Bachelor Thesis 2

Gaussia Luciferase Reporter Assay for Retinoic Acid Signaling

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ABSTRACT

Retinoic acid (RA) is an important developmental signaling molecule responsible for the pattering of multiple vertebrate tissues. RA is also a potent teratogen, causing multi-organ birth defects in humans. Endogenous RA levels must be therefore tightly controlled in the developing embryo.

This study was carried out in order to gain a more comprehensive understanding on the mechanism of RA signaling. In order for us to reach that goal different approches where carried out.

Our experimental approach basically consisted of making stable clones using a F9 cell line. The stable clones were transfected and assayed using the Gaussia luciferase assay kit to read the luminescence. The cells were normally harvested at 95% confluency and the assay was always carried out using a 96 well dish.

The Gaussia luciferase is a novel luciferase which is a 1000 fold brighter than firefly and Renilla luciferase. It is extremely sensitive and it is able to detect 1021mol levels of enzyme.

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ABBREVATIONS

RA	Retinoic Acid
Fgf	Fibroblast growth factor
RBP	Retinol binding protein
Adh	Alcohol dehydrogenase
Rdh	Retinol dehydrogenase
Raldh	Retinaldehyde dehydrogenases
CYP	Cytochrompe P
CRABP	Cellular Retinoic acid binding Protein
RAR	Retinoic Acid Receptor
RXR	Retinoic X Receptor
ATRA	All-trans-retinoic acid
RARE	Retinoic acid response element
ROL	Retinol
STRA	Stimulated by retinoic acid
LRAT	Lecitin retinol transferase
ER	Estrogen Receptor

AR	Androgen Receptor
GR	Glucocorticoid Receptor
VDR	Vitamin D Receptor
PPAR	Peroxisome- proliferator- activated receptor
LBD	Ligand binding domain
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
VADD	Vitamin A Deficiency Disorder
VAD	Vitamin A Deficiency
UAS	Upstream Activation Sequence
GLuc	Gaussia Luciferase
kDa	Kilo Dalton

1. INTRODUCTION

1.1. Retinoic Acid Signaling

Retinoic Acid (RA) is the active form of Vitamin A (Retinol). Vitamin A, is an essential nutrient that needs rigorous regulation during embryonic development, because Vitamin A deficiency could lead to congenital malformations in the heart, eye, circullatory, urogenital, and respiratory system, and the ecessive intake of Vitamin A is teratogenic [1]. Vitamin A is retrieved from provitamin A carotenoids, or as preformed retinyl esters from animal sources [2,3]. Unlike Fgf or Wnt that binds to the cell surface receptors, RA acts directly on target genes in the nucleus via its receptors. Retinol, transported in the the serum by retinol-binding protein 4 (Rbp4) to RA-generating tissue, is first converted to retinaldehyde by alcohol or retinol dehydrogenases (Adh or Rdh) it is then further converted to RA by retinaldehyde dehydrogenases (Raldh). Adhs are widely expressed whereas Raldhs show tissue-specific expression [4,5]. Regulation of RA is further achieved by CYP enzymes, which are responsible for the degradation of RA [6], and for the cellular RA-binding proteins (CRABP), which aid in RA uptake [7]. Upon entering its target cell RA can bind to two types of retinoid receptors; the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both theses receptors are part of the NR superfamily [8]. In mammals there are three types of RAR abd RXR receptors (α , β , γ) and multiple isoforms of each RAR performs various functions [9]. It has been reported that in zebrafish all 3 subtypes of RXR receptors can be found but only RAR α and γ . RAR binds all-trans-retinoic acid (ATRA) while RXR binds the 9-cis-RA isomer. RXRs are known to heterodimize with RARs, ligand binding to the RAR component of heterodimers is sufficient for the signal transduction in mice[10]. Couplinf of the ligand-

receptors complex with a RA response element (RARE) on a target gene's promoter mediates transcripton.

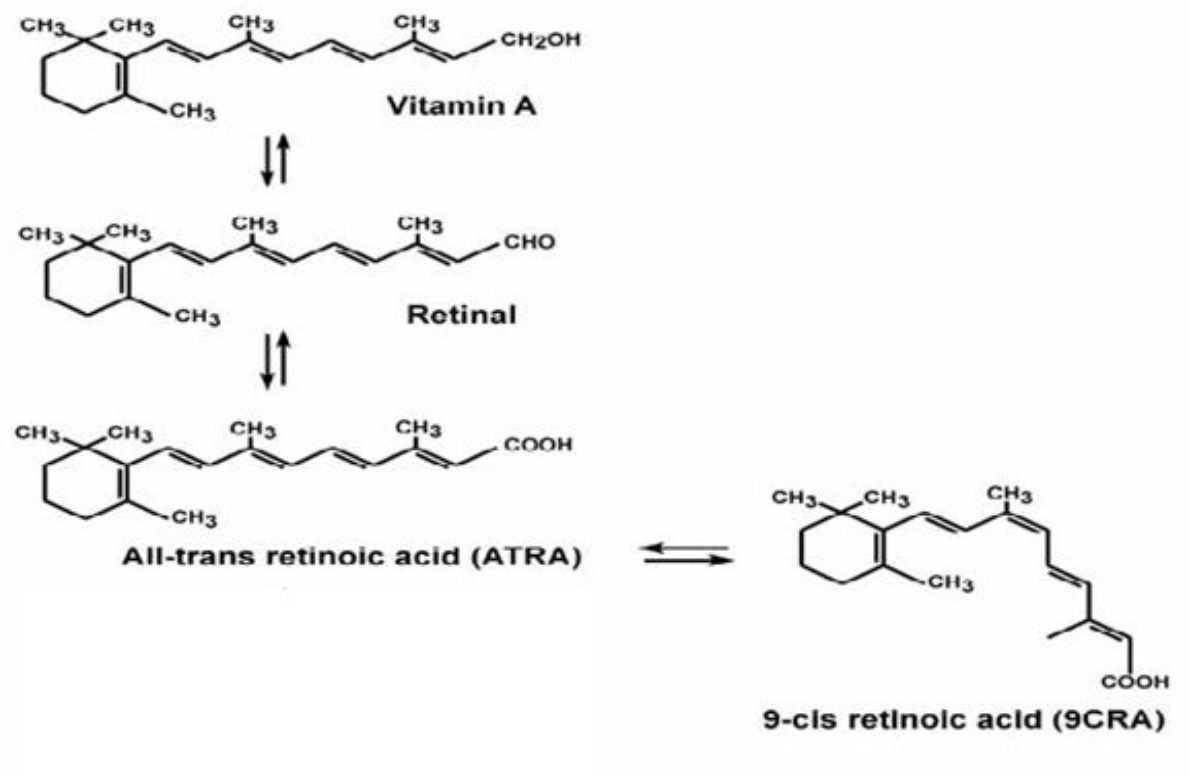

Figure 1: Shows the structural configuration of Vitamin A, Retinal, ATRA, and 9-cis retinoic acid which are all involved in Retinoic acid signaling

1.2 Retinoic Acid Metabolism

As mentioned before Retinoic acid is necessary for many different steps during embryonic development, but RA can not be freely transported through the body. Retinol (ROL), on the other hand, can be taken up through diets and shuttled through the blood stream in complex with retinoid binding proteins (RBPs). ROL is transported into the cell when it reaches its target tissue via the transmembrane receptor STRA6, in tandem with the retinol binding protein RBP4, after it reaches its target tissue it can be stored either

as rertinyl esters by Lecitin retinol transferase (LRAT) or metabolized [11]. The metabolism of ROL to RA involves a two enzymatic step. The first step is the synthesis of RAL via retinal dehydrogenase (RDH) enzymes which was mentioned earlier on. This reaction step is said to be the rate limiting step of vitamin A metabolism and it is reversible [11]. The second reaction step is the conversion of RAL to RA , which is catalyzed by retinaldehyde dehydrogenase (RALDH) enzymes. Once RA is synthesized, it can either be degraded by the cytochrome P540, family 26 (CYP26) enzymes, or it can bind to the retinoic acid receptors (RARs), which then dimerize with Retinoid x Receptors (RXRs) [11].

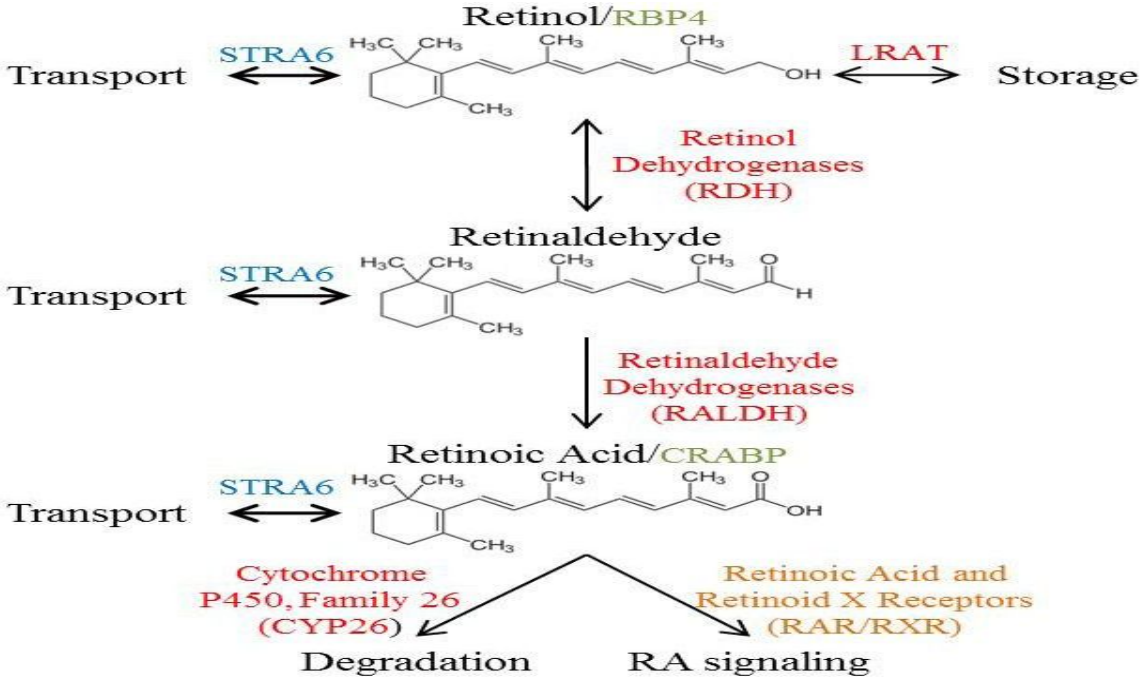

Figure 2: Shows the Vitamin A metabolism pathway. Retinol, is transported in complex with RBP4, is either stored or metabolized to RA via a two-enzymatic step. RA is then degarded or utilized to allow transcription factors, in complex with cellular retinoic acid binding protein (CRABP), to stimulate gene expression. The necessary enzymes are labeled in red. Transcription factors are shown in orange. Retinoid binding proteins are marked in green. Finally the Membrane bound transporters are labeled in blue.

1.3 Nuclear Hormone Receptors

The biological roles of the two active RA isomers which are the all-trans-RA and 9-cis isomer which are shown in figure 1 above, include regulation of cellular proliferation, differentiation and embryonic development, all of these functions are determined through the binding of two members of the family of nuclear hormone receptors, namely Retinoid X receptors (RXR) and Retinoic acid receptors (RAR) [12,13]. The nuclear hormone receptors are ligand-activated transcription factors that bind to specific DNA sequences in the promoter region of experimental genes and act to modify gene transcription[14]. The nuclear receptor family had been categorized into 6 subclasses based on sequence homology and evolutionary consideration [15]. The 3 subfamilies contains steroid receptors, such as estrogen receptor (ER), androgen receptor (AR), and glucocorticoid receptor (GR). The steroid receptor normally acts as homodimers and interact with the inverted DNA consensus sequences. The subfamilies 1 and 2 contain numerous receptors including the retinoid receptor RAR and RXR, vitamin D receptor (VDR), and the peroxisome-proliferator-acitvated receptor (PPAR) [15].

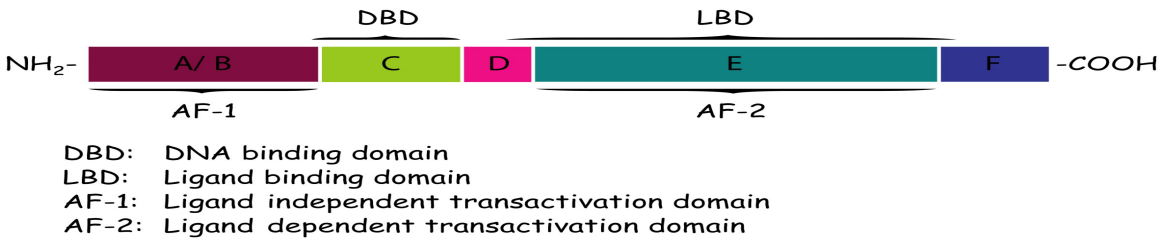

Figure 3: Illustration of functional domains of the nuclear receptor.

Nuclear hormone receptors have a similar structural architecture, which is composed of modular domains A-F which can be seen in figure 3 above. The amino terminal A/B

region contains one the receptors activation function which is responsible for basal activation (ligand-independent activation) while a second ligand-binding domain (LBD, region E) [12]. The LBD carries out multiple functions. It binds ligand, and contains a dimerization region as well as a region that mediates interactions with a variety of accessory proteins. Domain C contains two zinc finger pattern that allow for DNA binding by the receptor while the D domain is the hinge region conferrring protein flexibility. The F domain is present in only some of the nuclear hormone receptors including RAR, however its function is unknown [10].

Two class II nuclear hormone receptors, RAR and RXR, bind retinoic acids with specific ligand-binding precision. Both 9-cis-RA and all-trans-RA can be used to activate RAR, while RXR can only be activated by 9-cis-RA [16]. RAR and RXR receptors both have three isoforms as mentioned earlier on (α , β , γ); each expressed from different genes and display different expression profiles, suggesting that they have specific roles. Each isotypes can be found as a different isoform as a result of differential promoter usage and alternative splicing [17]. RAR-RXR heterodimers bind to a DNA response element which is comprised of a direct repeat of the hexanucleotide half-site 5'-AGGTCA-3' and separated by either two or five nucleotides [18]. Target genes of retinoid receptors, such as CYP26a, control the cellular processes which are involved in differentation, apoptosis, and cell cycle control. RXR behaves as a common binding partner for all class II nuclear receptors and is considered the ''master regulator'' for non-steroid receptor signaling [18].

1.4 Vitamin A deficiency

Vitamin A is an important nurtrient that is needed in small portions for the normal function of the visual system, and maintenance of cell function for growth, epithelial integrity, red blood cell production, immunity and reproduction. The body cannot synthesize some essential nutrients and therefore most be provided through a diet. When the dietary intake is chronically low, there would be insufficient vitamin A to support vision and cellular processes, which would lead to impraired tissue function. Low vitamin A intake during nutritionally demanding periods in life, such as infancy, childhood, pregnancy and location, greatly increases the risk of health consequences, or vitamin A deficiency disorder (VADD).

Dietary deficiency can begin early in life, with colostrum being discarded or breastfeeding beign inadequate, thereby denying infants their first critical source of vitamin A [19]. VAD occurs usally in an environment of ecological, social and economical deprivation, in which a chronically deficient dieatry intake of vitamin A coexists with severe infections, such as measles, and frequent infections which causes diarrhoea and respirtatory diseases that can lower the intake through loss of appetite and absoption, and deplete the body stores of vitamin A through excessive metabolism and excretion [20,21].

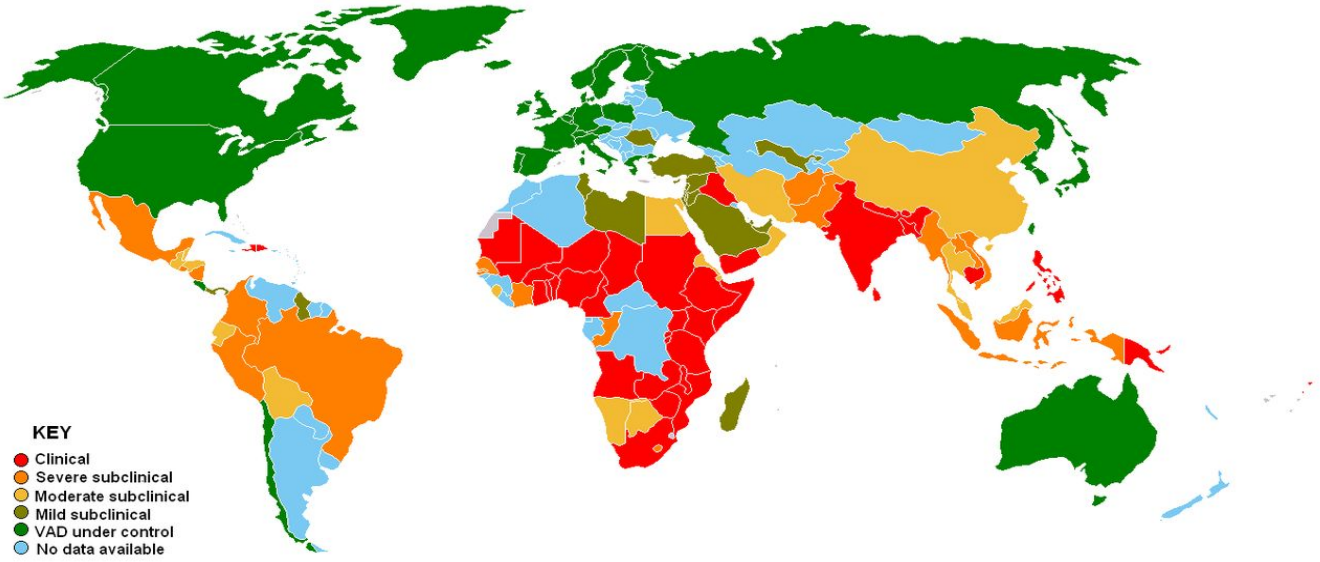

Figure 4: Countries Affected by Vitamin A deficiency (VAD)

Vitamin A deficiency decreases various functions, and as a result of this it can lead to different health consequences, to which infants, young childrens, and pregnant women appear to be at a larger risk. The most common VADD is Xerophthalmia, and it is the leading preventable cause of blindness in children throughout the world [22]. Night blindness usually occurs during pregnancy, a likely consequence of preexisting, marginal maternal vitamin A status superimposed by nutritional demands of pregnancy and intercurrent infections [23]. VAD can lead to anaemia in children and women, most likley due to multiple apparent roles of vitamin A in supporting iron mobilization and transport, and hematopoiesis [24]. Previous VAD appears to worsen infections [25], and it has been shown that vitamin A supplementation reduces the risk of death in 6-59 month old children by about 23-30% [26-28]. The has been a report that three trials from southern asia have been conducted and it showed that vitamin A supplementation reduced mortality by 21% in the first six months of life [29], two other studies have also

been conducted in africa and they showed no impact of this intervention [30,31]. One study on the other hand reported an approximate 40% reduction in maternal mortality following the everyday dietary supplementation with vitamin A during pregnancy [32]

Table 1: Showes the estimated requirement for Vitamin A (µg RE/day)

Age group	Mean requirement µg RE/day	Recommended safe intake µg RE/day
Infants and children		
0-6 months	180	375
7-12 months	190	400
1-3 years	200	400
4-6 years	200	450
7-years	250	500
Adolescents, 10-18 years	330-400	600
Adults		
Females,19-65 years	270	500
Males, 19-65 years	300	600
65+	300	600
Pregnant women	370	800
Lactating women	450	850

1.5. Gal4/UAS System

Gal 4 is a galactose-inducible gene regulator in yeast,saccharomyces cerevisiae [33]. The system is designed for targeting gene expression in Drosophila. Gal 4 allows for activation of any cloned gene in a broad range of tissue- and cell specific pattern [34]. Apart from Drosophila the Gal4/UAS system is also used in mice and has recently been introduced into xenopus and zebrafish [35-37]. Gal 4 regulates the transcription of the distinct transcribed Gal 10 and Gal 1 genes by directly binding to four related 17bp sites which are located between these loci [38]. These sites characterizes an Upstream

Activation sequence (UAS) element, which is comparable to an enhancer element defined in multicellular eukaryotes, that is important for the transcriptional activation of these Gal4-regulated genes. A large amount of Gal 4 insertion has been isolated by different laboratories and they are widely used to ectopically express genes of interest [39]. The UAS-target gene lines are generated separately. A cassette of UAS-miimal promoter target gene is constructed and used for generating target transgene lines.

In 1996 Roth [40] developed a method of screening for phenotypes caused by misexpression of endogenous genes. In this method, a minimal promoter under the control of UAS elements is imported into multiple sites in the Drosophila genome to drive endogenous genes flanking downstream the inserted UAS regulated promoter. When combined with a specific Gal4 line, the flanking endogenous gene will express ectopically in a pattern direction by the Gal4 activator. Roth et al. [41], developed 2300 independent UAS lines that were screened for dominant phenotypes in combination with various Gal 4 pattern lines. Dominant phenotypic abnormalities weree detected in 2-7% of the UAS depending on which Gal4 line was used.

The key feature of the Gal4 system is that the Gal4 gene and UAS-target gene are initially separated into two distinct transgenic lines. This ensures that the generated parental lines are viable since in Gal4 pattern lines, the expression of Gal4 alone has no detactable effects on development of organisms; in the UAS lines, the target gene is silent in the absence of Gal4 activator. Only upon crossing these two lines will the target gene be activated in the progeny of the cross, and can the phenotypically defective of convensently be studied

1.6 Gaussia Luciferase Reporter Assay

The Gaussia princeps which is also know as gaussia luciferase or Gluc is a marine organism with multiple secretory glands capable of producing luciferase in response to environmental signals [42-46]. It has been shown that this reporter is secreted naturally from mammalian cells in an active form and that the levels of Gluc in the given medium are with respect to cell number, growth and proliferation [42,46]. The Copepod is approximately 10mm in length and lives in the pacific ocean. The glands that are secreted from this organism are made from one single cell; further more its cytoplasm is packed with many secretory vesicles. The organism releases its secretory vesicles through a pore to the plasma membrane in response to nerve stimuli. It has been reported that the secretory vesicles of Gaussia princeps consist of both the luciferase and its substrate, coelentrazine. Luciferase and its substrate react only when Co-factors Ca^{2+} and O_2 are present [47].

Figure 5 : This figure illustrates the Photo-Oxidation catalyzed by Gaussia Luciferase.

It has been theorized that this organism uses bioluminescence as a means of defense mechanism when it is being approched by predators or to attract and draw attention of a mate.

The Gluc reporter assay is said to be a sensitive reproter in monitoring endoplasmic reticulum stress and the secretory pathway [42,43,45,48] , promoter activity [49], small interference RNA silencing and micro-RNA biogenesis [50,51], as well as protein-protein interaction [52]. Compared to other luciferase reporter assays,

the Gaussia luciferase is classified to be the smallest luciferase, consisting of 185 Amino acids and it has a molecular weight of 19.8 kDa [53]. Gaussia luciferase emits light at a peak of 480nm [54]. Like other luciferases, the Gaussia luciferase catalyzes the oxidation of coelenterazine in the presence of molecular oxygen forming coelenteramide and Co_2, with a simultaneous release of light as the excited coleneteramide falls to the ground state. The Gaussia luciferase is rapidly inactivated during reaction [55], and exhibits a flash-type emission profile. This is similar to other luciferases such as that from Renilla reniformis, where the bioluminescent coelenteramide product is known to act as a strong competitive inhibitor of luciferase [56]. In vivo, the Gluc reporter assay has been used to localize tumors and to monitor tumor growth and prolfieration using bioluminescence imaging [46,57].

As mentioned before Gluc is naturally secreted, its level in blood or urine can be used as a marker to oversee different in vivo biological events, like tumor growth and response to therapy, viral infections and replication, as well as the viability of circulating cells [44]. Performing this assay is extremely costly and requires a luminometer with a built in injector. Gluc is very stable in the conditioned medium, but a negative untreated cell control should always be included to which the results can be normalized. However, this is not a problem in vivo, as the Gluc half-life in circulation is very short. Being that the reporter assay is very sensitive, it is prone to pipetting errors [46]. The Gaussia luciferase assay has many advantages compared to other commercially available reporter assays. For example the Gaussia luciferase possesses a natural secretory signal, therefore upon expression it is secreted into the cell medium. This is very beneficial and time saving since the lysis of the cell is not required. The Gaussia

luciferase assay is also thermally stable and has extremely high activity in light production which allows for very sensitive assays.

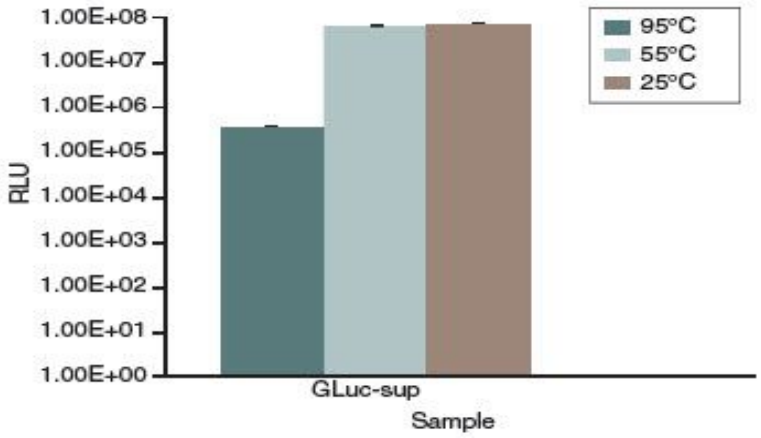

Figure 6: Stability of Gaussia Luciferase at various temperatures

Furthermore, it has also been reported that the bioluminescence produced by the Gaussia luciferase is 1000-fold stronger in signal intensity than other reporters such as Renilla and firefly luciferase, therefore making Gaussia luciferase an ideal transcriptional reporter [53].

2. Aim of the Research

The aim of my research was to gain better understanding on the mechanism and signaling of retinoic acid. To achieve this various methods were used. In one case, the development of an exquisitely sensitive one-hybrid nuclear reporter assay for studying the levels of retinoic acid in reporter cells was developed. The assay was validated and the transfected cells were cloned by limiting dilution.

METHODS AND MATERIALS

3. Materials

3.1 Chemicals

Table 2: Showing a list of the Chemicals

Name	Supplier
Agar	Sigma
DNA polymerase	New England Biolabs
Blue/orange Loading Dye x6	Promega
Dimethylsulphoxide (DMSO)	Sigma-Aldrich
dNTP mix	Bioline
DMEM medium	Invitrogen
GC buffer	New England Biolabs
Ethanol	Sigma
Fetal Calf Serum	Sigma
Ethidium Bromide	Sigma
Lipofectamin™2000	Invitrogen
TAE (Tris- Acetae EDTA)	Promega
TBE (Tris-Barate EDTA)	Sigma
Trypton	Fluka

Trypson	Sigma
UltraPure™ Agarose	Invitrogen
Yeast Extract	Fluka

3.2 Antibiotics

Table 3: Shows the Antibiotics

Ampicillin Sodium Salt	Sigma-Aldrich
Gentamycin	Invitrogen
Penicillin- Streptomycin mix	Invitrogen

3.3 Commercial Kit

Table 4: Shows all the commercial kits used

CloneJet PCR Cloning Kit	Thermo Scientific
Expand High Fidelity PCR system	Roche
Miniprep Kit	Eppendorf
Midiprep Kit	Eppendorf
QIAquick Gel Extraction Kit	Qiagen
Gaussia Luciferase Assay Kit	New England Biolabs
DNA ligation Kit	Roche

3.4 Growth Medium

Table 5: Shows the different Mediums and preparations

Bacterial growth medium

LB (lysogeny Broth) Agar	LB (lysogeny Broth) Medium
Preparation of 500ml	Preparation of 500ml
10g of Agar (2%)	5g of Trypton
5g of Tryptone	5g of NaCl
5g of NaCl	2.5g of Yeast Extract
2.5g of Yeast Extract	dH_2O to final volume of 500ml
dH_2O to final volume of 500ml	Sterilised by autoclaving
Sterilised by autoclaving	

3.5 Medium for Freezing Cells

Preparation of 50ml

45ml of FCS

5ml of Dimethylsulphoxide (DMSO)

3.6 Enzymes

Table 6: Indicating the enzymes

Bgl II	New England Biolabs

4. Methods

4.1 Polymerase Chain Reaction (PCR)

PCR was performed to amplify the DNA fragments in vectors to either verify the presence of specific sequences or as an integral part of the seamless cloning procedure.

General reaction mix for a typical PCR reaction

10x Buffer	= 5µl
dNTP mix	= 2µl
Forward Primer	= 5µl
Reverse Primer	= 5µl
DNA Template	= 1µl
DNA Polymerase	= 1µl
ddH_2O	= 31µl

In this study we used the Phusion protocol for the PCR and the reaction mix was as followed

ddH_2O	= 16.25µl
10mM dNTP (200µM)	= 0.5µl
5x GC Buffer	= 5µl
10^{-5} (1/10) dilution of primer stock combined	= 2.5µl
pFR Luc	= 0.5µl
Phusion polymerase	= 0.5µl

Thermocycling Condition for the PCR

STEP	TEMPATURE	TIME
1	4ºC	2min
2	98ºC	30sec
3	98ºC	10sec
4	60ºC	30sec
5	72ºC	15sec
Final step is Hold	4ºC	

A final last extension step should have been included but the product was only ~200bp.

4.2 Electrophoresis of DNA

The agrose gel electrophoresis was used to separate digestion products and to purify PCR products. To make the gel and achieve the desired percentage, the appropriate amount of agarose powder was mixed which was 1 gram of agarose powder and 50ml of TBE buffer which was then melted in a microwave until it was completely melted. The meleted agarose mix was allowed to cool down for 5-10min at room temperature. At this point, 1µl of ethidium bromide (10µg/µl) was added to the melted agarose to facilitate visualisation. The solution was then poured into an appropriate gel casting tray containing comb/s and it was allowed to cool down for atleast 15-20 minutes to solidify at room temperatures. After the gel was solidified, the comb/s was removed and 1xTBE

buffer was poured in to fill up the gel tank. After that 4µl of sample and 1µl of loading dye were loaded into wells of the gel. The Electrophoresis was performed with an appropriate voltage (60V) for 60minutes. At the end, the gel was photographed under a UV-light.

4.3 Gel Purification

Throughout this study purification was always carried out using QIAquick Gel extraction kit and it was done according to the manufacturer's instructions. The protocol was designed to extract and purify DNA of 70bp to 10kb from standard or low-melt agarose gels in TAE or TBE buffer.

The DNA fragments from the agarose gel were cut out with a clean, sharp scalpel and placed into a eppendorf tube. The gel slice was then weight and 3 volumes of QG buffer was added to 1 volume of gel (100mg~100µl). The eppendorf tube was then incubated at 50ºC for 10 minutes (or until the gel slice has completely dissolved). By vortexing the tube every 2-3minute during the incubation process the gel dissolved faster. After the gel dissolved 1gel volume of Isoproponol was added to the sample and mixed. A QIAquick spin column was placed into a 2ml collection tube and the sample mix was transferred to the spin column. The column mix was centrifuged at 13,000rpm for 30-60 seconds. Next, the flow -through was discarded and the spin column was placed back into the collection tube. 500µl of QG buffer was added to the QIAquick column and centrifuged for 1min, this step was done to remove all traces of agarose. To wash,750µl of buffer PE was added to the spin column and the column was centrifuged at 13,00rpm for 30-60seconds. The flow-through was discarded, and the spin column was placed back into the collection tube and centrifuged for an additional 1 minute. The

spin column was then placed into a microcentrifuge tube and 50µl of elution buffer EB were transferred to the center of the spin column. After 1minute of incubation at room temperature, the microcentrifuge tube was centrifuged at13,000rpm for 1minute. The purified DNA was then analyzed and the optical density (OD) was taken.

4.4 Ligation

The thermo Scientific Clone JET PCR Cloning Kit was used in this study to ligate the DNA fragments. The set up for the ligation was as followed

2x Reaction Buffer	= 10µl
Purified PCR products	= 2µl
PJet 1.2 Vector	= 1µl
ddH_2O	= 6µl
T4 ligase	= 1µl

After the ligation set up the following steps were carried out

- The ligation mix was vortexed and centrifuged for 3-5seconds
- Then the ligation mixture was incubated at room temperature for 30-60 minutes
- Finally the ligation mixture was used directly for transformation

4.5 Transformation of Z-competent Cells

The transformation were done according to the z-competent E.coli transformation kit and buffer set, which is an entirely new method to make competent E.coli cells for simple and highly efficient E.coli transformation. The following steps were carried out for the transformation of the competent cells.

- 1-5µl DNA was added and gently mixed
- The cells were then incubated on ice for a duration of 10-60 minutes

- 100µl of the cells were then spread on a prewarmed plate (plate warmed at 37ºC)
- The plate was then incubated at 37ºC for the colonies to grow

in this study only 40µl of the bacterial prep at 5µl of the ligation reaction was transformed, and 10ng of the pGluc TK 2 mini was transformed into 90µl of bacterial prep instead.

4.6 Transfection

The transfection of pGluc TK2 mini vector and pGluc TK2 mini plus PCR of Dhrs3 was done according to the transfection protocol from Lipofectamin^{R}2000 Transfection Reagent Invitrogen by life technologies. The steps that are as followed

- Two plates were prepared one with the pGluc TK2 mini vector alone and one with the vector and Dhrs3.
- 0.8µl of DNA was diluted in 50µl of DMEM and mixed gently
- 2µl of Lipofectamin^{R}2000 Transfection Reagent was mixed in 50µl of DMEM and incubated for 5 minutes at room temperature.
- After the 5 minutes incubation time the diluted DNA and the diluted Lipofectamin^{R}2000 Transfection Reagent were combined and mixed gently and incubated for 20 minutes at room temperature.
- After the 20 minutes incubation time 100µl of the mix was added to each well containing the vector and the vector plus Dhrs3 and mixed gently by rocking the plate back and forth.
- The plates were then incubated at 37ºC in a CO_2 incubator for 18-48 hours prior to testing.
- The cells were 95% or more confluent at the time of transfection.

- Each well on the plate had different levels of Retinoic acid (No RA,100nM and 1µM).

4.7 Gaussia Luciferase Assay

The assay was done according to the BioLux Gaussia Luciferase Assay Kit protocol, protocol 1 (Luminometers without injector). The following steps were done for the assay

- The Gluc assay solution was prepared by adding 0.5µl per sample of BioLux Gluc substrate to 50µl per sample of BioLux Gluc Assay Buffer immediately before performing the assay.
- It was then mixed well by inverting the tube several times (at this stage vortexing is not advised)
- After solution was well mixed the luminometer was set for 2-10 seconds of integration.
- The samples were then pipetted (5-20µl) into a 96-well white or black plate or a luminometer tube. In this study a white plate was used.
- For this assay different levels of Retinoic acid per µl were added to the Gluc assay solution and the luminescence was immediately measured.
- The previous step was repeated for all samples.

Before the assay was carried out 50µl were taking out from 2 Wells for the control. The RA treatment was carried out as followed:

Cells with Zero RA treatment got 2µl DMF in 2.5ml per 0.5µl/well

Cells with 500 RA treatment got 1µl DMF in 2.5ml per 0.5µl/well

Cells with 1000 RA treatment got 2µl DMF in 2.5ml per 0.5µl/well.

4.8 Miniprep of UAS pGLuc TK2 mini clones 1-14

The protocol used was the QIAprep Spin Miniprep Kit and it was used for the clones 1-14. This protocol is designed for the purification of up to 20µg of high-copy plasmid DNA from 1-5ml overnight culture of E.coli in Luria-Bertani (LB) medium. A single colony from the overnight plates was selected and placed in a tube containing 10ml of LB medium and 10µl of Ampicillin. The tubes were incubated overnight at 37ºC with a shaker set at 200rpm. 1.5ml of the overnight cultures were then centrifuged at 13000rpm for 5minutes. The supernatant fluid was discarded and the pellet was resuspended in 250µl of the P1 buffer which contains RNase which was then mixed a few times with a pipette. In order to lyse the bacteria 250µl of buffer P2 was then added. The tubes were mixed gently by inverting the tubes 4-6 times the tubes were so long inverted till the solutions became viscous and slightly clear. 350µl of buffer N3 was then added and mixed immediately and thoroughly by inverting the tubes 4-6 times again until the solutions became cloudy. The tubes were then centrifuged for 10 minutes at 13000rpm in a table-top microcentrifuge. At the end of the centrifugation process the supernatants was applied to a QIAprep spin column by gently decanting or pipetting. Centrifugation was then carried out again for 30-60 seconds and the flow through was discarded. The QIAprep spin column was then washed by addind 500µl of buffer PB and centrifuged for another 30-60 seconds. The flow through was discared and the QIAprep spin column was washed again by adding 750µl of buffer PB and centrifuged for 30-60 seconds. The flow through was once more discarded and the QIAprep spin coolumn was centrifuged for an additional 1 minute to remove all traces of residual wash buffer. The QIAprep column was then placed in a clean 1.5ml microcentifuge tube, and 50µl of buffer EB was added to the center of each QIAprep spin column. The tubes were then let to stand for 1

minute and finally centrifuged for 1 minute. At the end of the miniprep the optical density (OD) was taking from all the samples.

4.9 General Cultivation of Cells

The F9 cells were cultivated in a T-25 flask and fed with DMEM medium. Cells were grown until they reached ~90% confluency. They were then washed twice with 1xPBS and then treated with 1ml of 1x Trypsin for the T-25 flask in order to detach the cells. Cells were incubated with trypsin for 2-5 minutes and then observed under the microscope. When cells where fully detached 2 or 3 ml of resuspended cells depending on the required dilution were dispensed into a new flask and 8 or 12 ml of fresh medium was added to the flask depending on the size of the flask.

4.10 Cell Cloning by Serial Dilution in 96 Well plates

The cell cloning was done according to the single cell cloning protocol.

Procedure:

- The reagent dispensing tray was filled with 12ml of the cultured medium, then 100µl medium was added to all wells in the 96 well plate except well A1 which was left empty.
- 200µl of the cell suspension was added to well A1 and then 100µl were transfered from the first well to well B1 and gently mixed by pipetting up and down. This was repeated in a 1:2 dilution down the entire coloumn, 100µl was discarded from H1 so that it ends up with the same volume as the wells above it.

- An additional 100µl of medium was added to each well in column 1. Then 100µl from the wells were quickly transfered from the first column (A1 through H1) to those in the second column (A2 through H2) and mixed gently.
- The same process was repeated in a 1:2 dilution across the entire plate, discarding 100µl from each well in the last column (A12 through H12) so that all the wells end up with 100µl of cell suspension.
- The final volume of all the wells were brought to 200µl by adding 100µl of medium to each well. The plate was then labelled with date and cell type.
    - The plate was then incubated at 37ºC in a humidified CO_2 incubator.

5. RESULTS

5.1 Cloning by Serial Dilution

The technique used is widely used for clonal isolation of hybridomas and other cell lines that are not attachment dependent. The method is also very useful for cloning attachment dependent cells especially when the cell plating efficiency is very low, unknown, or unpredictable. The procedure started by cloning 12 clones of which 8 of the clones responded to Retinoic Acid, based on the value from the luminiscence reding the best 4 clones were picked for further cloning (clones 4,8,9 and 12). The 4 clones were frozen and clone 4 was then recloned into subclones.

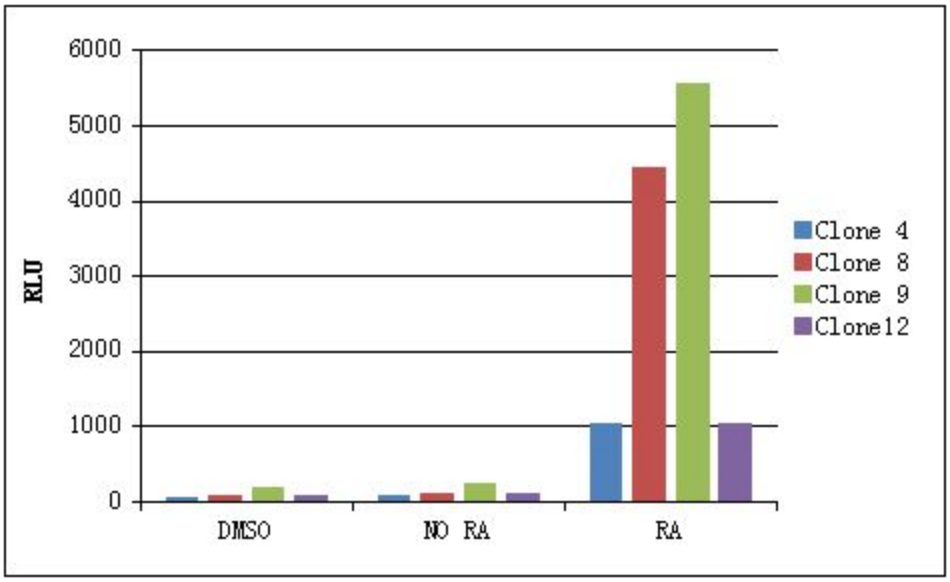

Figure 7: Shows the 4 clones that were picked out of the 8 that responded to Retinoic Acid the best .

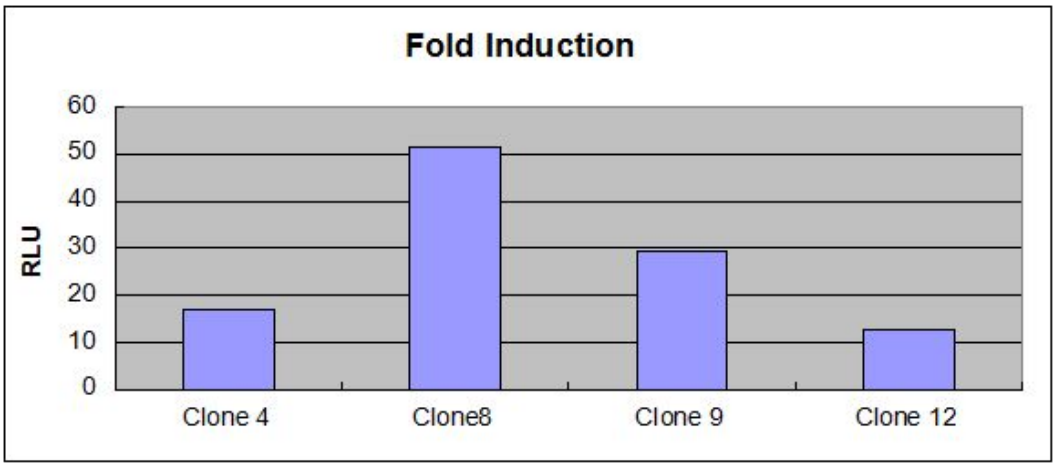

Figure 8: Shows the Fold induction of the 4 clones.

5.2 Subcloning Clone 4

From the 8 clones that responded to RA the best 4 were choosen and frozen as mentioned earlier before. The clone 4 was recloned into 4 sub and tested again with RA and without RA. The Clones with RA were given 50µl of the GLuc assay solution and

the clones without RA were left blank. The assay was then performed to see the concentration of RA.

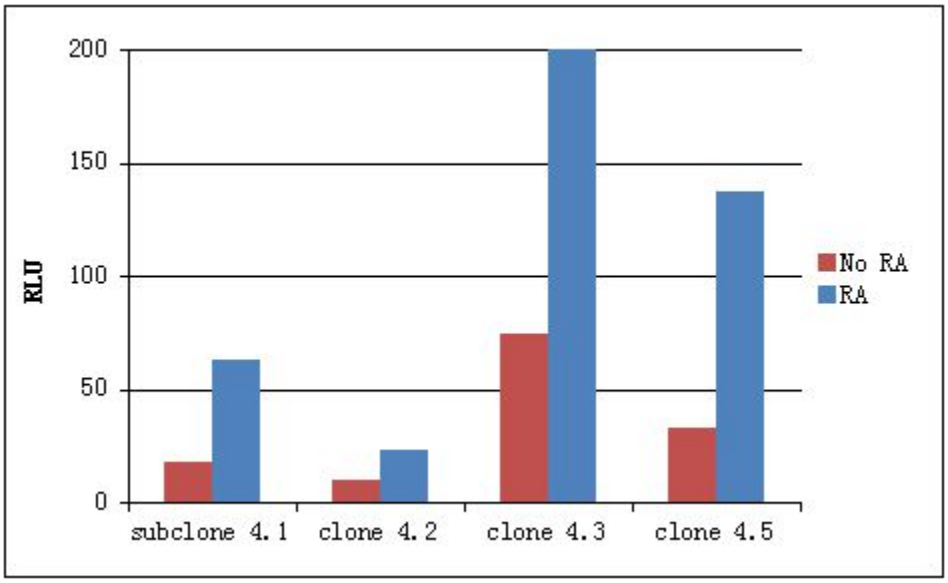

Figure 9: Shows the concentration of RA in the 4 subclones.

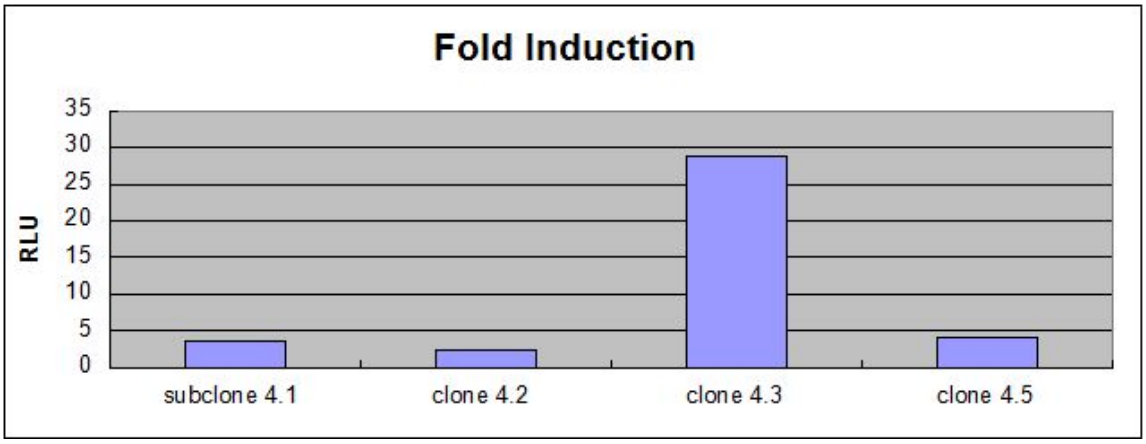

Figure 10: shows the Fold Induction of the subclones

5.3 Gaussia Luciferase Secreted Reporter

Cloning of the F9 cells was carried out by limiting dilutions. The PCR of the 5 tandem copies of the UAS (upstream Activation Sequence) from the pFR-Luc vector [58], was amplified and inserted into the Bgl2 site of pGluc mini-TK2 vector which was obtained

from NEB (New england Biolabs) [59]. The Insetion resulted in the generation of a Gaussia luciferase secreted reporter under the control of UAS-Gal4 system was was termed pGluc MIni-Tk2 UAS. In order to have carried out the assay, 5 reactions were set up, the reaction are as followed

- Dhrs 3 (3-12) PGluc Mini-TK2 clone 7
- $(UAS)_5$ PGluc Mini-TK2 clone 4
- PGluc Mini-TK2 vector alone
- $(UAS)_5$-PGluc Mini-TK2 + Gal4RAR
- $(UAS)_5$-PGluc Mini-TK2 clone 4 + VP16-Gal4RAR

The assay was carried out after the setup and the luminiscence was read.

Table 7: Showing the concentration of RA after performing the luminescence assay.

Dhrs 3 (3-12) PGluc Mini-TK2 clone 7	Without RA	RA
	17440	9136
	12369	11784
$(UAS)_5$ PGluc Mini-TK2 clone 4	Without RA	RA
	364	186
	245	406
PGluc Mini-TK2 vector	Without RA	RA

alone		
	1785	1537
	1742	1866
$(UAS)_5$-PGluc Mini-TK2 + Gal4RAR	Without RA	RA
	283	37631
	103	47315
$(UAS)_5$-PGluc Mini-TK2 clone 4 + VP16-Gal4RAR	Without RA	RA
	61823	81738
	30145	156929

5.4 Plasmid Construction

The Gal4-RAR α, β and γ chimeras were constructed by PCR amplification of the D to F region of the mouse RAR α, β and γ.

The F9 cells were transfected according to the transfection protocol which was explained under the methods and after twenty-four hours of transfection, the cells (subclone 4.3) was treated with various Retinoic acid concentrations for twelve to fifteen hours. After the treatment the Gal4-RAR Clone 4.3 was then assayed and the luminescence was read. The assay was done in triplicates for each concentration.

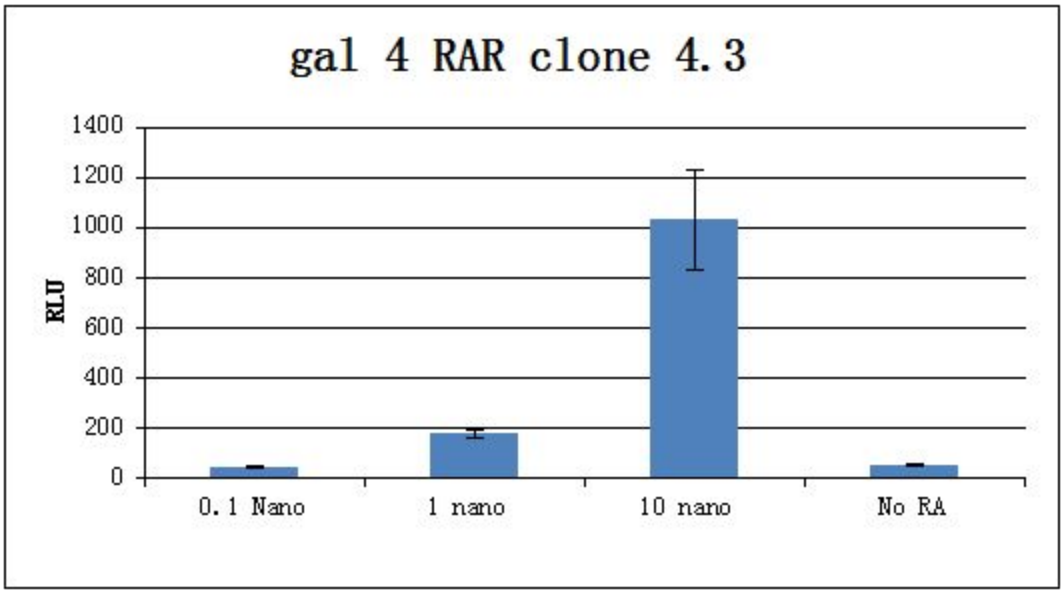

Figure 11: Shows the luminescence of the GAL4-RAR CLone 4.3 at different concentrations

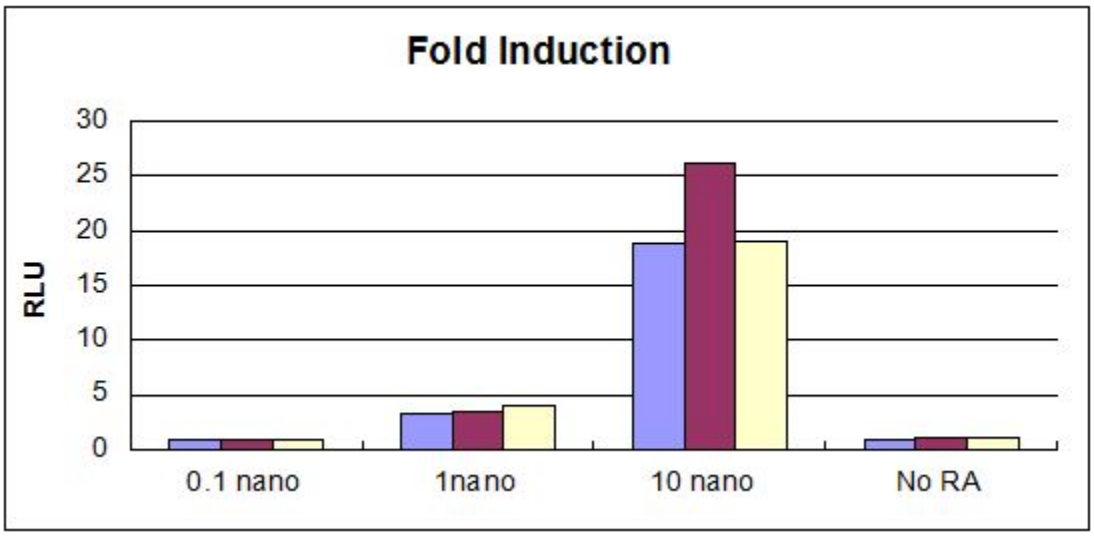

Figure 12: Shows the Fold Induction of the Gal4-RAR Clone 4.3 at various concentration.

5.5 Optical Density of UAS pGluc Tk2 mini clones 1-14

For this experiment a mini prep of the fourteen UAS pGluc Tk2 mini clones was performed. The QIAprep spin mini prep kit was used to performe the experiment. The QIAprep mini prep system provides a fast, simple, and cost-effective plasmid miniprep method for routine molecular biology laboratory applications. Plasmid DNA purified with QIAprep miniprep kit is immediately ready for use. Phenol extraction and ethanol precipitation are not required, and high-quality plasmid DNA is eluted in a small volume of Tris buffer or water. The procedure was done according to the protocol which was mentioned earlier on in the methods.

Table 8: Showing the Optical Density of the UAS pGluc Tk2 mini clones 1-14

UAS pGluc TK2 mini clones 1-14	Optical Density
1	198.1 ng/µl
2	294.5 ng/µl
3	290 ng/µl
4	284.5 ng/µl
5	265 ng/µl
6	254.1 ng/µl
7	256.4 ng/µl
8	290.6 ng/µl
9	304.1 ng/µl
10	204.4 ng/µl
11	318.1 ng/µl
12	276.7 ng/µl

13	248.2 ng/µl
14	269.1 ng/µl

5.6 Luciferase Assay

For the luciferase reporter assay the F9 cells were grown in DMEM. All assays were performed in a 96 Well dish, and the cells were approximately 95% confluent before retinoic acid was added to the cells. Each Well contained a different clone from the 12 clones which were obtained by serial dilution. On the third day the cells were harvested and the luminescence was read using the Biolux Gaussia Luciferase Assay Kit (NEB). The procedure for the luciferase assay is explained in details under the method section.

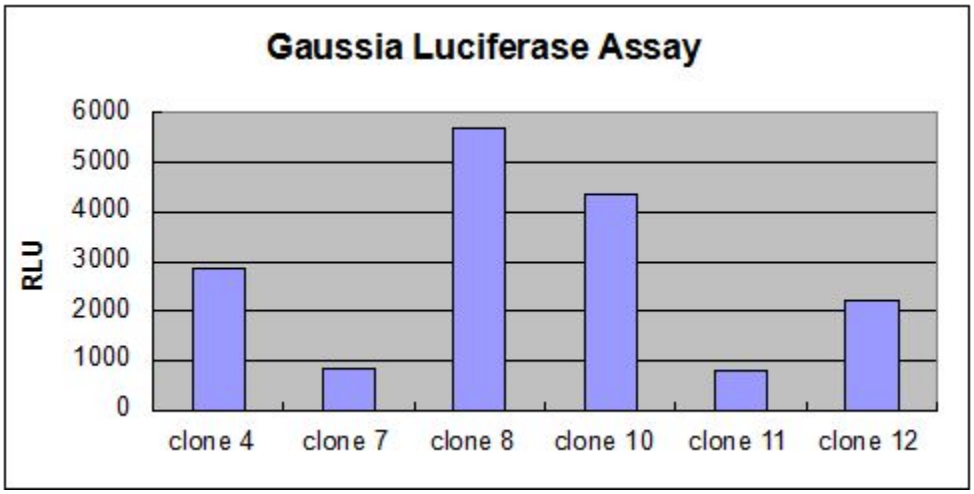

Figure 13: Shows the Luminescence reading from the Luciferase Assay

6. DISCUSSION

The goal of this study was to gain a better understanding on the role, regulation, signaling and mechanism of Retinoic acid in embryonic development. In order to prepare for this study, various research papers were discussed and different methods and protocols were compared in order to find a common starting point. The estimated requirment for Vitamin A intake per day can be seen in with various age groups (Table1) due to the lack of Vitamin A intake more than 500,000 deaths 250,000 cases of blindness are reported annually.

In this study F9 cells were used because the F9 teratocarcinoma cell line differentiates in vitro after treatment with retinoic acid and cAMP and has been a widely used model system for the study of the molecular events that are responsible for cellular commitment and differentiation during early development.

The cloning by serial dilution results (figure7) showed the best four clones that responded the most to Retinoic acid and in the fold induction (figure8) the clone 4 showed an enormous increase in luminescence signaling, there was a 30 times increase in luciferase signaling, when compared to the clone that was not treated with Retinoic acid. All the other clones that were treated with Retinoic acid showed an increase in luminescence signaling.

In the subcloning experiment the clone 4 was picked and cloned into four subclones (figure9) each of the subclones was treated with Retinoic acid and without Retinoic acid before the assay was performed. An enormous increase in clone 4.3 was observed compared to the clones that were not treated with Retinoic acid. The fold induction of

the clone 4.3 was also aprroximately 200 fold higher than other clones (figure10). The lack of increase in the other subclones could have been due to various factors that could have gone wrong during the experiment. One of which could have been pippetting errors before the luminescence was read.

The Gaussia luciferase secreted reporter results were all performed in duplicates (Table9) both were treated with Retinoic acid and without retinoic acid. Two of the reactions did not show a good increase in luminescence when compared to the untreated one. $(UAS)_5$-pGluc Mini-Tk2 plus Gal4-RAR vector showed an immense increase. It showed an 250-300 fold increase in luciferase signaling in the presence of 100nM retinoic acid. The $(UAS)_5$-pGluc Mini-Tk2 clone4 Plus VP16-Gal4-RAR had a very high constitutive level of activation even in the absence of Retinoic acid, this made it less dependent on the presence of a ligand for activity and it was thus a less reliable marker (Table9). The results could be compared to that of the construct by waxman [60] which described the original construct and their use to make transgenic zebrafish. There could have been several reasons why the other reactions didn't respond as well as the two reactions, this could have been due to pipetting errors or due to some environmental factors which had to be taking into consideration.

The luminescence reading of the plasmid construct with the Gal4-RAR Clone 4.3 showed a high luminescence signaling at the 10 nanogram concentration (figure11). The experiment was carried out in triplicates to ensure proper readings and the fold induction also showed an approximately 30 fold increase in luminescence signaling compared to the other concentrations.

The optical density of the UAS pGluc Tk2 mini clone 1-14 was measured using the nanodrop (Table 8). Some of the clones measured a higher optical density this could have been due to improper cleaning of the nanodrop after each reading. During each measurment cycle, the sample was assessed at both 1mm and 0.2 minute path, which provided an extensive dynamic range. This essentially eliminated the need to perform dilutions prior to the measurment.

The luciferase assay was done using the Gaussia luciferase assay kit (NEB) in a 96 well dish, It was observed that the clones 4,8,and 10 had a high luminescence signaling response compared to the other clones (figure 13). The low signal response could have been due to errors by the dilution because optimum dilution was neccessary as the plate reader could not measure accurately a luminescence signal which was too weak or too intense . The Gaussia luciferase could be compared to other luciferase assays (Renilla reniformis or firefly luciferase) and it has been proven that the Gaussia luciferase is up to a 1000-fold brighter than the other luciferase assays.

7. CONCLUSION

Overall it can be concluded that bioluminescence imaging using Gaussia Luciferase as the the light emitting enzyme is considered the most advantageous technique for this study, especially since Gaussia luciferase possesses a natural secretory signal which upon expression is secreted into the medium, this makes cell lysing in order to assay Gaussia luciferase activities not necessary .

In the last experiment of this study, we tried to replicate a well cited studies the sumoylation assay which showed no positive results (data not shown) even tho more

sophisticated methods were applied and the procedures were followed according to the protocols.

The experiments should be repeated in order to arrive at other results which can then be compared with those of this studies. Other cell lines could be used and more research papers should be discussed to find alternative methods which could be used and which could lead to our long-term goal in fully understanding the mechanism of Retinoic acid signaling.

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