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Role of microRNAs in Regulating Vitamin D Signalling in Colorectal Cancer

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1 Introduction

1.1 Vitamin D and colorectal cancer

1.1.1 Introduction

There are two major forms of vitamin D: (1) vitamin D_2 (Ergocalciferol), which is produced in plants and (2) vitamin D_3 (Cholecalciferol), which can be generated in the skin or ingested (*Figure 1*). The production of vitamin D_3 in the skin was unknown till 1980 (Holick et al. 1980), therefore it was classified as a vitamin in the first half of the 19th century because it was believed that ingestion of vitamin D through diet is its only source.

In this chapter, we focus on the steroid hormone vitamin D_3 (1,25-dihydroxyvitamin D_3 or 1,25-D_3; the most active metabolite of vitamin D_3), more precisely, its discovery, biosynthesis, influence on gene expression, different functions and actions in the cellular environment and role in colorectal cancer.

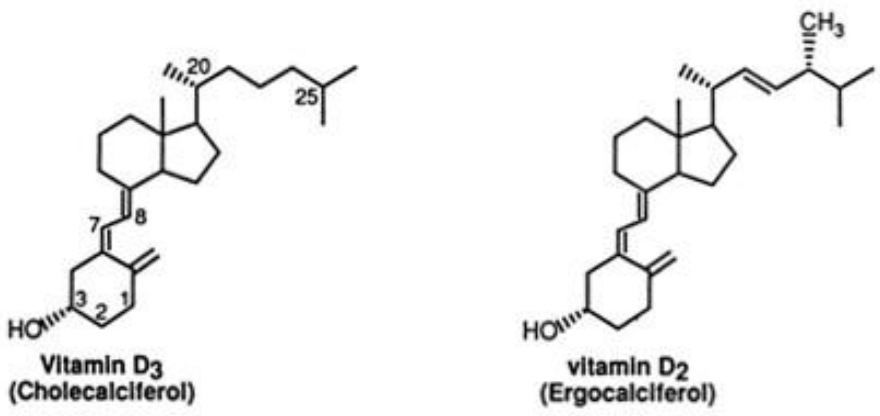

Figure 1: Forms of Vitamin D

Chemical structures of the nutritional forms of vitamin D: Vitamin D3 (Cholecalciferol) and Vitamin D2 (Ergocalciferol).

(Figure is from (Jones et al. 1998))

1.1.2 The history of vitamin D – A story of rickets, cod-liver oil and sunlight

In 1650, the anatomist and physiologist Francis Glisson described the childhood bone disease called rickets for the first time in a scientific way (Dunn 1998) and therefore, this essay represents the first characterization of a disease caused by vitamin D-deficiency. The etiology of rickets was not understood for a long time because at that point, the existence and importance of vitamins had not been discovered yet.

It took until 1906 that Frederick Growland Hopkins, who worked on animal feeding experiments, proposed the existence of essential substances for growth and survival in the animal's diet which he called 'accessory food factors' (Hopkins 1906). This was the discovery of a new group of substances and these factors were later called vitamins. In 1929, Hopkins (together with Christiaan Eijkman) was awarded the Nobel Prize in Physiology or Medicine for this work.

Eight years after the suggestion of the presence of the 'accessory food factors' in the diet, in 1914, Elmer Verner McCollum and Marguerite Davis reported that laboratory rats were not able to grow when their diets had only lard or olive oil as source of fat (McCollum and Davis 1914). Interestingly, these rats started to grow when soluble extracts of butter or eggs were added to the diet. McCollum and Davis were able to isolate the responsible, fat-soluble substance from butterfat and named it 'fat-soluble factor A' (McCollum and Davis 1914).

In 1919, Sir Edward Mellanby fed dogs, which were kept inside without exposure to sunlight, with a diet with low-fat milk and bread (Rajakumar 2003). As a result of this diet, the dogs were diagnosed with rickets. By adding cod-liver oil to the diet, the outbreak of the disease could be prevented. He wrote in his work (Mellanby 1919):

> *"Rickets is a deficiency disease which develops in consequence of the absence of some accessory food factor or factors. It therefore seems probable that the cause of rickets is a diminished intake of an anti-rachitic factor, which is either [McCollum's] fat-soluble factor A, or has a similar distribution to it."*

In 1922, McCollum and colleagues found out that 'fat-soluble factor A' consisted of 2 entities: one of them was later called vitamin A. He called the other entity vitamin

D because by that time, vitamin B and C were already discovered and named in a chronological way (McCollum et al. 1922).

In the meantime, various research groups demonstrated that (1) radiation (from the sun or artificial sources) of laboratory animals and (2) irradiation of their diets could cure rickets (Smith and Hume 1923). Although the reason for the cure was unknown, milk and bread were irradiated with ultraviolet light and this simple process caused a rapid reduction in the prevalence of rickets in children.

In 1925, Alfred F. Hess, Mildred Weinstock and F. Dorothy Helman isolated cholesterol from rat brain and hypothesized that it could be activated by exposure to ultraviolet light (Hess et al. 1925). He wrote:

> *" [...] it would seem quite possible that the cholesterol [nowadays it is known that this is 7-dehydrocholesterol] in the skin is normally activated by UV-irradiation and rendered anti-rachitic—that the solar rays and artificial radiations can bring about this conversion. This point of view regards the superficial skin as an organ, which reacts to particular light waves rather than as a mere protective covering."*

One year later, in 1926, Hess asked Adolf Windaus for a collaboration to identify the chemical structure of this anti-rachitic substance, thought to be cholesterol. Together with Otto Rosenheim, they were able to demonstrate that the precursor of vitamin D is not cholesterol itself, but a substance which is associated with it (Moon and Reich 1975). After testing 30 different steroid preparations for anti-rachitic activity upon irradiation, Hess and Windaus found out that irradiated ergosterol could cure rickets in rats (Windaus and Hess 1926). At the same time, Rosenheim together with Thomas Arthur Webster also suggested that ergosterol was the previtamin D and could be converted to vitamin D by UV irradiation (Rosenheim and Webster 1927).

In the next years, three research groups worked on the irradiation product of ergosterol: Webster and his team (Askew et al. 1931), in the Netherlands Engbert Harmen Reerink and Aart Van Wijk (Wijk and Niekerk 1931) and the group of Windaus (Windaus 1931). They named the product vitamin D-2 or ergocalciferol. Ergocalciferol had very strong anti-rachitic effect in rats. However, the question remained: how animals or humans can obtain their vitamin D by sunlight although ergosterol does not occur in the organisms.

The answer to the question was not solved until the middle 1930s when Windaus and colleagues isolated the missing link, 7-dehydrocholesterol, from swine skin and also found it in the skin of rats and humans (Holick 2010). They demonstrated that this substance is convertible by irradiation and then forms an anti-rachitic molecule which they called vitamin D_3 or cholecalciferol. It was also Windaus and colleagues who identified its structure.

Leon Velluz and Gaston Amiard clarified the complete photochemical and thermal reactions that converted ergosterol to calciferol in 1955. 25 years later, Holick and colleagues were able to elucidate the chronology of chemical reactions leading to the photoproduction of cholecalciferol in the human skin (Holick et al. 1980).

1.1.3 Vitamin D pathway

Upon exposure to ultraviolet B radiation (wavelength 290-315nm), pre-vitamin D_3 (Pre-D_3) is generated from 7-dehydrocholesterol in a photochemical reaction in the skin (Holick et al. 1980). In a time- and temperature-dependent way, pre-vitamin D_3 is isomerized to vitamin D_3 (cholecalciferol, D_3) (Holick and Tian 1995). Apart from the synthesis in the skin, there are also foods that are sources of vitamin D3, like fatty fish (the best source), cheese, liver, egg yolks, mushrooms and milk (contain smaller amounts). Dietary vitamin D_3 is absorbed in the small intestine (Haddad et al. 1993). Irrespective whether vitamin D_3 is synthesized in the skin or ingested and absorbed, it enters the bloodstream and binds to the multifunctional protein vitamin D-binding protein (DPB) which transports it to the liver (Cooke et al. 1979).

In the liver, vitamin-D_3-25-hydroxylases enzymatically hydroxylate D_3 at position C25 to generate the more stable metabolite 25-hydroxyvitamin D_3 (25-D_3, calcidiol) (Jones et al. 1998). Up to date, five vitamin-D3-25-hydroxylases are proposed to be involved in this reaction: the microsomal CYP2R1, CYP2J2, CYP3A4 and the mitochondrial CYP27A1 (Hobaus et al. 2013). CYP2R1 is thought to be the most active enzyme in hydroxylation of D_3 at position C25 (Cheng et al. 2004). 25-D_3 is the most abundant vitamin D_3 metabolite and its level in the serum is used to determine the vitamin D status of patients for more than 40 years (Hollis 2005). DPB binds 25-D_3 and transports it in the kidney for further modifications.

The enzyme CYP27B1 (25-hydroxyvitamin D_3 1-alpha-hydroxylase; a cytochrome P450 enzyme) is located in the proximal tubule of the kidney. It is expressed also in other tissues and cell types, like colon, prostate, brain and immune cells (Townsend et al. 2005). CYP27B1 hydroxylates 25-D_3 at position C1 and generates 1,25-dihydroxyvitamin D3 (Holick et al. 1971), the most active metabolite of vitamin D_3.

The mitochondrial enzyme CYP24A1 (1,25-dihydroxyvitamin D_3 24-hydroxylase; also a cytochrome P450 enzyme) initiates the degradation of 25-dihydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 via 24-hydroxylation. The reaction products are the metabolites 24,25-dihydroxyvitamin D_3 and 1,24,25-trihydroxyvitamin D_3 (Nykjaer et al. 1999) which get excreted (Beckman et al. 1996). The chemical structures of the different forms of vitamin D are shown in *Figure 2* and a schematic overview of the vitamin D metabolism is illustrated in *Figure 3*.

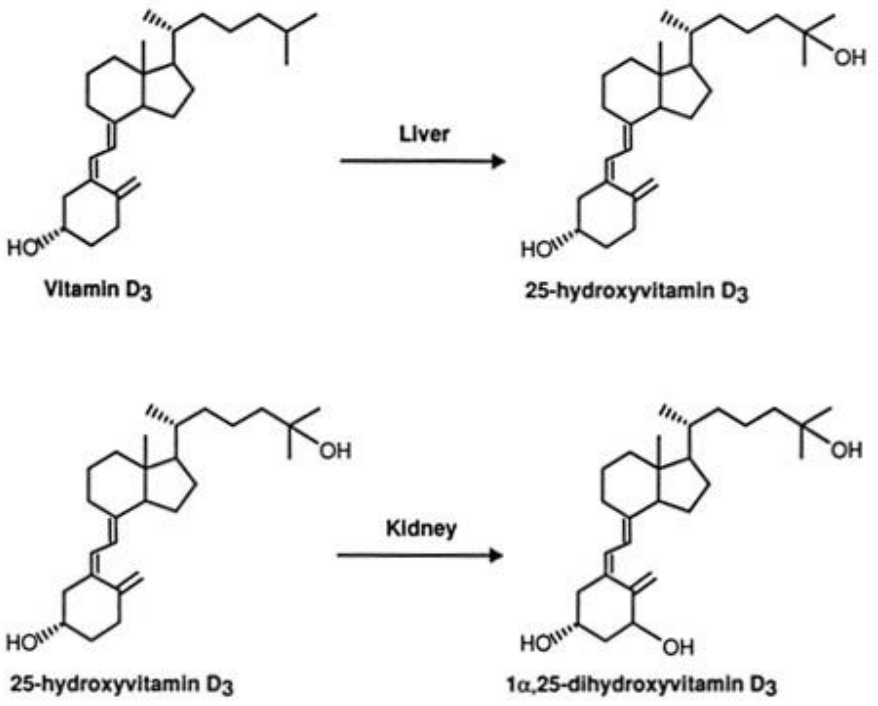

Figure 2: Biosynthesis of 1,25-dihydroxyvitamin D_3
In the liver, Vitamin D_3 (Cholecalciferol) is modulated to 25-hyfrocyvitamin D3 which is then converted to 1,25-dihydroxycitamin D_3 in the kidney.
(Figures adapted from (Jones et al. 1998))

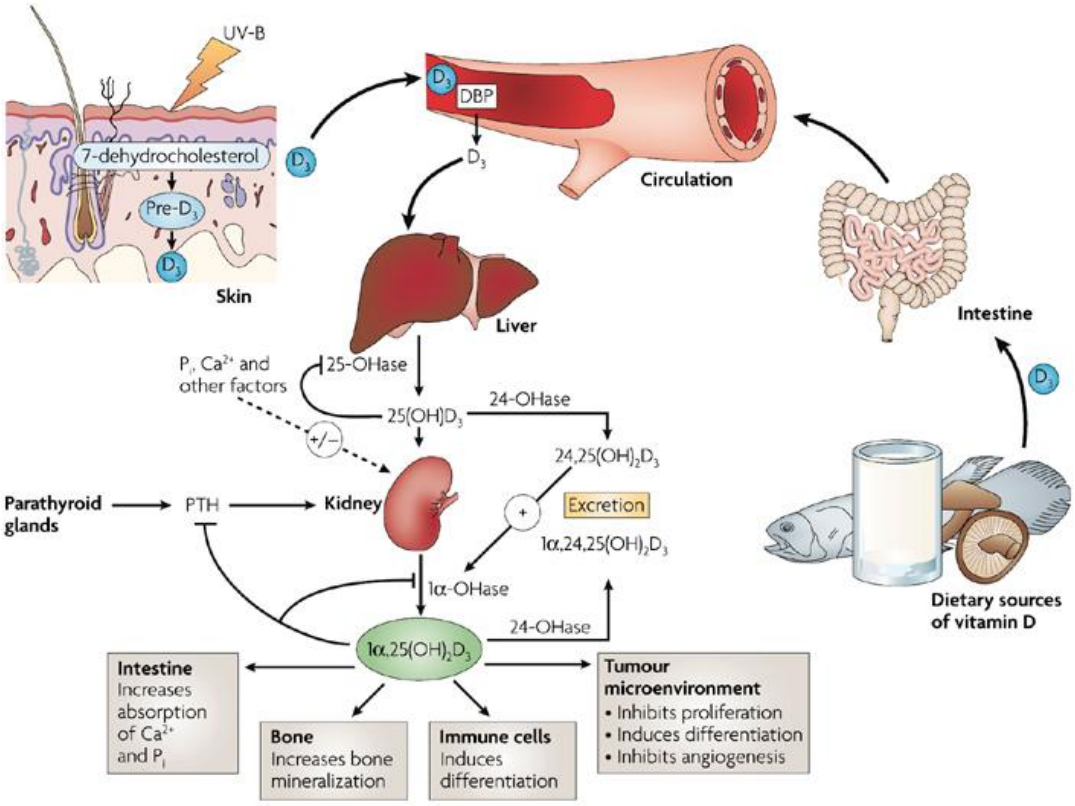

Figure 3: Vitamin D metabolism

Vitamin D_3 (D_3) can be either synthesized in the skin via a photochemical reaction or ingested through diet. In the bloodstream, D_3 binds to vitamin D-binding protein (DBP) and is transported to the liver. There, 25-OHase (a vitamin-D_3-25-hydroxylase) hydroxylates D_3 at position C25 to generate $25(OH)D_3$ (25-D_3 or calcidiol) which is transported to the kidney where 1α-OHase (CYP27B1) generates $1α,25(OH_2)D_3$ (1,25-D_3 or calcitriol), the most active form of vitamin D. This synthesis is stimulated by PTH (parathyroid hormone) and suppressed by 1,25-D3 itself.

24-OHase (CYP21A1) degrades 1,25-D_3 to 1α,24,25(OH2)D3 (1,24,25-trihydroxyvitamin D_3) which are consequently excreted.

(Figure from (Deeb et al. 2007))

1.1.4 1,25-dihydroxyvitamin D_3, vitamin D receptor and gene expression

1,25-D_3 associated with DBP (vitamin D-binding protein) enters a target cell and there, the two molecules dissociate. 1,25-D_3 migrates into the cell nucleus and interacts with the intracellular vitamin D receptor (VDR) (Haussler and Norman 1968). VDR belongs to the nuclear receptor family of transcription factors and is encoded by the VDR gene (Evans 1998). VDR forms heterodimers with another intracellular receptor, the retinoid X receptor (RXR). The VDR-RXR-1,25-D_3 complex binds to vitamin D response elements (VDRE) in the DNA, which are specific sequences in the promoter region of the 1,25-D_3 target genes, to activate or suppress their expression (Kimmel-Jehan et al. 1997). Recently, two studies examined the number of 1,25-D_3 stimulated VDR-binding sites in lymphoblastoids and monocytes via chromatin immunoprecipitation sequencing (Chip-Seq) technique which indicate the enourmous number of 1820 and 2776 1,25-D_3 target genes (Carlberg et al. 2012).

1.1.5 1,25-dihydroxyvitamin D_3 – A pleiotropic hormone

Since the discovery of the connection between rickets and vitamin D, the most studied role of 1,25-D_3 is the regulation of phosphate and calcium homeostasis. Together with parathyroid hormone (PTH), 1,25-D_3 is able to increase the efficiency of absorption of intestinal calcium and phosphate and plays an important role in bone mineralisation, bone health and maintenance (Holick 2007). Studies have demonstrated that VDR is expressed also in colon, brain, breast, bone marrow and immune cells, and other functions of 1,25-D_3 than calcium and bone homeostasis have been shown (Bikle 2009) which are partly described in the next paragraphs in this chapter.

Many studies highlight the important role of 1,25-D_3 in regulating the immune system. For instance, Toll-like receptor (TLR) activation of macrophages, which in turn leads to recognition of microbial infections and initiation of immune responses, upregulates VDR expression (Liu et al. 2006). Vitamin D supplements enhance the phagocytic functions of human macrophages (Martineau et al. 2007).

Recent findings have shown that 1,25-D_3 induces generation of antimicrobial peptides, like cathelicidin (Liu et al. 2007), that plays an essential role in the immune system in host defence against Mycobacterium tuberculosis (Shapira et

al. 2010). Vitamin D deficiency has been linked to a fivefold-increased risk for the development of tuberculosis (Talat et al. 2010). Furthermore, VDR polymorphisms have been associated with an increased risk for progression of tuberculosis (Larcombe et al. 2008),(Selvaraj et al. 2008).

Low vitamin D status has been connected with both type 1 and type 2 diabetes (Scragg et al. 2004),(Chiu et al. 2004). In an interesting study from Finland, children who received 2000 IU/d vitamin D during their first year of life, had over 80% reduction in risk of incident type 1 diabetes after 31 years, compared with the control group who did not receive vitamin D supplements (Hyppönen et al. 2001).

Vitamin D deficiency is also associated with multiple sclerosis (MS) (Ascherio et al. 2010), increased mortality (Skaaby et al. 2012), Parkinson's disease (Vinh Quôc Luong and Thi Hoàng Nguyên 2012) and some forms of cancer (Krishnan et al. 2010), including colon cancer.

Considering the connection between 1,25-D_3 and cancer, it is not surprising that 1,25-D_3 and VDR are thought to be involved in the regulation of cell proliferation and differentiation (Samuel and Sitrin 2008). Studies in VDR knockout mice have shown an increased epithelial proliferation in the descending colon (Kallay et al. 2001) and an abnormal, reduced epidermal differentiation during the first weeks of life (Xie et al. 2002). It has also been demonstrated that in kerationcytes, 1,25-D_3 downregulates various factors that are involved in cell proliferation, like keratin 16, c-myc and EGFR (Nagpal et al. 2005).

1.1.6 Vitamin D and colorectal cancer

According to EuropaColon (www.europacolon.com), a colorectal cancer community founded in 2005, CRC is the second leading cause of cancer-related deaths in Europe with 230 000 yearly deaths. Several risk factors were suggested to be important for the outbreak of CRC, like age, family history and life style, including dietary habits and physical activities (Kang et al. 2011).

In 1980, Frank and Cedric Garland hypothesised for the first time the connection between colon cancer and low vitamin D status based on ecological studies (Garland and Garland 2006). They observed a geographic distribution of colon cancer-related deaths in the United States with lower mortality rates in the south-

west compared to the north-west. According to these findings, Garland and Garland proposed that this inverse relation between CRC incidence and solar radiation could be caused by the lower vitamin D synthesis in areas with less sunlight. In 1989, the same authors published a study that results strongly supported their hypothesis by showing an inverse correlation between CRC and vitamin D in the USA (Pereira et al. 2012).

CYP24A1 gene encodes the enzyme 1,25-dihydroxyvitamin D_3 24-hydroxylase which degrades 1,25-D_3, the most active metabolite of vitamin D_3. Several studies show overexpression of CYP24A1 in different forms of cancer, including the colon (Bareis et al. 2001),(Anderson et al. 2006), ovarian (Anderson et al. 2006), cutaneous squamous cell (Reichrath et al. 2004), esophageal (Mimori 2004) and lung (Parise et al. 2006) carcinomas. These findings indicate a possible important role of CYP24A1 in the complicated, multistep process of tumorigenesis.

1.2 MicroRNAs

1.2.1 Introduction

MicroRNAs (miRNAs) are a relatively recently discovered group of small non-coding RNAs which regulate the expression of messenger RNAs (mRNAs) at post-transcriptional level. miRNAs are found in plants and animals but apart from their functions, they show differences in their biosynthesis and molecular mechanisms.

The role of miRNAs in various cellular pathways has been investigated for approximately 20 years. Over 1800 human miRNA species have been identified so far (according to www.mirbase.org). It is hypothesized that miRNAs are involved in every major pathway. This chapter describes the history of the discovery of miRNAs, their biogenesis pathways, their mechanisms to regulate gene expression, their role in cancer progression, with special focus on microRNA-22 and microRNA-125b.

1.2.2 History of microRNAs – It all began in a worm

In 1993, Victor Ambros and his colleagues, Rhonda Feinbaum and Rosalinde Lee, discovered a small non-coding RNA, called *lin-4*, in *Caenorhabditis elegans* (Lee et al. 1993). They detected that the gene *lin-4* which was already known to play an essential role in the temporal control of the larval development (Horvitz 1980), does not encode proteins but a pair of small RNAs of approximately 22 and 61 nucleotides in length.

At that time, it was also known that null mutations in the heterochronic gene *lin-14* led to an opposite phenotype of the *null-lin-4* mutations (Ambros and Horvitz 1984). Ambros's and Gary Ruvkun's laboratories recognized that the approximately 22 nucleotides long *lin-4* RNA has antisense complementarity to many regions in the 3'-UTR (3'-untranslated region) of the *lin-14* RNA (Lee et al. 1993), (Wightman 1993) and both working groups described a new regulatory mechanism of non-protein-coding RNAs, based on the ability of *lin-4* to regulate *lin-14* expression by binding to its 3'-UTR.

In the year 2000, a second small non-coding RNA (21nt), *let-7*, was detected in *Caenorhabditis elegans* (Reinhart et al. 2000). *let-7* is also involved in regulating developmental timing and is complementary to the 3'-UTR of the genes *lin-14, lin-28, lin-41, lin-42 and daf-12* but it is not homologous to *lin-4*. *let-7* RNAs were also detected in samples from different animals, including vertebrates, ascidians, hemichordate, molluscs, annelids and arthropods (Pasquinelli et al. 2000). This was the starting signal for a new area of research of a new class of small non coding RNAs, later called micro RNAs.

Recent findings of miRNAs in *Chlamydomonas reinhartii* (a unicellular algae) indicate that this class of RNAs might be evolutionary older than expected (Schwach et al. 2007),(Allen and Howell 2010) and this leads one to expect more exciting findings about miRNAs in the future.

1.2.3 Biogenesis of microRNAs

1.2.3.1 Introduction

The biogenesis of miRNAs in animals and plants shows similarities as well as differences in the major processing steps (Guleria et al. 2011),(Ul Hussain 2012)

and in this chapter, the focus will be on the synthesis of animal miRNAs. The synthesis of miRNAs can start in two different ways which only differ in the first steps that occur in the nucleus: (1) miRNAs are transcribed either from their own genes (canonical miRNAs) or (2) they are generated from introns via splicing in a unique manner (called mirtrons) (see *Figure 4*).

1.2.3.2 Canonical microRNAs:

RNA polymerase II transcribes a miRNA gene and this transcript is called primary microRNA (pri-miRNA) with a length of approximately 500-3000 nucleotides. The pri-miRNA forms a hairpin structure with an imperfectly paired double stranded stem and a terminal loop. Like a primary transcript from a protein-coding gene, pri-miRNA is capped and polyadenylated (Cai et al. 2004).

In the nucleus, a multiprotein complex containing the two core components Drosha and Di George Syndrome critical region gene 8 (DGCR8), called the microprocessor complex, recognizes the above described secondary structures (Gregory et al. 2004). Drosha is an RNAse III enzyme and DGCR8 contains an RNA-binding domain. The microprocessor complex cleaves the pri-miRNA at the base of the hairpin and generates a precursor miRNA (pre-miRNA) of approximately 65 nucleotides in length with usually a 2-nt overhang at the 3'-end (Han et al. 2006).

1.2.3.3 Mirtrons

The alternative miRNA biogenesis, the 'mirtron' pathway, combines intron splicing with dicing in a Drosha-independent manner. The first mirtrons were identified in Caenorhabditis elegans (Ruby et al. 2007) and Drosophila melanogaster (Okamura et al. 2007) but comparable loci were later identified in chicken (Glazov et al. 2008), cows (Glazov et al. 2009), rodents (Rnas et al. 2008) and primates (Berezikov et al. 2007).

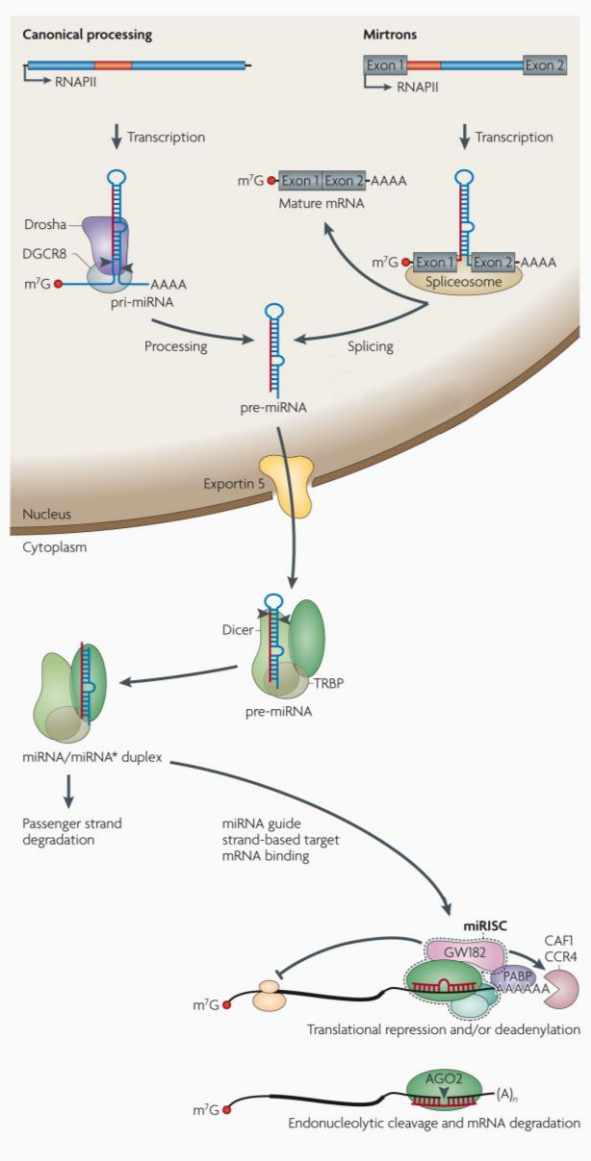

Figure 4: Biogenesis of microRNAs

RNA polymerase II (RNAPII) processes MicroRNAs (miRNAs) from genes or from introns. In the canonical pathway, Drosha and DGCR8 processes a primary miRNA (pri-miRNA) into a precursor-miRNA (pre-miRNA). In the mirtron pathway, pre-miRNA are generated via splicing and debranching from a intron. In both cases, Exportin 5 transports the pre-miRNA into the cytoplasma where it is cleaved by Dicer, assisted by TRBP, and generates a miRNA/miRNA* duplex. miRNA* is released and degraded whereas miRNA incorporated into an miRNA-induced silencing complex (miRISC). The miRISC–miRNA complex binds partial complementary to the 3'-UTR of a mRNA and so represses the translation or degrades the mRNA.

(Figure adapted from (Krol et al. 2010)

From a transcribed pre-mRNA, the spliceosome removes the mirtron which forms a lariat that is debranched by the lariat debranching enzyme (Ldbr). Then, the debranched mirtron forms secondary structures and folds into a pre-miRNA (Ruby et al. 2007),(Okamura et al. 2007).

1.2.3.4 Transport into the cytoplasm and processing of precursor microRNAs to generate mature microRNAs

The nuclear export receptor Exportin 5 recognizes the 3'-overhang of the double-stranded pre-miRNA which is then exported in a Ran-GTP dependent manner into the cytoplasm (Yi et al. 2003),(Bohnsack et al. 2004). The Dicer, an RNAse III enzyme, binds the 3'-end of the pre-miRNA and cleaves it near the terminal loop, approximately 22 nt away from the 3'-end. Through this cleavage which is assisted by transactivation-responsive (TAR) RNA-binding protein (TRBP), a miRNA-miRNA* duplex with 2 nt long 3'-overhangs at both ends, is generated (Bernstein et al. 2001),(Hutvágner et al. 2001). The miRNA*-strand gets often degraded and the miRNA-strand binds to the ribonucleoprotein miRNA-induced silencing complex (miRISC).

1.2.3.5 Actions of microRNAs

The miRISC–miRNA complex recognizes and binds partial complementary messenger RNAs (mRNAs), whereas only 2-8 nucleotides (called the seed region) match perfectly with the 3'-UTRs of the target mRNA (Brennecke et al. 2005),(Ameres et al. 2007). There are two different ways how the miRISC-miRNA complex can repress the translation from mRNA into protein: (1) by blocking the strand for translation into protein or (2) by degrading the mRNA strand (Ul Hussain 2012). Due to the fact that the essential seed region is only few nucleotides long to which the miRISC-miRNA complex binds, one miRNA regulates multiple mRNAs and one mRNA can be regulated by multiple miRNAs (Meltzer 2005). This leads to an enormous number of possible regulatory mechanisms in the process of gene expression.

1.2.4 MicroRNAs and cancer

1.2.4.1 Introduction

Recent findings have shown that miRNAs can be deregulated due to various genetic and epigenetic alterations, which might affect development of cancer (Ventura and Jacks 2009). Genetic alterations can occur as a result of chromosomal abnormalities such as deletion, amplification or translocation of DNA fragments containing the information for miRNAs (Calin and Croce 2006a). Various epigenetic events, like altered histone modifications, hypermethylation of tumour suppressor genes and hypomethylation of oncogenes might influence also the expression of miRNAs (Melo and Esteller 2011). Approximately fifty percent of miRNAs are associated with CpG islands, sequences containing high numbers of cytosine-guanine dinucleotides (Melo and Esteller 2011). Methylated CpG islands can silence gene expression (Fatemi et al. 2005). Thus, the expression of these miRNAs can be silenced by DNA hypermethylation.

1.2.4.2 MicroRNAs as oncogenes

miRNAs can act as oncogenes. In the last years, numerous miRNAs with oncogenic potential have been identified (Medina and Slack 2008). One such oncogenic miRNA is MicroRNA-21 (miR-21). Many studies have shown that miR-21 is overexpressed in various types of cancer, like in glioblastoma (Corsten et al. 2007), pancreatic (Roldo et al. 2006) breast (Si et al. 2007), lung (Li et al. 2011), esophageal squamous cell (Cai et al. 2012), hepatocellular (Meng et al. 2007) and colorectal (Bullock et al. 2013) cancer. Silencing experiments with antisense miR-21 showed an increased rate of apoptosis suggesting possible anti-apoptotic effects of miR-21 (Medina and Slack 2008) (Si et al. 2007).

Further, several tumour suppressors have been shown to be potential miR-21 targets (Medina and Slack 2008), like Phosphatase and Tensin homolog (PTEN) (Meng et al. 2007) and Tropomyosin 1 (TPM1) (Zhu et al. 2007). Therefore, miR-21 has been proposed as a possible biomarker for cancer diagnosis (Bowman et al. 2008).

1.2.4.3 MicroRNAs as tumor suppressors

miRNAs can act as tumour suppressors by inhibiting oncogene translation. The first identified miRNA in human, let-7, belongs to the let-7 microRNA family which is often downregulated in various forms of cancer (Wang et al. 2012b), such as in lung (Takamizawa et al. 2004), head and neck squamous cell (Childs et al. 2009), ovarian (Shell et al. 2007) and prostate (Liu et al. 2012) cancer.

Several studies have shown that let-7 might target and downregulate c-Myc, ras, high-mobility group A (HMGA), Janus protein tyrosine kinase (JAK), signal transducer and activator of transcription 3 (STAT3) and Np95/ICBP90-like RING finger protein (NIRF), which are critical oncogenes in tumour progression due to their involvement in regulating cell cycle, apoptosis and cell adhesion (Wang et al. 2012).

1.2.5 miR-22

The MicroRNA-22 (miR-22) gene is located on the short arm of chromosome 17 at position 17p13.3 (www.atlasgeneticsoncology.org). Many studies have shown that miR-22 is deregulated in various forms of cancer, for example it is downregulated in breast (Xiong et al. 2010), hepatocellular (Zhang et al. 2010), gastric (Wang et al. 2013), lung (Ling et al. 2012) and colorectal (Zhang et al. 2012) cancer and upregulated in advanced non small cell lung cancer (Franchina et al. 2013) and prostate cancer (Poliseno et al. 2010).

Apart from the possible role of miR-22 in cancer, this miRNA also plays an important role in multiple pathways that are involved in hematopoiesis and different cellular functions (Li et al. 2012), such as PTEN and Protein Kinase B (AKT) (Bar and Dikstein 2010), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Takata et al. 2011) and erstrogen receptor (ESR1) (Pandey and Picard 2009),(Li et al. 2012).

A recent study indicated a connection between miR-22 and vitamin D in colon cancer cells (Alvarez-Díaz et al. 2012). Different colon cancer cell lines were treated with 100nM 1,25-D_3 for 48 hours. The treatment increased the expression of miR-22 more than 2-fold in HT-29 cells. Further, the study showed that 1,25-D_3-induced miR-22 expression may have an inhibitory effect on the proliferation and migration of the used cells.

1.2.6 miR-125b

The MicroRNA-125b (miR-125b) gene is located on the long arm of chromosome 11 at position 11q24.1 (www.atlasgeneticsoncology.org) and is a putative homologue to lin-4 in Caenorhabditis elegans. As miR-22, many studies have indicated that miR-125b is often deregulated in different forms of cancer.

On the one hand, miR-125b is downregulated in breast (Iorio et al. 2005), oral squamous cell (Shiiba et al. 2013), hepatocellular (Jia et al. 2012) and bladder (Huang et al. 2011) cancer. It has been proposed that miR-125 triggers apoptosis by downregulating the expression of the pro-survival (anti-apoptotic) proteins Mcl-1 and Bcl-2 (Gong et al. 2012). Furthermore, it has been suggested that mit-125b downregulates CYP24A1 post-transcriptionally in breast cancer (Komagata et al. 2009a). This finding might explain why CYP24A1 is often very highly expressed in some of these tumours.

On the other hand, miR-125b is upregulated in neuroblastoma (Laneve et al. 2007), prostate (Shi et al. 2007) (Ozen et al. 2008), non-small-cell lung (Yuxia et al. 2012), thyroid (Vriens et al. 2012) and type II endometrial (Jiang et al. 2011) cancer. In addition, miR-125b might promote proliferation and migration of cancer cells by targeting the pro-apoptotic protein Tumor protein p53-inducible nuclear protein 1 (TP53INP1) (Jiang et al. 2011) and induce metastasis by targeting StAR-related lipid transfer domain protein 13 (STARD13) (Tang et al. 2012).

miR-125b can have pleiotropic effects in several cellular processes. miR-125b seems to be essential for various forms of cell differentiation. In embryogenesis, miR-125b is important for the proper differentiation of embryonic stem cells (ESC) by regulating Dies1 (Differentiation of ESCs 1) (Battista et al. 2013). In human neuronal differentiation, miR-125b regulates the development and differentiation of neuronal subtypes (Stappert et al. 2013). Further, miR-125b is a key mediator for Snail-induced stem cell propagation and chemoresistance (Liu et al. 2013).

In colorectal cancer, miR-125b has been described as tumourigenic. Studies have reported that miR-125b is upregulated in CRC (Lin et al. 2011), (Nishida et al. 2011). However, the precise role of miR-125b in CRC has to be investigated further.

2 Aim of this study

The two main aims of this diploma thesis were:

- To analyze the effect of 1,25-D_3 on the expression of miR-22 and miR-125b in human colorectal and prostate cancer cell lines.

- To determine the expression levels of miR-22 and miR-125b in human colorectal tumour samples and adjacent normal mucosa from the same patient.

Subaims were:

- To investigate the effect of 1,25-D_3 on the cell viability of human colorectal and prostate cancer cell lines.

- To analyze the basal expression of miR-22 and miR-125b in human colorectal and prostate cancer cell lines.

- To investigate the effect of Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) on the the expression of miR-22 and miR-125b in human colorecatal cancer cell lines.

3 Materials and Methods

3.1 Cell lines

3.1.1 Colon cancer cell lines

3.1.1.1 HT-29

The HT-29 cell line was derived from a 44 years old female Caucasian patient who suffered from a colorectal adenocarcinoma and it was isolated by Jorgen Fogh in 1964. HT-29 cells are hypertriploid, intestinal epithelial cells growing as adherent cells.

3.1.1.2 COGA-1A

The COGA-1A cell line is a subclone of the COGA-1 cell line which was isolated by Ernst Wagner and Alexandra Sinski from a G2 colorectal adenocarcinoma of a 64 years old female patient (Vécsey-Semjén et al. 2002). In cell culture, COGA-1A cells grow as adherent monolayer.

3.1.1.3 COGA-13

The COGA-13 cell line was established in our laboratory in collaboration with Ernst Wagner and Alexandra Sinski and derived from a G3 colon tumour. These cells are undifferentiated. COGA-13 cells express extremely high amounts of CYP24A1 and undetectable CYP27B1 levels (Lechner et al. 2007).

3.1.1.4 Caco-2/15

The Caco-2/15 cell line is a stable clone of the parent Caco-2, which is a heterogeneous colorectal adenocarcinoma cell line developed under the leadership of Jorgen Fogh. The Caco-2 cell line was isolated from a primary colonic tumour of a 72-years old Caucasian male patient.

3.1.1.5 Caco-2/AQ

The Caco-2/AQ cell line was established by Andrea Quaroni and is a subclone of Caco-2/15 cells. Caco-2/AQ cells divide and grow faster than Caco-2/15 cells. During the logarithmic growth phase, Caco-2/AQ cells have a population doubling time of approximately 24 hours, whereas Caco-2/15 cells show a cell division time of around 36 hours (Chirayath et al. 1998).

3.1.1.6 LT97

The human colon adenoma cell line LT97 was isolated from a small polyp of a patient suffering from familial polyposis coli, under the leadership of Brigitte Marian in 2002. LT97 cells have a ki-Ras mutation and do not express the tumour suppressor protein APC (Adenomatous polyposis coli) due to a loss of both alleles (Richter et al. 2002).

3.1.2 Prostate cancer cell line

3.1.2.1 DU145

The DU145 prostate cancer cell line was derived from a 69 year old Caucasian patient by Kenneth R. Stone in 1978 and is a hypotriploid cell line. DU145 cells are epithelial cells growing in as adherent cells. Due to the tumourigenic properties of DU145, this cell line is frequently used for xenotransplantation studies in immunodeficient mice.

3.2 Cell culture conditions

3.2.1 General

Cells were routinely cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Media was changed every second day and cell lines were routinely screened for mycoplasma.

3.2.2 Colon cancer cell lines

HT-29, COGA-1A, COGA-13, Caco-2/15 and Caco-2/AQ cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen).

LT97 cells were cultured in Ham's F-12 medium (Sigma) supplemented with 25% L-15 Leibovitz medium (Sigma), 2 mM L-glutamine (Invitrogen), 1,18 mg/ml NaHCO3, 44 µg/ml CaCl2xH2O (both Merck), 25 mM HEPES, 2% FCS, 10 µg/ml insulin, 2,4 U/ml penicillin, 2,4 µg/ml streptomycin, 30 ng/ml EGF (all from Invitrogen) and 20 µg/ml gentamycin (PAA).

3.2.3 Prostate cancer cell line

DU-145 cells grew in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 1mM sodium pyruvate (Sigma), 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen).

3.2.4 ITS media for 1,25-D_3 treatment

Due to the presence of substances (like vitamin D-binding protein and albumin) in FCS, which might influence the effect of 1,25-D_3, cells were incubated in FCS-free ITS media for duration of the treatment. ITS media is composed of DMEM supplemented with 10 µg/ml insulin, 5,5 µg/ml transferrin, 6,7 ng/ml sodium selenite (using Insulin-Transferrin-Selenium-Ethanolamine Solution (ITS-X), 100X from Gibco), 10 mM HEPES, 4 nM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen).

3.3 Viability assays

CellTiter-Blue® Cell Viability Assay (Promega) was performed according to the manufacturer's instructions. In short, the CellTiter-Blue® reagent added to the media contains the indicator dye resazurin which is converted into the fluorescent end product resorufin in metabolically active, living cells (*Figure 5*). The amount of resorufin can be measured with a fluorometer and indicates the metabolic activity of the cells.

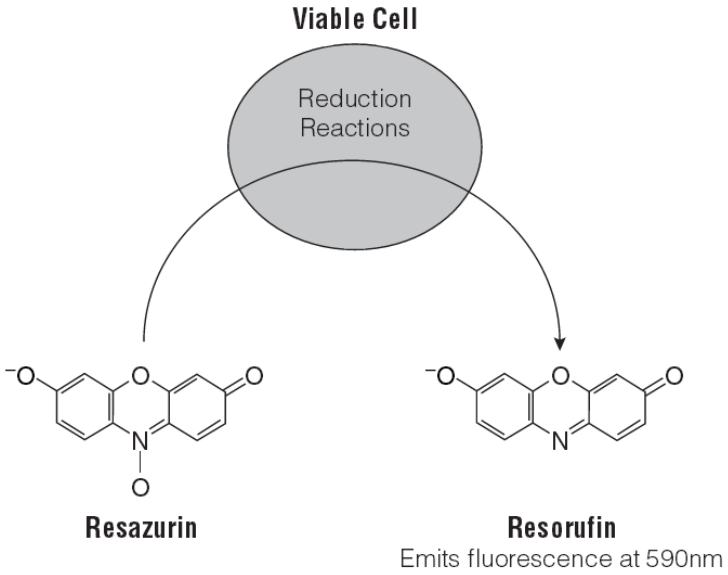

Figure 5: Conversion of resazurin to resorufin

Metabolically active cells convert resazurin to the highly fluorescent end product resorufin which emits fluorescence at 590nm. The measured fluorescence is proportional to the number of viable cells.

3.4 Colorectal tumour samples

After approval by the ethics committee (EK 258/2010 and EK 06-198-VK) of the Medical University of Vienna, fresh frozen tissue samples were collected at Rudolfsstiftung Hospital and the General Hospital of Vienna. After receiving a written consent from the patients, colorectal tumour tissue and adjacent mucosa from the same patients were collected by a pathologist who graded and classified the tumours according to the TNM system (T describes the tumour, N describes involved lymph nodes and M describes distant metastasis).

3.5 RNA extraction

RNA was extracted with Trizol® reagent (LifeTechnologies) according to the manufacturer's protocol and its integrity was determined by staining with Gel Red (Biotium) on an 1% agarose gel which was scanned and analyzed with a video camera imaging system under UV light (Herolab). The concentration and the purity of the isolated RNAs was measured with a full-spectrum spectrophotometer NanoDrop® ND-1000 (NanoDrop Technologies).

3.6 Reverse transcription

3.6.1 MicroRNAs

For analyzing the amount of miR-22, miR-125b and RNU6B (U6B small nuclear RNA; used as housekeeping gene), 10 ng of total RNA (including small RNAs) were reverse-transcribed into cDNA using TaqMan® MicroRNA Reverse Transcription Kit and the corresponding RT Primers (Applied Biosystems; assay 000398 for hsa-miR-22-3p, 000449 for hsa-miR-125b-5p and 001093 for RNU6B). 7 µl master mix (*Table 1*), 10 ng RNA and 3 µl 5X RT primers were added to each tube. RNAse-free dH_2O was added to reach a total volume of 15 µl. After spinning down and incubating on ice for five minutes, we performed the reverse transcription in a MyCyler™ Thermal Cycler (BIO-RAD). The parameters used are shown in *Table 2*.

Component	Volume
100mM dNTPs (with dTTP)	0.15 µL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00 µL
10X Reverse Transcription Buffer	1.50 µl
Rnase Inhibitor, 20U/µL	0.19 µl
Nuclease-free water	4.16 µl

Table 1: **Used RT-Mastermix for miRNAs for one reaction**

Step	Time	Temperature
Hold	30 minutes	16 °C
Hold	30 minutes	42 °C
Hold	5 minutes	85 °C
Hold	∞	4 °C

Table 2: **Used parameter values to program the thermal cycler for the reverse transcription of miRNAs**

3.6.2 Messenger RNAs

Two micrograms of the isolated RNA were reverse-transcribed with RevertAid H Minus Reverse Transcriptase and random hexamer primers (both Thermo

Scientific) into single-stranded cDNA. 1 µl random hexamer primers were mixed with 2 µg RNA and made up to 13 µl with RNAse-free dH_2O. After spinning down, the mixture was incubated at 70 °C for 10 minutes in a MyCyler™ Thermal Cycler (BIO-RAD). After 4 minutes on ice, 7 µl of the RT-Mastermix (*Table 3*) was added and the mixture was spinned down. Following incubation for 5 minutes at room temperature, the reverse transcription was performed in the thermal cycler. The used parameters are shown in *Table 4*.

Reagent	Amount
5X Reaction Buffer for RT	4 µl
10 mM dNTPs	2 µl
RevertAid H Minus RT	1 µl

Table 3: **RT-Mastermix for reverse transcription of mRNAs for one reaction (all reagents from Thermo Scientific)**

Step	Time	Temperature
Hold	60 minutes	42 °C
Hold	10 minutes	45 °C
Hold	15 minutes	70 °C
Hold	∞	10 °C

Table 4: **Used parameter values to program the thermal cycler for the reverse transcription of mRNAs**

3.7 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

3.7.1 General

Both quantitative reverse transcriptase polymerase chain reactions (qRT-PCRs) for miRNAs and mRNAs were performed either in a StepOnePlus™ Real-Time PCR System or in a 7900HT Fast Real-Time PCR System (both Applied Biosystems). It has to be mentioned that the analysis of one experiment was always performed in the same system. The temperature profile for the qRT-PCR is shown in *Table 5*.

Step	HOLD	HOLD	Cycle (40 cycles)	
			Denature	Anneal/Extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 minutes	10 minutes	15 seconds	60 seconds

Table 5: **Temperature profile for the qRT-PCR**

3.7.2 MicroRNAs

miR-22-3p and miR-125b-5p expressions were quantified in duplicates with qRT-PCR TaqMan® MicroRNA Assays (Applied Biosystems), namely assay 000398 for hsa-miR-22-3p and 000449 for hsa-miR-125b-5p, according to manufacturer's protocol. 1.7 µl nuclease free dH_2O, 17 µl master mix (*Table 6*) and 1.3 µl cDNA were added to each well. Samples were run in duplicates. The comparative cycle threshold (Ct) method was used to analyze the expression levels by calculating the $2^{-\Delta Ct}$. MiRNA expression levels were normalized using the 001093 assay (Applied Biosystems) for RNU6B snRNA (a component of the splicosome) as an internal control.

Component	Volume
TaqMan® Small RNA Assay (20X)	1.00 µL
TaqMan® Universal PCR Master Mix II (2X)	10.00 µL
Nuclease-free water	6.0 µL

Table 6: **Used qRT-PCR master mix for miRNAs for one reaction**

3.7.3 Messenger RNAs

mRNA levels of CYP24A1 were quantified using Power SYBR® Green PCR Master Mix (2X) (Applied Biosystems) and primers are listed in *Table 7*. Samples were run in duplicates according to the manufacturer's protocol. Fold changes of mRNA levels of the target genes were normalized to the expression of the housekeeping gene Beta-2 microglobulin (B2M), set relative to the calibrator using the $2^{-\Delta\Delta Ct}$ calculations.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
CYP24A1	AGTCTTCCCCTTCCAGGATCA	CAAACCGTGGAAGGCCTATC

Table 7: **Primer pairs used for the qRT-PCR**

4 Results

4.1 Viablity assays

To assess whether 1,25-D_3 influences the viability of the colon cancer cells HT-29, COGA-13, Caco-2/15, Caco-2/AQ, LT97 and the prostate cancer cell line DU145, cells were seeded in 96-well plates. All treatments described below were performed in 8 wells per group.

At 30% confluency, cells were transferred to ITS medium or ITS supplemented with 1% FCS medum for 24 hours. Then, cells were treated with 10 nM 1,25-D_3 for 24 and 48 hours and controls were treated with 0.01% ethanol (corresponding to the amount of ethanol added in 1,25-D_3 treatments).

Cells treated with the highly toxic dose of 5 µM 5-aza-2'-deoxycytidine (AZA) for 24 and 48 hours were used as negative control. We also had a control group without any treatment.

At the end of the treatments, 20 µl CellTiterBlue® Reagent was added to each well, the plate was shaken for 10 seconds. Cells were incubated for 4 hours at 37°C in the incubator. The fluorescence was measured at $560_{Ex}/590_{Em}$ nm.

1,25-D_3 treatment had only minor effect on viability in most cells studied (*Figure 6*). In HT-29 colon cancer cells, 1,25-D_3 treatment led to a slightly enhanced metabolic activity. Adding FCS to the ITS media affected only the viability of Caco-2/AQ cells. A minor inhibitory effect of 1,25-D_3 in these cells was seen only if the cells were grown in ITS. In the DU145 prostate cancer cells, no effect was detected relating to the treatment or the used media.

All cells showed very low metabolic activity after the treatment with AZA, as expected.

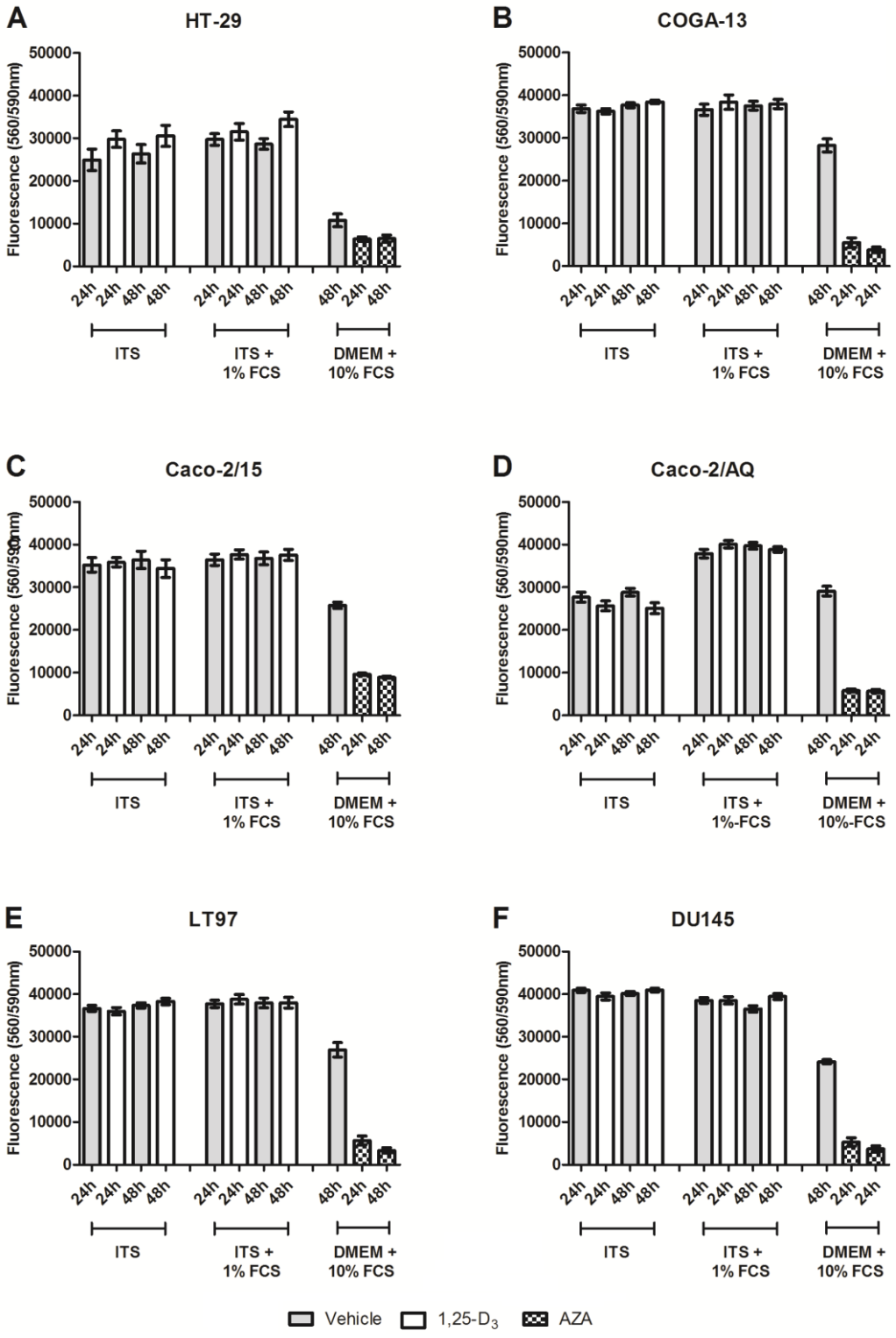

Figure 6: Effect of 1,25-D_3 on the cell viability

(A) HT-29, (B) COGA-13, (C) Caco-2/15, (D) Caco-2/AQ, (E) LT97 and (F) DU145 cells were treated with 10 nM 1,25-D_3 for 24 and 48 hours in ITS or ITS supplemented with 1% FCS media (n=8 ± SD).

4.2 Basal expression of miR-22 and miR-125b in HT-29, COGA-1A, COGA-13, Caco-2/15, Caco-2/AQ, LT97 and DU145 cancer cell lines

To determine the basal expression of miR-22 and miR-125b, HT-29, COGA-1A, COGA-13, Caco-2/15, Caco-2/AQ, LT97 colon and DU145 prostate cancer cells were cultured in 6-well plates. At 70-80% confluency, the expression levels of miR-22 and miR-125b were determined using qRT-PCR, and the U6 small nuclear RNA was used as an internal control (*Figure 7*).

The expression of miR-22 was considerable higher in the prostate cancer line DU145 compared with the colon cancer cells. HT-29, COGA-1A, COGA-13, Caco-2/15 and Caco-2/AQ colon cancer cells expressed miR-22 at similar levels while the adenoma cell line LT97 showed approximately 2-fold higher expression of miR-22 compared with the other colon cancer cells.

miR-125b was more than 100-fold higher expressed in COGA-13 cells compared with all the other examined colon cancer cells in which the expression was very low. The DU145 prostate cancer cells showed also a much higher expression of miR-125b compared with the colon cancer cells HT-29, COGA-1A, Caco-2/15, Caco-2/AQ and LT97.

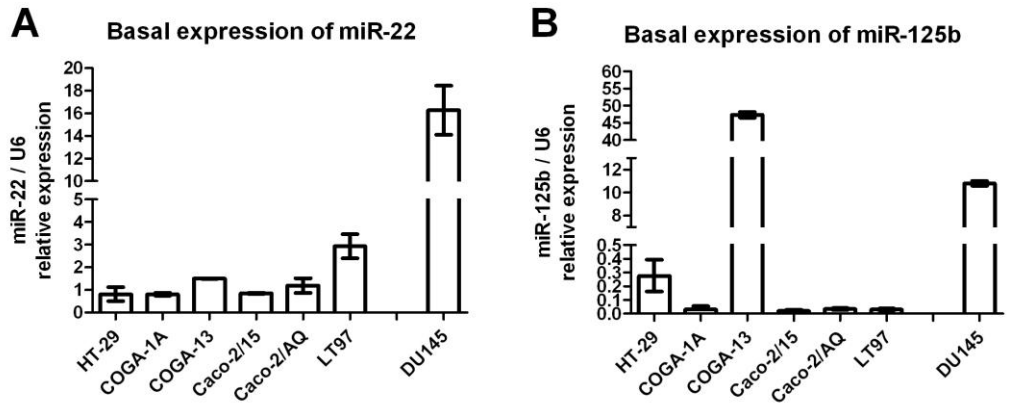

Figure 7: Basal expression of miR-22 and miR-125b

The relative expression levels of (A) miR-22 and (B) miR-125b in HT-29, COGA-1A, COGA-13, Caco-2/15, Caco-2/AQ, LT97 colon cancer and DU145 prostate cancer cells were measured by qRT-PCR, and the U6 small nuclear RNA was used as an internal control (n=3 ± SEM).

4.3 Basal expression of CYP24A1 in HT-29, DU145 and COGA-13 cancer cell lines

CYP24A1 is the vitamin D degrading enzyme and it is often upregulated in various forms of cancer, including CRC. Moreover, miR-125b has been suggested to be involved in the regulation of the expression of this enzyme. We analyzed the basal expression of CYP24A1 in the HT-29, DU145 and COGA-13 cell line using qRT-PCR, and B2M was used as an internal control. The expression levels of CYP24A1 were extremely high in the COGA-13 cells and very low in the HT-29 cells compared with the DU145 cells (*Figure 8*). All other cell lines studied have barely detectable basal CYP24A1 levels.

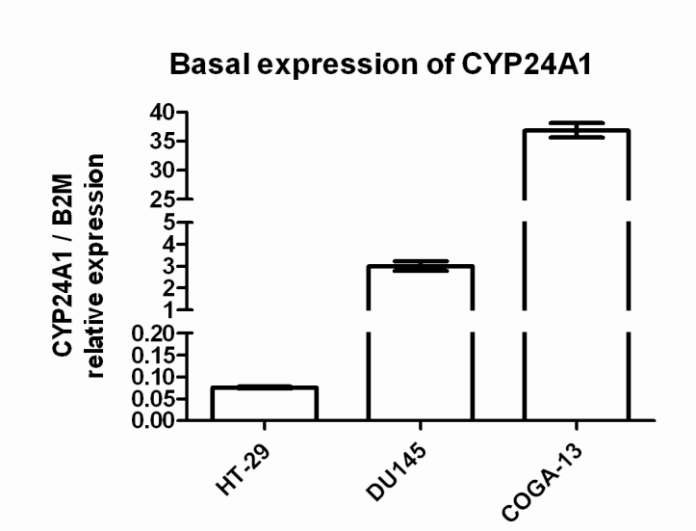

Figure 8: Basal expression of CYP24A1 in HT-29, DU145 and COGA-13 cells

The relative expression levels of CYP24A1 in HT-29 and COGA-13 colon cancer and DU145 prostate cancer cells were measured by qRT-PCR. B2M was used as an internal control (n=3 ± SEM).

4.4 Basal expression of Snail in HT-29, DU145 and COGA-13 cancer cell lines

Snail is suggested to play a critical role in cancer progression by inducing epithelial-mesenchymal transition. Since miR-125b was suggested to be a key mediator of Snail-induced stem cell propagation and chemoresistance, we analyzed the basal expression level of Snail in the COGA-13 and DU145 cells

because these cells express high basal levels of miR-125b. Because we also were interested in the expression levels of both miRNAs in HT-29 cells, we also measured the amount of Snail in these cells. The expression level of Snail in the colon cancer cells COGA-13 and HT-29 were very low compared to the prostate cancer cell line DU145 (*Figure 9*).

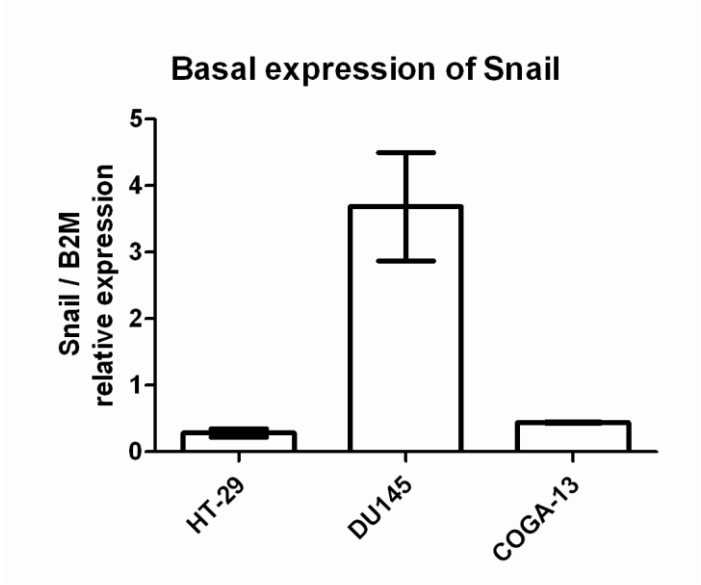

Figure 9: Basal expression of Snail in HT-29, DU145 and COGA-13 cells

The relative expression levels of Snail in HT-29 and C OGA-13 colon cancer and DU145 prostate cancer cells were measured by qRT-PCR. B2M was used as an internal control (n=3 ± SEM).

4.5 Role of 1,25-D_3 in regulating miR-22 and miR-125b in cancer cell lines

4.5.1 Effect of 1,25-D_3 on the expression of miR-22, miR-125b and CYP24A1 in COGA-13 colon cancer cells

4.5.1.1 Effect of 1,25-D_3 on the expression of CYP24A1 in COGA-13 colon cancer cells

CYP24A1 is a classical direct target gene of 1,25-D_3. Therefore, first we analyzed the expression of the vitamin D degrading enzyme CYP24A1 after the treatment with 10nM 1,25-D_3 for 5 hours, since it was suggested that miR-125b is involved in the regulation of the expression of this enzyme. CYP24A1 expression is extremely

high in these cells, and 1,25-D_3 treatment increases its expression only marginally (*Figure 10*).

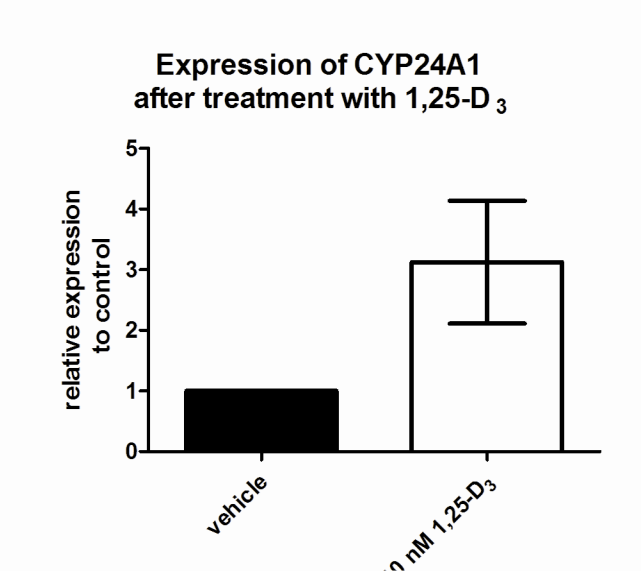

Figure 10: Effect of 1,25-D_3 on the expression of CYP24A1 in COGA-13 cells

Expression of CYP24A1 was analyzed after COGA-13 cells were exposed to 10 nM 1,25-D3 for 5 hours. Expression level after the treatment was set relative to the vehicle control (n=3 ± SEM).

4.5.1.2 Effect of 1,25-D_3 on the expression of miR-22 and miR-125b in the COGA-13 colon cancer cells

COGA-13 cells were seeded in 10 cm Petri dishes (12000 cells/cm²). 10 or 100 nM 1,25-D_3 were added for 1, 3, 5, 7, 10 and 24 hours. Controls were treated with 0.01% ethanol (corresponding to the amount of ethanol in the added 1,25-D_3). The expression levels of miR-22 and miR-125b were determined using TaqMan qRT-PCR.

Both miRNAs showed only minor differences in their expression levels after 1,25-D_3 treatment (*Figure 11*).

Although miR-22 was considered as a target gene for 1,25-D3, we observed no significant changes in its expression.

Neither 10 nM nor 100 nM 1,25-D3 had changed significantly the expression of miR-125b in COGA13 cells. There was a tendency of short transient upregulation after 3 hours followed by a decrease after 5 hours.

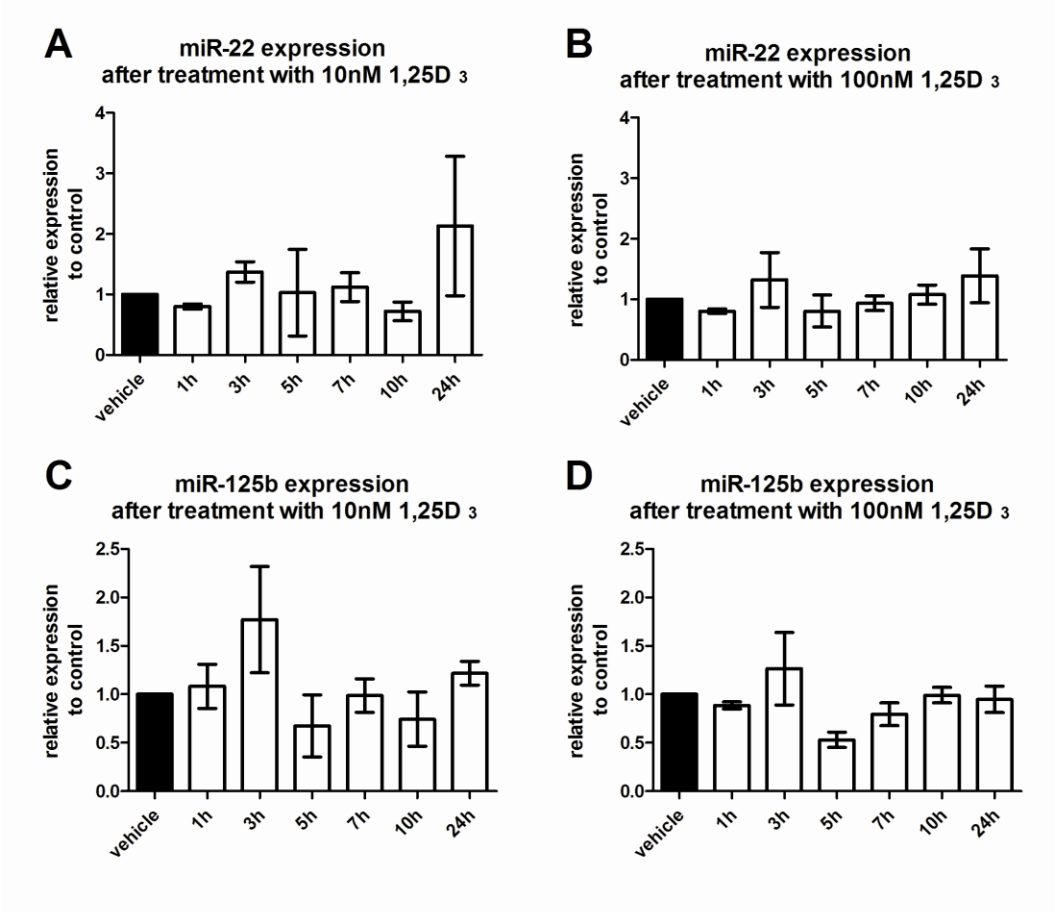

Figure 11: Effect of 1,25-D_3 on the expression of miR-22 and miR-125b in COGA-13 cells

Expression of miR-22 and miR-125b after treatment with 10 and 100 nM 1,25-D_3 for 1, 3, 5, 7, 10 and 24 hours were determined using TaqMan qRT-PCR. Expression levels after the treatments were set relative to the respective vehicle controls (n=3 ± SEM).

4.5.2 Effect of 1,25-D_3 on the expression of miR-22, miR-125b and CYP24A1 in DU145 prostate cancer cells

DU145 cells were seeded in 12-well plates (5000 cells/cm²). 10 nM 1,25-D_3 was added for 1, 3, 5, 7 and 9 hours. Controls were treated with 0.01% Ethanol (corresponding to the amount of ethanol in the added 1,25-D_3).

4.5.2.1 Effect of 1,25-D_3 on the expression of CYP24A1 in DU145 prostate cancer cells

DU145 cells express detectable levels of CYP24A1, although these have not reached the level of expression seen in COGA-13 cells. Treatment with 1,25-D_3 led to a significant upregulation of CYP24A1 already after 3 hours, peaking after 5 hours, when expression level of CYP24A1 was increased more than 25 times (*Figure 12*).

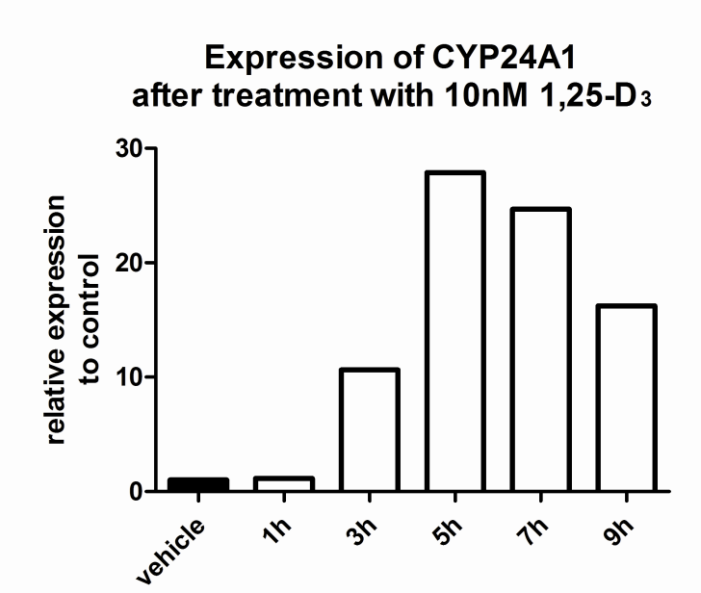

Figure 12: Effect of 1,25-D_3 on the expression of CYP24A1 in DU145 cells

Expression of CYP24A1 after treatment with 10 nM 1,25-D_3 for 1, 3, 5, 7 and 9 hours were determined using qRT-PCR. Expression levels after the treatments were set relative to the respective vehicle controls.

4.5.2.2 Effect of 1,25-D_3 on the expression of miR-22 and miR-125b in DU145 prostate cancer cells

We decided to analyze the expression of miR-22 and miR-125b after 1 and 5 hours treatment with 1,25-D_3. Both miRNAs were downregulated at both timepoints up to 50 percent (*Figure 13*). The downregulation of miR-125b after 5 hours and of miR-22 at both timepoints was significant.

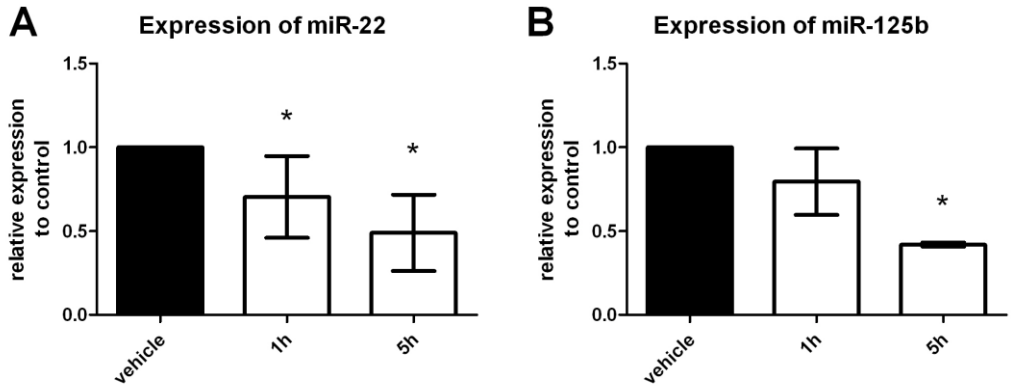

Figure 13: Effect of 1,25-D_3 on the expression of miR-22 and miR-125b in DU145 cells

Expression of miR-22 and miR-125b after treatment with 10 nM 1,25-D_3 for 1, and 5 hours were determined using TaqMan qRT-PCR. Expression levels after the treatments were set relative to the respective vehicle controls (n=3 ± SEM).

T-test was used for statistical analysis. * $P < 0.05$,

4.5.3 Effect of 1,25-D_3 on the expression of miR-22, miR-125b and CYP24A1 in HT-29 colon cancer cells

To assess the effect of 1,25-D_3 on HT-29 colon cancer cells, cells were seeded in 6-well plates (4210 cells/cm²). For testing the long-time and dose-dependent effects of 1,25-D_3, we treated the cells with 1, 10 and 100 nM 1,25-D_3 for 24 and 48 hours. In order to assess whether shorter treatments with 1,25-D_3 have already an effect on the expression of miR-22 and miR-125b in a time-dependent manner, we treated the cells for 1, 3, 5 and 7 hours with 10 nM 1,25-D3. Controls for each time point were treated with 0.01% ethanol (corresponding to the amount of ethanol in the added 1,25-D_3).

4.5.3.1 *Effect of 1,25-D_3 on the expression of CYP24A1 in HT-29 colon cancer cells*

We analyzed the expression of the vitamin D degrading enzyme CYP24A1 after the treatment with 10 and 100 nM 1,25-D_3 for 24 and 48 hours. CYP24A1 in the untreated cells is barely detectable. Treatment with 1,25-D3 increased the expression dramatically more than 100.000 times. (*Figure 14*).

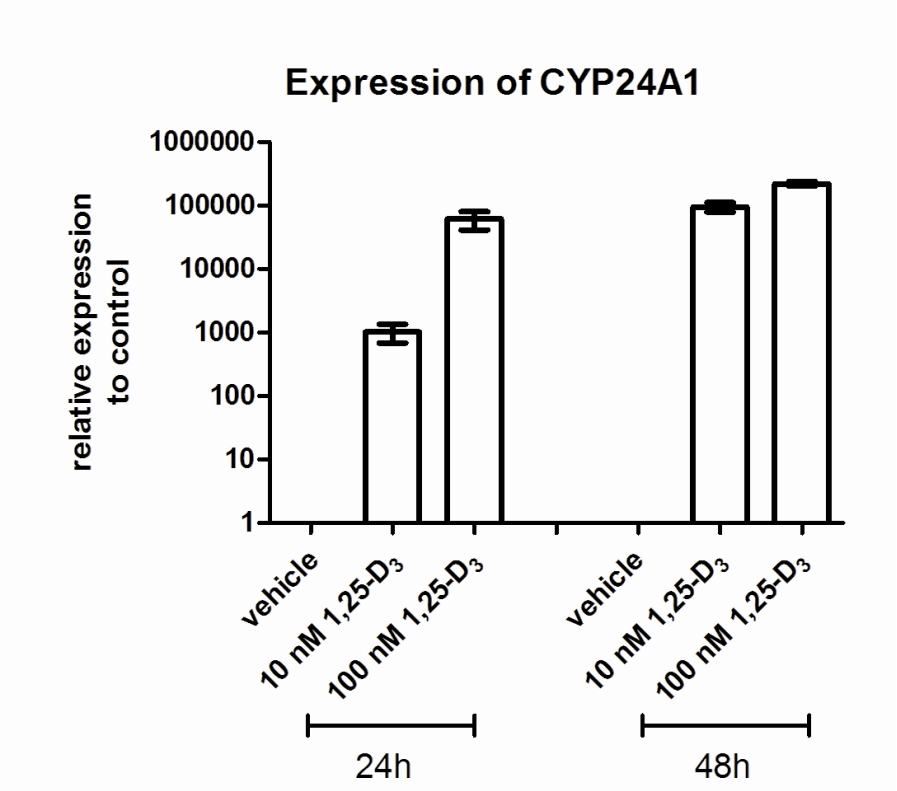

Figure 14: Effect of 1,25-D3 on the expression of CYP24A1 in HT-29 cells

Expression of CYP24A1 was determined after HT-29 cells were treated with 10 and 100 nM 1,25-D_3 treatment for 24 and 48 hours. Expression levels after the treatments were set relative to the respective vehicle control (n=3 ± SEM).

4.5.3.2 *Effect of 1,25-D_3 on the expression of miR-22 and miR-125b in HT-29 colon cancer cells*

Expression levels of miR-22 and miR-125b were determined using TaqMan qRT-PCR. miR-22 was upregulated up to approximately 100% upon treatment with 1,25-D_3 for 24 and 48 hours compared with the vehicle controls. The upregulation of the expression of miR-22 due to 100 nM 1,25-D_3 treatment reached significance

after 24 hours (*Figure 15*). Shorter treatments led to a downregulation of miR-22 after 1 and 3 hours, followed by an upregulation after 5 and 7 hours (*Figure 16*).

1,25-D_3 treatment for 24 hours led to an approximately 2-fold upregulation of miR-125b expression. Treatments with 100 nM 1,25-D_3 for 24 hours and treatments with 1 and 100 nM 1,25-D_3 for 48 hours resulted in a significant upregulation of miR-125b. HT-29 cells exposed to 10 nM 1,25-D3 for 48 hours showed a minor downregulation of miR-125b compared with the vehicle controls (*Figure 15*). After 1 hour of 1,25-D_3 treatment, miR-125b was slightly upregulated. Downregulation of miR-125b was seen only after 3 hours, after which the expression turned back to normal (*Figure 16*).

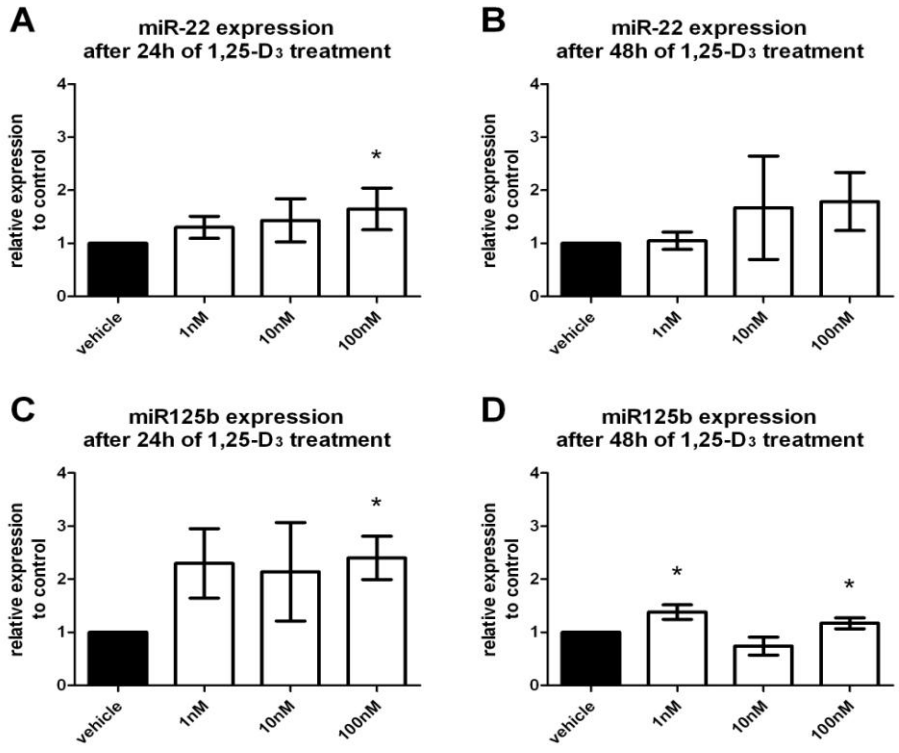

Figure 15: Long-time and dose-dependent effects of 1,25-D_3 on the expression of miR-22 and miR-125b in HT-29 cells

Expression levels of miR-22 and miR-125b were determined using TaqMan qRT-PCR after cells were exposed to 1, 10 and 100 nM 1,25-D_3 for 24 (A,C) and 48 hours (B,D). Expression levels after the treatments were set relative to the respective vehicle control (n=3 ± SEM).

Wilcoxon signed-rank test was used for statistical analysis. *$P < 0.05$

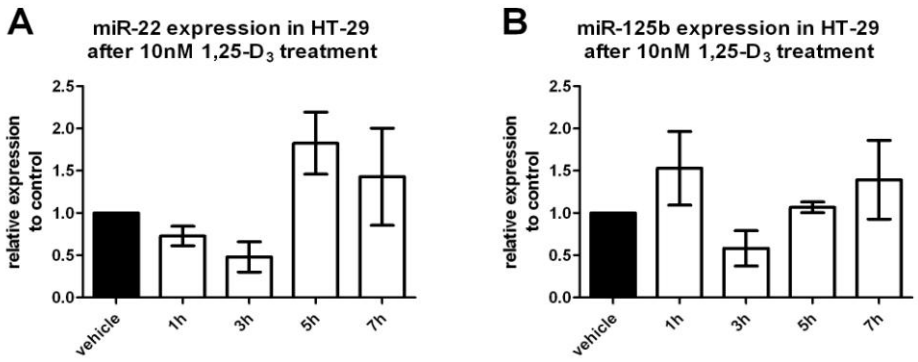

Figure 16: Short-time effects of 1,25-D_3 on the expression of miR-22 and miR-125b in HT-29 cells

Expression levels of (A) miR-22 and (B) miR-125b were determined using TaqMan qRT-PCR after cells were exposed to 10 nM 1,25-D_3 for 1, 3, 5 and 7 hours. Expression levels after the treatments were set relative to the respective vehicle controls (n=3 ± SEM).

4.6 Role of other factors in regulating miR-22 and miR-125b in Caco-2/AQ colon cancer cells

Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) play an important role in tumourigenesis. We tested whether these factors have an effect on the expression of mir-22 and miR-125b. We treated 2 week confluent Caco-2/AQ colon cancer cells with 100 ng/µl IL-6 and 50 ng/µl TNF-α for 6, 12, 24 and 48 hours. At each timepoint, controls were treated with 0,01% ethanol. The expression levels of miR-22 and miR-125b were analyzed using qRT-PCR and the U6 small nuclear RNA was used as an internal control.

4.6.1 The effect of Interleukin-6 (IL-6) on the expression of miR-22 and miR-125b in 2 weeks confluent Caco-2/AQ colon cancer cells

For preliminary test, we measured the expression of miR-22 and miR-125b after 6, 12, 24 and 48 hours treatment with 100 ng/µl IL-6. miR-22 was more than fifty percent downregulated after the IL-6 treatment for 6 hours, after which the expression turned back to normal. The expression of miR-125b was downregulated after 12 hours and upregulated after 24 hours of the treatment with IL-6 (*Figure 17*).

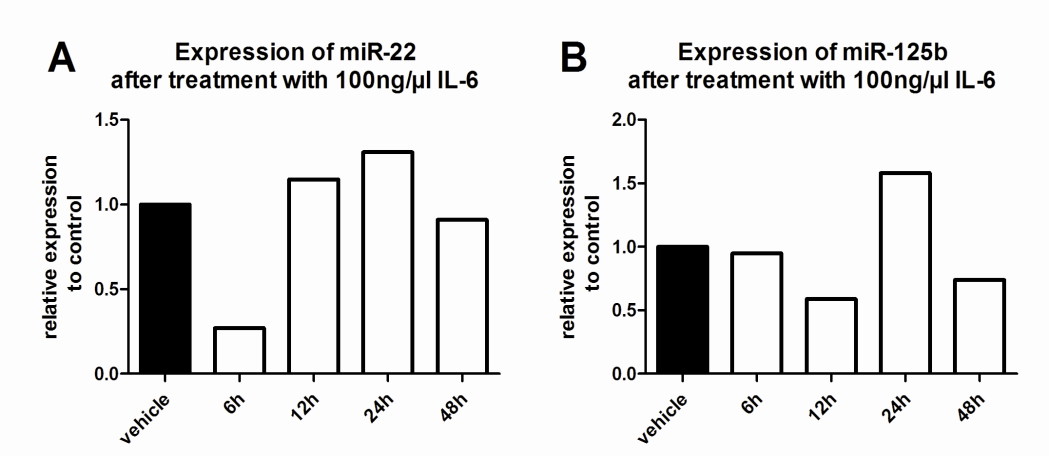

Figure 17: Effect of IL-6 on the expression of mir-22 and miR-125b in Caco-2/AQ cells (1 run)

Expression levels of miR-22 and miR-125b were determined after Caco-2/AQ cells were treated with 100 ng/µl IL-6 for 6, 12, 24 and 48 hours. Expression levels after the treatments were set relative to the respective vehicle controls.

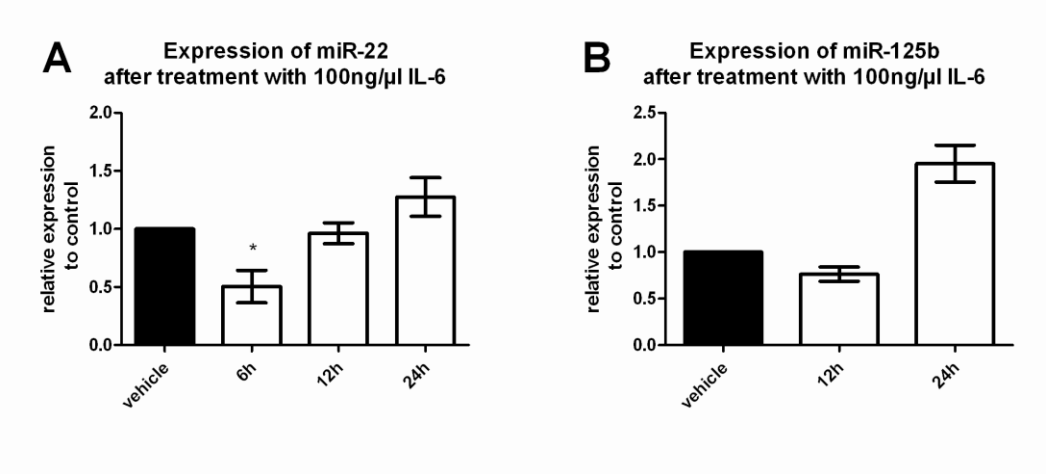

Figure 18: Effect of IL-6 on the expression of mir-22 and miR-125b in Caco-2/AQ cells

Expression levels of miR-22 and miR-125b were determined after Caco-2/AQ cells were treated with 100 ng/µl IL-6 for 6, 12, 24 and 48 hours. Expression levels after the treatments were set relative to the respective vehicle controls (n=3 ± SEM).

Paired samples test was used for statistical analysis. * $P < 0.05$

We decided to analyze further the expression of miR-22 after 6, 12 and 24 hours IL-6 treatment in all 3 runs. miR-125b expression was determined after IL-6 treatment for 12 and 24 hours.

We could confirm that IL-6 downregulated the expression of miR-22 significantly after 6 hours, whereas the expression turned back to normal after 12 hours (*Figure 18*).

IL-6 slightly downregulated miR-125b expression after 12 hours and doubled after 24 hours (*Figure 18*).

4.6.2 The effect of Tumour necrosis factor alpha (TNF-α) on the expression of miR-22 and miR-125b in 2 weeks confluent Caco-2/AQ colon cancer cells

We observed that miR-22 and miR-125b expression was only upregulated after 24 hours and at the other timpoints, the treatment with TNF-α had no effect (*Figure 19*).

Thus we decided to analyze further the expression of both miRNAs after treatment with TNF-α for 24 hours. We could confirm the effect of TNF-α of both miRNAs. miR-22 level was upregulated more than 1,5-fold and the expression of miR-125b more than 2-fold (*Figure 20*).

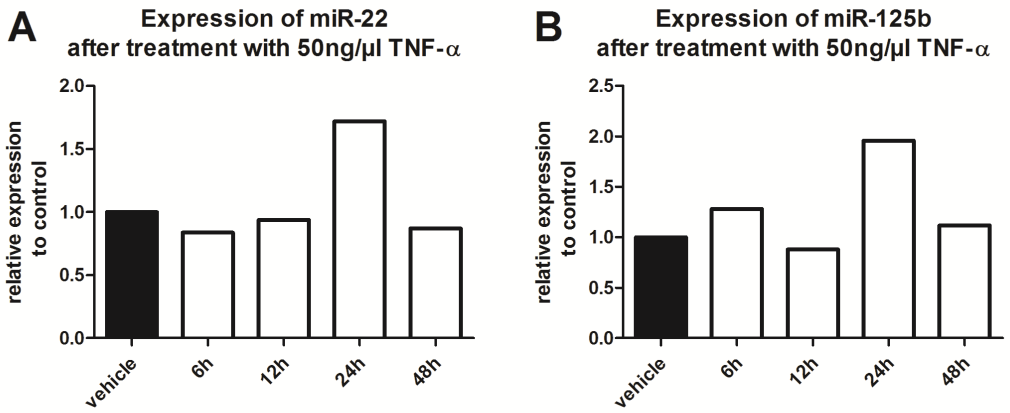

Figure 19: Effect of TNF-α on the expression of miR-22 and miR-125b in Caco-2/AQ cells (1 run)

Expression levels of miR-22 and miR-125b were determined after Caco-2/AQ cells were treated with 50 ng/µl TNF-α for 6, 12, 24 and 48 hours. Expression levels after the treatments were set relative to the respective vehicle controls.

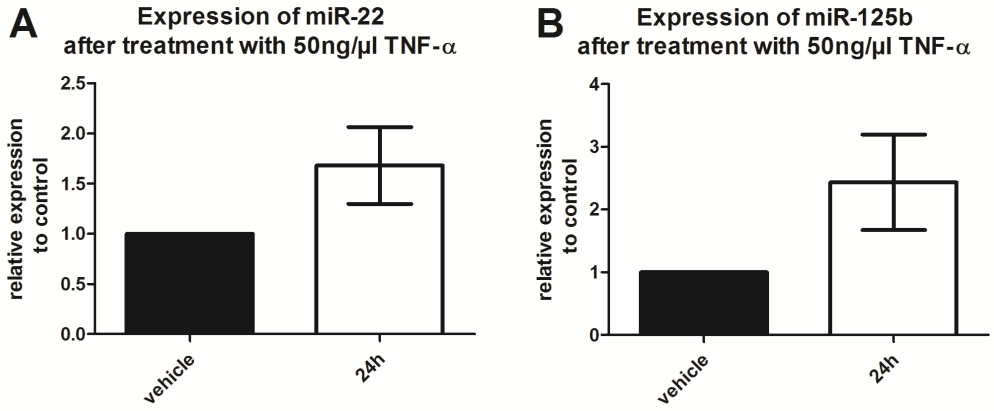

Figure 20: Effect of TNF-α on the expression of miR-22 and miR-125b in Caco-2/AQ cells

Expression levels of miR-22 and miR-125b were determined after Caco-2/AQ cells were treated with 50 ng/µl TNF-α for 24 hours. Expression levels after the treatments were set relative to the respective vehicle control (n=3 ± SEM).

4.7 Colorectal tumour samples

We evaluated further whether miR-22 or miR-125b expression is deregulated in colorectal cancer by qRT-PCR in 19 samples of human colorectal tumours and the adjacent mucosa from the same patient.

The expression of both miR-22 and miR-125b was similar in the tumour tissue compared with the respective adjacent mucosa. The amount of miR-125b was approximately 10-fold higher compared with the amount of miR-22 (*Figure 21*).

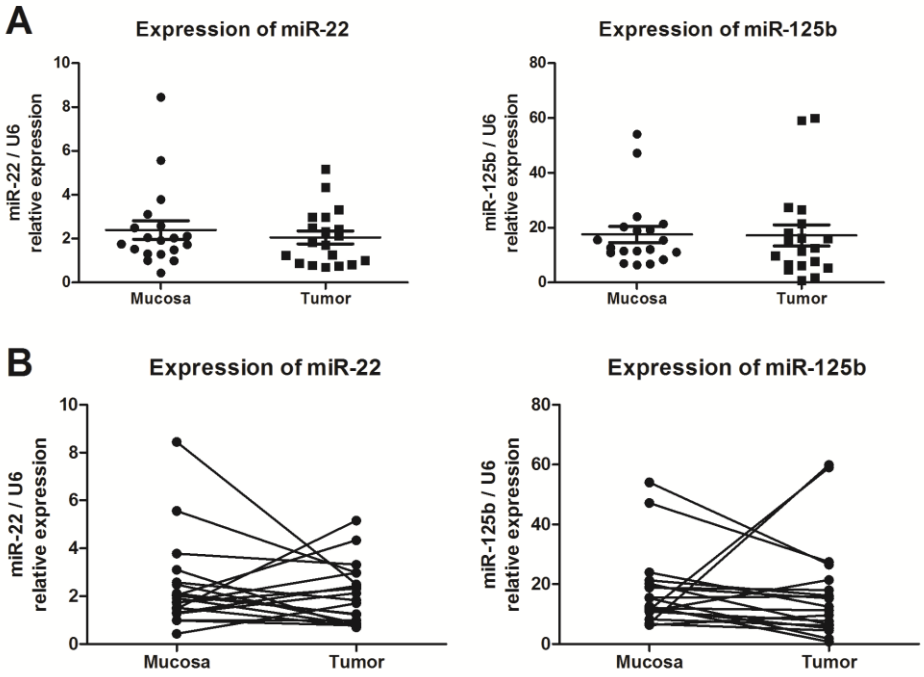

Figure 21: Expression of miR-22 and miR-125b in tissue samples

(A) The mean value of the expression of miR-22 and miR-125b in the mucosa and tumour samples. (B) Changes in miR-22 and miR-125b expression between the adjacent mucosa and the tumours in individual patients (n=19). Patients that have high levels of miR-22 were not the same patients with high levels of miR-125b.

5 Discussion

The two main aims of this study were (1) to investigate the effect of 1,25-D_3 on the expression of miR-22 and miR-125b in cancer cell lines, and (2) to analyze if these two miRNAs are deregulated in tumour samples from human colon cancer patients. We focused on these two miRNAs because it has been suggested that miR-22 expression is induced by 1,25-D_3 in colon cancer cells (Alvarez-Díaz et al. 2012) and miR-125b downregulates the vitamin D degrading enzyme CYP24A1 (Komagata et al. 2009b). Furthermore, both miRNAs are often deregulated in various forms of cancer. Studies have shown that miR-22 is downregulated in colon (Zhang et al. 2012) and upregulated in prostate (Lin et al. 2011) cancer patients. Our findings show that 1,25-D_3 modulates the expression of both miR-22 and miR-125 in colon as well as in prostate cancer cell lines. In the colon cancer cells COGA-13 and HT-29 both down- and upregulation of miR-22 and miR-125b occurred after the 1,25-D_3 treatment whereas in the prostate cancer cell line DU145, both miRNAs were downregulated. In the patients analyzed (n=19), the expression levels of both miR-22 and miR-125b were similar in the tumour and the adjacent mucosa of the same patient. Our study found no proof that mir-125b would regulate CYP24A1 expression, at least not in the cell lines analyzed.

In CRC, miR-22 becomes downregulated, while in prostate cancer it is upregulated (Poliseno et al. 2010). We could confirm that in the prostate cancer cells DU145, expression level of miR-22 was significantly higher than in all investigated colon cancer cells. The prostate cancer cells showed high basal expression of miR-125b as well, which is in accord with the findings of Shi and colleagues who reported that miR-125b is upregulated in prostate cancer cell lines (Shi et al. 2007). Interestingly, the basal expression of miR-125b was very low in all colon cancer cells analyzed with exception of COGA-13. This was surprising as we expected a higher expression of miR-125b in most colon cancer cells because it was suggested to be upregulated in patients suffering from CRC (Nishida et al. 2011). On the other hand, the very high level of miR-125b in COGA-13 cells was also unexpected, since it was suggested that miR-125b inhibits CYP24A1 expression (Komagata et al. 2009b) and COGA-13 cells express very high CYP24A1 levels. DU145 cells express CYP24A1 also at reasonably high levels, considering that in most cell lines CYP24A1 is barely detectable. We expected that

high expression level of miR-125b would correlate inversely with CYP24A1 level but this was not the case in COGA-13 and DU145 cells.

The transcription factor Snail has been shown to be highly expressed in CRC (Pena et al. 2005). Furthermore, it has been suggested that miR-125b is a key mediator of Snail-induced stem cell propagation and chemoresistance in breast cancer cells (Liu et al. 2013). In these cells, high expression levels of miR-125b correlated with high expression levels of Snail. Due to the very high basal expression of miR-125b in the COGA-13 and DU145 cells, and the moderate high expression in HT-29 cells compared with the other investigated cell lines, we also analyzed the basal expression of Snail in these 3 cell lines. Surprisingly, the expression level of Snail in the colon cancer cell lines HT-29 and COGA-13 was very low compared with the prostate cancer cell line DU145. Therefore, in our cells miR-125b expression seems to be independent of Snail expression. These results indicate that the high expression of miR-125b in the colon cancer cell line COGA-13 does not correlate with high expression of Snail. We concluded that the induction of Snail by miR-125b is tissue dependent and does not occur in our cancer cell lines.

We showed that the COGA-13 cell line expresses high levels of CYP24A1 and miR-125b. The 3-fold upregulation of CYP24A1 after 1,25-D3 treatment can be considered marginal if we compare with other cell lines. 1,25-D_3 had only minor effects on the expression of miR-22 and miR-125b also. We estimated that 1,25-D_3 does not play a major role in the regulation of either miR-22 or miR-125b in the COGA-13 cell line because 1,25-D_3 is degraded quickly due to the high levels of CYP24A1.

Following the high basal expression of miR-22 and miR-125b in the DU145 cell line, we analyzed the effect of 1,25-D_3 on the expression of both miRNAs. 1,25-D_3 had no effect on the expression of CYP24A1 after one hour as expected, but at later timepoints CYP24A1 was upregulated up to 25-fold. The highest upregulation was detected after 5 hours. Therefore, we analyzed the expression levels of miR-22 and miR-125b after 1 and 5 hours. At both timepoints, both miRNAs were downregulated. The downregulations increased in a time-dependent manner. After 5 hours, both miRNAs were downregulated significantly ($P < 0.05$). It has been suggested that miR-125b downregulates CYP24A1 in breast cancer (Komagata et al. 2009b). Our findings indicate that in the prostate cancer cell line DU145, 1,25-

D_3-induced upregualtion of CYP24A1 does not result in an upregulation of miR-125b. We estimate that the regulation of CYP24A1 by miR-125b is tissue dependent and may not occur in prostate cancer cells. Our results showed that 1,25-D_3 has a negative effect on the expression of miR-22 and miR-125b in the DU145 cell line.

A recent study has shown that in HT-29 colon cancer cells, miR-22 expression is induced in a dose- and time-dependent manner up to 3-fold due to 1,25-D_3 treatment (Alvarez-Díaz et al. 2012). Our results confirmed these findings. In HT-29 cells, miR-125b was also upregulated more than 2-fold after the treatment with 1,25-D_3 for 24 hours but this upregulation was partially lost after 48 hours treatment. The expression of CYP24A1 increased in a time- and dose-dependent manner up to 100.000-fold after the treatment with 100 nM 1,25-D_3 for 48 hours. This demonstrates that both CYP24A1 and miR-125b is induced by 1,25-D_3 treatment whereby the upregulation of CYP24A1 is 50.000-fold higher than the upregulation of miR-125b. Since it was suggested that miR-125b downregulates CYP24A1 (Komagata et al. 2009b), we estimate that miR-125b is upregulated when CYP24A1 level increases. These results indicate that miR-125b expression is not stimulated by the amount of CYP24A1. Therefore, we suggest that the expression of CYP24A1 in HT-29 cells is not regulated by miR-125b.

In a recent study published in April 2013, Padi and colleagues have shown that in HT-29 cells 1,25-D_3 treatment had a significant effect only on the expression of microRNA-627 (Padi et al. 2013). Interestingly, no expression of miR-22 and miR-125b were detected in this study. However, we estimate that further studies are needed to investigate the possible role of 1,25-D_3 on the expression of miR-22 and miR-125b.

Recent studies have shown that the deregulation of miRNA expression plays a critical role in cancer development (Calin and Croce 2006). Therefore, the analysis of miRNA expression patterns has become an important field in cancer research. The deregulation of miR-22 expression in cancer has been shown in many recent studies. miR-22 is downregulated in breast (Xiong et al. 2010), hepatocellular (Zhang et al. 2010), gastric (Wang et al. 2013), lung (Ling et al. 2012) and colorectal (Zhang et al. 2012) cancer and upregulated in advanced non small cell lung cancer (Franchina et al. 2013) and prostate cancer (Poliseno et al. 2010). miR-125b is downregulated in breast (Iorio et al. 2005), oral squamous cell (Shiiba

et al. 2013), hepatocellular (Jia et al. 2012), bladder (Huang et al. 2011) and colorectal (Lin et al. 2011), (Nishida et al. 2011) cancer and upregulated in neuroblastoma (Laneve et al. 2007), prostate (Shi et al. 2007) (Ozen et al. 2008), non-small-cell lung (Yuxia et al. 2012), thyroid (Vriens et al. 2012) and type II endometrial (Jiang et al. 2011) cancer. However, in this study we could not show any significant differences in the expression of miR-22 and miR-125b in colon tumours compared with the adjacent mucosa of the same patient. Both lower and higher expression of miR-22 and miR-125b in the tumours were detected compared with the mucosa. The expression level of miR-125b was around 10-fold higher than miR-22 in both tumour and mucosa samples. We conclude that expression of miR-22 and miR-125b were not altered in our patient cohort. All our patients samples derived from G2 grade tumours and we estimate that possible deregulations of miR-22 and miR-125b might occur in earlier or later stages of tumour progression. However, we think that due to our limited samples size, it would be disproportionate to draw definite conclusions about the expression levels of these miRNAs.

Interleukin-6 (IL-6) can operate both in a pro-inflammatory and anti-inflammatory manner. Tumour necrosis factor alpha (TNF-α) is involved in the regulation of cell proliferation, differentiation and apoptosis. IL-6 and TNF-α play important roles in tumourigenesis. Studies have shown an increased level of IL-6 in the serum of CRC patients (Galizia et al. 2002) and the level of IL-6 correlated with the size of the tumour (Chung and Chang 2003). Our results suggest that inflammatory cytokines might affect microRNA expression. Short-time treatment with IL-6 decreased the expression of miR-22 significantly by 50% ($P < 0.05$). The multifunctional cytokine TNF-α plays a key role in apoptosis, inflammation and immunity (Van Horssen et al. 2006). Treatment with TNF-α upregulated both miR-22 and miR-125b but these changes were not significant. These analysis of the effects of IL-6 and TNF-α on the expression of miR-22 and miR-125b indicated that IL-6 may play a role in the downregulation of miR-22 but further studies are needed.

1,25-D_3 was reported to show antiproliferative effects in various cancer cells lines (Kallay et al. 2001), (Xie et al. 2002), (Nagpal et al. 2005). We could not show this effect in our used cells. Interestingly, Caco-2/AQ cells showed considerable differences in their metabolic activity with regard to the used media. The presence

of 1% FCS in the ITS medium led to a mentionable increase in their viability compared with the ITS medium.

Based on the results of this study, we conclude that 1,25-D_3 modulates the expression of miR-22 and miR-125b in HT-29 and COGA-13 colon and DU145 prostate cancer cell lines. This effect is time-, concentration- and cell type dependent. On the one hand, long-time treatment with high concentration of 1,25-D_3 led to a significant upregulation of both miR-22 and miR-125b in the colon cancer cell line HT-29. On the other hand, short-time treatment with 1,25-D_3 decreased the expression of miR-22 and miR-125 in the prostate cancer cell line DU145. Since it was suggested that miR-125b downregulates the expression of CYP24A1, we expected that high expression level of CYP24A1 would lead to an upregulation of miR-125b. Our results show that miR-125b expression is not stimulated by the amount of CYP24A1 in colon cancer cells. Therefore, we suggest that the expression of CYP24A1 is not regulated by miR-125b in colon cancer cell lines HT-29 and COGA-13. Deregulations of miR-22 and miR-125b in patients suffering from CRC have been reported. However, we did not observe differences in their expression levels in our patient cohort when we compared their expression in the tumours with the adjacent mucosa of the same patient. We analyzed only G2 tumours (n=19) and maybe deregulations of both miRNAs occur in an earlier or later stage. However, we could show that IL-6 has a significant effect on the donwregulation of miR-22 in Caco-2/AQ colon cancer cells, indicating that IL-6 is involved in the regulation of the expression of miR-22.

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7 Abstract

Colorectal cancer (CRC) is one of the most common cancers in females and males and is the second leading cause of cancer-related deaths in Europe with 230.000 yearly deaths. Epidemiological studies point to a relationship between low serum 25 hydroxyvitamin D_3 (25-D_3) levels (the accepted measure of vitamin D in the body) and colorectal cancer risk indicating that Vitamin D may prevent tumour formation. Vitamin D metabolism is a highly regulated, multistep process that starts with the formation of previtamin D_3 in the skin mediated by ultraviolet radiation as well as intake from the diet. In the liver Vitamin D_3 is hydroxylated on position 25 to form 25-D_3. Circulating 25-D_3 is converted into 1,25 dihydroxyvitamin D_3 (1,25-D_3) which is the biologically most active vitamin D compound and has pleiotropic effects including regulation of cellular proliferation, differentiation and apoptosis. 1,25-D_3 is catabolised by the cytochrome P450 25-hydroxyvitamin D_3 24-hydroxylase which is encoded by the gene CYP24A1. Unbalanced high CYP24A1 levels were found in a variety of human malignancies, e.g. colorectal tumours.

MicroRNAs (miRNAs) are short non-coding RNAs with wide range of gene regulatory activity at the posttranscriptional level. miRNAs associate with several proteins in the RNA-induced silencing complex leading to mRNA degradation. Evidence is increasing that miRNAs play a key role in cancer progression. It has been shown that some become either over-expressed or silenced during tumour progression and metastasis, indicating a regulatory role in this process. Several miRNA species have been identified as 1,25-D_3 targets in human colon cancer cells, e.g. microRNA-22 (miR-22) which is induced by 1,25-D_3 and contributes to its antiproliferative, antimigratory and gene regulatory effects. It has been suggested that CYP24A1 is regulated post-transcriptionally by miR-125b. High expression levels of miRNA-125b are associated with enhanced malignant potential.

The aim of this study was to investigate the effect of 1,25-D_3 on the expression of miR-22 and miR-125b in cancer cell lines derived from colon (HT-29 and COGA-13) and prostate (DU145) tumours by Real-Time PCR and to assess the expression level of these two miRNAs in human colorectal tumour samples. Further we tested the effect of Interleukin-6 (IL-6) and tumour necrosis factor alpha

(TNF-α), which play an important role in tumourgenesis, on the expression of miR-22 and miR-125b.

Our results show that 1,25-D_3 treatment affects the expression of miR-22 and miR-125b in HT-29 colon and DU-145 prostate cancer cells in a time- and concentration-dependent manner. IL-6 and TNF-α also have a time-dependent effect on the expression of both miRNAs. In addition, we did not observe significant differences in miR-22 and miR-125b expression levels between human tumour and mucosa samples, indicating that these microRNAs were similarly expressed in our patient cohort.

8 Zusammenfassung

Kolorektaler Krebs (CRC) ist einer der häufigsten Krebserkrankungen bei Frauen und Männern und die zweithäufigste zum Tode führende Form von Krebs in Europa mit 230.000 Sterbefällen jährlich. Epidemiologische Studien zeigen einen Zusammenhang zwischen niedrigem 25-Hydroxy-Vitamin-D_3 (25-D_3) Serumspiegel (der Metabolit zur Messung von Vitamin D im Körper) und kolorektalem Krebs und deuten darauf hin, dass Vitamin D die Krebsentstehung verhindern könnte. Der Vitamin-D-Stoffwechsel ist ein hochregulierter, mehrstufiger Prozess, beginnend mit der Bildung von Prävitamin D_3, das in der Haut durch UV-Strahlung synthetisiert oder als Nahrung aufgenommen wird. In der Leber wird Vitamin D_3 an der Position 25 hydroxyliert und es entsteht 25-D_3. Zirkulierendes 25-D_3 wird zu 1,25-D_3 konvertiert, welches die biologisch aktivste Form von Vitamin D ist und pleiotrope Eigenschaften einschließlich der Regulierung von Zellproliferation, Differentiation und Apoptose besitzt. 1,25-D_3 wird durch das Cytochrom P450 24A1 katabolisiert, welches durch das CYP24A1 Gen kodiert ist. Unausgewogen hohe Werte von CYP24A1 werden in verschiedenen humanen, malignen Tumoren gefunden, wie zum Beispiel in kolorektalem Krebs.

MicroRNAs (miRNAs) sind kurze, nicht-kodierende RNAs mit großer Bandbreite von genregulierender Aktivität auf posttranskriptionaler Ebene. miRNAs verbinden sich mit einigen Proteinen des RNA-induced silencing complex, was zu einer Degradierung von mRNAs führt. Es gibt immer mehr Hinweise, dass miRNAs eine Schlüsselrolle bei der Krebsentstehung spielen. Es wurde gezeigt, dass einige miRNAs während der Entstehung von Krebs und Metastasen entweder hochreguliert oder stillgelegt werden, was auf eine regulatorische Rolle während dieser Prozesse hindeutet. Einige miRNAs wurden als Zielgene von 1,25-D_3 identifiziert, wie z.B. microRNA-22 (miR-22), welche durch Vitamin D induziert werden kann und zu dessen antiproliferativen, antimigratorischen und genregulierenden Eigenschaften beiträgt. Es wurde vorgeschlagen, dass CYP24A1 posttranskriptionell von miR-125b reguliert wird. Hohe Expression von miR-125b wird mit erhöhtem malignem Potential assoziiert.

Das Ziel dieser Studie war es, den Effekt von 1,25-D_3 auf die Expression von miR-22 und miR-125b in Krebszelllinien des Kolon (HT-29 und COGA-13) und der Prostata (DU145) mittels Real-Time PCR zu untersuchen und die

Expressionswerte von beiden miRNAs in humanen kolorektalen Tumorproben zu bestimmen. Weiters haben wir den Effekt von Interleukin-6 (IL-6) und Tumornekrosefaktor-α (TNF-α), welche eine wichtige Rolle bei der Tumorentstehung spielen, auf die Expression von miR-22 und miR-125b getestet.

Unsere Resultate zeigen dass 1,25-D_3 Behandlung die Expression von miR-22 und miR-125b in HT-29 Kolon- und DU145 Prostatakrebs Zelllinien in einer zeit- und konzentrationsabhängigen Weise beeinflusst. IL-6 und TNF-α haben auch einen zeitabhängigen Effekt auf die Expression von beiden miRNAs. Des Weiteren haben wir keine signifikanten Unterschiede in der miR-22 und miR-125b Expression zwischen den kolorektalen Tumoren und der Schleimhaut entdeckt, was darauf hinweist, dass diese microRNAs in unserer Patientenkohorte gleich expremiert werden.

9 Curriculum Vitae

Education

1991-1995	Elementary school
	Volksschule in Weissenbach an der Triesting, Austria
1995-2005	High school
	Bundesgymnasium und Bundesrealgymnasium Berndorf, Austria
2006-present	University of Vienna, Austria
	Biology – genetics - microbiology

Extracurricular Activities

June 2010	University of Vienna, Austria – Department of genetics in ecology
	Practical course on the topic: *Analysis of community composition and nitrification activity in ammonia-oxidizing archaea enrichment cultures under different growing conditions*
July 2011	Medical University of Vienna, Austria – Department of Pathophysiology
	Practical course on the topic: *Prevention of preneoplastic lesions by dietary vitamin D in a mouse model of colorectal carcinogenesis*

Publications

Hummel D., Thiem U., Höbaus J., Mesteri I., **Gober L.**, Stremnizter C., Graca J., Obermayer-Pietsch B., Kallay E. (2013) Prevention of Preneoplastic Lesions by Dietary Vitamin D in a Mouse Model of Colorectal Carcinogenesis. Journal of Steroid Biochemistry and Molecular Biology 136: 284-288

Scientific Symposia – Posters

Gober L., Höbaus J., Fetahu I., Hummel D., Kallay E. Role of microRNAs in Regulating Vitamin D Signalling in Colorectal Cancer
9th YSA PhD-Symposium, in Vienna, Austria, 19th-20th of June, 2013

Höbaus J., Thiem U., Hummel D., Fetahu I., **Gober L.**, Manhardt T., Kallay E. Possible Causes and Consequences of the Overexpression of the Vitamin D Catabolizing Enzyme CYP24A1 in Colorectal Cancer
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Höbaus J., Thiem U., Fetahu I., Hummel D., **Gober L.**, Manhardt T., Kallay E. Gene Amplification but not Epigenetic Alterations Cause Aberrant Vitamin D 24-Hydroxylase Expression in Colorectal Cancer
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