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The goal of this study was to determine what region of the Dkk-1 promoter was required for expression of the Dkk-1 gene under different growth conditions. Dkk-1 promoter activity was analyzed in NIH3T3 cell lines stably transfected with luciferase vectors containing 1068, 535, and 228 bp of Dkk-1 upstream sequence that were growing, confluent, serum starved and during the cell cycle. Dkk-1 promoter activity was also analyzed in the cell lines treated with the inhibitors aphidicolin, methotrexate, and U0126. In general, we found that Dkk-1 promoter activity is higher in a growing cell population than in a nondividing population. The 1068 Dkk-1 promoter construct does not appear to change dramatically during the cell cycle nor is it sensitive to an S phase inhibitor or U0126. Methotrexate treatment was found to decrease promoter activity. A sequence region between 1068 and 535 appears to be important for regulation of Dkk-1 promoter activity.

# REGULATION OF THE DICKKOPF-1 PROMOTER SEQUENCES UNDER VARIOUS GROWTH CONDITIONS

By

Jasmin Denee' Feimster

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Master of Science

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Approved by

_____
Committee Chair

To the Feimster, Cherry, Brown, Hyman, and Houpe families;

My gratitude for your support is immeasurable.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro

Committee Chair_____
Karen Katula

Committee Members _____
Amy Adamson

_____
Paul Steimle

_____
Date of acceptance by Committee

_____
Date of Final Oral Examination

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# CHAPTER I

# INTRODUCTION

The mechanisms controlling cell proliferation and differentiation are of fundamental importance. Significantly, knowledge of these mechanisms has broad applications to human health, in particular cancer and stem cell therapeutics. The Wnt signaling pathway is involved in cell fate and control of proliferation. The canonical Wnt pathway is antagonized by Dickkopf-1 (Dkk-1), which prevents the formation of an efficient Wnt receptor complex and thus has many consequences for the cell including decreased Wnt signaling and the hindrance of Wnt induced transcriptional regulation. This can affect cell growth, cell differentiation, and possibly lead to apoptosis or cell death. Dkk-1 has been shown to be involved in mesenchymal and epithelial stem cell proliferation and differentiation. It has also been shown that Dkk-1 can act as a tumor suppressor. If Dkk-1 is absent then there is an increase in uncontrolled cell growth, which can lead to tumor formation. The control of Dkk-1 expression is not completely understood. This knowledge is important because Dkk-1 has a central role in modulating the Wnt pathway and stem cell fate. In this study, the expression of the Dkk-1 gene under different growth conditions will be analyzed. The region of the Dkk-1 promoter required for its expression will then be determined. Potential transcription factor binding sites will be identified within these regions using bioinformatic approaches. These sites will eventually be mutated to determine if they are involved in Dkk-1 expression.

## Background

*Regulation of Cell Growth and Division*

The behavior of a cell is carefully regulated by the signals that it receives and responds to from its environment. These signals are responsible for initiating intracellular events such as metabolism, movement, proliferation, and differentiation. Cell proliferation is dependent on the cell cycle, a highly regulated division cycle that consists of four processes: cell growth, replication of DNA, distribution of chromosomes to daughter cells, and cell division. A eukaryotic cell divides about every 24 hours. Its cell cycle is divided into two parts: mitosis (nuclear division) and interphase (cell growth and DNA replication). Interphase is the period between two mitosis stages and includes $G_1$, S and $G_2$ phases. During $G_1$ (gap 1) the cell is growing and preparing for replication of DNA. $G_1$ is followed by the S phase (synthesis) where DNA is replicated. $G_2$ (gap 2) is next where the cell continues to grow and proteins are made in preparation for mitosis. The M phase (mitosis) corresponds to separation of the replicated chromosomes and is usually followed by cytokinesis. There are four distinct phases of mitosis: prophase, metaphase, anaphase and telophase.

The ability of a cell to progress between the stages of the cell cycle is controlled by extracellular signals and internal signals responsible for coordinating the processes that take place during the cell cycle. One of the central signaling pathways in controlling cell proliferation is the Ras pathway. It is an intricate signaling cascade that plays an important role in transmitting signals from growth factor receptors, regulating gene expression, and preventing apoptosis (McCubrey et al, 2006). Activation of this pathway

begins with the stimulation of growth factor receptors leading to the activation of Ras (a small GTPase) (Cooper and Hausman, 2007). Ras interacts with the Raf protein kinase. Raf phosphorylates MEK (MAP kinase/ERK kinase) and MEK phosphorylates ERK (extracellular signal regulated kinase). Once ERK is phosphorylated it is responsible for phosphorylating many other target proteins such as transcription factors and protein kinases (Cooper and Hausman, 2007). Many studies have shown that the Ras pathway becomes hyperactivated during human cancer development.

One important consequence of increased Ras signaling is upregulation of cyclin D expression. Cyclin D protein then complexes with its partner, cyclin dependent kinase (cdk) 4 or 6 which functions as a kinase, phosphorylating various substrates. Cyclin D-cdk 4/6 activity eventually leads to passage from $G_1$ phase into S phase. At this point the cell is independent of growth factors and proceeds through the cell cycle and mitosis. Progression through the cell cycle results from the sequential activity of different cyclin-cdk kinases. In order of activity these are cyclin D-cdk 4/6 ($G_1$), cyclin E-cdk 2 ($G_1$/S), cyclin A-cdk 2 (S), and cyclin B-cdk 1 (M).

The availability of growth factors in animal cells has an important role in the cell proliferation processes such as growth, DNA replication and mitosis. The regulation of these processes involves several checkpoints that control progression through the phases of the cell cycle. If growth factors are not available during $G_1$ then the cells enter $G_0$, a quiescent stage of the cell cycle. Most proliferation checkpoints are regulated in $G_1$ and along with regulating cell cycle progression they also are coordinated with one another to ensure that the events of the cell cycle take place in a sequential order (McCubrey, 2006).

*The Wnt signaling pathway and Control of Cell Growth and Differentiation*

Cell division within tissues of vertebrates is highly regulated and for the most part, occurs within the undifferentiated stem cell populations. Those stem cells respond to internal and external signals to tell them when to divide. One of the important signaling pathways involved in controlling stem cells is the Wnt pathway. The canonical Wnt pathway begins with the binding of Wnt ligands to the frizzled transmembrane receptor and LPR 5 and 6, which are two single-transmembrane protein members of the LDL receptor-related protein (LRP) family (Schweizer and Varmus, 2003) (Fig. 1A). Once frizzled is activated, it recruits the cytoplasmic bridging molecule Disheveled, which inhibits glycogen synthetase kinase 3 (Gregory et al, 2003). This inhibition decreases phosphorylation of β-catenin thus preventing β-catenin from being degraded by the ubiquitin pathway (Fig. 1A) (Kikucji, 2000; Huelsken and Birchmeier, 2001). β-catenin then moves to the nucleus where it forms a complex with the transcription factors leukocyte-enhancing factor (LEF-1) and T-cell factor (TCF-1), which then activates transcription of target genes (Liu et al, 2003). These transcription factors promote differentiation or proliferation (Liu et al, 2003). There are a large number of Wnt ligands that function in different cell types and during development (Miller, 2001).

One example of the role of Wnt signaling and the control of cell division and differentiation is in the intestinal epithelium. The Wnt signaling pathway is thought to play a major role in controlling stem cell proliferation in the gut, namely the crypt cell population (Crosnier at al, 2006). When the Wnt pathway is over activated by mutations in components such as β-catenin and adenomatous polyposis coli (APC), the downstream

targets of the pathway are activated. The crypts become enlarged and polyps form, leading to some colorectal cancers. If the Wnt pathway allows cells to proliferate indefinitely, a large number of cell cycles can result generating masses of giant crypt cells. Therefore, regulating the Wnt pathway is important to prevent polyps from growing without limit (Kuhnert et al, 2004).

Wnt signaling is also critical in mesenchymal differentiation and has been shown to be involved in differentiation of skeletal and cardiac muscle, adipocytes, bone and cartilage (Horowitz, 2004).

*Dkk-1 Structure and Function*

Dkk-1 is a secreted agonist of canonical Wnt signaling that interacts with Wnt coreceptors (Gregory, 2003). In humans, Dkk-1 mRNA is 1681 base pairs in length and codes for a 35 kDa protein (Kuhert et al, 2004). The binding of Dkk-1 to LPR-5/6 allows for the disassociation of LPR-5/6 from frizzled and prevents the formation of an efficient Wnt receptor complex (Gregory, 2003) (Fig. 1B). This in turn causes decreased Wnt signaling, an unstable β-catenin molecule, and hinders Wnt-induced transcriptional regulation (Gregory, 2003). This affects cell fate and cell growth and can lead to apoptosis (Fedi et al, 1999; Grotewold and Ruther, 2002).

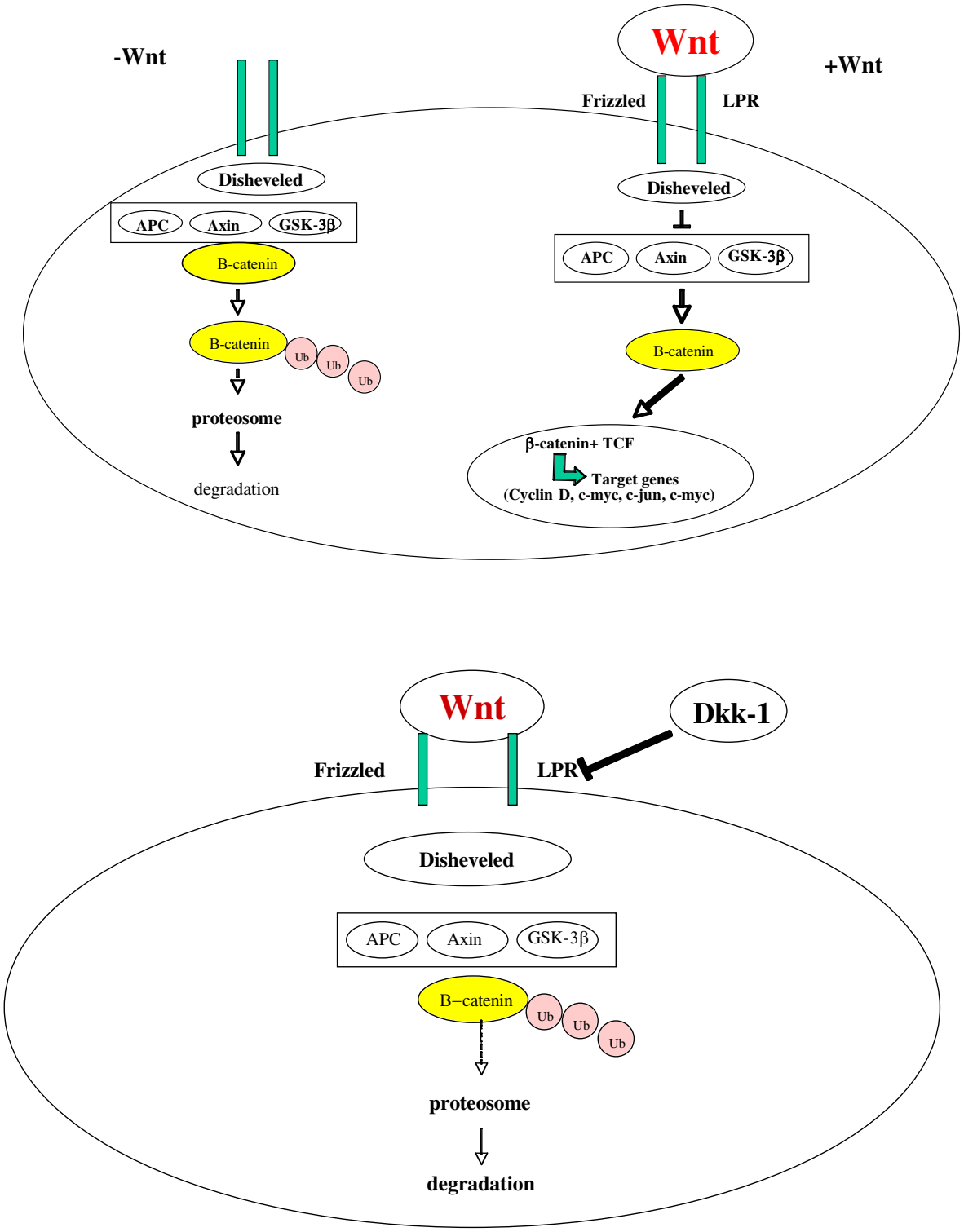



**Fig. 1** Wnt signaling pathway. **A)** Binding of Wnt ligands leads to stabilization of β-catenin, which moves to the nucleus and functions as a transcription factor. **B)** Inhibition of the Wnt pathway by Dkk-1. Dkk-1 binds to the LPR receptors and increases disassociation of the LPR receptors from the frizzled receptor.

*Role of Dkk-1 in Cell Proliferation*

Dkk-1 has been shown to be involved in human bone mesenchymal stem cell proliferation (Gregory et al, 2003). Mesenchymal stem cells give rise to a wide variety of cell types including cardiac muscle, fibroblasts (NIH3T3 cells), cartilage, bone, skeletal muscle, fat, stroma, and brain tissue (Horwitz, 2004). Synthesis of Dkk-1 causes the cells to re-enter the cell cycle by inhibiting the Wnt pathway. This was demonstrated using human

mesenchymal cell (hMSC) lines cultured from the bone marrow of human donors. The cell cycle was analyzed to determine the stages of cell growth and the effects of Dkk-1 on proliferation. Initially, hMSC growth during early log phase was arrested after replacing conditioned media with fresh media containing no Dkk-1. The addition of conditioned media from rapidly dividing hMSCs increased cell division (Gregory et al 2003). The level of secreted Dkk-1 protein was analyzed by methoinine labeling and shown to be high in the conditioned medium. The results demonstrate that the cells do not leave an extended log phase and reenter the cell cycle until they have synthesized Dkk-1 and the level of Dkk-1 has accumulated in a significant amount in the culture media. This was further proven by adding recombinant Dkk-1 to the hMSC cells in early log phase of growth. Cells treated with Dkk-1 protein showed increased proliferation compared to control without Dkk-1. As the hMSC cells moved from the log phase of cell growth to the stationary phase of cell growth, the level of Dkk-1 was found to decrease and the amount of Wnt-5A increased (Gregory et al, 2003).

In contrast, Dkk-1 has also been found to function as a tumor suppressor. This means that in the absence of Dkk-1 there is a lack of growth control and increased tumorigenesis. This was shown in two studies. In the paper by Gonzales-Sancho et al (2005), Dkk-1 expression was analyzed in 32 pairs of human colon tumors and in normal tissues. RT-PCR analysis showed that Dkk-1 was downregulated in colon tumors when compared to normal tissue. This decrease may in part, be due to inactivation of the Dkk-1 promoter by hypermethylation of the promoter (Aquilera et al, 2006). In another study, Dkk-1 was isolated from non-tumorgenic revertants of HeLa cells as a gene that increased in activity (Mikheev et al, 2004). In this study, overexpression of Dkk-1 was shown to increase apoptosis but not to affect cell growth. Also, this effect did not involve the canonical β-catenin pathway. These studies implicate Dkk-1 as a tumor suppressor.

*Regulation of Dkk-1 gene expression*

The Dkk-1 gene has been shown to be upregulated by β-catenin/TCF resulting in a negative feedback loop. In one study, a region of the Dkk-1 promoter containing nine binding sites for TCF was cloned to examine the regulation of Dkk-1 gene (Gonzales-Sancho et al, 2005). The roles of various TCF sites were then examined by generating deletion constructs made from the Dkk-1 promoter. The results showed that the TCF sites close to the initiation site and in the –909/-822 region contributed most significantly to the increase in Dkk-1 expression by activation of the Wnt pathway. The authors also examined Wnt proteins that signal in non-canonical and canonical pathways and how they induce the Dkk-1 promoter. They co-cultured cells that express Wnt-1 (canonical β-catenin) or Wnt-5A (non-canonical) with cells transfected with the Dkk-1 promoter

reporter construct. Co-culture with Wnt-1 led to the activation of Dkk-1 promoter but Wnt-5A did not. When cells expressing Wnt-1 were co-transfected with a Dkk-1 expression vector the activity from the Dkk-1 promoter was reduced, thus Dkk-1 protein can block transcription from its own promoter via negative feedback.

In another study, transcriptional targets of β-catenin were identified by microarray analysis (Chamorro et al, 2005). Dkk-1 was identified as a gene to be strongly induced. Further analysis showed, as in the study by Gonzales Sancho et al (2005), that the Dkk-1 promoter contains TCF binding sites that are required for this response to β-catenin activation. These studies provide strong support that Dkk-1 is upregulated by the activation of β-catenin.

Dkk-1 was found to be upregulated by p53. p53 is a tumor suppressor and plays a role in response to DNA damage and apoptosis. Messenger RNA from a cell line, p53-3, that expresses p53 was assayed and cDNA fragments were isolated (Wang et al, 2000). One clone was shown to be a derivative of the Dkk-1 gene. It was found that wild type p53 induces Dkk-1 and the amount of Dkk-1 in cells expressing p53 was 6-8 times higher than those cells not expressing p53 (Wang, 2000). Results also indicated that in cell lineages with endogenous wild type p53, Dkk-1 is induced by DNA damage. To test this, five cell lines were treated with camptothecin (a DNA damaging agent) and the levels of Dkk-1 and p21 were analyzed (Wang, 2000). Three of the cell lines, which carried the endogenous wild type p53 induced both Dkk-1 and p21. Dkk-1 was not induced in the other two cell lines, which carried a mutant p53 gene. It was also necessary to determine if Dkk-1 is regulated transcriptionally by p53. To do this the authors looked for a p53

responsive element in the Dkk-1 DNA sequence. A 3.4 kb region in the Dkk-1 gene was sequenced and a potential binding site for p53 was located 2.1 kb upstream from the Dkk-1 transcription start site. This site was shown to bind p53 and allow for p53 activation of a Dkk-1 luciferase reporter construct (Wang, 2000).

The Dkk-1 gene has also been shown to be induced by glucocorticoid (Ohnaka et al, 2004). The addition of dexamethasone to human osteoblasts increased the expression of Dkk-1 mRNA in a dose and time dependant manner. Dexamethasone also increased the activity of the Dkk-1 promoter as assayed with the Dkk-1 luciferase reporter. Three glucocorticoid response elements were identified in the Dkk-1 promoter between –837 and –314 base pair upstream (Ohnaka, 2004). More recently, Dkk-1 expression has been shown to be increased by progesterone in human endometrial stromal cells (Tulac et al, 2006).

Other transcription factors shown to activate Dkk-1 include BMP-4 and c-Jun (Grotewold and Ruther, 2002). In this study, these transcription factors upregulated Dkk-1 in the normal developing embryo and in embryos subjected to ultraviolet radiation and other genotoxic substances.

A diagram of the Dkk-1 upstream promoter region with the locations of characterized transcription factor binding sites is shown in Fig. 2. Overall, the above studies suggest that Dkk-1 is regulated by multiple factors under diverse conditions. However, the factors involved in regulating Dkk-1 expression during cell growth are still not known.

**Project Goals and Overview**

The goal of this study was to characterize the promoter of the Dkk-1 gene and determine what region of the promoter is required for regulated expression of the Dkk-1 gene under different growth conditions. To do this, we first analyzed the activity of the Dkk-1 gene under different growth conditions and during the cell cycle. The hypothesis was that the activity of the Dkk-1 promoter will vary expanding cell populations and low in confluent and serum starved, non-dividing cells depending on the growth state of the cell population. Second, we wanted to determine the region of the Dkk-1 promoter required for its activity under selected growth conditions. The hypothesis for this goal was that there is a specific region in the promoter sequence where transcription factors bind that are necessary for Dkk-1 activity under different growth conditions. When this region is removed or modified, we believed there would be little or no Dkk-1 activity, or a modified pattern of expression. Finally, we wanted to identify the transcription factors involved in regulating the activity of the Dkk-1 promoter under selective growth conditions. The hypothesis for this was that specific transcription binding sites would be identified in the regions of the Dkk-1 sequences required for its regulation. Mutation of those sites should alter expression of Dkk-1 under different growth conditions.

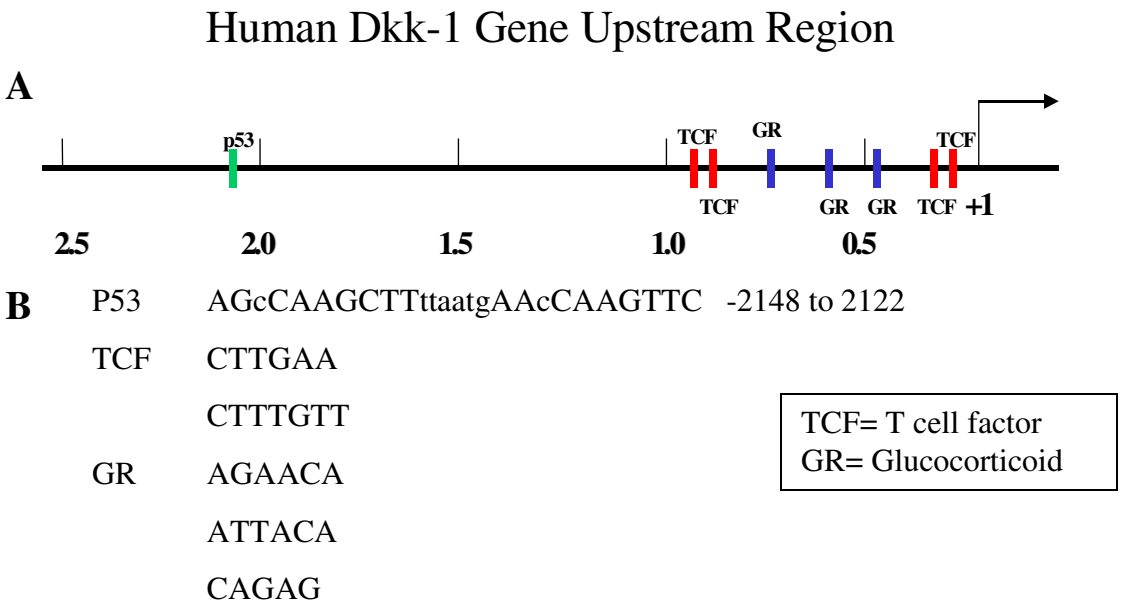



**Fig 2**. Dkk-1 Upstream Promoter Region with Transcription Factor Binding Sites. Binding sites for only those transcription factors that have been characterized are shown. The DNA sequences and positions for the factors are indicated below the diagram (B). Data was derived from Wang et al 2000 (p53), Chamorro et al 2005 (T Cell Factor), and Ohnaka et al 2004 (Glucocorticoid).

## CHAPTER II

## MATERIALS AND METHODS

Cell culture

The mouse fibroblast cell line NIH3T3 was obtained from the American Type Culture Collection (ATCC) and cells were cultured in DMEM-C at 37$^{o}$ Celsius in an incubator containing 5% carbon dioxide and 95% air. DMEM-C medium contains 88.8% (v/v) Dulbecco's Modified Eagles Medium (DMEM) with 3.7g/L sodium bicarbonate and 3.57 g/L HEPES, 10% Fetal Bovine Serum, 1.0% (v/v) Penicillin/Streptomycin (10,000 IU/ml and 10,000 μg/ml) and 0.1% (v/v) Amphotericin fungicide (250 μg/ml).

Preparation of Stable Cell lines

Dkk-1-luciferase plasmids (Dkk-1 LUC) containing 1068, 535, and 228 base pairs of upstream sequence and 48 base pairs downstream of the +1 site were obtained from Chamorro et al (2005). Stable lines of NIH3T3 mouse fibroblast cells were prepared using the Calcium-Phosphate method. The Dkk-1 LUC vectors were mixed with the neomycin (G418) selection vector (pMC1neopolyA; Stratagene) at a ratio of 4:1, respectively. The DNA was precipitated together by making the solution 0.2 M NaCl and adding two volumes of cold 95% ethanol and placing the samples at –20 $^{o}$ Celsius. The co-precipitated Dkk-1 LUC/neo DNA was pelleted and taken up in 100 μL of sterile

water at a concentration of 0.5 μg/μL. The NIH3T3 mouse fibroblast cells were collected and re-plated for transfection in 100 mm plates at $2x10^5$ cells per plate. The Dkk-1 LUC/neo DNA was transfected using the Calcium-Phosphate Profection Kit (Promega). Essentially, 10 μL (10 μg) of Dkk-1 LUC+neo was mixed with the 5M $CaCl_2$ solution and the mixture added to 2x HEPES buffer while vortexing. The sample was incubated at room temperature for 30 minutes and then added dropwise to the cells. The next day, the medium was changed to fresh DMEM-C. Three days after transfection, 25 μL of G418 (200 mg/mL) was added to each plate of cells to give a final concentration of 0.5 mg/mL. Selection continued until all of the cells lacking the neo-selection vector were killed and colonies began to form. During this period, fresh medium was added every three days along with G418. The resistant cells were collected by trypsinization and re-plated in T-75 flasks. Stocks of resistant cells were stored in liquid nitrogen. For storage, cells were collected and resuspended in DMEM-C and mixed 1:1 with 10% dimethyl sulfoxide in DMEM-C. These cells were transferred to freezing tubes and frozen for 24 hours at $-70^o$ Celsius before transferring them to liquid nitrogen.

Collection of Cells for Promoter Analysis Studies

Typically stably transfected Dkk-1 LUC cells were plated on a Friday for experiments the following week. Frozen stocks were plated in 12 mL of DMEM-C in a T-75 flask to which 10 μL of G418 (200mg/mL) was added. On Monday, the cells were collected and re-plated in the appropriate culture plate. To collect the cells, they were first washed with 4 mL of sterile Phosphate Buffered Saline (PBS). The PBS was removed and 4 mL of 1x Trypsin-EDTA (0.05%; 0.53 mM) was added to the flask. After

about one minute the cells were observed under a microscope to ensure that they had come off of the flask. An equal volume of DMEM-C was added and the entire contents of the flask were transferred to a 15 mL Falcon tube. The tubes were spun in a clinical centrifuge at setting #3 (1080 RPM) for 5 minutes. The supernant was removed and the pelleted cells were taken up in 5 mL of DMEM-C. The cells were counted using a hemacytometer and the appropriate cell density was plated for each experiment and culture plate.

Growing and Confluent Cell Populations

On the first day, collected 1068Dkk-1 LUC, 535Dkk-1 LUC, and 228Dkk-1 LUC-NIH3T3 cells were plated in DMEM-C in a 24 well culture dish at a density of $4x10^4$ cells per well. On the next day, the medium was changed to fresh DMEM-C. Cell lysates were collected from four wells, representing Day 1. Each day for the next four days, the medium was changed and the cell lysates were collected. The cells were assayed for luciferase activity and the protein concentration of the lysate determined. A cell line containing pGL3-Basic, the luciferase vector without a promoter, was used as a control.

Serum Starved and a Growing Population

On the first day, 1068Dkk-1 LUC, 535Dkk-1 LUC, and 228Dkk-1 LUC-NIH3T3 cells were plated in DMEM-C in two 24 well culture dishes at a density of $4x10^4$ cells per well. On the next day, the medium was changed in one plate (designated Low Serum) to DMEM-0.5% Fetal Bovine Serum and in the other plate to fresh DMEM-C. Cell lysates were collected from three wells of each plate (designated Day 1). Each day for the next

four days, the medium was changed to DMEM-C or Low Serum DMEM and the cell lysates were collected. The cells were assayed for luciferase activity and the protein concentration of the lysate determined. A cell line containing pGL3-Basic, the luciferase vector without a promoter, was used as a control.

Cell Cycle Analysis

On the first day, 1068Dkk-1 LUC, 535Dkk-1 LUC, and 228Dkk-1 LUC-NIH3T3 cells were plated in DMEM-C in a 24-well culture dish at a density of $4x10^4$ cells per well. On the next day, the medium was changed to DMEM-0.5% Fetal Bovine Serum (Low serum) to serum starved the cells for a period of 60-72 hours. After this period, the cells were released back into the cell cycle by changing the medium to DMEM-C containing 10% FBS. Cell lysates were collected beginning at 9 AM and then every 6 hours for 30 hours. The cells were assayed for luciferase activity and the protein concentration of the lysate determined. A cell line containing pGL3-Basic, the luciferase vector without a promoter, was used as a control.

Treatment with Inhibitors

*Aphidicolin*

On the first day, 1068Dkk-1 LUC, 535Dkk-1 LUC, and 228Dkk-1 LUC-NIH3T3 cells were plated in a 24-well culture dish in DMEM-C at a density of $6x10^4$ cells per well. The next day, the cells were treated with the DNA ploymerase inhibitor Aphidicolin (Calbiochem) from a 1 mg/mL stock in dimethyl sulfoxide (DMSO). To make a 1:4 dilution, 5 μL of stock was added to 15 μL of DMSO in a sterile microfuge

tube. The cells were treated with 0 μM, 0.5 μM and 2 μM aphidicolin for 24 and 48 hours. To the cells treated with 0 μM, 2 μL of DMSO was added, 2 μL of the 1:4 dilution was added to cells treated with 0.5 μM, and 2 μL of the stock solution was added to the cells treated at 2 μM. Twenty-four hours later, half of the cells were collected and the lysates stored at -20$^{o}$ Celsius. The next day, the remainder of the cells were collected and stored at -20$^{o}$ Celsius in microfuge tubes. The cell lysates were assayed for luciferase activity and the protein concentration of the lysates determined. A cell line containing pGL3-Basic, the luciferase vector without a promoter, was used as a control.

*Methotrexate*

On the first day, 1068Dkk-1 LUC, 535Dkk-1 LUC, and 228Dkk-1 LUC-NIH3T3 cells were plated in two 24-well culture dishes in DMEM-C at a density of 6x10$^{4}$ cells per well. The next day, the cells were treated Methotrexate (Fluka) from a 600 μM stock. To make a 600 μM stock solution, 2.37 mg of methotrexate was mixed with 2 mL of water. Five μL of 5N NaOH was added to bring the methotrexate into solution. The solution was brought up to 10 mL with water and it was filter sterilized. The cells were treated at concentrations of 0 μM (control), 1 μM, 10 μM, 20 μM, 50 μM, and 100 μM of Methotrexate by addidng 0 μL, 0.83 μL, 16.6 μL, 41.5 μL, and 83 μL of the 600 μM methotrexate stock to the appropriate wells. Incubation was for 24 and 48 hours. The next day, the cells in one of the plates were collected and the lysates were placed in microfuge tubes and stored at -20$^{o}$ Celsius. The following day, the cells from the remaining plate were collected, placed in microfuge tubes, and stored at -20$^{o}$ Celsius. The cell lysates were assayed for luciferase activity and the protein concentration of the lysate

determined. A cell line containing pGL3-Basic, the luciferase vector without a promoter, was used as a control.

*MEK 1/2 Inhibitor, U0126*

On the first day, 1068Dkk-1 LUC, 535Dkk-1 LUC, and 228Dkk-1 LUC-NIH3T3 cells were plated in a 24-well culture dish in DMEM-C at a density of $6x10^4$ cells per well. The next day, the cells were treated with the inhibitor MEK 1/2 U0126 (Promega). To the vial of U0126, 234 μL of DMSO was added to make a 10 μM stock (**A** stock). To make a 1:5 dilution, 40 μL of **A** was added to 160 μL of DMSO in a sterile microfuge tube (**B** stock). The cells were treated at concentrations of 0μM (control), 10 μM, and 50 μM. To the control, 2.5μL of DMSO was added, 2.5 μL of **B** stock was added to cells treated at 10 μM, and 2.5 μL of the **A** stock was added to the cells treated at 50 μM. The next day, half of the cells were collected and the lysates were placed in microfuge tubes and stored at -20$^o$ Celsius. The following day, the remaining cell lysates were collected, placed in microfuge tubes, and stored at -20$^o$ Celsius. The cells were assayed for luciferase activity and the protein concentration of the lysate determined. A cell line containing pGL3-Basic, the luciferase vector without a promoter, was used as a control.

<u>Collection of Cells for Luciferase and Protein Assay</u>

To collect the cells in preparation for luciferase assay, the medium was removed from each of the wells in the dish. Five hundred microliters of PBS was added to each and then removed by vacuum suction. One hundred and fifty microliters of 1x Reporter Lysis Buffer (Promega) was added to the cells and incubated at 37 $^o$ Celsius for 15

minutes. After incubation, the cell lysates were collected from the wells, placed in labeled microfuge tubes, and stored at -20 $^{o}$ Celsius.

*Luciferase assays*

Cells were assayed for luciferase activity using a Berthold luminometer (Lumat LB9501). Relative light units (RLUs) of luciferase activity were determined by placing 30μL of cell lysate in a tube. After inserting the tube into the luminometer, the arm was lowered to automatically inject luciferase substrate (luciferin) solution (Promega). The sample was read for 10 seconds. The activity levels were expressed as RLUs per μg protein.

*Protein Assay*

The level of protein was measured using a Bradford colormetric assay (BioRad). A standard curve was prepared using 1 mg/mL bovine serum albumin (BSA) as the standard. Six microfuge tubes were labeled. To the first microfuge tube, 800 μL of water were added. To the next, 1 μL of BSA (1 μg) was added to 799 μL of water. The third tube contained 5 μL (5 μg) of BSA and 795 μL of water. The fourth tube contained 10 μL of BSA (10 μg) and 790 μL of water. The fifth tube contained 15 μL of BSA (15 μg) and 785 μL of water. The last tube contained 20 μL of BSA (20 μg) and 780 μL of water. 200 μL of Bradford dye was added and each tube was vortexed. To prepare the samples, additional tubes were labeled and 780 μL of water was added to the sample tubes, along with 20 μL of the cell lysate and 200 μL of Bradford dye. Each tube was vortexed. The contents of each tube were transferred into spectrophotometer cuvettes. The standards and samples were read at 595 nanometers. A graph of the standard curve

was made using a hand held calculator and the amount of protein in each sample was extrapolated from the standard graph.

*Data Analysis*

The raw values of RLU's and μg protein were entered into Microsoft Excel 2003. The RLU/μg of protein was calculated and the four replicas were averaged. A graph was made using the average. Error bars indicate the standard error, which was calculated by taking the standard deviation divided by the square root of n (where n=number of replicas; which in most cases was 4).

Analysis of Potential Transcription Factor Binding Sites

First, the upstream sequences (2500 bp) of the Dkk-1 promoter were found using the UC-Santa Cruz website (http://genome.ucsc.edu/). Accessing the Dkk-1 Ref Seq gene was accomplished using the Accession number NM_012242. The sequence region from –1068 to –535 upstream from the +1 start site was copied and pasted into the Patch site (http://www.gene-regulation.com/pub/programs.html#match) for identification of potential transcription factor binding sites. The search conditions were set at 2 mismatches, no less than 5 nucleotide binding sites, and a lower score boundary of 87.5.

Transient Transfection of Dkk-1 Promoter Constructs

NIH3T3 cells were transfected with plasmid DNA using the *Trans*IT-3T3 Transfection Kit (Mirus). NIH3T3 cells were plated in a 24-well dish at a density of $1.5x10^5$ cells per well in DMEM- C. The next day, the cells were transfected with the plasmid DNA. First, 50 μL of serum free medium and 1.5 μl of *Trans*IT –3T3

Transfection Reagent was added to 12 sterile microfuge tubes and the contents were mixed. 0.5 μg of the 4 plasmid DNA constructs (pGL3, 228, 535, and 1068) was added to each of 3 tubes and mixed. 1 μL (10 ng) of SV40-Renilla luciferase reporter plasmid (Promega) was added to each tube and mixed. 0.5 μL of Authority Reagent was added and the tubes were incubated at room temperature for 30 minutes. After incubation, the *Trans*IT –3T3 Transfection Reagent/DNA/3T3 Authority Reagent mixture was added to the cells dropwise. The dish was rocked back and forth to distribute the complexes evenly. The cells were incubated for 48 hours.

The cells were collected as previously described. To assay the cells, Renilla Luciferase Assay Reagent was prepared (Promega) by adding 10 μL of Renilla substrate to 1 mL of Renilla Luciferase Assay buffer. The assay was performed in a Berthold luminometer (Lumat LB9501). Relative light units (RLUs) of Renilla activity were determined by placing 30μL of cell lysate in a tube. After inserting the tube into the luminometer, the arm was lowered to automatically inject Renilla substrate. The sample was read for 10 seconds. The cell lysates were then assayed for luciferase activity using luciferase substrate (luciferin) solution (Promega). Relative light units (RLUs) of luciferase activity were determined by placing 30μL of cell lysate in a tube. After inserting the tube into the luminometer, the arm was lowered to automatically inject luciferase substrate solution. The sample was read for 10 seconds. The activity levels for each construct (pGL3, 228, 535, and 1068) were expressed as Beatle RLU/ Renilla RLU. The raw values of Beatle and Luciferase RLU's were entered into Microsoft Excel 2003. The Beatle RLU/Renilla RLU was calculated and the 3 replicas were averaged. A graph

was made using the average. Error bars indicate the standard error, which was calculated by taking the standard deviation divided by the square root of n (where n=number of replicas).

# CHAPTER III

# RESULTS

Relative Expression Levels and Generation of cell lines for analysis of Dkk-1 promoter activity

Three Dkk-1 luciferase reporter vectors were obtained from Chamarro et al (2005). These contained 228 base pair, 535 base pair and 1068 base pair of upstream sequence plus 43 base pair of sequence downstream of the transcription start site (Fig. 3). These constructs were transfected into NIH3T3 cells along with a neo-poly A selection vector and selected with G418 to generate stable lines. The resulting resistant clones were collected as a group. The luciferase reporter vector pGL3-Basic was also stably transfected as a control. The relative levels of promoter activity from the plasmids were measured by transient transfection. Activity was normalized to *Renilla* luciferase activity from a co-transfected SV40-*Renilla* luciferase vector. As shown in Fig. 4, the activity from the 228Dkk-1 LUC construct is greater than the 535 construct but not significantly different from the 1068 construct.

Analysis of Dkk-1 expression under different growth conditions

Dkk-1 mRNA levels have been shown to vary in mesenchymal stem cell populations (Gregory et al, 2003). In expanding cell populations, Dkk-1 mRNA levels are high and the levels decrease in non-dividing confluent cells. Moreover, Dkk-1 mRNA levels decrease in serum starved cells (Gregory et al, 2005). In these studies, the activity

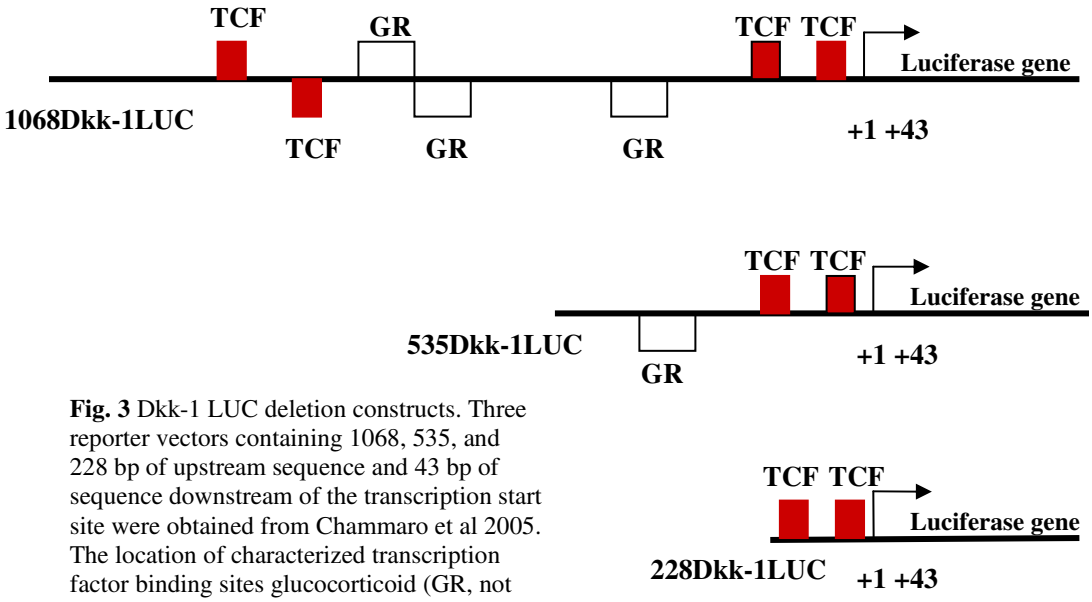



**Fig. 3** Dkk-1 LUC deletion constructs. Three reporter vectors containing 1068, 535, and 228 bp of upstream sequence and 43 bp of sequence downstream of the transcription start site were obtained from Chammaro et al 2005. The location of characterized transcription factor binding sites glucocorticoid (GR, not shaded) and T cell factor (TCF, shaded) are shown.

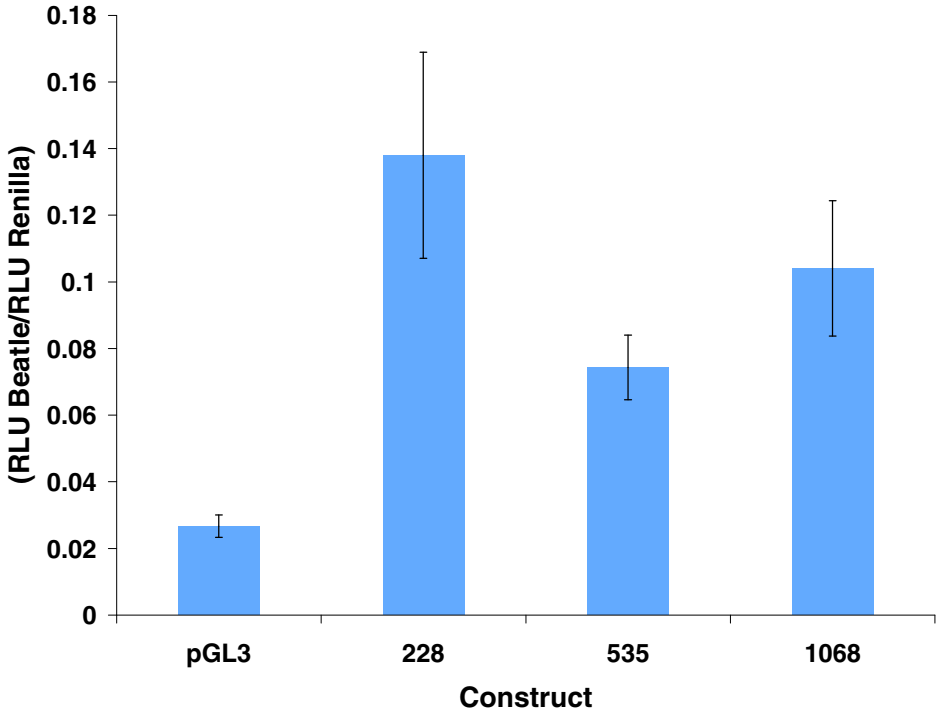



**Fig. 4** Transient Transfection of pGL3, 228Dkk-1 LUC, 535Dkk-1 LUC, and 1068Dkk-1 LUC. The cells were transiently transfected with each of the constructs and a SU40-Renilla luciferase control vector. Two days after transfection cells were collected and assayed for firefly and *Renilla* luciferase. The activity is expressed as Beatle RLU/Renilla RLU. The error bars represent the mean +/- standard error (n=3).

of the promoter was analyzed under different growth conditions. These included growing and confluent cells, normal and serum starved cells and different stages of the cell cycle.

*Growing and Confluent Cells*

Each of the NIH3T3 cell lines (1068, 535, 228, and pGL3) was plated in 24-well dishes at subconfluent density. The cells were allowed to grow for five days and the media was changed each day. The cells were collected each day at the same time. The luciferase activity and protein were determined as described and the promoter activity was expressed in relative light units per microgram of protein (RLU/μg protein).

The 1068Dkk-1 LUC cells had an activity level that increased up to Day 3 and then decreased from Day 3 to 5 (Fig. 5A). The pattern of Dkk-1 promoter activity in the 535 cell line was distinct (Fig 5A). The activity increased steadily throughout all five days. For the 228Dkk-1 LUC construct, the promoter activity increased at Days 1 through 3. There was a decrease in promoter activity from Day 3 to 5 (Fig 5B). Activity in the pGL3 control cell line increased at Days 1 through 4 (Fig 5B). At Day 5 the promoter activity decreases. Visual examination of the cells confirmed that the cells were confluent by Day 5 in all cell lines. These experiments were repeated one time with similar results.

*Growing and Serum Starved*

The stably transfected NIH3T3 cells (pGL3, 228, 535, and 1068) were plated in 24-well dishes at a sub-confluent density. The next day, the medium was changed in half of the wells to low serum. The medium was also changed in the other wells (complete

## Growing and Confluent

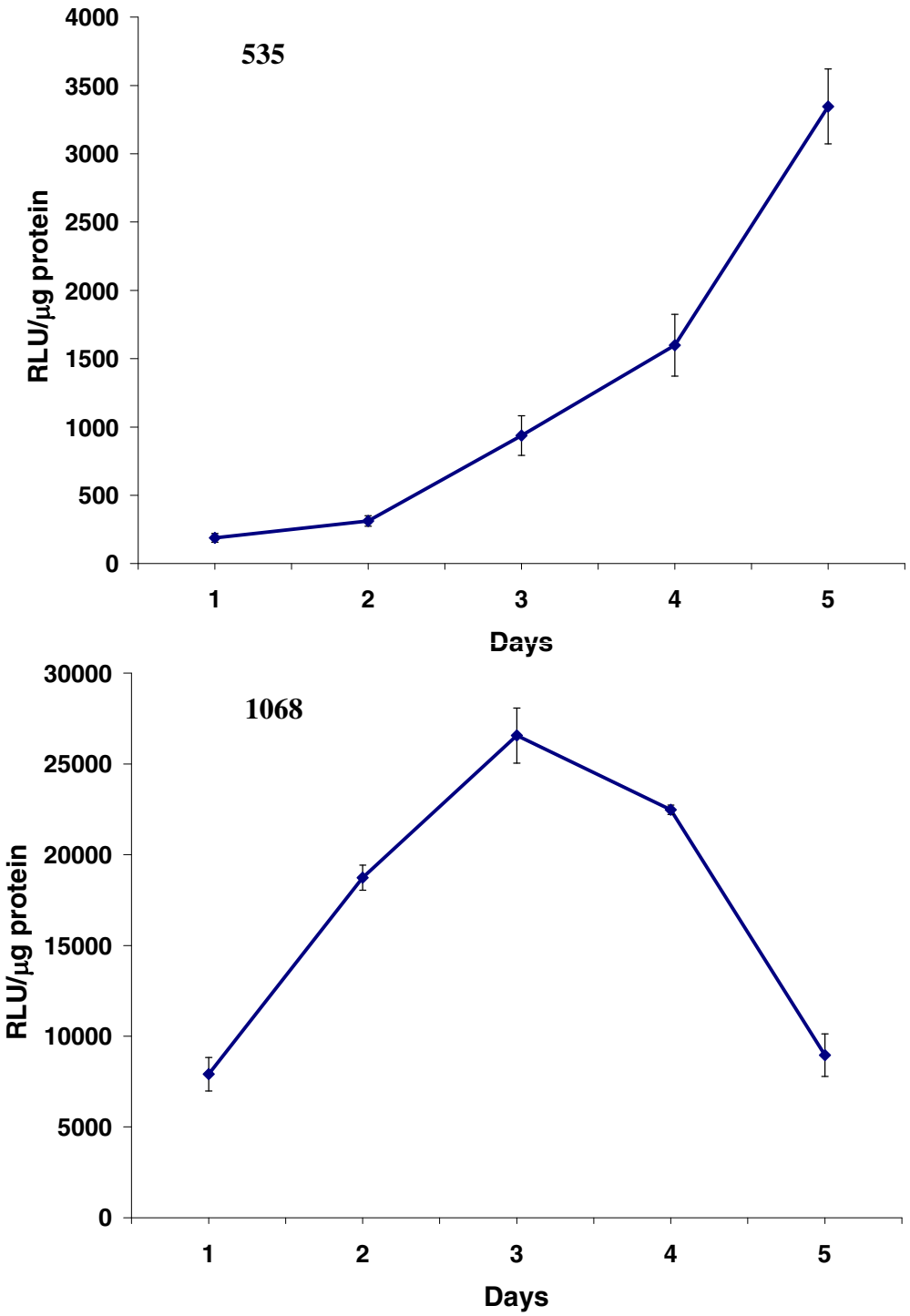



**Fig. 5A** Promoter activity in Growing and Confluent Cell Populations in 535Dkk-1LUC and 1068Dkk-1 LUC. Cells stably transfected with the indicated Dkk-1 luciferase reporter vectors were plated and allowed to grow over the days indicated. The lysates were collected each day for 5 days and assayed for luciferase (RLU) and total protein determined. The activity is expressed as RLU per µg of protein. The error bars represent the mean +/- standard error (n=4).

# Growing and Confluent

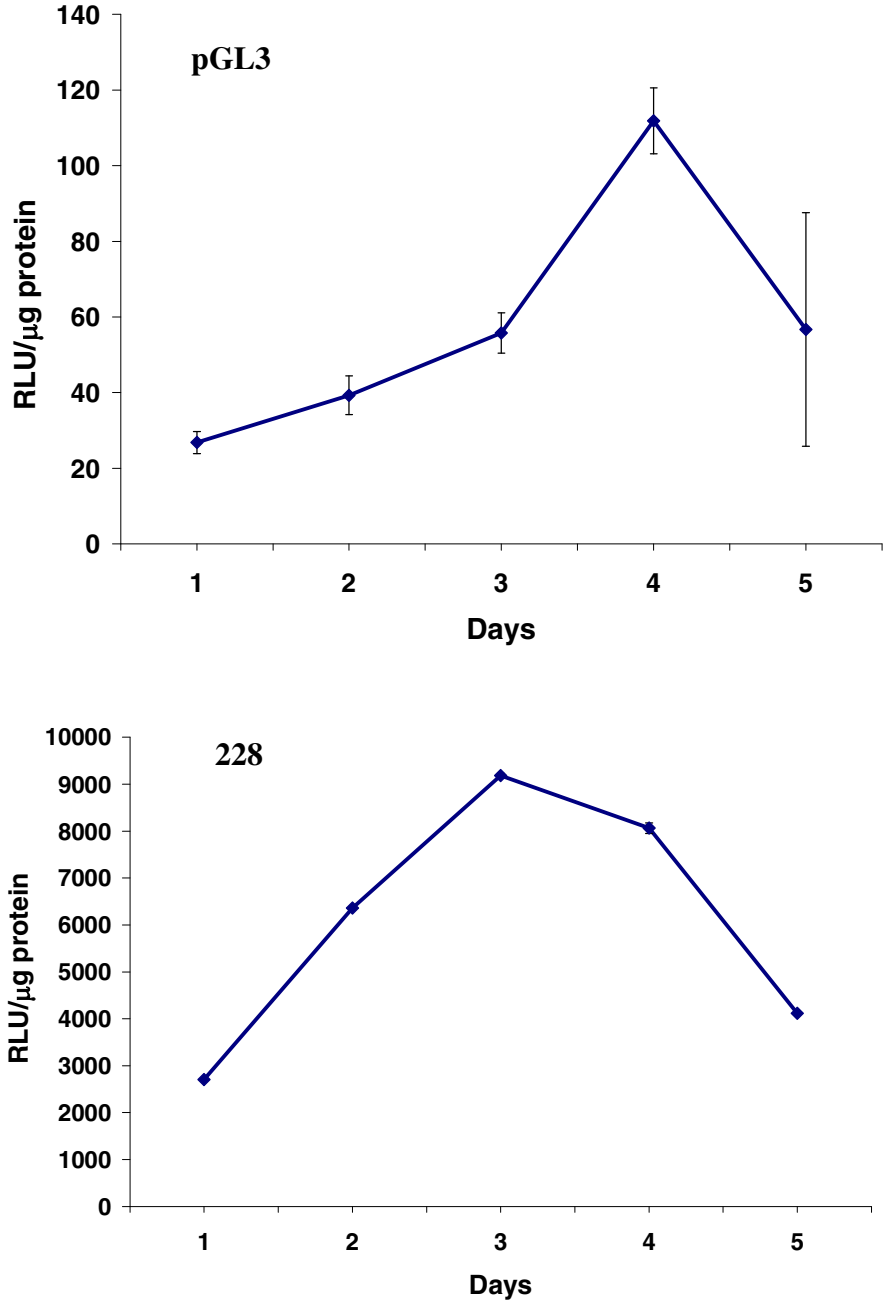



**Fig. 5B** Promoter activity in Growing and Confluent Cell Populations in 228Dkk-1 LUC and pGL3 Basic. Cells stably transfected with the indicated Dkk-1 luciferase reporter vectors were plated and allowed to grow over the days indicated. The lysates were collected each day for 5 days and assayed for luciferase (RLU) and total protein determined. The activity is expressed as RLU per µg of protein. The error bars represent the mean +/- standard error (n=4). Error bars for the 228Dkk-1 LUC are within the limits of the squares.

medium). Each day of collection, the medium was changed in both the low serum and complete medium wells. The cells were collected on Day 1 (day after replating), 2 and 3 in luciferase lysis buffer and relative light units (RLU) and total protein determined as described. Promoter activity was expressed as RLU/μg protein.

Figure 6 shows the promoter activity of the 1068-Dkk-1 LUC construct. The cells grown in low serum show an increase in promoter activity between Days 1 and 2. However, after Day 2, there is a significant decrease. The cells grown in normal serum show a steady and significant increase in activity over all three days. At Day 3 the level of activity in the cells grown in normal serum was more than 4 fold greater than the cells at low serum.

The 535-Dkk-1 LUC construct displayed a distinct and unexpected pattern of promoter activity in cells grown in low and normal serum (Fig. 6). The cells grown in low serum have a higher level of activity than those grown in normal serum with more than a two fold increase in activity. On Day 1, the level of activity is low and identical for both the serum starved and normal serum samples. However, cells grown in normal serum have very little increase in activity between days 1 and 2. There is an increase in activity between Days 2 and 3. At Day 3, the activity in low serum cells was 5 fold greater than that in normal cells. These results were confirmed in an independently conducted experiment.

Promoter activity in the 228-Dkk-1 LUC NIH3T3 cells increased over the three days for cells grown in normal and low serum medium (Fig. 7). However, the level of

## Serum Starved and a Growing Cell Population

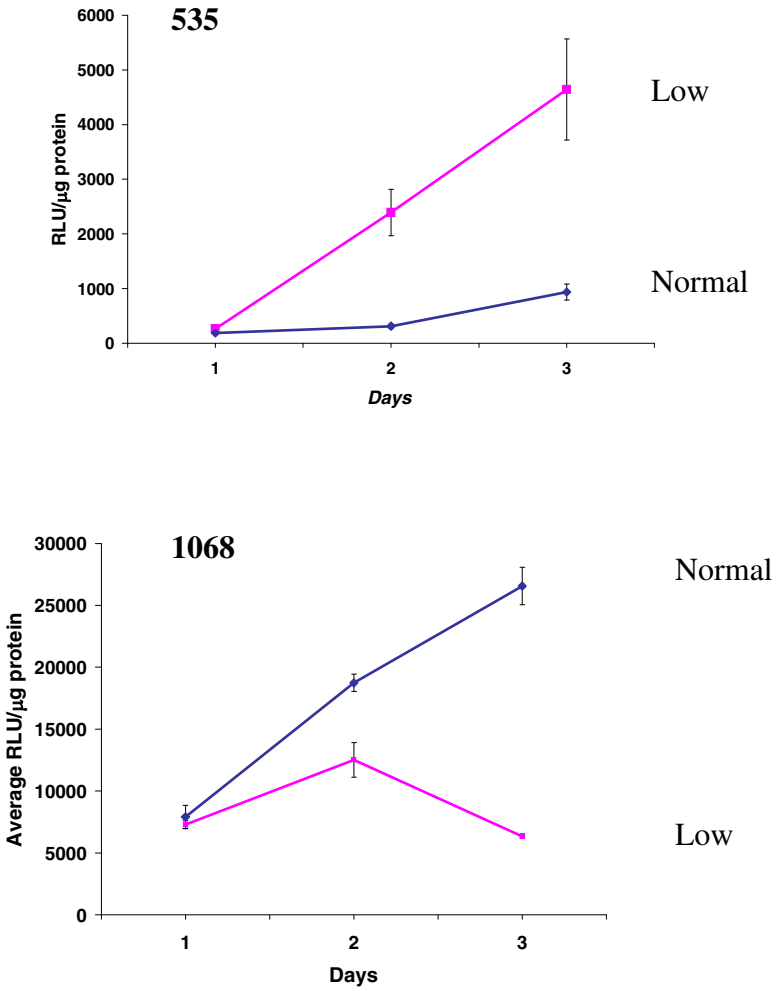



**Fig. 6** Promoter activity in Growing and Serum Starved Cell Populations in the 535Dkk-1 LUC and 1068Dkk-1 LUC. Cells stably transfected with the indicated Dkk-1 Luciferase reporter vector were plated and allowed to grow. At Day 1, the medium was removed and DMEM+0.5% FBS was added (serum starved) in one plate and fresh DMEM-C was added to the other plate (growing population). Cells were collected for 3 days and the lysates assayed for luciferase (RLU) and total protein determined. The error bars represent the mean +/- standard error (n=4). The activity is expressed as RLU per µg of protein. The dark diamond lines are normal serum and light squared lines are low serum.

activity in the low serum cells was less by 2 fold for Days 2 and 3, compared to the cells grown in normal medium.

Figure 7 also shows the promoter activity of the pGL3-Basic plasmid (control) over three days for growing and serum starved cells. On Day 1, the level of activity is low and almost identical for both the serum starved and normal serum samples, as would be expected. However, the cells grown in low serum have a significant increase in promoter activity by Day 2 (more than two-fold), which steadily increases until Day 3. The cells grown in normal serum also have in increase in promoter activity over all three days nearly identical to the cells grown in low serum.

These experiments were repeated once and similar results were obtained. The expression pattern for 1068 is as would be expected if Dkk-1 promoter activity corresponds to published data on Dkk-1 mRNA levels (Gregory et al, 2003); that is activity decreases in non dividing (serum starved) cells. The expression patterns for 535 and 228 are distinctly different in that promoter activity continues to increase rather than decrease with serum starvation. These results suggest that a region of the Dkk-1 promoter between 1068 and 535 contains DNA sequences required for regulating Dkk-1 transcription in growing and non-growing, serum starved cells.

*Activity of the Dkk-1 Promoter during the Cell Cycle*

Published data, as previously described, suggest that regulation of Dkk-1 gene expression is linked to the growth state of the cell population (Gregory, 2005). Indeed, our results show that expression from the full length (1068) Dkk-1 vector is lower in serum starved and confluent cells than in a growing population. We also wanted to

## Serum Starved and a Growing Cell Population

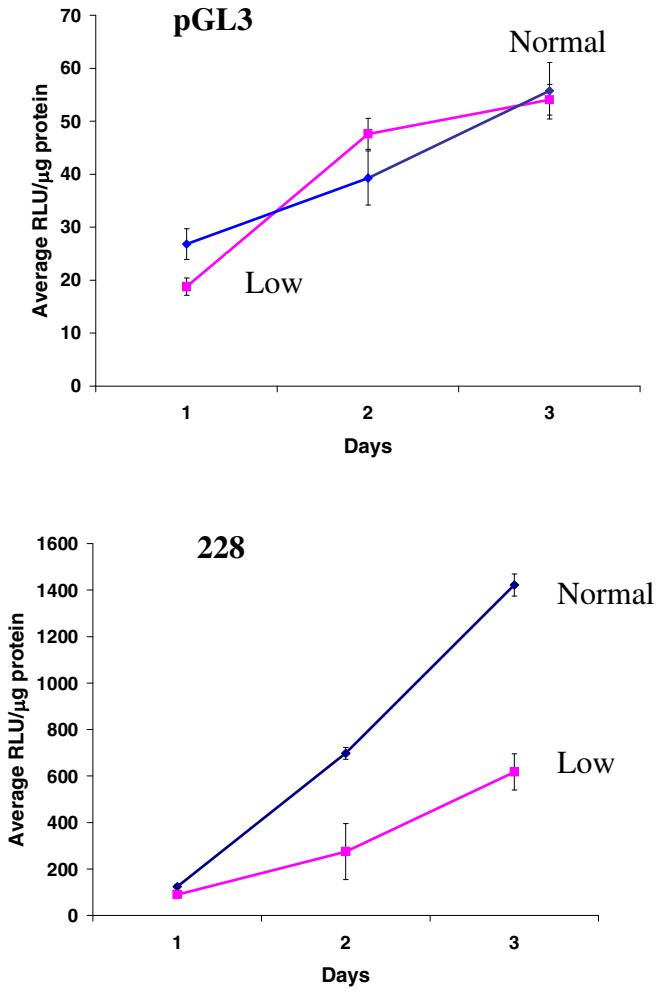



**Fig. 7**. Promoter activity in Growing and Serum Starved Cell Populations in 228Dkk-1 LUC and pGL3 Basic. Cells stably transfected with the indicated Dkk-1 Luciferase reporter vector were plated and allowed to grow. At Day 1, the medium was removed and DMEM+0.5% FBS was added (serum starved) in one plate and fresh DMEM-C was added to the other plate (growing population). Cells were collected for 3 days and the lysates assayed for luciferase (RLU) and total protein determined. The error bars represent the mean +/- standard error (n=4). The activity is expressed as RLU per µg of protein. The dark diamond lines are normal serum and light squared lines are low serum.

determine if Dkk-1 promoter activity varies during the cell cycle. To accomplish this goal the cell cycle pattern of Dkk-1 promoter activity was analyzed in the NIH3T3 cells with the stably transfected 1068, 535, and 228 reporter constructs. The stably transfected NIH3T3 cells (pGL3, 228, 535, and 1068) were plated in multi-well dishes and on the next day, the medium was changed to low serum. The cells were serum starved and then released back into the cell cycle by changing the medium to complete medium.

The 1068Dkk-1 LUC shows a distinct pattern (Fig. 8). The promoter activity displays a slight decrease from 0 hour to 24 hour, but is essentially flat. At hour 30 there is almost a 3-fold increase in promoter activity. This could indicate that the cells have completed a full cycle and have divided. It is not clear why such an increase was not detected in the other cell lines. Figure 8 also shows the promoter activity in the 535Dkk-1 LUC. Activity is highest at 0 hour and then the activity steadily decreases throughout the rest of the time course. The 228Dkk-1 LUC results are shown in Fig. 9. At hour 0, the level of activity is highest and then decreases. After 18 hour activity remains essentially flat. Figure 9 shows the promoter activity in the pGL3-Basic plasmid (control). The level of activity begins high at hour 0. It then decreases from 0 hour to18 hour and then slightly increases at 24 hour and remains essentially the same at 30 hour. Overall, these results indicate that there is little or no cell cycle regulation of Dkk-1 promoter activity.

Effects of cell cycle and growth inhibitors on Dkk-1 promoter activity

Cell populations respond to various external and internal signals that lead to cell division, withdrawal from the cell cycle, or possibly cell death. These signals include

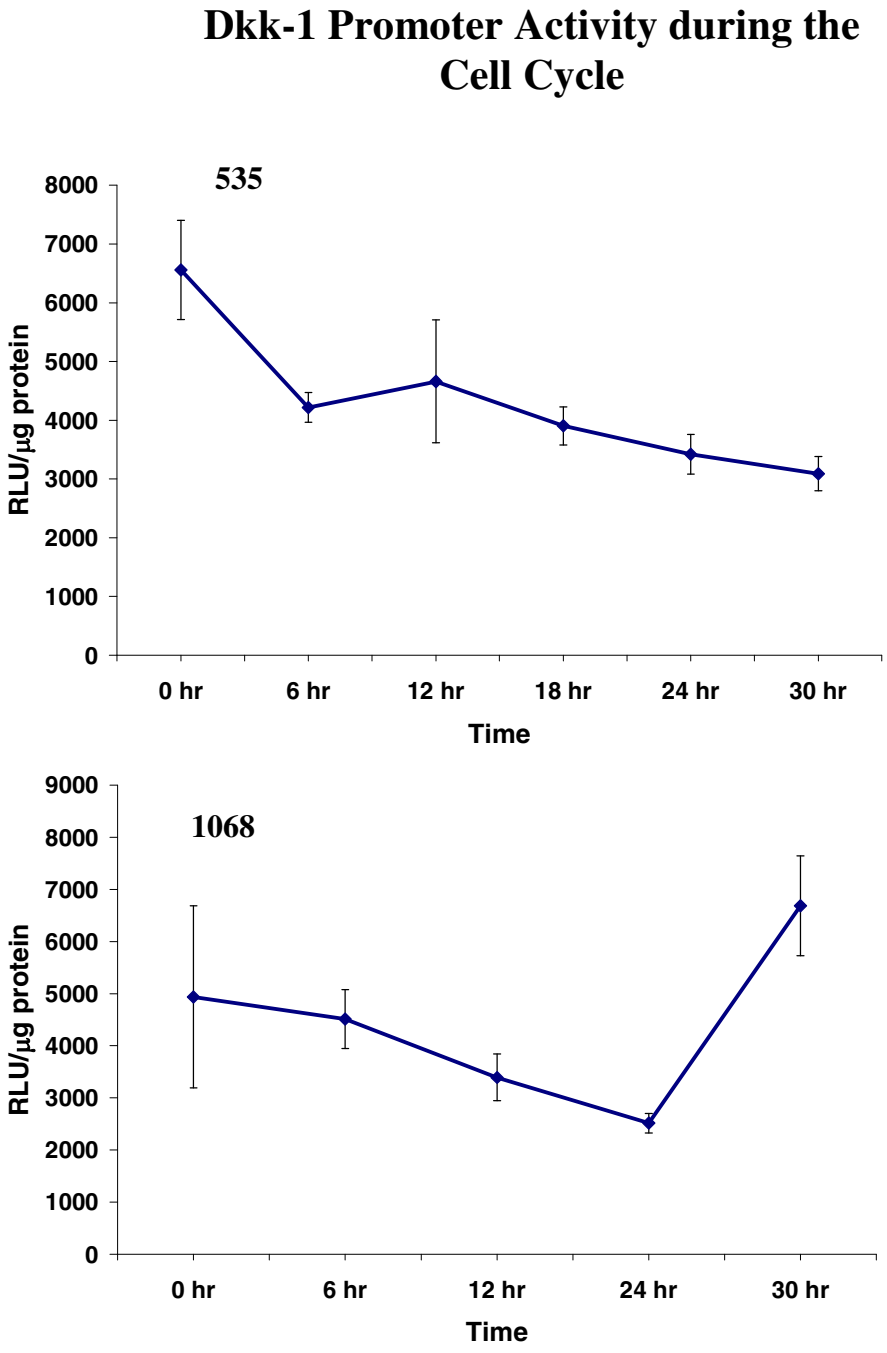
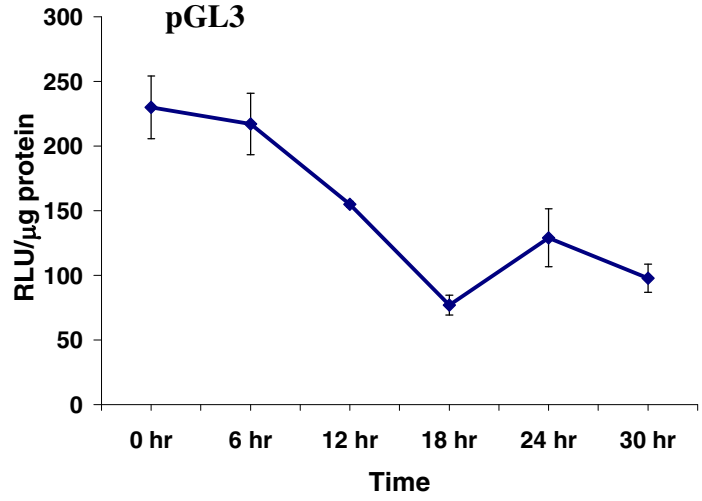



**Fig. 8** Cell cycle Expression in 535Dkk-1 LUC and 1068Dkk-1. NIH3T3 cells stably transfected with the 535Dkk-1 LUC and 1068Dkk-1 vector were plated and serum starved. At time 0, the medium was removed and complete DMEM-C was added. Cells were collected at the times indicated and the lysates were assayed for luciferase (RLU) and the total protein determined. The promoter activity is expressed as RLU per µg of protein. The error bars represent the mean +/- standard error (n=4).

# Dkk-1 Promoter Activity during the Cell Cycle



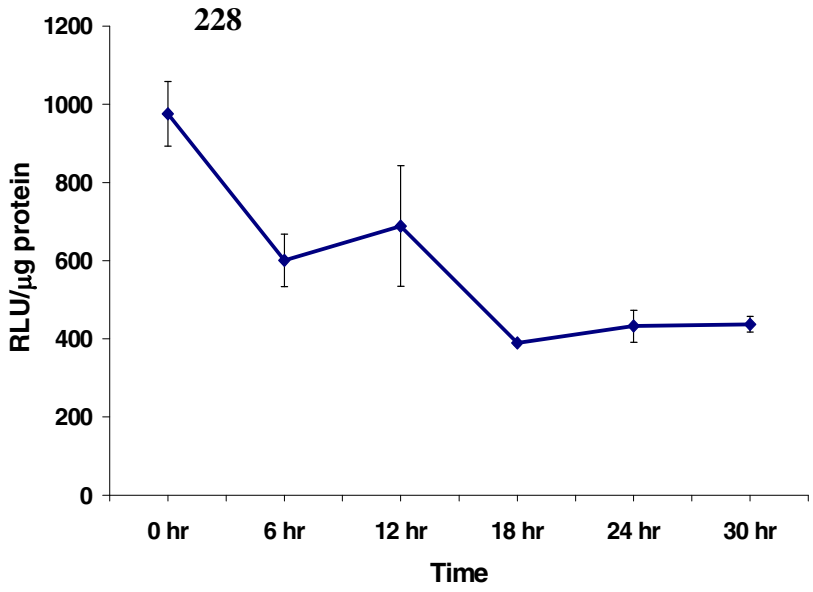




**Fig. 9** Cell cycle Expression in 228Dkk-1 LUC and pGL3 Basic. Cells stably transfected with the pGL3 and 228Dkk-1 LUC vector were plated and serum starved. At time 0, the medium was removed and complete DMEM-C was added. Cells were collected at the times indicated and the lysates were assayed for luciferase (RLU) and the total protein determined. The promoter activity is expressed as RLU per µg of protein. The error bars represent the mean +/- standard error (n=4).

various growth factors that are secreted by the cells, found in the serum, or arise from contact between individual cells. Our results indicate that the Dkk-1 promoter is sensitive to cell density and growth factors in the medium. However, it does not appear that Dkk-1 promoter activity is regulated during the cell cycle. In these studies, we asked if inhibitors of the cell cycle and cell growth affect Dkk-1 promoter activity. The three inhibitors used were the U0126, methotrexate, and aphidicolin. MEK 1/2 U0126 inhibits the kinase activity of MAP kinase kinase, MEK. It inhibits active and inactive MEK 1/2 and is responsible for downstream inhibition of ERK 1 and ERK 2 mediated responses. This inhibitor was used as a means to determine if the decrease in Dkk-1 promoter activity was linked to the Ras pathway. The rationale is that removal of serum causes a reduction in promoter activity, which is due to removal of Ras specific growth factors. Methotrexate inhibits the enzyme dihydrofolate reductase (DHFR), a critical enzyme in the folate pathway. Consequently, methtrexate treated cells are arrested in the S phase, due to lack of thymidine for DNA synthesis. This inhibitor was selected, in part, because previous studies have shown that Dkk-1 mRNA levels are reduced in folate deficient and methotrexate treated cells. Aphidicolin arrests cells in $G_1$ by blocking DNA synthesis and inhibiting DNA polymerase $\alpha$. We wanted to determine if stopping the cells at another point in the cell cycle other than $G_0/G_1$ would alter Dkk-1 promoter activity.

*MEK 1/2 Inhibitor U0126*

The stably transfected NIH3T3 cells (pGL3, 228, 535, and 1068) were plated in multi-well dishes. The next day, the cells were treated with U0126 at specified concentrations. The cell lysates were collected 24 and 48 hours after treatment. Figure

10 shows the 1068-Dkk-1 LUC construct. There was no distinct pattern of activity. At 24 hours after treatment there was a slight increase in promoter activity at 10 μg and a slight decrease at 50 μg. At 48 hours, there was about the same level of activity at 0 μg and 50 μg, but an increase in promoter activity in cells treated with 10 μg of U0126. Figure 10 also shows the 535-Dkk-1 LUC construct. At 24 hours, there is almost a 3-fold increase in promoter activity at 10 μg and 50 μg. The increase in promoter activity continues at 48 hour at both 10 μg and 50 μg. The 228-DKK-1 LUC construct is shown in Fig 11. Here, the activity increases as the concentration of U0126 increases both at 24 and 48 hours, however at 48 hour, there is a greater increase in promoter activity 50 μg. Figure 11 shows the promoter activity in the pGL3 Basic plasmid at 24 and 48 hours after treatment with MEK 1/2 U0126. At 24 hours, there is a decrease in activity in cells treated with 10 μg and 50 μg of U0126. At 48 hours, there is no significant decrease in promoter activity.

*Aphidicolin*

The stably transfected NIH3T3 cells (228, 535, and 1068) were plated in multi-well dishes and the next day, the cells were treated with aphidicolin at specified concentrations. The cell lysates were collected 24 and 48 hours after treatment. The relative light units (RLU) and total protein were determined as described. The 1068-Dkk-1 LUC construct is shown in Fig 12. At both 24 and 48 hours, there was essentially no difference between the control treated cells at both concentrations.

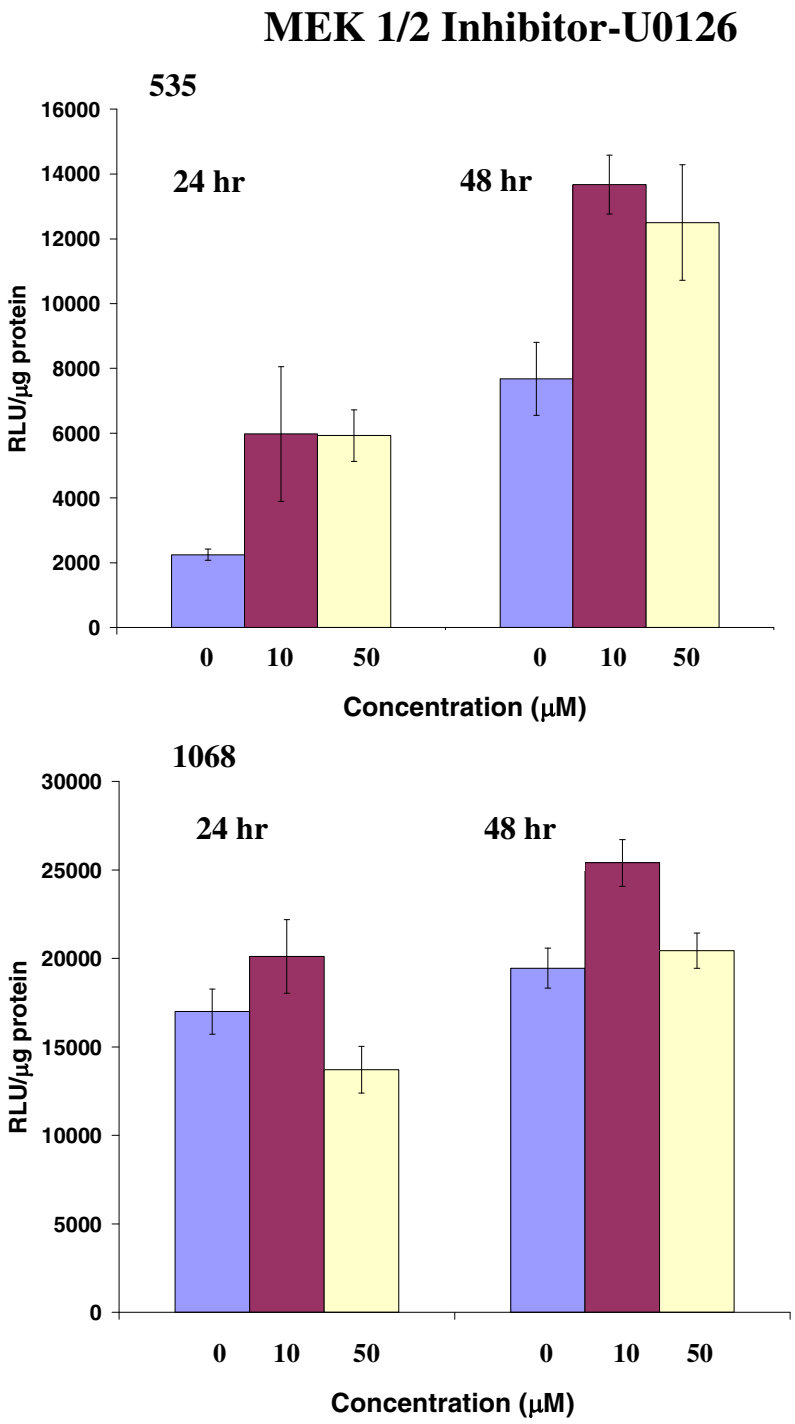



**Fig. 10** Promoter activity in the 535Dkk-1 LUC and 1068Dkk-1 LUC cells treated with MEK 1/2 U0126. The cells were treated with U0126 at the indicated concentrations and collected 24 and 48 hours after treatment. The cell lysates were assayed for luciferase (RLU) and total protein determined. The promoter activity is expressed as RLU per µg of protein. The error bars represent the mean +/- standard error (n=4).

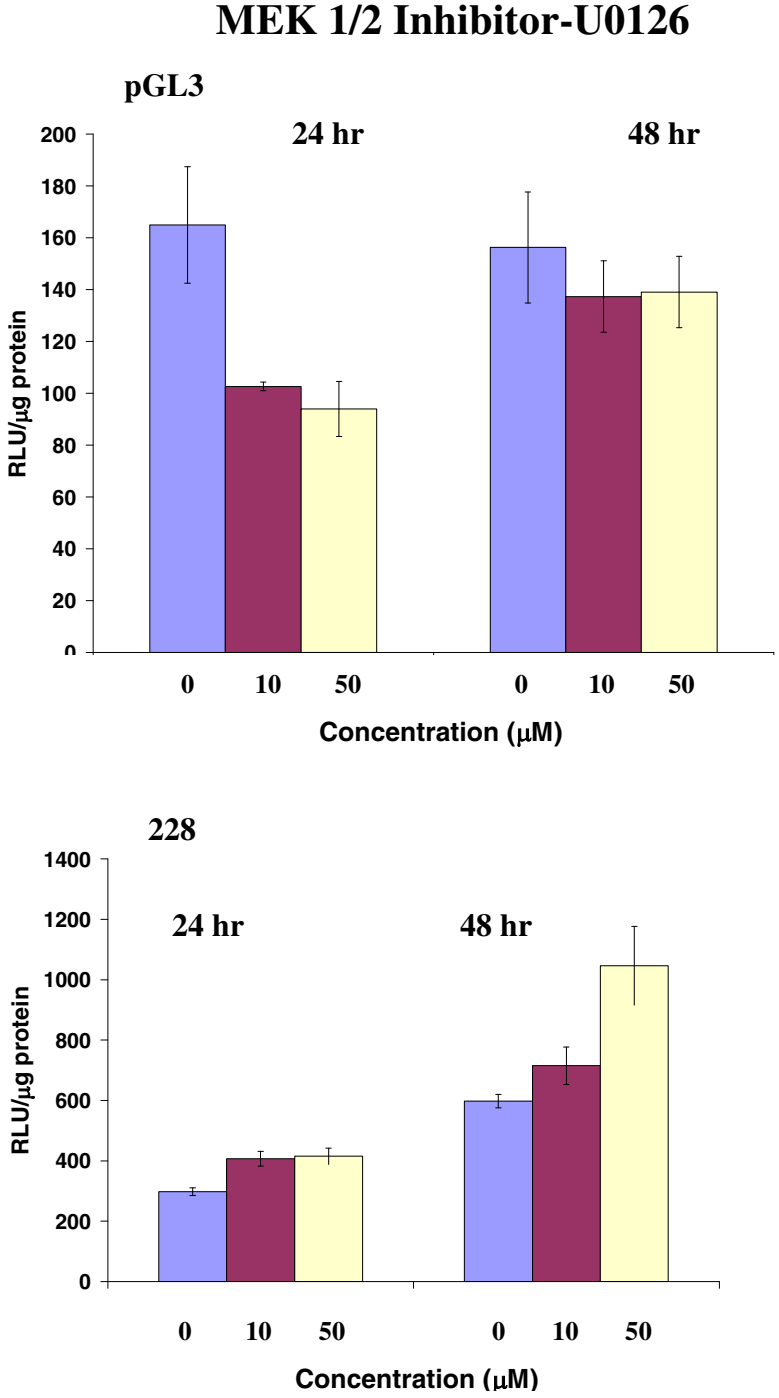



**Fig.11** Promoter activity in 228Dkk-1 LUC and pGL3-Basic cells treated with MEK 1/2 U0126. The cells were treated with U0126 at the indicated concentrations and collected 24 and 48 hours after treatment. The cell lysates were assayed for luciferase (RLU) and total protein determined. The promoter activity is expressed as RLU per μg of protein. The error bars represent the mean +/- standard error (n=4).

The decrease in overall promoter activity at 48 hour relative to the 24 hour is likely due to the cells becoming confluent. Figure 13 shows the promoter activity in the 535Dkk-1 LUC construct. At 24 hours, the activity was about the same at all concentrations. At 48 hours the activity at 0.5 μg was about the same as the control, but a nearly 2-fold decrease in activity was detected at 2 μg. The promoter activity of the 228Dkk-1 LUC construct is shown in Fig. 13. At 24 hours, the promoter activity increased as the concentration of aphidicolin increased. At 48 hours, the promoter activity decreased as the concentration increased, with the decrease being almost 2 fold.

*Methotrexate*

The stably transfected NIH3T3 cells (pGL3, 228, 535, and 1068) were plated in multi-well dishes and treated with methotrexate at specified concentrations for 24 and 48 hours. The cell lysates were collected at 24 and 48 hours after treatment. Overall the responses of the different constructs to methotrexate treatment are distinct. The promoter activity in the 1068-Dkk-1 LUC is shown in Fig. 14. After 24 hours of treatment, there was essentially no difference between the control and treated cells. However, at 48-hour promoter activity decreases to approximately the same level at all methotrexate concentrations relative to the control, untreated cells. Figure 14 shows the 535Dkk-1 LUC construct. At 24 hours, the activity is about the same except at 100 μg. At this concentration, there is a decrease in promoter activity to almost 3-fold.

# Aphidicolin

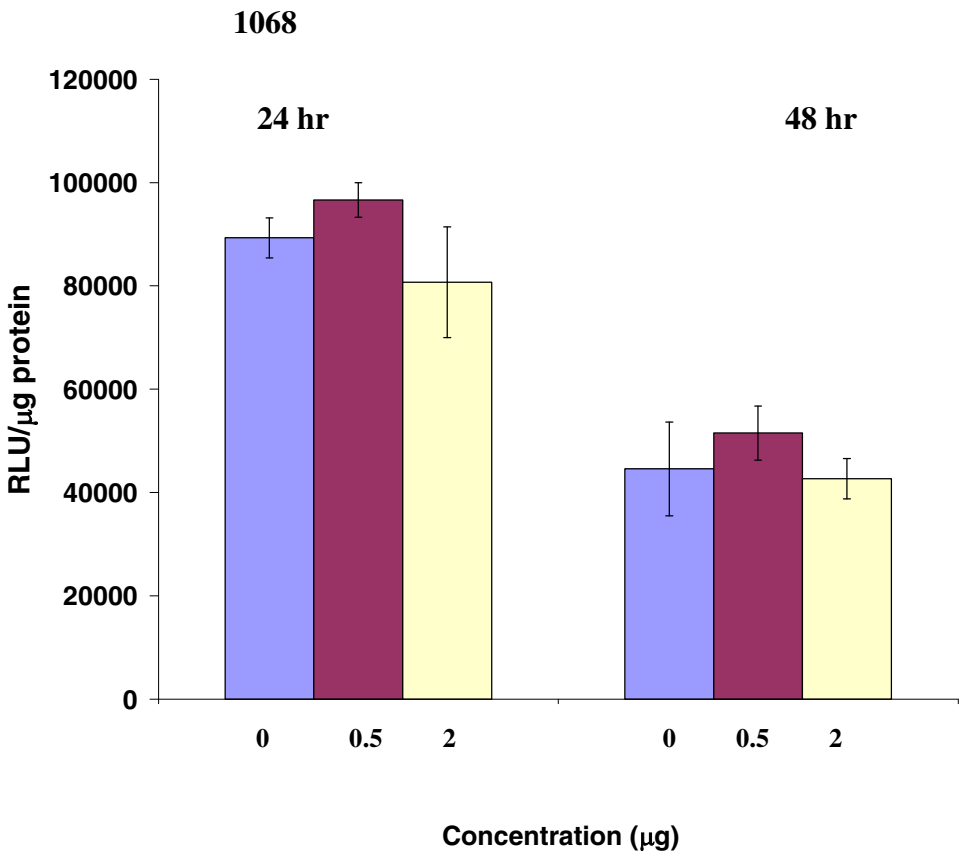



**Fig. 12** Promoter activity in1068Dkk-1 LUC cells treated with Aphidicolin. The cells were treated with aphidicolin at the indicated concentrations and collected 24 and 48 hours after treatment. The cell lysates were assayed for luciferase (RLU) and total protein determined. The promoter activity is expressed as RLU per μg of protein. The error bars represent the mean +/- standard error (n=4).

# Aphidicolin

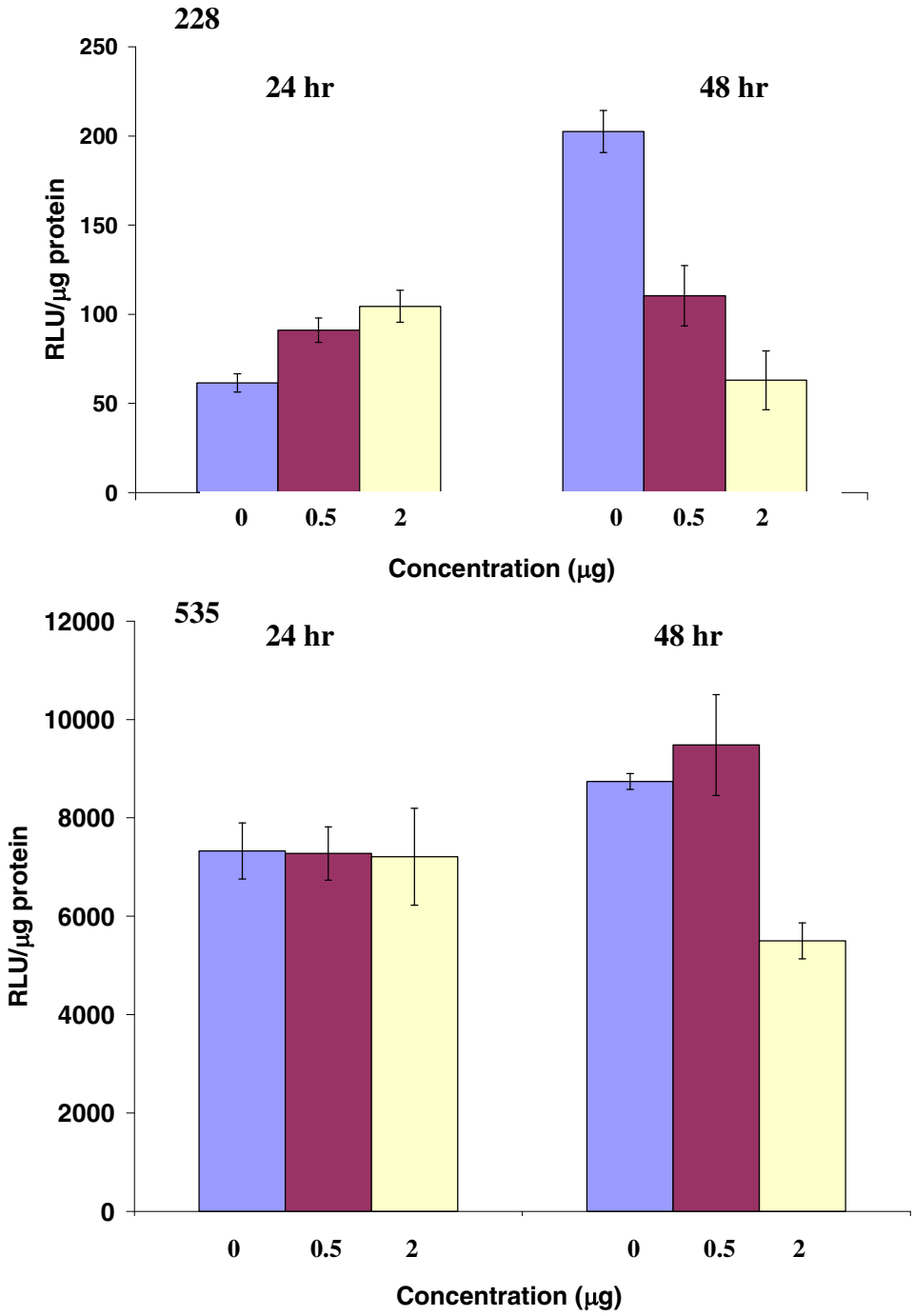



**Fig. 13** Promoter activity in 535Dkk-1 LUC and 228Dkk-1 LUC treated with Aphidicolin. The cells were treated with aphidicolin at the indicated concentrations and collected 24 and 48 hours after treatment. The cell lysates were assayed for luciferase (RLU) and total protein determined. The promoter activity is expressed as RLU per µg of protein. The error bars represent the mean +/- standard error (n=4).

At 48 hours promoter activity increases with increasing methotrexate concentration, being significantly higher at 20, 50, and 100μg. Overall, at 48 hours there was an approximately 2-3-fold increase in promoter activity. In Fig. 15 the 228Dkk-1 LUC construct is shown. There is no significant difference between the untreated control and treated cells at both 24 and 48 hour. The pGL3 Basic plasmid (Fig. 15) displays a decrease in activity as the concentration of methotrexate increased in the 24 hour treated cells. At 48 hours, there is no difference between the treated and untreated controls.

*Identification of Transcription Factor Binding Sites*

We wanted to analyze -1068 to -535 region of the Dkk-1 promoter to determine if there were any transcription factors that could be involved in regulating the activity of the Dkk-1 promoter under selective growth conditions. Using the UC-Santa Cruz website (http://genome.ucsc.edu/) we were able to access the Dkk-1 Ref Seq gene region from –1068 to –535 upstream from the +1 start site. This region was pasted into the Patch site (http://www.gene-regulation.com/pub/programs.html#match) for identification of potential transcription factor binding sites. Our search resulted in the identification of several transcription factor binding sites (Table 1).

# Methotrexate

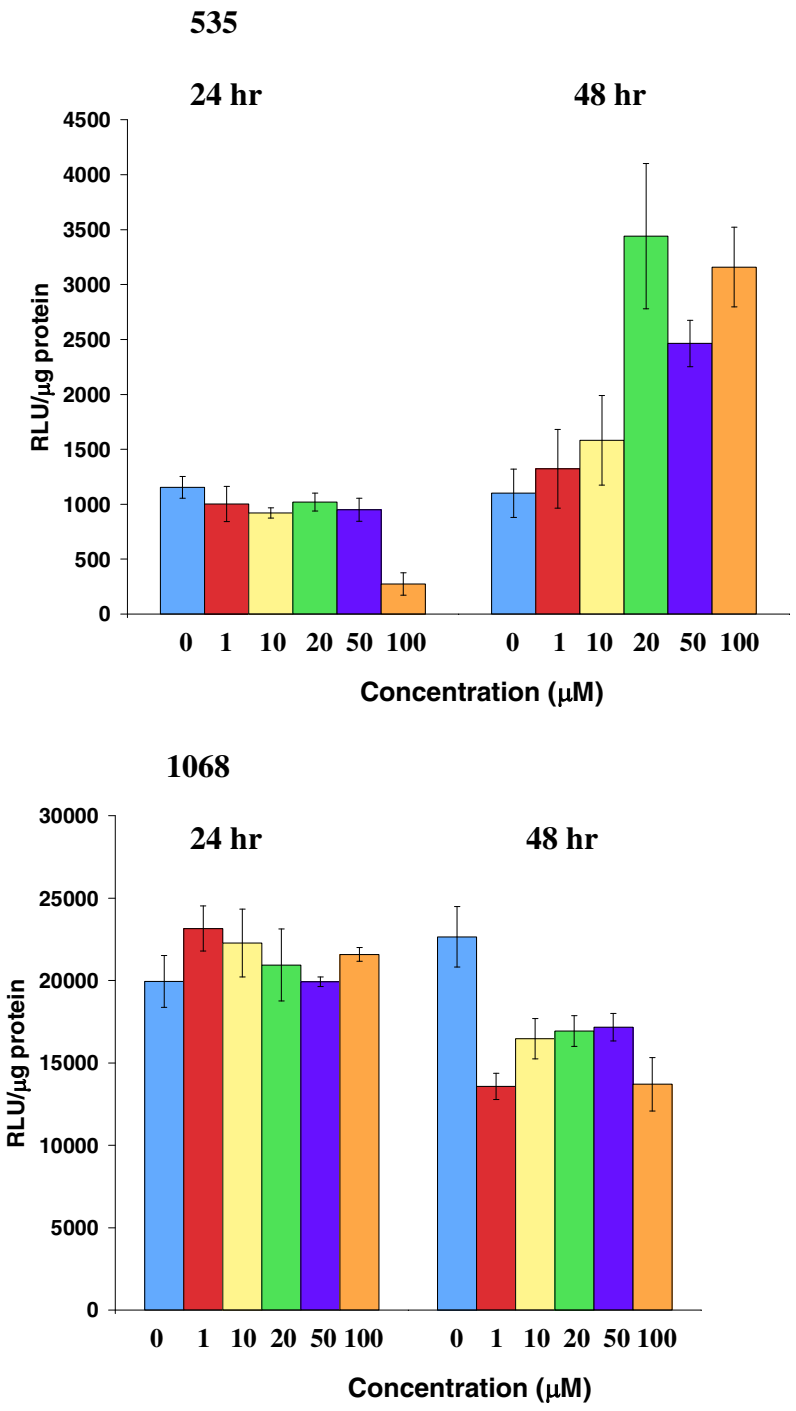



**Fig. 14** Promoter activity in 535Dkk-1 LUC and 1068Dkk-1 LUC cells treated with Methotrexate. The cells were treated with methotrexate inhibitor at indicated concentrations and collected 24 and 48 hours after treatment. The cell lysates were assayed for luciferase (RLU) and total protein determined. The promoter activity is expressed as RLU per µg of protein. The error bars represent the mean +/- standard error (n=4).

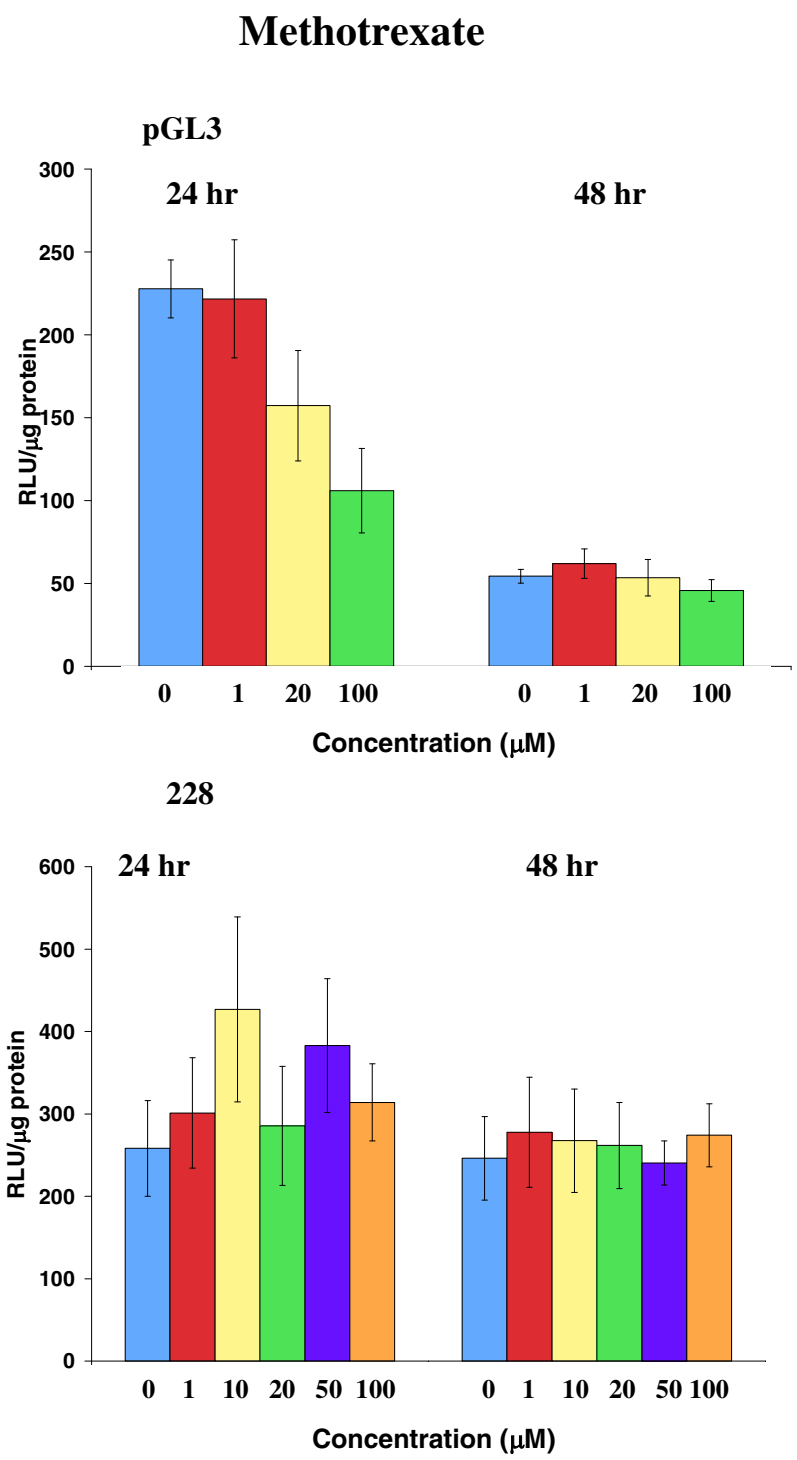



**Fig. 15** Promoter activity in 228Dkk-1 LUC and pGL3-Basic cells treated with Methotrexate. The cells were treated with methotrexate at indicated concentrations and collected 24 and 48 hours after treatment. The cell lysates were assayed for luciferase (RLU) and total protein determined. The promoter activity is expressed as RLU per µg of protein. The error bars represent the mean +/- standard error (n=4).

**Table 1** Identified Transcription Factor Binding Sites *

| Factor | Binding site | General feature |
|---|---|---|
| SF-1 | TCAAGGTAA | Steroidogenesis; male sexual differentiation |
| LUN-1 | TGGGA, TCCCA | Expressed in lung |
| c-Myb | CAGTTG, CAGTTG, CAACTG, CAACT | Required for G1/S transition |
| SP-1 | CCCCC; CCCCTCCCC | Phosphorylation; Interacts with NF-κ B |
| NF-AT4 | GGAAA | Transcription activating complex with AP-1 |
| NF-κ B | GAAATTTCC | Key regulator of genes involved in responses to infection, inflammation, stress |
| POU2F1 | TATGCAAAT | Part of the replication machinery of adenovirus |
| WT-1 | CGCCCTCTC | Expression stabilizes and partially inactivate p53; expressed in cells that undergo a mesenchymal |
| AP-2 α A, B | GGGAAGA | Negatively regulates trans-activation exerted by c-Myc |
| YY1 | CATTT | Interacts with c-Myc |
| TCF-4 | CTTTGAA; TTCAAAG; TTGAA; CTTTGAT | Vital element of the Wnt signaling cascade |
| LEF-1 | ATCAAAGTA; ATCAAAG | Interacts with β-catenin |
| TFIID | TATAT | TATA-box-binding complex of TBP and TAFs |
| Elk-1 | TTCCGA | Downstream target of Raf-1; activator |

The sequences between –1068 and –535 were analyzed using Patch (http://www.gene-regulation.com/pub/programs.html#match) as described in Materials and Methods. Binding sites for some of the transcription factors appear more than once.

# CHAPTER IV

# DISCUSSION

The goal of this study was to analyze the Dkk-1 promoter region under various growth conditions and to determine the region of upstream sequences required for its regulation. We also wanted to identify potential transcription factors involved in Dkk-1 regulation. To do this we first analyzed a construct that contained 1068 base pairs of upstream Dkk-1 sequence fused to the luciferase reporter gene. The level of luciferase activity in cells containing this plasmid indicated activity of the Dkk-1 promoter. NIH3T3 cells containing this construct were grown under different growth conditions, including expanding and confluent cell populations and serum starvation. Cell cycle expression was analyzed in NIH3T3 cells by first arresting the cells by serum starvation and then releasing them back into the cell cycle with complete medium. Finally, the cells were treated with various growth inhibitors: aphidicolin, MEK 1/2 inhibitor U0126, and methotrexate. The Dkk-1 promoter activity in the 1068Dkk-1 LUC NIH3T3 cells was analyzed under each condition. These experiments were repeated with deletion constructs containing 535 and 228 bp. A summary of the results can be found in Table 2. In general, we found that Dkk-1 promoter activity is higher in a growing cell population than in a nondividing population. The 1068 Dkk-1 promoter construct does not appear to change dramatically during the cell cycle nor is it sensitive to an S phase inhibitor or

**Table 2.** Summary of Results

| | **Experimental Treatments** | | | | | |
|---|---|---|---|---|---|---|
| **Cell Line** | **Confluence** | **Serum Starvation** | **Cell Cycle** | **Ras Inhibitor-U0126** | **Aphidicolin** | **Methotrexate** |
| 1068 | Increase up to Day 3 then decrease to Day 5 | Decreased by Day 3; activity less than control | Gradual decrease, increase at 30 hours | Slight decrease at 24 hrs; No effect at 48 hrs | No effect at 24 hrs or 48 hrs | No effect 24 hrs, decrease at 48 hrs |
| 535 | Continual increase up to Day 5, no decrease | Continual increase, no decrease; activity greater than control | Gradual decrease | Increase at both 24 hrs and 48 hrs | No effect at 24 hrs, decrease at 48 hrs | No effect 24 hrs, increase at 48 hrs |
| 228 | Increase up to Day 3 then decrease to Day 5 | Continual increase, no decrease; activity greater than control | Gradual decrease | Increase at both 24 hrs and 48 hrs | Increase at 24 hrs, decrease at 48 hrs | No effects at 24 or 48 hrs |
| pGL3 | Increase up to Day 4 then decrease at Day 5 | No reduction, continual increase, same level as control | Gradual decrease | Decrease at 24 hrs, no effect at 48 hrs | ND | Decrease at 24 hrs, no effect at 48 hrs |

U0126. A sequence region between 1068 and 535 appears to be important for growth regulation of this gene.

Dkk-1 Promoter Activity Decreases in Serum Starved and Density Arrested Cells

Proliferation of normal cells is controlled by the availability of growth factors and the cell population density. When the cells reach confluency they become quiescent and arrest in $G_o$. We wanted to see what effect cell density would have on Dkk -1 promoter activity. The 1068Dkk-1 LUC NIH3T3 cells were plated at a subconfluent density and allowed to grow in normal medium for five days during which time they reached confluence. NIH3T3 are immortalized but not transformed cells, hence they stop dividing when confluent. The promoter activity in the 1068Dkk-1 LUC cells increased more than two-fold between Days 1 and 2 and continued to increase until Day 3. After Day 3 the activity began a pattern of decrease and continued to decrease until Day 5. We believe that the decrease is due to the cells becoming confluent and entering an arrested growth state. These results are as would be expected. In the study by Gregory et al (2003), they found that Dkk-1 protein and mRNA levels decreased when mesenchymal stem cells reach a stationary phase. Because the medium was changed everyday, this decrease cannot be caused by reduced growth factors in the medium. Overall, these results suggest that 1068 base pairs is sufficient for down regulation of the Dkk-1 gene promoter in density-arrested cells.

We also wanted to analyze the promoter activity in the 1068Dkk-1 LUC construct under serum starved conditions and compare that to its activity when the cells are allowed to grow in normal medium. We again plated the cells at a subconfluent density and

allowed them to grow in reduced serum and normal complete medium for three days. The promoter activity in the cells grown in normal serum increased each of the three days. The promoter activity increased from Day 1 to Day 2 in the cells grown in low serum, but then decreased at Day 3. We believe that this decrease is due to the absence of growth factors in the low serum medium. This lack of sufficient growth factors is expected to reduce cell signaling, leading to diminished signal being sent to the cells telling them to divide. These results indicate that the Dkk-1 promoter responds to growth factor stimulation. Although the cells are subconfluent, the lack of growth factors causes the cells to enter $G_0$.

Dkk-1 Promoter activity shows little variation during the Cell Cycle

We also wanted to determine how the Dkk-1 promoter responds to re-entry into the cell cycle after serum starvation. The cells were plated and serum starved and then released back into the cell cycle by changing to complete medium plus serum. We found little change in promoter activity from 0 to 24 hours. However, after 24 hours, the activity increased sharply, more than 2 fold. It is difficult to determine why there was a decrease in activity in the beginning of the time course. It is likely that the spike in activity at 30 hours is due to the cells completing a full cell cycle and dividing. Although the cell number increases at this time, this increase cannot account for the increase in promoter activity since luciferase activity was standardized to protein content. Thus, the increase in promoter activity should reflect a real change. It is possible that initial stimulation of added serum to the cells is not sufficient to activate the Dkk-1 promoter. Only when the cells are growing more normally does the promoter activity increase.

The Dkk-1 Promoter Lacks Sensitivity to a Ras Pathway Inhibitor

We wanted to know what would happen to promoter activity in the 1068Dkk-1 LUC NIH3T3 cells when treated with various growth inhibitors. We began with the MEK 1/2 inhibitor, U0126. This compound is known to inhibit the Ras pathway by acting as noncompetitive inhibitors to the MEK1 and MEK2 kinases and binding to free MEK as well as MEK*ERK and MEK*ATP complexes (Reuter et al, 2000). Based on our finding that the Dkk-1 promoter activity decreases in serum-starved cells, it is expected that U0126 will also cause a decrease. To test the effects of U0126 on Dkk-1, we plated the 1068Dkk-1 LUC cells in normal serum and treated them with U0126 at different concentrations. At higher concentrations, other kinases in addition to MEK 1/2 may be affected. At 24 hours, we saw that cells treated with 50 μg of U0126 had the lowest amount of promoter activity. At 48 hours, promoter activity was the highest in cells treated with 10 μg of U0126. Inhibiting the central Ras pathway via U0126 causes a reduction in cell signaling. Therefore, we would expect a lower level of Dkk-1 promoter activity at higher concentrations and longer treatments. We did see that cells treated at 50 μg had the lowest level of activity at 24 hr. But this decrease was not seen at 48 hr. These results indicate that the Dkk-1 promoter is not very sensitive to inhibition of the Ras pathway. Thus, the decrease in promoter activity in serum-starved cells is probably not due to reduction in Ras signaling.

The Dkk-1 Promoter Does Not Respond to an Inhibitor of S Phase

We also treated 1068Dkk-1 LUC cells with aphidicolin. Aphidicolin interrupts DNA synthesis by inhibiting DNA polymerase α and causes arrest in the $G_1$/S phase. To

determine how aphidicolin affects Dkk-1, 1068Dkk-1 LUC cells were treated with different concentrations of aphidicolin for 24 and 48 hours. At 24 hours, we saw that aphidicolin had little effect on promoter activity. Looking at the data for the cell cycle expression, we would not expect that aphidicolin would have an effect on activity because there was little change in promoter activity except towards the very end of the cell cycle. These results suggest that the Dkk-1 promoter is responding to factors that determine if the cells enter $G_1$ (from $G_0$) and moves through the cell cycle rather than events within the cell cycle.

The Dkk-1 Promoter is Sensitive to a Folate Pathway Inhibitor

Finally, we treated the 1068Dkk-1 LUC cells with methotrexate. Methotrexate is an inhibitor of the folate pathway enzyme, dihydrofolate reductase. Treating cells with methotrexate will have multiple effects including slowing DNA synthesis in S phase. Our main interest in methotrexate derives from the finding that Dkk-1 mRNA levels are reduced in folate depleted cells (Katula et al, submitted). It is expected that methotrexate would cause a decrease in Dkk-1 promoter activity. We found that methotrexate had no effect at 24 hours but caused a reduction at all concentrations at 48 hours. One explanation is that it took additional time to reduce folate levels within the cell population. Combined with the aphidicolin data (which did not effect Dkk-1 promoter), these findings suggest that the effect of methotrexate on Dkk-1 promoter activity is not due to arrest in S phase.

The Region between -1068 and -535 Appears to be Important for Regulation of Dkk-1

Our next goal was to determine what region of the Dkk-1 upstream sequences was required for regulation of the Dkk-1 gene. We believe that there is a specific region of the promoter where transcription factors bind that is necessary for Dkk-1 regulated activity. To achieve this goal, we obtained deletion constructs of base pair lengths 535 and 228 and analyzed the activity of the promoter under various growth conditions and during the cell cycle. The same was done for the control vector pGL3. We expected that one or more of these deletion constructs would display an altered pattern of expression. For example, under serum starvation, the 535 or 228 would not display a decrease in promoter activity. This would indicate that the region between 1068 and 535 is required for correct regulation of Dkk-1 expression. The results of the 535 and 228 construct will first be compared to the 1068, then the control plasmid, pGL3 will be discussed. The promoter activity of the three cell lines (1068, 535, and 228) and pGL3-Basic were initially compared by transient transfection. Our results indicate that the level of promoter activity is similar for all three constructs (Fig. 4). This indicates that 228 bp upstream sequences are sufficient for maximum activity in a growing population. The control plasmid, pGL3, as expected, had very low activity.

The 535Dkk-1LUC NIH3T3 cells showed a distinct pattern of expression when allowed to grow to confluence for five days. Each day, there was an increase in Dkk-1 promoter activity. Unlike the 1068Dkk-1LUC, which displayed a sharp decrease after

day three, the 535 had an activity level that did not decrease. When the 535 cells were allowed to grow under serum starved conditions and in normal medium, there was also an unexpected pattern. The level of promoter activity increased in both the serum starved cells and in cells grown in normal serum. In the 1068 cells, promoter activity decreased under serum starvation. It is not clear why promoter activity is higher in the serum starved cells. These results suggest that DNA sequences in the 1068 to 535 region are necessary for suppressing Dkk-1 promoter activity in nongrowing cells. Similar to the 1068 construct, the 535 construct showed little variation in promoter activity during the cell cycle.

We treated the 535Dkk-1LUC NIH3T3 cells with various inhibitors of the cell cycle to see how they would affect the activity of Dkk-1 promoter. We saw that MEK ½ U0126 inhibitor had little effect on 1068. In contrast, the 535 cells displayed an increase in promoter activity when treated with U0126. This suggests that sequences downstream of 535 are responsive to the Ras pathway. When treated with the S phase inhibitor aphidicolin, we saw that the 535 cells showed no change in activity for any concentrations after 24 hours, similar to the 1068. However, at 48 hours promoter activity decreased at the highest concentration. The promoter activity in 535 cells treated with methotrexate was not affected at 24 hours, again similar to 1068. However, after 48 hours of treatment, activity increased in contrast to the decrease for 1068. In general, the inhibitor studies support the notion that sequences between 1068 and 535 contain sequences involved in regulating Dkk-1 promoter activity.

The 228Dkk-1LUC NIH3T3 cells were also analyzed under the same conditions as the 1068 and 535. When the cells were allowed to grow to confluence, we observed a pattern of activity almost identical to that of the 1068Dkk-1LUC NIH3T3 cells; the promoter activity increased until Day 3 and then decreased drastically afterwards (Fig. 5). This can be attributed to the cells reaching a stationary growth phase and ceasing to divide, causing a decrease in promoter activity. When the cells were grown in normal medium and serum starved, the results were more similar to 535; there was no decrease in activity. Unlike 535, the activity level was less than the control. Analysis of the 228Dkk-1 LUC cells during the cell cycle showed no consequential pattern; activity decreased over the entire time course. These results are similar to 535. When treated with U0126 inhibitor, the results were similar to 535; activity increased at both 24 and 48 hours. Contrary to the full length, the highest level of activity was at 50 μM. Cells treated with aphidicolin were also more similar to 535 than 1068. This pattern is unlike the 1068, which did not respond to aphidicolin. This is unexpected considering the cell cycle pattern of expression for 228. However the 228 construct is different and lacks upstream sequence, and within this region, is responsive to aphidicolin. When treated with methotrexate, an inhibitor of the enzyme dihydrofolate reductase the 228Dkk-1 LUC cells showed no change in promoter activity. In general, promoter activity in 228 cells is distinct but with some similarities to 1068 (confluence) and 535 (serum starvation, cell cycle, Ras inhibitor, and aphidicolin). This suggests other regulatory sites in the 535 to 228 region.

Expression from the Control Vector pGL3 Basic Varies

As a control, the vector pGL3 basic was used. It contains the luciferase vector without any promoter and was used to generate Dkk-1 promoter constructs. We would expect that under experimental conditions it would show little or no change in activity. However, in some experiments this was not the case. Under serum starved and growing and confluent conditions, the overall activity levels were low, as would be expected. There was little difference between the serum starved cells and cells grown in normal medium. The promoter activity was low and decreased throughout the cell cycle. When treated with the inhibitors, there was a strange and unexpected pattern. The pGL3 basic behaved in some instances like the Dkk-1 luciferase constructs, showing higher levels of activity at lowest concentrations with the U0126 inhibitor (24 hours) and methotrexate (24 hours). In other cases it behaved very differently from the Dkk-1 luciferase constructs. We believe that these behaviors can be attributed to some sequence in the pGL3 basic vector acting as transcription factor binding sites and transcription initiation sites (Promega Technical Services).

The Region between 1068 and 535 contains Multiple Binding Sites

Our results suggest that the region between 1068 and 535 contains transcription factor binding sites required for regulation of the Dkk-1 promoter. Within this region, two TCF sites have been identified (Gonzales-Sancho et al, 2005) (Fig. 2). It is possible these sites are required for regulation of the Dkk-1 promoter in serum starved and density arrested cells. This was not determined by Gonzales-Sancho et al. There also could be additional transcription factor binding sites within the region. To determine this we

analyzed the region between 1068 bp and 535 bp upstream using the sequence analysis tool Patch (*http://www.gene-regulation.com/pub/programs.html#match*.) Table 1 shows the results of this analysis. There are numerous potential binding sites based in this region. Some of these factors are involved in cell growth. However, it is not known if these factors actually bind to the Dkk-1 sequences. As a start, it will be most critical to analyze specific mutations of the TCF sites (in the 1068 construct) to determine if these sites are involved. If they are not involved, then other sites can be examined.

Experimental Concerns

One of the most important issues is that of the control vector used, pGL3. Because it showed some unusual activity, even though it did not contain any promoter sequence, it may be necessary to repeat these experiments using the reporter vector pGL4 The pGL4 vector lacks background activity (Promega Technical Services). However, this would require that all of the Dkk-1 upstream sequences be re-subcloned into this vector. Another concern is the 535Dkk-1 LUC NIH3T3 cells. The results from this cell line were unexpected and different from the others. It is possible a group of cells were selected with altered growth characteristics. We are currently generating entirely new stable transfected cell lines for all the contructs. Each of these will be assayed under different growth conditions.

Summary

These studies confirm that Dkk-1 promoter activity is higher in a growing cell population and decreases as the cells become arrested in $G_0$. These studies further suggest

that the region of the promoter between 1068 and 535 base pair upstream is required for regulated expression of the Dkk-1 gene under different growth conditions. We analyzed the activity under different conditions and the cell cycle and treated the cells with various inhibitors. We found that Dkk-1 promoter activity decreases in serum starved and density arrested cells and shows little variation during the cell cycle. When the Dkk-1 LUC NIH3T3 cells were treated with inhibitors, we found that the promoter is not sensitive to a Ras pathway inhibitor, MEK U0126, it does not respond to aphidicolin, an inhibitor of S phase and the promoter is sensitive to methotrexate, a folate pathway inhibitor. After analyzing the region we believe required for regulation we found that there are specific binding sites required for regulation and mutating those sites could alter Dkk-1 gene expression under different growth conditions. Since the Dkk-1 promoter was found not to be sensitive to a Ras pathway inhibitor, it is unlikely that reduced Ras signaling is responsible for the decrease in Dkk-1 promoter activity in serum starved cells. The Dkk-1 promoter does not appear to vary significantly during the cell cycle and is not affected by an inhibitor of S phase. Together these results suggest that the Dkk-1 promoter is regulated relative to the growth state of the cell population and not the cell cycle. However, the Dkk-1 promoter is sensitive to methotrexate. These studies further suggest that sequences between 1068 and 535 in the Dkk-1 promoter are important for regulation. Computer analysis reveals numerous potential transcription factor binding sites. Further studies will focus on this sequence region.

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