Insulin production in the mouse uterus

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Declaration

I hereby declare that the work embodied in this thesis is the result of original research and that this thesis does not contain any material that has been submitted for any degree or diploma to any university or institution.
Also, the best of my knowledge, it does not contain any material published by anyone, except where due reference is made.

(Signed)……

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I would like to thank my colleagues and the staff of the Aitken Laboratory for their assistance during this period of time. Of particular note is Dr Ben Curry who taught me PCR. I am also grateful for the companionship of my fellow students, Aleona Swegen, Brendan Houston, Jacinta Martin, Sally Hall, Miguel Xavier, Bettina Mihalas and Nicole Camlin and post docs Mark Baker, Mariana Neves, Elizabeth Bromfield and Zamira Gibb. I wish them all the best for their futures. A nicer group of people I could not hope to meet.

I am grateful to my parents for indulging me while I pursued my dreams.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAD	Bcl-2-associated death promoter
BMDC	Bone marrow derived cells
BSA	Bovine serum albumin
BWW	Biggers-Whitten-Whittingham media
CASA	Computer assisted sperm assessment
CNS	Central Nervous System
CT	Cycle threshold
CXCL1	CXC-motif ligand 1
DAPI	4',6-diamidino-2-phenylindole
dNTP	Deoxynucleotide triphosphate
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FRET	Förster resonance energy transfer
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GnRH	Gonadotropin-releasing hormone
GRB2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
HBSS	Hanks balanced salt solution
HTRF	Homogeneous Time Resolved Fluorescence
ICC	Immunocytochemistry
IGF	Insulin-like growth factor
InsP3	Inositol trisphosphate
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
LH	Lutenising hormone
MAP	Mitogen-activated protein kinase
PBS	Phosphate buffered saline
PC 1/3	Proprotein convertase 1
PCR	Polymerase chain reaction

PDK1	Phosphoinositide dependent kinase-1
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PTB	Phosphotyrosine-binding domains
qPCR	Quantitative polymerase chain reaction.
RHEB	Ras Homolog Enriched In Brain
RT-PCR	Real time polymerase chain reaction
SH2	Src homology 2
SOS	Son of Sevenless
TSC2	Tuberous sclerosis complex 2

Chapter 1:

Review of extrapancreatic insulin production and insulin signaling

Abstract

Traditional paradigms hold that insulin production is restricted to the β cells of the pancreas. However, this long held view of insulin genesis has been challenged by recent studies showing that insulin can be produced by alternative, non-pancreatic cells. In this context, insulin has been reported to be produced in the brain, liver, bone marrow and in some reproductive organs. The functional consequences of extrapancreatic insulin production remain uncertain but could have a wide range of implications varying from diabetes to regulation of fertility. Insulin is most well-known for its role in controlling glucose homeostasis, which it does through activation of signaling cascades encompassing the phosphoinositide 3-kinase (PI3K) pathway. However, insulin is also a growth factor that is capable of encouraging cell proliferation through the extracellular signal-regulated kinase (ERK) - mitogen-activated protein kinases (MAPK) pathway. In this review we consider the growing body of literature supporting instances of extrapancreatic insulin production, and seek to establish whether such sources play a conserved or divergent role to that of the more widely studied insulin produced in the pancreas.

1. 1 Introduction

The molecular origins of insulin have been traced back as far as the simplest unicellular eukaryotes (Ferrannini et al 1999). Its discovery in 1921-22 by Frederick G. Banting and colleagues led to the treatment of diabetes, a condition that until then was fatal. Perhaps not surprisingly, insulin has since been studied primarily in relation to its role in regulation of glucose metabolism. Insulin production has traditionally been thought to be restricted to the β cells in the pancreas. However, the insulin receptor (IR) is widely expressed in a multitude of cell types and insulin is able to activate pathways separate to glucose regulation (Bevan 2001). It is in these roles that our knowledge of insulin is less developed. Even less studied is insulin that is produced from an extrapancreatic source, that is; insulin synthesized outside of the pancreas.

Our laboratory has generated preliminary data showing that the mouse uterus contains insulin mRNA. To provide context and highlight the possible implications of this discovery, this review will examine the currently published literature of extrapancreatic insulin and provide an overview of insulin synthesis, release and signaling. The

literature is broad, going back decades, with many different organs and cell types having been implicated, but generally there is little follow up research which has sought to examine the molecular basis of insulin function within these cells. In the first part of this review, focus is placed on the different organs that have been shown to produce insulin. An interesting observation of the available literature is that these organs can be separated into those that produce insulin when subjected to high concentrations of glucose and those whose insulin production is apparently unaffected by glucose. The latter part of the review then seeks to examine the pathways that may be activated in response to the extra-pancreatic production of insulin. In this context, it is known that insulin signaling is initiated upon binding to its receptor, the insulin receptor (IR) that forms a key part of the tyrosine kinase family and is active on both the plasma membrane as well as displaying the potential to be internalised into endosomes (Bevan 2001). After docking with IR, insulin is able to activate numerous different pathways but the two most common, and the focus of this review, are the cell metabolism regulating phosphoinositide 3-kinase (PI3K) pathway and the pro-mitogenic extracellular signal-regulated kinase (ERK) - mitogen-activated protein kinases (MAPK) pathway.

1.2 Insulin production

1.2. 1 Insulin synthesis

Insulin is an extremely well-studied polypeptide hormone produced predominantly by β cells of the pancreas. The most important biological function of insulin relates to its role in the regulation of fat and carbohydrate metabolism by promoting the uptake of glucose from the blood to skeletal muscles and other tissues. Insulin also has been implicated in the promotion of cell growth, differentiation and protein synthesis. The mature insulin polypeptide comprises 51 amino acids and has a molecular weight of 5.8 kDa. However, the *Ins* gene encodes a 110-amino acid precursor known as preproinsulin. Most animals have only a single copy of the insulin gene, but rodents have two non-allelic insulin genes (insulin I and II), which differ in their number of introns and chromosomal locations (Soares et al 1985). Insulin production is regulated

at the transcriptional level by a number of transcription factors and post-transcriptionally by mRNA stability and factors that influence rates of protein translation (Zhuo et al 2013).

During translation, ribosomes feed the newly created preproinsulin directly into the rough endoplasmic reticulum. Preproinsulin contains a hydrophobic N-terminal signal peptide which interacts with cytosolic ribonucleoprotein signal recognition particles which allows it to cross the membrane of the rough endoplasmic reticulum into the lumen (Egea et al 2005). Upon entering the lumen it is immediately cleaved by signal peptidases to become proinsulin (Patzelt et al 1978, Jansen et al 1995). Proinsulin is then folded into a three dimensional conformation stabilized by the formation of three disulfide bonds (Huang and Arvan 1995). Upon assuming its folded state, proinsulin is transported out of the endoplasmic reticulum and into the Golgi apparatus. There it enters immature secretory vesicles and is cleaved by endopeptidases proprotein convertase 1 and 2. The endopeptidases cleave at two positions, release C-peptide and leave two peptide chains linked by 2 disulfide bonds, to form the mature insulin hormone (Figure 1.2.1.1). Accordingly, insulin and C-peptide are produced simultaneously and in equal proportion. The mature insulin is packaged into mature vesicles waiting for the appropriate metabolic signal that will trigger the vesicles fusion with the membrane and insulin release. Such signals are predominated by glucose, an evolutionary consequence of the mammalian diet. Glucose sensing is, in turn, performed by pancreatic β cells that, upon detecting an increase in glucose concentration, respond in two phases. The first phase of release proceeds rapidly in direct response to increased blood glucose levels, while the second is characterized by a sustained, slow release of newly formed vesicles that can be triggered independently of the sugar (Zhuo et al 2013).

Interestingly, the release of insulin can also be modulated by estrogen, which has been shown to enhance insulin secretion. Accordingly, estrogen receptors have been localized to the β pancreatic cells, and β-estradiol exposure leads to elevated insulin secretion (Nadal 1998). It has also been demonstrated that estrogen can increase insulin secretion in postmenopausal women (Brussard et al 1997) and *in vitro* in human and mouse pancreatic cell lines (Al-Majed et al 2005). This insulinotropic effect is mediated by potentiating glucose-stimulated insulin secretion (Ropero et al 2002, Nadal 2009). Upon

binding to its receptor (ER-α) (Alonso-Magdalena et al 2008), estrogen can significantly decrease KATP channel activity in a reversible manner (Nadal 1998). This leads to membrane depolarization and stimulates the opening of voltage-gated Ca^{2+} channels, thereby potentiating glucose-induced intracellular [Ca^{2+}] oscillations.

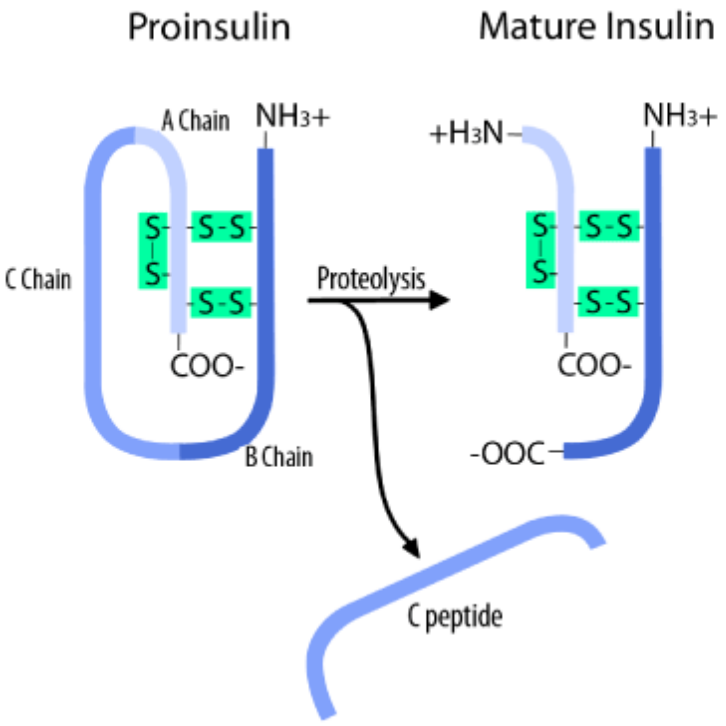


Figure 1.2.1.1 Arrangement of B-chain and A-chain before and after cleavage. Proinsulin is cleaved into insulin and C-peptide by cellular peptidases and the exoprotease carboxypeptidase E. The endopeptidases cleave at 2 positions, lysine-64 and arginine-65, and arginine-31 and -32 (Creighton 1993), free the central C-peptide region into circulation. The B- and A- chains remain linked by disulfide bonds at cysteine-7 and cysteine-7, and cysteine-20 and cysteine-19, and become insulin. Original figure (Thompson and Kanamarlapudi 2013)

1.2.2 Extrapancreatic Insulin Production

Having a crucial role in one of the most prevalent illnesses afflicting humanity, diabetes, insulin is among the most well studied molecules in science. It is perhaps surprising and somewhat controversial that organs other than the pancreas are capable of producing insulin. As mentioned previously, insulin production is traditionally thought to be limited to the β cells in the pancreas. However there is a small but growing body of literature reporting extrapancreatic production of insulin. There appear to be several characteristics in common among the cells that have been implicated in extrapancreatic insulin production; some organs produce insulin when exposed to high concentrations of glucose while other organs seems capable of producing insulin independent of glucose stimuli. The latter of these organs tend to comprise cells that are highly proliferative.

1.2.3 Glucose independent

1.2.3.1 Neuronal tissue

The brain and central nervous system (CNS) are not only an insulin target but also capable of synthesizing this hormone. Indeed, the *Ins* gene has been found to be expressed in several regions of the human brain (Mehran et al 2012). It has been known for decades that the rodent equivalent, *Ins2*, mRNA is also present in the brains of rats (Devaskar et al 1993) and choroid plexus (secreting epithelium) of mice (Lamotte et al 2004, Devaskar 1994). More recently, Molnár et al (2014) reported the detection insulin mRNA in neurogliaform cells in the rat cortex using single-cell quantitative RT-PCR. *In vitro* cell culture of neuronal cells has shown that mechanistically, there are several similarities between β pancreatic cells and neurons, particularly in relation to ATP-sensitive K^+ channel depolarization (Gerozissis 2003). In this context, both β cells and neurons are electrically excitable and respond to both hormonal stimuli and glucose challenge by depolarization and exocytosis. For instance, it has been demonstrated *in vitro* that primary cultures of neuronal cells are capable of impressive increases in insulin production, up to three fold, upon depolarization by potassium ions (Clarke et al 1986). Taken together, these studies provide evidence that mammalian neuronal cells are not just affected by insulin, they are also capable of insulin production and release. However, they are not without controversy given that other studies have failed to detect

any immunoreactive proinsulin or insulin positive cells in the brains of mice (Kojima et al 2004).

1.2.3.2 Reproductive tissue

Extending these observations, insulin synthesis has also been reported in reproductive cells and organs. Illustrative of this, both insulin transcripts and the mature protein have been detected in human spermatozoa (Aquila et al 2005) and testis (Aitken unpublished). In spermatozoa, insulin secretion was increased after the induction of capacitation, an important phase of functional maturation during which spermatozoa attain the ability to recognise an oocyte and engage in fertilization. Such observations raise the possibility that the hormone may play a previously unappreciated role in the autocrine regulation of sperm activation. In keeping with this notion, insulin secretion by spermatozoa was found to be influenced by exposure to exogenous glucose, possibly signifying that it forms part of the mechanism that modulates the cells energy requirements independent of systemic insulin (Aquila et al 2005). Furthermore, incubation of human spermatozoa with exogenous insulin has been shown to increase their motility, viability, production of nitric oxide (NO) and induce acrosomal exocytosis, all of which are prerequisites for successful fertilization (Lampiao & du Plessis 2008). In our own laboratory we have attained evidence that insulin transcripts, protein, the insulin receptor and C-peptide are all present in the spermatozoa of both humans and mice (Figure 1.2.3.2.1). Owing to the presence of both the C-peptide, that is normally cleaved from the maturing protein, and the encoding mRNA, we infer that spermatozoa are capable of producing their own insulin, presumably prior to their release from the germinal epithelium of the testes, rather than this hormone being delivered to the cell from systemic circulation. In terms of its functional significance, we have been able to demonstrate that co-incubation of human spermatozoa with insulin promotes a dose-dependent increase in their motility profile. This effect appears to be driven by interaction with the insulin receptor, which itself becomes phosphorylated after insulin exposure, and subsequently signals via an AKT/Bcl-2-associated death promoter (BAD) pathway. Owing to the anti-apoptotic action of the AKT/BAD pathway, it is suggested that insulin can act in an autocrine manner to prolong sperm longevity (Aitken, unpublished). A similar mechanism-of-action has been proposed for prolactin, which has been designated a pro-survival factor for human spermatozoa by virtue of its ability to stimulate the PI3K pathway and maintain the pro-apoptotic factor

BAD in a phosphorylated inactive state (Pujianto et al 2010; Koppers et al 2011) However, in contrast to the results reported by Aquila and colleagues (2005), our incubation of human spermatozoa with glucose failed to elicit any elevation in insulin secretion into the surrounding media. Insulin mRNA can also be detected in mouse uterus (Aitken, unpublished).

Various stem cells have been shown to be able to differentiate into insulin producing cells. Adult stem cells are rare, undifferentiated cells present in adult tissues and organs. These cells are extremely difficult to identify in tissue due to their rarity, lack of distinguishing morphological features, and few specific markers available for adult stem cells (Gargett et al 2009). They are instead defined by their functional characteristics, namely their substantial capacity for self-renewal, high proliferative potential and ability to differentiate into other cell types (Gargett et al 2009). The endometrium has been shown to contain endometrial stem-like cells and it is believed that they contribute to its highly regenerative nature (Chan et al 2004). In humans, epithelial and stromal adult cell stem cells are expected to be located in the basal layer of the endometrium due to regeneration starting from this layer after the top two-thirds are shed during menstruation (Gargett 2007, Padykula 1997). Stem cell-like cells have been isolated from menstrual blood, and these cells were found to be capable of differentiating into nine lineages including a pancreatic lineage that stained positive for insulin (Meng et al 2007). Studies have also shown that human endometrial mesenchymal stem-like, isolated from human endometrial tissue, can be differentiated into insulin producing cells *in vitro* (Sabramaria et al 2011, Li et al 2010). We have little understanding of the contribution of endometrial stem-like cells to the growth and proliferation of the endometrium during the menstrual cycle and into pregnancy. Their ability to differentiate into insulin producing cells, admittedly observed only under *in vitro* conditions, has the potential to contribute to the proliferative nature of the endometrium due to insulin's pro-mitogenic properties.

1.2.4 Glucose dependent

1.2.4.1 Hepatic tissue

Extrapancreatic insulin has been reported in the liver of mice, rats and humans (Chen et al 2010, Rosenzweig et al 1980). Such reports are consistent with demonstrations that

hepatic oval cells, the liver progenitor cells, can be activated and differentiated toward a pancreatic β cell phenotype when exposed to high glucose or liver injury (Chen et al 2010, Kim et al 2007, Vorobeychik et al 2008). Chen et al (2010) found insulin gene expression increases in liver tissue exposed to hyperglycaemia. In this study, diabetes was induced in mice using a common strategy of a single injection of the pancreatic β cell toxin streptozotocin. Subsequent assessment of *in vivo* insulin production was performed using a bioluminescence-imaging assay of firefly luciferase under a rat-insulin gene promoter prior to confirmation with PCR. This combined analysis revealed an approximate six fold increase in insulin production within the liver of mice eight days after the induction of hyperglycaemia. Similarly, other studies have also shown that liver cells can be induced to express insulin following viral-vector mediated ectopic expression of pancreatic transcription and differentiation factors (Ferber et al 2000, Tang et al 2006). Importantly, the level of insulin produced using these experimental paradigms has been sufficient to cure the diabetic symptoms of the mice.

Surprisingly, both insulin mRNA and protein expression have been found in multiple diabetic mouse and rat models (Kojima et al 2004). For instance, in a study by Chentoufi and Polychronakos, insulin mRNA was consistently detected in the thymus of diabetic mice (Chentoufi and Polychronakos 2002). This study also reported that bone marrow-derived cells (BMDC) were a major source of proinsulin producing cells in both bone marrow and in the liver. While, the mechanism as to how hyperglycaemia can lead to extrapancreatic insulin production is unclear, Kojima et al (2004) suggested that low level expression could be helpful in preventing the development of autoimmunity to insulin.

An alternative possibility is that insulin production could be an unintended consequence of hyperglycaemia. A mechanism to explain this is that hyperglycaemia changes the epigenetic environment which leads to the differentiation of stem cells into insulin producing cells. Normally insulin production is tightly regulated to produce only the exact amount necessary to meet bodily requirements. However, in a disease like type 1 diabetes where the consequences of prolonged elevated blood glucose levels are fatal it is not inconceivable that the signal to produce insulin would be deliberately increased in an attempt to avoid death. This signal could weaken the normally tight regulation of insulin production and stimulate non-pancreatic cells to produce insulin (Kojima et al.

2004). Organs in which insulin production has been reported such as the liver, bone marrow and uterus are all highly regenerative and also known to contain stem cell populations. Extending these findings, bone marrow and endometrium stem cells have both been successfully differentiated into insulin producing cells *in vitro*. To our knowledge there have been no *in vivo* studies that have investigated the effect of hyperglycaemia on stem cells in these organs.

There is a growing body of literature documenting epigenetic changes in diabetics. Epigenetic mechanisms such as DNA methylation, histone modifications and regulation of ATP-dependent remodelling of chromatin structure have been shown to affect the differentiation of stem cells (Zhou et al 2011). However more research regarding the consequence of diabetes induced epigenetic changes on stem cell differentiation is necessary before any real conclusions can be drawn. Nevertheless, the widespread occurrence of extrapancreatic insulin in diabetic and non-diabetic animals suggests that we should revise our thinking on the regulation of insulin gene expression.

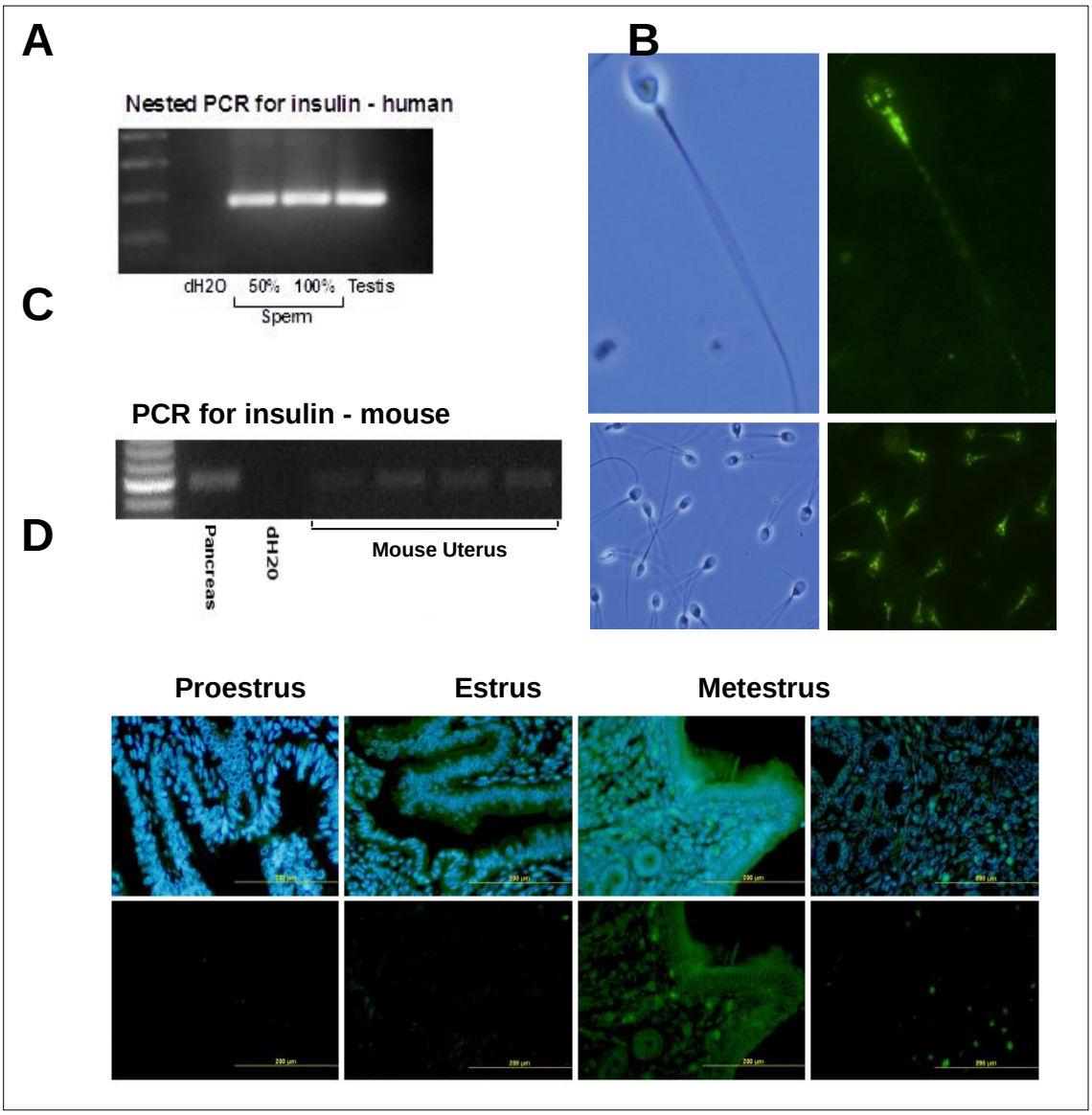


Figure 1.2.3.2.1: Insulin in human spermatozoa and mouse endometrium. (A) Human *Ins* mRNA was detected in human spermatozoa and testis using nested PCR, bands were sequenced and confirmed to be insulin. Immunocytochemistry for the insulin antibody in human spermatozoa found that it was predominantly localized to the midpiece (B). Mouse *Ins* mRNA was detected with RT-PCR in the mouse uterus (C) and confirmed by sequencing. (D) Immunocytochemistry for insulin showed that insulin protein expression (green) changed during the estrous cycle and was localized within the epithelial and stromal cells of the endometrium. Blue = DAPI nuclear counterstain.

1.3 Insulin signal transduction

Part of the purpose of this review is to challenge the long held belief that insulin production is limited to the pancreas and that insulin signaling is predominantly a feature of glucose metabolism. However, it is a possibility that extrapancreatic insulin is synthesised for a different purpose. Below I have set out some of the most common pathways

1.3.1 Insulin receptor

Insulin signaling is initiated at the plasma membrane via binding to the insulin receptor (IR), a receptor/tyrosine kinase tetramer that is composed of two α and two β subunits linked by disulfide bonds (Lee and Pilch, 1995). The α subunit is comprised of 723 amino acids and contains the sites for ligand binding. The α subunit also comprises a cysteine-rich domain that displays a high level of conservation with the insulin-like growth factor 1 (IGF-1) receptor as well as members of the epidermal growth factor (EGF) receptor family (Schlessing and Ullrich 1992). Using a systems biology approach some models for IR-ligand binding have been developed (Mkern et al 2006, Kiselyov et al 2008, Ward and Lawrence 2009*)*. In these models, the IR alpha subunit has two monomers each of which accommodate two insulin binding sites, thus enabling the simultaneous binding of four insulin molecules. The β subunit contains 620 amino acids and contains tyrosine residues that undergo autophosphorylation upon ligand binding to the IR (Lee and Pilch 1995). The binding of ligand to the α chains of the IR ectodomain induces structural changes within the receptor leading to autophosphorylation of various tyrosine residues within the β-chain. These changes promote the recruitment of specific adapter proteins such as the insulin receptor substrate proteins (IRS) (figure 1.3.1.1).

The IR is encoded by the *Insr* gene, from which two isoforms emerge due to alternative splicing during transcription. The two isoforms differ by the presence (IR-B) or absence (IR-A) of 12 amino acids at the carboxyl terminus of the α-subunit. Insulin binds with similar affinity to both isoforms, but IGFs have greater affinity for IR-A than IR-B (Siddle 2011).

The insulin receptor is predominantly active when located on the plasma membrane, bound and internalized receptors are thought to be inactivated by phosphotyrosine-specific phosphatases which are localized to the cytosolic face of the endoplasmic reticulum (Dube & Tremblay 2005). However, this model has attracted some controversy in view of evidence that internalized receptors still possess the ability to signal, particularly through the ERK-MAPK pathways (Jensen & De Meyts 2009). The activated ligand-receptor complex, initially at the cell surface, can be internalized into endosomes (Backer et al 1991, Goh & Sorkin 2013). Endocytosis of activated receptors is thought to have the dual effect of concentrating receptors within endosomes and allowing the IR tyrosine kinase to phosphorylate substrates that are different from those accessible at the plasma membrane (Bevan et al 2000). Some studies have also shown that IR signaling complexes are recruited to specific insulin-inducible gene loci (Nelson et al. 2011).

In addition to its receptor, insulin is able to bind to the IGF receptor and IGF-IR hybrid with a low affinity (De Meyts et al 1994, Soos et al 1993). However the affinity of interaction has been shown to change depending on the IR isoform involved (Pandini et al 2002). The physiological role for this form of binding is not currently known but it could ameliorate IGF signaling.

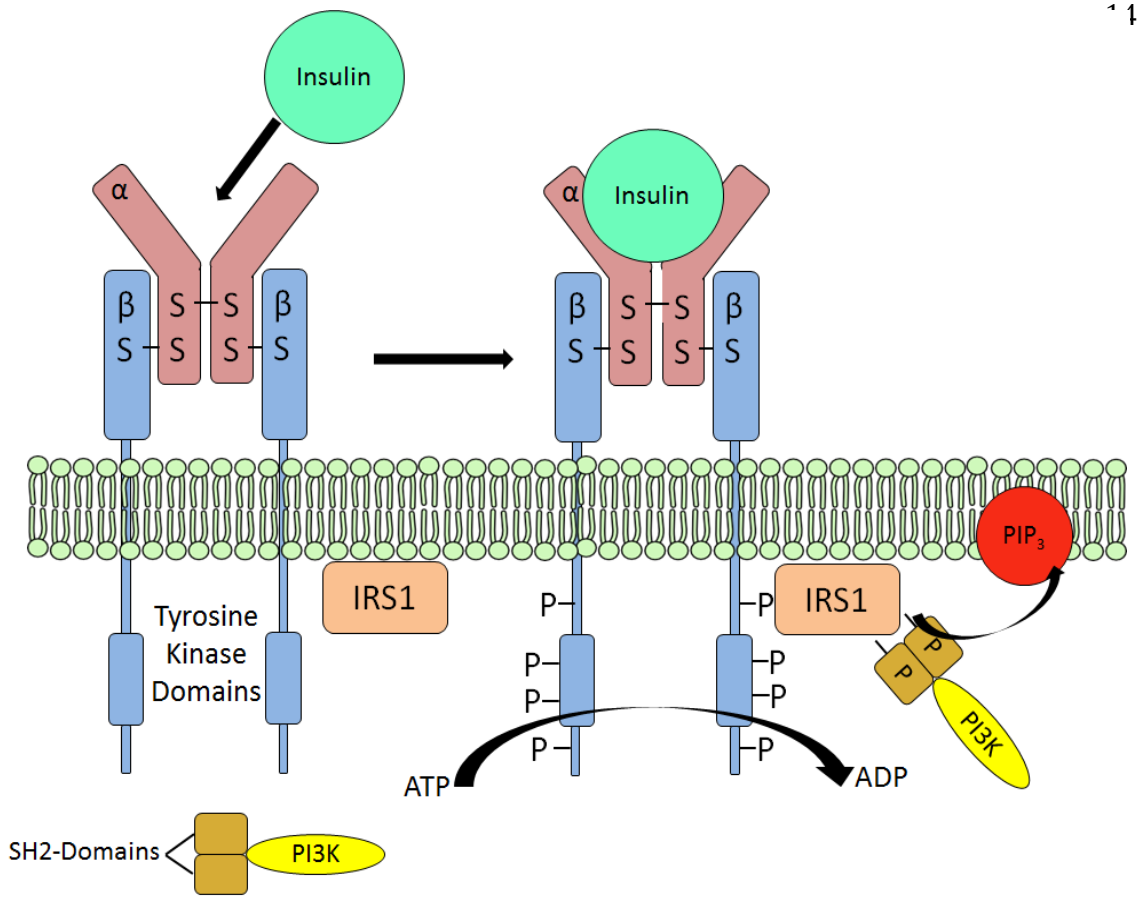


Figure 1.3.1.1 : Insulin activation of its receptor. Insulin binding to the α chain of the IR activates the external alpha units of the IR's tyrosine kinase which induce a conformational change and activates the kinase domain of the β intracellular subunits. The IR then phosphorylates the IRS-1 which in turn phosphorylates the SH2 domains on PI3K. PI3K catalyzes the addition of a phosphate group to the membrane lipid PIP2, converting in to PIP3. PIP3 is then able to bind to further downstream proteins such as AKT.

1.3.2 Insulin Signaling

Insulin has an incredibly broad range of functions, ranging from profound physiological influences on regulating blood glucose levels, through to promoting cell proliferation, protein synthesis and inhibition of apoptosis. The major signaling pathways for insulin are the phosphatidylinositol 3-kinase (PI3K) pathway and the extracellular signal-regulated kinase (ERK)- mitogen activated protein kinase (MAPK) pathway. Both play a central role in driving many of the phenotypic changes induced by growth factors. The pathways become interconnected downstream of activation, in certain situations having an inhibitory effect on each other (Figure 1.3.2.1). This cross talk could be necessary to maintain a balance between the output of each pathway, preventing hyperactivation of a single pathway and uncontrolled cell proliferation (Worster et al 2012). Since insulin shares these signaling pathways with IGFs and it can activate IGF receptors it is not always apparent which growth factor is used. Rather signal transduction varies between cell types and is determined partly by the expression of the receptors and downstream targets and partly by a sufficient quantity of ligand binding (Dumont et al 2002).

1.3.3 PI3K Pathway

Insulin signaling begins with a series of tyrosine phosphorylation steps. The binding of insulin to the IR α subunit leads to autophosphorylation of tyrosine residues on the β subunit. These phosphorylated residues are detected by phosphotyrosine-binding (PTB) domains of the insulin receptor substrate (IRS). Serine/threonine phosphorylation of IRS leads to inhibition while phosphorylation of certain tyrosine residues can potentiate the IRS (Siddle, 2011).

Phosphorylated tyrosines on the IRS are detected by the Src homology 2 (SH2) domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). The catalytic subunit of PI3K, p110, then phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) leading to the formation of phosphatidylinositol 3,4,5 triphosphate (PIP3) (Mendoza et al 2011). PIP3 can, in turn, initiate activation of protein serine kinase cascades by co-recruitment to the plasma membrane of phosphoinositide dependent kinase-1 (PDK1) and AKT.

Once active, AKT can lead to the most well know of insulin functions, regulation of glycogen synthesis and glucose uptake. Phosphorylated AKT leads to the phosphorylation of glycogen synthase kinase 3 (GSK3). A major substrate of GSK3 is glycogen synthase, an enzyme responsible for catalyzing the final step in glycogen synthesis (Wada 2009). Phosphorylation of glycogen synthase by GSK3 will lead to the inhibition of glycogen synthesis; therefore the inactivation of GSK3 by AKT promotes glucose storage as glycogen. Activated AKT also mediates insulin-stimulated translocation of GLUT4 glucose transporters in muscle and adipose tissue (Whiteman et al. 2002). GLUT4 is responsible for promoting the release, movement and docking and fusion of insulin vesicles with the plasma membrane (Larance et al. 2008).

PI3K activation influences cell division through the rapamycin (mTOR) pathway (Wang et al 2006, Cai et 2015). AKT phosphorylation of the tuberous sclerosis complex 2 (TSC2) releases TSC inhibition of the GTPase RAS homolog enriched in brain (RHEB). RHEB-GTP directly activates mTOR. The mTOR protein phosphorylates further downstream substrates that promote ribosome biogenesis and translation of cell growth and division proteins (Mendoza et al 2011). In the uterine epithelial cells the situation appears more complex. Estrogen is synthesized during the proliferative phase of the menstrual cycle in humans or the proestrus phase in mice while progesterone is synthesized in the secretory phase in humans and post-mating in mice. As in other cells growth factor signaling to mTOR is activated by AKT however, in the uterine epithelium AKT is stimulated by estrogen while being inhibited by progesterone (Chen et al 2005). This suggests that the PI3K activated AKT mechanism is not adequate in itself to promote insulin-dependent protein synthesis in the uterine epithelial cells.

Lastly, AKT activity can inhibit apoptosis through the inactivation of pro-apoptotic proteins. This pathway leads to the promotion of cell survival through the phosphorylation and inactivation of proapoptotic proteins such as BAD in spermatozoa and other cell types (Sen et al., 2003; Koppers et al 2011). A decrease in PI3K activity leads to the dephosphorylation of AKT1 which in turn leads to the dephosphorylation of BAD and the activation of apoptosis (Koppers et al. 2011).

1.3.4 ERK-MAPK Pathway

The other major insulin signaling pathway is that involving the ERK-MAPK cascade. The ERK-MAPK pathway is able to elicit prosurvival and mitogenic cellular responses, such as cell division and migration, by activating the RAS oncoprotein. It is through this pathway that insulin can act as a weak, but important, growth factor. As part of its mitogenic action, insulin is able to modulate the effectiveness of other growth factors.

As in the PI3K pathway, ERK-MAPK signaling is initiated upon insulin binding to its receptor and induction of IRS phosphorylation. However, in this instance, such events lead to the recruitment of growth factor receptor-bound protein 2 (GRB2), an adaptor protein that contains SH3 domains, which in turn associates with the guanine nucleotide exchange factor son-of sevenless (SOS) (Ogawa et al 1998). SOS is responsible for activating RAS, however for this to occur RAS must first be anchored to the plasma membrane. The process of RAS translocation to the plasma membrane involves the attachment of a farnesyl moiety, in a process known as farnesylation that is catalysed by the enzyme farnesyltransferase (Zhang and Casey 1996). When anchored, RAS is activated by SOS and then recruits and activates RAF at the plasma membrane. RAF activates MEK and MEK activates ERK.

The critical role insulin has in the ERK-MAPK pathway that other growth factors do not is in the farnesylation of RAS (Draznin 2010). Unlike other growth factors, insulin also activates farnesyltransferase by phosphorylating its alpha subunit. Phosphorylation and activation of farnesyltransferase leads to a larger concentration of membrane-bound, farnesylated RAS available for activation by other growth factors. The activation of farnesyltransferase by insulin also requires an intact insulin receptor, but not an IGF-1 receptor (Goalstone et al 1998). This suggests that insulin is important not just for activating the ERK-MAPK pathway by itself but for enhancing the mitogenic potency of other growth factors.

Due to its mitogenic properties ERK-MAPK pathway is frequently activated in cancers including various types of uterine cancer (Kourea et al 2015). In the context of this review the obvious question is can insulin cause cancer? In a healthy person the answer must be no. The question becomes more complicated in the case of hyperinsulinaemia,

such as that seen in type 2 diabetes. Several studies have shown an increased risk of developing cancer in patients treated with insulin or insulin secretagogues as compared with metformin (Gupta et al 2002, Bowker et al 2006). The increased insulin concentration in circulation in combination with insulin resistance appears to lead to an increase in cell proliferation and therefore an increased risk of cancer.

Owing to the anti-apoptotic ability of estrogen to promote insulin release and previous findings of insulin mRNA in the endometrium, the activation of the ERK-MAPK pathway is active in the uterus. A rise in ERK has been linked to a rise in estrogen during the proliferative phase of the menstrual cycle, with ERK expression found in both epithelial and stromal cells (Mohamed et al 2014). The ERK-MAPK pathway is also active during the process of stromal cell decidualisation. Early molecular interactions between the embryo and mother depend on chemoattractants, particularly chemokine CXC-motif ligand 1 (CXCL1), for a successful pregnancy (Hess et al 2007). Inhibition of the ERK-MAPK pathway led to a statistically significant decrease of CXCL1 protein secretion, with the ERK-MAPK pathway being necessary for the activation of CXCL1s' transcription factor NFκB p65 (Baston- Büst et al 2013). Compared to other cancer types there is less information available about the etiology of uterine cancers. However extensive studies of breast cancer, another hormonally regulated cancer, have shown molecular interactions of estrogen/ER with ERK-MAPK signaling (Lanzino et al 2009, Shupnik et al 2004).

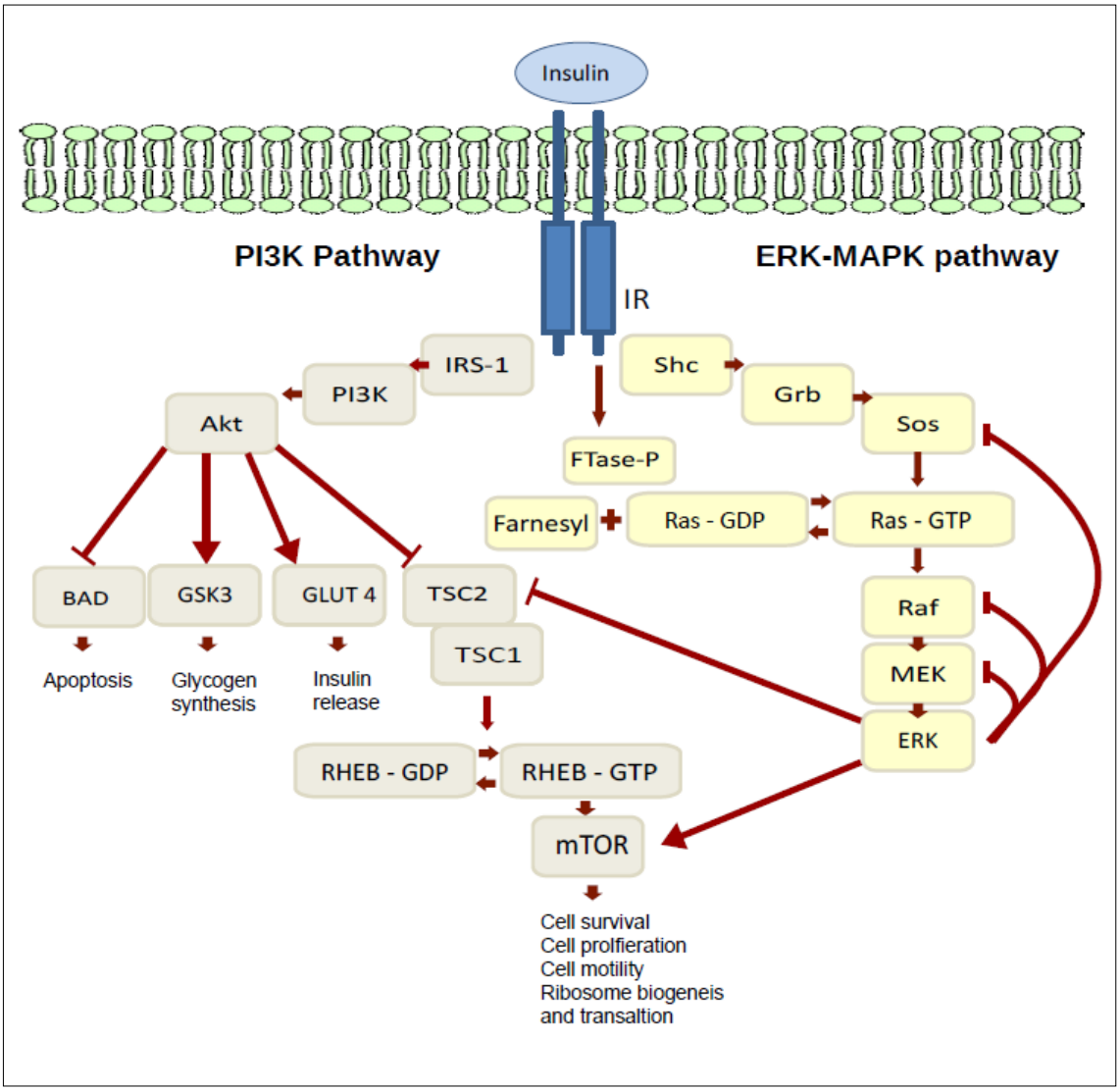


Figure 1.3.2.1: Insulin signaling can be broadly divided into two pathways. The PI3K pathway involves PI3K –mediated translocation and subsequent phosphorylation of AKT which regulates glucose metabolism by activation of GSK3 and GLUT 4. AKT can also inhibit apoptosis by inactivating BAD. The ERK-MAPK is involved in the regulation of cell proliferation. Grb associates and activates SOS. SOS promotes exchange of GTP for GDP but requires RAS to be bound to the plasma membrane. The first step of RAS translocation to the plasma membrane requires RAS to undergo farnesylation (attachment of a farnesyl moiety to cysteine residue of RAS) under the influence of farnesyltransferase (Ftase). Insulin binding to it's receptor phosphorylates Ftase thereby increasing the amount of farnesylated RAS. Anchored RAS is activated by SOS and then recruits and activates RAF at the plasma membrane. RAF activates Mitogen-activated protein kinase (MEK) and MEK activates ERK. Downstream there is some cross talk between the pathways as both AKT and ERK phosphorylation of the

TSC2 releases TSC inhibition of the GTPase Ras Homolog Enriched In Brain (RHEB). RHEB-GTP directly activates mTOR. The mTOR protein phosphorylates further downstream proteins that promote ribosome biogenesis and translation of cell growth and division proteins.

1.4 Conclusion

Extrapancreatic insulin producing cells can be broadly categorized into two groups. Those that can produce insulin independent of glucose concentration and those that produce insulin due to hyperglycaemia. Among the first group, insulin has been reported to be produced in the cells of the central nervous system, endometrium, testes and spermatozoa. Insulin in these cells is likely to be produced for a specific, but as yet unknown biological role. Insulin produced in the uterus seems to be linked to the estrous cycle with insulin mRNA increasing in parallel with estrogen during the follicular phase. Coinciding with such changes, the expression of the insulin receptors changes from being predominantly confined to the epithelial cells during the follicular phase to more expansive expression in the stromal cells during the luteal phase. Given the capacity of insulin to promote cellular proliferation through the combined PI3K and ERK-MAPK pathways it is tempting to speculate that it plays a role in the rapid endometrial cell growth that characterizes the menstrual cycle and may therefore be important in diseases featuring excessive cell growth such as uterine cancer and endometriosis.

There is considerable effort being expended in the pursuit of curing type 1 diabetes by transplanting new insulin producing cells derived from *in vitro* differentiated stem cells to compensate for the destroyed β cells (Bruin et al 2015). Considering the technical, and in some cases ethical, difficulty in acquiring a source of stem cells it would be beneficial to isolate naturally producing insulin cells. Our research suggests that such cells may exist in the endometrium and testes of rodents and they have potential to be used in place of stem cells.

In conclusion insulin is a protein most known for its role in glucose metabolism however it is also a growth factor and promotes cell proliferation by itself and/or in

tandem with other growth factors. The main location of insulin production is the β cells in the pancreas. Other organs such as the brain, testis, bone, thymus and liver have been reported to produce insulin under certain conditions. These observations have implications for regulation of insulin gene expression and could potentially offer a new approach for the generation of insulin-producing cells for diabetic treatment. In the future our focus will be on investigating insulin production in the mouse uterus, an organ that is vital for reproduction. The highly proliferative nature of the endometrial cells raises the possibility that uterine insulin acts to promote cell survival and mitogenesis as well as glucose regulation.

Chapter 2:

Localization of insulin in the mouse uterus

Chapter 2: introduction

2.1 Introduction

The aim of the following chapter is to explore when and where insulin is produced in the uterus. The inner mucous membrane lining the uterus is referred to as the endometrium. The endometrium is an incredibly plastic tissue that undergoes cyclic growth and degeneration. The endometrium consists of a single layer of columnar epithelium and rests on several layers of stromal cells. Tubular uterine glands form from an ingrowth of epithelial cells and stretch from the endometrial surface down to the base of the stroma, and contain spiral arteries. These physiological characteristics can be seen in figure 2.1.1

The experiments were performed using a mouse model. Reproduction in the mature female mouse involves a sequence of related and dependent hormonal and neural events. It begins with the hypothalamus secreting gonadotropin releasing hormone (GnRH) which stimulates cells in the anterior pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In the ovary the oocyte responds to LH and FSH and begins to develop. The oocyte is surrounded by follicular cells and these cells are next to an inner granulosa cell layer and an outer thecal cell layer. LH stimulates thecal cells to produce androgens, these androgens are converted to estrogens in granulosa cells through aromatase; FSH stimulates process by increasing levels of aromatase. Estrogen secretion will eventually lead to a surge of Gonadotropin-releasing hormone (GnRH) released from the hypothalamus, which in turn induces a surge of LH released from the pituitary. This LH surge leads to the rupture of the follicle and release of the oocyte. In mice the LH also induces a preovulatory surge of progesterone release from the ovaries. More progesterone is produced by mechanical vaginal stimulation and the formation of the corpus luteum.

The rhythmic interaction of pituitary and ovarian hormones are represented in the mouse estrous cycle, which can be divided into four stages (Allen 1922). The first stage is proestrus which is characterised by development of both the endometrium and ovarian follicles. Immediately after proestrus is the estrus stage, characterised by receptivity to mating under the influence of estrogen. The third stage is metestrus, characterised by sexual inactivity and the start of corpus luteum formation. The last

stage is diestrus, characterised by a dysfunctional corpus luteum that generates suboptimal amounts of progesterone due to the activity of luteal 20 α-hydroxy steroid dehydrogenase, which metabolises progesterone to the biologically inactive metabolite, 20 α-hydroxy progesterone.. Given the large amount of changes the uterus undergoes to prepare itself for an embryo we hypothesized that any insulin production would reflect the uterus' dynamic physiology.

The ejaculation of spermatozoa into the uterus as well as vaginal cervical stimulation during mating leads to a secretory response from the uterus (Fuxe and Nilsson 1963). The uterine secretions include a wide variety of compounds such as cytokines (Robertson et al 1992) and immunoglobulins (Parr and Parr 1985). Insulin has been shown to have a positive effect on spermatozoa and we want to examine if insulin is also released from the uterus after mating.

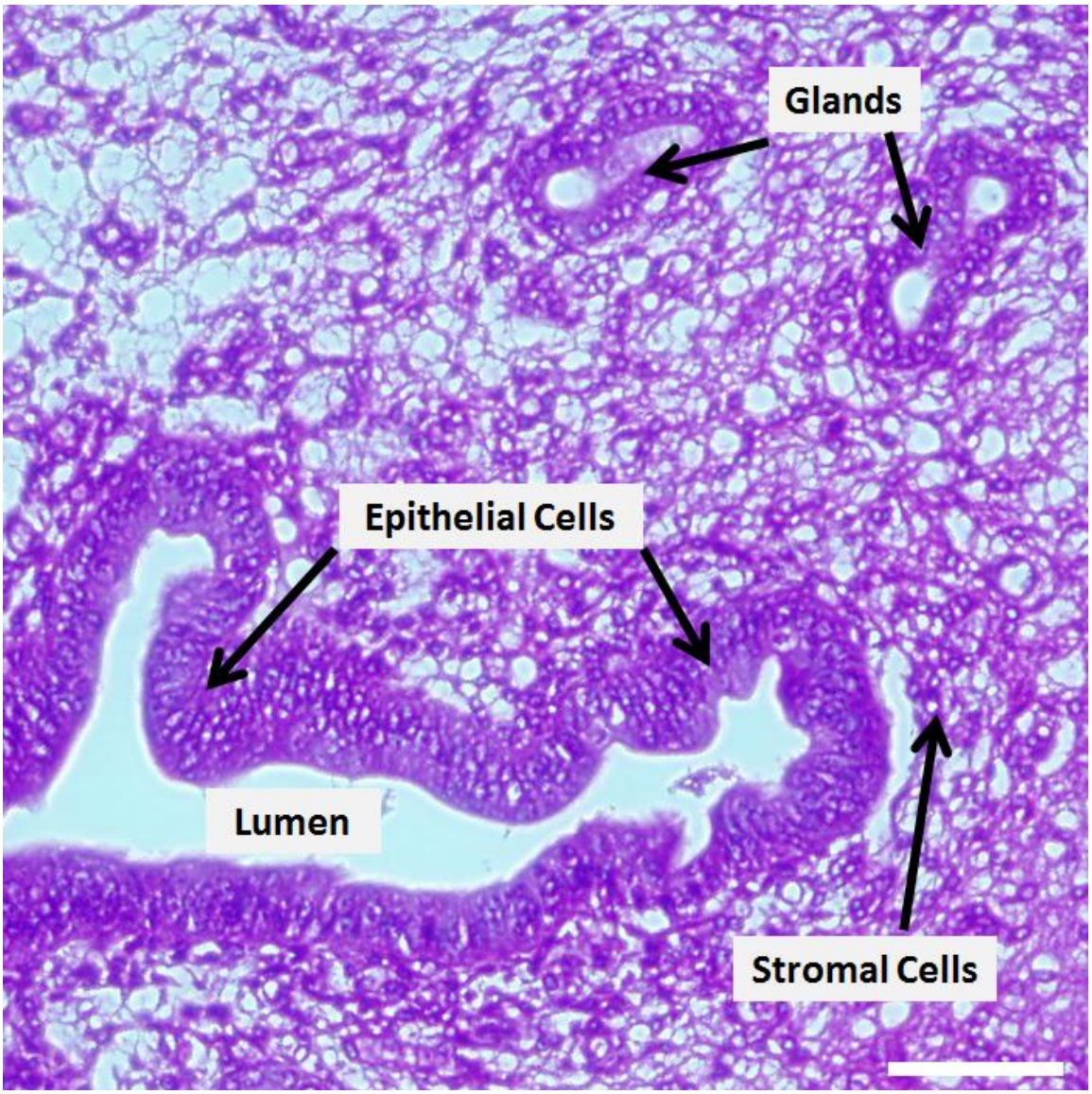


Figure 2.1.1: Hematoxylin and eosin stain of mouse endometrium. Endometrium contains a layer of epithelial cells resting on a mass of stromal cells. Throughout the stroma are glands that form from an ingrowth of epithelial cells. The endometrium experiences hormone driven cycle of proliferation and degeneration. Scale bar represents 200µm

2.2.1 Animals

Female Swiss mice (8-12 weeks). Mice were euthanised using CO_2 gas for 2-4 minutes. Mice were exsanguinated by perfusion with warm PBS. Stage of estrous cycle was determined by vaginal lavage (Maclean et al 2012). Uterine cells where collected by either gently rubbing the vagina of living mice with cotton bud or flushing the vagina of deceased mice with PBS. Cells were smeared on slides, allowed to dry and then stained with crystal violet. Examples of cells at different stages of estrous can be seen in figure 2.2.1.

2.2.2 RNA extraction and PCR

mRNA was extracted from homogenised mouse uterus using TRIzol (Ambion 15596-026). RNA was isolated using isopropanol. RNA yield and purity was measured using absorbance spectroscopy. All samples used had a 260/280 ratio of between 1.8 - 2.0. Three micrograms of total RNA was transcribed to generate cDNA using a cocktail of 3µl DEPC H20, 4µl RT buffer, 1µl 10mM dNTPs, 1µl RNAsin and 1µl DNASE and incubating at 42 °C for 3 h.

Reverse transcription PCR (RT-PCR) and nested RT-PCR was performed to amplify mouse INS mRNA using the following primers; Mouse *INS* forward 5'-CAGCCCCTCTGGCCATCTGC -3' and reverse 5'-GGGGCTTCCTCCCAGCTCCA -3', Mouse *INS* nested forward 5'-GCCTACCCACCCCACCTGGA-3' and reverse 5'-CACGGCGGGACTTGGGTGTG-3'. 1.5ul of cDNA was incubated with 26.8ul PCR H20, 10ul PCR buffer, 5ul 25mM MgCl2, 1ul 10mM dNTPS, 1ul forward primer, 1ul reverse primer, 0.2ul Taq polymerase for a total volume of 50ul. RT-PCR was verified by running product on agarose gel, 1% for RT-PCR and 2% for nested RT- PCR, and observing the location of the band. Bands were cut out and sequenced to confirm product was INS. Nested RT-PCR was verified by running product on 2% agarose gel and checking the location of the band. Bands were cut out and sequenced to confirm product was INS.

Quantitative PCR (qPCR) was performed using the following primers for insulin and the housekeeping genes GAPDH and Cyclophilin; mouse *INS* forward 5'-AGAGAGGAGGTACTTTGGACTATAA -3'

and reverse 5'-GCTTGCTGATGGTCTCTGATTA -3', *GAPDH* forward 5'-GAGATTGTTGCCATCAACGACC- 3' and reverse 5'- CCACGACATACTCAGCAC-3', *CYCLOPHILIN* forward 5'-CGTCTCCTTCGAGCTGTTT-3' and reverse 5'-ACCCTGGCACATGAATCCT-3'. Samples were cycled 50 times using the following conditions: 95 °C for 15 s, 64 °C for 1 min. Homogeneity of products from each reaction was confirmed by melt curve analysis. Melting curves can be seen in Figure 2.2.2. Insulin, GAPDH and Cyclophilin primers were optimised to have equal efficiency.

Three negative controls were used in qPCR to exclude the possibility of genomic DNA contamination: (1) no cDNA (water), (2) no RT enzyme was used, and (3) all primers had at least one intron in their annealing sequence. In some samples the no RT enzyme control would have some amplification very late in the cycle. Samples whose cycle threshold was within 5 cycles of the no RT enzyme control were excluded from results.

The delta threshold cycle (CT) methodology was used to calculate relative quantities of mRNA products from each sample by subtracting the CT of insulin from the CT of GAPDH. All samples were corrected for total input RNA by normalizing delta CT values to the delta CT value of a uterine sample at estrus that was used as a study calibrator.

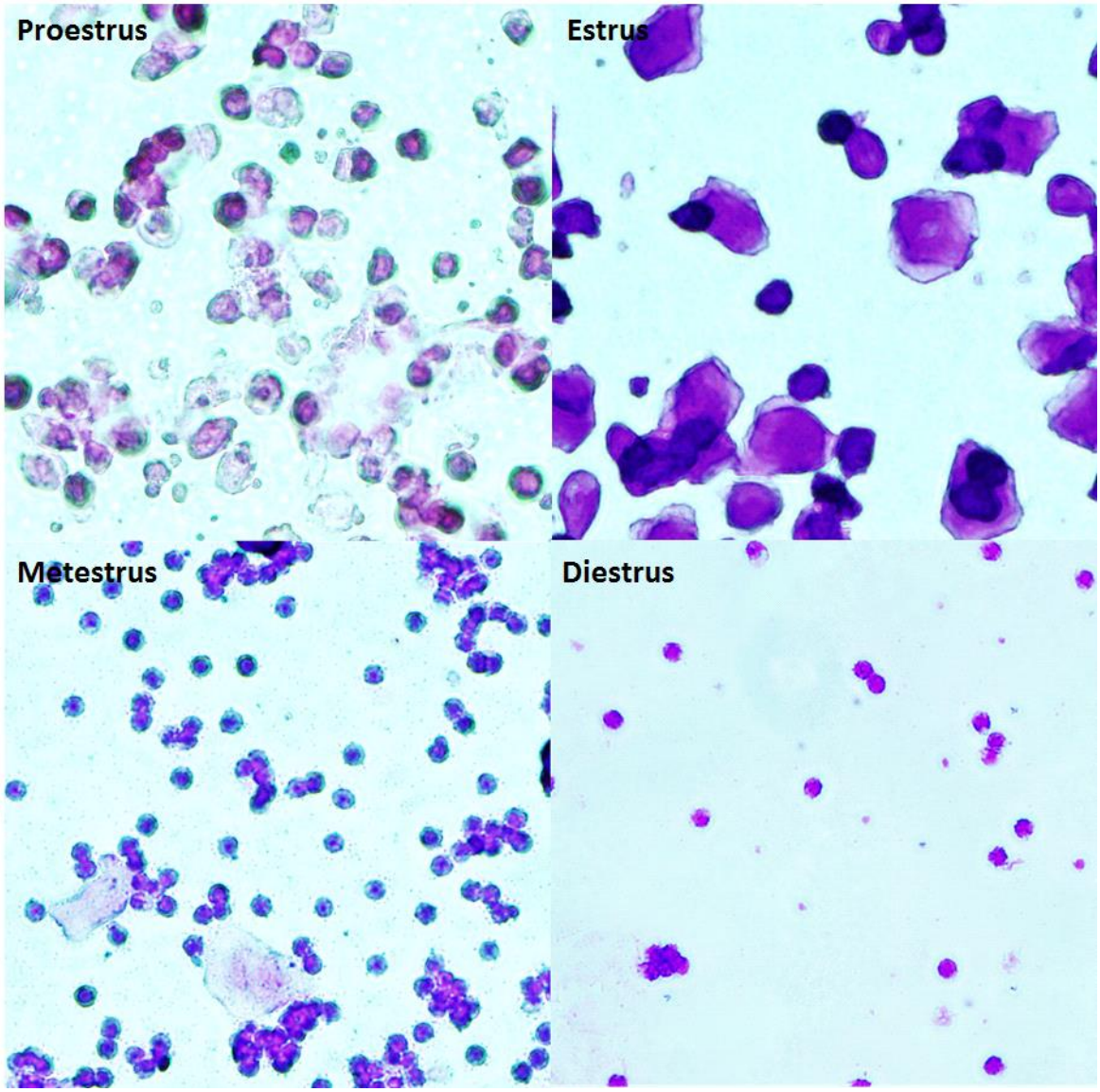


Figure 2.2.1: Examples of vaginal lavage stained with crystal violet. At proestrus, mostly nucleated and few cornified epithelial cells are present along with some leukocytes. In estrus only cornified epithelial cells are present. At metestrus, cornified epithelial cells and leukocytes are present. At diestrus, only leukocytes can be observed.

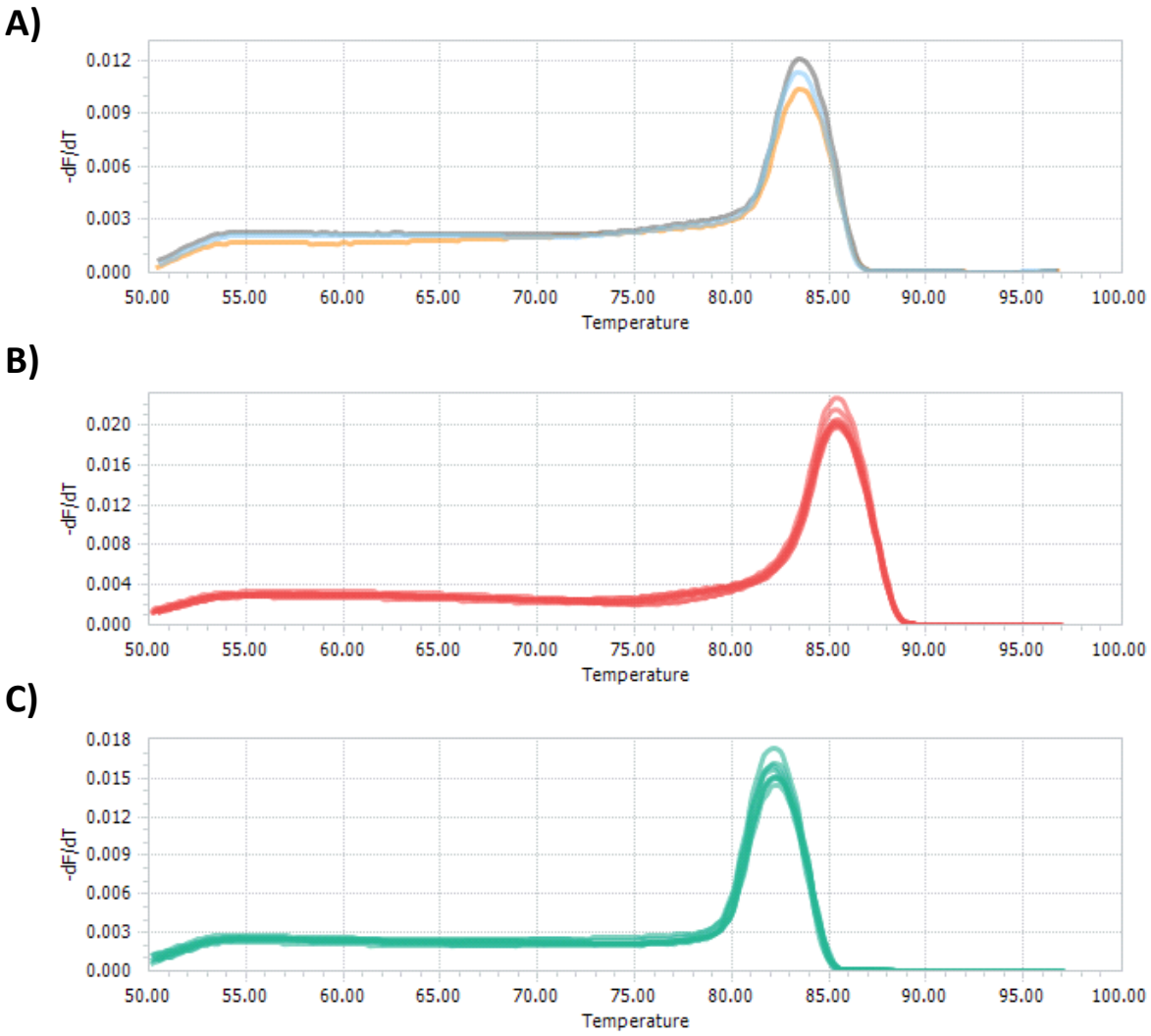


Figure 2.2.2: qPCR product melting curves. A) Insulin, B) GAPDH, C) Cyclophilin. Melting curves show that products were free of primer dimers. Experiments were run on a LightCycler® 96 System – Roche.

2.2.3 Immunocytochemistry of uterine tissue

Uterus sections were collected from mice and fixed in Bouins solution for 5 hours before being washed twice and stored in 80% ethanol. Tissue was embedded in wax and sectioned. After sectioning the tissue was de-waxed by three five minute xylene incubations. It was then rehydrated by washing for five minutes in different concentrations of ethanol, beginning with 100% ethanol and decreasing by 10% until a final wash of 50% ethanol. Heat based antigen retrieval was used. All antibodies except anti-insulin receptor used 10mM Na Citrate, pH6 with 3% tween. For anti-insulin receptor, 0.05M Tris, pH 10 with 3% tween was used. Slides were heated in a microwave set at high for five minutes. They were allowed to cool to room temperature over an hour before being washed three time in TBS. Slides were blocked with 3% BSA for one hour at room temperature then incubated with primary antibody overnight at 4°C. The following day the primary antibody was washed off with PBS and slides were incubated with Alexa Fluor 488 for one hour. Immunostaining of mouse pancreas with insulin, C-peptide and PC 1/3 localised immunofluorescence to the islets of Langerhans. Omitting the primary antibody and staining with the secondary antibody was also performed as control and did not show any staining (Figure 2.2.3).

Antibody	Manufacturer	Species	Dilution
Insulin monoclonal	Abcam	Rabbit	1:200
C Peptide polyclonal	Abcam	Rabbit	1:200
Anti-PC1/3 polyclonal	Abcam	Mouse	1:200
Insulin Receptor polyclonal	Abcam	Rabbit	1:200

Table 2.2.1: List of antibodies used in immunocytochemistry

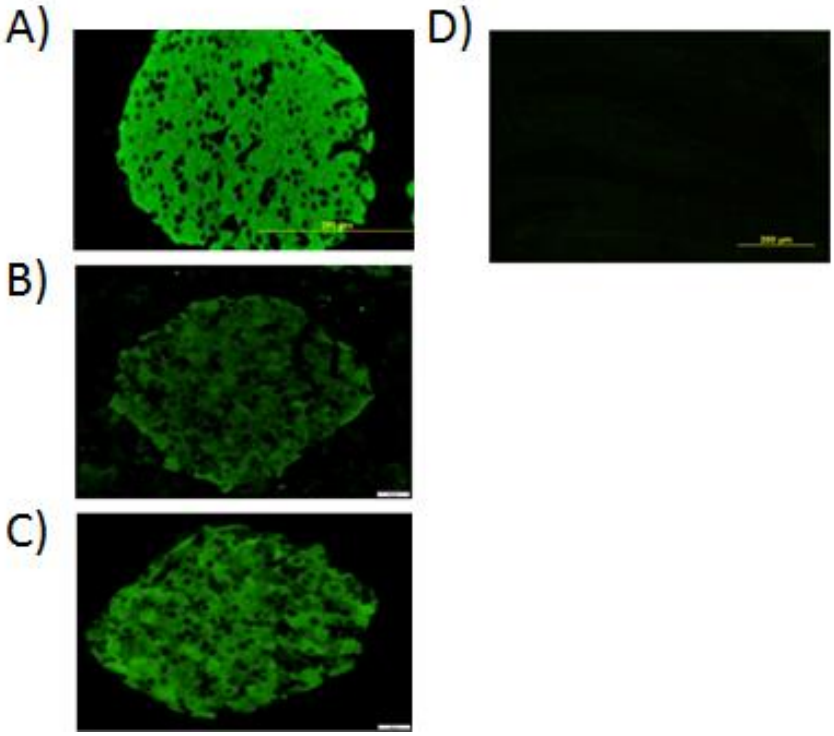


Figure 2.2.3: Positive and No Primary negative controls of antibodies. Mouse Islet of Langerhans used as a positive control for antibodies; A) Insulin, B) C-peptide, C) Proprotein Convertase 1. No primary negative controls; D) 1:200 Alexa Fluor 488 in mouse uterine tissue,

2.2.4 Collection of uterine fluid

Uteri were collected from Swiss mice on the morning after estrus. Mating was confirmed by the presence of spermatozoa pellet. Fine needle was inserted into the end of one uterine horn and 500ul of PBS was used to flush the uterus. Uterine flushing of mated mice contained spermatozoa and therefore was centrifuged at 2g for 15 minutes and the supernatant was collected for analysis.

2.2.5 Insulin measurement

Insulin was measured using a Cisbio HTRF® Insulin assay according to the manufacturer's instructions. Assay was previously validated and evaluated compared to other commercially available insulin ELISA assays (Hixon 2008). Briefly 10µl of sample or standard was incubated for two hours at room temperature in a 96 well plate with 5 µL anti-insulin Ab-cryptate and 5 µL anti-insulin Ab-XL665. Afterwards the plate was read using a BMG Labtech PHERAstar FS reader.

The assay measures insulin concentration by measuring the fluorescence released when the FRET phenomenon occurs. When an insulin molecule is bound by the antibodies the two antibodies come into close proximity with each other, allowing FRET to occur between the europium cryptate and the cross-linked allophycocyanin. This FRET phenomenon increases proportionally with the insulin concentration. Insulin concentration was worked out from a standard curve constructed using a manufacturer provided standard that was consecutively diluted 1:10 times in PBS.

2.2.6 Statistics

Experiments were replicated with material collected from at least three different animals, and the data are presented as mean ± SEM. Statistical analysis was performed with an analysis of variance (ANOVA). The existence of statistically significant differences between group means was tested using the Tukey Kramer pairs comparison test. Samples with a *P*-value <0.05 were considered statistically significant, one asterisk denotes a *P*-value <0.05, two asterisk denotes <0.001 and three denotes <0.0001.

2.3.1 Insulin transcript in the uterus changes according to the phase of the estrous cycle

To identify if the insulin transcript was present in the uterus, a combination of RT-PCR and nested PCR strategies were used (Figure 2.3.1.1). Insulin could be detected as a faint amplicon during proestrus and estrous using RT-PCR but not at metestrus and diestrus. In contrast, the use of a highly sensitive nested PCR analysis revealed a strong band for insulin at all stages of the estrous cycle. Each of the amplified PCR products were excised and confirmed by sequencing to be insulin. The uterus undergoes changes during the estrous cycle under hormonal regulation. To investigate if the amount of insulin transcript produced changed in association with the endocrine milieu, qPCR was used to quantify the insulin mRNA signal at different stages of the estrus cycle. Insulin could only detected consistently during the estrus phase. At proestrus only some samples had detectable insulin while none could be detected at metestrus and diestrus. An ANOVA analysis showed that the proestrus samples with detectable mRNA were not statistically different to the estrus the quantity of mRNA was not significantly different to estrus samples (p-Value = 0.3246). However at estrus there was more transcript (mean fold change1.15) compared to proestrus (mean fold change1.38). Both proestrus and estrus had significantly more transcript than at metestrus and diestrus (p-Value = <0.0001)

Detecting the insulin transcript in the uterus was a surprising find because of the rarity of insulin mRNA outside the pancreas, we next wanted to see if the transcript was translated and if so where in the uterus the protein was expressed.

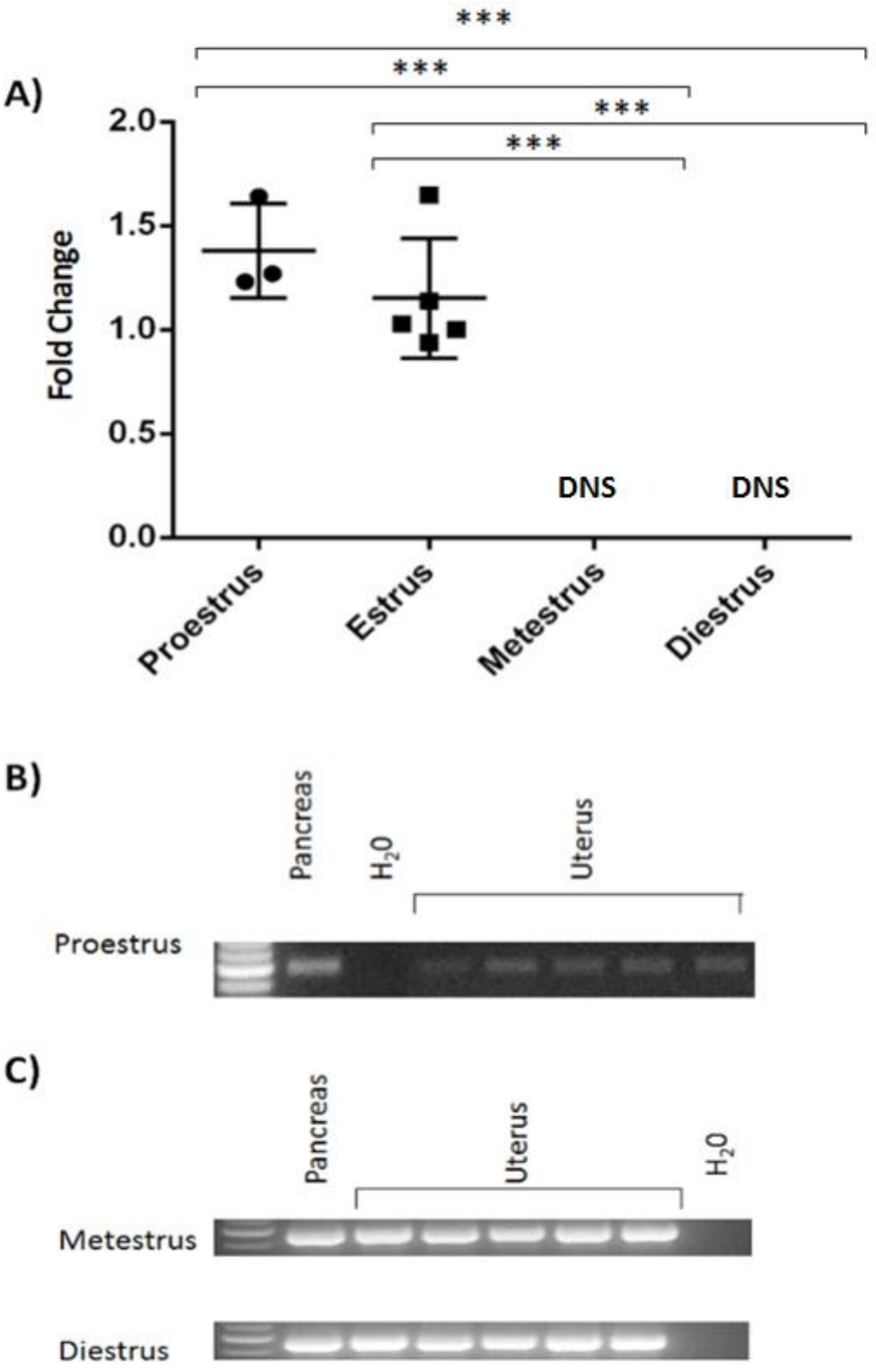


Figure 2.3.1.1: INS mRNA expression in mouse endometrium at different stages of the estrous cycle. A) The expression of *Ins* mRNA and the housekeeping genes *Cyclophilin* and *Gapdh* were examined by qPCR in mouse endometrium. The expression of *Ins* was normalised to a study calibrator sample at estrus. Quantitative

PCR demonstrated different expression levels of *Ins* mRNA. It could be detected in all samples at estrus, in some samples at proestrus and never at metestrus and diestrus. *Cyclophilin* and *Gapdh* were consistently found at all stages and in all samples. Statistical analysis was performed using the Tukey Kramer pairs comparison test. Asterisk represents value that was significantly different to other groups. Values are representative of five samples and three technical replicates. Non positive results from proestrus groups not included in statistical analysis as they would lower the fold change value, implying greater gene expression. B) RT PCR was able to detect a faint band in all proestrus samples but not metestrus and diestrus. C) Nested PCR was able to produce intense bands in samples from metestrus and diestrus. DNS = Data not significant

2.3.2 Localisation of insulin in the endometrium

Uterus sections at each of the four stages of the estrous cycle were probed for insulin using ICC. Insulin was strongly expressed in epithelial cells (fig 2.3.2.1) and glands (fig 2.3.2.2) at estrus and metestrus. The expression was weaker at diestrus and undetectable at proestrus.

Insulin was also localised to stromal cells (fig 2.3.2.3). It was strongly expressed in estrus, metestrus and diestrus however not all stromal cells expressed insulin. At estrus insulin was expressed in stromal cells closer to the epithelial cells, at metestrus insulin expressing cells were largely evenly distributed throughout the stroma but at diestrus insulin was only expressed in stromal cells furthest away from the epithelial cells. When the contrast of the images was increased small granule areas of high intensity fluorescence could be seen in the epithelial, glands and stroma. A similar phenomenon could also be seen in pancreatic β cells (fig 2.3.2.4). These results suggest that the insulin protein is first expressed at estrus, a day after insulin mRNA is detected, in epithelial and stromal cells and over time decreases, persisting longest in the stromal cells.

Detection of the insulin protein brought about the question of whether it was actually synthesised in the epithelial and stromal cells or if it was simply transported there from the pancreas. To resolve this issue we undertook an analysis of markers of insulin production, namely the protein synthesised as a by-product of insulin, C-peptide and the peptidase responsible for cleavage of the insulin precursor, PC 1/3.

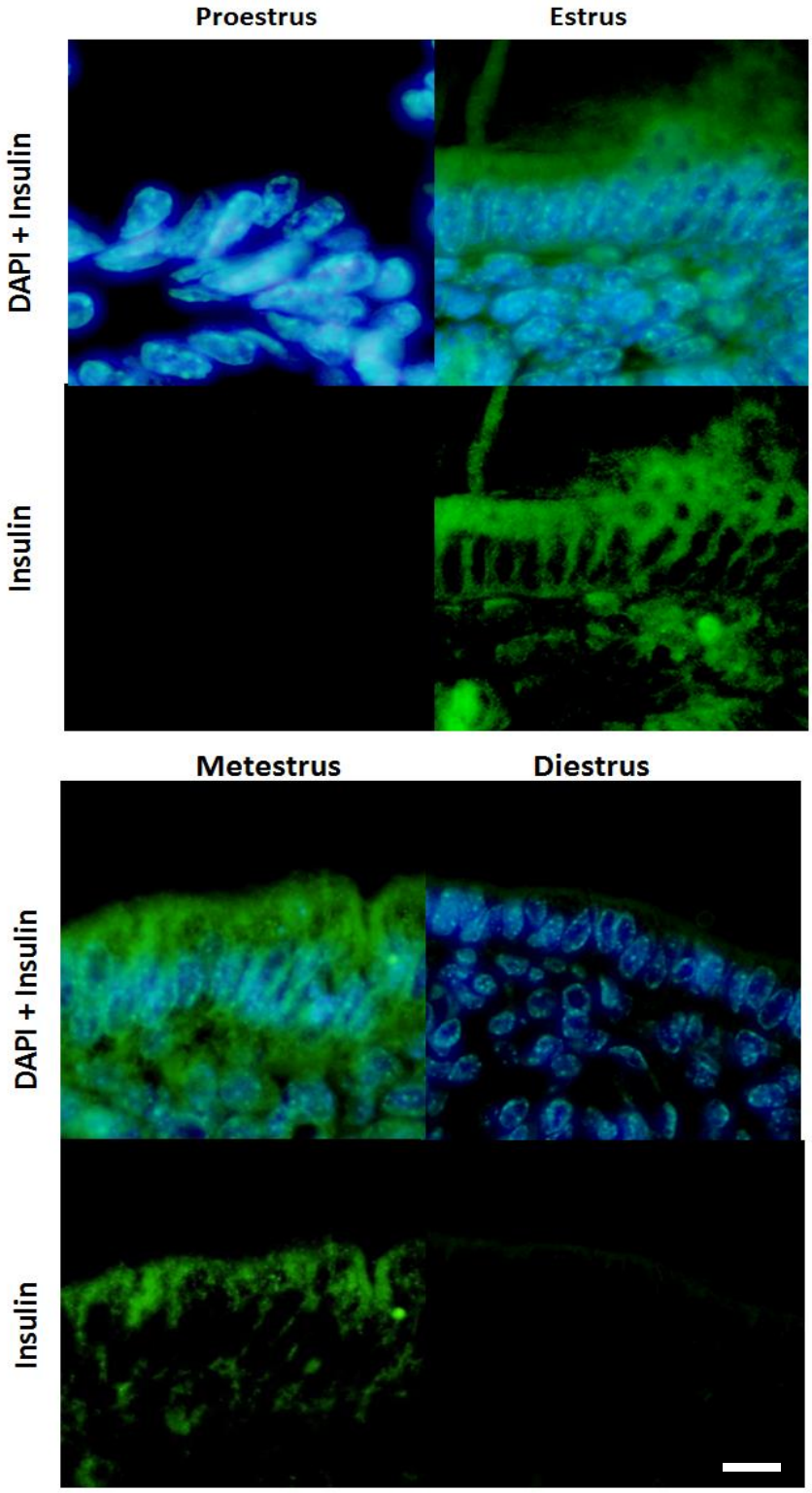


Figure 2.3.2.1: Localisation of insulin in mouse endometrial epithelial cells. Sections of mouse endometrium at each of the four stages of the estrous cycle showing fluorescent signal from DAPI (blue) and insulin antibody (green). Insulin is undetectable at proestrus, strongly visible at estrus and metestrus and faintly visible at diestrus. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2. Scale bar represents 40µm

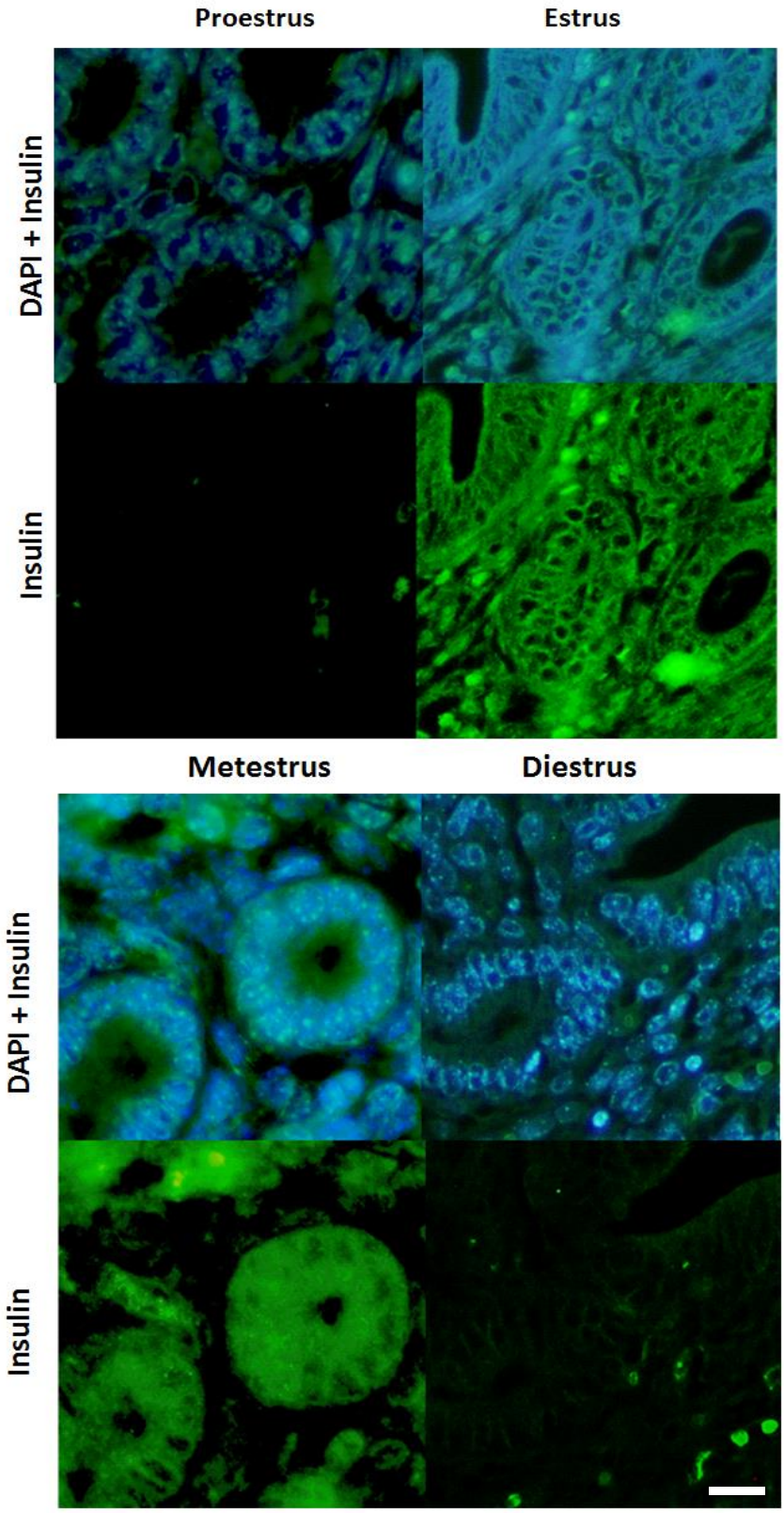


Figure 2.3.2.2: Localisation of insulin in mouse endometrial epithelial glands. Sections of mouse endometrium at each of the four stages of the estrous cycle showing fluorescent signal from DAPI (blue) and insulin antibody (green). Insulin is very weak at proestrus, strongly visible in the glands at estrus and metestrus and faintly visible at diestrus. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2. Scale bar represents 40µm

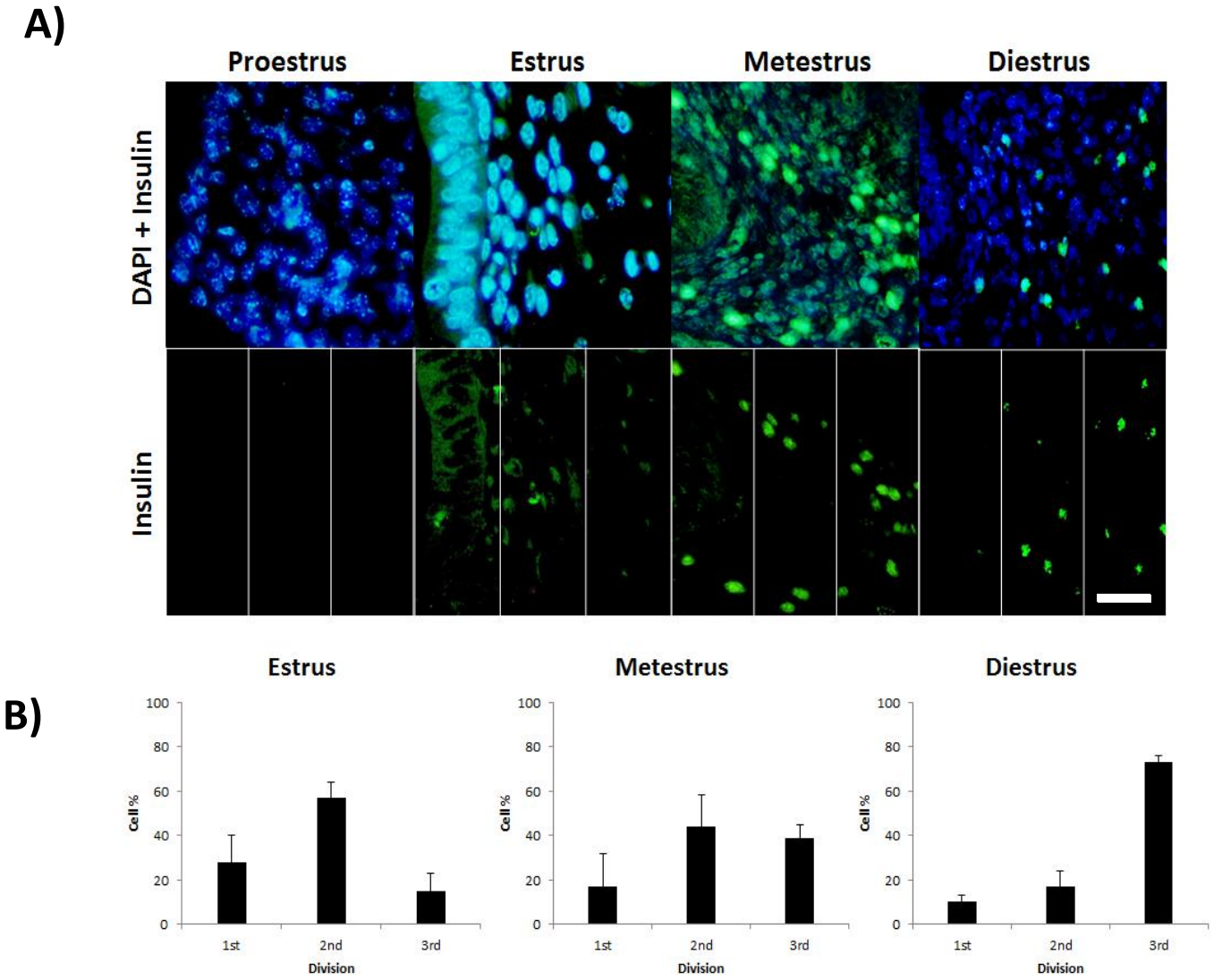


Figure 2.3.2.3: Localisation of insulin in mouse endometrial stromal cells. A) Sections of mouse endometrium at each of the four stages of the estrous cycle showing fluorescent signal from DAPI (blue) and insulin antibody (green). At estrus insulin is present mostly in those stromal cells closest to the epithelial cell. At metestrus insulin is visible throughout the stroma. At diestrus insulin is mostly present in those stromal cells further away from the epithelial cells. B) To quantify the above observations images were divided into three equal parts based on proximity to the epithelial cells. Number of fluorescent stromal cells in each division were counted and graphed as a percentage. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2. Scale bar represents 40µm

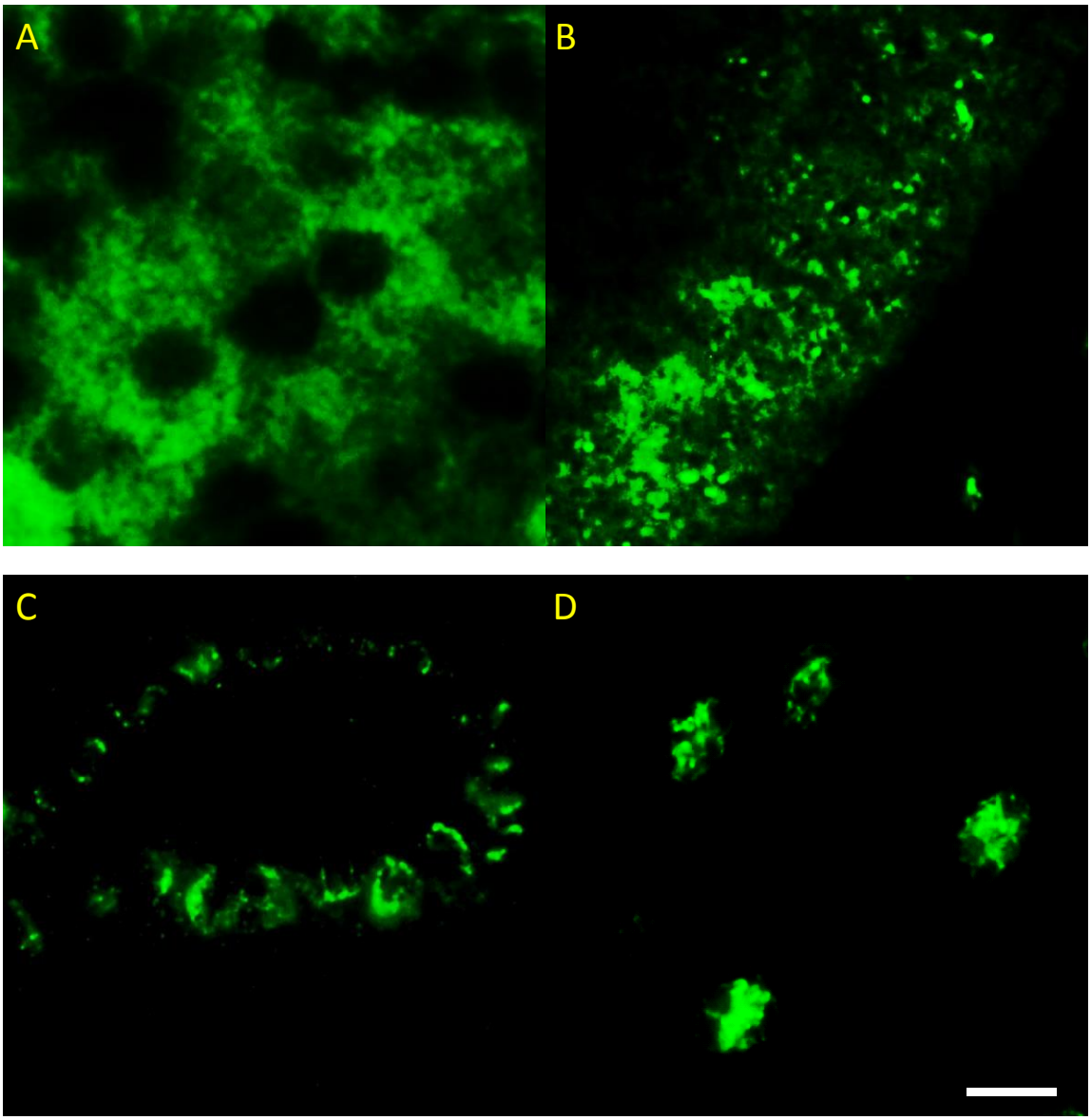


Figure 2.3.2.4: Visualisation of granules. Sharp green fluorescent dots could be seen in A) β cell of pancreas B) Endometrial epithelial cell C) Glands D) Stromal cells. Images had their colour curves manipulated in photo editing software, Gimp, to achieve contrast between areas of moderate and strong fluorescence. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2. Scale bar represents 50µm

2.3.3 Localisation of C-peptide in the endometrium

C-peptide was found to be strongly expressed in epithelial cells (fig 2.3.3.1) and endometrial glands (fig 2.3.3.2). In both epithelial cells and glands expression appeared to be strongest in the most distal parts of the cells. Measurements of pixel intensity showed that fluorescence was strongest at proestrus and decreased in intensity at each subsequent stage (figure 2.3.3.3). Contrary to what was seen with insulin, at proestrus through to metestrus nearly all stromal cells expressed C-peptide (figure 2.3.3.4). At diestrus only some cells had C-peptide expression but they were largely equally distributed through the stroma. Expression was strongest at proestrus and decreased with each subsequent estrous stage. A significant differences of *P*-value <0.0001 was seen between proestrus and diestrus, proestrus and metestrus, estrus and diestrus. A comparison of estrus and metestrus had a *P*-value of 0.004 and a comparison of proestrus and estrus had a *P*-value of 0.0034.

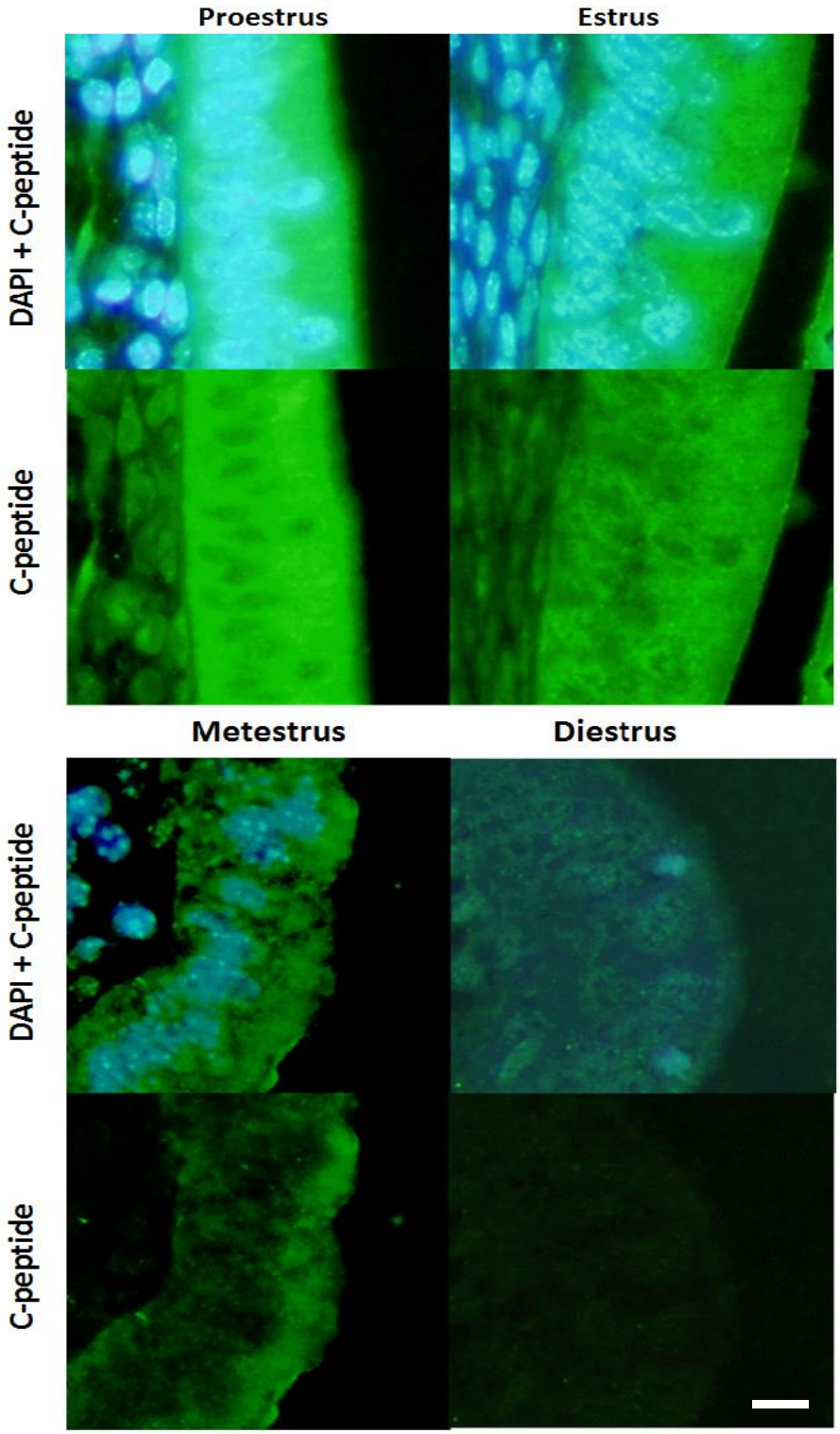


Figure 2.3.3.1: Localisation of C-peptide in mouse endometrial epithelial cells. Sections of mouse endometrium at each of the four stages of the estrous cycle showing fluorescent signal from DAPI (blue) and C-peptide antibody (green). C-peptide is very strongly expressed in the epithelial cells at proestrus and estrus, strongly expressed metestrus and weakly expressed at diestrus. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2. Scale bar represents 50µm

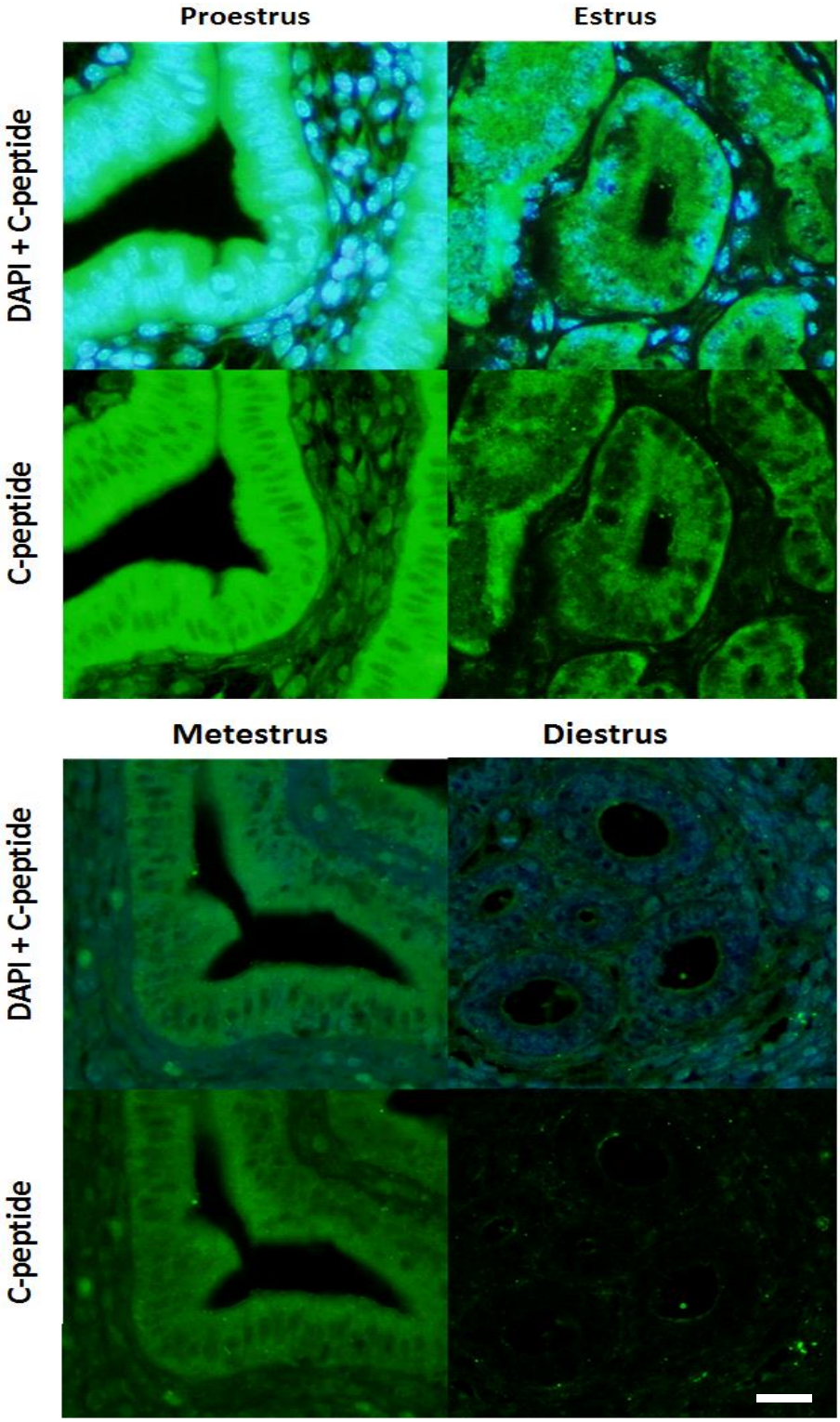


Figure 2.3.3.2: Localisation of C-peptide in mouse endometrial epithelial glands. Sections of mouse endometrium at each of the four stages of the estrous cycle showing fluorescent signal from DAPI (blue) and C-peptide antibody (green). C-peptide is very strongly expressed in the epithelial glands cells at proestrus and estrus, strongly expressed metestrus and weakly expressed at diestrus. Expression is strongest on the distal edge of the cells. Images are representative of three independent experiments. Scale bar represents 50µmSamples were prepared as described in methods 2.2

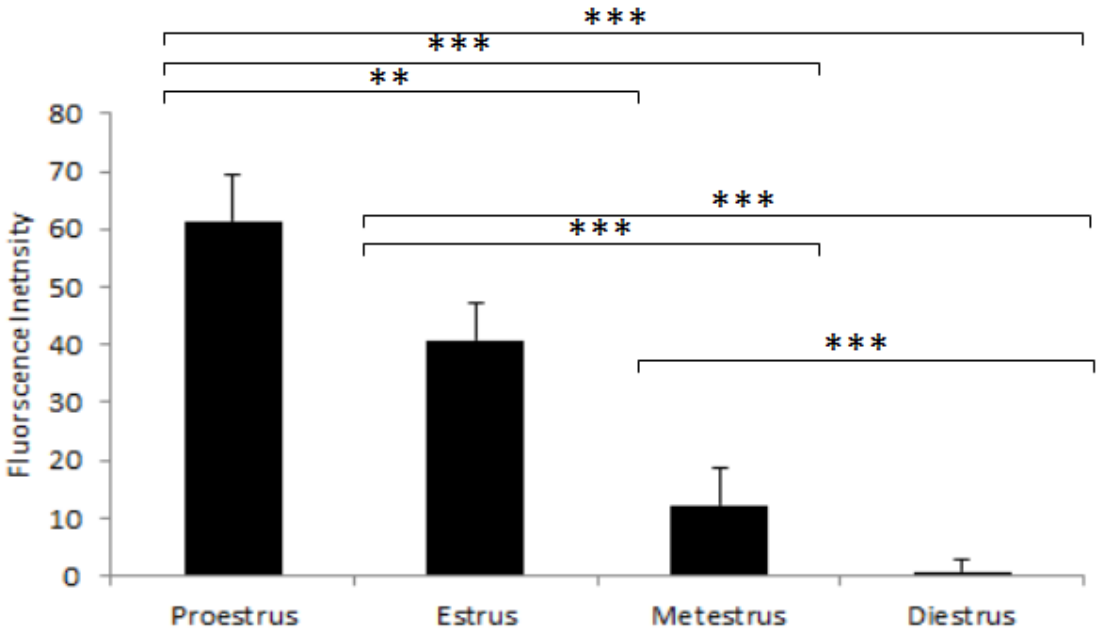


Figure 2.3.3.3: Pixel intensity measurements of C-peptide antibody fluorescence in epithelial cells. Expression was most abundant during proestrus and decreased at each subsequent stage of estrous. Pixel intensity was measured using the measurement function in Image J. Values indicate SEM of triplicate measurements from three representative experiments. Statistical analysis was performed using the Tukey Kramer pairs comparison test. Asterisk represents value that was significantly different to other groups.

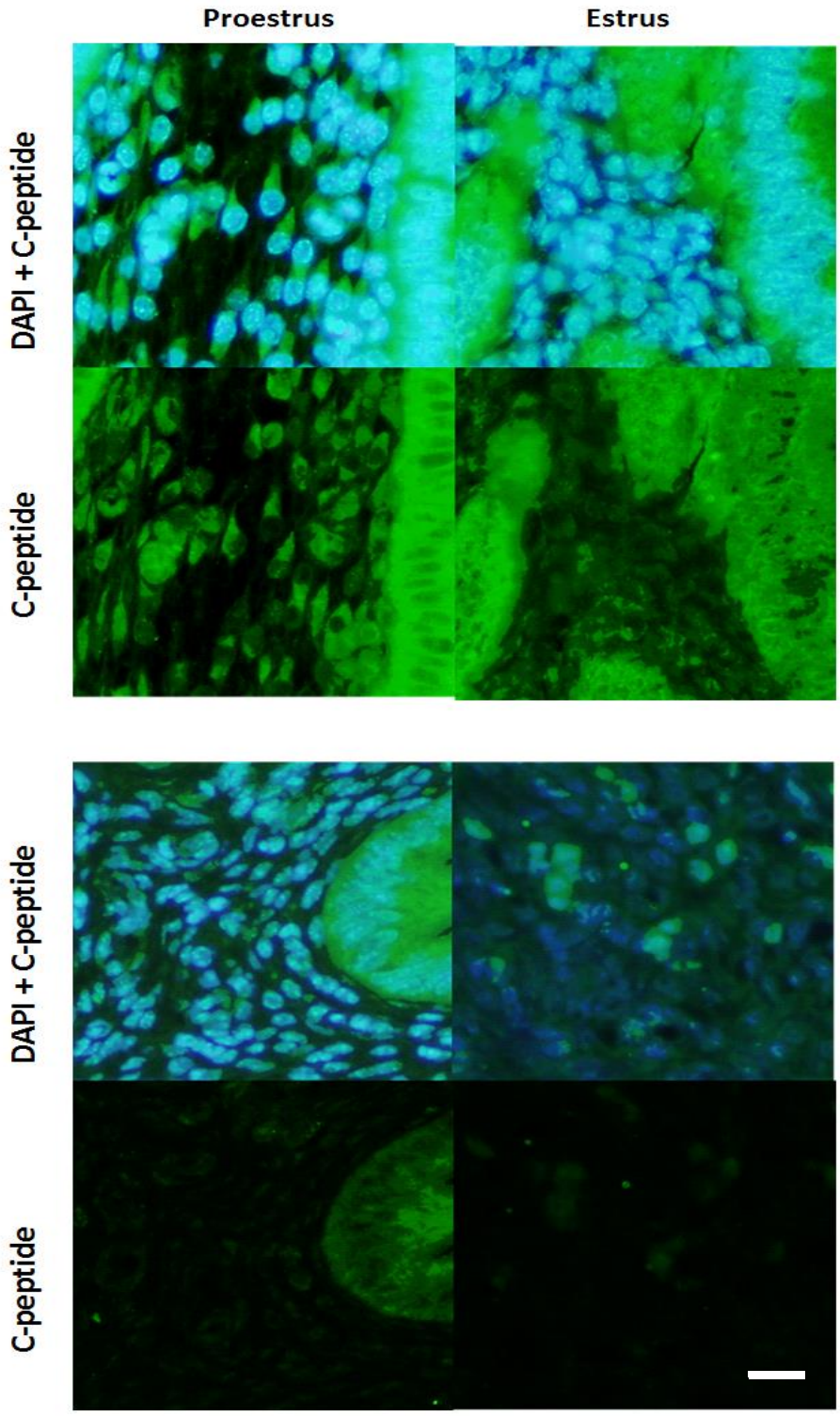


Figure 2.3.3.4: Localisation of C-peptide in mouse endometrial stromal cells. Sections of mouse endometrium at each of the four stages of the estrous cycle showing fluorescent signal from DAPI (blue) and C-peptide antibody (green). C-peptide is very strongly expressed in the epithelial cells at proestrus and estrus, strongly expressed metestrus and weakly expressed at diestrus. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2. Scale bar represents 50µm

2.3.4 Localisation of PC 1/3 in the endometrium

PC 1/3 was found to be very strongly expressed in epithelial cells at proestrus and diestrus before starting to fade in metestrus and diestrus (fig 2.3.4.1). This was confirmed my measuring pixel intensity of epithelial cells (fig 2.3.4.2). In stromal cells fluorescence was weaker but for the occasional stromal cell that had high fluorescence.

Our ICC localisation experiments of C-peptide and PC1/3 broadly support our findings of insulin with their expression being very strong in endometrial cells at proestrus/estrus and decreased as the cycle progressed to metestrus and diestrus. There was a strongly significant difference between proestrus and diestrus, and between proestrus and metestrus, *P*-value = <0.0001. A comparison between estrus and diestrus has a P-value = 0.0004 and a comparison between estrus and diestrus has a *P*-value of 0.0015. There was no statistically significant difference between proestrus and estrus, *P*-value = 0.101 and no significant difference existed between metestrus and diestrus, significant difference 0.4371. However there was a downward trend. In both C-peptide and PC 1/3 the epithelial cells had the brightest fluorescence at proestrus and estrus compared to stromal cells. This suggests that the epithelial cells and glands are where most of the insulin is produced.

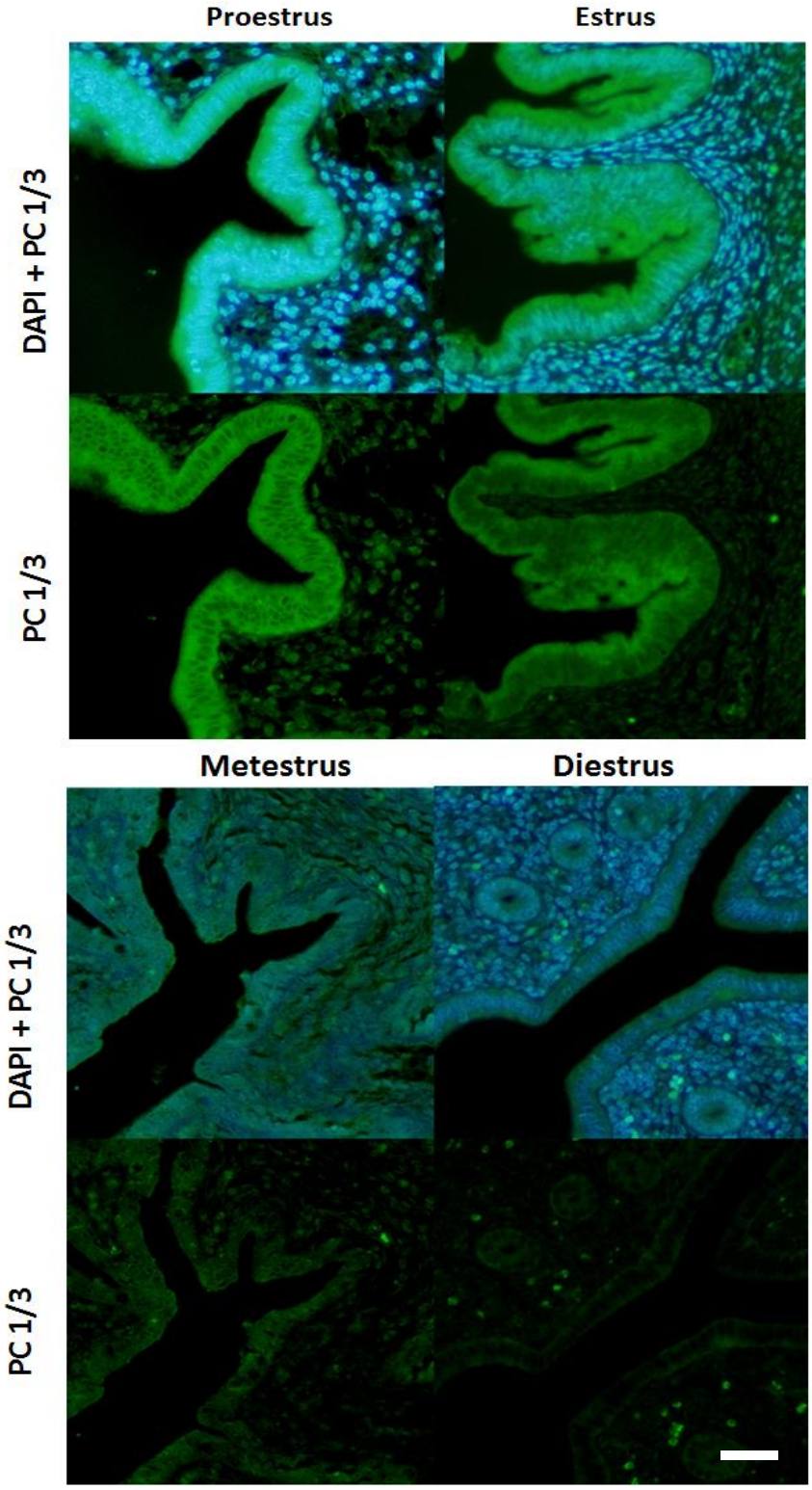


Figure 2.3.4.1: Immunofluorescence images of PC 1/3 in mouse endometrium at four stages of the estrous cycle. PC 1/3 is expressed in epithelial cells at proestrus and estrus. Expression decreases in metestrus and diestrus. PC 1/3 less expressed in stromal cells compared to epithelial cells at proestrus and estrus. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2 with the exception of being photographed with the 20x objective lens. Scale bar represents 100µm

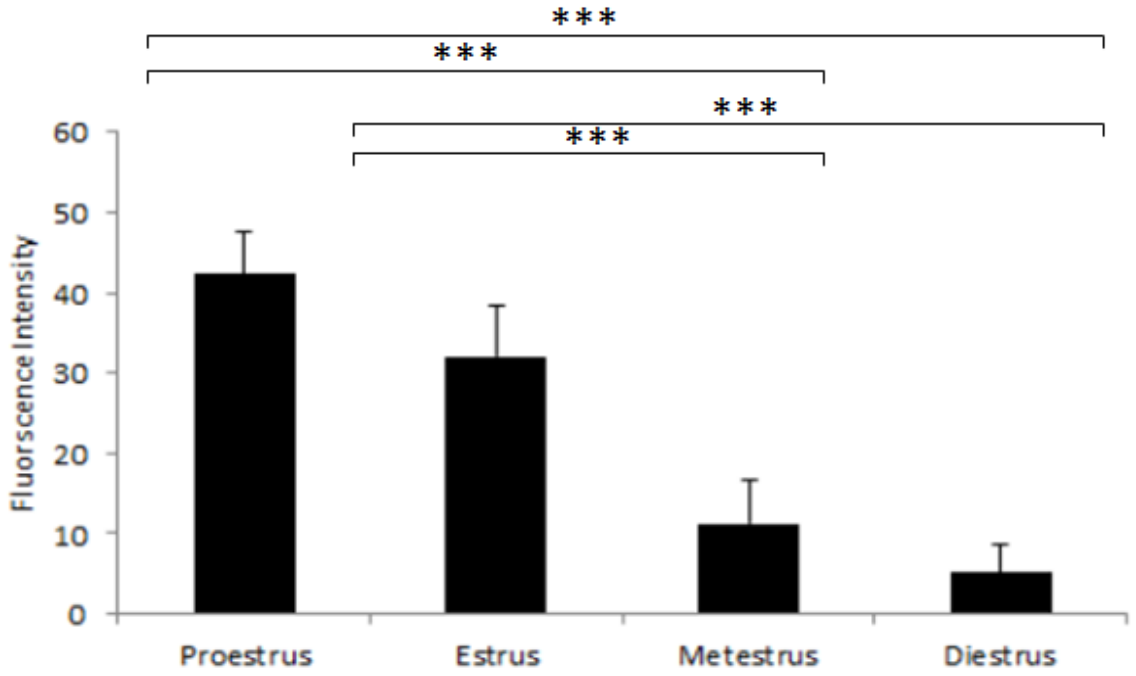


Figure 2.3.4.2: Pixel intensity measurements of PC 1/3 antibody fluorescence in epithelial cells. Expression was most abundant during proestrus and decreased at each subsequent stage of estrous. Pixel intensity was measured using the measurement function in Image J. Values indicate SEM of triplicate measurements from three representative experiments. Statistical analysis was performed using the Tukey Kramer pairs comparison test. Asterisk represents value that was significantly different to other groups.

2.3.5 Localisation of IR in the endometrium

ICC was performed for IR to see if the endometrium had a target for insulin binding. Tissue was permeabilised to also test for any internalised IR. The IR was strongly expressed in epithelial cells at all stages of the estrous cycle (fig 2.3.5.1). However in stromal cells expression of IR was seen in only a few stromal cells at proestrus and estrus. At metestrus and diestrus majority of stromal cells had expressed IR (fig 2.3.5.2). In both epithelial and stromal cells IR expression was stronger in the nuclear region compared to the plasma membrane.

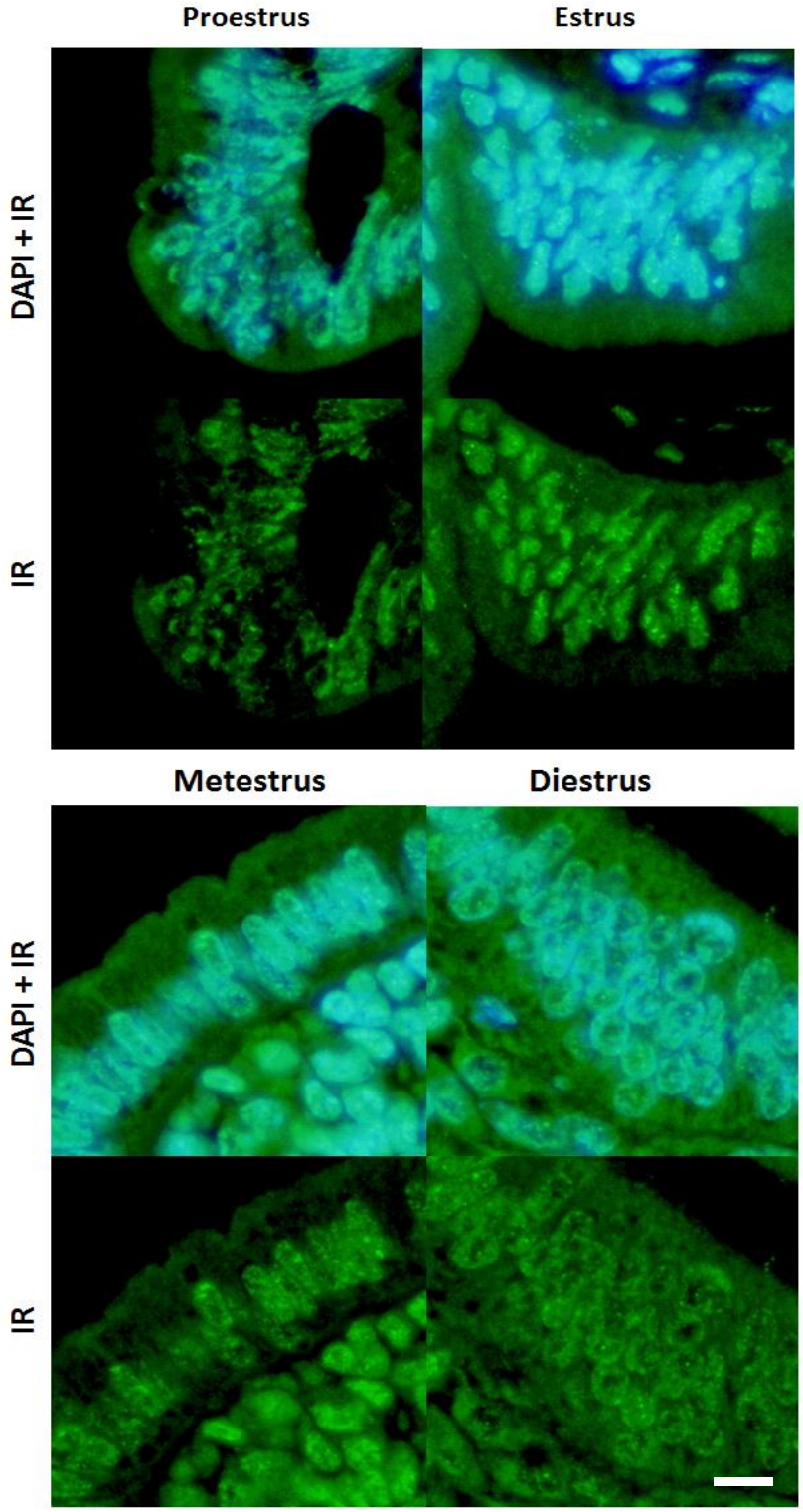


Figure 2.3.5.1: Immunofluorescence images of IR in mouse endometrial epithelial cells at four stages of the estrous cycle. The IR is strongly expressed in epithelial cells at all stage of estrous. As tissue was permeabilised IR can be seen in both plasma membrane and nucleus. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2. Scale bar represents 100µm

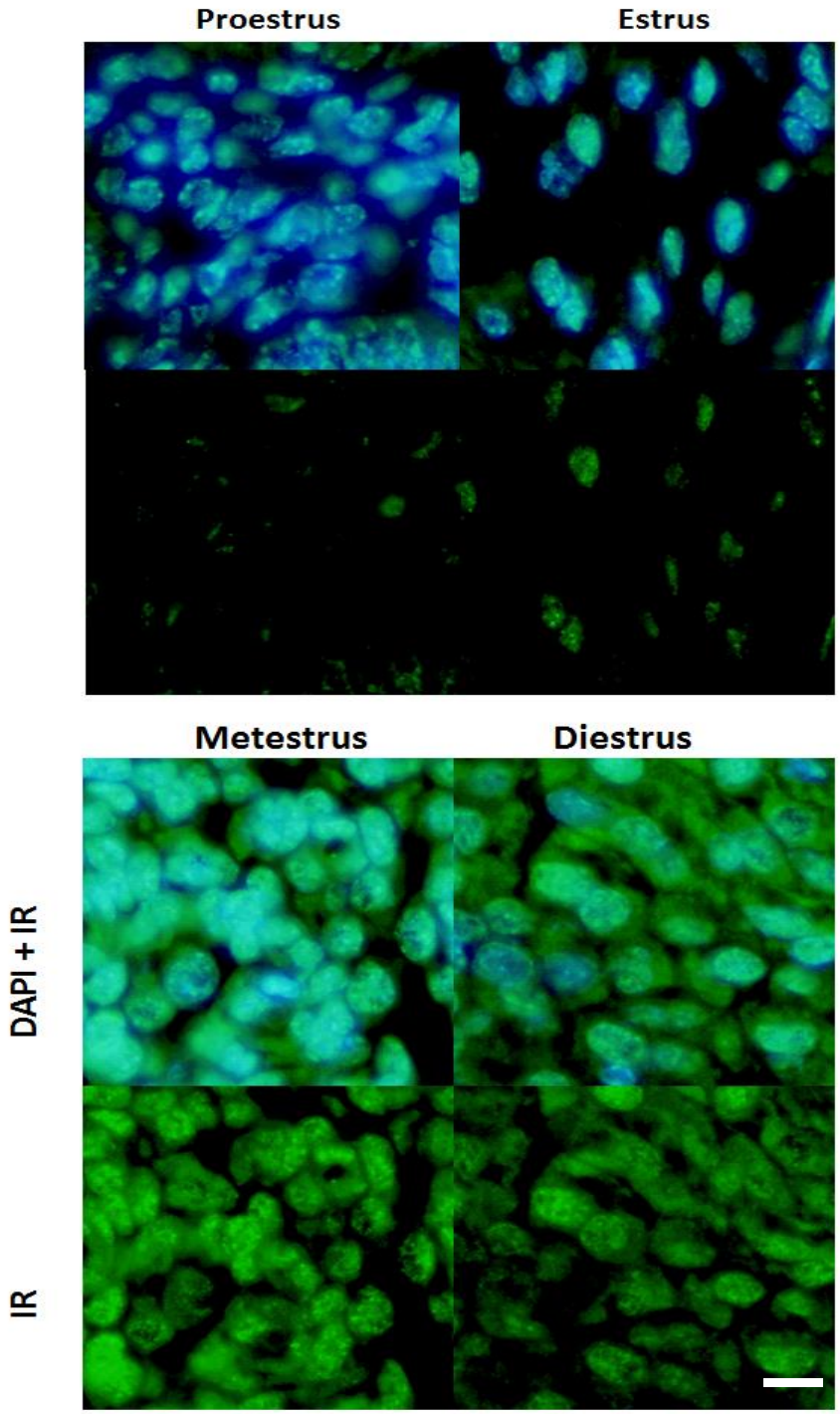


Figure 2.3.5.2: Immunofluorescence images of IR in mouse endometrial stromal cells at four stages of the estrous cycle. IR is IR is weakly expressed in stromal cells at proestrus and estrus. However it becomes strongly expressed in stromal cells at metestrus and diestrus. As tissue was permeabilised IR can be seen in both plasma membrane and nucleus. Images are representative of three independent experiments. Samples were prepared as described in methods 2.2. Scale bar represents 100µm

2.3.6 Measurement of insulin in mouse uterine flushings

PCR and ICC data suggested that insulin is present in the endometrium at estrus. As estrus is also the time during which female mice are receptive to mating and we investigated the possibility that insulin could be released from the endometrium at mating to the advantage of spermatozoa.

We collected and flushed the uteri of mice the morning after they mated. Mating was confirmed by the presence of a spermatozoa plug. Using a high sensitivity insulin assay uterine flushings of mated mice were compared to those of unmated mice at estrus. The assay was not able to detect any insulin in the uterine flushings of either mated or unmated mice. The experiment was replicated twice with three technical replicates of three biological samples of mated and unmated mice. These results led us to conclude that while insulin is produced in the uterus it is not detectable in the uterine fluid.

Chapter 1 discussion

2.4.1 Quantity of insulin transcript in mouse uterus changes with estrous cycle

We initially hypothesized that uterine insulin mRNA would change during the estrous cycle according to the cycling levels of hormones, particularly estrogen. Estrogen's effect is primarily trophic and its activity coincides with the proliferative activity of the endometrium during proestrus and estrus.

RT-PCR was used and amplicons were visible only during proestrus and estrus. However, nested PCR revealed additional amplified products in metestrus and diestrus samples, showing that insulin mRNA is present in the uterus and does indeed change with the phase estrous cycle. At this point it was still not clear during which stage insulin mRNA levels were highest. In the same samples used in RT-PCR, qPCR could only consistently detect insulin during estrus and only sometimes during proestrus. Estrogen levels begin to rise part way through proestrus, peaking in time with the LH surge just before the start of estrus. This could explain why only some of the proestrus samples had detectable insulin mRNA and supports the hypothesis that insulin mRNA synthesis is an estrogen driven event.

This is the first study to look at uterine insulin mRNA however previous work has looked at how pancreatic insulin mRNA changes over the estrous cycle. In rats there was a marked variation in insulin gene expression during the estrous cycle with the highest insulin expression observed during the proestrus day (Morimoto et al 2001). An explanation why insulin mRNA levels in our experiments were highest at estrus rather than proestrus could be in the uterine insulin has a different biological role to pancreatic insulin. If it is the case, as we suspect, that uterine insulin acts as a growth factor rather than for glucose metabolism then it would make sense that insulin is highest at estrus when the endometrium proliferates. Timing of tissue collection may also have a role. We collected tissue mid cycle from the animals when they were early in the cycle; usually rodents transition between stages of the estrous cycle in the middle hours of the night (Harkness et al 2009) while we were culling them in the early mornings. This may mean that we did not always collect tissue during the estrogen peak, however it is also important to point out there is considerable inter animal variation in the progression of the estrous cycle and collecting at night might not have guaranteed more effective capturing of each stage. Even when we gave ample time for the animal's cycles to

synchronise, it was not uncommon to find that several mice were at a different stage compared with their litter mates.

2.4.2 Insulin is produced in endometrial epithelial cells

We next looked for evidence of insulin synthesis to determine if the mRNA was transcribed. We found that insulin was expressed in both the epithelial cells, glands and stromal cells in the endometrium. At estrus and metestrus insulin could be clearly seen in all epithelial cells and some stromal cells. At diestrus it was very faint in the epithelial cells but some stromal cells had very clear expression. Interestingly at estrus the stromal cells closest to the epithelial cells and glands were more likely to express insulin. The opposite would occur at diestrus where the stromal cells furthermost from epithelial cells would express insulin. No insulin could be detected in any cells during proestrus.

The expression of the protein is not enough evidence to say that the endometrium is a site of insulin genesis. We looked for other markers of insulin production, the most common being C-peptide. As it is produced in equal proportion to insulin during the cleavage of preproinsulin, C-peptide is used as a proxy for insulin production when insulin is measured in people with type 1 diabetics to distinguish it from any injected insulin treatment (Khan et al 2011). Similarly, we used C-peptide to determine if and where the cleavage of preproinsulin occurs. C-peptide was expressed very strongly in the epithelial cells and glands. Measurements of pixel intensity showed that at proestrus expression was strongest and decreased at each subsequent stage. C-peptide could also be found in stromal cells with expression strongest at proestrus. Unlike with insulin, C-peptide was evenly distributed throughout the stroma independent of the cells proximity to epithelial cells.

For further evidence about the site of insulin production the tissue was probed for the peptidase responsible for cleaving preproinsulin, PC 1/3. Mouse endometrium did express PC 1/3 and its expression was strongest in epithelial cells at proestrus and estrus and decreased significantly at metestrus and diestrus. Our immunolocalisation experiments all showed that insulin, C-peptide and PC 1/3 are found in the endometrium and that expression is greatest at proestrus and estrus, suggesting that insulin production is influenced by estrogen. The epithelial cells and glands had higher levels of expression of insulin, C-peptide and PC 1/3 than stromal cells, marking them

as the most likely site of insulin production. Some stromal cells did express insulin in the later estrous stages but never C-peptide or PC 1/3 and this may mean that insulin was not produced by these cells, originating from the epithelial or pancreatic cells.

Small highly fluorescent granular areas suggest that insulin is tightly compressed inside the cell. In pancreatic cells insulin is stored in vesicles after production. The granules that can be seen in the endometrium suggest that in the endometrium insulin is also potentially stored in vesicles. However electron microscopy is needed to confirm if this is the case.

2.4.3 Insulin receptor expression in the endometrium.

Perhaps the most interesting observation is that the IR is very strongly expressed in the nucleus of the epithelial and stromal cells. This was very surprising as in the literature the IR is usually described as bound to the plasma membrane and we initially thought this was an error in our antigen retrieval protocol. After trying several different antigen retrieval methods the very strong nucleus staining persisted and we accepted it at a valid result. A recent study by Amaya et al (2014) provides some insight into the purpose of IR in the nucleus, suggesting that the IR is acting to facilitate insulin's mitogenic properties. In the liver insulin-induced increases in Ca^{2+} and cell proliferation required clathrin- and caveolin-dependent translocation of the IR to the nucleus where it would hydrolyze nuclear PIP2 and locally generate $InsP_3$-dependent Ca^{2+} signals there (Rodrigues et al 2008) Insulin's glucose regulating effects were independent of either of these events. Moreover, liver regeneration after partial hepatectomy also depended upon formation of $InsP_3$ in the nucleus but not the cytosol, whereas hepatic glucose metabolism was not affected by buffering $InsP_3$ in the nucleus. Further evidence that the nucleic IR is responsible for proliferation is that the receptor is activated by nucleoplasmic rather than cytosolic Ca^{2+} and that it's activation was necessary for hepatic cells to progress through the early prophase (Rodrigues et al 2007). As the uterus and the liver are both very highly regenerative organs it is possible that the function of nucleic IR in the endometrium is similarly mitogenic in nature.

The second observation is that while the IR is expressed in the epithelial cells at all stages of the estrous cycle, it is only expressed in stromal cells during metestrus and diestrus. This implies that there is less insulin signaling in stromal cells during proestrus and estrus. Richards et al using immunolocalization of P-Tyr and IRS-1 showed that it

was the epithelial cells of estrogen treated mice that were most reactive rather than stromal (Richards et al 1996). This supports the idea that insulin is mitogenic as epithelial cells proliferate under the influence of estrogen during proestrus and estrus. The expression on IR in both epithelial and stromal cells at metestrus and diestrus is perplexing. These stages are characterised by a release of progesterone, which inhibits endometrial growth (Kim et al 2013). Our immunocytochemical labeling showed that at metestrus and diestrus only a few stromal cells express insulin while nearly all cells express the IR, this may mean that even though the receptor is expressed there is no insulin available to activate it.

2.4.4 Detection of insulin in mouse uterine flushing.

To test if insulin is released at mating we analysed the uterine flushings of virgin mice in estrus and recently mated mice using a high sensitivity (0.2-10 ng/mL) HTRF based insulin assay. However no insulin could be detected. There are several explanations. Firstly insulin may be either bound to the spermatozoa or biologically broken down. Insulin has a half-life of 5-6 minutes in circulation (Duckworth et al 1998) and since mice mate generally mate in the middle of the night there was a period of 3-5 hours before we could collect the uterus. The second explanation is that insulin is transferred to the spermatozoa when they make contact with the epithelial cells rather than being indiscriminately secreted in to the uterine cavity. The last explanation is that uterine insulin is not secreted for the benefit of spermatozoa. In the next chapter we explore if insulin could affect mouse spermatozoa *in vitro*.

Chapter 3:

Effect of uterine insulin on spermatozoa and endometrial cells

Chapter 3 introduction

3.1 Introduction

The aim of this chapter is to better understand what role insulin could have on spermatozoa and the endometrium.

As previous reported by others in human (Lampiao & du Plessis, 2008) and porcine spermatozoa (Carpino 2008), we tested the hypothesis that insulin is beneficial to mouse spermatozoa, assessing their capacitation and longevity. Spermatozoa ejaculated from the epididymis are not ready or capable of fertilisation. They require time in the female reproductive tract to undergo a process called capacitation (Chang 1951). During capacitation the glycoprotein coat that surrounds the spermatozoa's membranes is removed, initiating changes in the surface charge of the membrane which gives them the ability to have increased hyperactivated motility, recognise and bind to the zona pellucida and to initiate the acrosome reaction (Florman and First, 1988, Aitken 1997). Capacitation can be induced *in vitro* by incubating spermatozoa in an appropriate media (Yanagimachi et al 1994). Capacitation is driven by reactive oxygen species (ROS) produced by the spermatozoa. The roles of ROS in capacitation is diverse and complex but initially during capacitation superoxide anion ($O2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) activate adenylyl cyclase that produces cAMP (De Lamirande and Gagnon 1995, Herrero et al 1999). This activates protein kinase A (PKA), which is necessary for the phosphorylation of tyrosine associated with successful sperm capacitation (Ecroyd et al 2003). Spermatozoa have very minimal oxidant defences and the accumulating levels of ROS will eventually overwhelm and induce apoptosis (Aitken et al 2014). Koppers et al have shown that apoptosis in spermatozoa is dependent on the activation of the PI3K/AKT pathway, when PI3K activity was inhibited the spermatozoa defaulted to an apoptotic state and a loss of motility (Koppers et al 2011). In our lab we previously discovered that the addition of insulin to human spermatozoa would preserve motility overtime and this was correlated to an increase in IR phosphorylation and PI3K activity (Aitken unpublished). A possible explanation is that insulin signals through the AKT/BAD pathways to prevent apoptosis. A similar mechanism has been suggested for prolactin, which has been designated a pro-survival factor for human spermatozoa as it stimulates the PI3K pathway and maintains the pro-apoptotic factor BAD in a phosphorylated inactive state (Pujianto et al 2010).

In the previous chapter we found that concentration of the insulin transcript was highest at proestrus and estrus. Similarly ICC showed that insulin, C-peptide and PC 1/3 were strongly expressed in epithelial cells at proestrus and estrus, these results all suggest that insulin production on the endometrium is mediated by estrogen. To further test this hypothesis we used a primary cell culture of mouse endometrium epithelial cells and incubated them with estrogen. We previously thought that mating could be the signal for insulin release however we were not able to detect any insulin in uterine flushings of mated mice. The traditional trigger for insulin secretion from the pancreas is glucose and therefore we aim to see if epithelial cells would react in a similar manner to glucose.

The other possibility is that insulin could have an autonomic signaling role in the endometrium. While glucose regulation is insulin's most important function we saw that the IR was more expressed in the nucleus of endometrial cells rather than the plasma membrane. This pattern of expression has been implicated as being important for cell proliferation (Rodrigues et al 2008, Amaya et al 2014). The major signaling pathways for insulin are the phosphatidylinositol 3-kinase (PI3K) pathway and the extracellular signal-regulated kinase (ERK)- mitogen activated protein kinase (MAPK) pathway. Both pathways are activated by growth factors and interact together in certain situations having an inhibitory effect on each other (Worster et al 2012). We hypothesised that insulin produced in the endometrium may autonomically activate the ERK-MAPK or PI3K pathways, and therefore we probed for phosphorylation of key proteins in these pathways. We used antibodies for pThr308Akt (Frank et al 1995) and pThr185/pTyr187 ERK (Cobb 1999) as we thought that these proteins would be activated early enough to not be ubiquitously expressed while at the same time not activated so late that they become influenced by other pathways.

Chapter 3 methods

3.2.1 Animals

This study used male and female Swiss mice (8-12 weeks). Mice were euthanised using CO_2 gas for 2-4 minutes. Female mice were exsanguinated by perfusion with warm PBS.

3.2.2 Isolation of spermatozoa

Immediately after adult male mice (>8 wk old) were euthanised, the epididymides were removed from the abdominal cavity carefully dissected free of fat and overlying connective tissue. The caudal region was isolated, blotted free of blood, and immersed under prewarmed water-saturated mineral oil. Spermatozoa were flushed out of the cauda by backflushing, a process that allows for collection of spermatozoa relatively free of leukocyte contamination. Backflushing involved placing the entire male reproductive track under a dissecting microscope with a magnification range in the order of 5-40X. A cannula was inserted into the vas deferens and a knot with black braided treated silk was tied around the cannulated vas deferens. A small opening was made in the cauda using a scissors through which spermatozoa can be expelled. Air was pushed through the cannula using a 3ml syringe, forcing spermatozoa out where they were drawn into the glass capillary by gentle suction. Spermatozoa were expelled from the glass capillary into 1 ml solution of BWW media (see appendix) (37 °C) by gently blowing into the mouthpiece.

3.2.3 Capacitation of spermatozoa

To capacitate spermatozoa were incubated in BWW and a positive control was incubated with BWW supplemented with 3mM pentoxifylline and 5mM dibutyryl cyclic AMP (dbcAMP). As a negative control BWW/-HCO_3^- was used as a negative control to generate a population of non-capacitated spermatozoa. 100Ng/ml, 500ng/ml and 1000ng/ml of insulin was added as a treatment.

Spermatozoa were capacitated by incubation for 90 min at 37°C under an atmosphere of 5% CO_2: 95% air. At regular intervals throughout the incubation, sperm suspensions

were gently mixed to prevent settling of the cells. At the end of this period capacitation was assessed by immunostaining spermatozoa for phosphorylated tyrosine.

3.2.4 Spermatozoa motility assessment

Spermatozoa motility was assessed by placing 10µl of the cell suspension on a clean slide and covering them with a cover slip. The slide was inserted into the Computer Assisted Sperm Assessment device and counted. Spermatozoa motility was expressed as total and progressive motility. Total motility refers to all moving spermatozoa while progressive motility refers to spermatozoa that are swimming from one location to another as opposed to twitching or circular swimming,

3.2.4 Endometrial epithelial cell extraction and cell culture

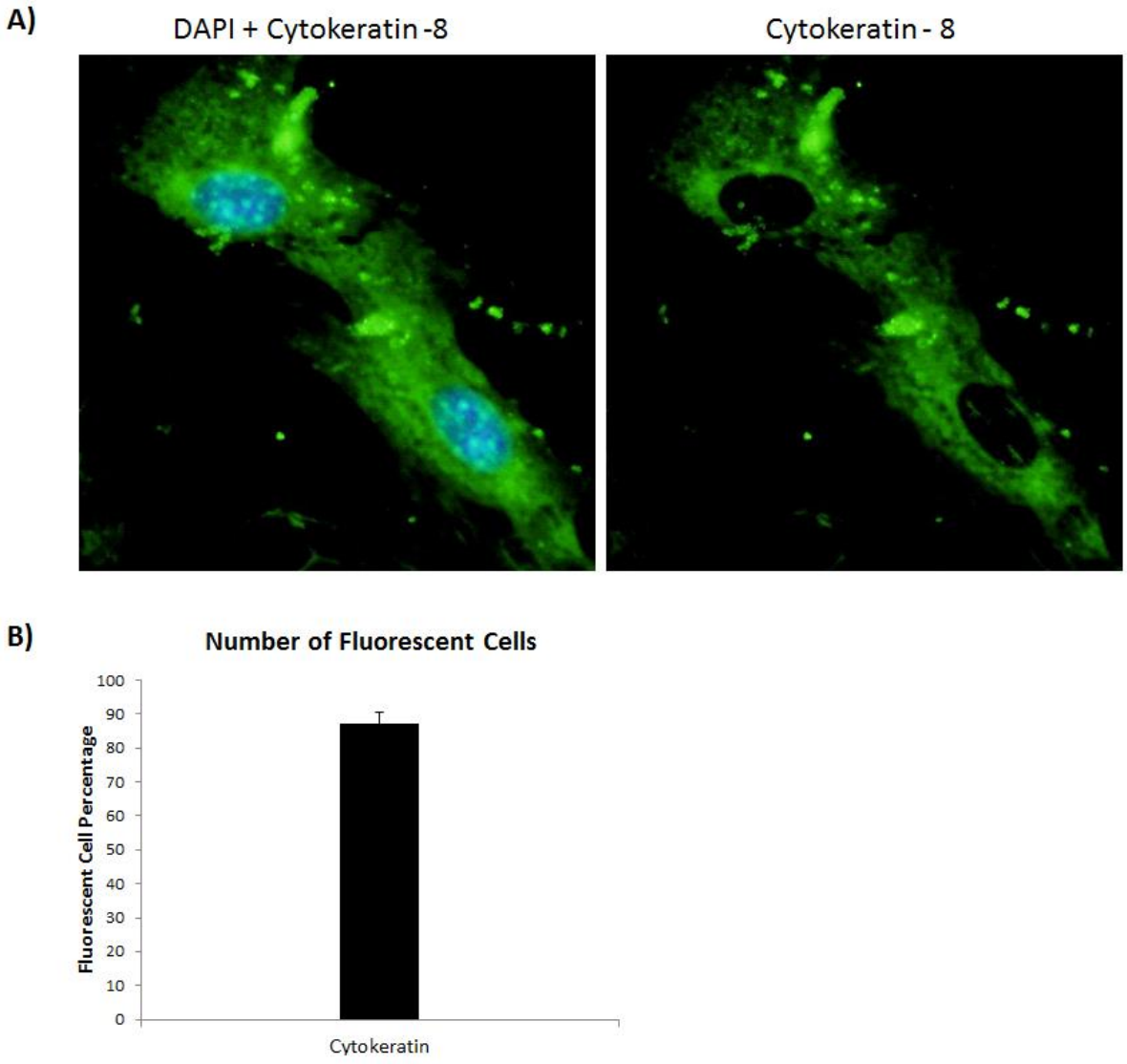
Endometrial epithelial cells were extracted and cultured using protocol developed by Toomyaka et al (1986) with some minor variations in the composition of the culture media.

Swiss mice (2-3 months of age) were perfused with PBS. Uteri were removed and cut open length wise. They were then incubated in 3.5% trypsin for 1h at -4°C and then 1h at room temperature before being transferred to ice cold Hanks Buffered Salt Solution (HBSS), 500ul of HBSS was suitable for two uteri. Uteri were then vortexed for 5 minutes to release sheets of epithelial cells. Afterwards uteri were transferred to fresh HBSS and the vortex process was repeated two more times. HBSS containing epithelial cells was centrifuged at 0.5g for 10 minutes to form an epithelial cell pellet at the bottom of the Eppendorf tube. Supernatant was removed and epithelial cells were re-suspended in DMEM:F12 + 10% Horse Serum and 5% penicillin streptomycin.

Epithelial cells were plated in a 24 well cell culture plate containing matrigel coated cover slips. Each repetition had five groups, an untreated control, three treatment groups and another untreated group that would act as a negative control for ICC experiments. Cells were cultured in DMEM:F12 + 10% Horse Serum and 5% penicillin streptomycin. The cells formed a monolayer on the matrigel within 1-2 days. After two days culture media was replaced with fresh culture media, also removing any detached cells. At this stage the cells were treated with either 40 pg/ml estrogen (β-estradiol), 15mg/ml glucose

or 40 pg/ml estrogen + 15mg/ml glucose. The old culture media was centrifuged at 0.5g to pallet the cells, which were then replated into their respective wells.

Purity of cell culture was verified by immunostaining cells for cytokeratin 8, protein exclusive to epithelial cells, and then counting number of fluorescent cells. Cell counts of four biological and technical replicates showed that our primary cell culture had a



purity of 85% (figure 3.2.1)

Figure 3.2.1: Immunofluorescence of cytokeratin-8 in mouse epithelial primary cell culture. A) Epithelial cells expressed cytokeratin -8. Mouse endometrial epithelial primary cells were isolated, plated in wells containing matrigel coated cover slips and given two days to adhere in DMEM:F12 + 10% FBS culture media. After two days

media was replaced with fresh culture media. After a further two day incubation cells were fixed in 4% paraformaldehyde, permeabilised with 3% Tween 20 in PBS and blocked with 3% BSA in PBS. They were then incubated with 1:100 anti-cytokeratin-8 antibody followed by 1:2000 DAPI. Images were visualised with fluorescence microscopy using a 40x objective lens and are representative of four independent experiments. B) On average 85% of cells stained positive for cytokeratin-8, implying that our culture was mostly pure. 100 cells were counted by eye and number of fluorescent cells recorded.

3.2.5 Immunocytochemistry of epithelial cell culture

Primary cultured epithelial cells were fixed with 4% paraformaldehyde for 10 min, permeabilised with PBS containing 2% BSA and 3% tween for 20 minutes. Cells were blocked with PBS + 3% BSA for one hour at room temperature then incubated with primary antibody overnight at 4°C. On the following day primary antibody was washed off with PBS and slides were incubated with Alexa Fluor 488 for one hour. Cells were again washed in PBS before being incubated with 1:2000 DAPI for 10 minutes. Cells were washed three times in PBS before being mounted on slides with 2µl mowiol. Purity of cell culture was checked by staining cells with cytokeratin-8 antibody, a protein that is exclusive to epithelial cells, and counting number of fluorescent cells. Epithelial cells composed 85% of the primary cell culture (figure 3.2.1). Omitting the primary antibody to create secondary only negative controls showed no to little nonspecific staining (figure 3.2.2)

Immunocytochemistry of spermatozoa

Spermatozoa cells were fixed with 4% paraformaldehyde for 10 min. They were then washed three time in PBS by centrifuging cells for minutes at 3000g and removing the supernatant. They were then permeabilised with PBS containing 2% BSA and 3% tween for 20 minutes and washed three more times as before. 50µl of solution containing spermatozoa was deposited on small round cover slips that were precoated with poly-l-lysine. Cells were allowed to settle overnight. The next day cells were permeabilised with 0.3% Triton-X for 15 minutes. They were then washed three times and then blocked for 1 hour in PBS + 3 % BSA for one hour at room temperature. Cells were then incubated with primary antibody overnight at 4°C. On the following day cells were washed three times with PBS and incubated with 1:200 Alexa Fluor 488 for one

hour. Cells were again washed in PBS before being incubated with 1:2000 DAPI for 10 minutes. Cells were washed three times in PBS before being mounted on slides with 2µl mowiol.

Antibody	Manufacturer	Species	Dilution
Insulin monoclonal	Abcam	Rabbit	1:100
C Peptide polyclonal	Abcam	Rabbit	1:200
Insulin Receptor polyclonal	Abcam	Rabbit	1:100
Anti- pThr185/pTyr187 ERK monoclonal	Sigma	Rabbit	1:200
Anti-pThr308 AKT	Abcam	Mouse	1:100
monoclonal	Sigma	Rabbit	1:100
Anti-Cytokeratin8 polyclonal	Sigma	Mouse	1:100
Anti-pTYR monoclonal			

Table 3.2.1: List of antibodies used for immunocytochemistry

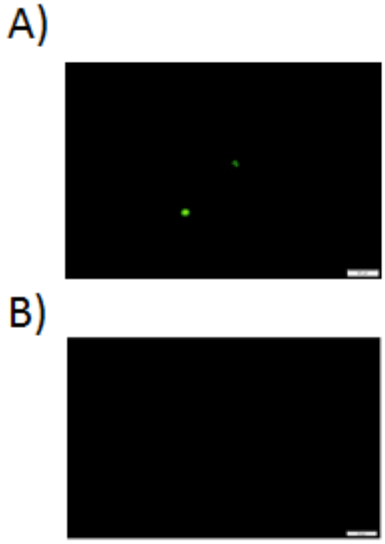


Figure 3.2.2: No Primary negative controls of antibodies. Images of 1:200 Alexa Fluor 488 in; A) primary epithelial cell culture, B) Spermatozoa.

3.2.6 Insulin measurement

Culture media was removed and stored for measurement. Cells were washed in 500µl ice cold PBS for five minutes. PBS containing the cells was aspirated into 1.3ml Eppendorf tube. 300ml of NP-40 was added to each tube and cells were agitated for 30 minutes at 4°C. Tubes were then centrifuged at 12,000 rpm at 4°C for 20 minutes.

Supernatant was collected and then put in a centrifugal evaporator for ~1 hours until around 100µl of supernatant remained.

Insulin was measured in supernatant using a Cisbio HTRF® Insulin assay as mentioned in Chapter 2.4.4

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3.2.7 Statistics

Experiments were replicated with material collected from at least three different animals, and the data are presented as mean ± SEM. Statistical analysis was performed using the analysis of variance (ANOVA) function in JMP 11 statistics software. The existence of statistically significant differences between control and variable groups was tested with a Control Dunnets comparison test. Samples with a *P*-value <0.05 were considered statistically significant, one asterisk denotes a *P*-value <0.05, two asterisk denotes <0.001 and three denotes <0.0001.

3.3.1 Effect of Insulin on mouse spermatozoa

Our initial hypothesis was that uterine insulin is released post-coitus to act as a pro-survival factor on spermatozoa. Previously co-incubation of insulin with human spermatozoa resulted in prolonged motility (Aitken unpublished). Due to species variation it is unknown if mouse spermatozoa would react in a similar manner.

ICC was used to assess the impact of insulin on capacitation by measuring tyrosine phosphorylation in the tail (Figure 3.3.1.1). The near absolute majority of spermatozoa in both the control and insulin treated groups had strong fluorescence in the tail. Negative control had no fluorescence. This result implies that insulin does not have a negative effect on capacitation.

Next we looked at the possibility that insulin could extend a spermatozoon's lifespan by measuring the effect insulin exposure has on motility (Figure 3.3.1.2**)**. Mouse spermatozoa were incubated for two and half hours in media containing 100µM, 10µM and 100nM insulin prior to assessment of total and progressive motility using computer assisted sperm assessment (CASA).

Addition of insulin did not preserve sperm motility. Instead insulin treated groups mostly had no statistical difference compared to control with some exceptions. At 120 minutes spermatozoa treated with10µM insulin were significantly more motile than control (*P*-value = 0.0037) however when treated with 100nM insulin they were significantly less motile (*P*-value = 0.0355). Progressive motility was similarly largely unaffected by insulin. At 120 minutes 10µM insulin group was short of being statistically significant (*P*-value = 0.21). At 150 minutes both the 10µM and 100nM insulin treatment groups were significantly lower compared to control (*P*-value = 0.006 and 0.0204).

We made the assumption that mouse spermatozoa expressed the IR but after not observing any positive effect of insulin we were less sure and needed to confirm the expression of the IR using ICC. In all cells there was very strong expression in the mid piece, and moderate expression in the tail (Figure 3.3.1.3).

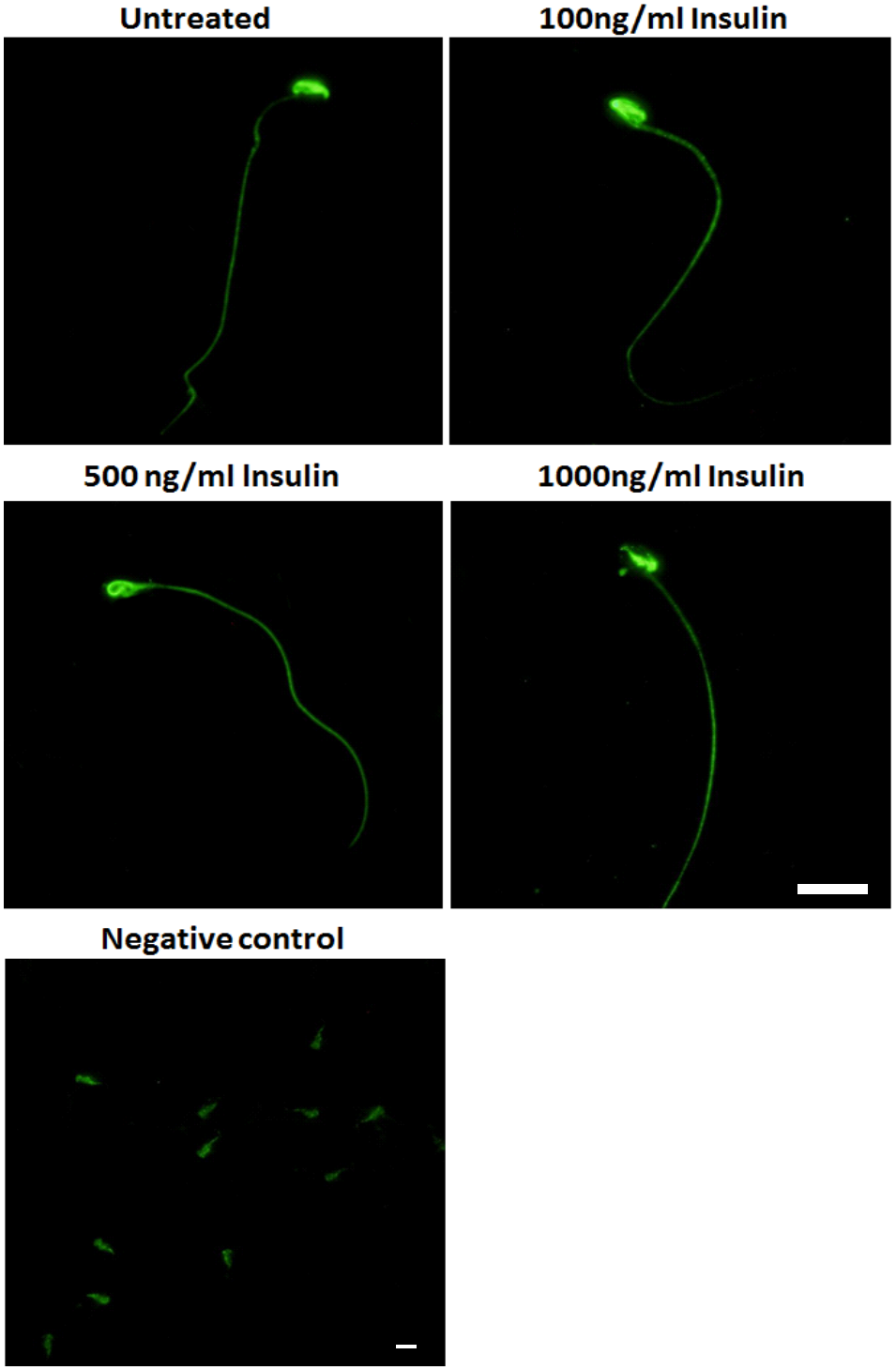

Figure 3.3.1.1 Effect of insulin dosage on capacitation. Spermatozoa in all treatment groups successfully underwent capacitation as shown by strong tyrosine phosphorylation in the tail. Capacitation was seen in just about all spermatozoa regardless of treatment. Negative control shows only fluorescence in the head. Spermatozoa were capacitated by incubation for 90 min in BWW at 37°C under an atmosphere of 5% CO2: 95% air, negative control was incubated with BWW/-HCO3-. Images represent average spermatozoa as seen in three separate experiments. Scale bar represent 10μm

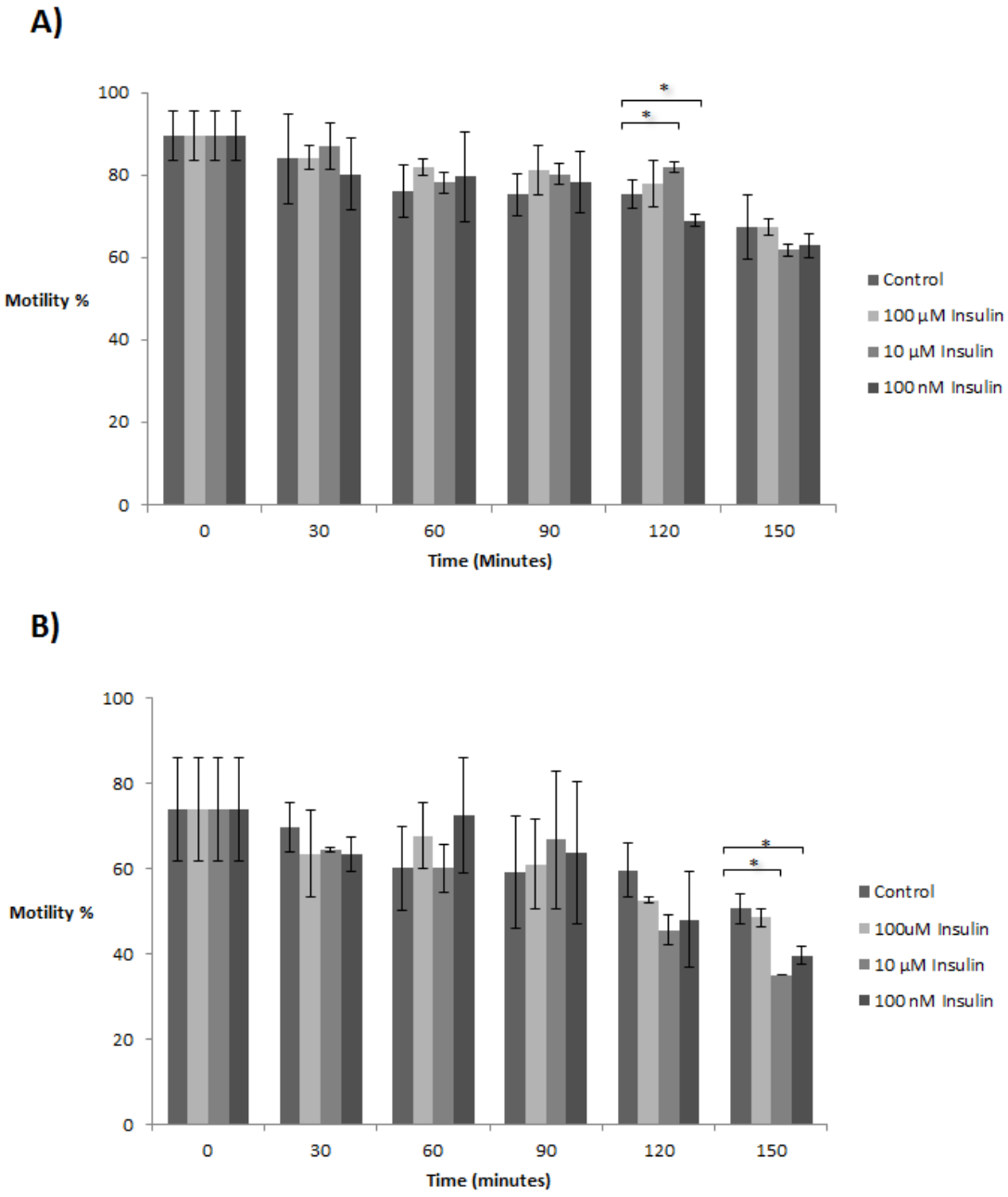

Figure 3.3.1.2: Effect of insulin on mouse spermatozoa motility and viability. Capacitated mouse spermatozoa were treated with either 100µM, 10µM or 100nM insulin. Motility, total (A) and progressive (B), was measured every 30 minutes for 150 minutes. There was generally little improvement in total motility compared to control with the exception of 10µM and 100M insulin at 120 minutes, though this did not remain at 150 minutes. Progressive motility generally declined, being statically lower at 150 minutes compared to control. Motility was assessed using CASA software.

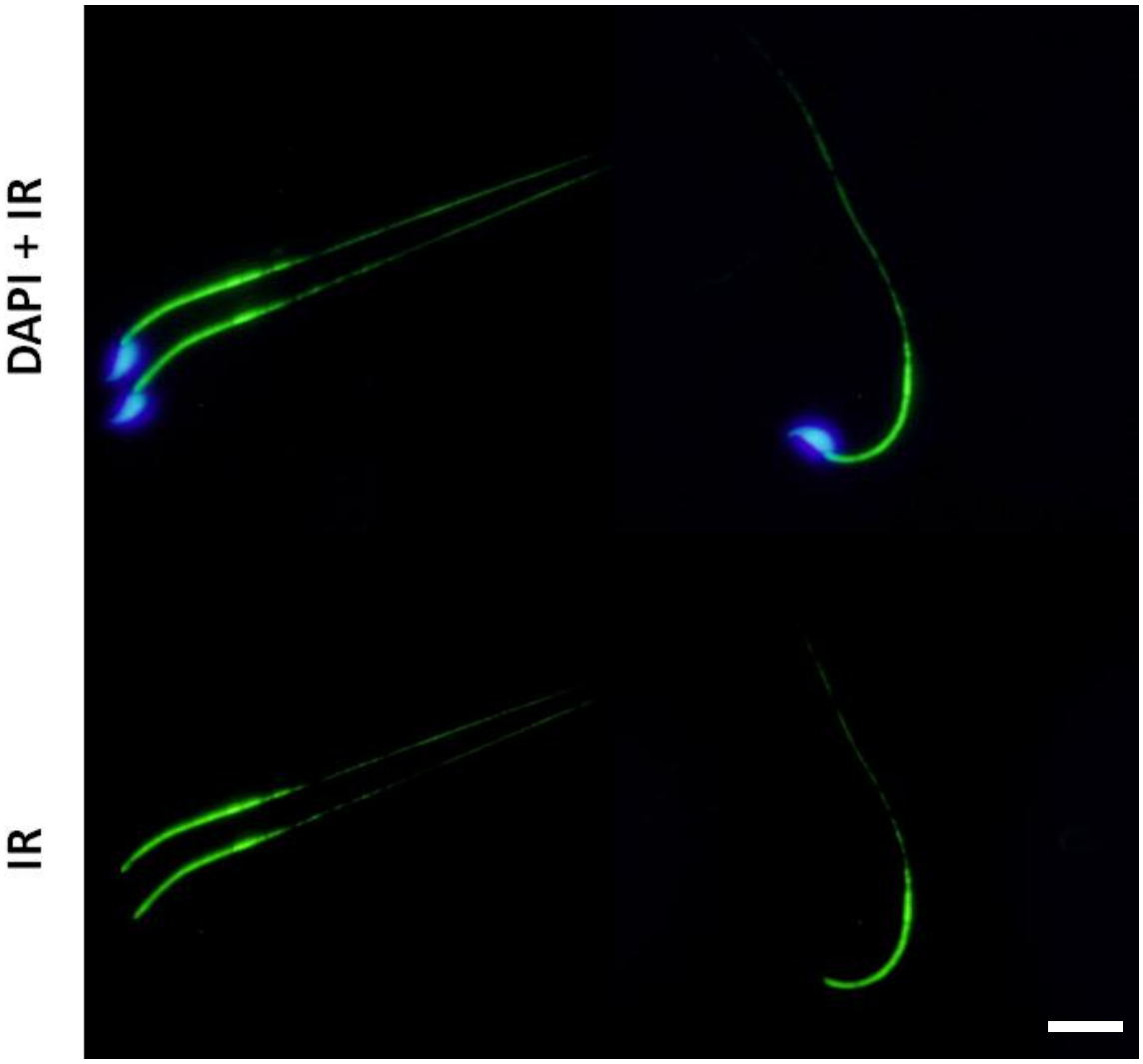

Figure 3.3.1.3: Immunofluorescence images of IR in capacitated mouse spermatozoa. Insulin was localised strongly to the mid piece and tail. No fluorescence was seen in the head. Spermatozoa cells were fixed with 4% paraformaldehyde for 10 min. Permeabilised with 3% Tween 20 in PBS and blocked with 3% BSA in PBS. They were then incubated with 1:200 anti-IR antibody followed by 1:2000 DAPI. Images were visualised with fluorescence microscopy using a 40x objective lens and are representative of three independent experiments. Scale bar represent 10μm

3.3.2 Insulin production in primary epithelial cell culture

We developed a primary cell culture of mouse endometrial epithelial cells and attempted to replicate the insulin expression we saw *in vitro* to better understand the effect insulin has on the endometrium. To test if estrogen is able to affect endometrial epithelial cells they were incubated with several concentrations of β-estradiol, 40pg/ml, a concentration at the higher end of that found in mouse circulation during estrus (Ström et al 2012) and two higher concentrations 100 pg/ml and 200 pg/ml. After two days of incubation with β-estradiol, ICC was performed to assess the presence of the mature insulin hormone (Figure 3.3.2.1). As shown, significantly more cells in the estrogen-treated groups labelled positive for insulin compared to that of the control cell population, 40pg/ml estrogen (P-value = 0.0174), 200pg/ml (P-value = 0.204). 100 pg/ml dosage was not significant (P-value = 0.114). There was no statistically significant difference between the different estrogen dosage groups. Although some cells were labelled in the control group, these tended to be smaller (<20μm) and separated from the majority of other cells, while the larger cells (>50um) among this population tended to have no/very weak fluorescence. In all cells, insulin staining was localised to the cytoplasm. Interestingly the strongest staining was around the peri-nuclear domain, a result that differs from what was previously seen in the uterus sections where the staining was strongest on the luminal border of the epithelial cells. Pancreatic cells respond to glucose by secreting insulin to test if epithelial cells would respond in a similar manner we supplemented the culture media with an additional 5mg/ml glucose for a total of 15mg/ml of glucose. Cell cultures supplemented with glucose had a modest, but not statistically significant, increase in the number of labeled cells compared to the control (Figure 3.3.2.2). Co-incubation with both estrogen and glucose led to more fluorescent cells then both the control and glucose supplemented group but were just outside significance (P-value = 0.0876). A HTRF based insulin assay was used to measure any insulin secreted into the media. However no insulin could be detected in the media (<0.35ng/ml). We next lysed the cells in our culture and measured the lysate. Insulin was detected in the control, estrogen and glucose treated groups (Figure 3.3.2.3). After normalizing the results according to protein quantification the estrogen treated cells were short of statistical significance compared to the control (P-value = 0.341), as were glucose treated cell (P-value = 0.901). Insulin in the glucose and estrogen treated group was detected at below the sensitivity range of the assay (<0.35ng/ml).

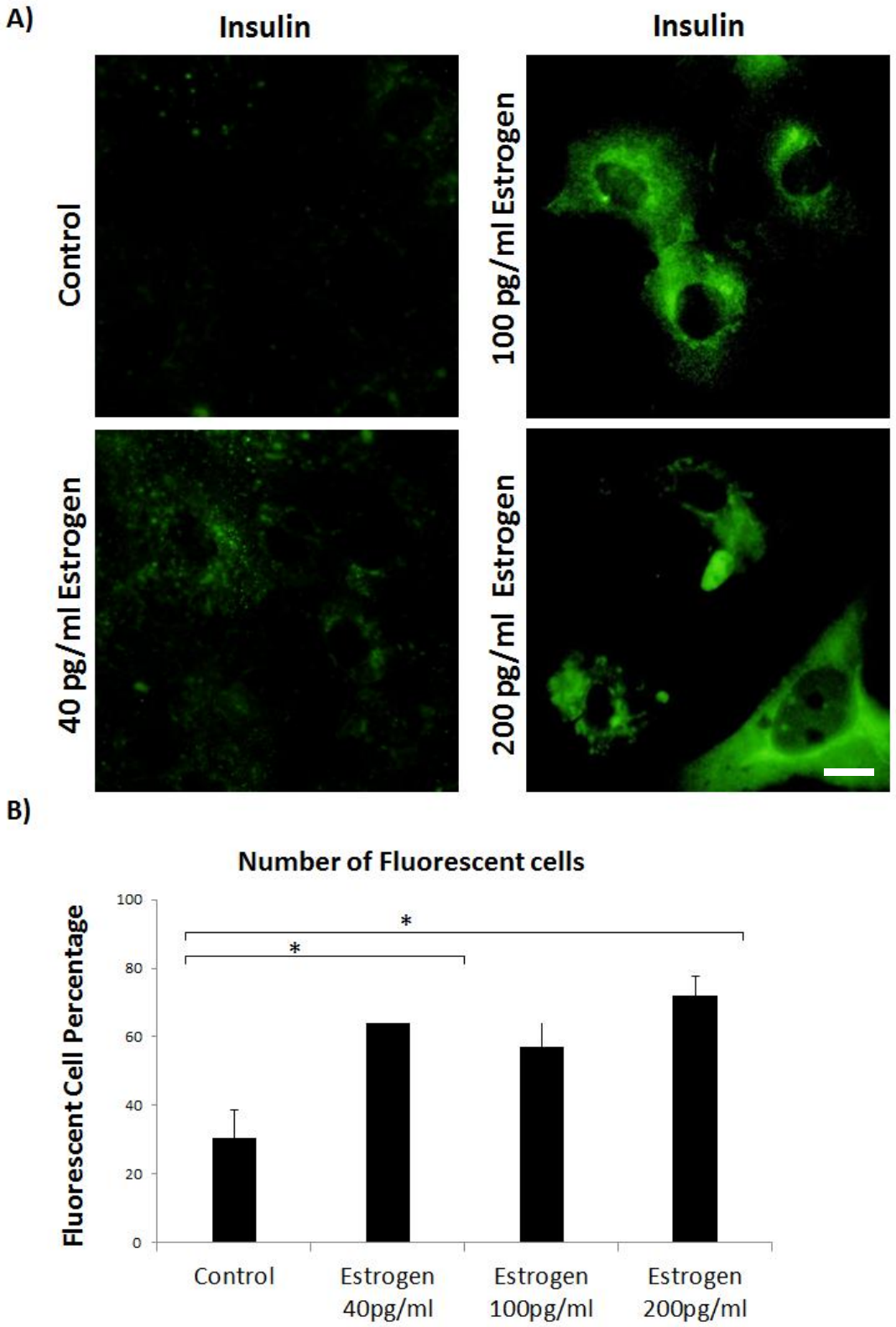

Figure 3.3.2.1: Immunofluorescence images of insulin in estrogen treated mouse epithelial primary cell culture. A) Estrogen treatment resulted in some epithelial cells expressing insulin. Mouse endometrial epithelial primary cells were isolated, cultured as described in chapter 3.2. After two days media was replaced with fresh culture media or fresh culture media containing either 40 pg/ml, 100 pg/ml or 200 pg/ml 17β-estradiol.

After a further two day incubation cells were fixed in 4% paraformaldehyde, permeabilised with 3% Tween 20 in PBS and blocked with 3% BSA in PBS. They were then incubated with 1:100 anti-insulin antibody followed by 1:2000 DAPI. Images were visualised with fluorescence microscopy using a 40x objective lens and are representative of three independent experiments. B) Estrogen treatment increased number of fluorescent cells. 100 cells were counted by eye from three independent experiments and number of fluorescent cells recorded. Asterisks denotes a statistically significant difference between groups, analysis was performed by an ANOVA followed by a Control Dunnets comparison test. Scale bar represents 20μm.

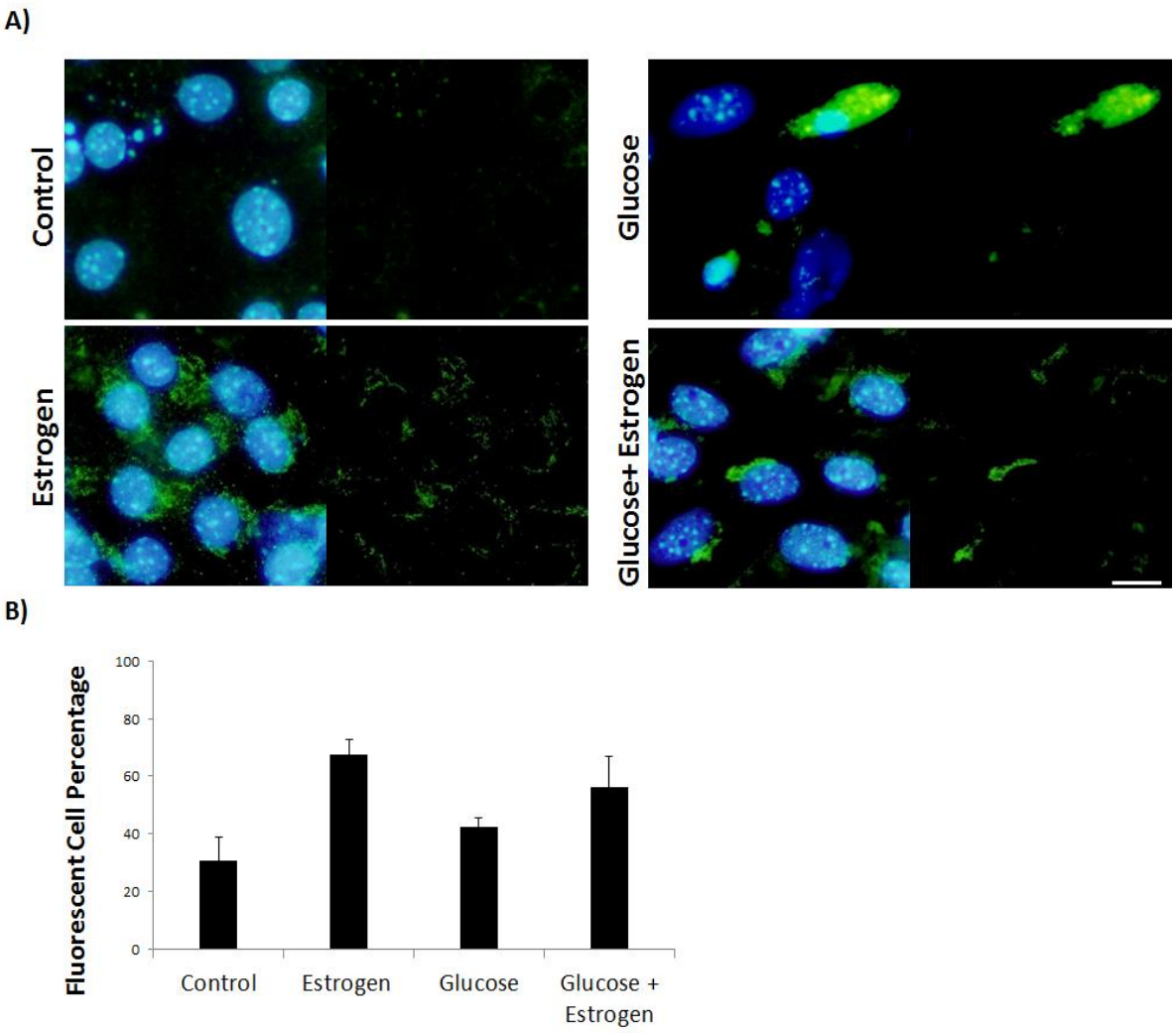

Figure 3.3.2.2: Immunofluorescence images of Insulin in mouse epithelial primary cell culture. A) Estrogen treatment resulted in some epithelial cells expressing insulin. Mouse endometrial epithelial primary cells were isolated, cultured as described in chapter 3.2. After incubation cells were fixed in 4% paraformaldehyde, permeabilised with 3% Tween 20 in PBS and blocked with 3% BSA in PBS. They were then incubated with 1:100 anti-insulin antibody followed by 1:2000 DAPI. Images were visualised with fluorescence microscopy using a 40x objective lens and are representative of three independent experiments. B) Estrogen treatment increased number of fluorescent cells. 100 cells were counted by eye from three independent experiments and number of fluorescent cells recorded. Asterisks denotes a statistically significant difference between groups, analysis was performed by an ANOVA followed by a Control Dunnets comparison test. Scale bar represents 20µm.

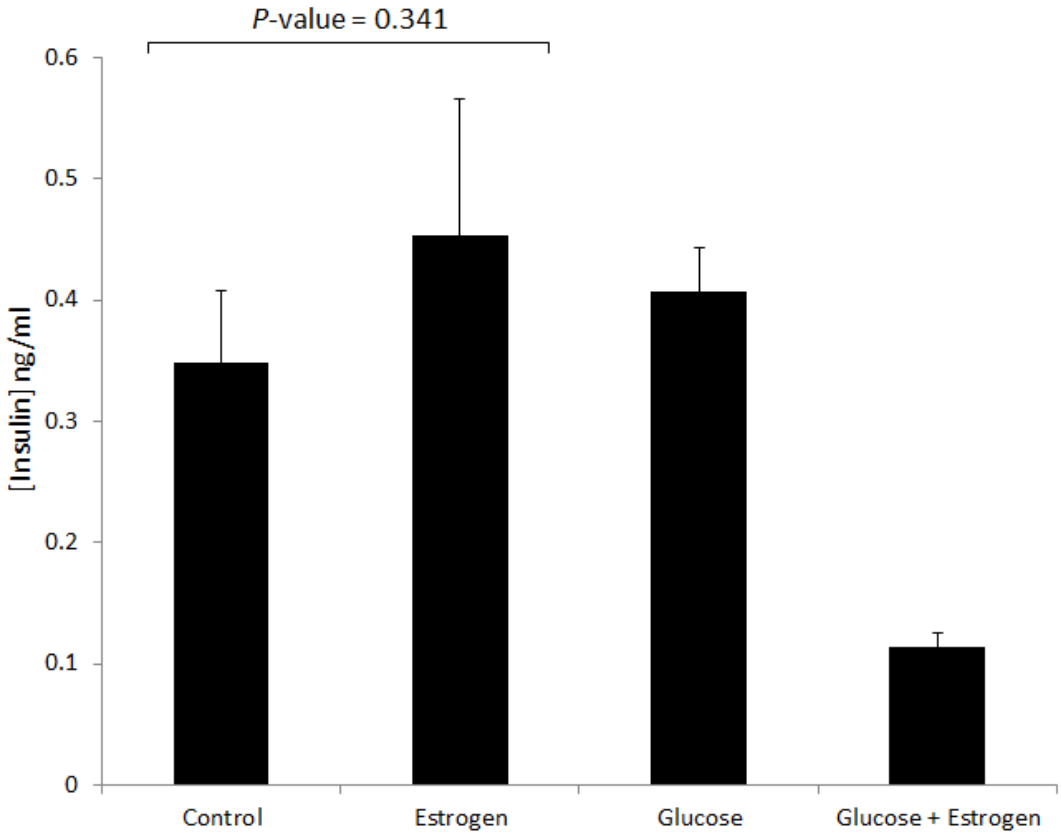

Figure 3.3.2.3: HTRF quantification of insulin from lysed mouse epithelial primary cells. Insulin could be detected in control and estrogen, and glucose treatment groups. While estrogen treated cells measured more insulin the increase was not statistically significant. Mouse endometrial epithelial primary cells were isolated, plated in wells containing matrigel coated cover slips and given two days to adhere in DMEM:F12 + 10% FBS culture media. After two days media was replaced with fresh culture media or fresh culture media containing either 40 pg/ml estrogen, 15mg/ml glucose or 40 pg/ml estrogen and 15mg/ml glucose. After a further two day incubation culture media was removed and cells were lysed in NP-40 before being quantified by a HTRF based insulin assay using a BMG Labtech PHERAstar FS reader. Values represent three biological replicates. Statistical analysis was performed by an ANOVA followed by a Control Dunnets comparison test.

3.3.3 C-peptide production in primary epithelial cell culture

It is possible that the insulin found in the epithelial cells originated from an outside source. To confirm that insulin is produced inside the cells ICC with anti-C-peptide antibodies was performed (Figure 3.3.3.1). In the estrogen group most cells showed fluorescence, though it was more frequent in smaller cells (<20µm) that clustered together in groups for 3-15. In the control group fluorescence was less common than in the estrogen group, limited to small cells clustered together. Interestingly C-peptide in the glucose groups was seen in the cytoplasm of both the smaller cell but also in the larger (>20µm), spaced cells. However there was no statistically significant difference in the number of fluorescent cells between the four groups but there appears to be a trend of more C-peptide fluorescent cells when exposed to estrogen and glucose. It is not immediately clear where C-peptide is localised as the cells overlapped but generally it appears to be in the cytoplasm.

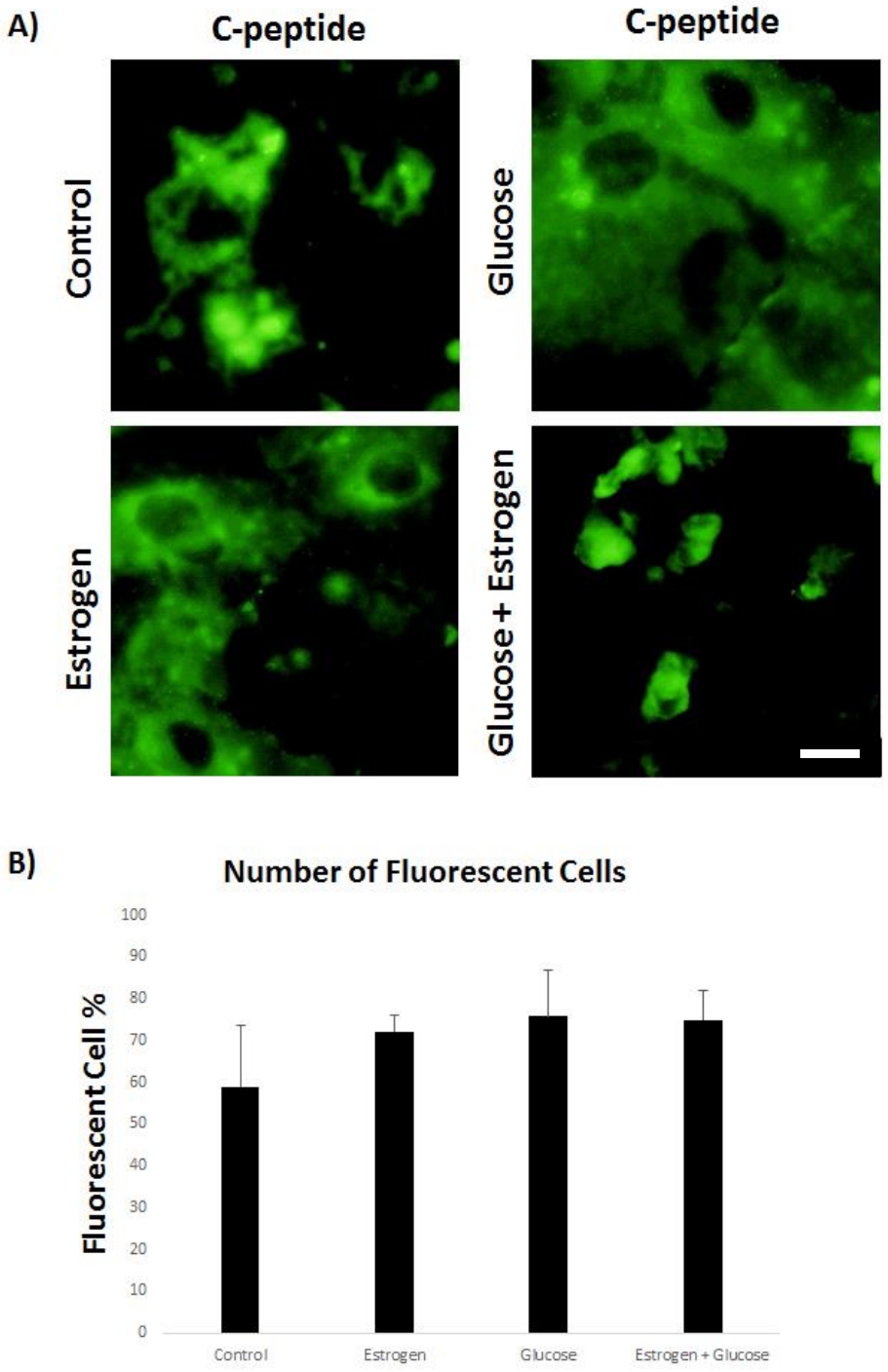

Figure 3.3.3.1: Immunofluorescence of C-peptide in mouse epithelial primary cell culture. A) Mouse endometrial epithelial primary cells were isolated, cultured as described in chapter 3.2. After incubation cells were fixed in 4% paraformaldehyde, permeabilised with 3% Tween 20 in PBS and blocked with 3% BSA in PBS. They were then incubated with 1:200 anti-C-peptide antibody. Images were visualised with fluorescence microscopy using a 40x objective lens and are representative of three independent experiments. B) Estrogen, glucose or estrogen and glucose treatment did not lead to a statistically significant increase in the number of fluorescent cells

compared to control. 100 cells were counted by eye from three independent experiments and number of fluorescent cells recorded. Analysis was performed by an ANOVA followed by a Control Dunnets comparison test. Scale bar represents 20μm.

3.3.4 IR expression in primary epithelial cell culture

To determine if insulin could have an autonomic effect we needed to know if the cells in our primary culture also expressed the insulin receptor (IR). The IR was localised to the cytoplasm in both control and estrogen treated cells (Figure 3.3.4.1). Some cells showed expression in the nucleus but this was nearly always only in smaller (<20µm) cells. The fluorescence was equally strong and widespread in both groups, implying that the IR is expressed independent of estrogen.

Because the IR was strongly expressed independent of treatment we conclude that any insulin produced by the epithelial cells will have a target for signaling and our next step was to try to determine which pathway becomes activated.

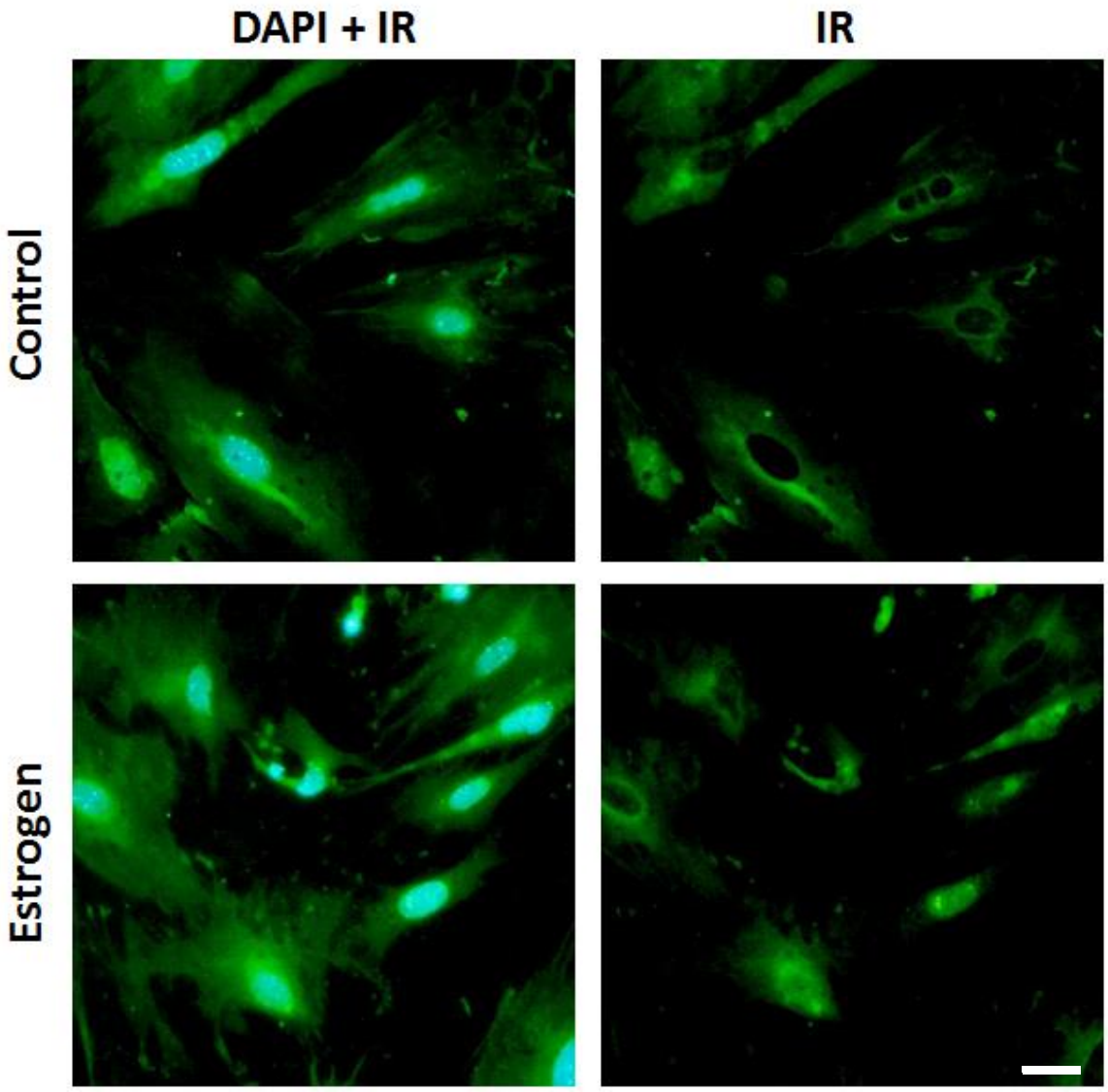

Figure 3.3.4.1: Immunofluorescence of IR in mouse epithelial primary cell culture. All (>95%) cells expressed IR in both control and estrogen supplemented media. Mouse endometrial epithelial primary cells were isolated, cultured as described in chapter 3.2. After incubation cells were fixed in 4% paraformaldehyde, permeabilised with 3% Tween 20 in PBS and blocked with 3% BSA in PBS. They were then incubated with 1:200 anti-IR antibody. Images were visualised with fluorescence microscopy using a 40x objective lens and are representative of three independent experiments. Scale bar represents 20μm.

3.3.5 Insulin signaling in primary epithelial cell culture

Almost all cells (>80%) expressed pThr308Akt independent of treatment. There was no statistically significant difference between the treatment groups and control (Figure 3.3.5.1). The fluorescence was localised to both the cytoplasm but also strongly in the nucleus.

Similarly pThr185/pTyr187 ERK was also widely expressed (Figure 3.3.5.2). There was no statistical significant differences between the treatment groups and control, the biggest difference was in the estrogen + glucose treatment (P-value = 0.1704). The fluorescence was localised to the cytoplasm and the nucleus.

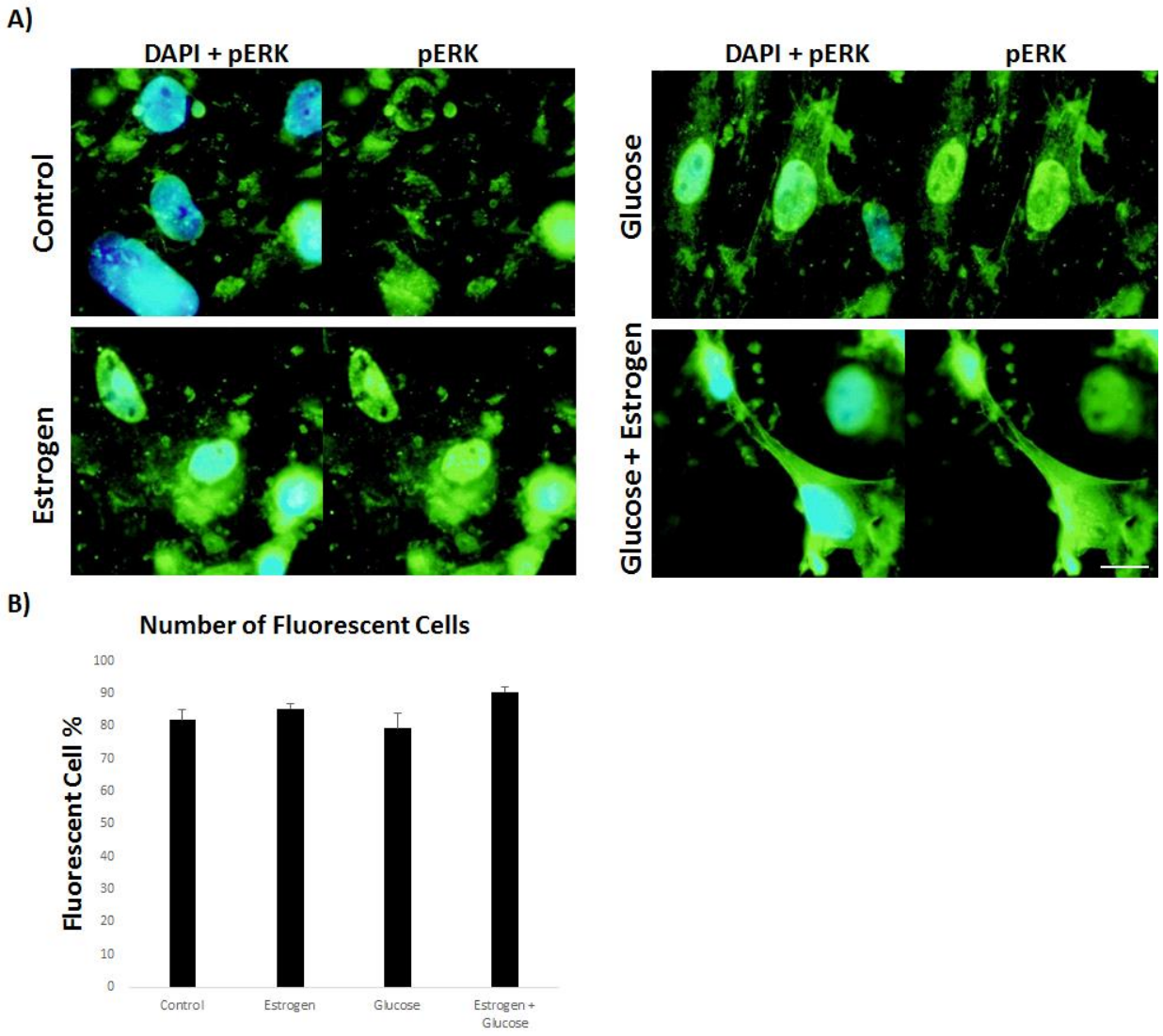

Figure 3.3.5.1: Immunofluorescence of pThr185/pTyr187 ERK in mouse epithelial primary cell culture. Epithelial cells expressed pThr185/pTyr187 ERK independent of treatment. Mouse endometrial epithelial primary cells were isolated, cultured as described in chapter 3.2. After four days cells were fixed in 4% paraformaldehyde, permeabilised with 3% Tween 20 in PBS and blocked with 3% BSA in PBS. They were then incubated with 1:100 anti- pThr185/pTyr187 ERK antibody. Images were visualised with fluorescence microscopy using a 40x objective lens and are representative of three independent experiments. B) There was no statistical difference in pThr185/pTyr187 ERK expression. Statistical analysis was performed by an ANOVA followed by a Control Dunnets comparison test. Scale bar represents 20μm.

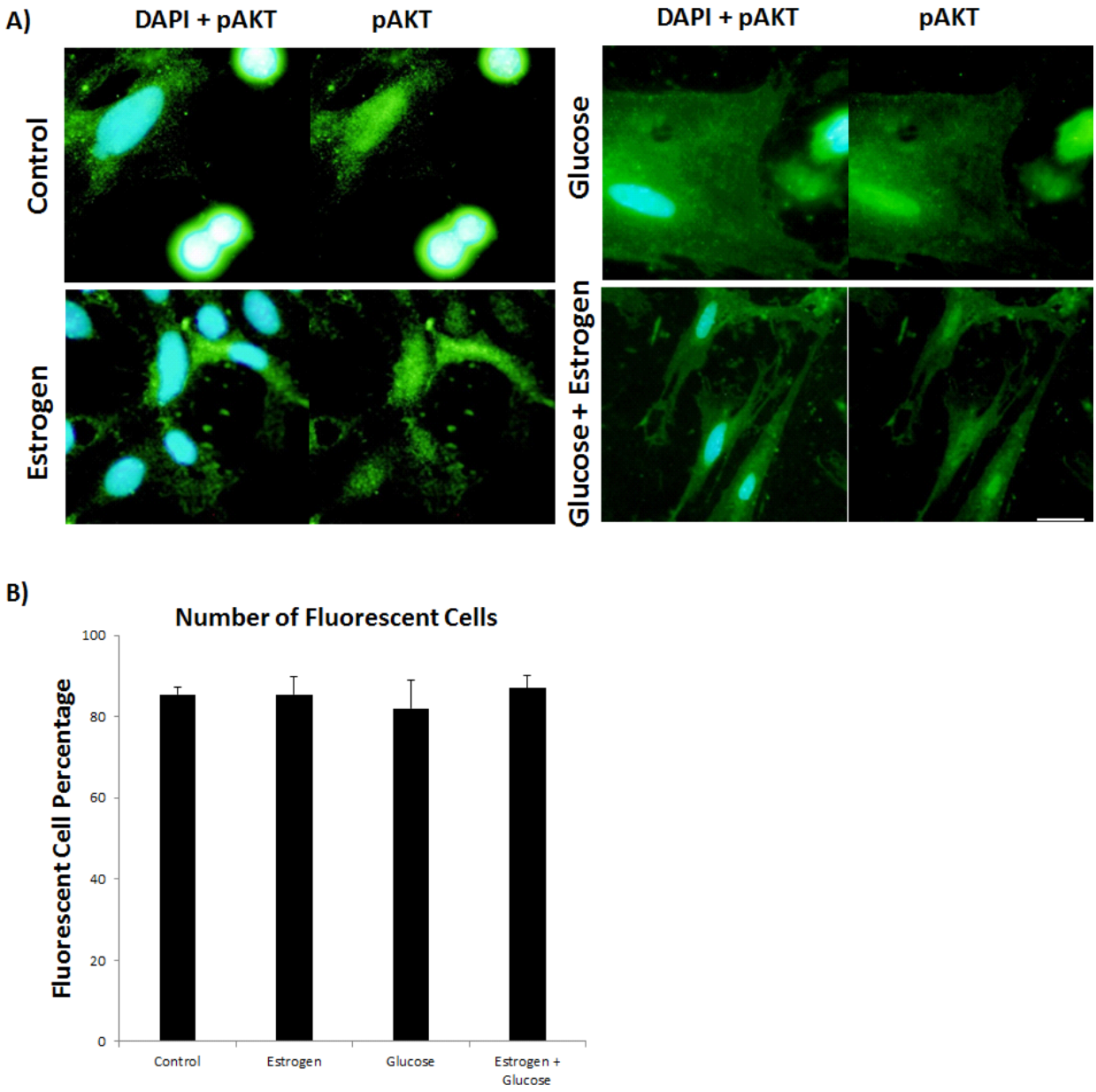

Figure 3.3.5.2: Immunofluorescence of pThr308 AKT in mouse epithelial primary cell culture. Epithelial cells expressed pThr308 AKT independent of treatment. Mouse endometrial epithelial primary cells were isolated, cultured as described in chapter 3.2. After a four days cells were fixed in 4% paraformaldehyde, permeabilised with 3% Tween 20 in PBS and blocked with 3% BSA in PBS. They were then incubated with 1:200 anti-pThr308 AKT antibody. Images were visualised with fluorescence microscopy using a 40x objective lens and are representative of three independent experiments. B) There was no statistical difference in pThr308 AKT expression. Statistical analysis was performed by an ANOVA followed by a Control Dunnets comparison test. Scale bar represents 20µm.

3.4.1 Mouse spermatozoa are unresponsive to insulin.

Spermatozoa need to undergo the process of capacitation to acquire the ability to have increased hyperactivated motility, recognise and bind to the zona pellucida and to initiate the acrosome reaction (Florman and First, 1988, Aitken 1997). However motility generates reactive oxygen species, gradually raising the levels of oxidative stress inside the spermatozoa until the cell is overwhelmed and undergoes apoptosis (De Lamirande and Gagnon 1995). Therefore there are only two possible fates for a spermatozoa once it has undergone capacitation, successfully fertilise an oocyte or undergo apoptosis. Prolonging the time a spermatozoa can remain motile before apoptosis will increase the chances of it encountering an oocyte.

Previously in our lab we found that human spermatozoa incubated with insulin would remain motile for longer. Increased IR and AKT phosphorylation were also seen, suggesting that insulin was responsible for the prolonged motility by activating the AKT/BAD anti-apoptosis pathway. Work by Lampiao & du Plessis also showed that insulin increases human spermatozoa motility, viability, acrosome reaction and nitric oxide (NO) production (2008). Studies of insulin's effects on porcine spermatozoa have yielded mixed results. Carpino et al have reported that insulin increased NO production and capacitation but made no reference to motility (2008). Cunha et al however found that the addition of insulin did not impact motility, sperm viability or the degradation rate of motility (2004).

We hypothesized that insulin produced in the mouse uterus would have a similar effect on spermatozoa and delay their apoptosis. We measured insulin's effect on capacitation and motility. Mouse spermatozoa were able to successfully undergo capacitation when incubated with insulin. We then measured total and progressive motility over time of mouse spermatozoa incubated with different concentrations of insulin. Mouse spermatozoa did not respond to insulin, instead motility generally decreased with time in both control and insulin treated groups. The poor response to insulin forced us to reassess the axiom that mouse spermatozoa should be affected by insulin. We had made the assumption that mouse spermatozoa possessed the IR but this has never been demonstrated in the literature. We used ICC to look for the IR and found that it was strongly expressed in mid-piece and tail of mouse spermatozoa. Previous work in our lab had localised the IR to the head, mid-piece and tail of human spermatozoa (Aitken

unpublished). In pigs the IR was localised to the mid-piece (Carpino et al 2010). This was first time anyone has localised the IR in mouse spermatozoa. Previously published mouse spermatozoa proteomes did not identify the IR in their analysis (Baker et al 2008, Chauvin et al 2012).

There are several possible explanations for non-responsiveness of mouse spermatozoa to insulin. The most likely is variation in spermatozoa between species. Mice have a naturally effective and efficient mating system and this could mean that their spermatozoa do not have a need for longevity. Mice have a shorter and more frequent reproductive cycle then either pigs (21 days) or humans (30 days) (Meziane et al 2007). The short time that a mouse is in estrus and willing to accept a male is well synchronized with ovulation (Meziane et al 2007). This is beneficial for mouse spermatozoa as the oocyte has been released and is ready for fertilization, compared to humans where spermatozoa maybe have to survive for a prolonged period of time in the females' reproductive tract waiting for ovulation to occur. Purely due to the small size of mice the distance that mouse spermatozoa need to swim to reach the oocyte is also much shorter than in the case of human or porcine gametes. The combination of synchronized mating and short travel distance to the oocyte means that mouse spermatozoa do not need to be particularly long lived. Therefore the mechanism through which insulin prolongs human sperm motility is not necessary and maybe absent in mouse spermatozoa.

It is also possible that the decline in motility is completely unrelated to insulin dosage. Mouse spermatozoa are more fragile than other species (Katkov and Mazur 1998). All effort was taken to carefully handle the cells but it's possible that at some point of the experiment the spermatozoa experienced a physical disturbance. The most likely traumatic event is covering the spermatozoa with a coverslip before measuring with the CASA. While all the glass slides and cover slips were prewarmed the change in environment may have been enough to influence the result. Furthermore, in mouse sperm, the cytoskeleton anchors the plasma membrane to the cell's internal structure, creating additional strains on the membrane under osmotic stress (Noiles et al. 1997). We did not check the osmolarity, assuming that our long standing protocols have been sufficiently optimised that osmolaric conditions were suitable.

An explanation for the benefits of insulin seen in other studies is that the insulin used was not a pure sample. In our study we used insulin from bovine pancreas purchased from Sigma Aldrich with the impression that the insulin powder was pure. In the product specifications sheet, the purity is not stated but only insulin is mentioned as a component of the powder, misleadingly implying that it is the only component. We dissolved the sample as per manufacturer's instructions and tested it with mass spectrometry. While the mass spectrometry was not quantifiable it was able to readily detect bovine serum albumin (BSA) in the insulin powder suggesting that BSA was abundantly present. BSA is very beneficial to spermatozoa in three ways. Firstly BSA leads to cholesterol release from spermatozoa promoting tyrosine phosphorylation and capacitation (Osheroff et al 1999). It also helps spermatozoa cope with oxidative stress by removing aldehydes from spermatozoa as well as containing cysteine residues that can be sacrificed to reactive oxygen species, thereby acting as antioxidants (Twigg et al 1998). Inadvertently incubating spermatozoa with BSA or albumin from another species could potentially be responsible for the improvements in capacitation and motility rather than insulin. At least one study (Aquila, 2013) showing insulin improved capacitation in porcine spermatozoa used Sigma insulin from porcine pancreas, which presumably would have been processed in a similar manner to ours. It is difficult to determine the purity of insulin used in other studies from just the information provided in their methods but potentially their samples may be inadvertently contaminated.

3.4.2 Epithelial cells produce insulin in response to estrogen in primary cell culture.

We created a primary cell culture of endometrial epithelial cells and incubated them with estrogen, glucose and estrogen + glucose to study insulin production and secretion. Insulin could be detected in the control and all treatment groups. However estrogen dosages of 40pg/ml and 200pg/ml significantly increased the number of fluorescent cells compared to the control. 100pg/ml had more fluorescent cells than the control but not significantly. These results support the concept of estrogen promoting insulin production in endometrial epithelial cells. Cells were also treated with glucose with the expectation that it may lead to insulin being release from the cells. Glucose treated cells also had more fluorescent cells compared to the control but not statistically significantly. Insulin measurement of the culture media after two day incubation could

not detect an insulin, the assay had a sensitivity range of 0.2 to 10 ng/mL. This suggests that epithelial cells had a different mechanism for insulin secretion to β cells. When the cells were lysed the assay was able to detect small quantities of insulin. While not statistically significant the estrogen treated cells had slightly more insulin.

C-peptide was readily found in the cultured epithelial cells, implying that pro-insulin cleavage and therefore insulin genesis is occurring. Both estrogen and glucose treated groups had more fluorescent cells compared to the control but the difference was not statistically significant. The presence of C-peptide in the untreated control challenges the hypothesis of estrogen induced insulin production. A possible explanation is that C-peptide is much less biologically active then insulin and therefore accumulates inside cells, resulting in higher cell counts. In circulation insulin has a half-life of five minutes compared to around 30 minutes for C-peptide and excess C-peptide is excreted from the body in urine (Wahren et al 2000). The cells grown *in vitro* may have a different rate of insulin consumption or be unable to remove C-peptide. More analysis of the culture media is needed to test if C-peptide is expelled from cells.

The IR was expressed in the majority of cells regardless of treatment. Interestingly only some cells had nucleic expression of IR. In endometrial tissue we saw very strong and widespread expression of nucleic IR. We assumed that the insulin produced is biologically active, though that still needs to be determined, and therefore available to start the PI3K or ERK-MAPK pathways. We looked at the expression of phosphorylated AKT and ERK. Both proteins were widely expressed in endometrial epithelial cells and incubation with estrogen, glucose or estrogen + glucose did not change the number of cells that expressed the proteins. These results suggest that in our primary cell culture there is wide spread expression of the IR and two signaling pathways associated with insulin. However we have not been able to clearly demonstrate a link between estrogen induced insulin production and up-regulation of either the PI3K or ERK-MAPK pathways.

3.4.3 Limitations of the cell-culture model

The primary cell culture experiments had two main limits. Firstly it was difficult to grow large amount of healthy epithelial cells. Most primary cell culture protocols require the culture media to be supplemented by insulin to promote cell growth (Alkhalaf et al 1991). As we were investigating insulin we did not supplement the

culture media. This led to more difficulty in growing cells but it also means that any insulin we detected most likely had come from the epithelial cells themselves rather than an outside source. It did occur to us that the insulin and C-peptide may have already been inside the cell when they were removed from the uterus rather than produced during cell culture. This is unlikely as after four days many of the original cells would have been replaced by new cells. Difficulty in growing large amounts of cells limited the amount of analytic approaches we could take. We initially wanted to measure *INS* expression using qPCR but could not extract enough mRNA. Similarly we could not extract enough protein for western blot analysis of AKT and ERK phosphorylation. The extraction of epithelial cells from the uteri of two mice, grown in culture over four days to form a monolayer of cells yielded around 2-3µg of protein after SDS extraction. Potentially using more mice would lead to more protein.

The second limitation is that endometrial epithelial cell function is closely tied to the layers of stromal cells on which they sit. As mentioned before estrogen leads to mass proliferation of epithelial cells *in vivo* however we and other researchers have struggled to see a large mitogenic response in *in vitro* (Alkhalaf et al 1991, Echima et al 1991). Epithelial cell proliferation may be induced by inductors originating in the neighbouring stromal cells in response to hormonal stimulation. Work by Pierro et al used a stromal and epithelial co-culture model to show that estrogen's proliferative properties in human endometrial epithelial cells are partly dependent on stromal cell secretions of IGF-1 (2001). What’s more they found the addition of insulin to the cell culture would only help proliferation if the stromal cells were active. In this context, it is possible that while we could use estrogen to induce insulin production in epithelial cells, insulin's ability to signal was limited by the lack of stromal cells.

Chapter 4:

Possible functions for uterine insulin

Final Discussion

Our data has shown that insulin and C-peptide production in the endometrium coincides with periods of estrogen release. In endometrial epithelial cells, estrogen synthesised at proestrus in mice leads to DNA synthesis and cell division (Tong et al 2008). Estrogen acts through different estrogen receptors (ER). The ERα is the best documented and is responsible for estrogen induced signaling (Strom et al 2004). How estrogen affects β-cell physiology is not understood but the literature suggests that estrogen either increases insulin production and/or increases insulin secretion. A study by Alonso-Magdalena et al showed that in mice, long term exposure to physiological concentrations of estrogen increased β-cell insulin content, insulin gene expression and insulin release (Alonso-Magdalena et al 2008). Estrogen can increase insulin secretion by decreasing K(ATP) channel activity (Nadal 1998) which leads to membrane depolarization and the opening of voltage-gated Ca^{2+} channels (Ropero et al 2002, Nadal 2009). The Ca^{2+} influx results in insulin containing vesicles fusing with the plasma membrane and releasing insulin. In mice the ERα receptor is present in the endometrial epithelium during the late proliferative phase and transitions to the stroma and decidua in the later stages of the menstrual cycle and pregnancy (Wang et al 2000). ERα knock-out mice are obese and insulin resistant (Heine et al 2000). The pattern of IR receptor expression, namely, first appearing in the epithelial cells at proestrus and estrus before becoming expressed in both epithelial and stromal at metestrus and diestrus, is similar to the expression of the ERα (Lecca et al 2001). This suggests that the IR follows the hormonal cycle, being expressed in epithelial cells under estrogen domination and stromal cells when progesterone is secreted. This observation together with PCR results showing that *INS* mRNA expression coincides with rises in estrogen provides tentative evidence that the insulin system in the endometrium is linked to estrogen.

The biological complexity of signaling in the endometrium means that it is very likely that insulin function is integrated with other separate pathways rather than operating in isolation. As insulin and IGFs have a similar chemical structure, ability to share receptors and can co-operate to activate ERK-MAPK pathways, looking at previous studies of IGFs in the endometrium could provide insight into our own observations. IGF-1 and 2 mRNA is present in pig and rat uterine tissue and are thought to have a role

in estrogen mediated cell proliferation (Simmen et al 1990, Norstedtet al 1989). In rats the levels of IGF1 mRNA change during the estrous cycle, being highest at the start of the estrous cycle, proestrus (Carlsson and Billig, 1991). The levels of IGF-1 receptors change in the uterus over the estrous cycle, being highest at proestrus (Ghahary and Murphy, 1989) and were localized using in situ hybridization to the luminal epithelial cells (Girvigian et al 1994). The levels of expression of IGF-I ligand, receptor, and biological responses in the uterus are up-regulated by estrogen (Girvigian et al 1994). These findings are not dissimilar to what we observed with insulin, with insulin mRNA and IR appearing at similar times and in the same cells as IGF.

This is very noteworthy because insulin can enhance the signaling of IGFs. Insulin and IGFs can both start the ERK-MAPK pathway. For the ERK-MAPK pathway to function a protein, RAS, needs to translocate to the plasma membrane. The translocation of RAS is dependent on insulin binding to its receptor (Goalstone et al 1998). While insulin is generally thought of only as a weak growth factor by itself, the signaling of more potent growth factors relies on insulin to translocate RAS, it can contribute to proliferation by supporting IGFs. Further evidence of the importance of insulin acting together with IGFs is that in human endometrial cells insulin can locally regulate the activity of the insulin-like growth factor system by decreasing the binding protein-1, thereby increasing the availability of IGF 1 for signaling (Irwin et al 1993). In cell culture for estrogen and insulin to have a proliferative effect on human endometrial epithelial cells IGFs had to be secreted from the stromal cells (Pierro et al 2001).

All these studies very strongly show a system in the endometrium that promotes cell proliferation and relies on insulin and IGF, rather than just IGF. We saw very strong nucleic expression of IR in our endometrial tissue and such expression has been associated with promoting proliferating in hepatic cells (Rodrigues et al 2007, Amaya et al 2014). When the nucleic IR was not activated the cell would become stuck in early prophase (Rodrigues et al 2007). In light of all this it seems very likely that insulin is produced in the endometrium to help with cell proliferation as opposed to any sort of glucose regulating function which is strictly dependent on pancreatic insulin. We propose the following mechanism (Figure 4.1.1).

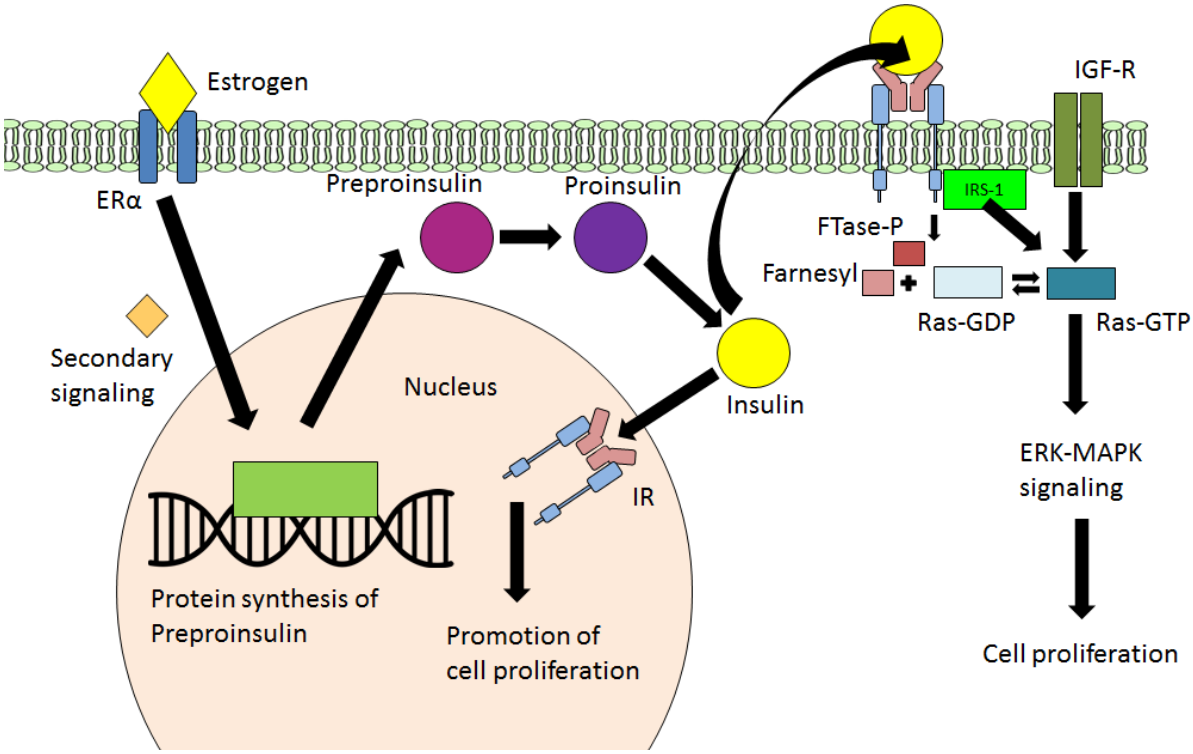

Figure 4.1.1 Proposed mechanism for uterine insulin. A rise in estrogen during proestrus and estrus leads to estrogen binding to its receptor and through a yet to be determined pathway induce the production of the preproinsulin protein. Preproinsulin is cleaved to proinsulin and that is cleaved to produce insulin. How insulin is packaged at this point is undetermined. Insulin can then potentially do one of two things, if epithelial cells function in a similar manner to hepatic cells then insulin can bind to the nucleic IR and promote cell proliferation. Alternatively it can support other growth factors, most likely IGF. Insulin binding to its receptor phosphorylates farnesyltransferase and increases the amount of farnesylated RAS Farnesylated RAS is necessary for ERK-MAPK signaling and appears to be depended largely on IR binding, as discussed in chapter 1.

A neglected aspect of the hormone driven cycle in our studies was progesterone. It strongly contributes to modulating the effect of estrogen and future studies are needed to elucidate it's impact on uterine insulin. In our opinion primary cell culture is not an adequate model to study such complex interactions between cells. A much better model would be to use ovariectomised mice. This way the uterine environment is largely preserved. Dosages of estrogen and progesterone can be injected into the mouse, giving better control of the experiment. Future studies can also look at insulin production during pregnancy to see how it changes throughout the development of the uterus.

Presumably estrogen suppression by progesterone would limit insulin production but this still needs to be tested.

In conclusion we have provided descriptive data showing that the mouse uterus contains the necessary components to produce insulin such as the insulin transcript and the peptidase responsible for cleavage of its precursor. The insulin and C-peptide proteins were localised to the epithelial and stromal cells in the endometrium and their expression changed with the estrous cycle. The IR is also widely expressed in the endometrium, particularly in the nucleus, providing a means for insulin signaling. Despite having a positive effect on human and porcine spermatozoa, mouse spermatozoa appear unaffected by insulin.

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APPENDIX: BUFFERS AND SOLUTIONS

	Constituents
3% Tween	3% v/v Tween-20 in PBS
0.2% Triton X-100	0.2% v/v Triton X-100 in PBS
4 % Paraformaldehyde	4g paraformaldehyde in 100ml 1x PBS (Adjust pH = 11 with NaOH) then adjust pH = 7 with H_2SO_4
10mM Na Citrate antigen retrieval	10mM Na Citrate, pH6 with 3% v/v tween.
05M Tris antigen retrieval	0.05M Tris, pH 10 with 3% v/v tween
Agarose Gel	1% or 2% v/v agarose gel in TAE
BWW stock solution	5.54mg/ml sodium chloride 0.35mg/ml potassium chloride 0.25mg/ml calcium chloride dehydrate 0.162 mg/ml potassium dihydrogen orthophosphate 0.294 mg/ml magnesium sulphate heptahydrate
BWW solution	105mg Sodium bicarb 500mg PVA 40mg Glucose 1ml HEPES 500µl Penicillin Streptomycin 185µl Sodium lactate 25µl Sodium pyruvate. Make up to 50ml with BWW stock solution.
BWW capacitation	105mg Sodium bicarb 500mg PVA 40mg Glucose 1ml HEPES 500µl Penicillin Streptomycin 185µl Sodium lactate 25µl Sodium pyruvate. 2.4g pentoxifylline 4.9g dibutyryl cyclic adenosine monophosphate Make up to 50ml with BWW stock solution.
BWW non-cap	86mg Sodium chloride 40mg Glucose 1ml HEPES 500µl Penicillin Streptomycin 185µl Sodium lactate 25µl Sodium pyruvate. Make up to 50ml with BWW stock solution.
Bouins Solution	75 m Picric acid (saturated) 25ml Formaldehyde (37-40%)

	5ml Glacial acetic acid
DEPC water	0.1 diethylpyrocarbonate added to Milli-Q H_20 and autoclaved twice.
dNTPs	10mM dATP 10mM dTTP 10mM dCTP 10mM dGTP in PCR water
ICC antibody buffer	1% w/v BSA in PBS
ICC blocking buffer	1% w/v BSA in PBS
MOWIOL	2.4g MOWIOL 4-88 6g glycerine 6ml d H_20 12ml 0.2M Tris (pH 8.5)
Primary cell culture media	DMEM:F12 10% v/v Horse Serum 5 v/v Penicillin Streptomycin
TAE buffer	40mM Tris 0.1% v/v acetic acid 50mM EDTA (pH 8.0)
TBS	0.1M Tris base 0.14M sodium chloride pH7.4