DIFFERENTIAL EXPRESSION OF GERM CELL NUCLEAR FACTOR (GCNF) IN HUMAN OVARIAN CANCER CELL LINES

A Thesis by

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The following faculty members have examined the final copy of this thesis for form and content, and recommended that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Biological Sciences.

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DEDICATION

To my father, husband and brother, for your support and guidance all through my life.
Specially in memory of my beloved mother.
ACKNOWLEDGEMENTS

I sincerely express heartfelt gratitude to my mentor Dr Jeffrey May, for giving me an opportunity to work in his lab. During this period I got a chance to learn so many scientific techniques and work with various equipment. Without his support and guidance it would not have been easy for me to complete my research work and produce this thesis. I would like to express to sincere thanks to my other committee members Dr William Hendry, Dr Bin Shuai and Dr Wimalasena. Thanks for being on my committee and offering valuable suggestions and guidance in completing my thesis. I would also like to thank Mr James Rouse and Rosanne Skinner of Kansas Biomedical Facility where all my DNA samples were sequenced.

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ABSTRACT

Germ Cell Nuclear Factor (GCNF) is the only existing member of nuclear receptor (NR) super family NR6A1, and an orphan receptor because of an unidentified ligand. Members of NR superfamily act as ligand activated transcription factors which regulate the target gene expression either by activating or repressing transcriptional activity. GCNF is expressed in both embryonic and adult stages in the human. In adults, expression of GCNF is restricted to testis and ovaries. GCNF is also found in other species such as Xenopus leavis, Zebrafish, rats, mice and hamsters.

GCNF mRNA and protein were recently found in our lab to be expressed in various human ovarian cancer cell lines; i) ES2 – a clear cell carcinoma, ii) PA1-teratocarcinoma, iii) TOV 112D – an adenocarcinoma and iv) OVCAR–3 another adenocarcinoma. Differences in morphologies and growth rates were observed in the above cell lines. According to the growth rate curve, ES2 cells have faster growth rate than other cancer cells.

According to previous studies in our lab, GCNF was also found to be expressed in a nontransformed cell line, MLEC (Mink Lung Epithelial Cells). When MLEC cells were cultured with varying amounts of TGF-β1, a decrease in amount of GCNF mRNA expression was observed. A proportionate decrease in rate of cell proliferation was also observed. Based on these two findings, a direct relationship between growth and GCNF expression was postulated. Based on this proposed relationship experiments were undertaken to establish the potential correlation between cell growth and levels of GCNF mRNA expression. Analysis of GCNF expressed in various cancer cells using Quantitative Real time-PCR proved that PA1 had the highest amount of mRNA expression and ES2 had the least. When growth rates were compared, ES2 had the fastest doubling time when compared to PA1,
TOV112D and OVCAR cells. This contradicts the hypothesis, in spite of having fastest growth rate, ES2 contained the least amount of GCNF mRNA. The results suggest that there is no direct, linear correlation between cell growth rate and the level GCNF mRNA expression.

Expression of GCNF protein in the cancer cell lines was demonstrated via western blot analysis. Newer more efficient polyclonal antibodies have been produced by Santa Cruz Biotechnology. When nuclear and cytoplasmic fractions from cancer cells were subjected to western blotting using the new antibodies, specific bands were exhibited in the range of 50 -75 KD. PA1 cells exhibited the bands with high intensity when compared with other cancer cell lines. To study the GCNF DNA binding properties quantitative analysis of GCNF protein was performed. It is mainly via electrophoretic mobility shift assay, but we could not attain consistency in results.

Preliminary studies had demonstrated the homology of the DNA binding domain in TOV112D (epithelial cancer cell) when compared with published human GCNF sequence. The main focus of this objective was to determine if there was any homology in the coding sequence (CDS) of GCNF in other ovarian cancer cells. Cancer cells selected to prove this objective were PA1 (germ cell tumor) and ES2 (epithelial cell tumor). Three different pairs of primers providing overlapping cDNA sequences to amplify the entire GCNF mRNA sequences following reverse transcription were used to illustrate homology. A standard PCR was performed using the different primers followed by gel electrophoresis. DNA was extracted and sent for sequencing. The results indicated that the PA1 cell GCNF RNA was amplified by all 3 primer pairs, whereas ES2 cells did not amplify one of the three overlapping primers (GCNF NH and GCNF DW). These results gave a new dimension to the objective. PA1 cells are of germ cell in origin whereas ES2 is epithelial in origin.
When the PA1 sequence was compared to the published human GCNF sequence it showed 98% homology in the CDS (Coding sequence).

The impact of GCNF siRNA on PA1 cells was examined. siRNA was utilized to achieve gene silencing or gene knock down. Previous studies indicated an effect of GCNF siRNA on cancer cells of epithelial origin (TOV 112D and ES2). In terms of growth inhibition, the results showed a proportional decrease in the cell proliferation and GCNF mRNA expression. When cells of (epithelial and germ cell cancers) were compared, similarity of effect was demonstrated. This result demonstrates that GCNF is required for growth. When growth was suppressed sufficiently by siRNA it appeared to directly affect GCNF mRNA expression.

Results of the experimentation manifest discrepancies in the expression of GCNF in human ovarian cancer cell lines. Nuclear receptors bind to ligands which can be altered serve as potential pharmacological targets. As GCNF still exists as an orphan nuclear receptor, discovery of the ligand will pave the way for creating new drugs which will be helpful in the future to cure some of the life threatening diseases like Alzheimers, Diabetes and Cancer etc.
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LIST OF ABBREVIATIONS/NOMENCLATURE

AF  Activation Function
Abs Etoh  Absolute ethyl alcohol
BLAST  Basic Local Alignment Search Tool
BMP-15  Bone Morphogenetic Protein
bp  Base pair
BSA  Bovine Serum Albumin
C  Celsius
CDS  Coding Sequence
CER I  Cytoplasmic Extraction Reagent I
CER II  Cytoplasmic Extraction Reagent II
CO₂  Carbondioxide
CTE  Carboxy Terminal Extension
DBD  DNA Binding Domain
DEPC  DiEthylPyroCarbonate
DMSO  DiMethylSulfoxide
DNA  Deoxyribonucleic acid
EDTA  Ethylene diamine tetraacetic acid
EtOH  Ethyl alcohol
FCS  Fetal Calf Serum
GCNF  Germ Cell Nuclear Factor
GDF-9  Growth Differentiating Factor-9
HRE  Hormone Response Element
kDa  Kilo dalton
LBD  Ligand Binding Domain
LIST OF ABBREVIATIONS/NOMENCLATURE (continued)

LSB  Lammeli Sample Buffer
M    Molar
ml   millilitre
MLEC Mink Lung Epithelial Cells
mRNA messenger Ribonucleic acid
NaOAc Sodium Acetate
Na$_2$HPO$_4$ Sodium Hydrogen Phosphate
NCBI National Center for Biotechnological Information
NER  Nuclear Extraction Reagent
ng   Nanogram
Oct-4 Octamer -4
PBS  Phospahate Buffered Saline
PCR  Polymerase Chain Reaction
Pmol Pico mol
PVDF PolyvinylindeneFluoride
RNA  Ribonucleic Acid
RPM Revolutions Per Minute
RT   Reverse Transcription
SDS  Sodium Dodecyl Sulphate
SF-1 Steroidogenic Factor-1
siRNA small interfering Ribonucleic acid
TBS  Tris Buffered Saline
TTBS Tween Tris Buffer Saline
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<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor beta 1</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<td>µl</td>
<td>Microlitre</td>
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CHAPTER 1

INTRODUCTION

1.1 Cell signaling

Cell signaling is a convoluted and elaborate process by which cells interact with other cells and their environment. Intercellular communication within multicellular organisms is a highly synchronized process which is responsible for sustaining different physiological activities in the body. Environmental and physiological stimuli received by the cells are processed by the specific signal transduction pathways via receptors. Hydrophilic signaling molecules (ligands) like growth factors, neurotransmitters, and peptide hormones bind directly to the cell surface receptors (membrane receptors). Hydrophobic signaling molecules like steroid hormones (estrogen, progesterone and vitamin D), bind to intracellular and often intranuclear receptors [1]. Ligand binding to the specific receptor initiates conformational changes in the ligand receptor complex followed by interactions with transcriptional cofactors and DNA binding which result in stimulation or inhibition of the transcriptional process leading to gene expression or repression [2, 3]. Because of the nuclear location of members of the nuclear receptor family, they are an intensely studied area of molecular biology.

1.2 Nuclear Hormone Receptors

Nuclear receptors form a superfamily of proteins which act as transcriptional regulators in (animals) metazoans, responsible for regulating diverse biological processes like reproduction, development, homoeostasis and metabolism [4, 5, 22, 23]. The nuclear receptor superfamily consists of nuclear hormone receptors (NHR) and orphan nuclear receptors (ONR) which are mainly ligand-activated transcription factors [1, 4].

Molecular cloning of cDNA encoding the glucocorticoid receptor (GR) was considered a critical advancement in the field of molecular endocrinology in 1985. Since
then, 60 nuclear receptors have been found [6, 7, 8, 9]. Members of the nuclear receptor family are activated by small hydrophobic molecules (ligands) such as steroid hormones (androgens, progesterones, estrogens, glucocorticoids, mineralocorticoids), thyroid hormone, and-retinoids, etc. [10] Transcriptional regulation occurs through the interaction of proteins with chaperones, ligands, corepressors, coactivators and DNA [11, 12, 13, 14, 15]. The proteins bind to the nuclear receptors through specific amino acid sequences which are either ligand dependant or independent. The proteins bind to the DNA response elements as monomers, homodimers, or heterodimers [16, 17]. In general, ligand binding to nuclear receptors causes an exchange of coactivators for corepressors to facilitate transcription. Coregulators (Corepressors or Coactivators) induce chromatin modification by histone acetylation/histone deacetylation, and methylation [1, 18, 19]. NHRs are activated by binding to their cognate hormones which leads to conformational changes in the receptor protein complex. Receptor ligand binding leads to interaction of the receptor region called the DNA binding domain which bind to DNA binding sequences called hormone response elements (HRE) on the DNA[20].

Transcriptional regulation by activated NRs comprise of series of steps; 1) Binding of NRs to DNA binding sites in promoter regions, 2) Modification of chromatin by coregulators and associated factors, 3) Control in binding of Polymerase II and promoter region [33, 34, 35, 36, 37]

1.2.1 Evolution & Diversity of NR

The NR superfamily is considered to be the largest family of transcription factors. A wide range of variety within the family is due to individual gene duplications and mutations which were selected throughout the process of evolution. Genomic duplications were experienced by the early metazoans which was responsible for the distribution of the mammalian nuclear receptors all throughout the genome [21]. The most up-to-date estimation
of the number of NR genes in the human genome is thought to be 49 (with three NR pseudogenes) and is based on phylogenetic analysis and sequence alignment [24]. NR genes present in other species such as *Drosophila melanogaster* which consists of 21 genes [25], whereas *Caenorhabditis elegans* exhibits 270 genes [26]. NR share common conserved structural regions in spite of having a diverse range of members.

1.2.2 Significance and Classification

Nuclear receptors are primarily ligand-activated transcription factors which have been consigned to six subfamilies based on evolutionary studies [27]. Several members of nuclear receptor family have been identified through sequence similarity to known and receptors for which ligands have been unidentified and are known as orphan nuclear receptors [23]. Ligands are generally small molecules which can be modified easily through drug design. These changes result in controlling functions which cause major diseases like diabetes, cancer, osteoporosis etc. So they serve as promising pharmacological targets. Since orphan nuclear receptors are proteins in search of ligands, they developed into a new field of interest for identification of novel signaling pathways [28, 29].

Nuclear receptors were classified into four classes based on dimerization status, DNA binding specificity, and chemical ligands [4]. Class 1 receptors include steroid hormone receptors which function as homodimers and bind to halfsite RE inverted repeats. Class 2 receptors mainly function as heterodimers with retinoid receptors. Class 3 receptors such as GCNF function as homodimers by binding to direct repeats (DR) of DNA sequence [30, 31, 32]. Class 4 receptors such as SF1, RORα, ERRs function as monomers which bind to half site sequences [38]. Most of the receptors of class 3 and 4 are classified as orphan nuclear receptors because of unidentified ligands.
1.2.3 Structural organization of Nuclear Hormone Receptor

Nuclear receptors typically have a common structural organization which contains six modular domains (A-F). Starting with a N terminal region, there exists a variable modulatory domain or A/B domain, the highly conserved DNA binding domain, the hinge region, the C-terminal region or the ligand binding domain and a F domain which is not found in all NRs and whose function is unknown [39].

N Terminal region, A/B domain, Hyper variable amino terminus, Modulatory domain

The amino terminal (N) region is comprised of an activation function (AF-1) which serves ligand-independent transcriptional activation [40]. The NTD is likely involved in multiple– protein interactions and the activity of AF1 region has been associated with the length of the domain for members of nuclear receptor family [41, 42]. The NTD is a less conserved region, having variable size and amino acid sequence [11, 43]. The AF-1 region exhibits specificity for promoters, which are modified by its phosphorylation through several signaling pathways [44, 45].

DNA Binding Domain

Irrespective of the diversity within the NRs, structural similarities are shared. The DBD is the highly conserved region which is primarily responsible for DNA response element recognition. The DBD consist mainly of two zinc finger motifs that include ~ 80 amino acid residues. Ligand binding causes alteration in the receptor protein complex. Nuclear receptors bind to specific regions of DNA called Hormone Response Elements (HRE). NRs bind to HREs as monomers, homodimers or heterodimers [17]. The HREs consists of consensus half site sequences which are direct repeats, inverted repeats or everted repeats. The desired half site sequences for class 1 receptors or steroid receptors is 5’-AGAACA-3’ while the desired sequence for other members of the receptors 2, 3, and 4 which include estrogen receptors, thyroid hormone receptors and orphan nuclear receptors etc
is 5'-AGGTCA-3'. Generally half site sequences in HREs which bind as monomers are lead by 5' flanking sequences rich in A/T [46]. Structural studies of DBDs which are bound to HREs designate that half site recognition contains two specific regions a P box and D box. The P box is situated at the end of amino terminal (proximal) region of zinc finger with five amino acids and the D box is situated at the amino terminal (distal) region of the zinc finger and is responsible for the DBD HRE recognition by identifying spacing between the two half sites [47,48,49].

**Hinge Region**

This region serves as linker sequence between the DBD and the LBD. This domain plays an important role in stabilization of LBD and is also considered as the most variable region.

**Ligand Binding Domain**

The LBD is a multifunctional domain which is involved in dimerization, nuclear localization, gene silencing or activation (cofactor binding) and HSP (Heat Shock Protein) binding [4, 5, 9]. The LBD is a moderately conserved domain with an activation function 2 (AF-2) which works in presence of ligand by coactivator recruitment [40]. Structurally, the LBD is formed by 12 packed α helices which are intruded by three β strands which are folded into antiparallel sheets [13, 22, 50]. Structures of LBD which are apo (ligand free) and holo (ligand occupied), either alone or associated with coactivators, offers important information regarding ligand induced modifications and protein-protein interactions. Apo and holo structures revealed that the LBD is variable and consists of a ligand binding pocket (LBP) [51]. Following binding of ligand to the LBP, structural changes in the helices occur bringing the AF-2 region close to the helices. This was called a mouse trap model which explains that AF-2 functions as a lid to cover LBP [52].
Structural representation of Nuclear Hormone Receptor

NH2  Amino terminus region
A/B  N Terminal Region
C    DNA Binding Domain
D    Hinge region
E    Ligand Binding Domain
F    Unknown
COOH Carboxy Terminus Region
1.3 Orphan Nuclear Receptors

The nuclear receptor superfamily represents the largest family of transcription factors. Nuclear receptors without known ligands were designated as orphan nuclear receptors [53, 54].

1.3.1 Expression and functions of GCNF

Germ Cell Nuclear Factor is the one and only existing member of the nuclear receptor sixth subfamily (NR6A1) [9]. GCNF is also known as RTR and NCNF (Retinoic Acid Related Testis Associated Receptor and Neuronal Cell Nuclear Factor) respectively. [55, 56] GCNF was originally cloned from mouse and was classified as a unique orphan nuclear receptor in mouse germ cells [30, 57].

GCNF expression is observed in human, Xenopus leavis, mouse and zebrafish [58, 59, 60, 61]. GCNF functions as a transcriptional repressor and targets a gene by binding as a homodimer to the DR0 direct repeats or extended half sites of consensus sequence 5’-AGGTCA-3’ with zero spacing between them [62, 63, 64]. GCNF exhibits a somewhat restricted pattern of expression. It is mainly seen in germ cells of the gonads (ovaries and testis) [30, 55, 65-68]. In mouse ovaries, GCNF is expressed in primary, secondary and preovulatory follicles and also in oocytes. In mouse testis, GCNF is expressed in post-meiotic round spermatids, whereas in humans, it is seen in pachytene spermatocytes [30, 53, 66, 67].

GCNF expression was observed in early mouse embryos during gastrulation. It is required for normal embryogenesis, development of anterio-posterior axis, and organogenesis in mouse and Xenopus Laevis [57, 69, 70]. GCNF exhibits spatio-temporal control on the developing nervous system and is also seen in post-mitotic neurons of the adult mouse brain [65]. Levels of GCNF are high in developing nervous system but the levels decrease during gastrulation. GCNF directly suppress the activity of the following members of the TGF-β, family of growth factors including bone morphogenetic protein 15 (BMP-15) and Growth
Differentiating Factor 9 (GDF-9) in the ovary [71, 72, 73, 74]. Ovarian function and female fertility are influenced by GCNF by controlling the paracrine interaction between oocytes and somatic cells through various signaling pathways, GCNF is reported to suppress BMP15 and GDF9 [80]. Regulation of GCNF is also seen on mouse protamine 1 and protamine 2 genes involved in spermatogenesis [75]. Embryonic stem cells and embryonal carcinoma cells express GCNF [76, 77]. GCNF expression was proven to be necessary for the suppression of Oct 4. This was demonstrated in mouse embryos by knocking out the GCNF gene [78]. Oct-4, a POU transcription factor, is crucial for the maintenance of pluripotency of embryonic stem cells (ES) [79]. ES and embryonal carcinoma cells (EC) express high levels of Oct 4 and upon differentiation, the gene is down regulated (101, 102). Nanog is a new transcription factor which is also responsible for maintenance of pluripotency. Similar to Oct 4, Nanog is required for development of embryo. Expression of Nanog is restricted to inner cell mass in preimplantation embryos and embryonic ectoderm in the post implantation embryos.

1.3.2 Structural Organization of GCNF

GCNF gene is located on chromosome 9 at locus q33-34.1 in the human and consists of 10 exons, whereas the mouse GCNF gene contains 11 exons and is located on chromosome 2 [53, 58, 76, 87]. Structurally GCNF consists of N terminal domain, DBD, hinge region LBD, and C-terminal region. Based upon sequence analysis, GCNF homologs exhibit a great deal of homology in the DBD and LBD among different species [58, 64]. The DBD of GCNF is the highly conserved region which is 60% identical to RXRa and RARa [30]. The P box consists of three amino acids which are responsible for binding site recognition [88, 89]. GCNF binds as a homodimer to direct repeats of the half site sequence AGGTCA with repeats separated by zero spacing (DR0). Steroidogenic factor 1 binds to target gene with zero nucleotide spacer DR0 element, HS (aggTCAGGTCA), GCNF binds to target gene as a homodimer and a monomer [30, 61], which indicates that the A box of
GCNF is involved in DNA binding. Homodimerization of GCNF is responsible for its function as repressor along with binding to DR0 sequences which was dependant on three separate domains [61]

1.3.3 Mechanism of action and regulation of GCNF

GCNF, a transcriptional repressor, binds to its cognate response elements (GCNF-RE) as a homodimer or oligomer [81]. A GCNF-RE consists of a direct repeat consensus motif PuGGTCA with zero spacing (DR0) [82]. GCNF does not have activation function (AF-2) which act as activators of transcription but it consists of a particular region which plays a role in dimerization and enrolling corepressors [61,62]. GCNF networks with the nuclear co-repressors, and also suppressor RAP 80 which is highly expressed in testis [62, 78, 83]. Repression of GCNF is arbitrated by interactions with the corepressors N-CorR and SMRT in the absence of ligand [54]. GCNF is mainly believed to function in the following manner: I) Regulation of transcription occurs by binding of protein to GCNF–RE of the target genes which causes transcriptional interference by competing for the same the transcription factors. II) Regulation of transcription also might occur indirectly by controlling components of cell signaling pathways or contending for transcriptionally active multiprotein complexes [79]. NRs associate with different elements involved in transcription, corepressors, coactivators and cointegrators like CBP [CREB (c-AMP) response element binding) -binding protein], p300 and PCAF (p300/CBP-associated factor) and also steroid receptor RNA activator (SRA) [84, 85, 86]. Ligand binding induces change in the LBD and causes disassociation of corepressors and recruiting coactivator complexes [19].
CHAPTER 2

RESEARCH OBJECTIVES

According to previous studies, a relationship between growth or proliferation of cells and GCNF expression was suggested. GCNF mRNA expression was demonstrated in several human ovarian cancer cell lines which exhibited varied growth rates and morphology. The relative and comparative amount of GCNF mRNA produced by these human ovarian cancer lines is unknown. This prompted us to investigate the association of cell growth rate to GCNF mRNA expression. The research objectives of the project are as follows,

- To document the expression of GCNF mRNA in human ovarian cancer cell lines (ES2, PA1, TOV 112D, and OVCAR) using 187 bp Realtime PCR primers and also investigate the correlation between cell growth rate and GCNF mRNA expression using Real time quantitative PCR.

- To demonstrate the presence of GCNF and cellular localization of protein in human ovarian cancer cell lines by performing western blotting with newer available polyclonal antibodies available. Nuclear and cytoplasmic fractions of all four cell lines were prepared using a commercially available kit.

- Previous studies compared the GCNF mRNA sequence homology of the GCNF DBD in TOV 112D cells to the published human GCNF sequence. An important objective of this research is to establish the base sequence of GCNF mRNA in PA1 (germ cell tumor) using several sets of primers. These overlapping primer pair allows us to establish sequence homology spanning the entire GCNF mRNA coding sequence. cDNA of PA1 cell line was subjected to standard PCR using primers stated below and the isolated PCR
products were sent to University Medical Center Biotechnology Facility in Kansas City, KS for sequencing

- GCNF NH & GCNF DW 628bp
- GCNF UP & GCNF RT DW 1001bp
- GCNF RT UP & GCNF COOH 560bp

➢ To demonstrate the impact on cell growth rate when GCNF gene expression is knocked down (gene silencing) using siRNA in PA1 (germ cell tumor) cancer cells. Cells receive treatment with GCNF siRNA or control siRNA as per protocol and extent of gene silencing is assessed by reverse transcription and standard PCR. A series of bands will be obtained as a result of gel electrophoresis. These results would be compared and contrasted to siRNA transfection in TOV 112D (epithelial cell tumors). The main reason for looking into PA1 cells is to see if knocking down GCNF gene using siRNA, in this germ cell tumor line will suppress proliferation as it does in epithelial cancer cells.
CHAPTER 3
MATERIALS AND METHODS

3.1 Culture of Human Ovarian Cancer Cells

The American Type Culture Collection (ATCC) was the source of all the human cancer cell lines used in this work. Cancer cell lines of epithelial origin include ES2 (CRL-1978), a clear cell carcinoma, TOV112 D (CRL 11731), a malignant adenocarcinoma, and OVCAR-3 (HTB-161) an epithelial adenocarcinoma. A germ cell tumor line, PAI (CRL1572), was also utilized. Upon arrival, the cells were thawed rapidly in a 37°C waterbath and transferred into 75 cm$^2$ flask containing 25ml of appropriate medium supplemented with Fetal Calf Serum (FCS) and other necessary contents as per ATCC instructions (culture media varies with different cell lines). All the cell culture steps were performed in a sterile laminar flow cabinet. The cell culture flasks were kept at 37°C in a water-jacketed incubator in a humidified atmosphere containing 5% CO$_2$ and 95% air. When near confluent growth was observed, the cells were trypsinized using Trypsin/EDTA (0.25% trypsin, 1mM EDTA) which is used to detach the cells from the bottom of the flask. Respective medium containing FCS was added to stop the trypsin activity in the flask. The whole medium was transferred into 16 ml sterile tubes and centrifuged to pellet the cells. The supernatant was discarded and the cells were resuspended with specific amounts of culture medium containing and 5% DMSO and they were transferred into cryotubes which were appropriately labeled. These tubes were kept in a Styrofoam cooler, placed into a -80°C freezer overnight. The following day, the tubes were transferred into liquid nitrogen at-196°C and stored there until used. Whenever needed, vials of the cells were taken from liquid nitrogen, and subjected to rapid thawing at 37°C and transferred into 75cm$^2$ flasks with respective media to propagate the cells.
Epithelial cell tumors

ES2  McCoy’s 5A medium supplemented with 10% FCS
TOV 112D  Ham’s F12: DMEM (1:1, FD) medium supplemented with 15% FCS
OVCAR3  RPMI medium supplemented with 20% FCS and 1µl/ml of insulin

Germ Cell tumor

PA1  FD medium supplemented with 20% FCS

3.2 GCNF Primers

Primers are short oligonucleotide segments which serve as starting points of DNA amplification. The primers which are human-specific sequences used in the project were as follows

GCNF NH& GCNF COOH
GCNF UP& GCNF DW (351 bp product)
GCNF RT UP & GCNF RT DW (187 bp product)

**Human Germ Cell Nuclear Factor (GCNF)**

Image source: Laboratory Data
Primers were designed based on the regions exhibiting similarities between human and mouse GCNF sequence. This process was determined using BLAST from NCBI website. Primers were checked for any formation of inter or intrachain primer dimer formation and also hair pin stem loop structures using www.premier biosoft.com/netprimer. Primers were produced by Sigma Genosys and were diluted to 100 pmol/µl vials which were stored at -80°C. Primers were further diluted to 10pmol/µl which was used as working stocks. The human and mouse GCNF exhibited significant homology, so the primers designed should work with cancer cell lines obtained from human. These GCNF UP and GCNF DOWN primers generate a 351 bp PCR product, GCNF UP primer (sense strand) 5’ GAACAAAGCAACCTGTCTCAT 3’ and GCNF DW primer (antisense strand) 5’ ATGGTTGCTCCAGTGATTG 3’. In order to perform real time PCR, human GCNF primers were specifically designed which generates a product which is 187 bp in length, GCNF RT UP primer 5’ GCTAATCTGCTGTCTTCCC 3’, and GCNF RT DOWN primer 5’ ACTCCTCGTTGCTGACCTTT 3’. In order to determine the complete sequence homology GCNF primers used were GCNF NH, 5’ GCTCCTGACAACCTCCTCC 3’ and GCNF COOH, 5’ GCCTCCATCTTGGTCTCTCTG 3’ which hybridize to the 5’ and 3’ untranslated region.

3.3 Isolation of Total RNA

This process involves several steps including i. Extraction ii. Precipitation iii. Washing and iv. Quantification

3.3.1 RNA Extraction

Ultraspec RNA isolation reagent from Biotecx labs was used to isolate total cellular RNA. It was taken out from 4°C and kept at room temperature for 30 minutes. Cell culture flasks were removed from the incubator and the media poured into waste receptacle. This was
followed by addition of 3ml/75 cm² flask of Ultraspec reagent followed by swirling the reagent over the flask while on ice for five minutes. The reagent dissolves the cells and initiates the isolation of total RNA. 3 ml of lysate was equally distributed into three DEPC treated, 1.5ml microfuge tubes. 200µl of chloroform was added to each tube which was shaken vigorously by hand for 15 seconds. The tubes were kept on ice for next five minutes and then subjected to centrifugation at 12,500 RPM for 15 minutes at 4ºC.

3.3.2 Precipitation

As a result of centrifugation, two layers of liquid formed in the microfuge tube. The clear, upper aqueous phase was removed using a micropipettor and placed (500µl) in to a clean, appropriately labeled DEPC treated microfuge tubes. Equal amounts of ice cold isopropanol was added and gently mixed and tubes were stored at -20ºC for 24 to 48 hours to precipitate the RNA.

3.3.3 Washing

The tubes were taken out of the freezer and subjected to centrifugation in a sorvall microcentrifuge for 30 minutes at 4ºC @ 12,500 RPM. The supernatant was discarded without disturbing the RNA pellet. 1.0ml of 75%ethanol and tubes were vortexed to suspend the pellet. The tubes were then subjected to centrifugation for 5 minutes at 7,500 RPM. The step was repeated one more time and care was taken to remove as much as possible. The tubes were covered with parafilm, to which several holes were punched and vacuum dried for 10 minutes. The RNA pellet turns transparent and appears as a drop. The RNA pellet was suspended in 25µl of nuclease free water and kept at 4ºC for 2 hours. Later 1µl of the above was added to the 99µl of Na₂HPO₄ for the quantification of total RNA.

3.3.4 Quantification

RNA content was determined by spectroscopy using an Eppendorf Biophotometer at 260/280. The readings were taken relative to a blank and the purity of RNA was observed.
The values range from 1.8-2µg/µl. Total RNA is expressed as same and the total amount of RNA prepared. The samples were stored in the freezer -20ºC until utilized for reverse transcription.

3.4 Reverse Transcription (RT)

RT was performed using two different types of kits based up on the experimental objective. RT was performed by a) Reverse Transcription System by Promega for standard PCR b) RT kit by Invitrogen (SuperScript™III First Strand Synthesis SuperMix for qRT-PCR) for real time PCR

3.4.1 Reverse Transcription System (Promega)

The total RNA samples and respective reagents for RT were thawed on ice. 1µg of the RNA was placed in the DEPC treated tube and kept in water bath for 10 minutes at 70ºC followed by rapid cooling and brief spinning. Primers used in the RT process were Oligo dT and random hexamers which were included in the kit. Master Mix was prepared according to the protocol and added to the sample and incubated at room temperature for 10 minutes. Samples were incubated at 42ºC for 45 minutes and followed by 94ºC for 3 minutes. The RT products were stored at -20ºC until utilized for the PCR.

3.4.2 Standard PCR

cDNA from RT was subjected to standard PCR using a Promega PCR Core System I kit and a Thermolyne Amplitron II thermal cycler. Several programs were created for running the samples. The programs were created based on the melting temperatures of the primers. Initial denaturation was performed at 94ºC for 2 minutes. This was followed by 35 cycles of: Denaturation at 94ºC for 1 min, primer specific annealing at 55ºC for 1 minute; extension at 72ºC for 2 minutes. After 35 cycles, a final extension at 72ºC for 12 minutes was performed followed by a hold at 4ºC. Samples were stored in the refrigerator until utilized for gel electrophoretic analysis. RT/PCR samples were subjected to agarose gel electrophoresis.
using 2% agarose gel with ethidium bromide, as a DNA intercalating agent. DNA bands were seen when the gel was exposed to the UV light. The gel was photographed using Kodak EDAS Photodocumentation System.

3.4.3 DNA Extraction

The dsDNA PCR products were extracted from the agarose gel using QIAEX II Agarose Gel Extraction Kit. DNA readings were taken using Eppendorf Biophotometer.

3.4.4 DNA Purification

To the known volume of DNA sample, 0.1 volume of 3M NaOAc was added. 2μl of pellet paint was added to the tubes, followed by twice the volume of absolute ethylalcohol. DNA was allowed to precipitate at -20°C for 2 hours. The tubes were centrifuged at 13,000 RPM for 20 minutes. Supematant was removed and 1.0 ml of 70 % ethyl alcohol was added. The tubes were subjected to centrifugation at 7500 RPM for 5 minutes. Supematant was removed and the pellet was dried in vacuum dryer for 10 minutes. The product was resuspended in 10mM tris/EDTA. Readings were taken in an Eppendorf Biophotometer. DNA samples and the relevant primer pairs were diluted to 25ng/µl and 2pmol/µl respectively and sent for DNA sequencing to Kansas University Medical Center Biotechnology Facility in Kansas City, KS.

Results of sequencing were compared to published human GCNF sequence for any similarities and differences. BLAST analysis from the NCBI website was used to align the known and experimental sequences.

3.4.5 Reverse Transcription System (Invitrogen)

1µg of RNA was placed in to DEPC-treated tubes along with master mix prepared as per the protocol provided by Invitrogen. The samples were incubated at 25°C for 10 minutes, 50°C for 30 minutes, the reaction was terminated at 85°C at 5 minutes and kept on ice. This
was followed by the addition of 1µl of E.coli RNase H and incubated at 37ºC for 20 minutes. The cDNA product was stored at -20ºC until utilized for Real time-PCR

3.4.6 Real Time PCR

The RT samples were subjected to real time-PCR using kit by Takara in a Cephid Smart Cycler II System. All reagents and samples were thawed on ice and a master mix was prepared as per the instructions on protocol provided by Takara Bio Inc. The reagents along with the samples were added to special tubes made for the smart cycler II for use in real time-PCR. SYBr green (DNA intercalating dye) was used in the Real time PCR. SYBr green binds to the amplified product and emits fluorescence. The tubes were centrifuged before they were loaded into Smart Cycler II System. Real time PCR requires less time to complete when compared to Standard PCR.

Initial denaturation occurred at 95ºC for 3-5 seconds. This step was followed by primer annealing (the temperature is variable depends of the specific primers used) at 65ºC for 10-20 seconds. Finally extension 72ºC for 6-15 seconds was performed. Based on the melting temperatures of the primers, a program was specifically designed to run with real time primers. The program runs through 40 cycles and the system has a function that it can terminate the reaction as soon as it detects the amplified product. As a result it yields quicker analysis and also prevents excess amplification of the product before melt curve analysis. The results obtained were plotted on a graph with concentration of DNA on X axis and cycle threshold values (Ct values) on Y axis. The Ct or cycle threshold value is the cycle number at which the fluorescent signal within the reaction crosses a specified threshold value relative to background. Amplification curve represent values of fluorescence vs cycle threshold values. The threshold value is the level of signal that represents a statistically significant increase over baseline signal. The threshold differentiates between amplification signal and
background fluorescence. The Tm is the melt temperature at which DNA hybridization reverses and fluorescence declines.

3.5 Protein Analysis

The proteins were analyzed by several steps such as extraction of nuclear and cytoplasmic fractions, protein assay, western blot and EMSA.

3.5.1 Extraction of Nuclear and Cytoplasmic fractions

The extractions were performed according to the protocol in a kit obtained from Pierce Biotechnology. Cancer cell lines were cultured in 75cm² flasks with appropriate medium and when they exhibited near confluent growth, flasks were taken out of the incubator for extractions. Cells were rinsed with 10 ml of sterile PBS. 1ml of PBS was added to the flask and cells were detached with a rubber scrapper and transferred into DEPC treated microfuge tubes and they were subjected centrifugation in a microfuge for 1 min. As a result, a pellet was formed which gives the approximate volume of cells range from 20 -50µl. If the volume of the cells was 20 µl, 200µl of ice cold Cytoplasmic Extraction Reagent I was added to the pellet along with 2µl of protease inhibitors. The tubes were vortexed vigorously and incubated on ice for 10 minutes. Followed by addition of 11µl of ice cold cytoplasmic extraction reagent II and vortexed for 5 seconds and incubated on ice for 1 minute. Following incubation the tube was vortexed at 16,000xg for 5 minutes. The supernatant (cytoplasmic fraction) was transferred into clean and prechilled tube. The pellet was resuspended with 100µl of nuclear extraction reagent along with 1µl of protease inhibitor. The tube was incubated on ice for 40 minutes and vortexed for every 10 minutes. Following the incubation the tube was centrifuged for 10 minutes at 16,000xg. The supernatant (nuclear fraction) was transferred into a clean and prechilled tube. The nuclear and cytoplasmic extracts were placed in -80ºC for storage until utilized.
3.5.2 Protein Assay

Protein content in the nuclear and cytoplasmic fractions were determined using BRC protein assay kit from BioRad. A Beckman Spectrophotometer was used to obtain the absorbance at 750nm relative to a standard curve generated using BSA which were read at a wavelength of 750nm with visible light source. The samples were frozen until subjected to western blot analysis.

3.5.3 Western Blotting

Nuclear and cytoplasmic fractions of ES2, PA1, TOV112 D, and OVCAR cells were thawed on ice. Equal amounts of protein were added to DEPC treated tubes along with 7µl of 5X Laemmli Sample Buffer (LSB), a nondenaturing buffer and boiled in a water bath for five minutes. LSB was used to minimize changes in the GCNF structure. The tubes were taken out and centrifuged briefly. Precise protein gradient (4-20%) gels (10-12 wells) were taken out of refrigerator and warmed to room temperature for fifteen minutes. Gel electrophoresis apparatus was set up and BioRad Prestained standards and Precision Plus standards were added to wells 1 and 2, respectively. Equal concentrations of the protein (50µg in 35µl) of each sample were loaded on to the gel. Initially the gel was run at low voltage 25mV for the samples to enter in to the gel, later it was run at 50 mV for 2 to 3 hours. The gel was taken out and carefully assembled for the transfer on to PVDF membrane in the electrophoretic transfer apparatus overnight at 4ºC. This was followed by blocking of the membrane with 20 ml of TTBS + 1%BSA for 60 minutes. Primary antibody treatment followed which includes treatment of membrane with normal rabbit serum (5µl) which serves as control or anti hGCNF rabbit polyclonal antibody which was diluted 1:500 in TTBS and incubated for one hour and washed twice with TTBS and once with TBS for 15 minutes. Secondary antibody treatment was followed by 1:500 dilution, in which both the membranes were treated with bovine anti rabbit polyclonal antibody linked to horse radish peroxidase for one hour and
fifteen minutes. 5µl of streptactin was added to the secondary antibody solution. Streptactin is responsible for viewing Precision Plus standard molecular weight markers. Both of the membranes were washed and treated with Luminol reagent (Santa Cruz Biotechnology) for one minute and viewed under BioradVersadoc digital Photoimaging system which revealed chemiluminescent bands.

3.5.4 Electrophoretic Mobility Shift Assay (EMSA)

Qualitative analysis of protein was done in order to study the GCNF DNA binding properties. Cancer cell lines were grown in respective medium and nuclear and cytoplasmic fractions were extracted using a kit from Pierce Biotechnology. Protein concentrations in each of the fraction were determined using BRC Biorad Assay. EMSA is the technique done to identify proteins in protein-DNA complex. Native GCNF DNA binding oligos were prepared by adding equal amounts of GCNF forward and reverse oligos in a PCR tube. It was kept in a hot water bath at 94ºC for 3 minutes. Followed by slow cooling and the tubes were kept at 4ºC. GCNF DNA binding oligos were ran on 3% agarose gel along with GCNF forward oligos. 4x EMSA buffer was freshly prepared. Specific amounts of buffer, probe, sample and nuclease free water was added to the DEPC treated tubes which made up to 20µl of reaction volume and incubated at 37ºC for 30 minutes. 1µl of gel red was added to each tube and incubated for 10 minutes at room temperature. The samples were loaded on to 8-16% gradient gel, was run at 30 mV initially for 15 -20 minutes and later voltage was increased up to 50mV which run for 2-3 hours. After 3 hours gel electrophoretic apparatus was turned off. The gel was carefully removed from plastic case and placed under UV light for observing gel shift in the procedure.
Components of 4x EMSA Buffer (1000µl)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM Tris</td>
<td>400µl</td>
</tr>
<tr>
<td>1M NaCl</td>
<td>200µl</td>
</tr>
<tr>
<td>1M DTT</td>
<td>4µl</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>8µl</td>
</tr>
<tr>
<td>Glycerol</td>
<td>200µl</td>
</tr>
<tr>
<td>Water</td>
<td>188µl</td>
</tr>
</tbody>
</table>

Native GCNF DNA Binding Site

5' C TGG GTA AGG TCA AGG CTA TTC TAA AGT CGA 3'
3' G ACC CAT TCC AGT TCC GAT AAG ATT TCA GCT 5'

3.6 siRNA Transfection

The control siRNA, GCNF siRNA, transfection medium, transfection reagent, and all the necessary requirements were provided by a GCNF siRNA from Santa Cruz Biotechnology. This process utilized 3, double stranded oligonucleotides for gene silencing. They were 5’ UUC UUG UCA CGA CUG CAU Ctt -3’, 5’ UCU UCU CUG AUA GCC UUC Ctt -3’ and 5’-UUCAAU UGU UCC AGC UGU Gtt -3’. A 75cm² flask of PA1 cells was grown in the specific medium and allowed to attain ~90% confluency upon which cell counts were performed. Based on the cell counts, a six well tissue culture plate was setup with 200,000 cells per well along with antibiotic-free growth medium with FCS. The tissue culture plate was kept in the CO₂ incubator for 24 hours. The plate was checked for viability of the cells, which is the important factor in performing transfection experiment. The following solutions were prepared to carry out transfection for one well.
Solution A

This was prepared by diluting 8µl of siRNA duplex (control siRNA or GCNF siRNA) into 100µl of transfection medium which was mixed gently and incubated for five minutes.

Solution B

This was prepared by diluting 8µl of siRNA transfection reagent into 100µl of siRNA transfection medium which was mixed gently and incubated for five minutes.

After five minutes, both solutions A and B were gently mixed to form siRNA-siRNA transfection reagent complex and incubated for 30 minutes at room temperature. The cells were subjected to washing with 2ml of transfection medium which was immediately aspirated.

800µl of siRNA transfection medium was added to each tube containing control or GCNF siRNA with transfection reagent mixture. Both the solutions were gently mixed and the mixture was superimposed on the cells which were incubated for 9½ hours at 37°C in a CO₂ incubator. After the incubation, 1ml of growth medium was added to the cells with twice the amount of serum and antibiotic concentration without removing the transfection mixture. If toxicity was evident, the transfection mixture was removed and replaced with normal growth medium. The cells were incubated for 18-24 hours.

Following this the medium was aspirated and replaced with fresh growth medium and the cells were incubated for 24-72 hours. At the end of incubation, the cells were subjected to trypsinization and cell counts were performed using coulter counter. Total RNA was isolated from the same, reverse transcription was performed using Promega Kit and standard PCR was performed using the 351bp primer set (GCNF UP& DOWN). Agarose gel electrophoresis of the samples was performed to observe the GCNF PCR product.
CHAPTER 4

RESULTS

4.1 Correlation between Growth and GCNF mRNA expression

Preliminary studies demonstrated the expression of GCNF in human ovarian cancer cell lines. Results also suggested a relationship between cell growth and GCNF. Based upon differences in growth rates among cancer cells, population doubling times were expected to reflect differences in GCNF mRNA expression. Initially we started by confirming that the primers used were efficient and relevant. GCNF real time primers and GCNF primers were used to amplify GCNF message. The results obtained in Figure 1 clearly indicate that the primer sets produced at 187bp and 351bp GCNF PCR products respectively, using various cancer cell cDNA.

4.1.1 Standard Curve

A standard curve was constructed in order to prove the linearity of the real time assay over a range of DNA (concentrations). It was also used to determine the reproducibility of the PCR. Total RNA was isolated from ES2 cells and subjected to RT, followed by standard PCR with primers (GCNF RT Up and GCNF RT Down primers), gel electrophoresis was performed. DNA bands were cut from the gel and dsDNA (PCR product) was extracted, purified and subjected to spectroscopy to quantitate the amount of DNA. Dilutions of DNA were made ranging from 10,000pg-0.1pg (10ng-100fg). These dilutions were subjected to real time quantitative PCR with GCNF real time UP and DOWN primers. Table 1 gives the range of the DNA values utilized and Cycle threshold (Ct) values obtained. Figures 2 and 3 represent standardization of the data (amplification curve) and melt temperature curves. Figure 4 represents the DNA standard curve of four ovarian cancer cells, when the cycle threshold (Ct) values were plotted against the log concentration of the DNA. The slope and Y intercept of the graph was calculated along with correlation coefficient, which was -0.994
which indicates the graph was linear and also proves the efficiency and useful range of the Real time PCR.

4.1.2 Real Time PCR

Equal amounts of RNA (1µg) from all four cancer cell lines was subjected to reverse transcription using a kit from Invitrogen designed for use with real time PCR. cDNA from all the cancer cell lines was subjected to real time PCR with real time primers. The result (real time PCR fluorescence curve for ovarian cancer cells) obtained is shown in the Figure 5. Figure 6 illustrates the melt curve when all four ovarian cancer cells were used. Based on the Cycle threshold values obtained, PA1 cells express higher levels of GCNF mRNA than other cell lines. When different reaction volumes (varying amounts of cDNA, 0.2µl and 1µl) were tested, a similar result (PA1 cells showed high levels of mRNA when compared with other cell lines) was obtained. Figures 7 and 8 represents the Ct values and Melt curves respectively, for varying reaction volumes. A graph (Figure 9) was plotted illustrating the relationship between reaction volume and cycle threshold values. A graph illustrating the growth rates of cancer cells from previous data (Figure 10) was compared with the graph representing the levels of GCNF mRNA (Figure 9) to illustrate any correlation between cell proliferations or growth and levels of GCNF mRNA. The results indicate that GCNF mRNA expression did not directly correlate with growth as hypothesized.

4.2 Expression of GCNF protein by ovarian cancer cells

Expression of protein in all four cancer cell lines was estimated by quantitative and qualitative methods. Quantitative analysis was performed by Western Blotting technique. Qualitative analysis was performed by EMSA.

4.2.1 Western Blot or Immunoblot

Nuclear and cytoplasmic fractions of ES2, PA1, TOV112D and OVCAR cells were extracted and were subjected to western blot analysis using a GCNF antibody. Following the
exposure to primary and secondary antibodies, the PVDF membrane was treated with Luminol reagent and viewed under Biorad Versadoc Photo Documentation System to view the chemiluminescence generated. Precision Plus Protein (PPP) standards were used to estimate the protein size. PPP standards range from 10 kDa – 250 kDa. As per published values, the molecular weight of GCNF was supposed to be 60kDa. When nuclear and cytoplasmic fractions were subjected to western blot analysis all four cell lines expressed coherent bands which were consistent in showing up at 60kDa (Figure 11, 12). Normal non immune rabbit serum Ig was used as a control. The membranes probed with control Ig did not show any specific bands at corresponding size represented in the Figure 13. This indicates the specific presence of GCNF protein in the lysates.

4.2.2 Electrophoretic Mobility Shift Assay (EMSA)

Western Blot analysis of GCNF protein of cancer cell lysates indicated that the molecular weight of GCNF was 60kDa. In order to study the DNA binding properties of the GCNF protein, qualitative analysis was performed. EMSA was used to exhibit gel shift in the samples through the interaction of GCNF protein with a ds oligonucleotide probe containing the GCNF DNA binding site.

Nuclear and cytoplasmic fractions were obtained as per the protocol. Protein concentrations of the samples were assessed by BRC Biorad protein assay. Samples were loaded on to a precast gradient gel (4-20%) and ran slowly at 30mV for 15-20 minutes. The voltage was increased to 50mV and ran for 3 hours. At the end of three hours the gel electrophoresis was stopped and the gel was carefully removed from the plastic casing and placed under UV light to observe gel shift in nuclear and cytoplasmic fractions in the samples. Initially, the procedure was performed with ES2 cells and TOV 112 D cells which showed a gel shift, specifically in the nuclear fractions. When the same procedure was repeated with ES2 and PA1 cells no specific gel shifts were observed. Further it proved
difficult to reproduce the original shifts. Despite of making several changes in the procedure, there was no consistency in the appearance of the gel shift. The conclusion here is that we could not demonstrate the GCNF protein reproducibly using EMSA.

4.3 GCNF mRNA sequence in CRL 1572 (PA1 cells)

PA-1 germ tumor cells were cultured and total RNA was extracted. RT was performed using the Promega kit and subjected to standard PCR using three different primer pairs which overlapped and spanned the GCNF mRNA sequence. Figures 14 represent the amplification of ovarian cancer cell GCNF mRNA using the overlapping primer pairs. Figure 15 represents the regions of the GCNF mRNA sequence amplified by the overlapping primer sets. Samples were subjected to gel electrophoresis, bands were cut out, and DNA was extracted. Isolated DNA samples were sent for sequencing along with their respective primers. The DNA sample concentration for sequencing was 25ng/µl and the primer concentration was 2pmol/µl. The results of sequencing were analyzed through Chromas Lite software. This provided us with individual sequences using overlapping primers and the ability to construct the overall GCNF mRNA sequence. The experimental sequence was compared to the published human GCNF by using BLAST search tool in NCBI webpage (ncbi.nih.nlm.gov). Using the above information obtained of PA1, coding sequence (CDS) was configured. Figure 17 contains the individual GCNF sequence of PA1 cells generated from the overlapping primer sets. When the entire sequence was compared with human GCNF transcript variant 2 it exhibited 98% homology. Figure 18 represents homology between the experimental and published sequences.

The above procedure was repeated with ES2 cells. ES2 cells did not reproduceably express one of the regions encompassing overlapping primers (Figure 16). After a series of repetitions using the overlapping primers, we found that ES2 cells did not express the sequence spanned by the, first primer pair (GCNF NH and GCNF DOWN) the amino
terminus. Strong bands were expressed when ES2 cells were treated with the other overlapping primers. Figure 14 and 16 represents ES2 cells with overlapping primers. So these samples were subject to gel electrophoresis and DNA bands were cut and DNA was extracted and sent for sequencing with the concentrations mentioned above. The results were processed through Chromas lite software and sequences obtained were compared. Apart from first primer pair, other overlapping primers showed 100% homology when compared to the published human GCNF sequence. Information obtained was not sufficient to construct the GCNF entire mRNA sequence of ES2 cells.

4.4 GCNF Gene knock down in PA1 cells using siRNA

Previously, GCNF siRNA was used to knock down TOV 112 D GCNF mRNA. It was decided to investigate this in the PA1 germ cell tumor line. PA1 cells were cultured and plated on a six-well plate and duplicates were maintained and followed as per the protocol provided in the kit by Santa Cruz Biotechnology. Wells received either control siRNA, GCNF siRNA or were left untreated. Post incubation, the cell counts were performed using Coulter Counter and total RNA was isolated and RT/PCR was performed following gel electrophoresis. Table 3 represents the cell counts obtained with the different treatments and the significant decrease in their numbers with siRNA. There was a corresponding decrease in the GCNF mRNA expression as shown in Figure 19. The decline in the GCNF mRNA expression and resultant cell count was particularly silencing the GCNF siRNA treated cells. Preliminary studies proved that siRNA transfection of TOV 112 D cells (epithelial cell origin) exhibited a gradual decrease in the cell numbers, which reflected in the decrease in the GCNF mRNA expression. Results of siRNA transfection of TOV 112D (epithelial cell origin) when compared PA1 (germ cell origin) suggest that decline in GCNF expression ultimately effects the cell proliferation (Figure 20 and 21). Control siRNA also showed a minimal but real effect when compared to the GCNF siRNA which caused decrease in levels
of GCNF mRNA. The explanation for this is unknown at present but not found in the earlier studies.
CHAPTER 5
DISCUSSION

GCNF is a novel orphan receptor and belongs to the large and diverse family of nuclear receptors. It exhibits unique properties, and exists as the sole member of the nuclear receptor subfamily NR6A1 [55,65]. The nuclear receptor superfamily is subdivided into 6 subfamilies and 26 groups of receptors based on phylogenetic studies [90]. GCNF is classified as an orphan nuclear receptor because of an unidentified ligand. GCNF is closely related to members of nuclear receptor family 5 which consists of steroidogenic factor (SF1/NR5A1) and liver receptor homologous protein 1 (LRH-1/NR5A2) [91]. Orphan nuclear receptors such as GCNF, SF1 and LRH-1 are emerging as factors for regulating gene expression in the oocyte and somatic cells within the ovary, which impacts female reproduction [92].

Expression of GCNF in human ovarian cancer cells was a novel observation, which originated from studies using epithelial cell and germ cell lines. Previous studies strongly suggested a relationship between cell growth and GCNF expression. The main focus of the project was to demonstrate a direct correlation between the level of GCNF mRNA expression and the proliferation rate of cancer cells both of epithelial and germ cell origin.

Quantitative analysis of cancer cell mRNA was performed by Real time-PCR. Growth rates were compared to GCNF mRNA expression levels. The data indicated a lack of direct correlation between the GCNF mRNA level and cell proliferation. A standard curve was constructed to demonstrate the linearity of the real time PCR assay range. According to the hypothesis, faster growing cells would have high levels of GCNF mRNA than slow growing cells. I expected high levels of GCNF mRNA in ES2 cells which exhibit a fast growth rate. But when growth rate of four ovarian cancer cell lines was compared to GCNF mRNA expression data in ovarian cancer cell lines, it disproved the hypothesis even though there
was difference in the GCNF mRNA expression. PA1 cells had the highest levels of GCNF mRNA followed by TOV112 D, OVCAR and ES2 cells. This reveals that there is no direct correlation between cell growth or proliferation and expression of GCNF mRNA.

Analysis of the GCNF protein was performed via western blot. Expression of GCNF protein was observed in cancer cells when nuclear and cytoplasmic fractions were utilized. Specific bands were found in both fractions. All the cell lines exhibited strong bands. Newer GCNF rabbit polyclonal antibodies proved to be efficient in detecting GCNF protein relative to antibodies used previously. Precision Plus Protein standards were used as a reference to estimate the size of the expressed GCNF in all cancer cell lines. From the western blot results, we detected the expression of GCNF in all four ovarian cancer cells with a molecular weight of 60 kDa. PA1 expressed a bright and coherent band when compared to other cells. Basically it implies the presence of high amounts of the target protein when compared to other cells in spite of using same amount of total extract protein. It is unknown that, if the high amount of protein levels obtained in PA1 germ cell origin relates to the cell proliferation rate. As GCNF is nuclear receptor, we expect to see it predominantly in the nuclear fraction. But it was also visible in cytoplasmic fraction indicating it might be due to contamination occurring during the cell fractionation procedure. Possibility of cross contamination in nuclear and cytoplasmic fractions is high so it must be checked before protein analysis. Specific selective markers could be utilized for this process. Accordingly the correlation between GCNF protein levels and cell growth or proliferation is still in question.

Qualitative analysis of GCNF protein was performed by EMSA (Electrophoretic Mobility Shift Assay). Nuclear and cytoplasmic fractions were mixed with EMSA buffer and specific probe. When the gels were observed under ultra violet light inconsistent appearance of gel shift was noticed. Even after modifications in the procedure, we could not attain strong evidence for gel shift. We tried by increasing the period of incubation from 30 minutes to 1hr,
2hr. A possible candidate for EMSA problems was the reducing agent, DTT (Dithiothreitol) used in EMSA buffer. This may have changed the conformation of the GCNF thus affecting its binding to the DNA probe. Nuclear hormone receptors require a ligand for activation. Lack of identified ligand in GCNF might be responsible for inconsistent bands. NRs also require Zn\(^{++}\) to be functional because of Zinc finger motifs on DBD. Ligand and Zn\(^{++}\) ions are responsible for binding reactions in EMSA. EDTA used in the buffer might also be a reason. EDTA might interfere with binding reactions because it acts as a strong chelator.

EMSA has its own limitations. Mostly the samples are not at chemical equilibrium during electrophoresis step. Dissociation of complexes during electrophoresis prevents their detection and also binding density and also most of the complexes are stable in gel than in free solution [93, 94, 95]. Other limitations includes electrophoretic mobility of protein DNA complex is dependent on several other factors other than the molecular weight of protein [96]. Electrophoretic mobility of protein and DNA complex does not provide direct exact location of the nucleic acid complexes which are occupied by proteins [96]. As we did not observe gel shift, other techniques such as CHIP (Chromatin Immuno Precipitation) might be useful to study GCNF DNA binding sites.

Complete sequence of GCNF gene in PA1 cells was determined using overlapping primers. We did expect to see differences in the GCNF message in cancer cells originating from a germ cell tumor. When PA1 GCNF sequence was compared to published human GCNF sequence, 98% homology was exhibited. The homology was seen in the coding sequence (CDS) which ranges from 179-1621bp. PA1 sequence was indicated by black arrow (Query) and the published human GCNF sequence was indicated by blue arrow (Subject) (Figure 14). Several small gaps were found in the sequence which overall yielded significant similarity with human GCNF transcript variant 2. A few mismatches were found in the sequence. Mostly gaps were found in the N-terminal region which is responsible for the gene
regulation. When ES2 cell GCNF DNA was treated with overlapping primers, the cells did not express PCR product for first primer pair (GCNF NH and GCNF DW). Several repetitions of this attempt generated the same result. The N terminal domain is considered less conserved region in the nuclear hormone receptor family. A/B domain consists of transactivation function (AF-1). This implies that the N Terminal Domain may be potentially involved in protein-protein interactions and the length of the domain positively regulates with the activity of AF1 for different members of nuclear receptor superfamily [41, 42]. Alternative splicing may also be one specific reason. Post translational modifications such as phosphorylation play a regulatory role in maintaining the activity of AF-1 region (97, 98).

100% Sequence homology was observed when other primer pairs were used. Gene expression is regulated dynamically in a spatio-temporal manner by which cells respond to environmental and physiological stimulus. Several important factors like DNA-binding transcription factors, coregulators and RNA Polymerase II are responsible for mediating gene expression (99, 100).

Gene silencing by GCNF siRNA transfection on PA1 cells resulted a decrease in cell proliferation which was reflected in levels of GCNF mRNA expression. The graph in Figure 20 exhibits a significant decline in rate of cell proliferation. When the PCR product was ran on gel, there was an obvious decrease in the expression of GCNF mRNA providing evidence of gene silencing. Comparative analysis between cancer cells of germ cell origin (PA1) (Figure 20) and epithelial cell origin (TOV 112 D) (Figure 21) exhibited uniformity in gene knock down. Even though one of the objectives proved that there is no direct correlation between growth rate and levels of GCNF mRNA, when PA1 cells were subjected to gene silencing we observed a knock down in its expression. Based on the results obtained, it confirms GCNF is required for growth. These results suggest that there might be threshold
levels of GCNF which are required for transcriptional repression leading to growth. GCNF gene at high levels might drop the level to less than threshold levels.

Ovarian cancer exhibits one of the highest mortality rates in women. Ovarian cancers are classified based on their origin from epithelial cells, stromal cells or germ cells. Tumors originating from epithelial cells are considered to be more malignant than tumors originating from germ cells. Nuclear receptors which have putative ligands are considered potential pharmacological targets. These ligands can be subjected to chemical modifications and new drugs can be synthesized which can fight against several malignant cancers. GCNF still remains an orphan nuclear receptor because its ligand has yet to be discovered. Discovery of the ligand for orphan nuclear receptors will help us in better understanding of its structure and mechanism not only limited to transcriptional regulation but also in its use in pharmaceutical industries to make novel drugs.
CHAPTER 6
SUMMARY AND CONCLUSION

GCNF was expressed in ovarian cancer cells of from both epithelial cells and germ cells origin. Previous studies suggested a relationship between growth and GCNF expression. The relative levels of GCNF mRNA expression among these cancer cells were not determined. Quantitative analysis of the cancer cells was done using Real time PCR to know the levels of GCNF mRNA expression. The results indicate differences in the expression levels with regards to mRNA using equal amounts of cDNA from all samples. A hypothesis was developed from growth rate curve obtained in the previous studies. When cancer cell growth rates were related to levels of GCNF mRNA expression, a direct correlation between them could not be demonstrated.

Newer, more targeted antibodies were used to analyze GCNF protein among samples. Nuclear and cytoplasmic fractions subjected to western blot. The size of GCNF was determined to be 60 kDa. Strong bands were shown in all samples. We were unsuccessful in demonstrating the GCNF protein using EMSA.

Complete sequence of PA1 (germ cell tumor) was attained using overlapping primers. When the sequence was compared to published human GCNF transcript variant 2 it showed 98% homology. When ES2 cells (epithelial cell tumor) were used we had issues with the one of the overlapping primer pair (GCNF NH and GCNF DOWN).

GCNF siRNA transfection in PA1 cells indicated there was a decrease in both cell counts and GCNF expression. When results of siRNA transfection of both epithelial cells (TOV 112 D) and germ cell tumor (PA1) were compared, decrease in GCNF expression and cell counts were similar.
CONCLUSION

Based on the results obtained, no linear correlation between cell growth rate or proliferation and levels of GCNF mRNA expression was observed. But when gene was knocked down there was clear evidence of decrease in cell counts and GCNF mRNA expression. This suggests the decrease in relative levels of GCNF mRNA in PA1 cells might be due to threshold levels. This explains a relationship between growth rate and levels of GCNF mRNA within the threshold levels.
REFERENCES


87. Susens, U., and Borgmeyer, U (2000) Genome Biol 1, Research 0006-1 _0006-3


APPENDIX
**Table 1**: Above table represents data for constructing DNA standard curve. Different dilutions of DNA ranging from 1:10-1:1000000 were subjected to Realtime PCR with real time primers. Ct represents the cycle threshold value.

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Figure 1 – Expression of GCNF mRNA in human ovarian cancer cell lines. Cell lines were cultured as per the protocol. Total RNA was isolated; reverse transcribed and subjected to standard PCR using real time and GCNF primers. When GCNF UP and Down primers were used all the cell lines (1.ES2, 2.PA1, 3.TOV, 4.OVCAR) showed a coherent band at 351bp. S represents the standard DNA 100bp ladder for reference. When GCNF RT UP and RT DOWN primers were used all cell lines exhibited clear band at 187 bp.
**Figure 2:** Real time PCR exhibiting amplification using increasing amounts of DNA (Table 1). Samples cross the threshold values based upon the amount of DNA present.

**Figure 3:** Melt curve for samples illustrated in Figure 2. Results indicate the presence of PCR product.
Figure 4: This represents the DNA standard curve constructed using data from purified dsDNA PCR product. The cycle threshold values were plotted against varying concentrations of DNA ranging from 10ng-100fg.
Figure 5: Real time PCR for the ovarian cancer cell lines. Results indicate different threshold values using the same amount of RNA (1µg). The graph indicates that PA1 cells express highest amount of RNA expression whereas ES2 express the least and ranks last.
**Figure 6**: Melt curve for PCR products illustrated in Figure 5 for all four ovarian cancer cells lines. The high melt temperature (85°C) indicates product.
**Figure 7**: Repeat of Real time PCR using 0.2 and 1.0µl of cDNA for all four ovarian cancer cell lines. Comparative linearity of Real Time-PCR for all 4 cancer cells, when equal amount of RNA was used. **Table 2** below represents Cycle threshold values obtained were shown below for varying amounts of cDNA.

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Figure 8: Melt curve for Real Time PCR with 0.2 and 1.0µl volume of cDNA prepared from the four ovarian cancer cell lines
Figure 9: Different reaction volumes (varying amounts of cDNA) of all cell lines were subject to Real time-PCR. A graph was plotted between volumes of cDNA utilized and threshold values obtained during Real time PCR.
Figure 10: Indicates growth rate curve obtained from preliminary data. The data suggest that ES 2 cells have faster growth rate when compared to other cell lines. Doubling time for ES2 cells is less when compared to PA1, TOV 112 D and OVCAR.

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OVCAR>TOV=PA1>ES2

Image source: GCNF as a growth regulator in normal and neoplastic cells. Thesis by Soumya Srikanthan. Wichita State University Library (103)
Figure 11, 12: Expression of GCNF protein in the nuclear and cytoplasmic fractions of all cell lines. Western blot analysis was performed on all the cell samples as per the protocol. S in the above figure represents Biorad precision plus standards ranging from 250 kDa- 10 kDa. The arrows above show the bands which are 75kDa and 50 kDa. All the samples 1-4 (PA1, ES2, TOV 112d and OVCAR) showed a clear band at 55-60kDa.

Figure 13: Treatment of the membrane with control (normal immune rabbit serum)
Figure 14: Expression of GCNF in ES2, PA1, TOV112D and OVCAR cells using overlapping primers sets that span the GCNF coding sequence.

Figure 15: Schematic illustration of the region in the GCNF mRNA sequence amplified by the overlapping primer sets.
Figure 16: Expression of the overlapping GCNF sequences in ES2 and PA1 cells (Repeat)
Figure 17: GCNF sequence of PA1 cells obtained from sequencing which was processed through Chromas lite software.

78 GCTCCTGACAACTC
93 CTCCCCCTCGGCGGAGCAGACCACAGCGCCGCTAGGCGCGGCGACAGTACGCGGAGCAACAAAC
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813 AAGATGAGAAGGAGATCTGTCATGAAAGCAATTAACTTCCTAAATCAAGATATCGGG
873 ATCTGAGCGAGCTCAGCAGGAGGCCAGGAGGCTCTACAGGCTACAGGCTAC
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**Figure 18**: Comparison of PA1 sequence (Black arrow) with published human GCNF sequence Transcript variant 2 (Blue arrow)

**PA1 Sequence**

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**Coding Sequence = 179-1621**
Figure 18 (continued)

PA1 sequence (Continued)

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**Figure 19:** GCNF mRNA levels following gene knock down by siRNA in PA1 cells. PA1 cells were plated in 6 well plate and duplicates were maintained for each treatment. First set of wells receive no treatment, second set of wells were treated with control siRNA, and the third set of wells were treated with GCNF siRNA. The results indicate that there was decrease in the GCNF mRNA in the wells which received GCNF siRNA. The values below represent the intensity of the bands. Band in lanes 2, 3 showed a clear decrease in intensity, with the GCNF siRNA being the most reduced.

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Figure 20: Graph representing the effect of gene knock down in PA1 cell counts. A decline in the GCNF mRNA expression was observed in the Figure 15. Table 3 represents the cell counts, mean /SEM values of when PA1 cells received different siRNA treatments.

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<td>2562</td>
<td>2890</td>
<td>2749</td>
<td>2733</td>
<td>95</td>
<td>..........</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>2178</td>
<td>2362</td>
<td>2256</td>
<td>2265</td>
<td>53</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>GCNF siRNA</td>
<td>1246</td>
<td>1494</td>
<td>1397</td>
<td>1379</td>
<td>72</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 3: Effect of Gene silencing by GCNF siRNA on PA1 cells. The values above represent the cell counts of PA1 cells when subjected to treatments with siRNA. Decline in cell counts can be observed. The P values obtained were P<0.0001 and are considered extremely significant.
**Figure 21:** Effect of gene knock down in TOV 112 D cells and a decrease in cell counts and GCNF mRNA expression.

Image source for 21: GCNF as a growth regulator in normal and neoplastic cells. Thesis by Soumya Srikanthan. Wichita State University Library (103)