fewer cells in the three highest concentrations of harmine (8, 32 and 64 µM, data not shown). This was microscopically already visible before harmine treatment. The amount of live cells per well was almost equal in the vehicle control and 0.015625, 0.125, and 0.5 µM treated wells. Because of the high levels of toxicity in 32 and 64 µM of harmine these concentrations show no potential in influencing HPC proliferation. Therefore, these concentrations were discarded in further experiments. The dilution series selected for the concentration optimisation, based on the readout of this viability pilot, was 0 (vehicle control), 0.05, 0.25, 0.5, 1, 2, 4, 8 and 16 µM harmine.

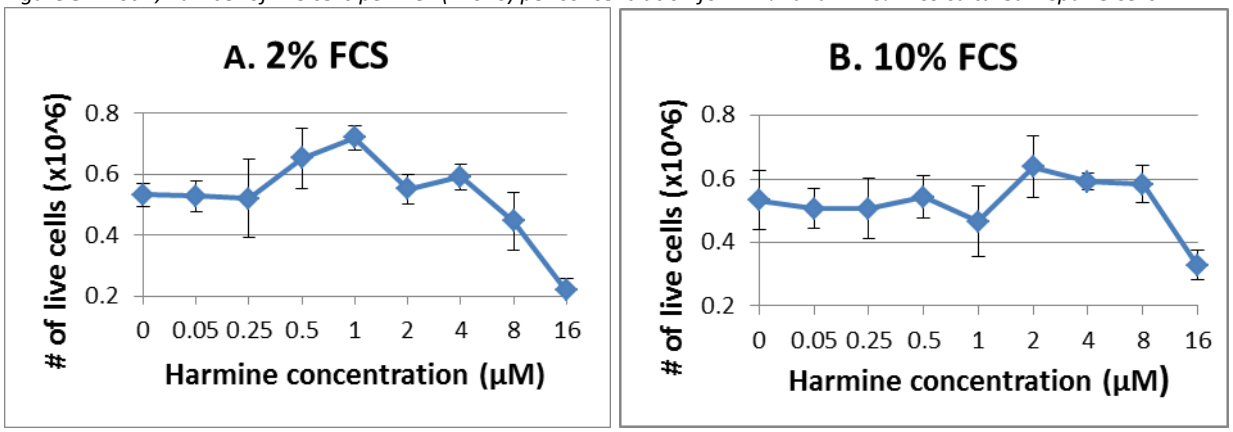
Figure 2. Percentage of viable cells as measured with trypane blue viability assay



Effect on proliferation

During the concentration optimisation in the first pilot the cells were microscopically assessed to determine the optimal time point for harvesting. After 96 hours the vehicle controls of 10% FCS cells were nearly 100% confluent, so these were harvested at this time point. The 2% FCS cells grew slower, so when the first wells reached 100% confluency after 120 hours they were harvested. For both 2% and 10% FCS cultured HepaRGs there was no difference between the 0.05 µM, 0.25 µM and the vehicle controls. Higher concentrations did not show an apparent increase in cell number compared to the vehicle controls. The highest concentration of 16 µM showed a decrease in cell number (figure 3).

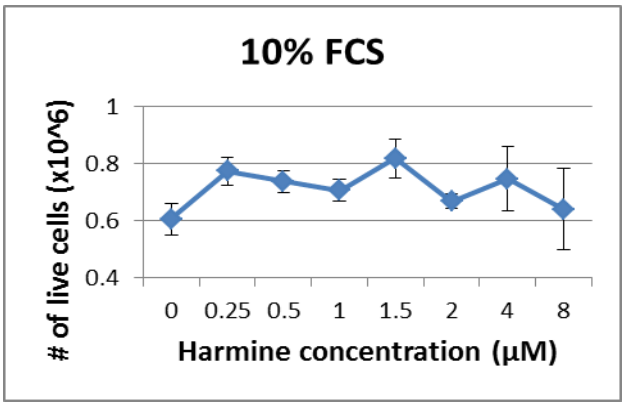
Figure 3. Pilot 1, number of live cells per well (x10^6) per concentration for A. 2% and B. 10% FCS cultured HepaRG cells



From these data it was not possible to draw a conclusion on whether or not harmine increases proliferation of HepaRGs, and if so, what the optimal concentration was. The data were inconclusive, especially in cells cultured on 10% FCS medium. Therefore this part of the experiment was repeated. Because the 16 µM harmine showed a drastic decrease in cell number, this concentration was left out of the second experiment. The 0.05 µM concentration was also excluded from the second experiment, as it shows little to no difference compared to both the vehicle control and 0.25 µM condition. The 1 µM and 2 µM showed potential to affect proliferation, so an extra concentration of 1.5 µM was added.

In the second pilot microscopic assessment of the wells on 24 hours after seeding (before harmine treatment) showed that the distribution of cells within each well is uneven. This impeded an accurate estimation of confluency. However, 96 hours after seeding some parts of the fuller wells had reached 100% confluency so the cells were then harvested. As in the first pilot, there is no definite increase in cell number after harmine treatment (figure 4).

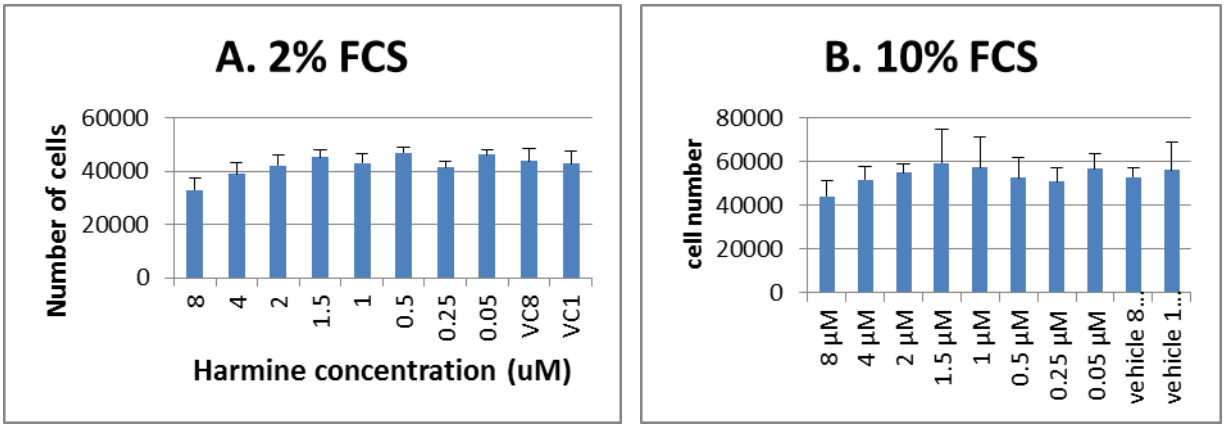
Figure 4. Pilot 2, number of live cells per well (x10^6) per concentration of 10% FCS cultured HepaRGs



CyQuant assay

The CyQuant analysis shows that there was no increase in cell number after treatment with different concentrations of harmine (figure 5). This was the same for both 2% and 10% FCS cultured cells on all time points (see attachment 4, figures 20 and 21).

Figure 5. Number of cells per well on 72 hours for A. 2% FCS and B. 10% HepaRG cells

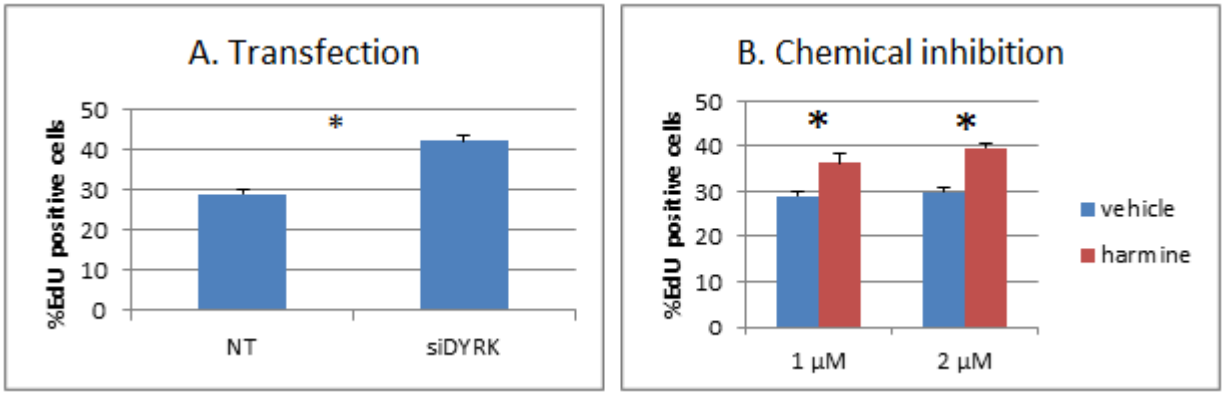


Effect of DYRK1A knockdown and inhibition on HepaRG cells

EdU incorporation

The use of siRNA mediated gene silencing showed that DYRK1A knockdown by siRNA mediated gene silencing resulted in a significant increase in EdU incorporation compared to the negative control. This was the case for both the 2% and 10% FCS culture conditions and after 48 and 72 hours of knockdown (figure 6, and attachment 4, figure 22). The results of both 1 µM and 2 µM harmine treated wells were the same as that of the siRNA mediated gene silencing (figure 6, and attachment 4, figure 23).

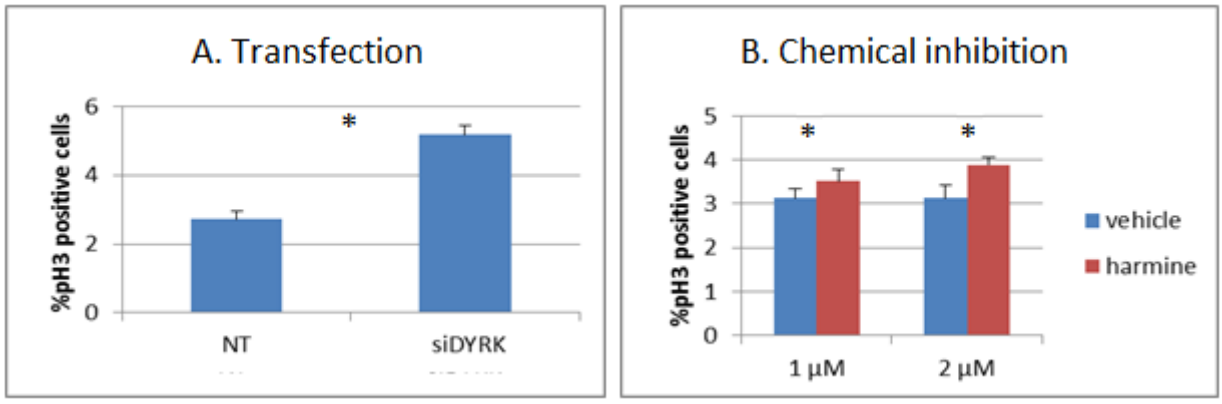
*Figure 6. % of EdU positive cells of Non Target vs. siDYRK1A treated wells for 2% on 48 hours. * = statistical significance of P<0.05*



pH3 phosphorylation

Data on the percentage of cells with phosphorylated histone H3 showed the same trend as in the percentage of EdU positive cells (figure 7). siRNA mediated gene silencing resulted in a significant increase of pH3 positive cells for all conditions (attachment 4, figure 24). Treatment with 1 µM harmine resulted in a significantly increased population of pH3 positive cells on 2% FCS cultured cells on 72 hours and on 10% FCS cultured cells on 48 hours. 2% FCS cultured cells treated with 2 µM harmine had a significantly increased percentage of pH3 positive cells on both time points, while there was no significant increase in 10% FCS cultured cells (attachment 4, figure 25).

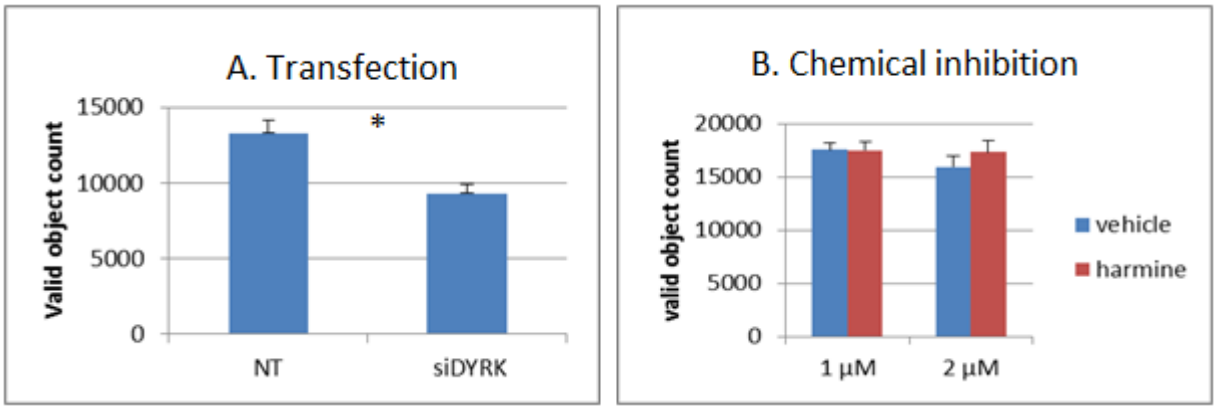
*Figure 7. % of pH3 positive of 10% FCS HepaRGs on 72 hours for A. siRNA mediated gene silencing and B. Chemical inhibition. * = statistical significance of P<0.05*



Valid object count

As the entire surface of the well was scanned it was possible to get an accurate estimation of the total cell number per well from the valid object count. This is a measurement of all nuclei in the well. In order to do this the assumption had to be made that there is no difference between the percentage of binuclear cells between the conditions. On 72 hours for both culturing conditions siRNA mediated gene silencing resulted in a significant decrease of cells. For all other conditions gene silencing and chemical inhibition of DYRK1A did not result in a difference in the number of nuclei per well (figure 8). This is the case for both 2% and 10% FCS cultured cells on all time points (see Appendix 3, figures 26 and 27).

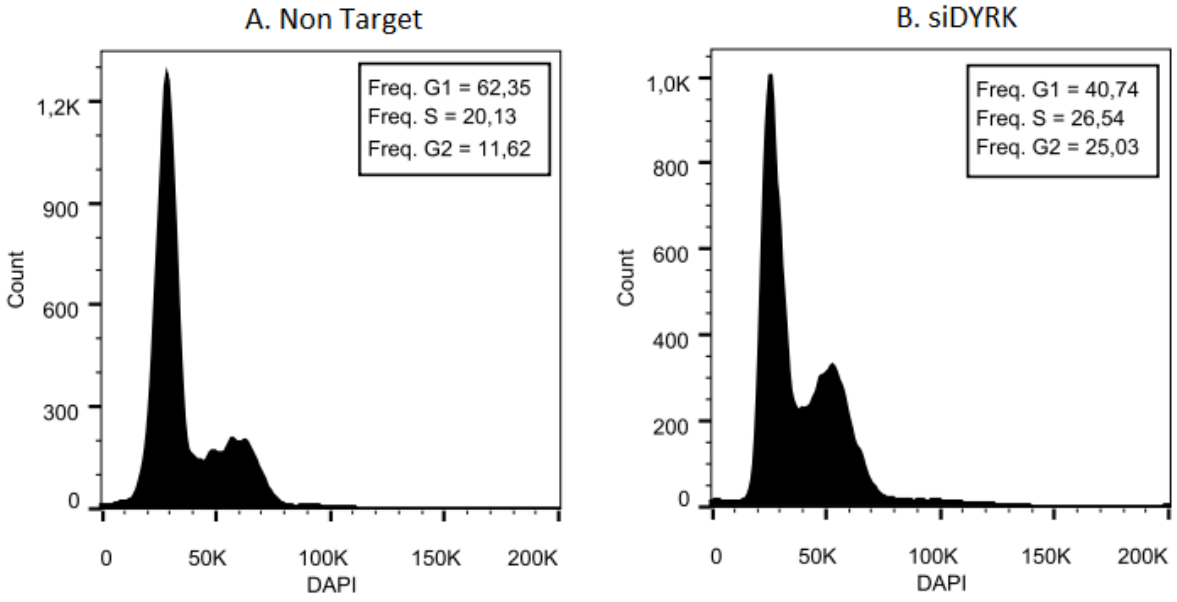
*Figure 8. Valid object count of 10% FCS HepaRGs on 72 hours for A. siRNA mediated gene silencing and B. Chemical inhibition. * = statistical significance of P<0.05*



Cell cycle distribution

DYRK1A knockdown and chemical inhibition caused a shift in the cell cycle distribution, where the population of cells in G_1 phase decreased and the populations of cell in S phase and G_2 phase increased (figure 9). This was found in all conditions in the siRNA mediated gene silencing. The harmine inhibited wells also show this effect, though considerably less pronounced than in knockdown wells. This effect was not found in 10% FCS cultured HepaRGs after 72 hour treatment with 1 µM harmine. See attachment 4, figures 28-33.

Figure 9. Histogram of DNA content (DAPI intensity) for A. Non Target and B. siDYRK for 2% FCS with the percentage of cell in G_1, S and G_2 phase (data on sub-G_1 and super-G_2 are not shown), using the Dean Jett Fox model



Discussion

The results indicate that harmine in high concentrations is toxic to HepaRG cells. When treated with 8 µM or higher concentrations of harmine the cells show signs of toxicity after 24 hours of incubation. Even though this toxic effect of harmine was microscopically visible, there no decrease in viability as measured by trypane blue was observed after treatment with high concentrations of harmine. This phenomenon can be explained by the fact that the wells are washed with HBSS before trypsinising, thus washing away dead cells. It is necessary to perform this step, because the FCS in the medium antagonises the trypsin needed to harvest the cells. Chemical inhibition of DYRK1A did not result in an apparent increase in the number of cells per well, as the difference between the treated cells and the vehicle controls was not as pronounced as expected. Because our detection method might not have had accurate sensitivity to measure the phenotype another approach was used. The number of cells in a well can also be determined by measuring the amount of DNA per well. This was done by performing a CyQuant assay. In this assay it is assumed that there is no difference in the amount of binucleated cells between the harmine treated wells and vehicle controls. The CyQuant assay reveals that there is no difference in DNA content and thus cell number between wells treated with harmine in different concentrations and the appropriate vehicle controls. Chemical inhibition of DYRK1A does not result in an increase in proliferation of HepaRG cells.

An increased percentage of EdU positive cells was observed after gene silencing and chemical inhibition of DYRK1A, both after 48 and 72 hours and upon culturing with 2% and 10% FCS. An increased incorporation of EdU means that during the 3 hour pulse more cells were going through S-phase. The percentage of pH3 positive cells also increases after knockdown, both for 2% and 10% FCS HepaRG cells and on 48 and 72 hours. This effect can also be observed in the chemically inhibited wells, though it is not as pronounced as in the siRNA mediated gene silencing. The fact that this M-phase marker is increased after knockdown (and for the 2% FCS cultured HepaRGs also after chemical inhibition) of DYRK1A means that more cells are going through M-phase. These cells have duplicated their DNA, cell

organelles and cytoplasm and are dividing from one 4n cell into two 2n daughter cells. However, the knockdown and inhibition of DYRK1A did not result in an increase in cell number, indicating there is no effect on proliferation. Thus, our hypothesis that it is possible to enhance the rate of proliferation of HPCs by inhibiting DYRK1A had to be rejected.

DAPI staining intensity was used as a parameter for DNA content per nucleus. Analysis of the DNA content has shown that the distribution of the cells over the different phases of the cell cycle changes after DYRK1A knockdown and inhibition. DYRK1A knockdown resulted in an increase in the percentage of cells found in S-phase and G2-phase and a decrease in the percentage of cells in G1-phase. This effect is more pronounced in siRNA mediated gene silencing than in chemical inhibition. A possible explanation for this is the fact that chemical inhibition might be a less specific and/or potent way to inhibit DYRK1A.

The concentrations at which harmine is toxic are comparable to what other studies have shown. Göckler et al found that harmine shows minimal toxicity in concentrations up to 1 µM in HeLa and HEK293 cells, which is the concentration resulting in >90% inhibition of DYRK1A. Toxic effects were found in concentrations exceeding 3 µM (40). However, Litovchick et al used a concentration of 10 µM on a glioblastoma cell line. This resulted in a significant decrease in substrate phosphorylation by DYRK1A, without described toxic effects (41).

Literature available on the effect harmine has on proliferation is also contradictory. On the one hand Song et al found that treatment with high concentrations of harmine resulted in a decrease in proliferation of actively dividing HeLa, MCF-7, and SW480 cells. On cells that are in a non-dividing and quiescent state, such as stationary fibroblasts, harmine showed little effect (42). On the other hand Litovchick et al found that inhibition of DYRK1A (using siRNA or harmine) resulted in higher levels of DNA synthesis in a serum starved glioblastoma cell line. Additionally they found that not inhibition but overexpression of DYRK1A resulted in a suppression of proliferation in osteosarcoma cells. This was on the condition that the kinase site, responsible for substrate phosphorylation, was active (41). The proliferation parameter was measured using a BrdU incorporation assay, which is comparable to EdU (16, 41). The increase in BrdU incorporation after DYRK1A knockdown found by Litovchick et al fits the increase in EdU incorporation as a result of knockdown or inhibition of DYRK1A found in this research project. Now also in the HepaRG cell line it has been shown that the increase of EdU incorporation was due to a shift in the cell cycle distribution instead of an increase in proliferation. A specific mechanism has been proposed that links DYRK1A to a specific protein complex, the DREAM complex, in the cell cycle machinery. Upon inhibition of DYRK1A DREAM assembly is disrupted and cells will more readily enter S phase, even in an unfavourable growth condition such as serum deprivation.

As this shift in the cell cycle distribution has not yet been shown in liver cells it is an interesting finding that requires a follow-up. The high throughput screen and the follow up until now were performed in a cell line. The results are therefore not 100% interchangeable with HPCs *in vivo*. In order to validate that this phenotype is also relevant in the same cell type *in vivo* it is necessary to repeat the experiment in primary cells. The model used is that of the Organoid Derived Progenitor Cell (ODPC). This will be described in the second part of this report. In this part of the project similar experiments were performed on primary cells, with HepaRG cells as a positive control. Additionally a second method of DNA content analysis (flow cytometry) was employed, as the Arrayscan does not take binucleated cells into account. In conclusion, further study is required to elucidate the effect of DYRK1A in hepatic progenitor cells.

Chapter 3.

The effect of DYRK1A knockdown and inhibition on proliferation and cell cycle in Organoid Derived Progenitor Cells

Abstract

Background and aims – Studies in HepaRG cells have shown that DYRK1A inhibition causes a shift in cell cycle distribution, both by siRNA mediated gene silencing and chemical inhibition. This effect has not yet been described before in liver cells and warrants follow up study in primary liver cells. The aim of this part of the project was to validate the results found in HepaRG cells in a primary liver stem cell culture.

Materials and methods – Mouse Organoid Derived Progenitor Cells (ODPCs) are used as an *in vitro* model for HPCs. Dyrk1A was knocked down by siRNA mediated gene silencing and chemically inhibited with harmine. Knockdown was verified on mRNA level with qPCR and on protein level with Western blot. Proliferation was measured by assessing EdU incorporation and pH3 phosphorylation. Cell cycle distribution was measured using flow cytometry and subsequently analysed using FlowJo software. HepaRGs were taken up in this experiment as a positive control, as it has already been shown that a phenotype can be established in this cell type.

Results – In ODPCs knockdown and chemical inhibition of Dyrk1A did not affect proliferation, nor was there an effect on cell cycle distribution. Knockdown was confirmed on mRNA and protein level on 48 hours and on mRNA level on 72 hours. Knockdown of DYRK1A in HepaRGs by siRNA mediated gene silencing resulted in a shift in the cell cycle distribution. Chemical inhibition by harmine did not have an effect on the cell cycle.

Conclusion – The effect on cell cycle distribution of siRNA mediated gene silencing of DYRK1A as found in the high throughput screen and part A of this project was confirmed by flow cytometry and FlowJo analysis. This effect is not found in chemically inhibited wells in HepaRGs. In ODPCs there is no effect of Dyrk1A knockdown on the cell cycle, nor is there an effect of chemical inhibition. The fact that the phenotype found in HepaRGs cannot be validated in a primary HPC culture needs to be further investigated. Neither cell types showed an effect of chemical inhibition on the cell cycle distribution.

Introduction

The results in HepaRG cells show that DYRK1A knockdown has an effect on the cell cycle. Both knockdown by siRNA mediated gene silencing and chemical inhibition of DYRK1A result in an increase in EdU incorporation and pH3 phosphorylation, with a more pronounced effect of gene silencing. This fits the measured increase in S-phase and G2/M-phase. This effect has not yet been shown in hepatic cells and requires a follow up to validate that this phenotype is not cell line specific, but can be found *ex vivo* and *in vivo* in HPCs as well. In order to do this the previously performed experiments have to be repeated in a primary cell line. The model used for this is that of the Organoid Derived Progenitor Cell (ODPC). ODPCs are derived from hepatic organoids. The culture of these three dimensional structures of primary HPCs can be sustained for over 12 months. During the entire culture time their differentiation potential is maintained, which is unique for stem cells (28). Organoids can be used for stem cell transplantation or as a model for the *in vitro* study of diseases. In this research project organoids are used as a model for *in vitro* HPCs. The effect of DYRK1A knockdown or inhibition is validated using this primary cell culture.

The introduction of hepatic Organoids is accompanied by several technical difficulties. Due to their spherical shape, which is an essential feature of organoids, it is difficult to accomplish knockdown by siRNA mediated gene silencing. Additionally, it is more difficult to score cells after immunocytochemical or –fluorescent staining. In order to overcome these practical objections the organoids plated out on plastic culture plates as single cells. The ODPCs are acquired by incubation of organoid fragments with trypsin to digest the fragments to single cell level. These cells are plated out in expansion medium, after which they can be used directly in an experiment, or they can be subcultured as ODPCs. In this research project the ODPCs were created *de novo* from three dimensional organoid cultures for each experiment, and were not subcultured as ODPCs.

Required optimisations

Though the ODPCs overcome some of the technical hurdles that organoids pose, the use of these cells does not come without difficulties. The protocols we have for HepaRG cells are not by definition suitable for use on ODPCs. For example, the procedure of siRNA mediated gene silencing in seeded mouse ODPCs (forward transfection) is not yet optimised. The first step in this part of the research project is to ensure that Dyrk1A is expressed in OPDCs on both mRNA level. This will be done by quantitative Polymerase Chain Reaction (qPCR). The second step is optimising transfection efficiency. This will be done using siGLO Green Transfection Indicator (siGLO, Thermo Scientific). This is a fluorescent oligonucleotide duplex that concentrates its signal in the nucleus to permit visual assessment of uptake into mammalian cells. siGLO is used in this optimisation experiment to determine the ideal concentrations of siRNA and lipofectamin, as well as the optimal confluency of the transfected well. Important to acknowledge is the fact that siGLO is a qualitative indicator of transfection efficiency, and cannot be used for quantitative determination of siRNA uptake. The last step is to verify that there is knockdown after transfection. For this purpose cells were transfected with a siRNA against Dyrk1a (siDYRK). The negative control is a non targeting siRNA (NT). As a positive control siRNA against E2F7 (siE2F7) was included. This siRNA has successfully silenced E2F7on mRNA level in mouse embryonic fibroblasts (43). This positive control is included to ensure that ODPCs are suitable for transfection and that it is possible to establish knockdown in ODPCs.

In order to confirm knockdown on protein level the Western blot protocol needs to be optimised. This consists of several components. What needs to be ascertained is whether or not there is baseline expression of Dyrk1A in ODPC cells, and whether or not the antibody against human DYRK1A is also suitable for mouse. The last step is to optimise the staining protocol.

For the EdU incorporation assay cells are pulsed prior to fixation, and stained with fluorescent azide after fixation (16). As it is not an antibody-mediated reaction, the protocol is interchangeable with

different cell types. The most important aspect to focus on is the duration of the EdU pulse, which depends on the duration of the cell. Cells that divide faster will suffice with a shorter EdU pulse (e.g. 3 hours), while cells that pass through the cell cycle slower require a longer pulse (e.g. 6 hours) in order to label a subpopulation of cells. The ODPCs grow with approximately the same speed as HepaRGs, thus a pulse of the same duration (3 hours) was given to the ODPCs as well. The protocol for pH3 staining required optimisation, as the efficiency of this protocol might differ between different cell types. DAPI staining protocols are interchangeable between different cell types. The staining intensity, amongst many other cytological features, was measured using the Arrayscan VTI (600 series). This automated microscope is capable of recognising nuclei and separating them from any debris in the well. In order to do this an algorithm needs to be developed in which the characteristics of a nucleus are specified. In this algorithm the size, shape, segmentation, object area, DAPI intensity and many other parameters are included to ensure that each nucleus is treated as such. As the nuclei of each cell type differ in these parameters, a new algorithm is required for every cell type. Cell lines are very homogenous, and thus algorithms for these cells tend to be easier to optimise than for primary cells. The latter often show a heterogeneous population of cells and nuclei, making it difficult to develop an algorithm with characteristics of 'the' nucleus.

Materials and methods

Organoid and ODPC culture

ODPC cells used for all experiments described were obtained by *de novo* synthesis from organoids. Organoids were thawed from liquid nitrogen storage at passage number 6, mixed with Matrigel (BD Bioscience, San Jose, CA, USA) and seeded in a 48 wells culture plate (Greiner). After the Matrigel is solidified culture medium was added. Culture medium was based on Advanced DMEM/F12 (Invitrogen) supplemented with B27 (without vitamin A), N2, N-acetylcysteine, gastrin and a specific set of growth factors as previously described (29). In short, this set consisted of mouse EGF, Rspo1, FGF10, HGF and nicotinamide (29). Organoids were passaged once a week. The organoids were removed from the Matrigel, mechanically disrupted into fragments and seeded in fresh Matrigel. The passage ratio was 1:4-1:8 once per week, depending on the requirements of scheduled experiment. ODPCs were made *de novo* for each experiment by incubating organoid fragments with trypsin. The fragments were digested to single cell level, after which the cells were suspended in culture medium containing 10% FCS to enhance attachment and plated on plastic culture plates. After 24 hours medium was replaced with FCS free medium. Culture medium for ODPCs had the same composition as for organoids.

Optimisations

Measurement of Dyrk1A gene expression in ODPCs

ODPCs were seeded 10,000 cells per well in a 48 wells plate. The cells were incubated with maintenance medium (containing 10% FCS) for 24 hours, after which RNA was isolated using Buffer RLT containing β-mercapto-ethanol (Qiagen Benelux BV, Venlo, The Netherlands), after which it was processed using a RNeasy mini kit (Qiagen). RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Biorad). The reaction was performed in a final volume of 20 µL, which included iScript reaction mix, iScript reverse transcriptase and 0.5 µg RNA template. The reaction mixtures were heated at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. cDNA was stored at -20°C.

qPCR was performed using the CFX Connect™ Real-Time PCR Detection System (BioRad) in a final volume of 10 µL, which included SYBR green master mix, an optimised concentration of each primer and 4 µL of the reverse transcription product containing cDNA. Normalisation was ensured by use of independent reference genes. For ODPC these were β-Actin, GAPDH and RPS18. For HepaRG these were

HPRT and RPLT. Relative gene expression of each gene product (delta-cQ method) was used for comparison of mRNA levels. Undetectable gene expression levels were arbitrarily set to cQ 45.

Measurement of Dyrk1A protein expression in ODPCs

Trypsinised cells were washed with HBSS, centrifuged and resuspended in 350 µL RIPA buffer (1 % Igepal, 1 mM, PhenylMethylSulfonyl Fluoride, 1 µg/ml aprotinine, and 1 mM sodium orthovanadate, Sigma Aldrich) and incubated for 30 minutes at 4°C. Protein concentration of the lysate was determined using the DC protein assay. Proteins were denatured for 2 minutes at 95°C. Samples were loaded in each lane (for concentrations see attachment 5, table 2) of a 15% Criterion Tris-HCl gel (Biorad). Proteins were then transferred onto a Hybond-C Extra Nitrocellulose membrane (Amersham Biosciences Europe, Roosendaal, The Netherlands). The membrane was blocked in 10% non-fat dry milk in PBS for 1 hour at room temperature and incubated with different concentrations (0, 1:250, 1:375 and 1:500)of polyclonal antibody against DYRK1A (Sigma-Aldrich, HPA015810) overnight. After washing with TBS-Tween (0.1%) 3 times for 5 minutes the membrane was incubated with goat-anti-rabbit secondary antibody (Dako) for 1 hour at room temperature (omitted in negative control to check for fluorescent label on primary antibody). Blots were washed 3 times for 5 minutes with TBST and developed with an ECL kit (Amersham Biosciences Europe) following the producer's instructions. ChemiDoc XRS Chemi Luminescent image capture (Biorad) was used to capture the images, after which density of the immunoreactive bands was determined using Quantity one (Version 4.6.9, Biorad).

Forward transfection

To optimise transfection efficiency of lipofectamin mediated transfection ODPCs were seeded 7500 cells/well in a 48 wells plate. On day 1 the cells were transfected with 50 or 100 nM siGLO (Thermo Scientific) and 3 or 6 µL/mL lipofectamin RNAiMAX transfection reagent (Invitrogen). After 24 hours the transfection medium was removed, after which the percentage of siGLO positive cells was determined. Additionally, the effect of confluency on transfection efficiency was determined using siGLO. On day 0 ODPC cells were seeded in 5000, 7500 and 10000 cells per well in a 48 wells plate, and after 24 hours maintenance medium was replaced with transfection medium containing 50 nM siGLO and 6 µL/mL lipofectamin RNAiMAX transfection reagent. After 24 hours the transfection medium was removed, after which the percentage of siGLO positive cells was determined.

To determine knockdown efficiency ODPCs were seeded 5000 cells/well in a 48 wells plate (Greiner). At a confluency of 5-10% maintenance medium was replaced with transfection medium with 5 nM siRNA (NT, siDYRK or siE2F7, Thermo Scientific) and 6 µL/mL lipofectamin RNAiMAX transfection reagent. After 24 hours RNA was isolated using Buffer RLT containing β-Mercapto-ethanol. Gene expression was measured to verify knockdown on mRNA level. In a second experiment knockdown efficiency of Dyrk1A was determined on 24, 48 and 72 hours after transfection.

EdU-pH3-DAPI immunofluorescence and automated microscopy

The pH3 stain was optimised for ODPCs. Cells were seeded 1000 cells/well in a 96 wells plate and fixed and permeabilised with 3.7% PFA and 0.5% Triton fixating solution. The normal goat serum (NGS, Dako) concentration (5 and 10%), blocking time (30 and 60 minutes), rabbit anti-phospho-histone-H3 primary antibody (Millipore) concentration (1:500 and 1:300) and goat anti-rabbit-AF568 secondary antibody (1:200, Dako) were varied independently. The cells were counterstained with DAPI (1:4000, Invitrogen). Staining was assessed by fluorescence microscopy.

A triple stain was performed on ODPCs. Cells were stained using EdU staining buffer (containing 100 mM Tris, 1 mM $CuSO_4$, 5 µM fluorescent AF488-azide and 100 mM ascorbic acid). For the pospho-histone-3 stain a serumblock of 1 hour was performed using 10% NGS. Cells were incubated for 1 hour at room temperature (RT) with primary antibody (rabbit anti-phospho-histone-3, 1:300), followed by a

washing step with PBST and incubation with secondary antibody (goat anti-rabbit-AF568, 1:200) for 1 hour at RT. DAPI (Invitrogen) was used as a nuclear counter stain in a dilution of 1:4000 for 30 minutes at RT. Plates were scanned using the Arrayscan and a new algorithm for OCPCs was developed using vHCS view software.

Flow cytometry

Single cell HepaRGs were fixed by overnight incubation in 70% ethanol (200,000 cells/mL) at 4°C. Cells were washed twice with ice cold PBS, after which each tube was treated differently. Either whole cells were stained with PI buffer (5 µg/mL Propidium Iodide and 250 µg/mL RNAse in PBS) with or without passing through a 70 µM filter, or the cells were first digested to nuclear level with pepsin in different concentrations (0.5, 0.25 or 0.125 mg/mL).

For practical reasons the two best protocols on HepaRGs were tested on ODPCs to determine the optimal staining protocol. ODPCs were fixed by overnight incubation in 70% ethanol (2 tubes of 40,000cells/tube) at 4°C. Cells were washed once with ice cold PBS. Either whole cells were stained with PI buffer without filtering, or the cells were first digested to nuclear level with pepsin (0.5 mg/mL). Nuclear staining intensity was assessed using the FACSCalibur flow cytometer.

siRNA mediated gene silencing and chemical inhibition

Transfection

At a confluency of 5-15% ODPCs were incubated in transfection medium containing 5 nM siRNA (Non Targeting or siDYRK) and 6 nM lipofectamin RNAiMAX transfection reagent. After 24 hours transfection medium was replaced with maintenance medium. At 48 and 72 hours after transfection cells were harvested.

Chemical inhibition

ODPCs were incubated with maintenance medium containing 1 or 2 µM harmine or the appropriate vehicle controls. Medium containing harmine or vehicle was replaced after 48 hours (for the 72h time point). After 48 and 72 hours cells used for flow cytometry were trypsinised to a single cell suspension, after which they were fixed overnight in 70% ethanol. At the same time points other wells were pulsed with EdU for 3 hours (EdU omitted in the negative control) and fixed and permeabilised with a 3.7% PFA and 0.5% Triton fixating solution for 10 minutes at room temperature. Plates were stored at 4°C until further use.

Automated microscopy

On both the transfected and the chemically inhibited plates an EdU-pH3-DAPI triple stain was performed. Cells were stained for 30 minutes at RT with EdU staining buffer (containing 100 mM Tris, 1 mM $CuSO_4$, 5 µM fluorescent AF488-azide and 100 mM ascorbic acid). A serum block was performed for 1 hour at RT with 10% NGS. After this the cells are incubated for 1 hour at room temperature with primary antibody (rabbit anti-phospho-histone-3, 1:300), followed by a secondary antibody (goat anti-rabbit-AF568, 1:200) under the same conditions. DAPI (Invitrogen) was used as a nuclear counterstain in a concentration of 1:4000 for 30 minutes. The Arrayscan VTI (600 series) was used to measure the percentage of EdU positive cells, the percentage of pH3 positive cells, the average intensity of each nucleus of EdU and pH3, and the total intensity per nucleus of DAPI. Because the entire surface of the well was scanned the number of nuclei per well was also determined.

Flow cytometry
Both HepaRG and ODPC cells were washed with HBSS, harvested using trypsin, spun down and incubated in 70% ethanol overnight at 4°C for fixation. Cells were washed with ice cold PBS, spun down and resuspended in PI buffer (5 µg/mL Propidium Iodide and 250 µg/mL RNAse in PBS). PI staining intensity was measured using the BD FACSCalibur flow cytometer and used as a parameter for DNA content. Acquired data were analysed with FlowJo analysis software. This software was used to make histograms of DNA content per nucleus. Additionally it fit the curve based on Dean Jet Fox model to calculate the distribution of the nuclei over the phases of the cell cycle.

Knockdown confirmation on mRNA level
RNA was isolated using SPR at 48 and 72 hours after transfection. This was reverse transcribed using the iScript cDNA synthesis kit. Gene expression of Dyrk1A as measured by qPCR was normalised against the expression of β-actin, GAPDH and RPS18 (ODPC) or HPRT and RPL19 (HepaRG). Undetectable gene expression levels were arbitrarily set to cQ 45.

Knockdown confirmation on protein level
On 48 and 72 hours after transfection cell were washed with Hank's BSS, centrifuged and resuspended in 350 µL of RIPA buffer and incubated at 4°C for 30 minutes. Protein concentrations were determined using a DC Protein Assay (Biorad). Proteins were denatured for 2 minutes at 95°C. Samples were loaded in each lane of a 4-15% gradient Tris-HCl polyacrylamide gel (Bio-Rad) and separated with electrophoresis. Proteins were transferred onto Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences Europe). Membranes were blocked in 4% non-fat dry milk (Biorad) in TBS-Tween(0.1%) for 1 hour at room temperature and then incubated with polyclonal antibody against DYRK1A (1:250, Sigma-Aldrich) overnight. After washing with TBS-Tween (0.1%) 3 times for 5 minutes, membranes were incubated with goat-anti-rabbit secondary antibody (Dako) in a 1:5000 dilution for 1 hour at room temperature. Blots were washed 3 times for 5 minutes with PBST and developed with an ECL kit according to the manufacturer's protocol (Amersham Biosciences Europe). As a loading control Beta-Actin antibody (Pan Actin AB-5, Neomarkers, Fremont, USA) in a 1:2,000 dilution was used. ChemiDoc XRS Chemi Luminescent image capture (Biorad) was used to capture the images, after which density of the immunoreactive bands was determined using Quantity one (Version 4.6.9, Biorad). The measured density was corrected for background staining and normalised against density of B-Actin.

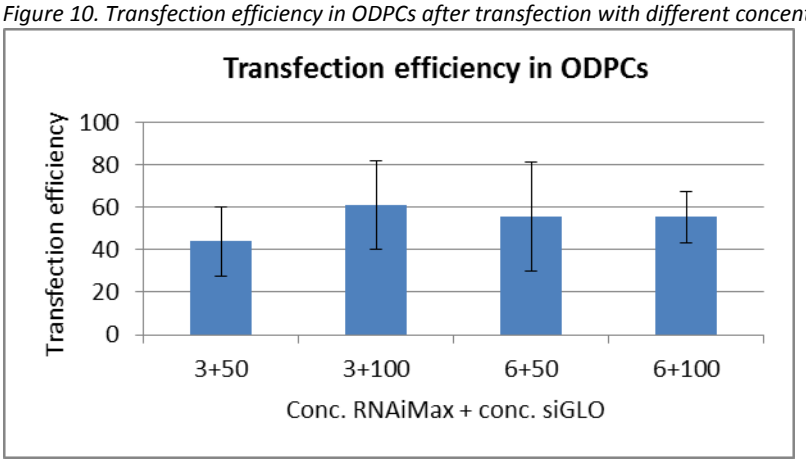
Statistical analysis
As there were not enough measurements to prove that the data were normally distributed, it was assumed that they were not normally distributed. Therefore statistical analysis was performed using a Mann-Whitney U test. All data were analysed using R software library (version2.11.1).

Results
Optimisations
Gene expression and protein levels of DYRK1A in ODPCs
qPCR on ODPCs and organoids samples showed expression on mRNA level of DYRK1A and E2F7. Expression on protein level was assessed using Western blot, with positive results in ODPCs as well as in HepaRGs (unpublished data). The anti-DYRK1A antibody (Sigma-Aldrich) is specific against human (HepaRG) and mouse (ODPC). The optimal concentration of primary antibody was 1:250, DYRK1A was stained properly without too much background staining. The negative controls (1st antibody, 2nd antibody or both antibodies omitted) showed no staining.

siRNA mediated gene silencing

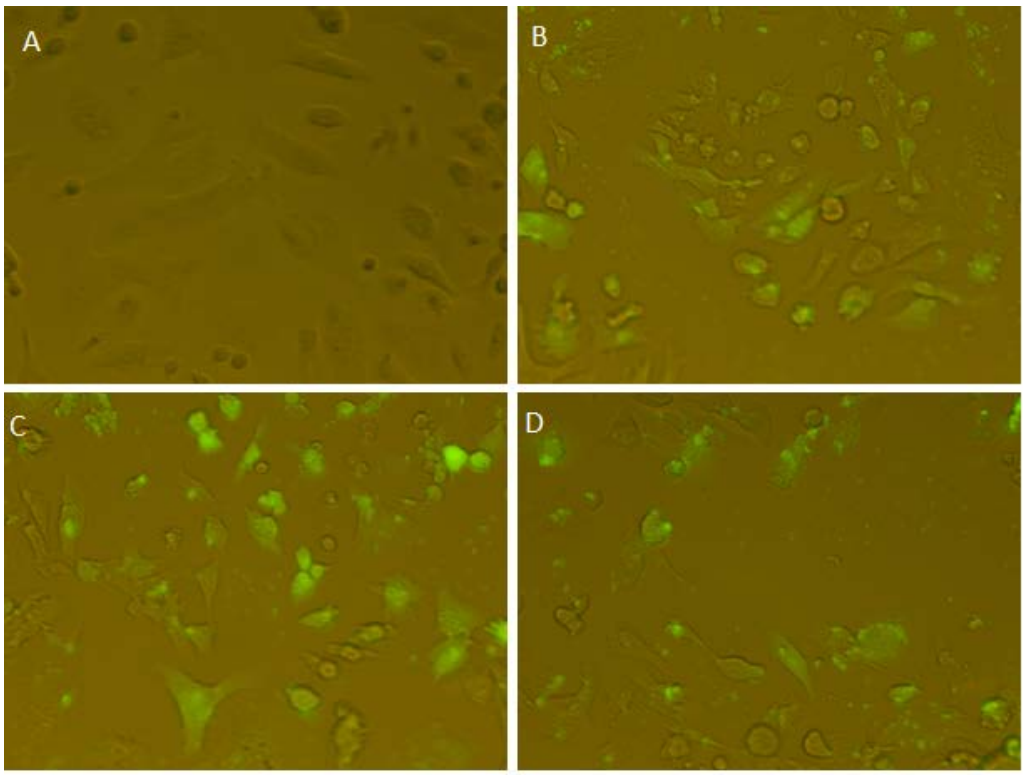
The 6 µL/mL RNAiMAX showed a more consistent uptake of siGLO (figure 10). As there were no toxic effects of this concentration it was used in further protocols. There was no effect of a higher concentration of siGLO, so the lowest concentration was chosen.

Figure 10. Transfection efficiency in ODPCs after transfection with different concentrations of siGLO and RNAiMAX



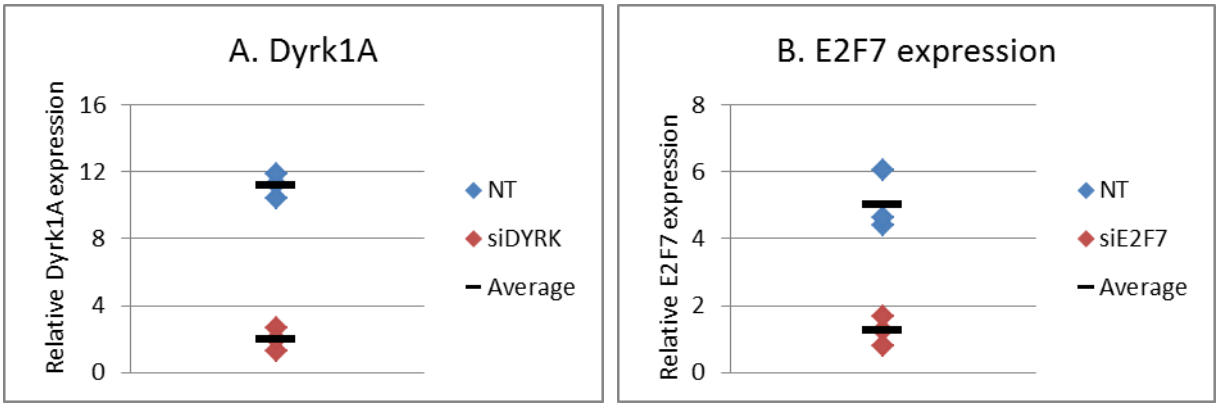
The effect on confluency was not visible between the wells. Transfection efficiency was 50-60% in all conditions (figure 11). However, within each well there was a great difference. In parts of the well where confluency was 10-15% the transfection was successful in up to 80% of the cells. Parts of the well that were highly confluent (50-100%) only 0-10% of the cells were transfected.

Figure 11. Pictures from fluorescence microscope > A. 5000 (negative control), B. 5000, C. 7500 and D. 10,000 cells per well



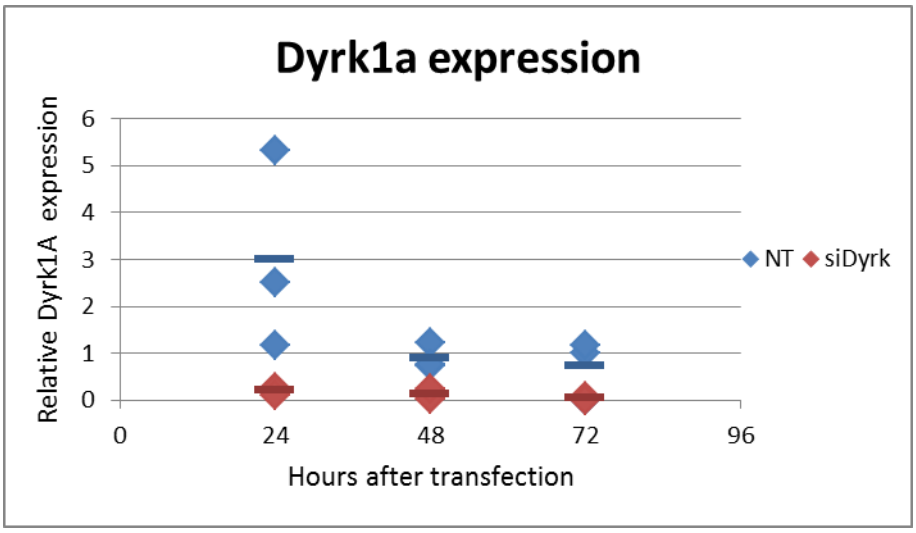
The knockdown efficiency was tested for both siDYRK1A and siE2F7. The expression of Dyrk1A decreased from a relative expression of 100% in non-target treated cells to 17% in siDYRK treated cells (knockdown of 83%). The relative expression of E2F7 decreased from 100% to 23% (knockdown of 77%, figure 12).

Figure 12. Gene expression on mRNA level of A. Dyrk1A and B. E2F7 in ODPCs at 24 hours after transfection



In a second experiment the knockdown was tested on 24, 48 and 72 hours after transfection. The knockdown was 93.3% on 24 hours, 85.5% on 48 hours and 94% on 72 hours after transfection (figure 13).

Figure 13. Gene expression on mRNA level of Dyrk1A at 24, 48 and 72 hours after transfection



pH3 phosphorylation

Optimal staining was assessed as having little background staining, high staining intensity of nuclei and lowest signal to noise ratio. The protocol resulting in the best pH3 staining included a 10% NGS serum block of 1 hour, followed by incubation with primary antibody (1:300) for 1 hour at room temperature (RT) and after washing with PBST, incubation with secondary antibody (1:200) for 1 hour at RT. After performing an EdU-pH3-DAPI triple stain according to the new protocol cells were imaged using the Arrayscan. An algorithm for ODPCs was developed by adjusting the 'smooth factor', 'segmentation factor', 'object area' and 'average intensity'. Due to the heterogeneity of the nuclear shape and size of the ODPCs, the algorithm was difficult to develop and remained suboptimal when compared to for example HepaRG cells.

Flow cytometry

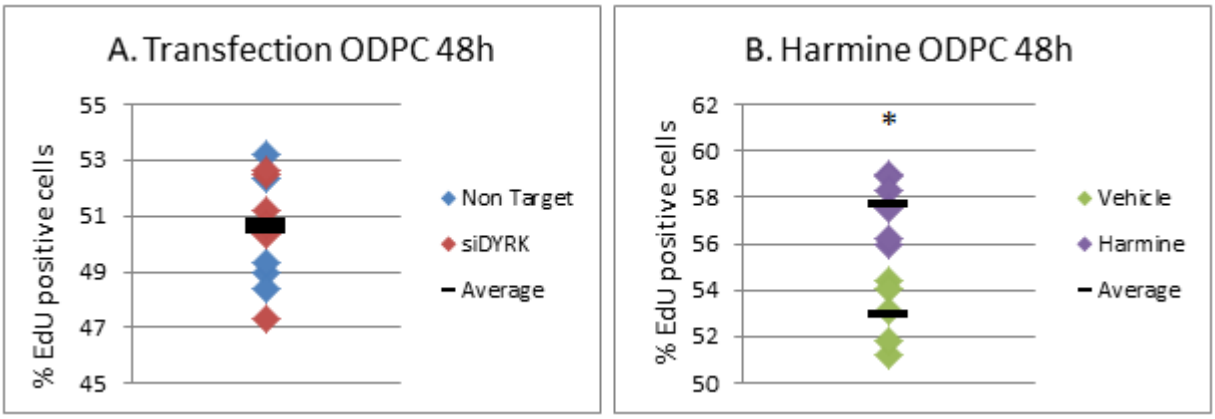
The staining of whole cell HepaRGs resulted in better flow cytometric results, as pepsin digestion resulted in the degradation of nuclei as well as cellular membranes. The pepsin digestion in different concentrations showed similar results, where many degraded cell debris disturbed the measurement of intact nuclei. Filtering the cells through a 70 µM filter resulted in the loss of cells, and there were little to no clumps of cells in the unfiltered condition. Therefore the optimal protocol for PI stain on HepaRG cells is direct resupsension in PI buffer after washing with ice cold PBS. For ODPCs this protocol was tested, as well as the protocol including pepsin digestion (0.5 mg/ml). In ODPCs pepsin digestion resulted in even more cellular fragments, while whole cell staining and flow cytometry gave better results. Thus, the same protocol as for HepaRGs was selected for ODPCs.

Effect of DYRK1A knockdown and inhibition on ODPC cells

EdU incorporation

There was no significant increase in the percentage of EdU positive cells after knockdown of DYRK1A in ODPCs. Chemical inhibition of DYRK1A resulted in an increase in the percentage of EdU positive cells on respectively 48 and 72 hours (figure 14). Knockdown of DYRK1A in HepaRG cells resulted in a significant increase of in the percentage of EdU positive cells (see attachment 5, figure 34). Chemical inhibition of DYRK1A significantly increased the percentage of EdU positive cells in HepaRG cells on 72 hours.

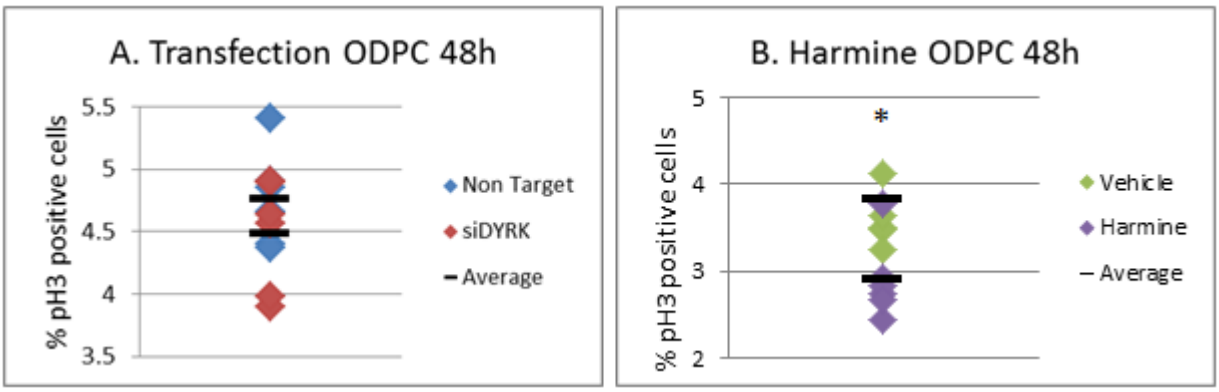
*Figure 14. Percentage of EdU positive cell in ODPCs on A. 48 hours and B. 72 hours after transfection. * = statistical significance of P<0.05*



pH3 phosphorylation

In ODPCs the percentage of pH3 positive cells did not differ between DYRK1A knockdown wells and Non Targeting controls. In harmine treated wells there was a decrease in the percentage of pH3 positive cells after 48 and 72 hours of chemical inhibition (figure 15). In HepaRGs chemical inhibition of DYRK1A on 48 hours resulted in a significant increase in the percentage pH3 positive cells, siRNA mediated gene silencing did not. On 72 hours both transfection and chemical inhibition of DYRK1A resulted in a significant increase in the percentage of pH3 positive cells (attachment 5, figure 35).

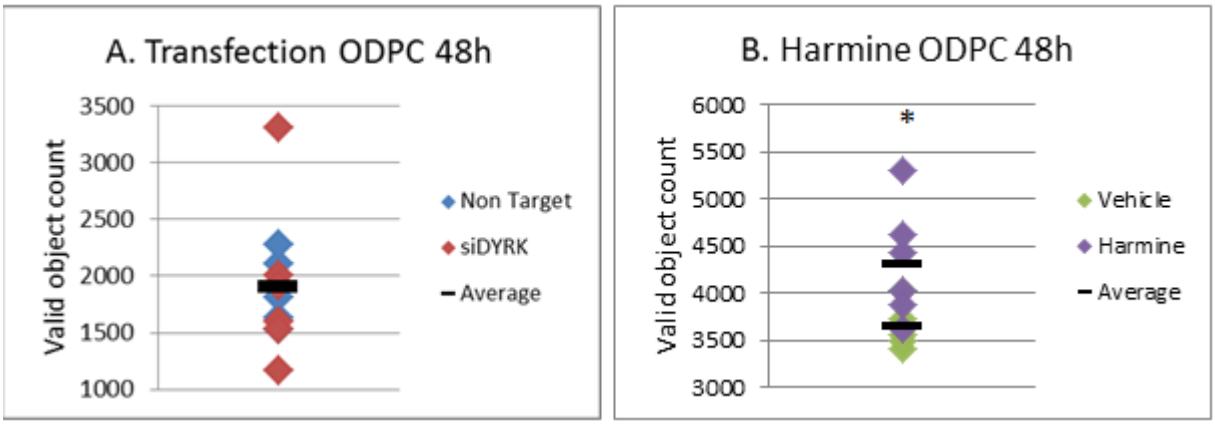
Figure 15. Percentage of pH3 positive cell in ODPCs 48 hours for A. NT vs. siDYRK and B. vehicle vs. harmine



Valid object count

Dyrk1A knockdown did not result in changes in cell numbers in ODPCs. Chemical inhibition gave a significant increase in cell number on 48 hours. In HepaRG cells DYRK1A knockdown resulted in a decrease of cell number on 48 hours. Chemical inhibition of DYRK1A did not result in changes in cell number (figure 16 and attachment 5, figure 36).

Figure 16. Cell number of ODPC cells on 48 hours for A. NT vs. siDYRK and B. vehicle vs. harmine

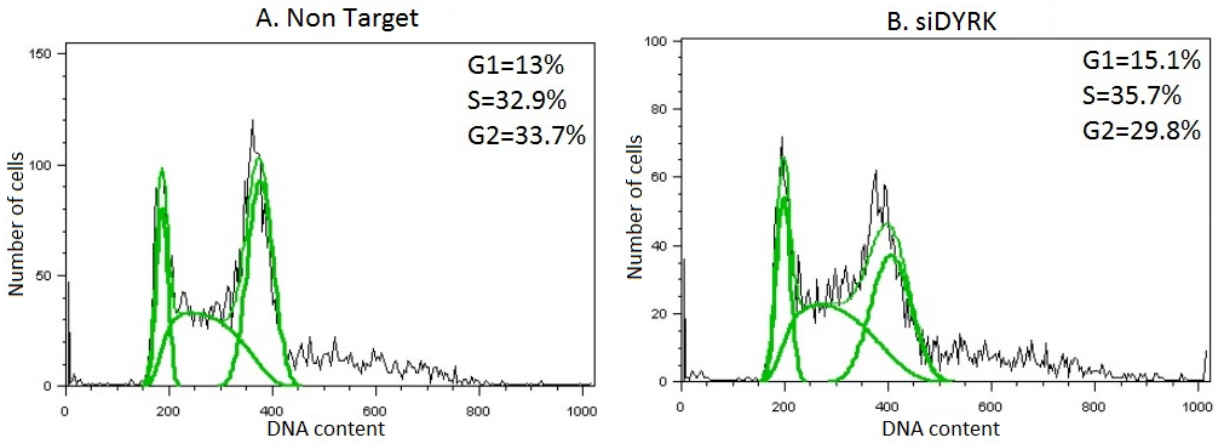


Cell cycle distribution

In ODPCs cell cycle distribution remained unchanged after knockdown or chemical inhibition of DYRK1A (figure 17 and attachment 5 figures 39 and 40).

In HepaRG cells there was a clear phenotype after knockdown of DYRK1A. The percentages of cells in S phase and G_2 phase significantly increased while the percentage of cells in G_1 phase decreased, at both 48 and 72 hours after transfection. Chemical inhibition of DYRK1A did not result in a phenotype, neither on 48 hours nor on 72 hours (attachment 4, figures 37 and 38).

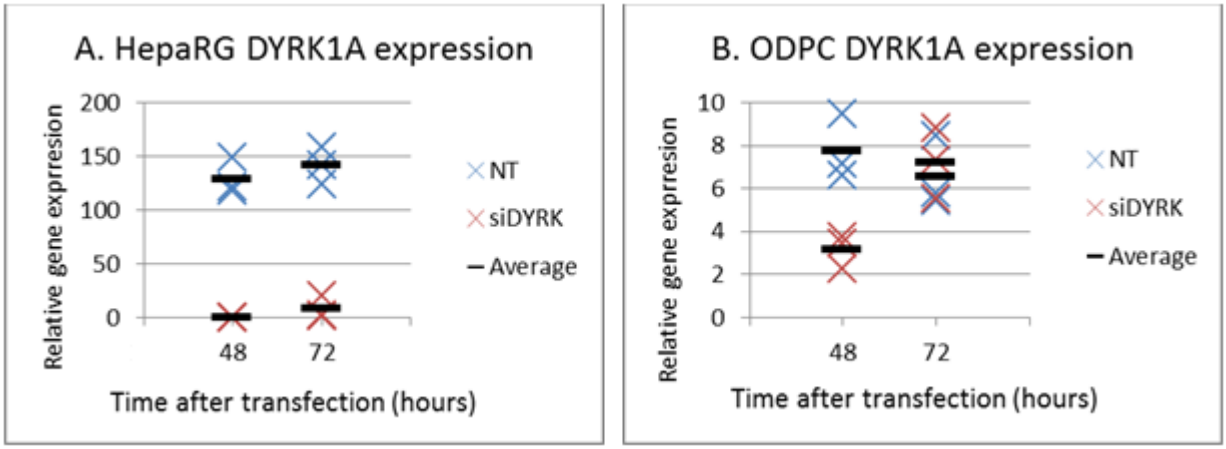
Figure 17. Cell cycle distribution of ODPC cells at 72 hours of A. Non Targeting and B. siDYRK with the percentage of cell in G_1, S and G_2 phase (data on sub-G_1 and super-G_2 are not shown). Green lines represent curve fits using the Dean Jett Fox model



Knockdown confirmation on mRNA level

siRNA mediated gene silencing in HepaRG cells resulted in 99% and 95% knockdown at respectively 48 and 72 hours after transfection. In ODPCs a knockdown of 59% was established at 48 hours, while there was no knockdown measured on mRNA level at 72 hours (figure 18).

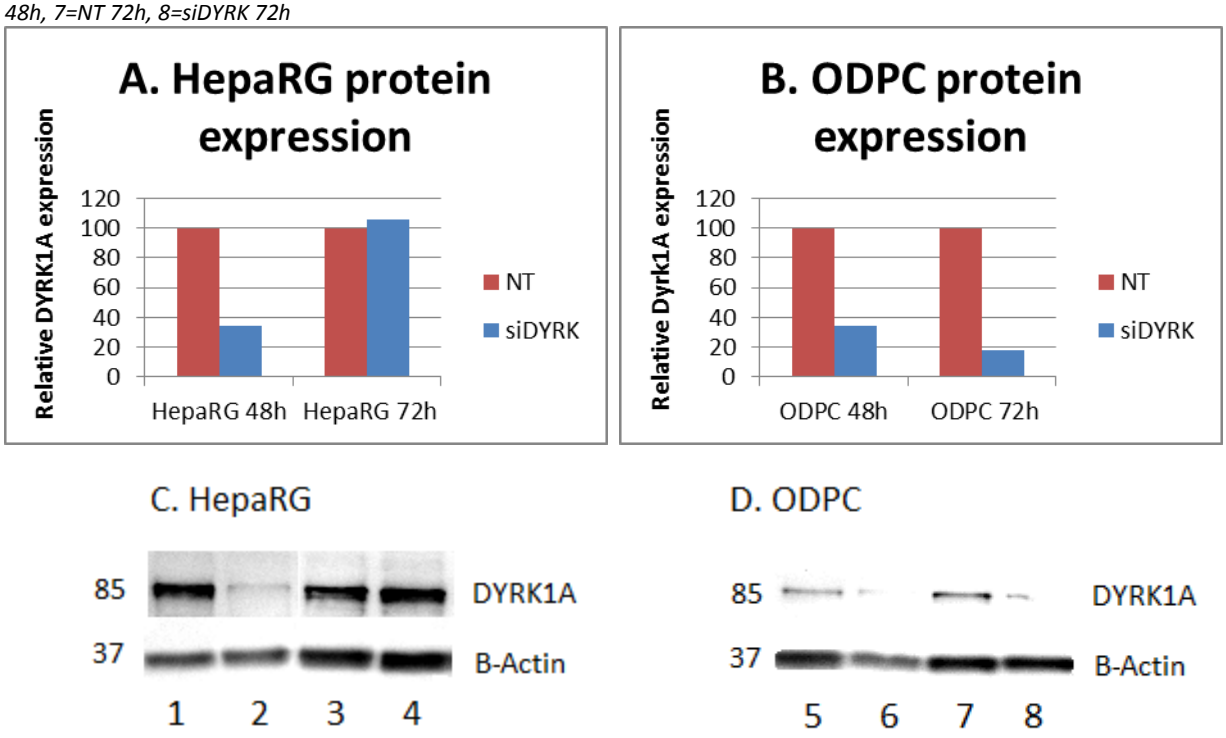
Figure 18. Relative gene expression of DYRK1A (and reference genes) in A. HepaRG (HPRT and RPL19) and B. ODPC (B-actin, GAPDH and RPS18)



Knockdown confirmation on protein level

On protein level siRNA mediated gene silencing in HepaRGs resulted in 65% knockdown on 48 hours after transfection. On 72 hours there was no knockdown on protein level. For ODPCs knockdown was 65% on 48 hours after transfection and 82% on 72 hours (figure 19).

Figure 19. Relative protein levels of DYRK1A in A. HepaRG and B. ODPC normalised against protein levels of β-Actin. Western blot data of C. HepaRG samples (1=NT 48h, 2=siDYRK 48h, 3=NT 72h and 4=siDYRK 72h) and D. ODPC samples (5=NT 48h, 6=siDYRK 48h, 7=NT 72h, 8=siDYRK 72h



Discussion

In HepaRGs there was an increase in the percentage of EdU positive cells, as well as an increase in pH3 positive cells in DYRK1A knockdown wells compared to the negative controls. There was no difference in cell number between these two conditions, so there was no effect on proliferation. However, knockdown did result in a shift in cell cycle distribution, as expected based on the results acquired in the high throughput screen and part A of this research project. Knockdown of DYRK1A resulted in a decrease of the population of cells in G_1 phase and an increase in S phase and G_2 phase populations. Knockdown was verified for the duration of the experiment on mRNA and protein level but did not correspond at 72h. This could be a technical artefact, as there was still a clear phenotype. This phenotype caused by DYRK1A knockdown is supported by own, unpublished data acquired in the high throughput screen and in part A of this research project, as wells as by Litovchick et al, in their paper on the role of DYRK1A in DREAM complex assembly (41). The fact that there was no effect of chemical inhibition of DYRK1A is contradictory to what was found by Litovchick et al. In this second part of the project a more sensitive technique was used for the cell cycle analysis, flow cytometry. Using this technique the results in HepaRG cells are repeated, with a shift in the cell cycle distribution as a result of DYRK1A knockdown by siRNA mediated gene silencing. Chemical inhibition of DYRK1A in HepaRGs did not result in a shift in the cell cycle, neither on 48 nor on 72 hours. This is in contrast with data found by Litovchick et al. In their research both chemical inhibition of DYRK1A by harmine and siRNA mediated gene silencing of serum starved cells resulted in higher levels of DNA synthesis when compared to the negative controls, suggesting that DYRK1A is required for entry into G_0/quiescence (41).

Results indicate that in ODPCs knockdown and chemical inhibition of DYRK1A no increase in the percentage of EdU positive cells, no increase in the percentage of pH3 positive cells, no increase in cell number and no shift in cell cycle distribution. Ultimately, there is no phenotype after knockdown of

DYRK1A in ODPCs. Knockdown is confirmed on protein level on both 48 and 72 hours, while knockdown on mRNA level was present on 48 hours. However, in previous experiments knockdown of over 85% has been accomplished on both 48 and 72 hours. Furthermore, in Western blot the band of ODPC siDYRK treated on 72 hours did not span the entire width of the lane. Though taken into consideration when determining knockdown, this technical artefact may have influenced the results. Therefore it is necessary to repeat this experiment and ensure that there is high mRNA and protein knockdown before drawing definitive conclusions about whether or not knockdown of Dyrk1A results in a phenotype in ODPCs.

If knockdown is validated and again the phenotype found in HepaRGs is not repeatable in ODPCs it needs to be investigated whether or not ODPCs are an appropriate model for primary HPCs. If this is not the case, a different model for primary HPCs has to be used to validate the phenotype found in HepaRGs. Organoids express stem cell markers and have the potential for self-renewal and differentiation (29). The three dimensional structure is an essential characteristic of organoid culture, and could be required to maintain its stem cell characteristics. It is imaginable that seeding ODPCs in a monolayer causes changes in gene expression, reducing its stem cell character. It is therefore required to conduct further investigations regarding the ODPCs to determine whether or not these cells are a suitable model for primary HPCs. For example, gene expression studies could be done to see whether short- and long term two dimensional culture affects the expression of stem cell markers. Additionally differentiation studies should be performed, to examine if the potential to differentiate towards fully functional hepatocytes persists. In conclusion, it is necessary to further study ODPCs as a stem cell model.

Chapter 4.

Summary and discussion

Summary

In part A the effect of chemical inhibition by harmine and siRNA mediated gene silencing of DYRK1A on the HepaRG cell line was determined. This revealed that neither chemical inhibition nor siRNA mediated knockdown of DYRK1A had an effect on proliferation in these cells, as there was no measured increase in cell number compared to the respective negative control. However, DYRK1A knockdown and inhibition both resulted in a shift in the cell cycle distribution when measured after 48 and 72 hours. There was an increase in the percentage of cells in S phase and G_2 phase, while the percentage of cells in G_1 phase decreased. This effect is more pronounced upon siRNA mediated gene silencing of DYRK1A than with chemical inhibition using harmine.

In part B the effect of DYRK1A inhibition on the cell cycle was evaluated in primary Hepatic Progenitor Cells (HPCs). ODPCs were used as a model for primary HPC culture, with HepaRG cells as a positive control. Proliferation of ODPCs is not influenced by Dyrk1A knockdown or inhibition. Additionally, siRNA mediated gene silencing and chemical inhibition did not have an effect on cell cycle distribution. Knockdown on mRNA was found on 48 hours, on protein level there was knockdown on 48 hours and 72 hours after transfection. In HepaRGs there was no effect of DYRK1A inhibition and knockdown on proliferation of HepaRGs. Additionally the shift in the cell cycle distribution found in part A was also detected using flow cytometry. Therefore, the lack of phenotype in ODPCs is not due to methodological problems.

Conclusions

The experiments in this research project have shown that siRNA mediated gene silencing and chemical inhibition of Dyrk1A do not have an effect on proliferation or cell cycle distribution in OPDCs. In HepaRG cells neither DYRK1A knockdown nor chemical inhibition had an effect on proliferation. In part A siRNA mediated gene silencing was a more potent means of DYRK1A inhibition, but both methods resulted in a shift in the cell cycle. In part B only siRNA mediated gene silencing had an effect on the cell cycle.

Discussion

Chemical inhibition of DYRK1A by harmine

Inhibition of DYRK1A by harmine did not result in a phenotype in HepaRGs and ODPCs when analysed with flow cytometry. As several researchers have shown that DYRK1A is a specific inhibitor of protein kinase DYRK1A, this is unexpected (30, 40, 44). A possible explanation for this is that the concentration of harmine used is insufficient for this cell type. If there is low uptake of harmine there is not enough inhibition of DYRK1A to establish a phenotype. Another potential problem is the localisation of DYRK1A, as this can be cytoplasmic and nuclear (19, 20). If the latter is the case and harmine cannot enter the nucleus, there is no DYRK1A inhibition. Therefore it needs to be investigated whether or not phosphorylation of specific substrates such as Lin52 by DYRK1A is inhibited by harmine. This can be done by Western blotting of the phosphorylated and unphosphorylated substrate after DYRK1A knockdown and inhibition, as performed by Litovchick et al. If this shows that there is no functional effect of harmine in the current concentrations in these cell types, higher concentrations need to be tested, potentially in combination with permeabilisation of the cell membrane or the nucleus. Another possibility is the use of a different chemical inhibitor of DYRK1A, such as ECGC. If harmine turns out to be functional in the used concentrations, it can be concluded that DYRK1A inhibition by harmine does not result in a phenotype in HepaRGs and ODPCs. This is contradictory to results in other cell lines and primary cell cultures previously published (19, 22, 27, 30, 40-42).

ODPCs as a model for primary HPC culture

It has been shown that siRNA mediated gene silencing is a successful means of DYRK1A inhibition, resulting in a shift in the cell cycle in human HepaRG cells. This effect was not found in mouse ODPCs.

There are several possible explanations for this difference. The first potential explanation is a species difference. Data published on DYRK1A inhibition and knockdown are on human cells (30, 40-42), but research on mouse (19, 22, 27) and chicks (27, 45) has also been performed. As an effect of DYRK1A inhibition or knockdown was found in all species, it seems unlikely that the lack of effect in ODPCs is due to a species difference. A second explanation is that the effect found on the cell cycle distribution is cell line specific. Normal cells have the capacity to divide a limited number of times before becoming senescent. A cell line has been transformed or immortalised, meaning that it can divide indefinitely. This differs from primary (stem cell) culture, where cells are isolated from tissue and cultured under the appropriate conditions. Unlike somatic cells that are limited in their division, stem cells isolated through this method maintain their potential for long-term self-renewal. Therefore the primary culture of stem cells can be maintained over longer periods of time (46). As primary stem cell culture does not involve immortalisation, this step could influence the phenotype caused by DYRK1A inhibition. However, different studies have used different types of cells, ranging from cell lines (19, 40-42) to primary cell culture (22) and embryo culture (27, 45). In these different cell types an effect of DYRK1A inhibition or knockdown was found, suggesting that immortalisation is not a factor required for an effect of DYRK1A inhibition. A third possibility for the difference in results between HepaRGs and the ODPCs is a difference in the degree of maturation of both cells types. Organoids and ODPCs are Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5) positive (29), while HepaRGs do not express this stem cell marker. Differentiation of organoids results in decreased expression of Lgr5 (29). In order to investigate this, the differentiation potential of ODPCs must be determined, and after developing a more mature stem cell model the effect of siRNA mediated gene silencing should be re-evaluated. Additionally, it is possible that ODPCs lose their stem cell characteristics, as the three dimensional structure is essential to organoid culture. This needs to be researched by measuring expression of stem cell markers, as well as markers that indicate differentiation and epithelial-mesenchymal transition. The last explanation for our lack of phenotype in ODPCs is that the knockdown is not as high as expected based on the results. On mRNA level there was knockdown on 48 hours after transfection, on protein level there was knockdown on both time points. However, Western blot data showed that the band on ODPC siDYRK on 72 hours did not span the entire width of the lane, suggesting a technical error. Therefore, ideally, this experiment has to be repeated to ensure that there is knockdown on protein level on 72 hours.

The role of DYRK1A in the cell cycle

DYRK1A has been implicated to play a role in cell cycle control in mammalian cells, suggesting it may play an important role in suppression of proliferation (41). According to Litovchick et al, DYRK1A directly phosphorylates the DREAM subunit LIN52 on S28, a step required for DREAM complex assembly. Preventing this phosphorylation by chemical inhibition or siRNA mediated gene silencing results in the loss of the cells ability to enter quiescence or senescence (41).

If DYRK1A inhibition prevents cells from entering G_0, it could enhance the possibilities for the culture of (primary) cells that now pose difficulties due to natural quiescence, for example primary hepatocytes. It is not possible to sustain primary hepatocytes in culture (47), as their quiescence means there is no proliferation. If DYRK1A prevents this, it enables the use of primary hepatocytes as a culture model for disease or drug testing.

Recommendations for future research DYRK1A

If DYRK1A knockdown only affect more mature HPCs, it is still an interesting therapeutic target. Immature HPCs are quiescent, while more mature cells are reactive and proliferating. Though it is interesting and perhaps necessary to stimulate the quiescent population of cells, it could also be of therapeutic value to enhance the proliferation rate of already activated HPCs. In order to investigate this phenomenon experiments as performed on HepaRGs should be repeated in a less matured HPCs, in

order to investigate if maturation is an important factor in the effect of DYRK1A knockdown or inhibition. This can be done using less mature HPCs, such as the THLE5b cell line.

Also, a different model that can be used for study of DYRK1A is an *in vivo* mouse model of Dyrk1A knockout or overexpression, as described by Lepagnol et al (22). Liver organoids derived from these mice do not need in vitro treatment for DYRK1A knockdown or overexpression, so they could be used as a positive or negative control. The effect DYRK1A overexpression or knockdown has in these mice can be measured by harvesting cells for organoid culture, and perform flow cytometry and FlowJo analysis on these cells. Also, rescue of overexpression could be attempted by chemical inhibition of siRNA mediated gene silencing of DYRK1A. In this experiment the effect of rescue on cell number and cell cycle distribution needs to be assessed as performed in the HepaRGs and ODPCs.

Concluding remarks

In this year of research a lot of questions have been answered. However, every answer raises a new question that requires follow up experiments or literature research to answer. Research as described in this discussion is needed to elucidate the effect DYRK1A (inhibition) has on the cell cycle of HPCs, *in vitro, ex vivo* and *in vivo.*

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Attachments

Attachment 1 – List of used abbreviations

ATP	Adenosine triphosphate
DAPI	4',6-diamidino-2-phenylindole
DBC	Department of Biochemistry and Cell Biology
DCSCA	Department of Clinical Sciences of Companion Animals
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	Department of Pathobiology
DYRK1A	Human Dual-Specificity Yak Related Kinase 1A
Dyrk1A	Mouse Dual-Specificity Yak Related Kinase 1A
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FGF10	Fibroblast growth factor 10
HBBS	Hank's buffered saline solution
HGF	Hepatocyte growth factor
HPC	Hepatic progenitor cell
HTS	High throughput screen
iPS cell	induced Pluripotent Stem cell
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
NGS	Normal goat serum
NT	Non target
ODPC	Organoid derived progenitor cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
pH3	Phospho-histone-H3
PI	Propidium iodide
qPCR	quantitative Polymerase Chain Reaction
RM	Regenerative medicine
Rspo1	Rspondin 1
RT	Room temperature
TR	Tissue Repair

Attachment 2 – Courses attended during the Honours Programme

Statistics course

Date: January and February 2013
By: Hans Vernooij and Jan van den Broek
ECTS: 0
Summary: In this statistics course we have learned different methods of analysing results and in which cases these methods need to be used. Furthermore, we learned how to program R to perform the different methods of analysing. On the last day of the course we had to present our own statistical analysis of our data. Because I did not have enough data at the time, I gave a presentation of how I would like to analyse my future data.

The following methods were discussed

- General
  - T-Test
  - Chi-square test
  - Correlation
  - Regression
- Linear models (continuous data)
  - ANOVA
  - Regression analysis
  - ANCOVA
- Logistic regression (binary data)
  - Linear regression
  - Odds ratio
  - Likelihood
- Longitudinal/dependent data
  - Model with fixed and random effects
- Survival analysis
  - Kaplan Meier
  - Cox proportional hazard

Writing for an academic publication in the veterinary and life sciences

Date: February, March and April 2013
By: Linda McPhee
ECTS: 3
Summary: In this writing course I have learned about nearly everything that is required to write a good academic article, as well as how to analyse articles written by others. Besides an elaborate explanation on the proper structure of an article, we have also learned background information on copyright, the correct use of illustrations and how the reader reads your work. The latter is important, as I have come to learn that this is very different from the way you read your own work as a writer. Not only did we learn what a reader thinks of what you have written, we also learned how to adjust our text, so that everyone reads what you meant to write. I am currently working on an article, which is to be submitted for publication.

Attachment 3 – Techniques mastered during the Honours Programme

RNA

- RNA isolation
- cDNA synthesis
- Primer design
    - Optimization for qPCR
- Quantitative Polymerase Chain Reaction (qPCR)

Protein

- Protein isolation
- DC Protein assay
- Western blot

Cell culture

- Cell culture
    - HepaRG cell line
    - Three dimensional mouse organoids
    - Mouse Organoid Derived Progenitor Cells (ODPCs)
- Cell count
    - Bürker-Türk counting chamber
    - Biorad trypane blue assay
    - CyQuant analysis
- Lipofectamin mediated siRNA transfection

Immunohistochemistry and immunocytochemistry: immunofluorescence (IF)

- Paraffin tissue samples
    - Ki67-CK19-double stain
- HepaRG cell line
    - DYRK1A-DAPI-double stain optimization
- Arrayscan
    - EdU incorporation assay
    - EdU-pH3-DAPI triple stain
- Flow cytometry
    - Propidium-Iodide stain

Attachment 4 – Additional information chapter 2

CyQuant

Figure 20. Number of cells per well for 2% FCS HepaRG on 24 (A), 48 (B), 72 (C) and 96 (D) hours

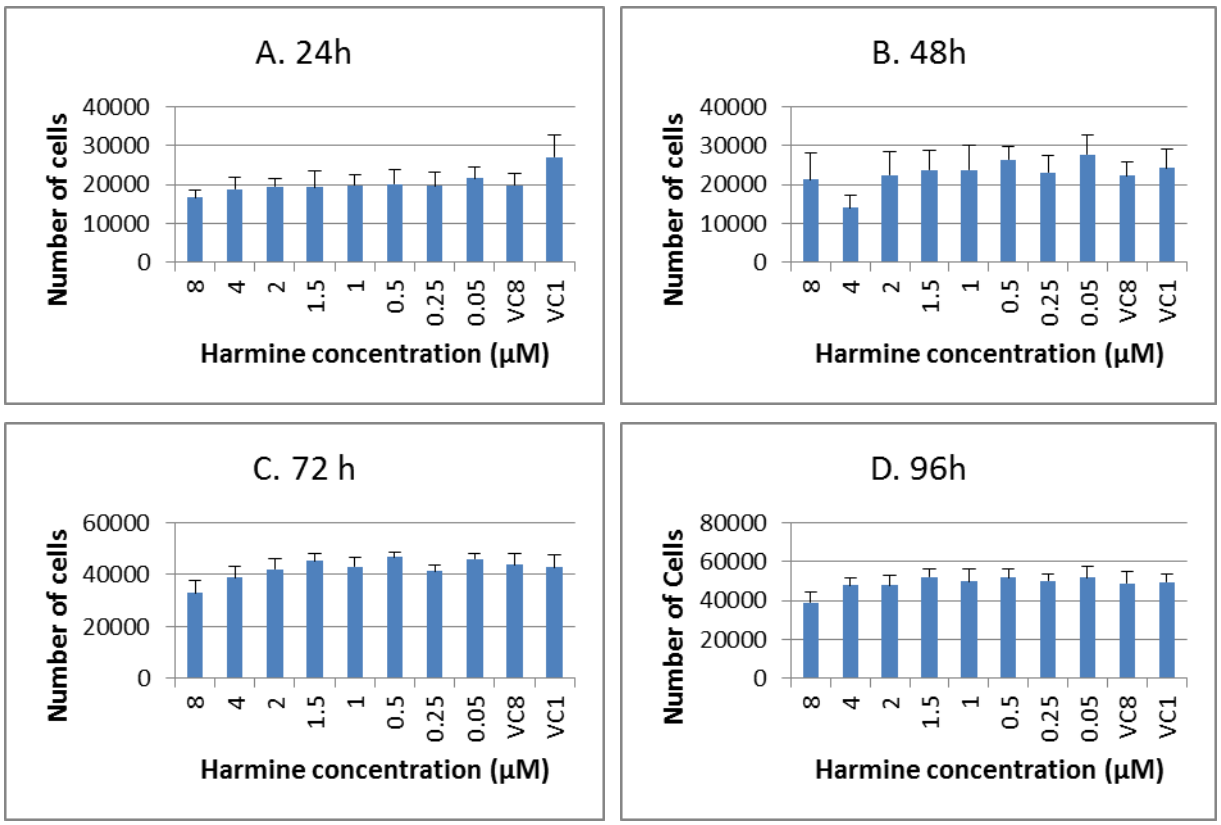
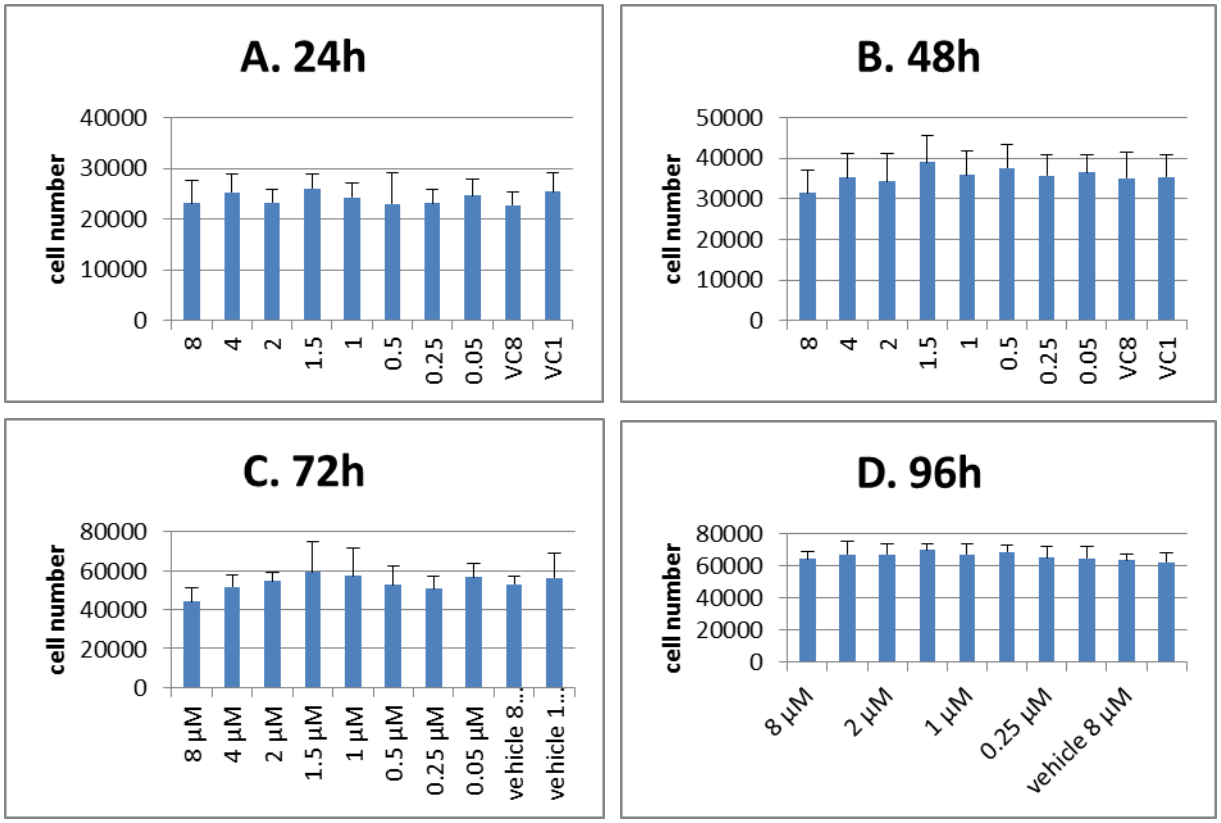


Figure 21. Number of cells per well for 10% FCS HepaRG on 24 (A), 48 (B), 72 (C) and 96 (D) hours



EdU incorporation

*Figure 22. % of EdU positive cells of Non Target vs. siDYRK1A treated wells for 2% and 10% FCS HepaRGs on 48 and 72 hours. * = statistical significance of P<0.05*

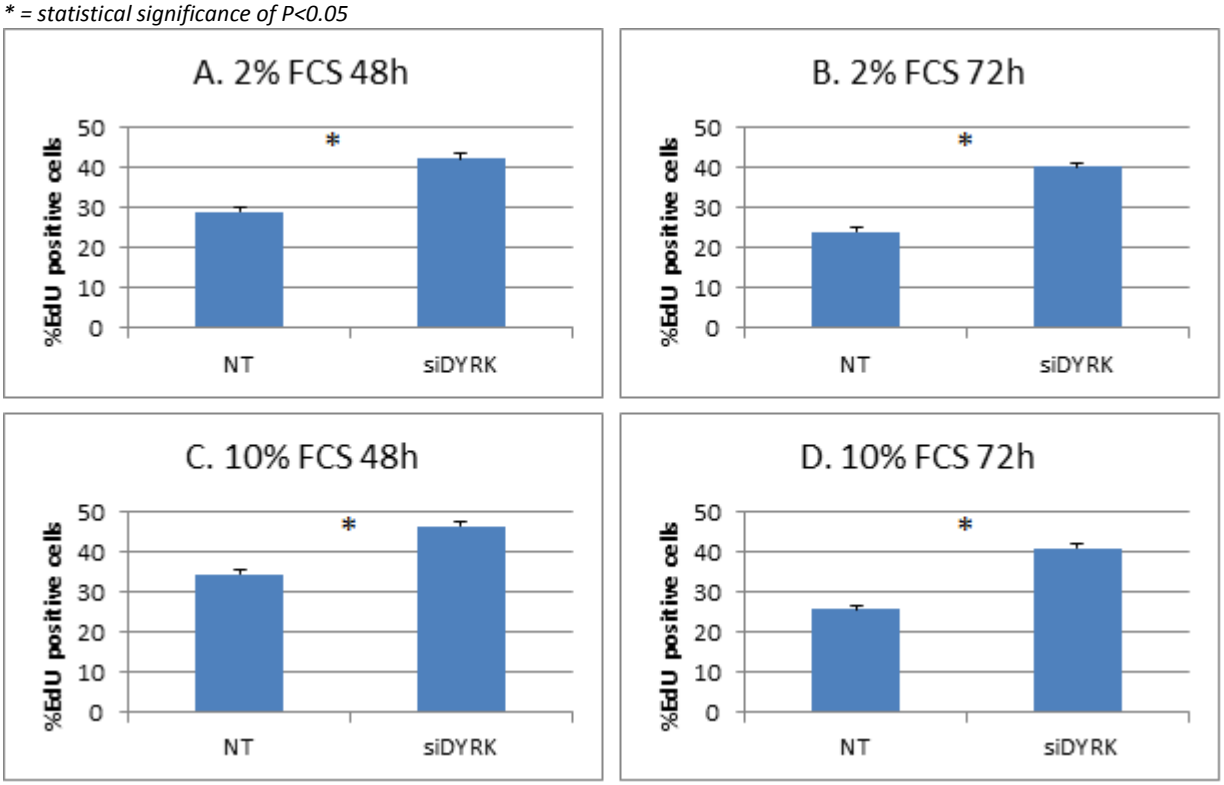
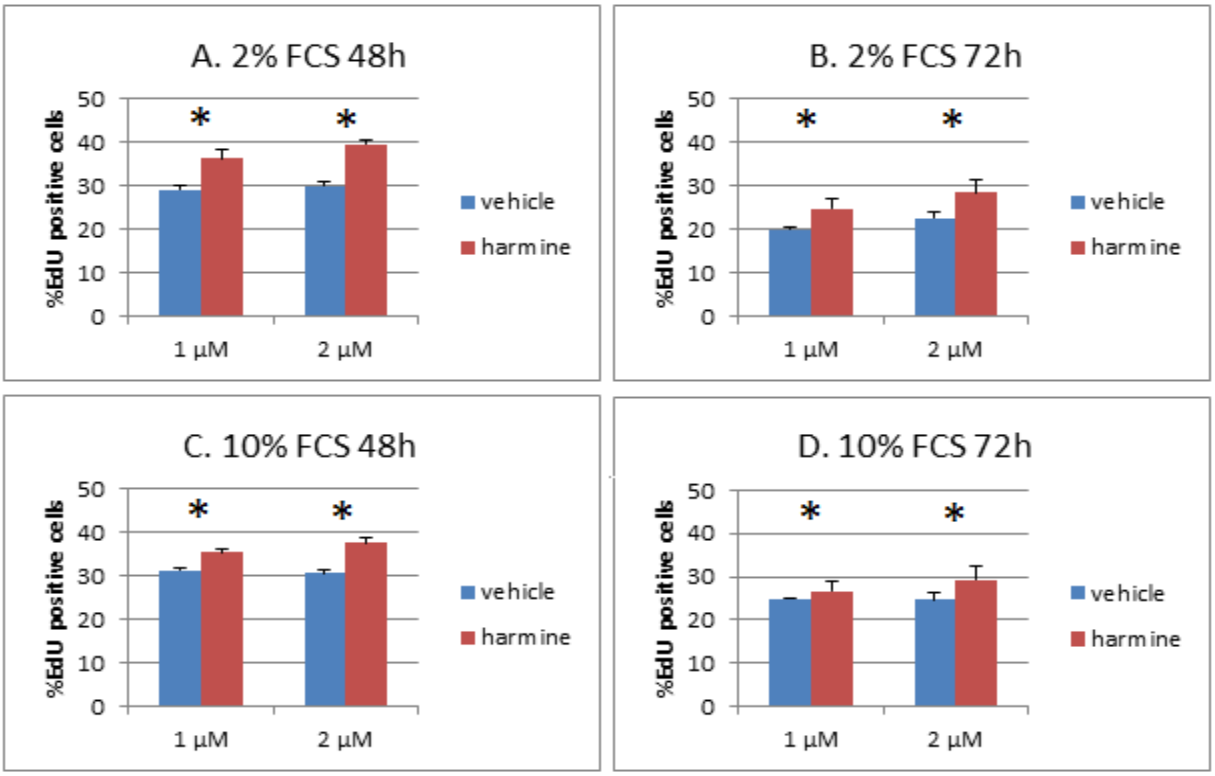


*Figure 23. % of EdU positive cells of vehicle versus harmine treated wells for 2% and 10% FCS HepaRGs on 48 and 72 hours. * = statistical significance of P<0.05*



pH3 phosphorylation

Figure 24. % of pH3 positive cells of Non Target vs. siDYRK1A treated wells for 2% and 10% FCS HepaRGs on 48 and 72 hours
** = statistical significance of P<0.05*

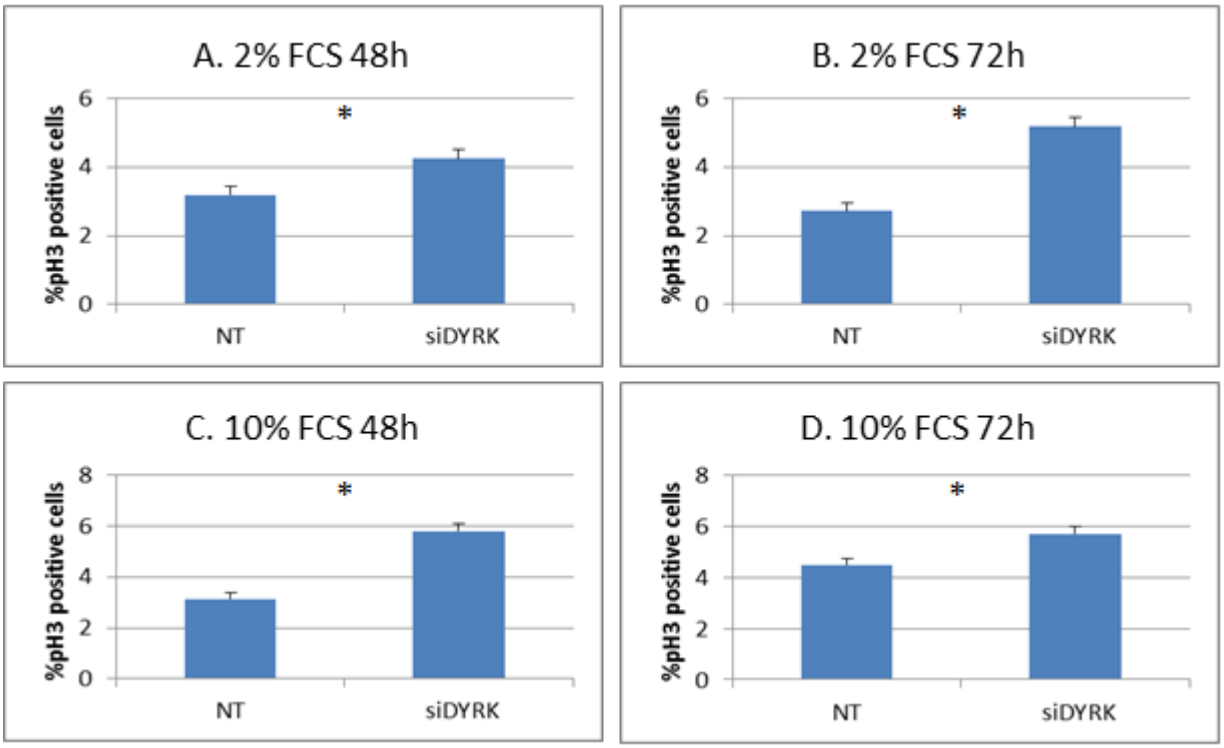
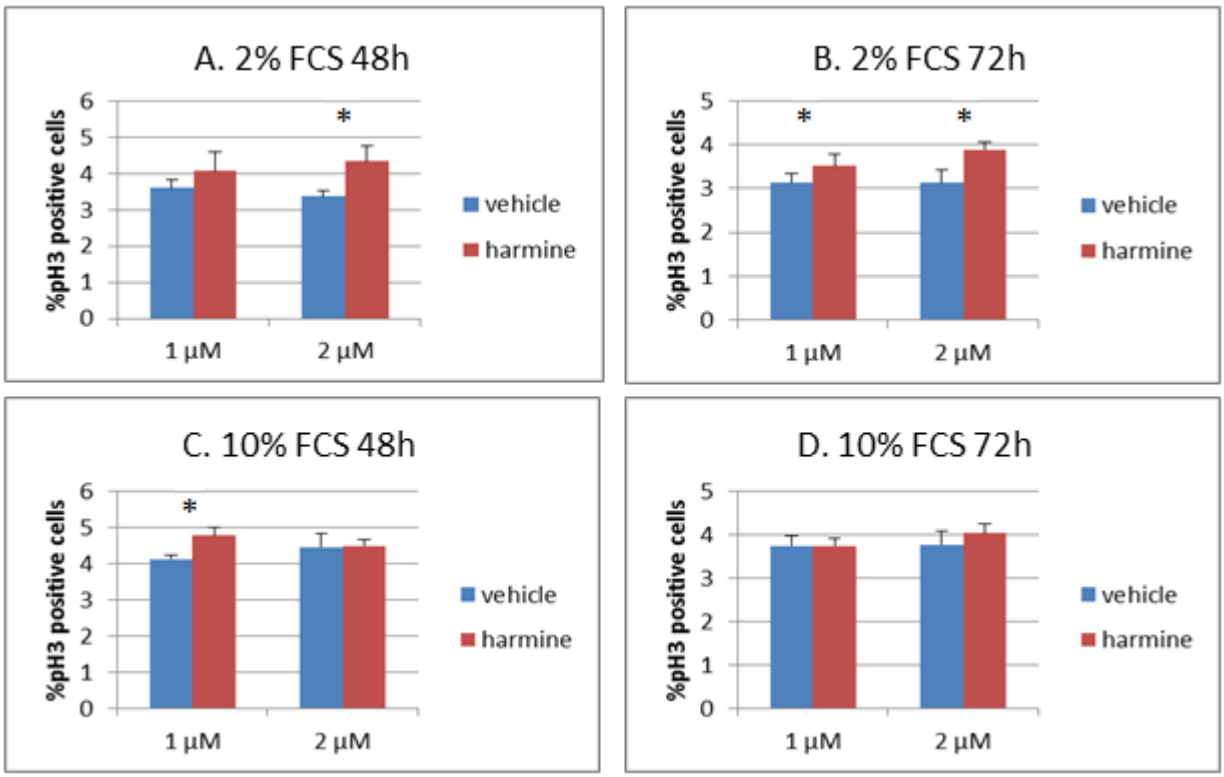


Figure 25. % of pH3 positive cells of vehicle vs. harmine treated wells for 2% and 10% FCS HepaRGs on 48 and 72 hours
** = statistical significance of P<0.05*



Valid object count

Figure 26. Valid object count of Non Target vs. siDYRK1A treated wells for 2% and 10% FCS HepaRGs on 48 and 72 hours
** = statistical significance of P<0.05*

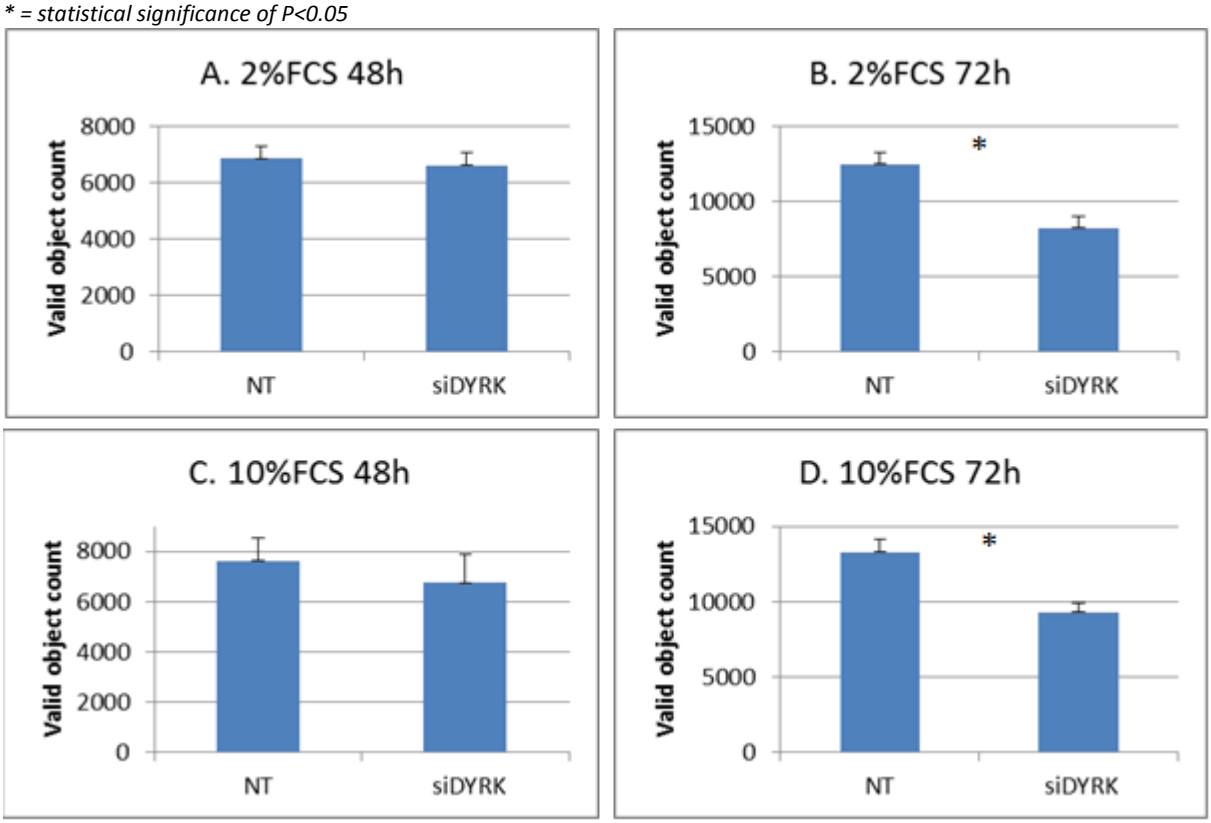
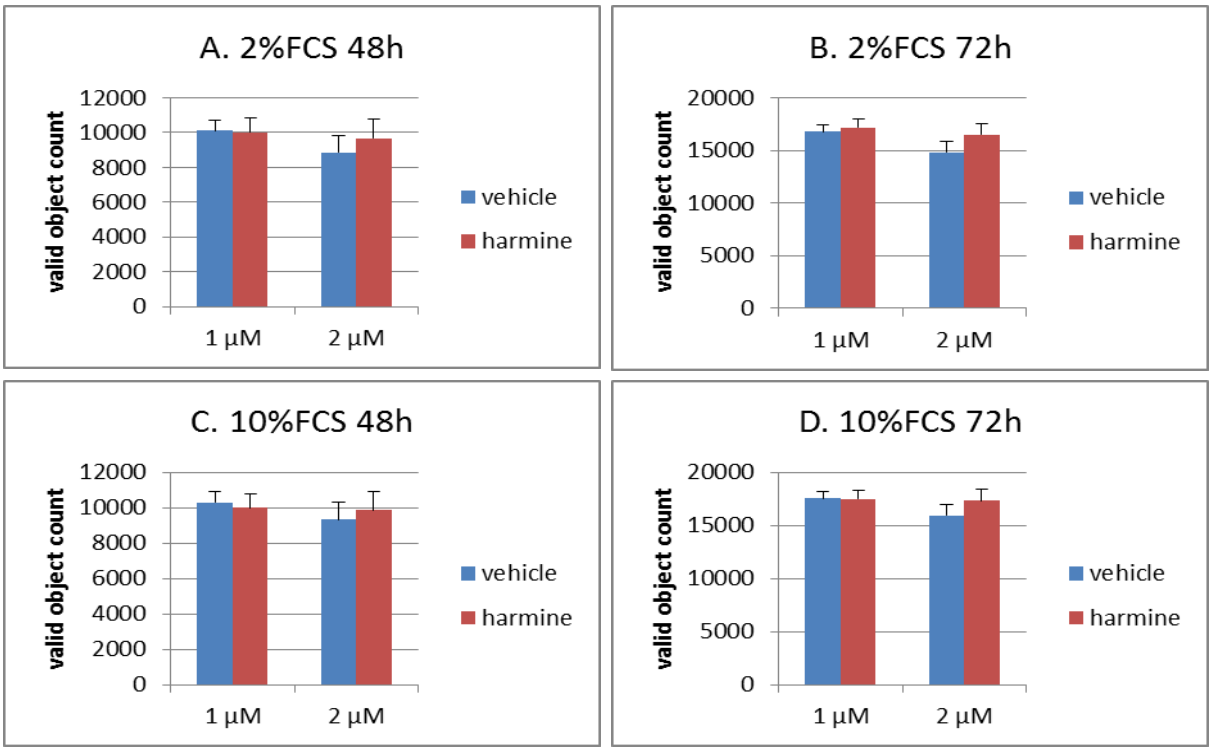


Figure 27. Valid object count of vehicle vs. harmine treated wells for 2% and 10% FCS HepaRGs on 48 and 72 hours
** = statistical significance of P<0.05*



Cell cycle analysis

Figure 28. Histogram of DNA content and cell cycle distribution of siRNA mediated gene silencing in 2% FCS cultured HepaRGs of A. NT on 48 hours, B. siDYRK on 48 hours, C. NT on 72 hours and D. siDYRK on 72 hours after transfection.

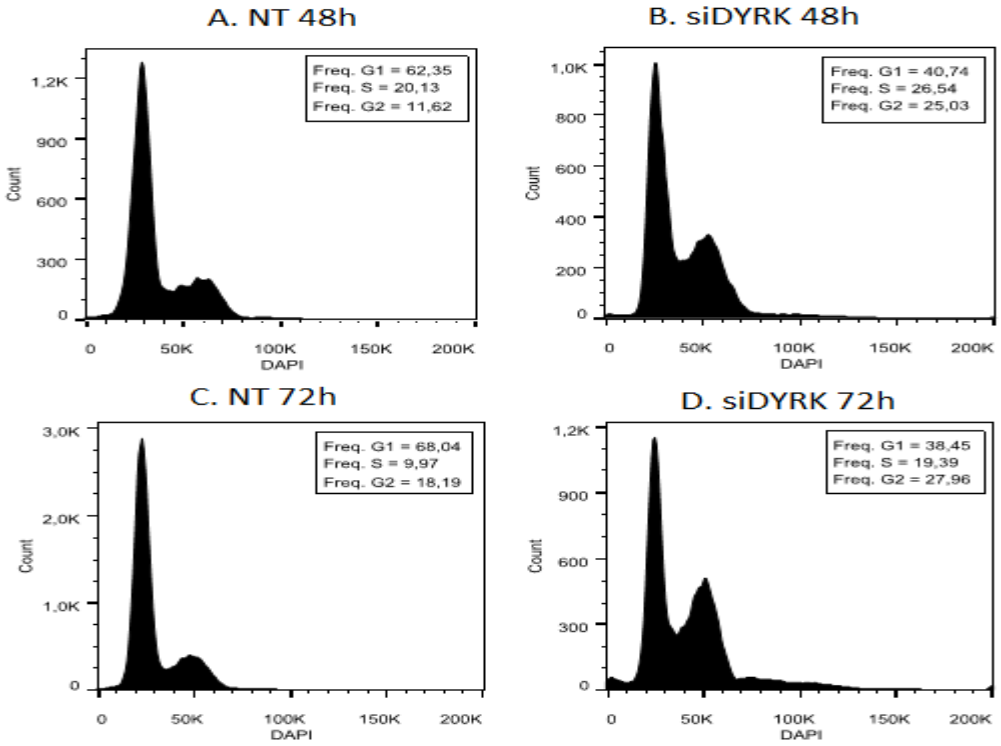


Figure 29. Histogram of DNA content and cell cycle distribution of siRNA mediated gene silencing in 10% FCS cultured HepaRGs of A. NT on 48 hours, B. siDYRK on 48 hours, C. NT on 72 hours and D. siDYRK on 72 hours after transfection.

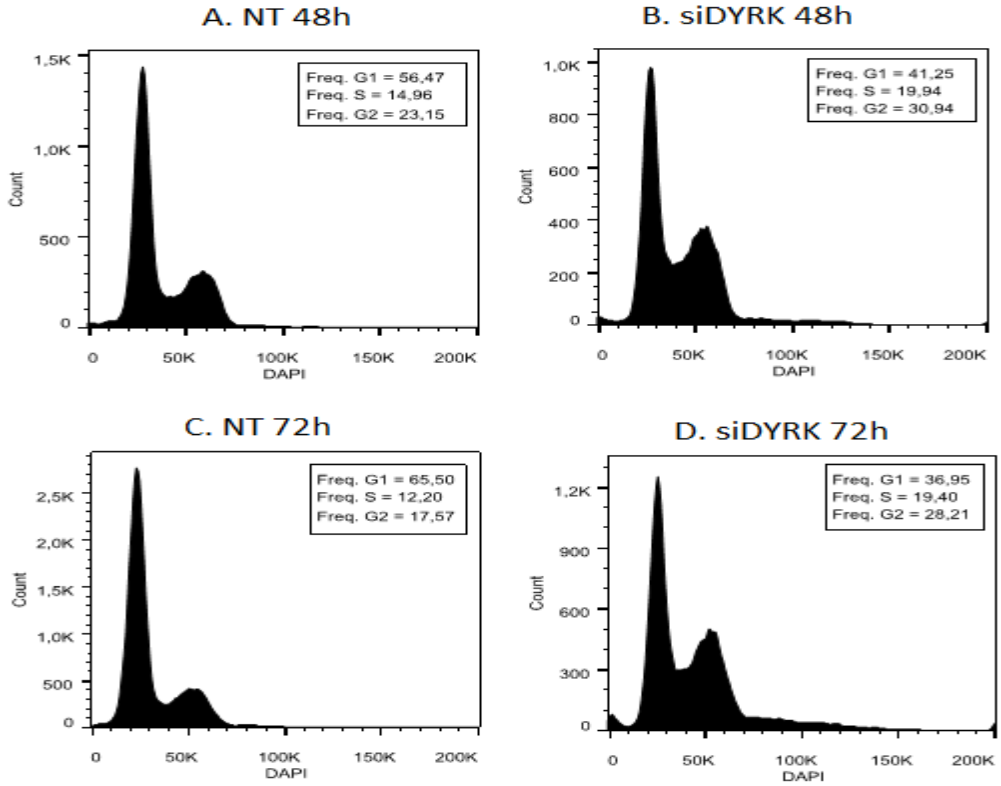


Figure 30. Histogram of DNA content and cell cycle distribution of chemical inhibition in 2% FCS cultured HepaRGs on 48 hours of A. Vehicle control 1 µM, B. Harmine 1 µM, C. Vehicle control 2 µM and D. Harmine2 µM

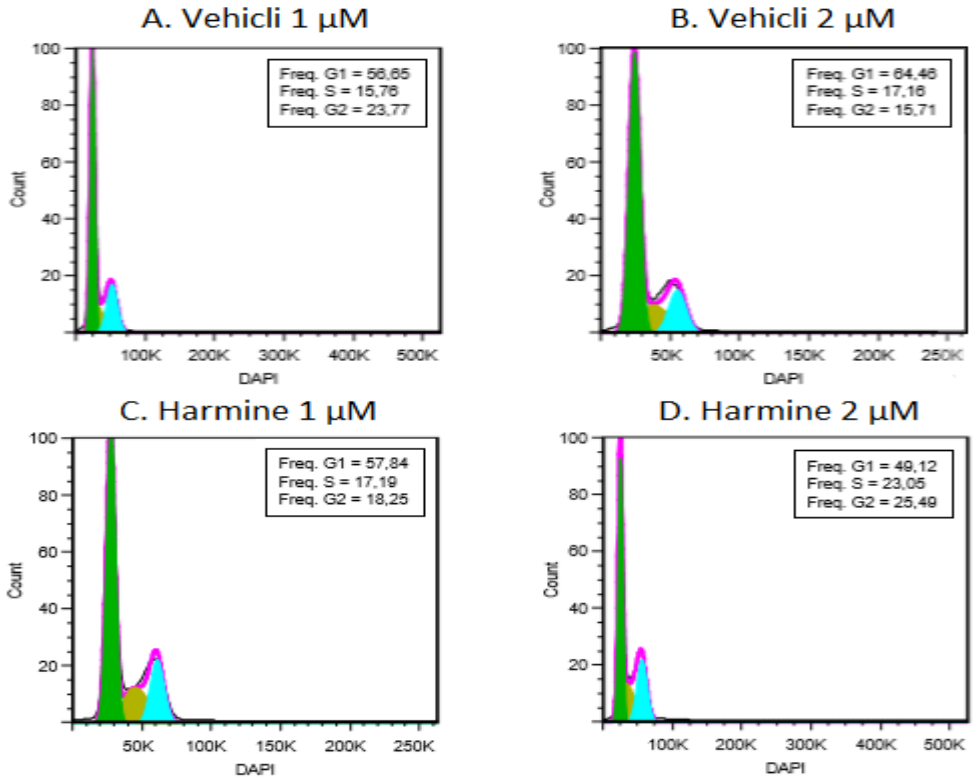


Figure 31. Histogram of DNA content and cell cycle distribution of chemical inhibition in 2% FCS cultured HepaRGs on 72 hours of A. Vehicle control 1 µM, B. Harmine 1 µM, C. Vehicle control 2 µM and D. Harmine2 µM

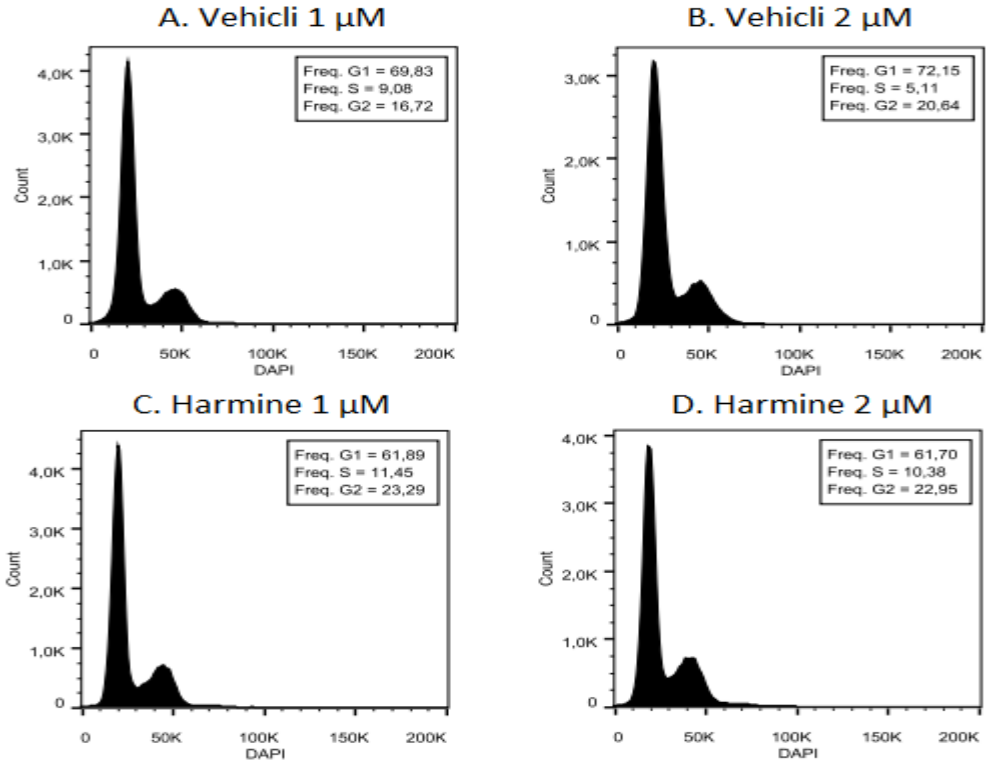


Figure 32. Histogram of DNA content and cell cycle distribution of chemical inhibition in 10% FCS cultured HepaRGs on 48 hours of A. Vehicle control 1 µM, B. Harmine 1 µM, C. Vehicle control 2 µM and D. Harmine2 µM

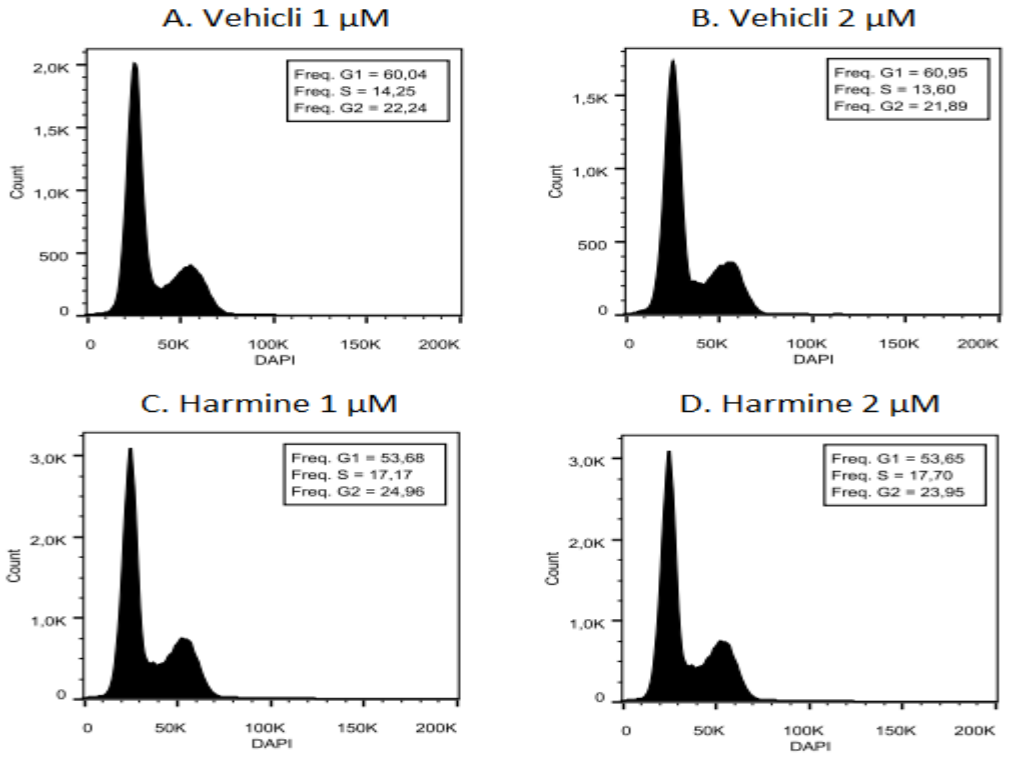
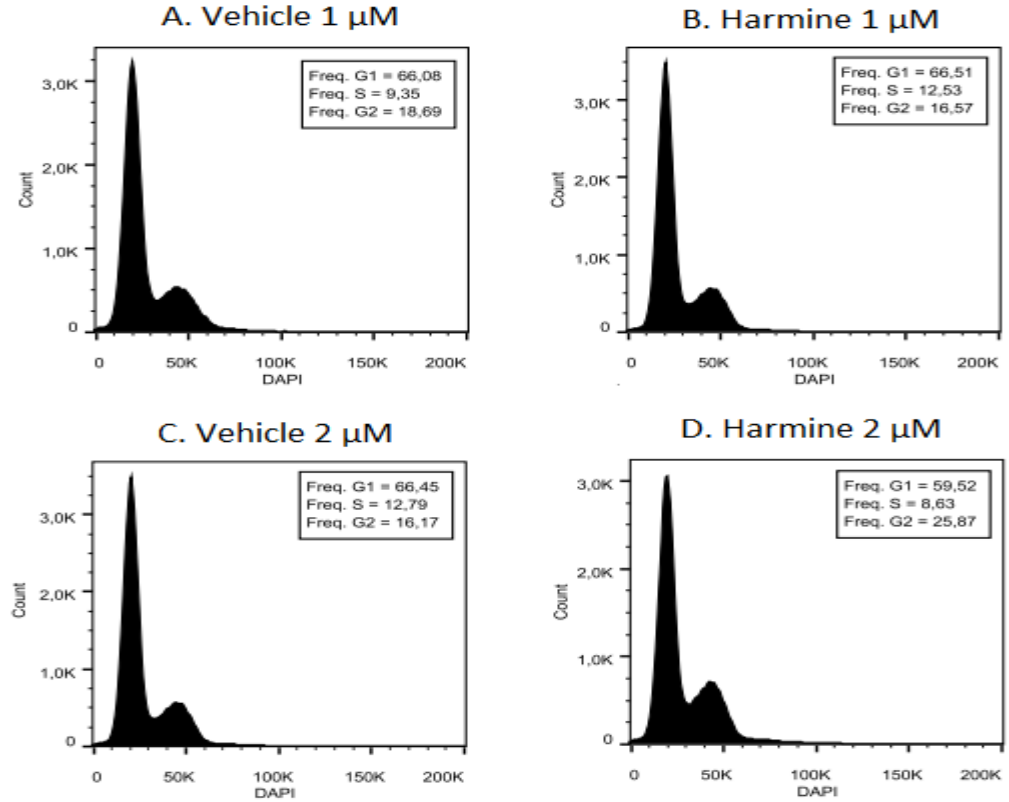


Figure 33. Histogram of DNA content and cell cycle distribution of chemical inhibition in 10% FCS cultured HepaRGs on 72 hours of A. Vehicle control 1 µM, B. Harmine 1 µM, C. Vehicle control 2 µM and D. Harmine2 µM



Attachment 5 – Additional information chapter 3

Table 1. qPCR primer information

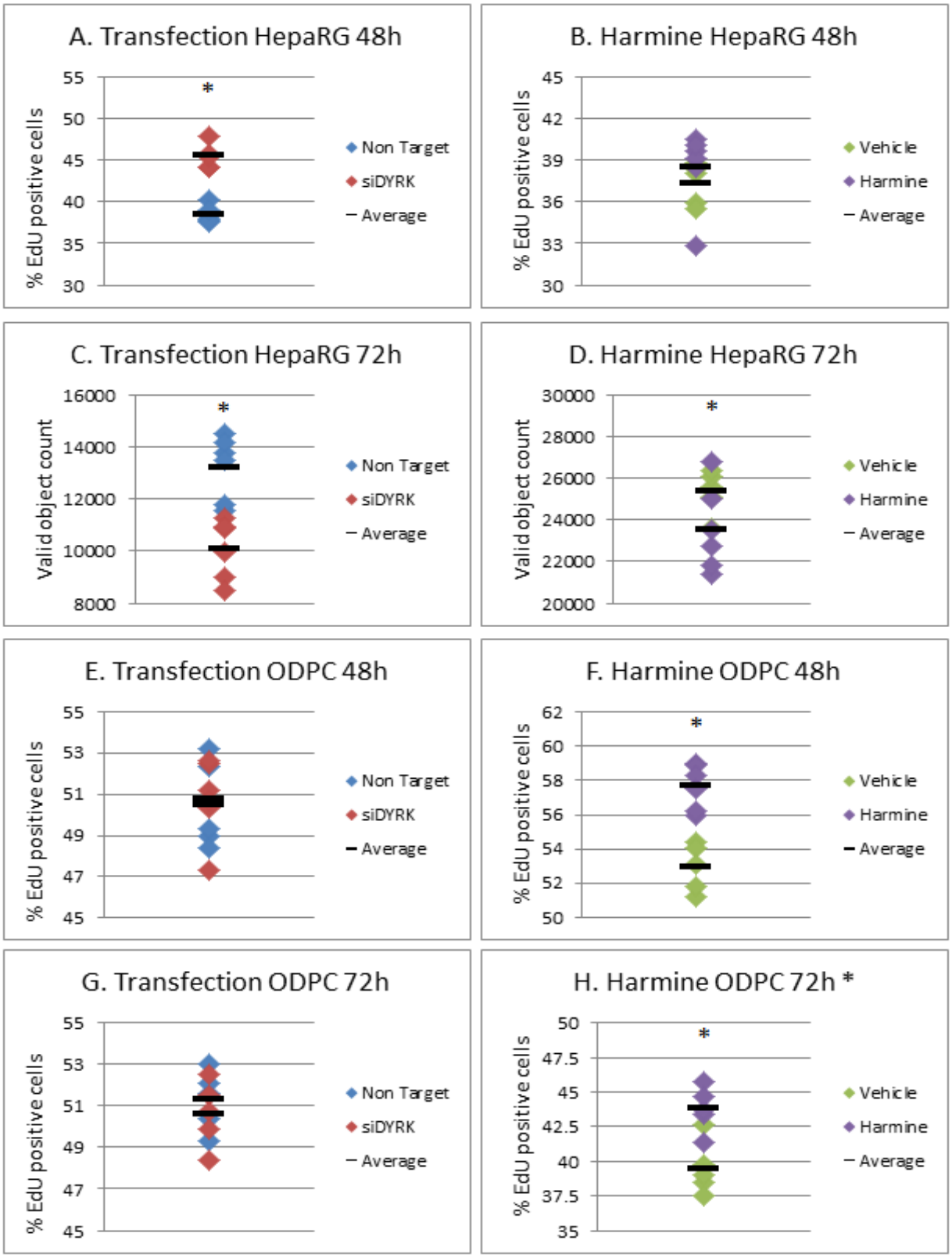
Primer		**Sequence**	**Temperature**
DYRK1A Human	Forward	TTGACTCCTTGATAGGCAAAGGT	60°C
	Reverse	CATTCTTGCTCCACACGATCAT	
HPRT Human	Forward	ATAAGCCAGACTTTGTTGGA	60°C
	Reverse	CTCAACTTGAACTCTCATCTTAGG	
RPL19 Human	Forward	ATGAGTATGCTCAGGCTTCAG	64°C
	Reverse	GATCAGCCCATCTTTGATGAG	
Dyrk1A Mouse	Forward	GTGTCTGCCTTACCATATTCTG	61°C
	Reverse	TGCTGGATCACGGAAGG	
E2F7 Mouse	Forward	CTCCTGTGCCAGAAGTTTC	64°C
	Reverse	CATAGATGCGTCTCCTTTCC	
B-Actin Mouse	Forward	AGCTCCTTCGTTGCCGGTCCA	57°C
	Reverse	TTTGCACATGCCGGAGCCGTTG	
GAPDH Mouse	Forward	GAAGGTCGGTGTGAACGG	61°C
	Reverse	TGAAGGGGTCGTTGATGG	
RPS18 Mouse	Forward	GATCCCTGAGAAGTTCCAGCAC	57°C
	Reverse	ACCACATGAGCATATCTCCGC	

Table 2. Protein concentrations for Western blot

Cell type	Sample	Concentration
HepaRG 48hours	Non Target	0.547 mg/mL
	siDYRK	
HepaRG 72 hours	Non Target	0.702 mg/mL
	siDYRK	
ODPC 48h	Non Target	0.409 mg/mL
	siDYRK	
ODPC 72 hours	Non Target	0.567 mg/mL
	siDYRK	

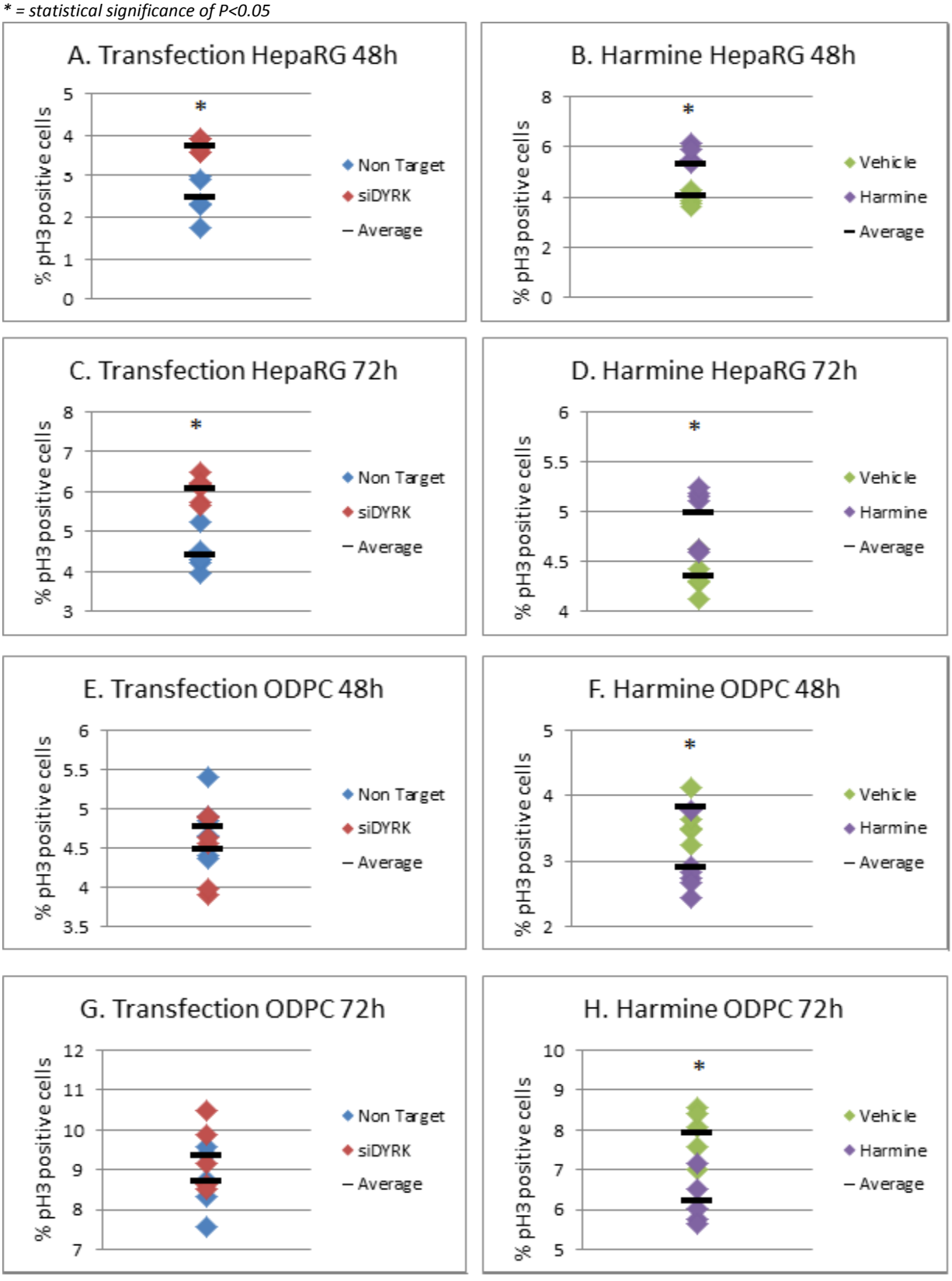
EdU incorporation

Figure 34. % of EdU positive cells of NT versus siDYRK and vehicle versus harmine for HepaRGs and ODPCs on 48 and 72 hours
** = statistical significance of P<0.05*



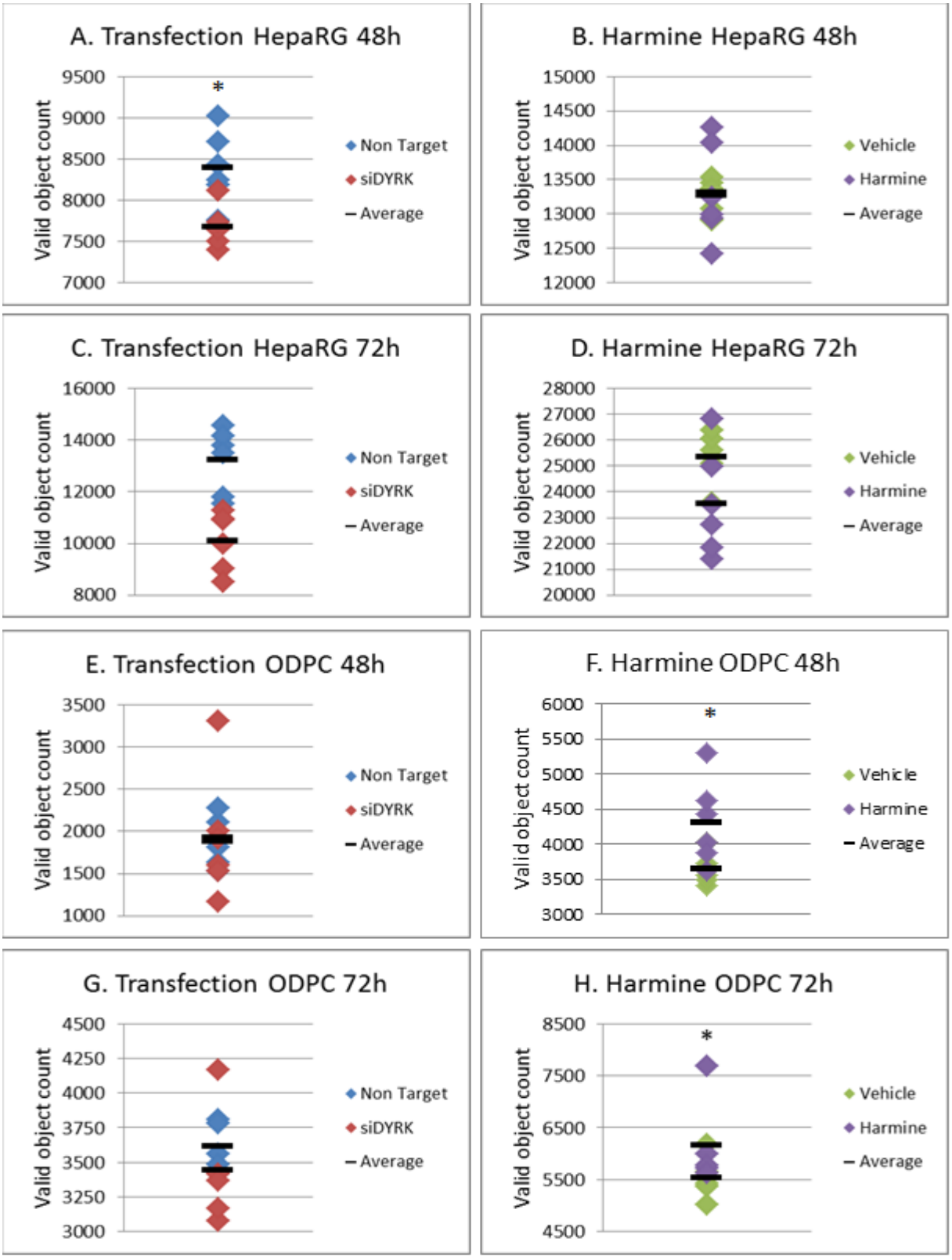
pH3 phosphorylation

Figure 35. % of pH3 positive cells of NT versus siDYRK and vehicle versus harmine for HepaRGs and ODPCs on 48 and 72 hours
** = statistical significance of P<0.05*



Valid object count

Figure 36. Cell number of NT versus siDYRK and vehicle versus harmine for HepaRGs and ODPCs on 48 and 72 hours
** = statistical significance of*
P<0.05



Cell cycle analysis

Figure 37. Histogram of DNA content and cell cycle distribution of HepaRG cells on 48 hours of A. Non Target, B. siDYRK, C. vehicle control and D. harmine. Green lines represent curve fits using the Dean Jett Fox model

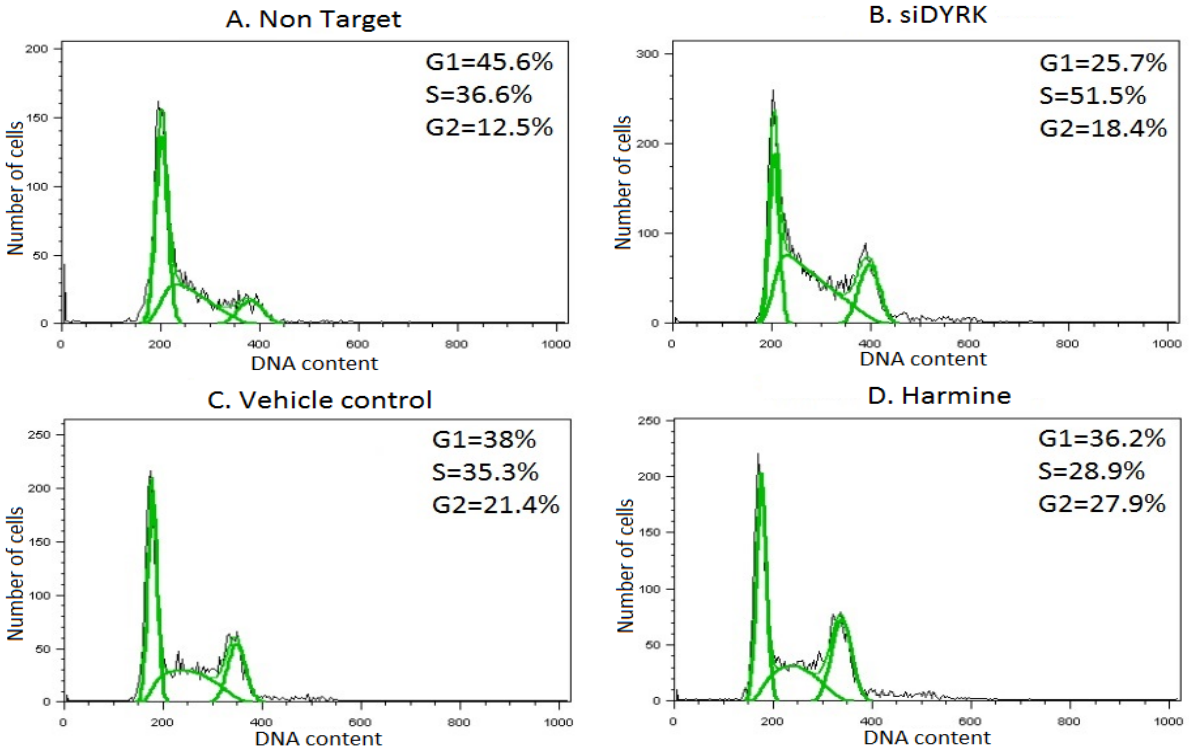


Figure 38. Histogram of DNA content and cell cycle distribution of HepaRG cells on 72 hours of A. Non Target, B. siDYRK, C. vehicle control and D. harmine. Green lines represent curve fits using the Dean Jett Fox model

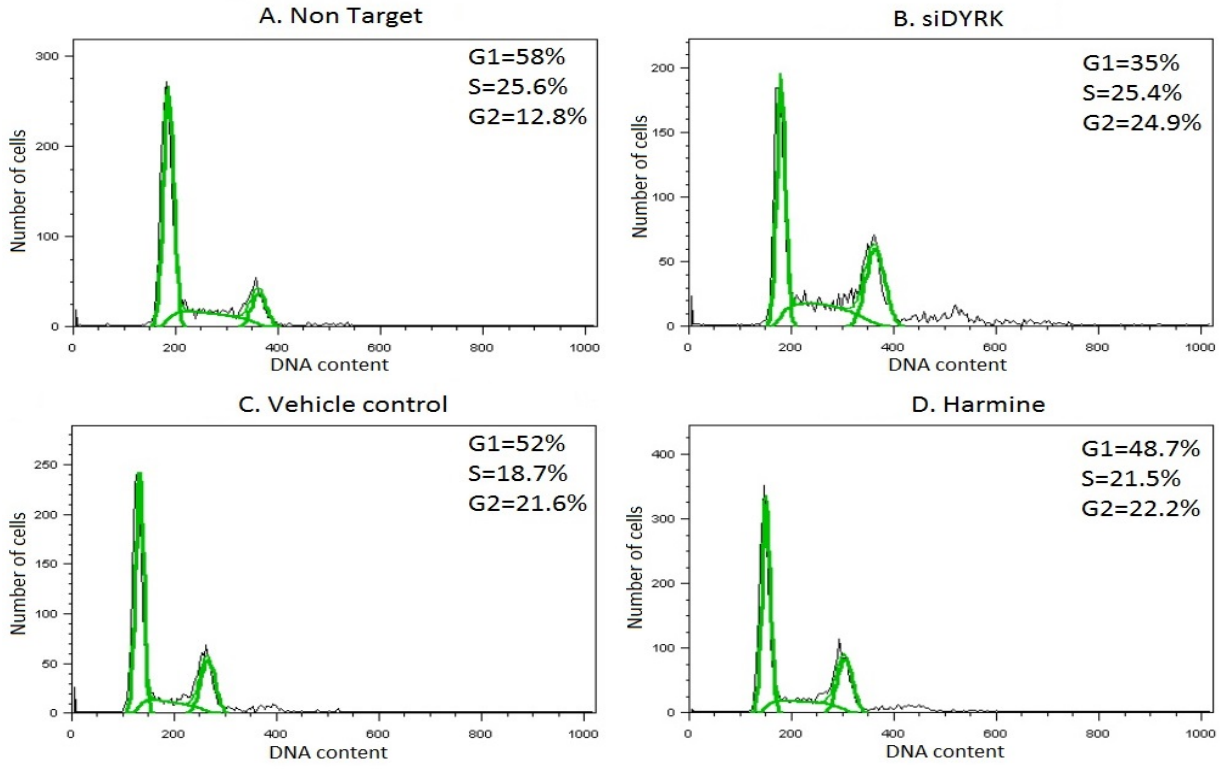


Figure 39. Histogram of DNA content and cell cycle distribution of ODPC cells on 48 hours of A. Non Target, B. siDYRK, C. vehicle control and D. harmine. Green lines represent curve fits using the Dean Jett Fox model

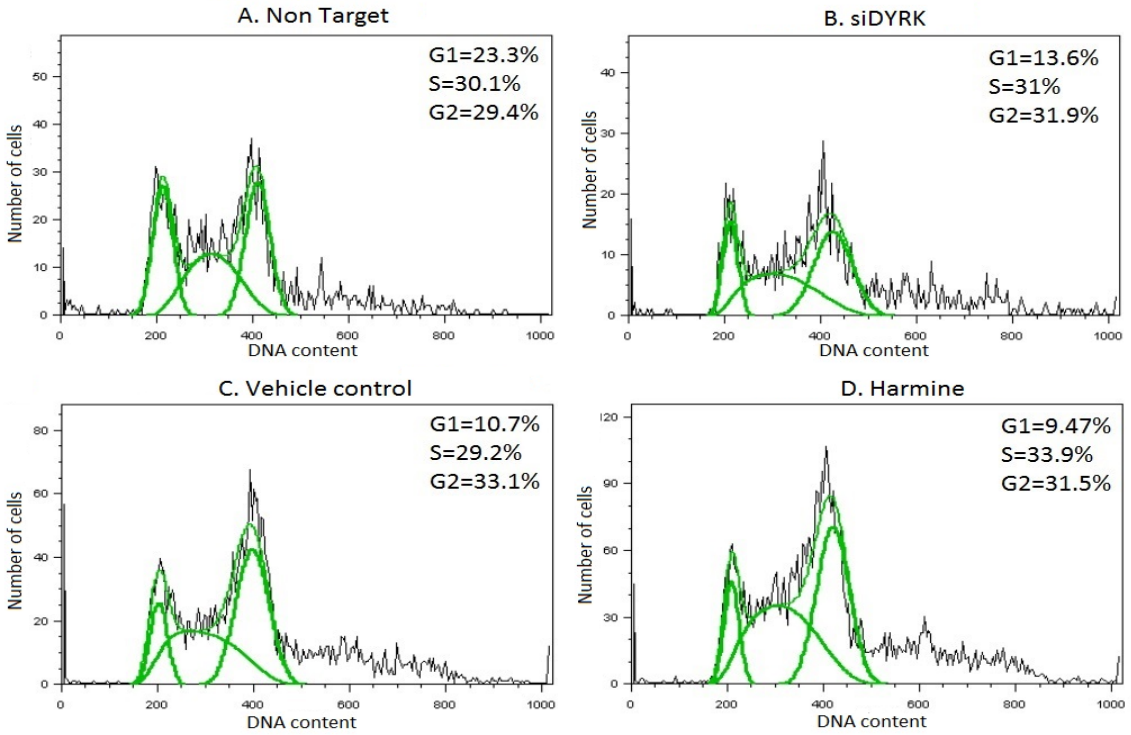


Figure 40. Histogram of DNA content and cell cycle distribution of ODPC cells on 72 hours of A. Non Target, B. siDYRK, C. vehicle control and D. harmine. Green lines represent curve fits using the Dean Jett Fox model