THE ROLE OF FILAMIN IN GONADOTROPIN RELEASING HORMONE RECEPTOR SIGNALLING AND EXPRESSION

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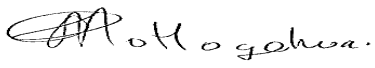
A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2020

Declaration

I, Lawrence Katlego Motlogelwa declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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(Lawrence Katlego Motlogelwa)

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Abstract

Gonadotropin Releasing Hormone (GnRH) is the central regulator of reproductive development and function. When GnRH binds to its receptor it results in signal transduction cascades which eventually culminate in gonadotropin synthesis and secretion. These hormones in turn regulate steroidogenesis and gametogenesis. The GnRH receptor can activate various signalling pathways in pituitary gonadotropes, however the $G\alpha_{q/11}$ protein kinase C (PKC) dependent extracellular signal-regulated (ERK) pathway remains the main pathway through which gonadotropin synthesis and secretion are mediated. The mechanisms underlying the GnRH receptor ERK pathway have not been fully defined, it is not clear how signal specificity of this pathway is maintained. One of the explanations is the presence of scaffolding proteins and signalling complexes (signalosomes), which have been shown to involve the cytoskeleton and associated proteins.

Filamin A, an actin-binding cytoskeletal protein has been shown to modulate trafficking and signalling of several receptors. Filamin A organises complex cellular signals from the plasma membrane, cytoplasm to the nucleus. Filamin A has also been shown to interact with upstream effectors of ERK, namely PKC and mitogen activated protein kinase kinase (MEK 1). However, the role of filamin A in GnRH receptor signalling has not yet been studied. We investigated the role of filamin A in GnRH receptor signalling. Filamin A siRNAs were designed and synthesised to reduce filamin A protein expression in GnRH receptor-transfected HEK 293 cells, then GnRH receptor-mediated ERK phosphorylation was measured by western blotting. There was no significant difference in HEK 293 cells with normal and reduced protein expression of filamin A. GnRH receptor-mediated ERK phosphorylation was also measured in GnRH receptor-transfected filamin A replete (M2A7) and deficient (M2) melanoma cells. GnRH receptor-mediated ERK phosphorylation kinetics varied between M2A7 and M2 cells. ERK phosphorylation in the M2 cells reached its maximum after 15 minutes and started to decrease after 30 minutes. In contrast ERK phosphorylation in the M2A7 cells was elevated from 1 minute and sustained through to 60 minutes.

Furthermore, ligand affinity and cell surface receptor expression were assessed using competition binding assays, effector coupling was assessed by measuring GnRH receptor-mediated IP production using IP assays. Filamin A is not required for GnRH receptor-mediated ERK phosphorylation, but its presence results in rapid and sustained GnRH receptor-mediated ERK phosphorylation. Furthermore, the latter is not due to changes in GnRH ligand binding affinity, cell surface receptor expression or G protein coupling. These results suggest that filamin A is part of the GnRH receptor signalosome and is required for enhanced ERK phosphorylation, thus filamin A may have a role in the regulation of reproductive development and function.

Dedication

Dedicated to Onneile and Olehile who perpetually inspire me to become a better version of myself.

Acknowledgements

This work was funded by grants obtained from the Faculty Research Committee Funds of the University of the Witwatersrand, the Medical Research Council (MRC) group for Receptor Biology, the National Research Foundation.

I extend my heartfelt gratitude to my supervisors; Dr Michael Madziva and Dr Colleen Flanagan, for their support and dedication to this study even when it seemed improbable that we would reach this stage. I will also like to thank my colleagues Mohlalefi Makara, Owen Karimanzira, Ashmeetha Manilall, Eleanor Cave for eye opening discussions, for helping to hone my molecular techniques skills and for the light moments enjoyed during this study. I am also very grateful to my life partner, Matebogo Mangaba, Ms Damaris Matabane and Dr Anthony Kinghorn for being supportive during the writing process and Prof Fumihiko Nakamur, Harvard Medical School, Boston, USA for sending us the human melanoma culture M2A7 and M2 cells.

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ABD: actin-binding domain

B_0: total ligand binding

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CaCl: calcium chloride

CREB: cAMP response element-binding protein

CPM: counts per minute

DAG: diacylglycerol

DMEM: Dulbecco's-modified Eagle's medium

E. coli: Escherichia coli

ERK: extracellular signal-regulated kinase

E_{max}: maximal effect

EC_{50}: concentration of a drug that gives half-maximal response

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FBS: foetal bovine serum

FSH: follicle-stimulating hormone

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GDP: guanosine diphosphate

GnRH: gonadotropin-releasing hormone

GPCR: G protein-coupled receptor

GTP: guanosine triphosphate

HEPES: N-2-hydroxyethylpiperazine-N-ethanesulfonic acid

HRP: horseradish peroxidase

IC_{50}: half-maximal inhibitory concentration

IP_3: inositol 1,4,5-triphosphate

JNK: Jun-N-terminal kinase

KCl: potassium chloride

LB: Luria broth

LH: luteinising hormone

$MgCl_2$: magnesium chloride

MAPK: mitogen activated protein kinase

MEK: mitogen activated protein kinase kinase

NaCl: sodium chloride

PBS: phosphate buffered saline

PIP_2: phosphatidylinositol 4,5-bisphosphate

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PVDF: polyvinylidene difluoride

TBS: Tris-HCL buffered Saline

TNF: tumour necrosis factor

TRAF2: tumour necrosis factor receptor-associated factor 2

VGCC: voltage-gate calcium channels

Chapter 1: Introduction

1.1 The physiological importance of gonadotropin releasing hormone

1.1.1 Gonadotropin-releasing hormone regulates reproduction in humans

Gonadotropin-releasing hormone (GnRH) is released by the hypothalamus (Millar *et al*., 2004). GnRH binds to its receptor on the anterior pituitary gland gonadotrope cells to stimulate synthesis and release of the gonadotropins, luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Millar *et al*., 2004). LH and FSH in turn are secreted from vesicles into the systemic circulation and act on gonads to stimulate the production of spermatozoa, ova and sex steroids which are required for pubertal development and gonadal function (Kaiser *et al*., 1997; Jablonka-Shariff and Boime, 2004; Coss, 2018).

There are different GnRH pulse frequencies and amplitudes, and these result in varying patterns of LH and FSH secretion. The pulsatile release of GnRH is important to maintain the responsiveness of the gonadotropes for LH and FSH secretion, because perpetual release of GnRH leads to brief release of LH and FSH and eventually inhibition of GnRH release (Clayton, 1989). High frequencies of GnRH have been shown to favour LH secretion, while low frequencies result in the preferential secretion of FSH (Thompson and Kaiser, 2014). These differential frequencies of GnRH release which control the patterns of LH and FSH secretion are critical for the regulation of reproduction (Thompson and Kaiser, 2014), as the timing of ovulation due to LH surge caused by a surge in GnRH has to be perfect in relation to maturation of the follicles (Thompson and Kaiser, 2014).

In males FSH promotes the maturation of testes while LH stimulates production of testosterone by the testes (Kaiser *et al*., 1997; Coss, 2018). Testosterone stimulates the production of sperm and the development of masculine characteristics in males (Kaiser *et al*., 1997; Coss, 2018). In females both FSH and LH have a role in the development of ova in the follicle cells of the ovaries,

LH also regulates the induction of ovulation, and both hormones stimulate the production of oestrogen and progesterone by the ovaries (Kaiser *et al*., 1997; Coss, 2018). Oestrogen and progesterone control the menstrual cycle, and prepare the body for pregnancy (Kaiser *et al*., 1997). Oestrogen is also responsible for the development of secondary female sex characteristics (Kaiser *et al*., 1997). By modulating the synthesis and release of LH and FSH, GnRH regulates sexual development and reproduction in humans (Gharib *et al*., 1990; Burger *et al*., 2004). A lack of GnRH results in low gonadotropin levels and consequently the absence of pubertal maturation and infertility, a disorder termed hypogonadotropic hypogonadism (Ciccone and Kaiser, 2009)

1.1.2 Clinical pathology of GnRH production

The central role that GnRH plays in reproduction regulation has made GnRH receptor signalling fundamental science worth unravelling. A better understanding of GnRH signalling and thus the modulation of LH and FSH secretion would therefore have clinical benefits. Treatments for infertility and of sex steroid-dependent cancers could be improved.

In addition to being expressed in the pituitary, several research studies have shown that the GnRH receptor and its ligand are expressed in other tissues, such as breast, ovary, testis, endometrium, placenta and prostate tissues (Harrison *et al*., 2004). However, the significance of GnRH on extra pituitary tissues is not yet known, as studies on the physiological function of the GnRH receptor and its ligand in these sites are not conclusive (Harrison *et al.*, 2004). However, several studies have been able to identify GnRH receptor and its ligand in cancers of the prostate, ovary, endometrium, breast, urinary bladder, and non-reproductive cancers like glioblastoma and pancreatic cancers (Gründker and Emons, 2017). GnRH receptor and its ligand have been shown to regulate cell proliferation in these cancer cells (Harrison *et al.*, 2004; Gründker and Emons, 2017).

GnRH analogues, both agonists and antagonists have been successfully used to treat GnRH receptor positive cancers, including prostate (Harrison *et al.*, 2004), breast, ovarian, and endometrial cancers (Gründker and Emons, 2017). The main effect of GnRH analogues is the down-regulation of the hypothalamic–pituitary–gonadal axis resulting in decreased production of steroids, which are mitogenic for these cancers. GnRH analogues exert anti proliferative and in other cases apoptotic effects (Harris *et al.*, 2003; Harrison *et al.*, 2004; Naor and Huhtaniemi, 2013; Gründker and Emons, 2017).

GnRH has many effects in humans. Further investigations are needed to elucidate GnRH receptor signalling in the pituitary, extra pituitary tissues and cancer cells; and to show how these differ. We do not fully understand how the GnRH receptor can induce different physiological responses in the gonadotropes, mediating differential LH and FSH secretion(Stamatiades and Kaiser, 2018); and inhibiting growth of gonadal steroid-dependent cancer cells. Specific studies to clarify the molecular mechanisms of GnRH receptor function in all aspects including ligand binding, effector coupling and signalling cascades are needed. These will give us a better understanding of GnRH action on pituitary and extra-pituitary tissues. This will in turn provide a context for the developing innovative treatment approaches.

1.2 GnRH ligand and the GnRH receptor

The GnRH ligand is 10 amino acids long and has amino and carboxyl termini that are modified such that they do not have the usual positive and negative charges (Millar *et al.*, 2004). Numerous structural variants of GnRH have been identified in protochordates and vertebrates (Millar *et al.*, 2004). Three types of GnRH ligands and their cognate receptors have been identified in vertebrates (Millar *et al.*, 2004). The structural analysis of genes which encode GnRHs is supportive of the classification of GnRHs into three types (White *et al.*, 1998). The GnRH which is processed in the hypothalamic neurons is designated GnRH I, while the

other form which was isolated from chicken brain is designated GnRH II. GnRH III is expressed only in teleosts, a class of ray-finned fishes (White *et al.*, 1998).

The GnRH receptors are part of the family of G protein coupled receptors (GPCRs); they have an extracellular amino-terminus followed by seven trans-membrane domain spanning α-helical segments which are connected by three intracellular and three extracellular loops (Millar *et al.*, 2004; Flanagan and Manilall, 2017). The GnRH receptor is constituted of 327 amino acids, (Sealfon *et al.*, 1997). The extracellular domains contribute to ligand recognition and binding and the intracellular domains contribute to binding intracellular signalling proteins and desensitisation (Dohlman *et al.*, 1987; Millar *et al.*, 2004; Flanagan and Manilall, 2017). The mammalian GnRH receptor type I is unlike other GPCRs, including the non-mammalian GnRH receptors and the mammalian GnRH receptor type II, in that it lacks a cytoplasmic carboxy-terminal tail (Reinhart *et al*., 1992; Millar *et al.*, 2004). The lack of the cytoplasmic carboxy-terminal tail has been shown to result in slower rates of receptor desensitisation and internalisation post ligand stimulated receptor activation (Millar *et al.*, 2004; Madziva *et al.*, 2015).

GnRH I, GnRH II (with the exception of mouse) and GnRH receptor type I are universally conserved, while GnRH receptor type II gene is switched off in several species including man (Millar *et al.*, 2004). However, GnRH receptor type I can bind with high affinity to both the GnRH I and GnRH II ligands but displays different physiological and pharmacological effects. This has been shown to be due to the receptor's ability to confer different signalling pathways (Millar *et al.*, 2004), depending on the ligand the GnRH receptor binds. The GnRH receptor can assume several active conformations, this allows the receptor to couple to different G proteins resulting in varying intracellular signalling pathways (Millar *et al.*, 2004, 2008). For my study I used GnRH I and the GnRH receptor type I, to which I refer hereafter as GnRH and the GnRH receptor respectively.

1.3 GnRH receptor signalling

1.3.1 Activation of the GnRH receptor

When GnRH binds to the GnRH receptor the conformation of the receptor changes from inactive to active (Millar *et al*., 2004, 2008). The active conformation consists of an agonist, the receptor and a heterotrimeric G protein, these together are known as the ternary complex. The G protein is trimeric, it has three subunits; Gα, Gβ and Gγ (Millar *et al*., 2004). When GnRH binds to the receptor, guanosine diphosphate (GDP) is exchanged for guanosine triphosphate (GTP) in the G protein complex (Millar *et al*., 2004). The Gα is activated by the exchange of GDP for GTP which results in the dissociation of the trimeric G protein to Gα and Gβγ units (Denis *et al*., 2012; Naor and Huhtaniemi, 2013), which in turn activate downstream signalling effectors (Denis *et al*., 2012; Naor and Huhtaniemi, 2013), whilst the hydrolysis of GTP converts the G protein to an inactive form which is stabilised by GDP (Millar *et al*., 2004).

The Gα proteins are categorised into four main groups ($G\alpha_{q/11}$, $G\alpha_{s}$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$), this is based on the primary sequence of the Gα subunit and the effectors it activates (Denis et al., 2012). The $G\alpha_{q/11}$ family, comprising of $G\alpha_{q}$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$ isoforms, stimulate phospholipase Cβ, which hydrolyses phosphatidyl 4,5-diphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Denis *et al*., 2012; Naor and Huhtaniemi, 2013). IP_3 is responsible for calcium mobilisation from intracellular stores, while DAG activates protein kinase C (PKC) (Denis et al., 2012; Naor and Huhtaniemi, 2013). The Gαs family is composed of four isoforms, which stimulate transmembrane adenylyl cyclase leading to the production of cyclic adenosine monophosphate (cAMP) (Denis et al., 2012; Naor and Huhtaniemi, 2013), which leads to subsequent activation of protein kinase A (PKA) and cAMP response element-binding protein (Denis et al., 2012; Naor and Huhtaniemi, 2013). The $G\alpha_{i/o}$ family comprises the $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, $G\alpha_{oB}$, $G\alpha_{z}$, $G\alpha_{t1}$, $G\alpha_{t2}$ and $G\alpha_{gust}$; they all inhibit adenylyl cyclase activity and decrease intracellular cAMP levels (Denis *et*

al., 2012), furthermore most isoforms can also activate potassium channels or inhibit calcium channels (Denis *et al*., 2012). And lastly, the $G\alpha_{12/13}$ family which comprises $G\alpha_{12}$ and $G\alpha_{13}$, regulate Rho family GTPase signalling and control cell cytoskeletal remodelling, thereby regulating cell spreading and migration (Denis *et al*., 2012; Naor and Huhtaniemi, 2013).

The capability of the GnRH receptor to recruit different signalling pathways depending on bound ligand suggests that the GnRH receptor can couple multiple G proteins. This has indeed been shown to be the case by Stanislaus and colleagues, who used palmitoylation to a measure G protein activation (Stanislaus *et al*., 1998). The GnRH receptor was shown to couple $G\alpha_i$, $G\alpha_s$, and $G\alpha_{q/11}$ (Stanislaus et al., 1998). Furthermore, they showed that coupling to multiple G proteins is not cell line dependent, but rather GnRH receptor specific (Stanislaus et al., 1998). This means GnRH receptors are capable of coupling to multiple G proteins *in vivo* and *in vitro* in various cells.

$G\alpha_q$ and $G\alpha_s$ have been shown to have additive effects in regulating gene expression of LH in primary pituitary cell cultures and an immortalised pituitary cell line, while Gi played no significant role in signalling in these cells (Stanislaus *et al*., 1997; Liu *et al*., 2002). There has been no conclusive results on the role of Gi with regards GnRH receptor function except in pathophysiology, GnRH receptor coupling to Gi has been demonstrated in several cancers (Millar et al., 2004); ovarian carcinomas, uterine leiomyosarcomas, uterine endometrial carcinomas, and prostate cancers (Millar et al., 2004).

1.3.2 Proximal and distal signalling of the GnRH receptor

In pituitary cells, the predominant G protein the GnRH receptor couples is $G\alpha_{q/11}$. When GnRH binds to its receptor it stabilises an active conformation which results in the interaction of the intracellular domains of the receptor with $G\alpha_{q/11}$ activating PLCβ which hydrolyses PIP_2 to generate the second messengers; IP_3

and diacylglycerol (DAG) (Berridge 1993; Kaiser et al., 1997). IP_3 and DAG subsequently mobilise calcium from intracellular stores (Berridge 1993; Kaiser et al., 1997) and activate PKC respectively (Berridge 1993; Millar *et al*., 2004). These signalling events which occur closer to the GnRH receptor, can be regarded as the proximal signalling of the GnRH receptor (Davidson *et al.*, 2004) (**Fig. 1**). The physiological significance of GnRH receptor proximal signalling is the increase in calcium which results in exocytosis of stored LH and FSH (Stojilkovic, Reinhart and Catt, 1994).

Signalling events which occur further downstream of the receptor; post calcium mobilisation and PKC activation in the case of the GnRH receptor may be regarded as distal signalling (Davidson *et al.*, 2004) (**Fig. 1**). While the mobilised calcium causes exocytosis of stored LH and FSH (Stojilkovic, Reinhart and Catt, 1994), the activation of PKC leads to the phosphorylation of the mitogen activated protein kinases (MAPKs), such as the extracellular signal-regulated kinase (ERK), jun-N-terminal kinase (JNK) and p38 (Naor *et al*., 2000; Benard *et al*., 2001) and the big MAPK (BMK) (Naor, Benard and Seger, 2000). The phosphorylated and thus activated MAPKs then stimulate the synthesis of the gonadotropins by activating transcription factors required for the synthesis of LH and FSH (Liu *et al*., 2002; Bonfil *et al*., 2004).

In summary, PLCβ activity consequential to $G\alpha_{q/11}$ activation leads to IP_3 production which through mediation of calcium mobilisation regulates the acute response of the cells to GnRH, that is the release of LH and FSH. Whilst more downstream signalling agents, the phosphorylated MAPKs regulate the long-term effect of GnRH, by controlling the synthesis of LH and FSH genes. GnRH receptor activation can therefore be determined by measuring IP_3 and phosphorylated MAPKs through inositol phosphate (IP) assays and western blotting assays of phosphorylated MAPKs respectively. Although the GnRH receptor can activate several signalling pathways in pituitary gonadotropes the $G\alpha_{q/11}$/PKC dependent ERK pathway is the main pathway through which gonadotropin synthesis is mediated (Perrett and Mcardle, 2013).

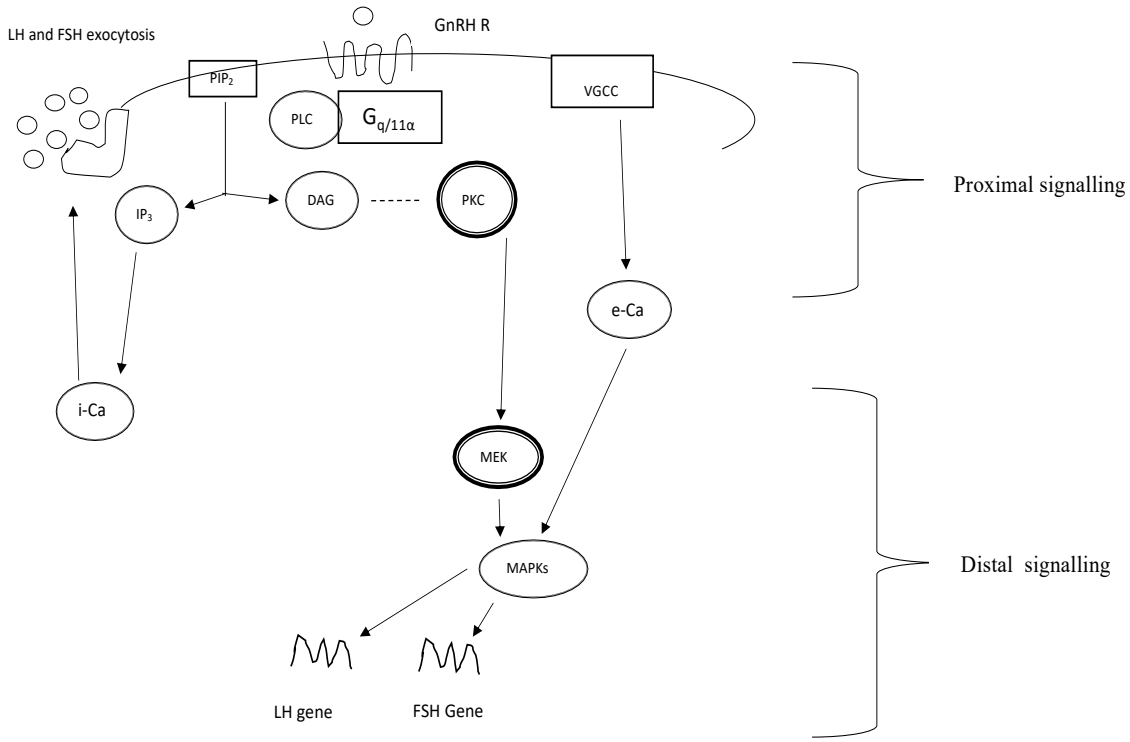


Figure 1: Schematic representation of the GnRH receptor signalling pathway

The figure depicts GnRH receptor signalling pathways post ligand binding. This figure is adapted from Lin et al., 2001 and Coss 2018 figures. Phosphatidyl 4,5-diphosphate (PIP_2); inositol 1,4,5-triphosphate (IP_3); diacylglycerol (DAG) protein kinase C (PKC); mitogen-activated protein kinase kinase (MEK); mitogen activated protein kinases (MAPKs); Ca=extracellular calcium; i-Ca=intracellular calcium. VGCC=voltage-gate calcium channels.

1.3.3 The PKC-dependent ERK pathway is the main distal GnRH receptor signalling pathway

GnRH receptor-mediated ERK activation in pituitary cells is PKC- and calcium-dependent (Reiss *et al.*, 1997; Kraus *et al.*, 2001). Mitogen-activated protein kinase kinase (MEK) is the upstream activator of ERK (Reiss *et al.*, 1997). The pathway connecting GnRH to ERK therefore, involves sequential activation of $G\alpha_{q/11}$ followed by PLCβ, PKC, and MEK (Naor, Benard and Seger, 2000).

In pituitary cells, phosphorylated (activated) ERK has been shown to translocate to the nucleus, an observation that supports the assertion that ERK has a role in regulating the transcription of gonadotropins (Roberson *et al.,* 1995; Haisenleder *et al.,* 1998; *Kraus et al*., 2001; Liu *et al*., 2002). There is evidence to suggest that the PKC-ERK pathway regulates the gene expression of the common α gonadotropin subunit (Roberson *et al.*, 1995), LHβ subunit (Harris *et al.*, 2002, 2003; Liu, Austin, *et al.*, 2002) and the FSHβ subunit (Vasilyev *et al.*, 2002). Furthermore, in mouse and rats, GnRH induced transcription of the GnRH receptor is primarily regulated through the PKC-ERK signalling pathway (Janjic, Stojilkovic and Bjelobaba, 2017). Therefore, ERK in contrast to other MAPKs such as JNK, p38 and BMK has been shown to be involved in the regulation of the LH, FSH and the GnRH receptor genes.

1.4 Allosteric modulation of receptor function

The GnRH receptor like other GPCRs has been shown to have allosteric qualities, it has multiple binding sites on which other molecules other than its natural ligand can bind (Naor and Huhtaniemi, 2013). This means the receptor's activity may be modulated by various molecules other than its natural ligand (Naor and Huhtaniemi, 2013). These receptor-activity modulating molecules may be membrane bound or cytosolic, and are generally regarded as binding partners. These molecules have an ability to induce receptor changes and affect receptor function (Milligan and White, 2001; Naor and Huhtaniemi, 2013).

Functions that these modulators can affect include, the interaction of the receptor with the ligand, transitions between selective conformations in turn affecting the type of G protein the receptor associates with, thereby culminating into selective signalling pathways (Naor and Huhtaniemi, 2013). These molecules secondary to the ligand may therefore be central to the observed differential receptor mediated physiological responses. This may well be true for the GnRH receptor, which has been shown to stabilise selective conformations, recruiting different G proteins resulting in different responses; mediating synthesis and release of gonadotropins in the pituitary and death in GnRH-dependent cancer cells. Therefore there is a possibility that different modulators associate with the GnRH receptor in different cellular contexts resulting in different selective conformations and physiological responses (Naor and Huhtaniemi, 2013).

Membrane or cytosolic proteins can form macromolecular complexes otherwise referred to as signalosomes in the membrane or in lipid rafts, and are able to modulate receptor activity modulators (Naor and Huhtaniemi, 2013). Scaffold proteins belong to this group of receptor-activity modulators (Naor and Huhtaniemi, 2013). β-arrestin is one of the widely studied receptor-activity modulators as it modulates GPCR function (Zheng, Loh and Law, 2010). The muscarinic M1 and angiotensin II type 1a receptors use the β-arrestin pathway to activate ERK (Scott *et al.*, 2006). The GnRH receptor due to its unique absence of a cytoplasmic carboxy-terminal tail does not interact with β-arrestin (Flanagan and Manilall, 2017). The latter explains the lack of GnRH receptor arrestin-dependent desensitisation, internalisation and signalling (Flanagan and Manilall, 2017). The GnRH receptor is peculiar, while most GPCRligands utilise several receptors for mediation of different biological events (Naor and Huhtaniemi, 2013); the GnRH receptor seems to be the only receptor that mediates GnRH induced gonadotrope migration, gonadotropin synthesis and release (Naor and Huhtaniemi, 2013). Perhaps the latter can be explained by involvement of several signalosomes that associate with the GnRH receptor in different cells and stabilise the GnRH receptor in selective confirmations leading to diverse biological responses (Naor and Huhtaniemi, 2013).

GnRH receptor signalling specificity is thought to be due to the presence of scaffolding proteins and signalosomes which bring together the receptor and signalling molecules (Navi *et al*., 2017). A signalosome for ERK phosphorylation by GnRH is localised to lipid rafts, and involves the GnRH receptor, c-Raf kinase, Ca^{2+} -calmodulin, and ERK (Navratil *et al*., 2003), and another localised at focal adhesions includes focal adhesion kinase (FAK) and c-Src (Davidson *et al*., 2004). The role of the FAK-cSrc signalosome is to sequester GnRH receptor-mediated phosphorylated ERK to activate FAK and paxillin and thus mediate cell migration (Navi *et al*., 2017). This action requires engagement of the actin cytoskeleton which has been shown to be remodelled upon GnRH stimulation (Davidson *et al*., 2004; Navratil *et al*., 2007). Furthermore, actin polymerisation is required for GnRH signalling to ERK (Davidson *et al*., 2004; Navratil *et al*., 2007).

1.5 Role of the actin cytoskeleton in GnRH receptor signalling

The actin cytoskeleton is involved in several important cellular functions including, cell shape, migration, and protein trafficking (Pollard and Cooper, 2009; Porat-Shliom *et al*., 2013). As indicated above GnRH stimulation results in active polymerisation in gonadotropes (Davidson *et al*., 2004; Navratil *et al*., 2007). It is well established that packaging of LH and FSH into secretory vesicles and trafficking of these to the membrane for release into circulation requires an intact actin cytoskeleton (Adams and Nett, 1979; Stojilkovic *et al*., 1988; Jablonka-Shariff and Boime, 2004; Izumi, Kasai and Gomi, 2007). Furthermore, GnRH signalling to ERK, the fundamental pathway for the regulation of gonadotropin synthesis and secretion and ultimately fertility requires actin polymerisation (Davidson *et al*., 2004; Navratil *et al*., 2007). The disruption of the actin cytoskeleton results in a loss of GnRH receptor-mediated ERK phosphorylation and consequently a reduction in LH secretion (Edwards *et al*., 2017).

However, the signalling mechanisms linking GnRH receptor activation and actin polymerisation are not well defined (Navratil *et al*., 2014). A signalling intermediate that links GnRH receptor activation to actin remodelling and ERK phosphorylation is possibly a scaffolding protein that interacts either directly or with associated signalling molecules of the GnRH receptor-ERK signalosome. Actin-binding proteins such as filamin A, due to their size and interactions with signalling molecules (Komaletdinova and Pinaev, 2006), may be the missing piece of the puzzle. Filamin A may be part of the GnRH receptor signalosome, linking the GnRH receptor with actin and other signalling molecules required for ERK phosphorylation.

1.6 The role of filamins in GPCR signalling and expression

1.6.1 Filamin structure

Filamins are homodimers of 240 to 280 kDa subunits, which associate via their carboxy termini (Gorlin *et* al., 1990; Van der Flier *et al*., 2001; Stossel *et al.*, 2001; Pundas *et al*., 2005). Each filamin subunit is made up of an amino-terminal actin-binding domain which is followed by 24 β-pleated sheet immunoglobulin-like domains interrupted by two flexible hinge regions (Gorlin *et* al., 1990; Van der Flier *et al*., 2001; Stossel *et al.*, 2001; Pundas *et al*., 2005). Three isoforms of filamin exist, filamin A, B and C. Filamin A and B are ubiquitously expressed while filamin C is found primarily in cardiac and skeletal muscle cells (Kim and McCulloch, 2011). Filamin A is expressed in most cell types (Kim and McCulloch, 2011), thus this isoform has been most studied.

1.6.2 Filamin function

The function of filamin is that of a cytoskeletal structural protein, which organises actin filaments by attaching actin filaments to the plasma membrane (Komaletdinova and Pinaev, 2006). Filamin also regulates actin-myosin

interaction which is fundamental for cellular morphology, motility and adhesion (Komaletdinova and Pinaev, 2006) and have additional diverse cellular functions (Komaletdinova and Pinaev, 2006). There is evidence that filamin A functions as a promiscuous scaffolding protein, organising complex cellular signals from the cytoplasm to the nucleus (Komaletdinova and Pinaev, 2006; Deng *et al.*, 2012). Over 90 binding partners, including receptors (Awata *et al.*, 2001; Lin *et al.*, 2001; He *et al.*, 2003; Huang *et al.*, 2006; Jiménez-Baranda *et al.*, 2007; Onoprishvili *et al.*, 2008; Minsaas *et al.*, 2010; Najib *et al.*, 2012), ion channels (Petrecca, Miller and Shrier, 2000), intracellular signalling molecules (Loo, Kanner and Aruffo, 1998; Ohta *et al.*, 1999; Leonardi *et al.*, 2000; Scott *et al.*, 2006; Popowicz *et al*., 2006), Rho family of GTPases (Ohta *et al*., 1999), the tumour necrosis factor receptor-associated factor-2 (TRAF2) (Leonardi *et al*., 2000), β1 integrin (Loo *et al*., 1998), tissue factor (Müller *et al*., 1999) and transcription factors (Kwon *et al.*, 2014) have been identified to interact with filamin A.

1.6.3 Filamin A and GPCR signalling

Filamin A has been shown to interact with four GPCRs, the calcium receptor, mu opioid receptor, chemokine (C-C motif) receptor 2B and the somatostatin receptor 2. Filamin A interacts with the calcium receptor and is required for calcium receptor-mediated ERK phosphorylation (activation) (Awata *et al.*, 2001; Huang *et al.*, 2006). The mu opioid receptor also interacts with filamin A, but this interaction is not required for ERK activation; since mu opioid receptor-transfected filamin A replete and deficient cells can both mediate ERK activation post stimulation, however the kinetics of ERK activation vary between these cells (Onoprishvili *et al.*, 2008). The mu opioid receptor transfected into filamin A-deficient cells shows a sustained ERK phosphorylation profile, with ERK being active for over 60 minutes, whereas filamin A-replete cells exhibit rapid ERK dephosphorylation after 30 minutes (Onoprishvili *et al.*, 2008). Minsaas and colleagues did not assess the functional significance of filamin A in downstream

signalling of the chemokine (C-C motif) receptor 2B (Minsaas *et al*., 2010). The somatostatin receptor 2 was shown to require filamin A to mediate ERK phosphorylation inhibition (Peverelli *et al*., 2014). Since the role of filamin A in distal signalling, as indicated by ERK activation varies between GPCRs, this means the functional consequences of filamin on downstream signalling of GPCRs is not predictable.

1.6.4 Interaction of filamin A with signalling proteins and its role in the PKC-ERK pathway

Since filamin A is a key cytoskeleton regulator it should not be surprising that it interacts with several signalling proteins central to cytoskeleton remodelling. Filamin A binds small GTPases, Ras-related small GTPases: Cdc42, Rho, Rac, and RalA; which are central to the formation of actin-rich surface structures in cells (Ohta *et al.*, 1999). The interaction with RalA is GTP-dependent and central to regulating actin cytoskeleton remodelling (Ohta *et al.*, 1999). However, filamin A-signalling protein interactions are not limited to those implicated in cytoskeleton reorganisation. Filamin A has been shown to bind to other types of signalling proteins whose function are broader than cytoskeleton remodelling.

Filamin A has been shown to be associated with several kinases, including protein kinase A and protein kinase C (Stossel *et al.*, 2001). The filamin A interaction with PKC, a key second messenger required for GnRH receptor-mediated phosphorylation of MAPKs including ERK, has been shown to be required for optimal PKC-mediated cellular signalling and thus ERK phosphorylation (Kim and McCulloch, 2011). Filamin A has also been shown to interact with mitogen-activated protein kinase kinase 1 (MEK 1), an upstream activator of ERK; and the interaction was shown to result in enhanced ERK phosphorylation in filamin A-replete cells relative to filamin A-deficient cells (Scott *et al.*, 2006). Thus, in summary filamin A interacts with PKC and MEK1 which are upstream activators of ERK, and its interaction with these signalling proteins enhances signalling.

Since filamin A has been shown to directly interact with other GPCRs, it may modulate receptor conformation or stability.

1.6.5 Filamin A effect on GPCR expression and ligand affinity

Studies have shown that filamin A plays a role in trafficking of GPCRs to the cell surface. Filamin A interaction with the chemokine receptor- CCR2B has been shown to be key for its internalisation (Minsaas *et al.*, 2010), while the interaction of filamin A with dopamine receptor subtypes D_2 and D_3 appears to be required for trafficking of these receptors to the plasma membrane, based on increased cell surface expression of D_2 receptors (Lin *et al.*, 2001; Kim and McCulloch, 2011). This is also the case for HIV receptors, where filamin A interacts with CD4 and the coreceptors (CCR5 or CXCR4) and regulates their clustering on the cell surface, which is thought to be essential for infection (Jiménez-Baranda *et al.*, 2007). Filamin A interaction has also been shown to be key for the stabilisation of the somatostatin receptor 2 following prolonged agonist stimulation (Peverelli *et al*., 2014). The effect of filamin A on cell surface expression of the GnRH receptor is yet to be studied.

Although receptor-filamin A interactions had no effect on ligand binding affinity of the dopamine D_2 (Li *et al.*, 2000), mu opioid receptor (Onoprishvili *et al.*, 2003) and CCR2B (Minsaas *et al.*, 2010) receptor, effector coupling was not investigated. By employing radioligand binding assays it was shown that the ligand affinity was the same for filamin A-replete and filamin A-deficient cells. To our knowledge no study has assessed the effect of filamin A on GnRH binding affinity. Should filamin not affect binding affinity, then the radioligand binding assays can be used to assess plasma membrane receptor expression by determining B_0 as an indicator of total receptor binding.

1.7 Investigating the role of filamin A in receptor signalling and expression

Two methods have been used to investigate the effect of decreased filamin A expression on functions of its protein binding partners, namely a pair of melanoma cell lines that are respectively filamin A-deficient and filamin A-replete, these two cell lines are used as a control and experimental groups; or knocking down filamin A by an RNA interference method using short interfering RNA (siRNA). Often these study models are employed concurrently. The pair of melanoma cell lines, which consists of; filamin A-deficient M2 cells and the filamin A-replete M2A7 cells, which are M2 cells stably transfected with filamin A (Cunningham *et al*., 1992), are a model that has been widely used. When studying the effect of filamin A in receptor function, a DNA plasmid with a receptor cDNA of interest is transfected into both cell lines and varying molecular techniques, including western blotting, fluorescence microscopy or radioligand binding assays are employed to assess functional consequences of filamin A on receptor activity and immunoprecipitation assays have been used to verify the interaction with filamin A (Loo, Kanner and Aruffo, 1998; Hjälm *et al.*, 2001; Zhang and Breitwieser, 2005; Mammoto, Huang and Ingber, 2006; Jiménez-Baranda *et al.*, 2007; Onoprishvili *et al.*, 2008; Minsaas *et al.*, 2010).

However, the melanoma cell model has some limitations because filamin A deficient cells have been shown to have abnormal Golgi network, dysfunctional cytoplasmic trafficking, protein sorting, localisation and reduced adhesion strength. Thus, the deficiency of filamin A has multiple consequences which may not be directly related to a variable being studied. Hence RNA interference is used as an alternative model to verify melanoma cell model results. RNA interference is commonly applied with the widely used cell culture lines such as HEK 293 cells (Ohta *et al.*, 1999; Awata *et al.*, 2001; Hjälm *et al.*, 2001; Onoprishvili *et al.*, 2008, 2003; Zhang and Breitwieser, 2005; Nakamura, Stossel and Hartwig, 2011; Tirupula *et al.*, 2015). Knocking down of a protein by RNA interference using siRNA requires that the siRNAs are designed to be complementary to the

gene of which expression is to be reduced (Walton et al., 2010). siRNAs function as follows, the silencing siRNAs are delivered to the cytoplasm via transfection where they are recognised by the proteins of the RNA-induced silencing complex, loading complex, Dicer, Argonaute 2 and TAR RNA-binding protein (Walton et al., 2010). The loading complex selects one strand from the siRNAs which acts as a guide strand, the loading complex with the guide strand and Argonaute 2 form the active RNA-induced silencing complex (Walton *et al*., 2010). This active RNA-induced silencing complex then recognises its target mRNA by complementarity between the guide strand and the region on the mRNA (Walton *et al*., 2010). This results in cleaving of the mRNA, leading to reduced levels of intact mRNA (Walton et al., 2010). The protein knock-down results from the normal degradation of protein, which cannot be replaced due to reduced levels of intact mRNA (Walton et al., 2010). For control purposes, non-specific non-silencing siRNAs are used, these siRNAs do not result in cleaving of any mRNA (Minsaas et al., 2010).

1.8 Study aim and objectives

Filamin A has been shown to be a scaffold protein that organises complex cellular signals from the plasma membrane, cytoplasm to the nucleus; through connecting with receptors and signalling proteins. The role of filamin A in GnRH receptor signalling has not yet been studied. Therefore, the aim of the study was to investigate whether filamin A has a role in GnRH receptor signalling as part of the GnRH receptor signalosome.

Specific objectives were to:

- express the GnRH receptor protein in HEK 293 cells and M2 and M2A7 cells
- develop a filamin A knock-down assay using siRNAs in HEK 293 cells
- measure GnRH receptor-mediated ERK phosphorylation in cells with varying filamin A expression to determine the role of filamin A in GnRH receptor distal signalling

- measure ligand binding affinity and cell surface expression of the GnRH receptor in cells with varying filamin A expression to determine if filamin A has a role in modulating GnRH receptor conformation or stability
- measure GnRH-stimulated IP production in cells with varying filamin A expression to determine if filamin A has a role in GnRH receptor proximal signalling

Chapter 2: Materials and Methods

2.1 Preparation of GnRH receptor DNA stocks

The human GnRH receptor cDNA previously subcloned (Flanagan et al. 2000) into the pcDNAI/Amp expression vector (*Invitrogen, San Diego, USA*) was used. DNA stocks of this GnRH receptor plasmid were made by transforming DH5α *Escherichia coli* (*E. coli*) cells (Invitrogen, Carlsbad, California) and extracting the plasmid DNA using a Pure Yield™ Plasmid Maxiprep Kit (*Promega, Madison, USA*). Plasmid DNA (10 ng of stock DNA) was added to the competent DH5α cells (Invitrogen, Carlsbad, California) and incubated on ice (20 min), heat shocked (42°C, 48 sec) and incubated on ice (3 min). Luria Broth [1% Tryptone, 1% sodium chloride, 0,5% yeast] (LB, 0.5 ml) medium was added and the mixture was incubated with shaking (13 500 x*g*, 90 min, 37°C) and cells were spread on LB agar plates with ampicillin (1 μg/ml, Sigma Aldrich, Saint Louis, Missouri) and cultured (16 hr, 37°C). Colonies were picked from the LB-ampicillin agar plate using a pipette tip, which was dipped and swirled in LB ampicillin (100 μg/ml) that was cultured with shaking (3.5 hr, 37°C). This was then used to inoculate a larger volume of LB ampicillin which was cultured with shaking (16 hr, 37°C).

Transformed DH5α cells (Invitrogen, Carlsbad, California) were pelleted by centrifugation and resuspended in Cell Resuspension Solution (*Promega, Madison, USA*) (12 ml) followed by an addition of Cell Lysis Solution (12 ml). The mixture was incubated (3 min, room temp), before adding Neutralising Solution (12 ml) and mixing. The lysate was centrifuged (13 500 x*g*, room temp, 30 min) and the supernatant was passed through the Pure Yield™ clearing column and binding column under vacuum. The binding column was washed with Endotoxin Removal Wash (5 ml) followed by Column Wash (20 ml) and a vacuum applied until the binding column membrane was dry. The column was placed in a clean 50 ml centrifuge tube and DNA was eluted with 1.5 ml of pre-warmed nuclease-free water (13 500 x*g*, room temp, 30 min). The plasmid concentration was determined by UV spectrophotometry (260 nM). A restriction enzyme analysis of the construct was undertaken to verify the sizes of plasmid

vector and insert using EcoR I and Xho I enzymes and agarose gel electrophoresis.

2.2 Filamin siRNA design and synthesis

Published filamin A siRNA sequences (Jiménez-Baranda *et al*., 2007; Minsaas *et al.*, 2010) were adapted to suit the Ambion siRNA kit requirements (**Table 1**). These siRNA sequences would target three different regions of the filamin A mRNA as shown in figure 2. The Ambion siRNA kit requires primer sequences to start with an AA dinucleotide, be 21 nucleotides long and have a GC content that was between 30 and 50 percent. BLAST (from the NCBI server) was used to retrieve the human filamin A cDNA (the DNA equivalent of the target mRNA sequence) and the DNAMAN software (Lynnon Biosoft, Quebec, Canada) was used to complete the siRNA design. The 8-nucleotide sequence (CCTGTCTC) complimentary to the T7 promoter primer required for efficient transcription of the siRNA was added to the 3' end to give the antisense oligonucleotide sequence (**Table 1**) that was synthesised (Inqaba Biotech (Pretoria, South Africa) for siRNA synthesis. To design the sense oligonucleotides, the reverse complimentary sequence of the identified cDNA sequence was generated (**Table 1**). An AA dinucleotide sequence was added to the 5' end and the 3' TT dinucleotide sequence was removed from the sense oligonucleotide before addition of the T7 promoter sequence-CCTGTCTC (**Table 1**). The DNA template oligonucleotides were dissolved in nuclease free water to a concentration of 100 µM. The design for siRNA 3 was slightly adapted since GC content was low, base A was replaced with C to increase GC content from 28.6% to 33.3%, since recommended GC content is at least 30% as per Ambion Silencer siRNA design instructions.

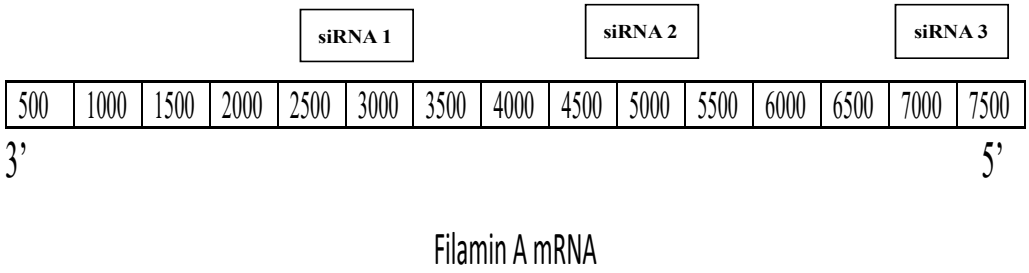
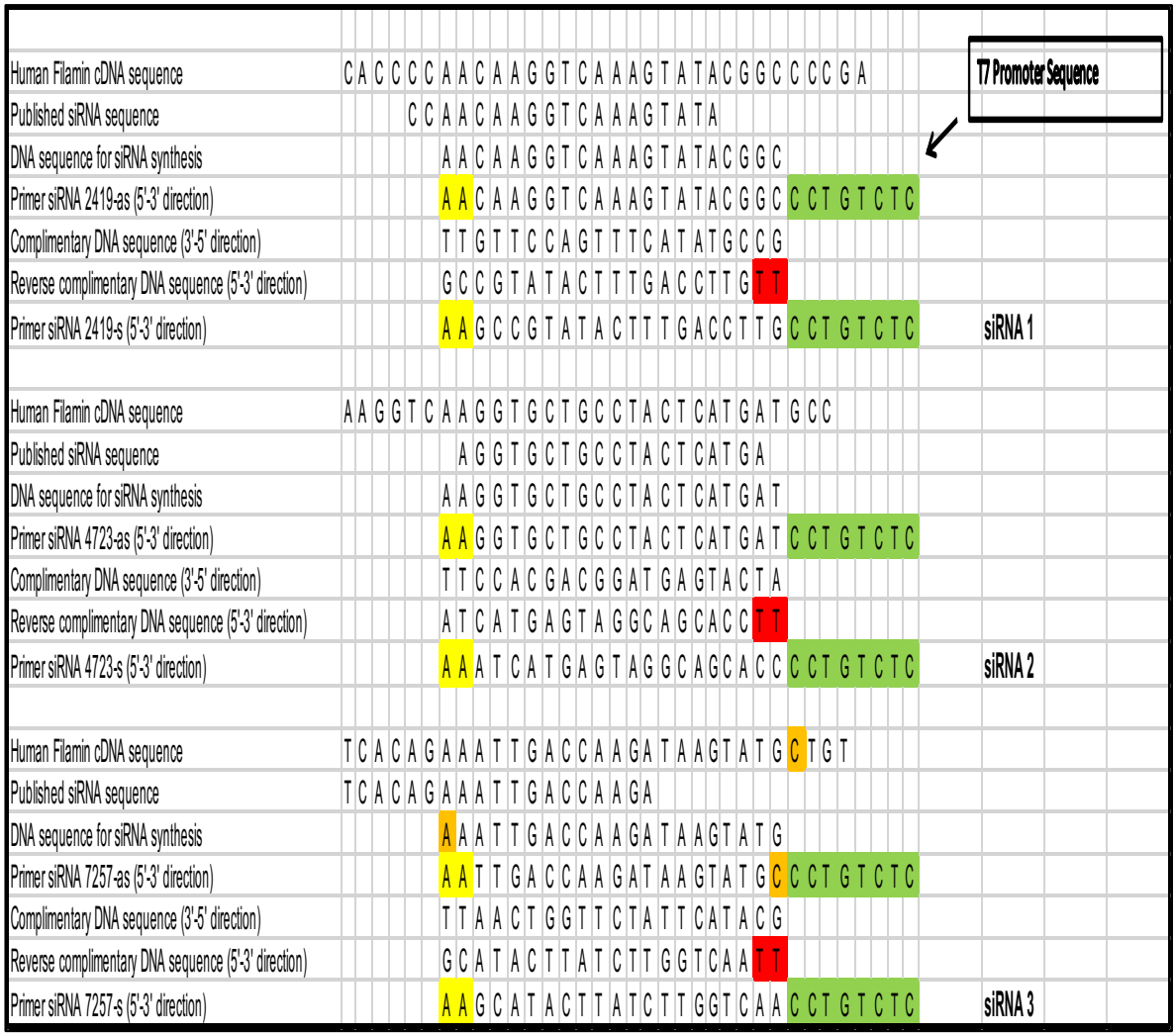


Figure 2: Schematic representation of siRNAs and filamin A mRNA.
Figure indicating three regions of the filamin A mRNA that the designed siRNA would target for silencing

Table 1: Identification of human filamin A DNA sequences for the design of DNA primers for siRNA synthesis.



Previously published human filamin A siRNA sequences were aligned with the human filamin A cDNA extracted from BLAST and used to design DNA primers for the synthesis of siRNA as described in the text. The double adenine nucleotides are highlighted in yellow for both the sense and antisense siRNA primers, while the double Thymine nucleotides which are removed from reverse complimentary DNA sequence to form the sense siRNA primer are highlighted in red. Nucleotides highlighted in orange, indicate a change made to the DNA sequence to meet kit instruction with regards GC content required for siRNA synthesis.

Briefly, to make double-stranded DNA (dsDNA) templates, oligonucleotides were hybridised to the T7 promoter primer separately by mixing each template (2 µl, 100 µM), T7 promoter primer (2 µl) and DNA Hybridisation buffer (6 µl) in a micro-centrifuge tube, heating (70°C, 5 min) and cooling (room temp, 5 min).

Then 10X Klenow reaction buffer (2 µl), 10X dNTP Mix (2 µl), nuclease free water (4 µl) and Exo-Klenow (2 µl) were added to the hybridised oligonucleotide templates, the mixture was gently vortexed, centrifuged (10 s) and incubated in a cabinet incubator (37°C, 30 min).

To make RNA, 2 µl of the dsDNA (double stranded DNA) was mixed with nuclease free water (4 µl), 2X NTP mix (10 µl), 10X T7 reaction buffer (2 µl) and T7 enzyme mix (2 µl) by slow vortexing and centrifugation. The reactions were then incubated (37°C, 3 hr). To allow transcription a cabinet incubator was always used for incubations longer than 5 minutes instead of the heating block to prevent condensation, which would in turn reduce siRNA yields. Then the sense and antisense transcription reactions were combined in a micro-centrifuge tube and incubated (37°C, 24 hr) to hybridise the newly-synthesised RNA strands which form the siRNA duplex. The incubation times for the transcription and hybridisation reactions were increased from the 2 and 16 hours recommended in the Ambion Silencer kit to 3 and 24 hours respectively to increase siRNA yields.

To remove the single stranded T7 promoter sequence, separate siRNA from enzymes and DNA oligomers and any salts the siRNA duplexes were mixed with digestion buffer (6 µl), nuclease-free water (48.5 µl), RNase (3 µl) and Dnase (2.5 µl), slowly vortexed and incubated (37°C, 2hr). After addition of siRNA binding buffer (400 µl) and incubation (room temp, 4 min) the mixture was applied to a pre-wetted filter cartridge and centrifuged (13 500 x*g*, 1 min). The filter cartridge was washed twice by applying wash buffer (500 µl) and centrifugation (1 min). The filter cartridge with the siRNA was transferred to a clean tube and the siRNA was eluted by adding pre-warmed (75°C) nuclease free water (100 µl), incubating for 2 minutes (21°C) followed by centrifugation (13 500 x*g*, 2 min).
The siRNA size and integrity were assessed by agarose gel electrophoresis. 1% agarose gel was prepared in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). Products were run at 65 V for 1 hour, stained with ethidium bromide and visualised under ultraviolet light using a transilluminator. The synthesised siRNA were aliquoted and stored at -80°C until transfection. siRNA

preparation was performed in the sterile laminar flow hood to minimise nucleotide degradation by Rnases and an Rnase decontamination solution (RnaseZap, Life Technologies, Carlsbad, USA) was used to reduce Rnase activity.

2.3 Cell culture and transient transfections of HEK 293 cells and M2 and M2A7 melanoma cells

Two different cell culture approaches were used to study the role of filamin A in GnRH receptor signalling and expression. HEK 293 cells as the widely used system to study GnRH receptor function and one of the easy to transfect cell lines was used. However, HEK 293 cells express filamin A so to study the role of filamin A in GnRH receptor signalling and expression the RNA interference method was applied. HEK 293 cells were transfected with the GnRH receptor and siRNA to vary filamin A protein expression. The second approach used was the melanoma cellular pair system, filamin A deficient M2 melanoma cells and filamin A replete M2A7 melanoma cells which were transfected with GnRH receptor.

HEK 293 cells were maintained at 10% carbon dioxide, 37°C in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS). M2 and M2A7 melanoma cells were maintained at 10% carbon dioxide, 37°C in Dulbesco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and G148 (200 µg/ml, added only to be M2A7 cells, important for maintaining filamin A expression (Nakagawa *et al*., 2010). All cells were passaged by washing and incubating (5 min) in 0,5% (v/v) PBS/EDTA, centrifuged (5 min) then resuspended in 10 ml of DMEM, a fraction (often 10% for routine cells maintenance) of resuspended cells were replaced into the flask with fresh medium to maintain as stock.

2.3.1 Transfection of HEK 293 cells with siRNA and the GnRH receptor

Initially the HEK 293 cells were transfected once with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA (control), which lacks significant sequence homology to the filamin transcript) or with filamin A specific siRNA. However, this did not result in satisfactory decrease of filamin A, so combined siRNA transfections (using more than one siRNA), multiple transfections with combined siRNA (using more than one siRNA and transfecting cells more than once) and varying transfected siRNA incubation times pre-experiment were undertaken to decrease filamin A expression. The siRNA double transfections were performed as follows: HEK 293 cells were cultured in 10 cm^2 dishes until 60% confluent, transfected with complexes of siRNAs (1.5 or 4.5 µg) and Fugene HD (40 µl) in DMEM (500 µl) and cultured overnight (10% CO_2, 37°C). In the evening of the day, 6 to 8 hours after the first siRNA transfection the cells were transfected again with siRNA without medium change. 48 or 72 hours after the first siRNA transfection the cells were counted using a Neubauer haemocytometer and plated into the 6 wells (0.3×10^6 cells per well) and 12 wells (0.15×10^6 cells per well) plates for western blotting.

In order to simultaneously knock down filamin A and express the GnRH receptor, HEK 293 cells were cultured in 10 cm^2 dishes until 60% confluent, transfected with complexes of siRNAs (4.5 µg) and Fugene HD (40 µl) in DMEM (500 µl) and cultured overnight (10% CO_2, 37°C). 12 to 16 hours after the first siRNA transfection, the cells were transfected with the GnRH receptor plasmid (10 µg), which was mixed with serum free medium (500 µl) and Fugene HD (40 µl, Promega, Madison, USA) and incubated (15 min), before being added drop-wise to cells and incubated (10% CO_2, 37°C, 16 hr), and the cells would be transfected again with siRNA 6 to 8 hours after the GnRH receptor plasmid transfection. 72 hours after the first siRNA transfection the cells were counted using a Neubauer haemocytometer and plated into the 6 well plates (0.3×10^6 cells per well)- or 12 well plates (0.15×10^6 cells per well) for ERK activation assays.

2.3.2 Transfection of M2 and M2A7 melanoma cells with GnRH receptor

The human melanoma cell line M2, which lacks filamin A expression (Cunningham *et al*., 1992) and the melanoma cell line M2A7, which stably expresses full length filamin A (both cell lines were provided by Fumihiko Nakamura, Harvard Medical School, Boston, USA) were transiently transfected with the GnRH receptor plasmid DNA. Cells were transferred to 10 cm^2 dishes and cultured until 80% confluent. DNA (10 µg) was added to serum free medium (500 µl), before addition of Fugene HD (40 µl, Promega, Madison, USA) and incubated (15 min) to form the complete transfection complex. The transfection complex was then added drop-wise to cells and incubated (10% CO_2, 37°C, 16 hr). 16 hours after the transfection medium was replaced. 48 hours after the transfection the cells were detached, counted using a Neubauer haemocytometer and plated into the 6 well plates ($0.3 x10^6$ cells per well) or 12 well plates ($0.15 x10^6$ cells per well)for ERK activation assays, IP production assays or competition radioligand binding assays.

2.4 Radioligand competition binding assays

Initial binding (B_0) and the 50% inhibitory concentration (IC_{50}) were measured in GnRH receptor-transfected M2 and M2A7 cells. B_0 is not a formal measure of total binding site number, but it is used as an indicator for cell-surface receptor expression for receptors with the same ligand affinity. A radio-iodinated GnRH receptor agonist, ^{125}I-His^5-D-Tyr^6 GnRH was prepared by Dr C. Flanagan using the chloramine-T protocol as previously described (Flanagan *et al*. 1998). GnRH receptor-transfected M2 and M2A7 cells were seeded into 12-well plates, incubated (16 hr, 37°C, 10% CO_2) and washed with cold 0.1% (w/v) BSA then incubated (4 hr) on ice in medium containing 100 000 counts per minute (cpm) of the radio-iodinated GnRH receptor agonist and varying concentrations of unlabelled cold GnRH peptide. The incubation was stopped by washing the cells three times with cold PBS. The cells were solubilised in 1 M NaOH (1 ml) and radioactivity in the lysate was counted in a γ-scintillation counter.

2.5 Inositol Phosphate assays

GnRH receptor-transfected M2 and M2A7 cells were plated into 12 well plates (0.15×10^6 cells per well), cells were radio-labelled with 3H-myo-inositol (1 µCi/ml) (Amersham, Buckinghamshire, England) in DMEM supplemented with 2% FCS and incubated (37°C, 10% CO_2, 16 hr). This incorporates 3H-myo-inositol into phosphatidylinositol 4,5-biphosphate (PIP_2), which is the substrate of phospholipase C (PLC). When GnRH receptor stimulation of G_q protein activates PLC it breaks down labelled PIP_2 into diacylglycerol (DAG) and [^{3}H]-IP_3. The labelling medium was aspirated, and cells were preincubated in warmed buffer I (NaCl 140 mM, KCl 4 mM, HEPES 40 mM, Glucose 8.6 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, 0.1% (w/v) BSA, LiCl 10 mM, pH 7.4, 1 ml/well, 15 min, 37°C). Cells were stimulated with different doses of GnRH (10^{-12} M to 10^{-7} M) in buffer I (60 min, 37°C). The GnRH solutions were aspirated and the cells were lysed by incubation with cold formic acid (10 mM, 1 ml/well, 30 min). The cell lysates were added to 500 µl of DOWEX-1 resin (8%, 50-100 mesh, Sigma-Aldrich, Johannesburg, South Africa) in 5ml borosilicate tubes (Kimble, Lasec, Johannesburg, South Africa) and vortexed. The resin was allowed to settle, and the supernatant was aspirated. The resin was then washed with water (4 ml) to remove unbound radioactivity. The bound [^{3}H]IP was eluted with 1 M ammonium formate-0.1 M formic acid (1 ml). The eluate (800 µl) containing [^{3}H]IP was mixed with scintillation liquid (3 ml) (Optiphase Hisafe, Perkin, Elmer) and counted using a β-counter. Dose-response curves were calculated using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

2.6 ERK activation assay

72 hours after the first siRNA transfection (of HEK 293 cells) and 48 hours after GnRH receptor transfection (of HEK 293, M2 and M2A7 cells), cells were stimulated with GnRH (10 µM, 1 ml per well) in HEPES-buffered DMEM for varying times. Stimulation was stopped by discarding the GnRH-containing medium and placing the plates on ice. The cells were washed with cold lysis

buffer [sodium chloride 150 mM, Tris-hydrochloric acid (pH 8.0) 150 mM, 1% Triton-X, 0,5% EDTA (pH 8.0), 0,1% SDS, complete protease inhibitor 100 mM, sodium fluoride 50 mM, sodium pyrophosphate 30 mM, sodium orthovandate 100 µM and then incubated in the lysis buffer (30 min). A scraper was used to detach the cells from the plate's surface and cell lysates were transferred to a microcentrifuge tube, vortexed to lyse the cells further and centrifuged (4°C, 13 500 xg, 15 min) to separate the cytoplasmic proteins from insoluble materials. The supernatant was mixed with SDS sample buffer [Tris-HCL (pH 6.8) 25 mM, 10% (v/v) glycerol, 8% (w/v) SDS, a small amount of bromophenol blue, β-mecaptoethanol 0.9 M] incubated (95°, 5 min), 10 µl of a sample was loaded onto 8% SDS-PAGE gels [3% (v/v) acryl-bisacrylamide mix, 1.5 M Tris (pH 8.8), 1% (w/v) SDS, 1% (w/v) ammonium persulfate, TEMED 6 µl] and electrophoresed (40 min, 120 V). The gel was incubated (15 min) in transfer buffer (tris 5.82 g/l, glycine 2.93 g/l, SDS 0.375 g/l, 200 ml methanol and 800 ml of distilled water) and proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane that was wetted in methanol (1 min) and pre-soaked (15 min) in transfer buffer. A stack comprising blotting paper (soaked in transfer buffer), the polyacrylamide gel and the membrane was placed in a semi-dry electro-blot transfer apparatus (BioRad, Hercules, USA) and proteins were transferred from the gel to the PVDF membrane by electrophoresis (45 min, 25 V). The PVDF membrane was incubated (60 min, room temp) with shaking in blocking buffer (5% non-fat milk in Tris-buffered saline).

Following blocking the membrane was incubated with specific antibodies; anti-filamin A (#4762, Cell Signalling, Beverley, USA), anti-phospho-ERK (#4377, Cell Signalling, Beverley, USA) or anti-ERK (#4695, Cell Signalling, Beverley, USA) (5 µl/5 ml in blocking buffer, overnight, 4°C). Total ERK was probed using the same PDVF membrane and quantified for normalisation purposes, thus ensuring even loading. Following the primary antibody incubation, the membrane was washed three times with Tris-buffered saline (5 min per wash), incubated in secondary antibody, anti-rabbit horseradish peroxidase (HRP) (#7074, Cell Signalling, Beverley, USA) (2 µl/4 ml in blocking buffer, 2 hr, room temp)

washed four times with Tris-buffered saline (5 min per wash) and visualised with chemiluminescence (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare, Little Chalfont, UK) using a ChemiDoc (BioRad). The measurements of the density of the specific protein bands were determined using the Quantity One software imaging programme (BioRad).

2.7 Data analysis

B_0 and IC_{50} values for the competition binding assays and E_{max} and EC_{50} values for GnRH-stimulation IP production were determined using GraphPad Prism 5.0 (GraphPad Software Inc., *La Jolla*, *CA, USA*). For IP assays unpaired t-tests were used to compare, E_{max} and EC_{50} values. Nonlinear regression was used in GraphPad Prism to calculate E_{max} and EC_{50}. Two-way ANOVA was used to compare GnRH receptor-mediated ERK phosphorylation in GnRH receptor transfected M2 and M2A7 cells and in siRNA-GnRH receptor transfected HEK 293 cells. For comparison of radioligand binding results used to assess cell surface GnRH receptor expression of the transfected cells, t-tests were used to compare B_0 and IC_{50} in GnRH receptor transfected M2 and M2A7 cells.

Chapter 3: Results

3.1 The role of filamin A in GnRH receptor function using RNA interference in HEK 293 cells

3.1.1 Filamin A knock-down protocol optimisation

To test the efficiency of the designed siRNAs to knockdown Filamin A in HEK 293 cells, HEK 293 cells were transfected once with GAPDH siRNA (control siRNA, which lacks significant sequence homology with filamin A mRNA so does not alter filamin A protein expression) or with filamin A-specific siRNA. However, this resulted in no measurable decrease of filamin A. Filamin A expression in cells transfected with filamin A specific siRNA and in the cells transfected with GAPDH siRNA was the same (**Table 2**). Thus, multiple transfections with single or combined siRNAs and varying the time of experiment after the first siRNA transfection were undertaken to determine the efficiency of the different protocols. Filamin A protein expression was determined by western blotting. Table 2 below indicates filamin A protein expression observed with different protocols (**Fig. 3**) leading up to the optimised siRNA transfection protocol.

There was no visible difference in filamin A expression among cells transfected with GAPDH and filamin A siRNA; single or multiple siRNA constructs when lysates were processed for immunoblotting 24 hours after a single siRNA transfection. When western blots were performed 48 hours post transfection with single or multiple siRNA constructs filamin A expression was knocked down to about two-thirds of the GAPDH-transfected controls (**Fig. 4 A and C**). The difference between knockdown achieved with the single siRNA 2 construct or multiple siRNA constructs was minimal (**Table 2**). When the two transfections method for a 72 hours incubation time was undertaken, this means lysates were processed for immunoblotting 72 hours after the first siRNA transfection, filamin knockdown was better compared to the two transfections 48 hours incubation protocol. In this 72-hour protocol more than 80% knockdown was observed (**Fig. 4 B and D**). However, like with the 48 hours incubation protocol filamin A

protein expression was not noticeably different for cells transfected with filamin A single construct siRNA 2 or multiple filamin A siRNA constructs (**Fig. 4 B**). The filamin A multiple constructs siRNA work slightly better compared to a single construct siRNA, however only slightly 6% and 3% more knockdown for the 48- and 72-hours post transfection protocols respectively. Greater knockdown of filamin A through siRNA was achieved with a longer time of active transfection, more filamin A was knocked down with the 72 hours post first siRNA transfection protocol relative to the 48 hours protocol.

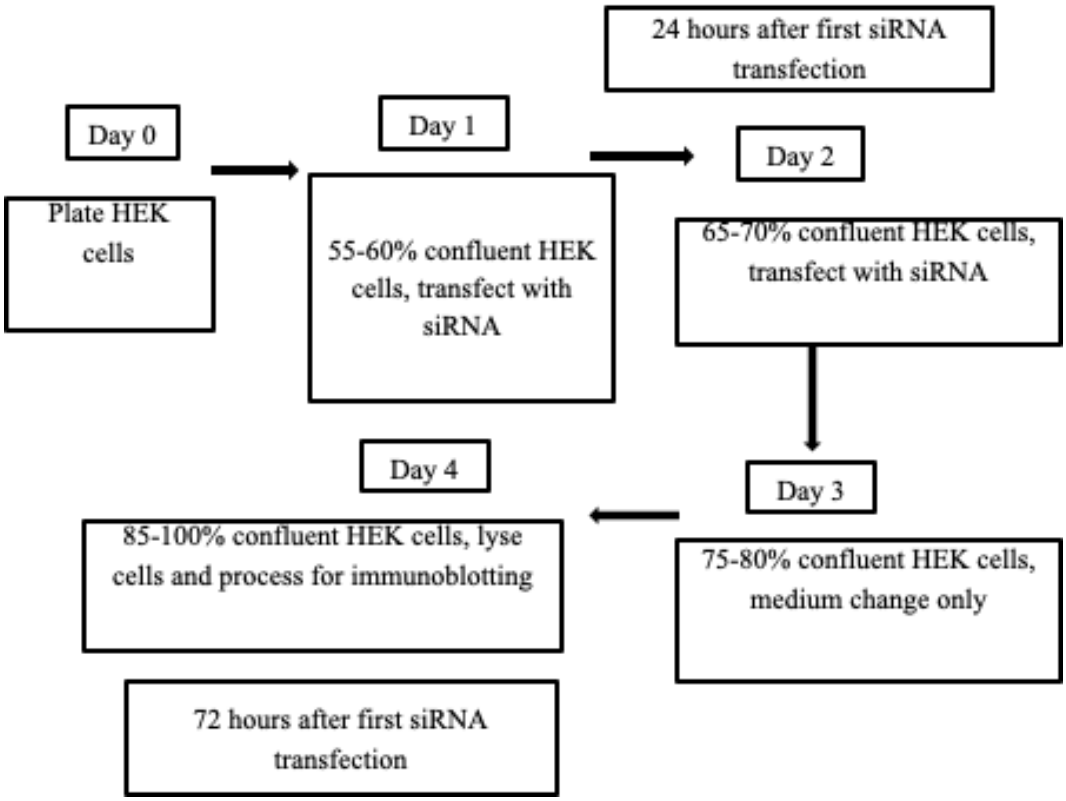


Figure 3: Optimised protocol for filamin A knockdown in HEK 293 cells.
HEK 293 cells were cultured in 10 cm^2 dishes until 55 to 60% confluent, transfected with complexes of siRNAs and cultured overnight (10% CO_2, 37°C). 6 to 8 hours after the first siRNA transfection the cells were transfected again with siRNA. 72 hours after the first siRNA transfection the cells were counted using a Neubauer haemocytometer and plated for western blotting.

Table 2: Optimisation of the siRNA transfection protocol

Time and number of transfections	Control (GAPDH)	siRNA 1	siRNA 2	siRNA 3	siRNAs 1, 2 and 3
24 hours post first siRNA transfection One Transfection	+++++ (5)	+++++ (5)	+++++ (5)	+++++ (5)	+++++ (5)
48 hours post first siRNA transfection One Transfection	+++++ (5)	+++++ (5)	++++ (4)	+++++ (5)	++++ (4)
48 hours post first siRNA transfection Two Transfections	+++++ (5)	Not performed	+++ (3)	Not performed	+++ (3)
72 hours post first siRNA transfection One Transfection	+++++ (5)	Not performed	+++++ (5)	Not performed	+++++ (5)
72 hours post first siRNA transfection Two Transfections	+++++ (5)	Not performed	++ (2)	Not performed	++ (2)

"+" sign in table 2 above is used as an indicator of observed filamin A protein concentration based on band densities in immunoblots. The densest band on each blot was allocated 5 "+" signs and other bands were scored relative to this, note that the numbers indicated in brackets are not units of density per se but are included to allow easier interpretation of the observed immunoblots at the time. Worth noting is that bands of cells transfected with GAPDH siRNA were

consistently denser, indicating lack of filamin A knockdown. This mean GAPDH siRNA was a good control.

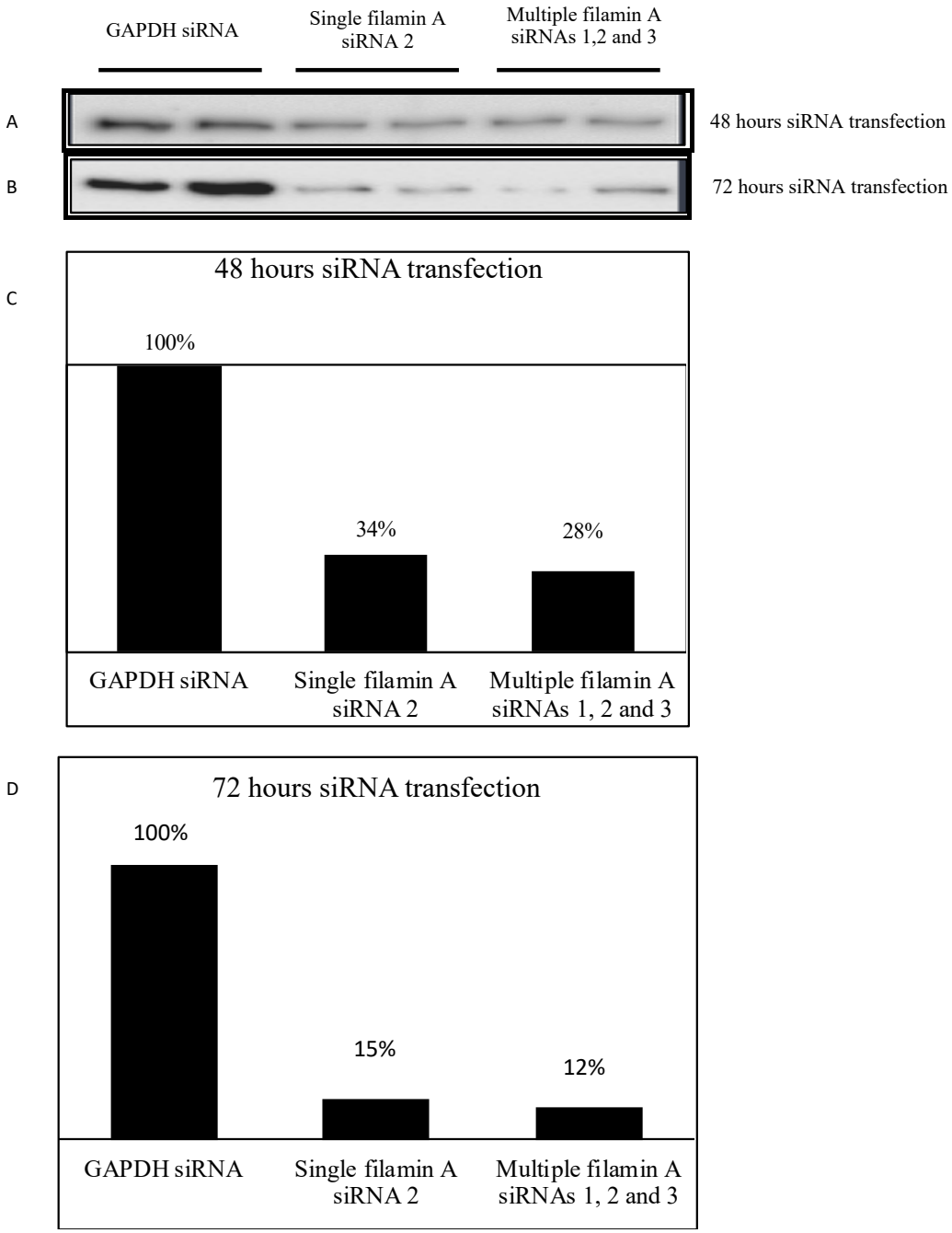


Figure 4: Filamin A knockdown in HEK 293 cells transfected with siRNA

HEK 293 cells were transfected with siRNA as indicated. Cells were lysed and used to prepare protein samples for immunoblotting 48 hours (A, C) or 72 hours (B, D) after the first siRNA transfection. Filamin A protein expression was determined by immunoblotting with anti-filamin A antibody. GAPDH density (mean value) normalised to 100%, filamin A siRNA transfected cells %= density

of filamin A siRNA transfected cells (mean value)/ GAPDH density (mean value). The results are a single independent experiment performed with duplicate samples.

3.1.2 GnRH receptor-mediated ERK phosphorylation in GnRH receptor-transfected HEK 293 cells

After successful knockdown of filamin A in HEK 293 cells, HEK 293 cells were transfected with the GnRH receptor plasmid. To examine the efficiency of the receptor transfection protocol and the functionality of the GnRH receptor ERK phosphorylation was determined by western blotting. GnRH receptor-transfected HEK 293 cell samples were processed for immunoblotting using the specific anti-phosphorylated ERK antibody. GnRH receptor-mediated ERK phosphorylation in GnRH receptor-transfected HEK 293 cells reached its maximum at 5 minutes and started to deactivate 15 minutes after stimulation (**Fig. 5**). The total ERK was relatively similar across all samples processed (**Fig. 5**). These results indicate that the transfection method works, and that the GnRH receptor is functional.

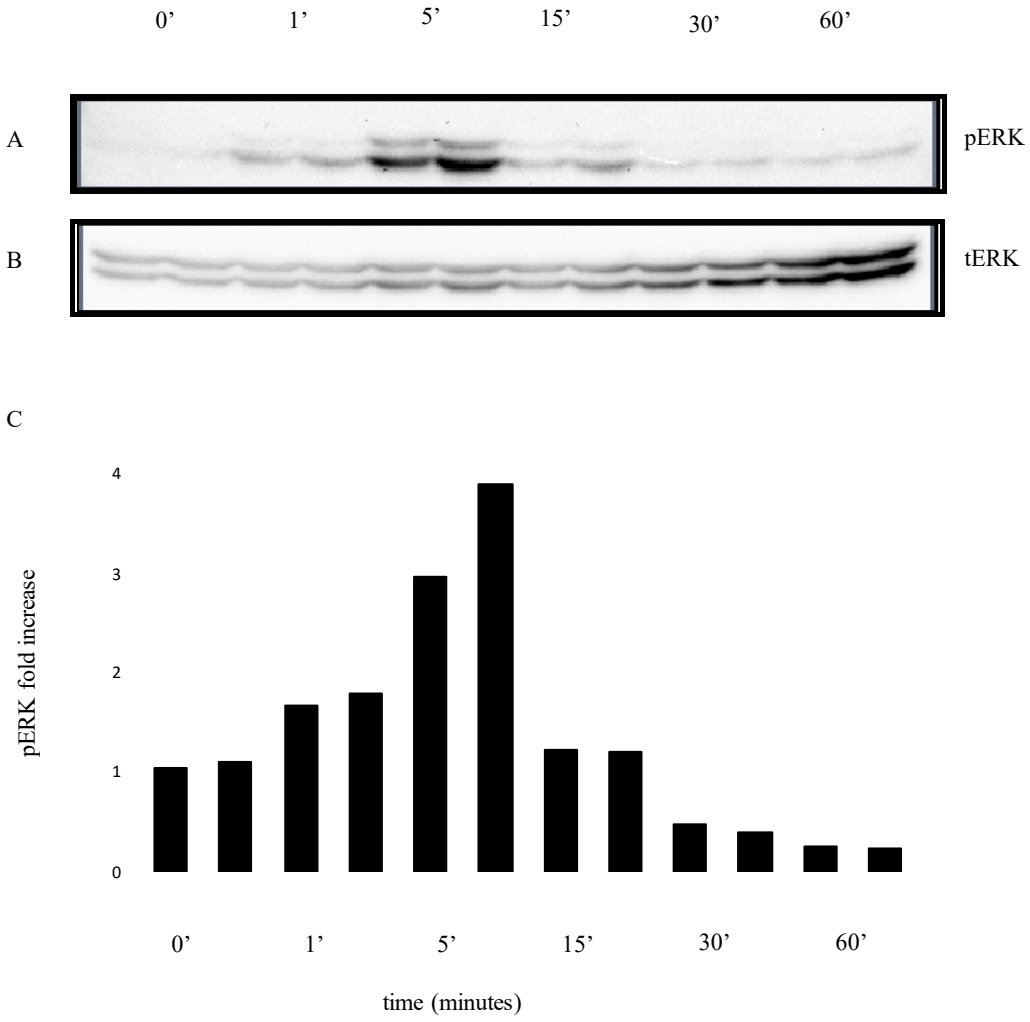


Figure 5: Time course of GnRH receptor-mediated ERK phosphorylation in GnRH receptor-transfected HEK 293 cells

GnRH-receptor transfected HEK 293 cells were stimulated with GnRH (1 µM) for the time periods indicated. Cells were lysed and used to prepare protein samples for immunoblotting. The activation of ERK and total ERK were determined by immunoblotting using anti-phospho-ERK and anti-MAPK 1/2 (total ERK) antibodies.. The results are from a single experiment. pERK fold increase=pERK/tERK.

3.1.3 GnRH receptor-mediated ERK phosphorylation in siRNA and GnRH receptor transfected HEK 293 cells

Following optimisation of the RNA interference method and success with measuring GnRH receptor-mediated ERK phosphorylation, the next step was to determine whether filamin A has a role in regulating GnRH receptor-mediated ERK phosphorylation, and therefore a role in GnRH receptor signalling. The effect of decreased filamin A expression on GnRH receptor-mediated ERK phosphorylation was determined. We assessed GnRH receptor-mediated ERK phosphorylation in HEK 293 cells transfected with GAPDH or filamin A siRNA and with the GnRH receptor.

Filamin A expression was reduced in HEK 293 cells transfected with filamin A siRNA compared to HEK 293 cells transfected with GAPDH siRNA(**Fig. 6**). GnRH receptor-mediated ERK phosphorylation was similar in cells transfected with GAPDH and filamin A siRNA (**Fig. 6**). These results indicate that filamin A may not play a role in regulating GnRH receptor distal signalling in HEK 293 cells. Since GnRH receptor-mediated ERK phosphorylation was similar in cells transfected with GAPDH and filamin A siRNA we did not assess GnRH receptor proximal signalling, ligand affinity and receptor expression in HEK 293 cells.

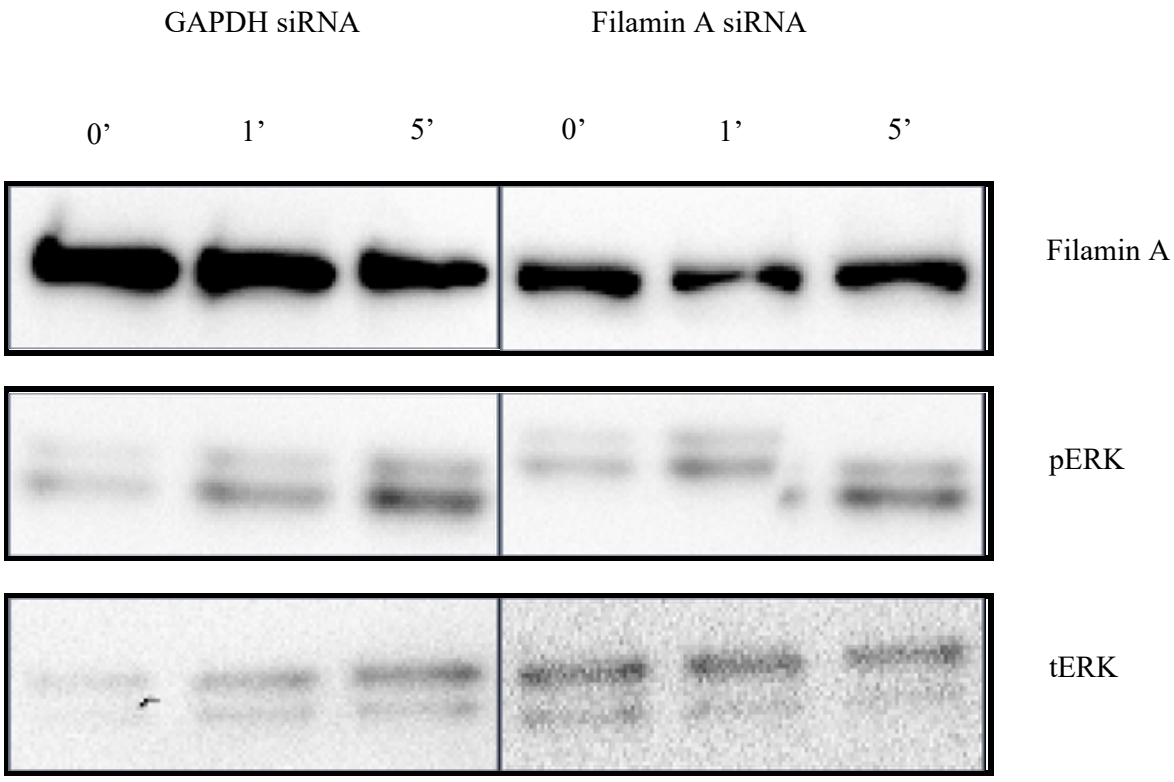


Figure 6: GnRH receptor-mediated ERK phosphorylation in siRNA and GnRH receptor-transfected HEK 293 cells

HEK 293 cells transfected with GAPDH or Filamin A siRNA and the GnRH receptor were stimulated with GnRH (1 μM) for the time periods indicated. Cells were lysed and used to prepare protein samples for immunoblotting. Filamin A protein expression, activation of ERK, and total ERK were determined by immunoblotting using anti-filamin A, anti-phospho-ERK, anti-MAPK 1/2 (total ERK) antibodies. The results are from a single independent experiment.

3.2 The role of filamin A in GnRH receptor signalling and expression in human melanoma cells

An alternative model to RNA-interference, the melanoma cellular pair of M2 cells (lacking filamin A) and M2A7 cells (expressing full-length filamin A) were used to investigate the role of filamin A in GnRH receptor signalling. Firstly, we had to verify that the M2 cells lack filamin A and M2A7 cells express filamin A. M2 and M2A7 cells were transfected with the GnRH receptor; GnRH receptor-mediated ERK phosphorylation was measured to determine if filamin A has a role in GnRH receptor distal signalling.

3.2.1 M2 cells lack filamin A and M2A7 cells express filamin A

To verify that M2 cells lack filamin A and M2A7 cells express filamin A; filamin A protein expression was determined by western blotting. Filamin A protein was not detected in M2 cell samples but was present in M2A7 cell samples (**Fig. 7**), whereas total ERK expression was similar in both cell lines (**Fig. 7**).

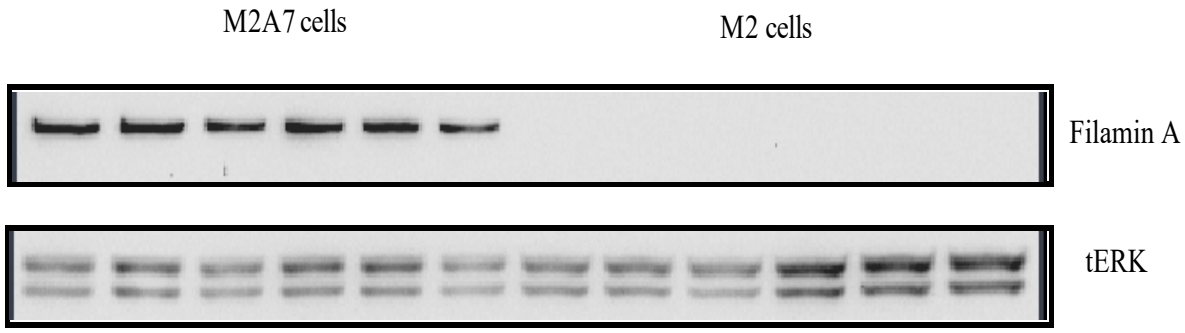
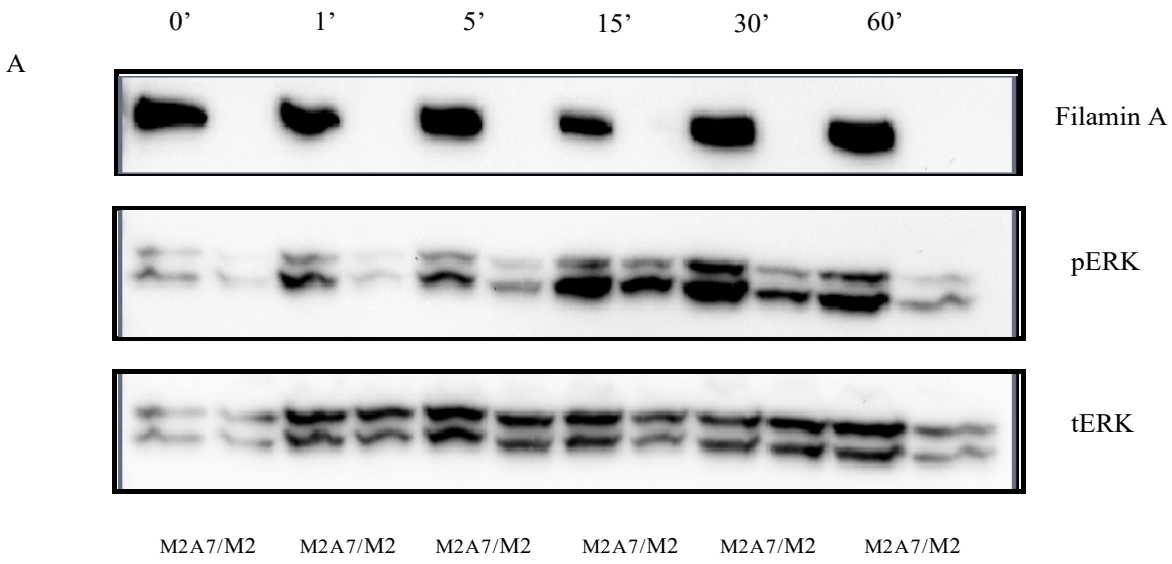


Figure 7: Filamin A protein expression in M2 and M2A7 cells

M2 and M2A7 cells were lysed and used to prepare protein samples for immunoblotting. Filamin A protein expression and total ERK were determined by immunoblotting using anti-filamin A and anti-MAPK 1/2 (total ERK) antibodies respectively. The results are a representative of a single independent experiment.

3.2.2 The role of filamin A in GnRH receptor-mediated ERK phosphorylation in GnRH receptor-transfected M2 and M2A7 melanoma cells

GnRH stimulation resulted in ERK phosphorylation in both the M2 and M2A7 cells transfected with the GnRH-receptor (**Fig. 8).** However, the kinetics of ERK phosphorylation over a 60 minutes period differed between the two cell lines (**Fig. 8)**. ERK phosphorylation in the filamin A-deficient M2 cells reached its maximum after 15 minutes and started to decrease after 30 minutes (**Fig.** 8). In contrast ERK phosphorylation in in the filamin A-replete M2A7 cells was elevated from 1 minute and sustained through to 60 minutes (**Fig. 8**). These results show a trend that indicates that filamin A is not required for GnRH receptor-mediated ERK phosphorylation but that filamin A modulates the kinetics of ERK phosphorylation in melanoma cells.



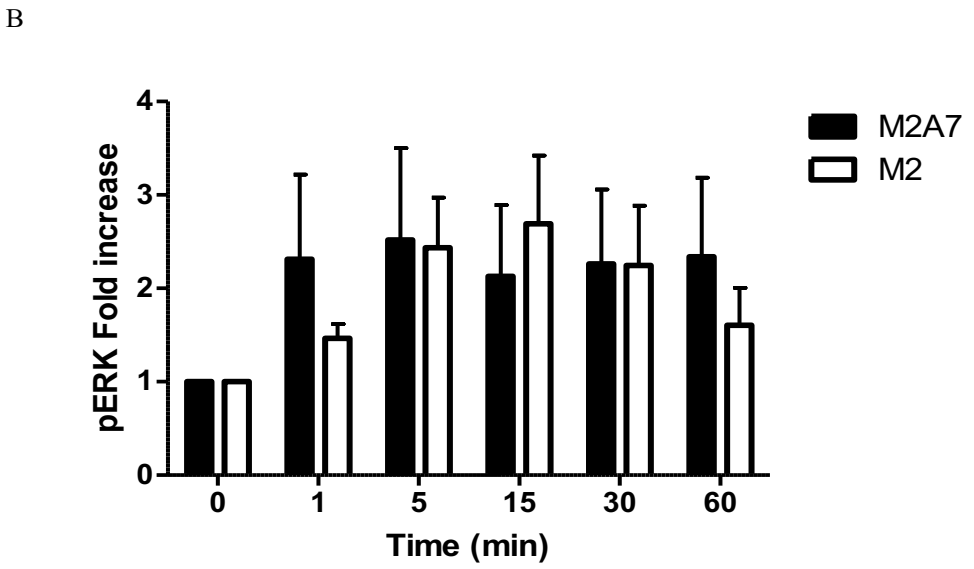


Figure 8: Time course of GnRH receptor-mediated ERK phosphorylation in GnRH receptor-transfected M2 and M2A7 melanoma cells

GnRH receptor-transfected M2A7 and M2 cells were stimulated with GnRH (1 µM) for the time periods indicated, the results are a representative of five independent experiments (A). Filamin A levels were determined by immunoblotting with anti-filamin A antibody, the activation of ERK was determined by immunoblotting with anti-phospho-ERK antibody, and total ERK with anti-ERK antibody. Two-way ANOVA was used to compare GnRH receptor-

mediated ERK phosphorylation in GnRH receptor transfected M2 and M2A7 cells. Data are fold increase means. pERK/tERK at 0 minutes was normalised as 1 per cell line and used as a reference point for subsequent data points. n=5 (B).

3.2.3 The role of filamin A in GnRH ligand binding and its effect on GnRH cell-surface receptor expression

Competition radioligand binding assays were used to determine GnRH receptor ligand binding affinity and cell-surface receptor expression in GnRH receptor-transfected M2 and M2A7 cells. The affinity of the GnRH receptor for GnRH was same in M2 and M2A7 cells. Log IC_{50} values of M2 and M2A7 cells were -8.16 ± 0.15 and -8.19 ± 0.14, respectively. Since binding affinity for the GnRH ligand was similar for GnRH receptor-transfected M2A7 and M2 cells, relative cell surface receptor expression levels could therefore be inferred from the B_0 values measured in the absence of unlabeled GnRH (**Fig. 9**). B_0 values for both M2 and M2A7 cells were similar in M2 and M2A7 cells (**Fig. 9**). This means GnRH receptor expression in M2 and M2A7 cells was similar. These results indicate that filamin A has no effect on cell surface expression of the GnRH receptor or GnRH binding affinity in GnRH receptor-transfected M2A7 and M2 cells.

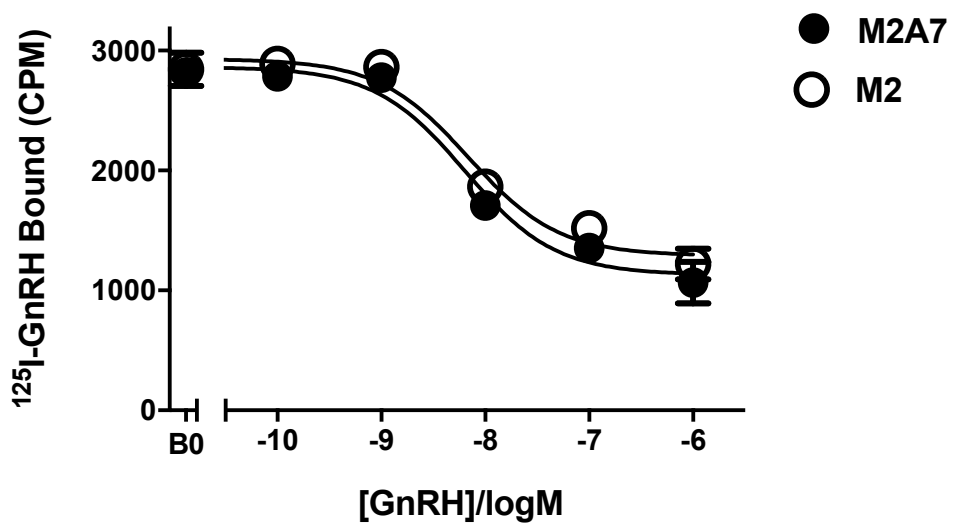


Figure 9: Competition radioligand binding assays in GnRH receptor transfected M2 and M2A7 melanoma cells

GnRH receptor transfected M2 and M2A7 cells seeded into 12 well plates were washed, incubated for 4 hours in medium containing 100 000 cpm of ^{125}I-[His^{5}-D-Tyr^{6}]-GnRH and varying concentrations of unlabeled GnRH peptide as indicated. The cells were then solubilised and radioactivity in the lysate was counted in a γ-scintillation counter. T-tests were used to compare B_0 and IC_{50} in GnRH receptor transfected M2 and M2A7 cells. The data are means and ranges from a single biological repeat performed in duplicate. p value= 0.1328.

3.2.4 GnRH receptor coupling to $G\alpha_{q/11}$ is filamin A independent

IP assays were performed to assess whether filamin A affects GnRH receptor coupling to the $G\alpha_{q/11}$ G protein. The IP responses elicited by GnRH in the M2 and M2A7 cells were similar. Maximal effect, E_{max} and the half-maximal response, EC_{50} values were not significantly different (**Fig. 10**). These results show that the GnRH receptor couples to $G\alpha_{q/11}$ in melanoma cells and that filamin A does not affect GnRH receptor proximal signalling in M2A7 and M2 melanoma cells.

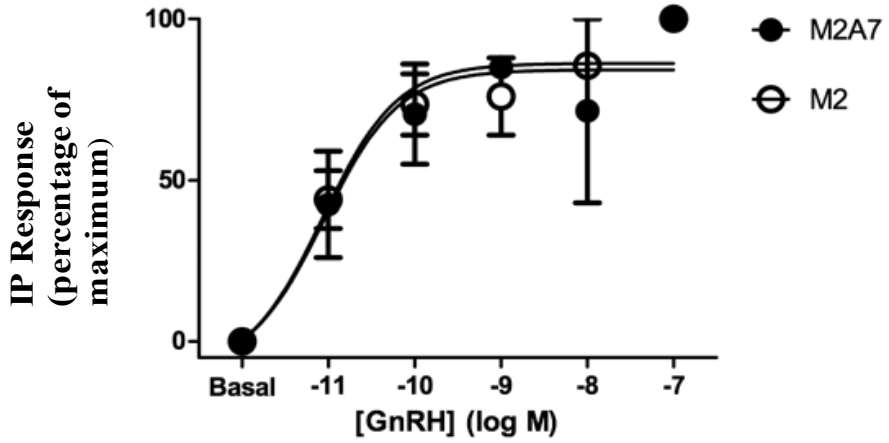


Figure 10: GnRH receptor-mediated IP production in GnRH-receptor transfected M2 and M2A7 melanoma cells

M2 and M2A7 cells transiently transfected with the GnRH receptor plasmid DNA, were radio-labelled with 3H-myo-inositol and stimulated with different doses of GnRH (10^{-11} M to 10^{-7} M) for 60 min. IP production was determined by counting radioactivity using a β-counter. Unpaired t-tests were used to compare, E_{max} and EC_{50} values. Nonlinear regression was used in GraphPad Prism to calculate E_{max} and EC_{50}. The data are means and ranges of three independent experiments performed in duplicate.

Chapter 4: Discussion

In the present study two methods were used to investigate the effect of decreased filamin A expression in GnRH receptor signalling. While the results were not statistically significant the trend suggests that filamin A may be a key signalling protein that links the GnRH receptor to ERK, and that this interaction results in rapid and sustained ERK phosphorylation in melanoma cells, but not in HEK 293 cells.

Through RNA interference I was able to vary filamin A protein expression in the experimental cell group (HEK 293 cells transfected with filamin A siRNA and GnRH receptor) relative to the control cell group (HEK 293 cells transfected with GAPDH siRNA and GnRH receptor) and measure GnRH receptor-mediated ERK phosphorylation in these cells. Observed GnRH receptor-mediated ERK phosphorylation kinetics and levels were similar between the control and experimental groups. These results suggest that filamin A may not have a role in GnRH receptor distal signalling.

Using the melanoma cell lines, the GnRH receptor protein was expressed in filamin A deficient M2 cells and filamin A replete M2A7 cells, then GnRH receptor-mediated ERK phosphorylation was measured. GnRH elicited ERK phosphorylation in both cell lines, however ERK phosphorylation quantified in these cells was suggestive of different kinetics. ERK phosphorylation in the filamin A-deficient M2 cells reached its maximum after 15 minutes and started to decrease after 30 minutes. In contrast ERK phosphorylation in the filamin A-replete M2A7 cells was elevated from 1 minute and sustained through to 60 minutes. These results suggest that filamin A is not required for GnRH receptor-mediated ERK phosphorylation, but its presence results in rapid and sustained ERK phosphorylation. The difference in GnRH receptor-mediated ERK phosphorylation in filamin A-deficient M2 cells and filamin A replete M2A7 cells suggested that filamin A might interact with the GnRH receptor which could affect GnRH ligand binding affinity, GnRH receptor expression and/or coupling to G proteins. Competition radioligand binding assays were used to determine GnRH receptor ligand binding affinity and cell-surface receptor expression in

GnRH receptor-transfected M2 and M2A7 cells. The binding affinity for the GnRH ligand and cell surface receptor expression level were similar for GnRH receptor-transfected M2A7 and M2 cells. These results indicate that filamin A does not have an effect on GnRH binding affinity or cell surface expression of the GnRH receptor in GnRH receptor-transfected M2A7 and M2 cells. IP assays were performed to assess whether filamin A affects GnRH receptor coupling to the $G\alpha_{q/11}$ G protein. The IP responses elicited by GnRH in the M2 and M2A7 cells were similar. These results indicate that the GnRH receptor couples to $G\alpha_{q/11}$ in melanoma cells and that filamin A has no effect on GnRH receptor G protein coupling.

In summary we have shown that filamin A is not required for GnRH receptor mediated ERK phosphorylation, however its presence may result in rapid and sustained ERK phosphorylation in melanoma cells. Furthermore, we have shown that the rapid and sustained ERK phosphorylation is not due to changes in GnRH ligand binding affinity, cell surface receptor expression or G protein coupling. These results suggest that filamin A may be a part of the GnRH receptor signalosome.

4.1 GnRH receptor-mediated ERK phosphorylation kinetics differ due to filamin A

The GnRH receptor has been shown to mediate ERK phosphorylation following receptor-ligand binding in several cell lines; CHO-K1, COS-7 ,αT3-1 and LβT2 cells, and rat pituitary gonadotropes (Roberson *et al.*, 1995; Harris *et al.*, 2003; Thompson and Kaiser, 2014; Mugami *et al.*, 2018). This is in line with our findings, GnRH stimulation resulted in ERK phosphorylation in GnRH receptor-transfected HEK 293 cells and in M2 and M2A7 melanoma cells transfected with the GnRH receptor.

I showed that filamin A multiple siRNAs work slightly better compared to single siRNA. However, the differences were small, 6% and 3% more knockdown for the 48 hours and 72 hours transfection protocols respectively (**Fig. 4**). The main factor for greater knockdown of filamin A was due to time, with longer time of active transfection, more filamin A was knocked down. There was better knockdown with the 72 hours post first siRNA transfection protocol relative to the 48 hours protocol (**Fig. 4**).

I was unable to replicate the knockdown when the HEK 293 cells were transfected with GnRH receptor plasmid to assess GnRH receptor-mediated ERK phosphorylation. GnRH receptor-mediated ERK phosphorylation was similar in HEK 293 cells with normal filamin A protein expression (control group) and in HEK 293 cells with less filamin A protein expression (experimental group). The GnRH stimulation time course to assess the effects of reduced filamin A on GnRH receptor-mediated ERK phosphorylation in HEK 293 cells was 0 to 5 minutes. We used the 0 to 5 minutes GnRH stimulation time course since ERK phosphorylation reaches its maximum at 5 minutes in HEK 293 cells (**Fig. 5**), thus we expected to notice a difference (if any) in ERK phosphorylation due to reduced filamin A within this timeframe. To our knowledge no study has assessed the effects of filamin A on GnRH receptor-mediated ERK phosphorylation. However, the role of filamin A in receptor-mediated ERK signalling has been studied for

several other receptors, including the GPCRs, calcium receptor (Awata *et al*., 2001; He *et al*., 2003; Zhang and Breitwieser, 2005; Huang *et al*., 2006) and the mu opioid receptor (Onoprishvili *et al.*, 2008; Simon and Onoprishvili., 2010). Only one study measured receptor-mediated ERK phosphorylation in HEK 293 cells (Huang *et al.*, 2006). In HEK 293 cells transfected with the calcium receptor and filamin A siRNA (Huang *et al.*, 2006), filamin A siRNA caused a 65% reduction in filamin A protein expression which resulted in a decrease in calcium receptor-mediated ERK phosphorylation (Huang *et al.*, 2006). Since our results for GnRH receptor-mediated ERK phosphorylation were similar in control and experimental HEK 293 cell groups, it is likely that the knockdown of filamin A was not sufficient to have functional consequences. This result is not conclusive, more biological repeats are required to affirm this assertion.

GnRH stimulation in GnRH receptor-transfected filamin A-deficient M2 cells and the filamin A-replete M2A7 cells led to ERK phosphorylation. However, the trend of GnRH receptor-mediated ERK phosphorylation suggests that the kinetics between the two cell lines were different. GnRH receptor-mediated ERK phosphorylation in the filamin A-deficient M2 cells reached its maximum after 5 minutes and started to decrease after 30 minutes. In contrast ERK phosphorylation in the filamin A-replete M2A7 cells was elevated from 1 minute and sustained through to 60 minutes. These results suggest that filamin A is not required for GnRH receptor-mediated ERK phosphorylation, but that the presence of filamin A causes different ERK phosphorylation kinetics. This result is relatable to the findings of Onoprishvil, where ERK phosphorylation is elicited in both M2A7 and M2 cells transfected with the mu opioid receptor, but the kinetics of ERK phosphorylation are different (Onoprishvili *et al.*, 2008). Mu opioid receptor-transfected filamin A replete M2A7 cells show rapid dephosphorylation of ERK, while filamin A deficient M2 cells show slower dephosphorylation of ERK after stimulation with [D-Ala2 ,N-Me-Phe4 ,Gly5 -ol]-enkephalin (DAMGO) (Onoprishvili *et al.*, 2008).

Mu opioid receptor stimulation by DAMGO utilises the β-arrestin pathway to induce ERK activation (Zheng, Loh and Law, 2010). β-arrestin is a binding partner of filamin A (Scott *et al.*, 2006), and their interaction is key for the rapid inactivation of mu opioid receptor-mediated ERK phosphorylation (Onoprishvili *et al.*, 2008). Due to the absence of a cytoplasmic carboxy-terminal tail the GnRH receptor does not signal via β-arrestin (Flanagan and Manilall, 2017), this could explain the sustained ERK phosphorylation observed in GnRH receptor-transfected filamin A replete M2A7 cells. This does not however fully account for the rapid ERK phosphorylation observed in filamin A replete M2A7 cells, a slower ERK phosphorylation in the filamin A-deficient M2 cells relative to M2A7 cells and deactivation of ERK after 30 minutes in M2 cells.

The pathway that connects GnRH to ERK follows sequential activation of $G\alpha_{q/11}$ followed by PLCβ, PKC, and MEK (Reiss *et al.*, 1997; Naor, Benard and Seger, 2000; Kraus *et al.*, 2001). Filamin A interacts with PKC (Kim and McCulloch, 2011) and MEK 1 (Scott *et al.*, 2006) which are upstream activators of ERK. The filamin A interaction with PKC has been shown to be required for optimal PKC-mediated cellular signalling and thus ERK phosphorylation (Kim and McCulloch, 2011). Furthermore, the interaction of filamin A with MEK 1 results in enhanced ERK phosphorylation in filamin A replete cells relative to filamin A deficient cells (Scott *et al.*, 2006). Since filamin A interacts with PKC and MEK1 and its interaction with these signalling proteins enhances signalling, rapid and sustained GnRH receptor-mediated ERK phosphorylation observed in GnRH receptor-transfected filamin A replete M2A7 cells could be due to the signalling complex or signalosome formed by the GnRH receptor, filamin A, PKC and MEK 1 linking to ERK. GnRH receptor-mediated ERK phosphorylation in GnRH receptor-transfected filamin A deficient M2 cells could be explained by other signalling pathways. The GnRH-PKC pathway is not the only pathway that lead to ERK phosphorylation, GnRH receptor-mediated ERK phosphorylation depending on cellular context may be via lipid rafts (Navratil *et al*., 2003), focal adhesion complexes (Maudsley *et al*., 2007), or transactivation of the epidermal growth factor receptor (EGFR) (Shah *et al*., 2003). Further investigations

measuring GnRH receptor-mediated ERK phosphorylation whilst varying the filamin A-PKC and filamin A-MEK1 complexes through silencing of PKC and MEK1 proteins as well as co-immunoprecipitation studies should be undertaken to elucidate how the GnRH-ERK pathway is associated with filamin A, and whether this includes engagement of actin.

These results indicate that although filamin A is not required for GnRH receptor-mediated ERK phosphorylation, its presence results in rapid and longer duration of ERK phosphorylation. This may be central to short- and/or long-term effects of GnRH, which include gonadotropin secretion, transcription factors activation and consequently the regulation of LH, FSH and the GnRH receptor gene expression.

The difference in GnRH receptor-mediated ERK phosphorylation M2A7 and M2 cells suggested that filamin A might interact with the GnRH receptor. The interaction of filamin A with the GnRH receptor could affect GnRH ligand binding affinity, GnRH receptor expression and/or coupling to G proteins. Competition radioligand binding assays were used to determine GnRH receptor ligand binding affinity and cell-surface receptor expression in GnRH receptor-transfected M2 and M2A7 cells. IP assays were performed to assess whether filamin A affects GnRH receptor coupling to the $G\alpha_{q/11}$ G protein.

4.2 Filamin A has no effect on ligand affinity and GnRH cell surface receptor expression

Functional GnRH receptor studies have generally focused on ligand-receptor interactions to elucidate receptor conformational changes. Filamin A is widely recognised as a scaffolding protein of note because of its capacity to interact with a wide variety of proteins, including receptors. Filamin A interactions with receptor and signalling molecules have been shown to have functional consequences. By employing radioligand binding assays Li showed that the affinity of different agonists and antagonists was the same for M2A7 and M2 cells

transfected with the dopamine D_2 receptor, meaning filamin A had no effect on ligand affinity (Li *et al.*, 2000), this was the same with the mu opioid receptor (Onoprishvili *et al.*, 2003) and CCR2B (Minsaas *et al.*, 2010). We show similar results for GnRH receptor-transfected melanoma cells (**Fig. 9**).

Our results indicate that filamin A has no effect on ligand-receptor interactions, the capacity of GnRH to bind to its receptor is not affected by filamin A. This seems to be a general trend as shown with other GPCRs; dopamine D_2 receptor, the mu opioid receptor and CCR2B. This result however does not prove or disapprove that filamin A may physically interact with the GnRH receptor, but rather that if there is an interaction, it has no effect on ligand-receptor binding. The latter is possible as shown with the dopamine D_2 and the mu opioid receptors, physical interaction of filamin A with these receptors had no influence on ligand affinity (Li *et al.*, 2000; Onoprishvili *et al.*, 2003; Minsaas *et al.*, 2010).

Since GnRH binding affinity was the same in M2A7 and M2 cells transfected with the GnRH receptor, relative cell surface receptor expression levels could therefore be inferred from the B_0 values measured in the absence of labelled GnRH. GnRH receptor expression was the same in M2 and M2A7 cells (**Fig.** 9). This finding is like what Onoprishvili and Minsaas found for the mu opioid receptor and CCR2B, filamin A had no effect on receptor membrane localisation and expression (Onoprishvili *et al.*, 2003; Minsaas *et al.*, 2010). However Li showed for another GPCR, the dopamine D_2 filamin A contributes to proper clustering and expression of this receptor on the cell surface (Li *et al.*, 2000), Lin and colleagues showed the same for the dopamine D_2 and D_3 receptors (Lin *et al.*, 2001). Thus, our results taken together with other existing literature suggest that filamin A influence on receptor binding affinity and cell surface expression is variable. In future studies, instead of making a conclusion regarding receptor cell surface expression on ligand binding assays other types of assay like confocal microscopy, enzyme-linked immunosorbent assay (ELISA) or immunoblotting can be utilised.

4.3 GnRH receptor coupling to $G\alpha_{q/11}$ in melanoma cells is filamin A independent

After showing that filamin A does not affect GnRH binding affinity and cell-surface receptor expression, we assessed whether filamin A may have a role in effector coupling. It is well established that the GnRH receptor predominantly couples with $G\alpha_{q/11}$, this has been shown in rat pituitary gonadotropes, CHO-K cells, COS-7 cells, and immortalised gonadotrope cell lines, αT3-1 and LβT2 cells (Naor, 2008; Naor and Huhtaniemi, 2013; Thompson and Kaiser, 2014). GnRH receptor-$G\alpha_{q/11}$ coupling leads to the activation of PLCβ which hydrolyses PIP_2 to generate the second messengers; IP_3 and diacylglycerol DAG (Kaiser, Conn and Chin, 1997). A measure of IP production therefore reflects PLCβ activity and in turn coupling of the receptor to $G\alpha_{q/11}$ and its activation.

We show that filamin A has no effect on GnRH receptor-mediated IP production in melanoma cells. Since GnRH receptor transfected M2A7 and M2 cells both produced IP when stimulated with GnRH, furthermore the maximal IP response and the EC_{50}, indicating the half maximal effective concentration were not significantly different between the two cell groups. These results indicate that the GnRH receptor in melanoma cells signals via $G\alpha_{q/11}$ and that filamin A has no effect on GnRH receptor coupling to $G\alpha_{q/11}$.To our knowledge, our study is the first to assess if filamin A has an impact on effector coupling.

Chapter 5: Conclusion

In conclusion, I optimised a protocol for silencing filamin A in HEK 293 cells using siRNAs and showed that lower filamin A protein expression in GnRH receptor-transfected HEK 293 cells does not affect GnRH receptor-mediated ERK phosphorylation. Furthermore, using filamin A replete M2A7 and filamin A deficient M2 melanoma cells I demonstrated that filamin A has an influence on GnRH receptor-mediated ERK phosphorylation kinetics. Furthermore, I showed that the difference in GnRH receptor-mediated ERK phosphorylation kinetics due to filamin A is independent of ligand binding affinity, cell surface receptor expression and $G\alpha_{q/11}$ coupling.

I propose that the observed ERK phosphorylation kinetics in the GnRH receptor transfected melanoma cells is due to the filamin A-PKC and filamin A-MEK1 complexes as shown in figure 10 below. Since filamin A is a binding partner of both PKC and MEK1 which are ERK upstream activators, a difference in filamin A expression could result in the observed differential ERK phosphorylation kinetics (Reiss *et al.*, 1997; Scott *et al.*, 2006; Kim and McCulloch, 2011). Further investigations measuring ERK activation by varying the filamin A-PKC and filamin A-MEK1 complexes through silencing of PKC and MEK1 proteins as well as co-immunoprecipitation studies should be undertaken to elucidate how the GnRH-ERK pathway is associated with filamin A, this would include assessing whether there is a direct interaction between the GnRH receptor and filamin A. In addition, to assess the physiological significance of the GnRH-ERK and filamin A signalosome studies measuring gonadotropin synthesis and secretion are required to further elucidate if this signalling complex regulates short term, long term or both effects of GnRH on gonadotropin function. A better understanding of the GnRH-ERK and filamin A pathway may be informative for developing pharmacological therapies to enhance or inhibit fertility.

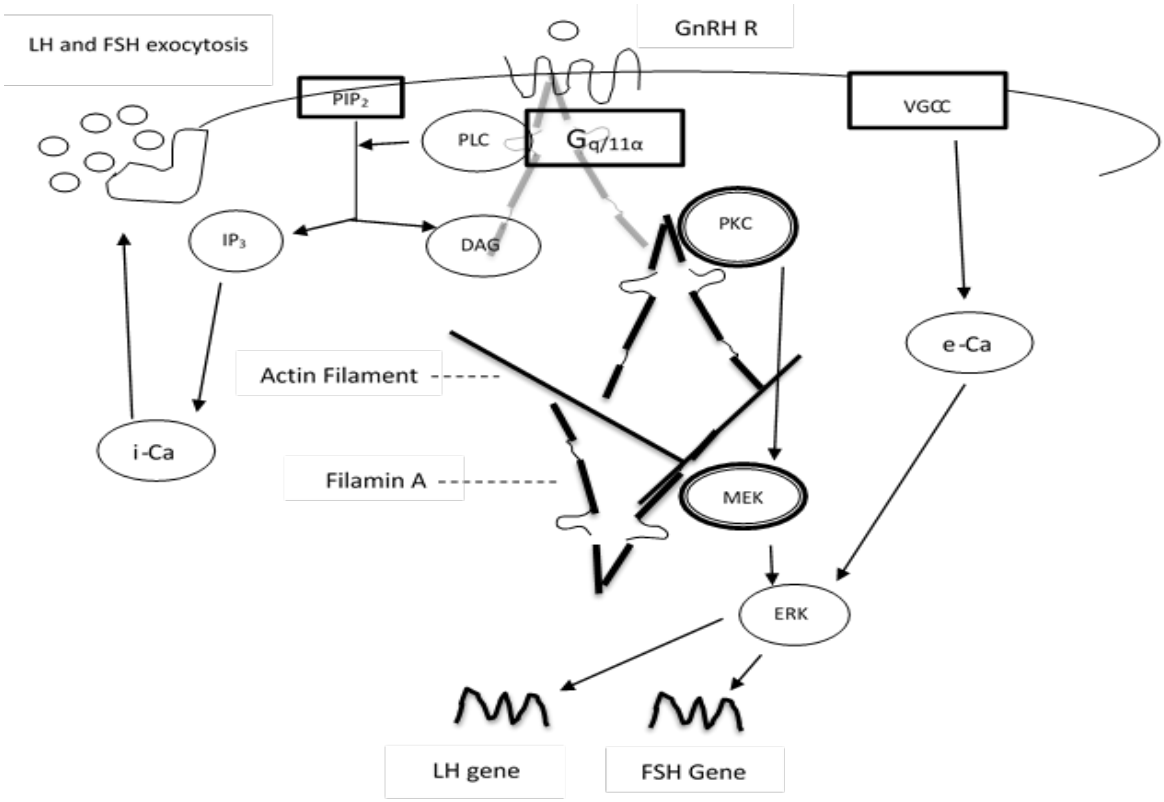


Figure 11: Schematic representation of the proposed filamin A interaction with the GnRH receptor signalling pathway

The model predicts that filamin A interacts with the GnRH receptor and stabilises the receptor in an active conformation linked with coupling to $G_{\alpha q/11}$. Furthermore, the model shows that filamin A interacts with protein kinase C (PKC) and mitogen-activated protein kinase kinase 1 (MEK1); and that the filamin A-PKC and filamin A-MEK complexes facilitate protein-protein interactions and therefore enhance GnRH receptor-mediated extracellular signal-regulated kinase (ERK) phosphorylation. This figure is adapted from Lin et al., 2001 and Coss 2018 figures. e-Ca=extracellular calcium; i-Ca=intracellular calcium. VGCC=voltage-gate calcium channels.

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Lawrence Katlego Motlogelwa

CLEARANCE CERTIFICATE	**M110566**
PROJECT	The Role of Filamin in GnRH Receptor Signalling and Localisation
INVESTIGATORS	Mr Lawrence Katlego Motlogelwa.
DEPARTMENT	School of Physiology
DATE CONSIDERED	06/05/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 06/05/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Michael T Madziva

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

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