Insulin and the early bovine embryo

Influences on *in vitro* development, gene expression, and morphology

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Abstract

Metabolic imbalance is a problem in the dairy industry because the metabolic demands of increased milk production can lead to decreased fertility, and more knowledge about improving the management and physical conditions of the cow (the links between fertility, nutrition, milking, and dry period) is needed. Insulin is an important hormone regulating the energy balance in the body, and insulin concentrations change in situations of energy deficiency or excess, both of which are known to decrease fertility in cows as well as in humans. Hyperinsulinemia is associated with decreased fertility by impairing the developmental potential of embryos, but the underlying reasons for this remain unclear. Our aim was to investigate insulin-induced changes on development, morphology and molecular signature in bovine blastocysts on Day 8 (BC8).

An *in vitro* model was used and morphology and gene expression were analysed by combining confocal microscopy and microarray-based transcriptome studies. Blastocysts were produced *in vitro* according to standard methods using oocytes that were supplemented with three different insulin levels (INS10 =10 µg/ml; INS0.1= 0.1 µg/ml; INS0=control) during maturation. The transcriptome profile of BC8 was obtained and embryo quality grades, developmental stages and morphologies were further assessed in terms of F-actin, DNA, and active mitochondria. Significant differences were observed in developmental rates and morphology after insulin exposure. The observed changes were reflected by increased expression of genes involved in cell division and structure, mitochondrial activation, lipid metabolism, and oxidative stress.

Combining all of the results, it was shown that elevated insulin impairs the developmental potential of the embryo. This work contributes to new knowledge about the molecular background of embryos developing under metabolic stress conditions such as hyperinsulinemia. Moreover, the studies are of comparative value for humans where impaired fertility is often related to metabolic disorders.

Keywords: Oocyte maturation, bovine blastocyst, transcriptome, metabolic syndrome, diabetes, hyperinsulinemia, embryo *in vitro* production, embryo morphology, lipids

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To my parents Gudrun and Olaf, and my beloved family and friends

"Und jedem Anfang wohnt ein Zauber inne, Der uns beschützt und der uns hilft, zu leben."

"A magic dwells in each beginning, protecting us, telling us how to live."

Hermann Hesse ("Stufen")

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Laskowski, Denise; Sjunnesson, Ylva; Gustafsson, Hans; Humblot, Patrice; Andersson, Göran; and Båge Renée (2016). Insulin concentrations used in *in vitro* embryo production systems - a pilot study on insulin stability with an emphasis on concentrations measured *in vivo*. *Acta Veterinaria Scandinavica* 58 (Suppl.1): 66.

II Laskowski, Denise; Båge, Renée; Humblot, Patrice; Andersson, Göran; Sirard, Marc-André and Sjunnesson, Ylva (2017). Insulin during *in vitro* oocyte maturation has an impact on development, mitochondria, and cytoskeleton in bovine Day 8 blastocysts. *Theriogenology* 101, 15–25.

III Laskowski, Denise; Sjunnesson, Ylva; Humblot, Patrice; Sirard, Marc-André; Andersson, Göran; Gustafsson, Hans and Båge, Renée (2016). *In vitro* bovine oocyte maturation changes blastocyst gene expression and developmental potential. *Reproduction, Fertility and Development* 29, 876-889.

IV Laskowski, Denise; Andersson, Göran; Humblot, Patrice; Sirard, Marc-André; Sjunnesson, Ylva; Ferreira, Christina; Pirro, Valentina; Båge, Renée (2017). Lipid profile of bovine blastocysts exposed to insulin during *in vitro* oocyte maturation. *Reproduction, Fertility and Development* (submitted manuscript).

The contribution of Denise Laskowski to the papers included in this thesis was as follows:

I Literature review, initial planning of the pilot study (including choice of measurement methods), the IVF laboratory work, and preparation of samples. Main responsibility for writing the manuscript.

II Performed most of the laboratory work, microscopy imaging and data analysis and interpretation together with supervisors. Main responsibility for writing the manuscript.

III Planning and performance of the laboratory work together with supervisors and collaborators, performed the IVF work, performed molecular biology methods under supervision, took part in data analysis and performed data interpretation. Main responsibility for writing the manuscript together with collaborators and supervisors.

IV Took major part in planning of the lipid study, establishing contact with collaborators, and developing methods. Interpretation of gene analysis data together with supervisors and collaborators, main responsibility for writing the manuscript.

Additional publications related to the thesis

- Laskowski, Denise; Sjunnesson, Ylva; Humblot, Patrice; Andersson, Göran; Gustafsson, Hans; and Båge, Renée (2016). The functional role of insulin in fertility and embryonic development - What can we learn from the bovine model? *Theriogenology,* 86(1), 457-464.

- Laskowski, Denise; Humblot, Patrice; Sirard, Marc-André; Sjunnesson, Ylva; Jhamat, Naveed; Båge; Renée; Andersson, Göran (2017). Elevated insulin changes DNA methylation pattern in bovine *in vitro* blastocysts. Submitted to *Reproduction.*

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Abbreviations

ACAA1	Acetyl-CoA acyltransferase 1
ACP5	Acid phosphatase 5
ADIPOR2	Adiponectin receptor 2
AGC	Automatic gain control
Akt/PKB	Protein kinase B
APLP2	Amyloid precursor-like protein 2
APOA1	Apolipoprotein A 1
aRNA	Anti-sense ribonucleic acid
ART	Assisted reproductive technologies
BC8	Blastocyst day 8
BSA	Bovine serum albumin
CAM	Camera image of the epifluorescence microscope
CC	Cumulus cells
COC	Cumulus-oocyte complexes
COMT	Catechol-O-methyltransferase
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
DESI MS	Desorption electrospray ionization mass spectrometry
DET	Differentially expressed transcript
DG	Diacylglycerol
DHCR7	7-Dehydrocholesterol reductase
DMR	Differentially methylated region
EGA	Embryonic genome activation
EHD1	EH domain containing 1
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FA	Fatty acid
FADS2	Fatty acid desaturase 2

FSH	Follicle stimulating hormone
gDNA	Genomic DNA
GLUT-4	Glucose transporter type 4
GnRH	Gonadotropin releasing hormone
GRB2	Growth factor receptor-bound protein 2
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase
HSPA1A/B	Heat shock protein family A (Hsp70) Member 1A
ICM	Inner cell mass
IETS	International Embryo Technology Society
IGF	Insulin-like growth factor
IGF2R	Insulin-like growth factor 2 receptor
INSIG1	Insulin induced gene 1
IRS	Insulin receptor substrate
IVF	*In vitro* fertilization
IVP	*In vitro* production
LAMP1	Lysosomal-associated membrane protein 1
LCFA-CoA	Long-chain acyl-coenzyme A
LDLR	Low density lipoprotein receptor
LH	Luteinizing hormone
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
mRNA	Messenger RNA
mSOF	Modified synthetic oviductal fluid
mTALP	Modified Tyrode's albumin lactate pyruvate
MVD	Mevalonate diphosphate decarboxylase
m/z	Mass number/ion charge number
NEB	Negative energy balance
NEFA	Non esterified fatty acids
NR1H2	Nuclear receptor subfamily 1 group H member 2
NR3C1	Nuclear receptor subfamily 3 group C member 1
PBS	Phosphate buffered saline
PEB	Positive energy balance
PIK3	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PNLIP	Pancreatic lipase

PPARα	Peroxisome proliferator-activated receptor alpha
PTPases	Tyrosine-specific protein phosphatases
PVA	Polyvinyl alcohol
Raf	Rapidly accelerated fibrosarcoma
Ras	Ras protein family
RIN	RNA integrity number
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Reverse transcribed quantitative PCR
SCAP	Sterol regulatory element-binding protein cleavage-activating protein
SH2/3	Src Homology 2/3 domain
SH2/ShC	Src homology 2 domain-containing
SoS	Son of Sevenless
SREBP	Sterol regulatory element binding protein
TAG	Triacylglycerid
TCM	Tissue culture medium
TNF	Tumour necrosis factor
VIM	Vimentin
W/V	Weight per volume

1 Introduction

1.1 Bovine reproduction and metabolic disorders

Reproductive performance is a key factor for cost efficiency in the dairy industry and cow reproduction and early embryonic development are well-studied topics. The oocyte matures in the follicle surrounded by follicular fluid, and this process is influenced by metabolic and nutritional changes in the blood (Leroy *et al.*, 2004, 2011; Aardema *et al.*, 2013). The concentrations of these metabolites might differ significantly depending on the maternal nutritional state. The oocyte appears to be very sensitive to metabolic stress, which might decrease its developmental potential. Moreover, there is even a risk that the offspring of an obese mother later in life will suffer from metabolic diseases because metabolic programming occurs early in life through epigenetic changes that can remain throughout the entire life (Heerwagen *et al.*, 2010).

Follicular growth and oocyte maturation takes 8 -12 weeks in the dairy cow (Beam & Butler, 1997, 1999). This means that the oocyte in the growing follicle that will be subjected to the first inseminations after parturition undergoes several metabolically challenging periods until final maturation. Often, dairy cows are over-conditioned in the dry period (Rukkwamsuk *et al.*, 1999), which leads to elevated circulating insulin levels and impaired insulin sensitivity (Locher *et al.*, 2015). After calving, the metabolic profile of dairy cows changes due to the increasing energy demands for milk production (Fleischer *et al.*, 2001). Most dairy cows go through a period of negative energy balance while insulin levels are low (Butler *et al.*, 2004), and non-esterified fatty acid (NEFA) concentrations are elevated due to lipid mobilization (Bossaert *et al.*, 2008). The dairy cow suffers from ketosis, a state during which β-hydroxybutyric acid levels are increased, a sign for severe energy deficiency leading to secondary diseases due to immune system deficiencies (Ingvartsen, 2006). Circulating insulin levels

increase when positive energy balance is restored (Gong *et al.*, 2002; Humblot *et al.*, 2008), and this has positive effects on reproductive functions. This switch from energy excess to energy deficiency might negatively affect the developmental competence of the oocyte because the oocyte metabolism and gene expression have to be well balanced to allow for optimal oocyte development during that time period (Leroy *et al.*, 2011). It is known that subfertile dairy heifers rapidly gain weight and become overconditioned after a period of repeated, unsuccessful inseminations (Gustafsson, 1985). These heifers are characterized by hormonal aberrations and by impaired oocyte quality and reduced early embryonic developmental competence (Båge, 2002; Båge *et al*., 2003; Awasthi *et al.,* 2010). If fat heifers finally manage to conceive, they will be at high risk of dystocia at parturition along with retained placenta and metritis, which in turn will increase the risk for fertility problems in the insemination period.

It has become evident that both increases and decreases in circulating insulin levels have a potential role in the health of the embryo developing under such conditions.

Moreover, similarities between the human and bovine species provide a fruitful research field because new insights in the relation between metabolic and reproductive disorders could help to improve fertility in both species. The suitability of a bovine model for human embryonic development can be explained by analogies in ovarian reserve, follicular dynamics and embryonic metabolism (Ménézo & Hérubel, 2002; Campbell *et al.*, 2003a).

1.2 Insulin

The hormone insulin was discovered in 1921 by Banting and Macleod and this marked a major breakthrough in medicine (Quianzon & Cheikh, 2012). Long before that, it was already hypothesized that there must be a substance secreted by the pancreas controlling carbohydrate metabolism (Bliss, 1993). Macleod isolated insulin from cow pancreases, and the first diabetic patient was successfully treated in 1922. Since then, insulin has been in focus for understanding the pathophysiological mechanisms of diabetes and related diseases, and the successful therapy of patients suffering from diabetes was honoured with the Nobel Prize in 1922.

Insulin is a peptide hormone produced by the β-cells in the islets of Langerhans (Sonksen & Sonksen, 2000), and it functions as a key metabolic regulator of energy homeostasis in the body. Insulin acts on multiple levels of lipid and glucose metabolism (Saltiel & Kahn, 2001), and its metabolic effects include stimulation of DNA synthesis, protein synthesis, transmembrane

transport, and lipogenesis (Kahn & White, 1988). Insulin is proteolytically converted from its precursor polypeptides in several steps, and the final activation step occurs by cleaving C-peptide from proinsulin (Steiner, 2008). The biologically active insulin molecule consists of 51 amino acids forming the A and B chain that are connected via three disulphide bridges (Brange & Langkjœr, 1993). Insulin is highly evolutionarily conserved in its structure among species; for example, bovine and human insulin only differ in three amino acid residues (Smith, 1966).

The interest in understanding the action and regulation of insulin has not decreased in recent years, and research on insulin is still of high relevance because diabetes and diabetes-related diseases are increasing worldwide (Seidell, 2000). Knowing about the importance and history of insulin explains our aim to contribute with our research to add one more piece to the puzzle in better understanding insulin-related pathways during early embryonic development.

1.2.1 Insulin receptor signalling and functions

Insulin action is transmitted through its binding with different affinities to both the insulin receptor and the related insulin-like growth factor (IGF)-1 receptor (Rechler & Nissley, 1985). The insulin receptor belongs to the family of ligand-activated receptors with tyrosine kinase activity, and it contains transmembrane signalling domains with two alpha and two beta chains (Lee & Pilch, 1994). Both insulin and IGF receptors auto-phosphorylate tyrosine residues on the receptor upon ligand binding, and their biological response is transmitted by phosphorylation of intracellular proteins that activate different signalling cascades (Sibley *et al.*, 1987; Häring, 1991; Tsakiridis *et al.*, 1999).

Two main intracellular pathways lead to either metabolic or mitogenic post-receptor actions through mitogen activated protein kinase (MAPK) or phosphatidyl-inositol-3-kinase (PI3K) (Shepherd et al., 1998; Figure 1, schematic illustration). In brief, insulin binding activates the insulin receptor tyrosine kinase to phosphorylate insulin receptor substrates (IRSs). IRS proteins are recruited to the receptor and phosphorylated on tyrosine residues, leading to binding sites for molecules with a Src-homology 2 (SH2) domain such as PI3K (Myers *et al.*, 1992), Shc –proteins, and growth factor receptor-bound protein 2 (GRB2) (Pelicci *et al.*, 1992; Sasaoka *et al.*, 1994; Sun *et al.*, 1997; Boucher *et al.*, 2014). Activated PI3K generates phosphatidylinositol (3,4,5)-trisphosphate ($PIP_{3)}$ from phosphatidylinositol 4,5-bisphosphate ($PIP_{2),}$ and this second messenger leads to an activation cascade of different protein kinases, especially protein kinase B (PKB/Akt), a serine kinase. PKB is involved in the translocation

of glucose transporter type 4 (GLUT-4) vesicles to the cell membrane, the most well-studied insulin action allowing glucose uptake. PKB also leads to increased protein synthesis (Pessin & Saltiel, 2000; Bevan, 2001; Lizcano & Alessi, 2002).

The signal transduction proteins leading to mitogenic functions are GRB2, a protein with SH2 and SH3 domains, and Shc (Pelicci *et al.*, 1992; Sasaoka *et al.*, 1994; Chow *et al.*, 1998). Shc proteins are able to bind GRB2 after tyrosine phosphorylation which leads to activation of Ras and, the beginning of a phosphorylation cascade and the consequent activation of MAPKs (Giorgetti *et al.*, 1994). MAPKs are proteins involved in the regulation of cell growth, division, and apoptosis and are able to phosphorylate nuclear proteins and several protein kinases that interact with transcription factors and thus influence gene expression (Sturgill *et al.*, 1988; Bevan, 2001; Hilger *et al.*, 2002).

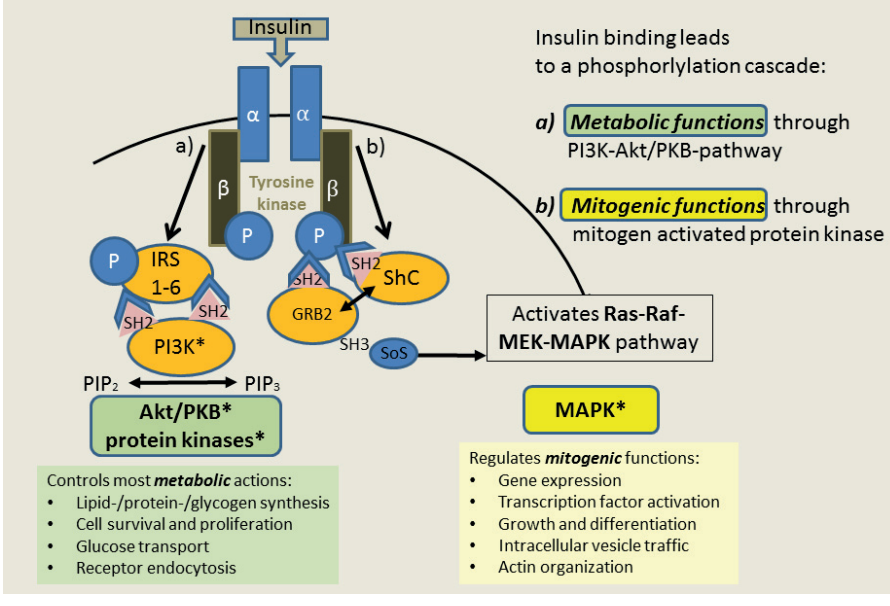

Figure 1. Insulin signalling pathway (described in detail in text).

Akt/PKB = Protein Kinase B, GRB-2 = Growth factor receptor-bound protein 2, IRS = Insulin receptor substrate, MAPK = mitogen-activated protein kinase, MEK = Mitogen-activated protein kinase kinase, P = Tyrosine phosphorylated state, PI3K = Phosphatidylinositol-4,5-bisphosphate 3-kinase, PIP_2 = Phosphatidylinositol 4,5-bisphosphate, PIP_3 = Phosphatidylinositol (3,4,5)-trisphosphate, Raf = Rapidly accelerated fibrosarcoma, Ras = Ras protein, SH2 = Src homology 2 domain-containing, ShC = ShC protein, SoS = son of sevenless, *activated

1.2.2 The role of insulin in the metabolism of ruminants

The different functions of insulin are tissue-specific, and the induced post-receptor mechanisms and responses differ in liver, adipose tissue, and skeletal muscle (Zachut *et al.*, 2013). In contrast to humans, the glucose metabolism of ruminants is not entirely dependent of insulin, and only a limited amount of the available glucose derives from direct intestinal absorption (Aschenbach *et al.*, 2010). In general, peripheral glucose concentrations are lower and insulin responses are weaker in ruminants (Kaske *et al.*, 2001). Even if the same mechanisms – including insulin-induced translocation of the glucose transporter GLUT4 towards the cell membrane to facilitate the glucose uptake in the cell – exist, lower GLUT4 and IRS-1 availability lead to a weaker response of glucose metabolism to insulin stimuli (Sasaki, 2002). Thus, only a small amount of glucose is used for lipogenesis in the adipose tissue, which uses acetate as a preferred substrate for lipid synthesis (Hanson & Ballard, 1967).

The glucose uptake in the mammary gland can be up to 92% of the total glucose consumption (Rose *et al.*, 1997). In the mammary gland, glucose uptake is independent of insulin signalling, and GLUT4 is not detectable (Komatsu *et al.*, 2005), and this is further evidence for the tissue specific characteristics of insulin and glucose metabolism.

Different from humans, the glucose requirements of ruminants must always – not just during fasting – be covered by gluconeogenesis from non-carbohydrate sources. The most abundant substrates derive from microbial activity in the rumen in form of short fatty acids (Brockman, 1978; Brockman & Laarveld, 1986) that are synthesized to glucose in the intermediate metabolism. The regulation of catabolic and anabolic actions is transmitted through glucagon and insulin where the major glucogenic substrates are propionate, lactate, pyruvate, amino acids and glycerol (Bergman, 1973). Propionate and other short-chained fatty acids and lactate have their origin in microbial fermentation. Lactate can originate from anaerobic glucose oxidation while glycerol together with NEFAs originate from fat mobilization (De Koster & Opsomer, 2013).

Insulin stimulates glucose uptake in muscle cells and adipose tissue, while glucagon has its primary target in the liver to stimulate gluconeogenesis (Brockman, 1978). The insulin response in different tissues varies through insulin receptor and GLUT4 availability, which changes during different periods of lactation. One explanation for this regulatory system is that glucose demands vary significantly depending on milk production and can increase up to four fold from late pregnancy to early lactation (Bell, 1995). The metabolism needs to adapt to the varying requirements by increasing expression of gluconeogenic enzymes in the liver (Graber *et al.*, 2010). At the same time, insulin stimulation is reduced (due to low circulating insulin levels postpartum or due to peripheral

insulin resistance) which leads to lipid mobilization and thus higher availability of substrates important for gluconeogenesis.

At the hepatic level, elevated insulin decreases glycogenolysis, i.e. the conversion of glycogen to glucose-6-phosphate, and decreases lipolysis, while it increases glycogen synthesis (Donkin & Armentano, 1995). This is due to the fact that the energy substrate availability is sufficient in the presence of high insulin concentrations, and this is a condition in which storage of energy would be favourable (McDowell, 1983). Some studies report a suppressive effect of insulin on gluconeogenesis (Donkin & Armentano, 1995; De Koster and Opsomer, 2013), while other authors state that gluconeogenesis from propionate is less regulated by insulin but mainly dependent on feed intake (Brockman, 1978; Danfær *et al.*, 1995). This makes sense because in ruminants, glucose has to be synthesised from its precursors, even in situations with high substrate availability.

1.2.3 Insulin and metabolic imbalance

Circulating levels of insulin serve as an indicator of energy balance in the body. Situations with short or long term hyper- or hypoinsulinemia exist in cattle as well as in other mammals including humans (Butler *et al.*, 2003; Eckel *et al.*, 2005).

In humans, the incidence of obesity, metabolic syndrome, and type 2 diabetes is increasing, all of which are examples of hyperinsulinemic conditions associated with positive energy balance (PEB)(Seidell, 2000). In the dairy cow, PEB is often maintained during the dry period when the cow is not lactating (Holtenius *et al.*, 2003), implying a risk of developing obesity. The change from PEB to negative energy balance (NEB) is especially dramatic in cows in good body condition, and many cows fail to adapt to the transition from an anabolic situation during the dry period to a catabolic postpartum condition (Roche *et al.*, 2000). The failure of adapting to the increasing energy demands for lactation leads to ketosis. It is possible to distinguish two different types of ketosis, e.g. using existing differences regarding the insulin levels measured in blood (Herdt, 2000; Oetzel, 2007). In brief, ketosis 1 or "underfeeding ketosis" often occurs 3 -6 weeks after calving at the peak of lactation and in thin animals, while ketosis 2 is present in fat cows and involves liver pathologies due to fat accumulation. Ketosis 2 occurs one or two weeks after calving, with an initial depression in food intake followed by fat mobilization, and it has a poorer prognosis than ketosis 1. This "fat cow syndrome" type of ketosis has similarities to type 2 diabetes in humans because the glucose and insulin levels initially are high and insulin sensitivity low, and this leads to the development of insulin resistance

(Oetzel, 2007). Interestingly, different cow breeds show variation concerning their sensitivity to metabolic perturbations and in how quickly PEB can be restored (O'Hara *et al.*, 2016; Ntallaris *et al.*, 2017), and there are strategies to change feeding regimes to avoid the negative effect of low insulin and glucose levels (Holtenius *et al.*, 1996; Gong *et al.*, 2002).

In conclusion, insulin-raising feeding regimes have a beneficial effect on cow health, while fat supplementation should be avoided because it decreases feed intake and is unfavourable for metabolic functions because lipid metabolism is already overloaded by lipids from body fat mobilization. Insulin is an important regulator of the growth hormone – insulin-like growth factor (GH-IGF) axis and thus has a role in lipid mobilization. It stimulates hepatic expression of GH receptors and IGF-1, which leads to high peripheral IGF-1 concentrations, while it inhibits GH receptors and IGF-1 in adipocytes (Butler *et al.*, 2003). High circulating levels of IGF-1 and insulin reduce circulating GH concentrations which reduces lipolytic mobilization of body energy reserves. This is the reason why it is beneficial to feed diets that increase circulating insulin levels after calving, thus reducing the negative cascade of metabolic disturbances linked to lipid breakdown. During situations of NEB (Lucy, 2006; Kawashima *et al.*, 2007), such as in early lactation of the dairy cow or other conditions when energy intake is lower than energy requirements for maintaining body functions and all physical activity, the opposite occurs and increasing GH concentrations lead to lipid mobilization while insulin is low. Metabolic stress conditions in the form of energy excess or deficiency have shared metabolic characteristics. The elevated levels of lipids in the blood can be dietary induced or can develop through fat mobilization (Leroy *et al.*, 2015). The over-conditioned cow in the dry period has a greater risk of suffering from NEB and secondary diseases after parturition, and this is why the extreme and abrupt change from PEB to NEB is reported to be a high stress factor that the individual has to cope with (Rukkwamsuk *et al.*, 1999). Insulin functions in carbohydrate and lipid metabolism are well studied and described (Saltiel & Kahn, 2001), but the consequences of elevated insulin exposure during early development might differ from its effects on differentiated tissues such as the liver, adipose tissue and intestine. Oxidative stress and mitochondrial damage have been described in the context of metabolic stress (Ceriello & Motz, 2004; Furukawa *et al.*, 2004; Roberts & Sindhu, 2009; Wu *et al.*, 2015) and might thus also be one explanation for the detrimental effect of changes related to elevated insulin concentrations during oocyte maturation (Figure 2).

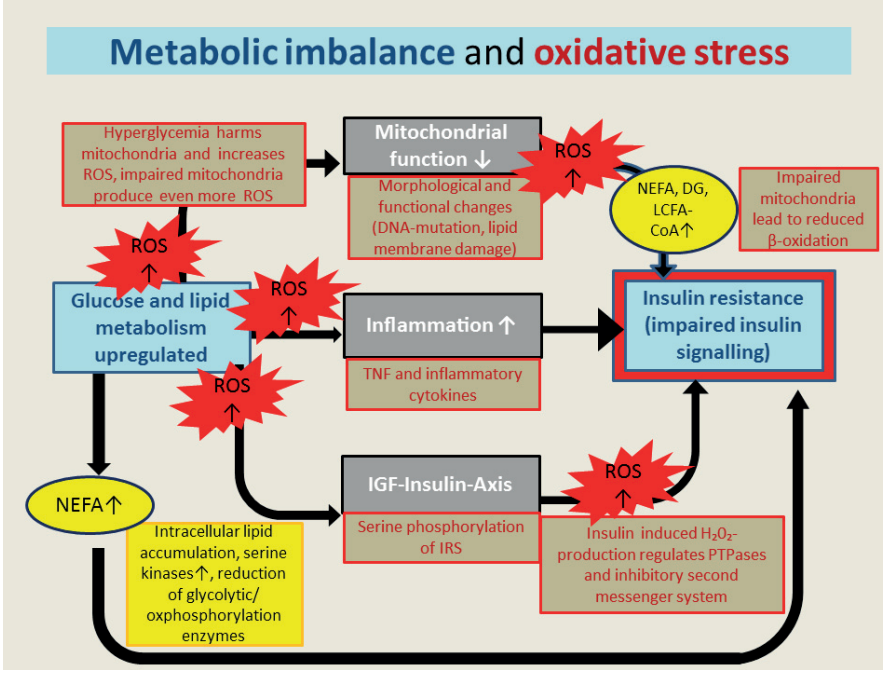

Figure 2. Involvement of ROS in mitochondrial dysfunction and insulin resistance.

ROS = Reactive oxygen species, IRS = Insulin receptor substrate, H_2O_2 = Hydrogen peroxide, PTPase = Tyrosine-specific protein phosphatases, DG = Diacylglycerol, LCFA-CoA = Long-chain acyl-CoA, TFN = tumour necrosis factor, NEFA = Non-esterified fatty acids.

1.2.4 Insulin resistance

Insulin resistance is the state when physiological concentrations of insulin no longer lead to normal responses in the target cells (Kahn, 1978).

It is important to be aware that insulin resistance can be tissue-specific and a transient condition in the dairy cow (Kahn, 1978; Muniyappa *et al.*, 2008). The most well-described form of "classical" insulin resistance is the reduced sensitivity of muscle and adipose tissue which can occur during pregnancy, obesity, and type 2 diabetes in humans (Häring, 1991; Pessin & Saltiel, 2000). In the dairy cow, insulin resistance is most common during late pregnancy and early lactation, most probably linked to hormonal and metabolic changes during lactation where glucose is the favourable substrate for the udder (Bell & Bauman, 1997). Moreover, this natural regulation mechanism might coincide with a massive increase in NEFAs which are thought to reduce insulin sensitivity and furthermore contribute to insulin resistance in the peripartum period (Oikawa & Oetzel, 2006; Pires *et al.*, 2007) by IRS-1 phosphorylation (Le Marchand-Brustel *et al.*, 2003).

The insulin receptor expression pattern is regulated in a similar way in all insulin sensitive tissue types, and insulin exposure leads to receptor downregulation that is regenerated within four hours after the end of insulin exposure (Marshall & Olefsky, 1981). This might be one of the pathophysiological mechanisms of insulin resistance in chronic hyperinsulinemia where the physiological receptor regeneration no longer occurs (Wigand & Blackard, 1979). The mechanisms that are involved in deactivating insulin action are very important for controlling metabolic pathways. Here, phosphatases are described as the main players to dephosphorylate the tyrosine on the activated receptor and IRS (Goldstein *et al.*, 1998).

Other factors that are known to participate in decreased insulin sensitivity are increased circulating NEFAs, hormones, proinflammatory cytokines, and reactive oxygen species (ROS), all of which are present in obese conditions and are involved in detrimental actions on post-receptor cascades (Reaven *et al.*, 1988; Boden, 1997; Houstis *et al.*, 2006; Shoelson *et al.*, 2006). One mechanism of insulin resistance at the post-receptor level is serine/threonine phosphorylation of insulin IRS-1 and 2 instead of tyrosine phosphorylation, which leads to reduced activation of PI3K and the subsequent glucose transport activation steps (Figure 3 and Czech & Corvera, 1999; Farese, 2002).

Accumulation of lipids – both if originating from nutritional sources (PEB, overfeeding, obesity) or from body-fat breakdown (NEB) – has toxic effects ("lipotoxicity"). Interestingly, overconditioned subfertile heifers not only have an excess of subcutaneous fat, but they also have a parallel, detrimental accumulation of fat in their oocytes (Awasthi *et al.*, 2010). Lipotoxicity can explain some mechanisms responsible for insulin resistance, and increased fatty acid accumulation in the cell can directly reduce insulin sensitivity. In addition to this direct effect, increased lipid influx can also indirectly contribute to insulin resistance by inducing oxidative stress during fatty acid breakdown. Mitochondrial oxidative phosphorylation produces ROS that have to be neutralised. Intracellular fatty acid metabolites like diacylglycerol (DAG) can directly activate serine/threonine kinases that phosphorylate IRS-1 and 2 with the consequence of decreased PI3K activation and impaired downstream signalling of the insulin receptor (Randle *et al.*, 1963; Sesti, 2006; Boucher *et al.*, 2014).

Moreover, high energy intake implies an increased production of ROS leading to activation of various cellular stress-response pathways, which can interfere with the physiological cellular signalling pathways (Bloch-Damti & Bashan, 2005). If fatty acids accumulate to a large extent, anti-oxidative molecules and pathways cannot be regenerated in time, which leads to

accumulation of ROS that emerge when excessive NADH, an electron donor in Acetyl-CoA- synthesis from glucose or fat, cannot be regenerated and single electrons are transferred to oxygen (Maechler *et al.*, 1999). The development of insulin resistance might thus represent a compensatory mechanism protecting the cell against further insulin-stimulated energy substrate uptake in an attempt to reduce oxidative stress (Ceriello & Motz, 2004). Increased circulating ROS have further detrimental effects leading to inflammatory responses, mitochondrial dysfunction, and insulin resistance (Figure 2).

In humans, decreased insulin sensitivity that finally leads to insulin resistance can often be diagnosed in overweight or obese persons (DeFronzo & Ferrannini, 1991). Here, long-term overexposure to insulin due to excess energy intake (hyperinsulinemia) is detrimental for the functioning of physiological insulin signalling and can, as a consequence, lead to the manifestation of type 2 diabetes with chronic hyperinsulinemia but a lack of response to insulin in the target cells (Kahn *et al.*, 2006).

In the dairy cow, insulin signalling is different from humans in some aspects due to changes in insulin sensitivity linked to the lactation period. During late pregnancy and continuing postpartum, the cow develops insulin resistance in adipose tissue and muscle (Bell, 1995). This is explained by the excessive energy requirements during early lactation where most glucose goes to the mammary gland where glucose uptake is mostly regulated through GLUT1, and this form of glucose transport is insulin independent (Komatsu et al., 2005, Rose *et al.*, 1997).

In contrast to insulin resistance in over-conditioned cows or during obesity – where hyperinsulinemic conditions appear – circulating insulin and glucose concentrations are low after parturition (De Koster & Opsomer, 2012). This adverse condition where hypoinsulinemia and insulin resistance appear simultaneously highlights the complexity of different mechanisms involved in insulin signalling and its pathophysiological mechanisms. The beneficial or detrimental effect of elevated insulin concentrations always needs to be put in the context of the nutritional situation, and this explains why both high and low insulin levels can imply stress for the individual.

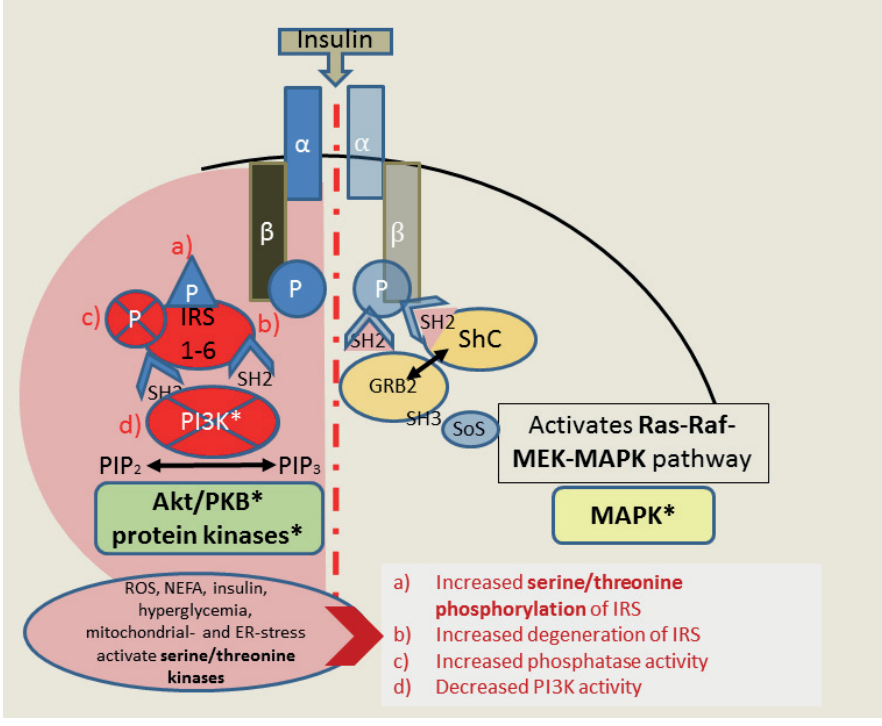

Figure 3. Insulin signalling during insulin resistance.

Akt/PKB = Protein kinase B, ER = Endoplasmic reticulum, GRB2 = Growth factor receptor-bound protein 2, IRS = Insulin receptor substrate, MAPK = Mitogen-activated protein kinase, MEK = Mitogen-activated protein kinase kinase, NEFA = Non-esterified fatty acids, P = Tyrosine phosphorylated state, PI3K = Phosphatidylinositol-4,5-bisphosphate 3-kinase, PIP_2 = Phosphatidylinositol 4,5-bisphosphate, PIP_3 = Phosphatidylinositol (3,4,5)-trisphosphate, Raf = Rapidly accelerated fibrosarcoma, Ras = Ras protein, ROS = Reactive oxygen species, SH2 = Src homology 2 domain-containing, Shc = Shc protein, SoS = son of sevenless, *activated

1.2.5 Insulin and insulin-like growth factors

Insulin-like growth factors (IGFs) are mitogen polypeptides, so-called survival factors that have structural similarities and functional interactions with insulin and insulin signalling pathways. Both IGF-1 and 2 have important roles both in pre- and postnatal growth (DeChiara *et al.*, 1991; Baker *et al.*, 1993). IGF-1 is expressed at low levels in the embryo and thus is seen as more important for postnatal development and growth control than during the embryonic and foetal period. However, mice nulliparous for IGF-1 have been shown to have more than 60% decreased birth weight compared to wild type mice (Powell-Braxton *et al.*, 1993).

IGF-2 is maternally imprinted and is mainly involved in prenatal growth through IGF-1 and insulin receptor. This shows that the insulin receptor, besides its important role in insulin signalling by transmitting metabolic effects, acts on growth control during embryogenesis (Louvi *et al.*, 1997).

In the bovine, transcripts of IGF-1 and 2 and receptors for insulin, IGF-1, and IGF-2 are detectable during the entire preimplantation period, highlighting their importance for growth control. Because all of these growth factors can bind to all the different receptor types (see Figure 8), the different actions are dose and time dependent.

1.2.6 Insulin and fertility

Insulin is an important hormone in the regulation of fertility. The interaction of insulin with other reproductive hormones is necessary because insulin serves as a sensor for metabolic balance, and a certain minimum energy supply needs to be available for reproductive functions such as ovulation (Hill *et al.*, 2008). This is evolutionarily explainable because energy supply must reflect the requirements needed throughout the pregnancy.

Insulin plays a central role in reproduction through direct signalling in the brain. It connects the regulation of the energy status to reproductive functions in the ovary (Bruning *et al.*, 2000). This happens through the hypothalamus–pituitary–ovarian axis where insulin can influence the gonadotropin-releasing hormone (GnRH) release pattern from hypothalamic neurons in the brain (Sánchez *et al.*, 2012). This interaction is mediated together with growth hormone and IGF. If circulating IGF and insulin levels are low due to maternal malnutrition or undernutrition, basal GnRH secretion is suppressed and/or its pulsatile secretion is depressed (Butler *et al.*, 2003), and this can delay important reproductive events, including the onset of puberty, and can impact on reoccurring events such as ovulation (Garcia-Garcia, 2012).

Importantly, both high-energy conditions as well as undernutrition can have detrimental effects on fertility. Because the ovarian pool is fixed at birth, all oocytes will go through possible periods of metabolic imbalance and possible negative impacts might be retained. This is why the consequences of metabolic stress on fertility might last longer than the stress situation itself. The consequences of cumulus-oocyte complex (COC) or embryo exposure to insulin vary depending on the targeted period of life and the metabolic status of the mother, and this has been shown by several authors. In the study of Armstrong (Armstrong *et al.*, 2001), a diet leading to higher peripheral insulin concentrations was beneficial for the growth of the dominant follicle but impaired oocyte quality. Also, high circulating insulin levels have been shown

to be beneficial for the resumption of cyclicity after parturition (Gong *et al.*, 2002) while this was not valid for heifers with already elevated body weight. A feed regimen designed to lower body weight gain was shown to lead to better embryo yield and quality after collection when compared to heifers with high body weight gain (Freret *et al.*, 2006). Moreover, oocyte quality was reduced in hyperinsulinemic heifers (Adamiak *et al.*, 2005a), and chronic dietary restriction resulted in a reduction in dominant follicle growth rate, diameter, and persistence, and the animals became anoestral (Diskin *et al.*, 2003). These studies highlight the importance of a feeding strategy taking into account the energy requirements for follicular growth without compromising oocyte quality and conception rates (Garnsworthy *et al.*, 2009).

In the ovary, insulin is distributed through the ovarian blood flow and reaches the follicular fluid and thus comes into direct contact with the oocyte via transudation (Poretsky *et al.*, 1999). Insulin receptors are present in all ovarian compartments, such as granulosa and theca cells, as well as in the stroma and on the oocyte (Poretsky *et al.*, 1985), and this shows the importance of insulin signalling on the different ovarian cells. In humans, insulin concentrations in the follicular fluid are positively related to progesterone levels (Diamond *et al.*, 1985) and insulin is thus, as in other mammals, presumed to be involved in oocyte maturation (Totey *et al.*, 1995). In cattle, insulin concentrations in the follicular fluid are dependent on follicular stage, and insulin tends to be higher in preovulatory compared to subordinate follicles. However, the basal level of circulating insulin is influenced by diet, independently of follicle stage (Landau *et al.*, 2000).

Besides a direct role of insulin in follicular development, it can have gonadotropic functions and stimulate steroidogenesis, and the gonadotropic effect of insulin becomes evident in ovarian hypofunction that can be observed in women with type 1 diabetes (Poretsky & Kalin, 1987). Insulin can potentiate the response of ovarian cells to gonadotropins on three levels: 1) through increased LH binding capacity on granulosa cells (Adashi *et al.*, 1985), 2) by sensitizing the pituitary gland to GnRH (Soldani *et al.*, 1994), and 3) by influencing the GnRH secretion of the hypothalamus (Sánchez *et al.*, 2012).

In particular, it is well studied how insulin stimulates steroidogenesis both *in vivo* and *in vitro*. Most of the functions of insulin on the ovary are closely linked to the IGF system, including the locally produced IGF binding-proteins (Spicer & Echternkamp, 1995). Hyperandrogenism in the insulin-resistant state is probably partly transmitted through the IGF receptor that binds insulin if insulin is applied in supra-physiological doses (Poretsky, 1991; Monget & Bondy, 2000). Thus, diet-induced hyperinsulinemia could lead to dysregulation of insulin's physiological ovarian functions as a mitogen and its effects on the

stimulation of progesterone production in granulosa cells, androgen production in theca cells and progesterone production in luteal cells. (Spicer & Echternkamp, 1995).

The role of insulin in the final stage of oocyte maturation is based on several facts. Insulin signalling is dependent on the responsiveness of the target tissue through increased expression of insulin receptors. In the preovulatory follicle, insulin receptors are upregulated, and this is associated with increased estradiol production, which supports insulin's action in final maturation and ovulation. The increased expression of receptors in the preovulatory period could thus be a crucial factor in transitioning the follicular development to the preovulatory stage (Gong *et al.*, 1991; Shimizu *et al.*, 2008). In humans, insulin acts as a local factor through the insulin receptor, initially appearing in granulosa cells of the preantral follicle (Samoto *et al.*, 1993).

Circulating insulin levels vary during the oestrous cycle with a peak on the day of ovulation, and feeding a diet that leads to hyperinsulinemia significantly reduces the steady-state distribution of insulin receptors in healthy follicles (Armstrong *et al.*, 2001).

1.2.7 Insulin and *in vitro* embryo production

Insulin signalling components are expressed in cumulus cells (CCs) and oocytes (Acevedo *et al.*, 2007), and insulin signalling is highly important in the very first stages of embryonic development. Transcripts for the insulin and IGF-1 and 2 receptors are detectable at all stages of cattle embryo development from zygote to the blastocyst, which shows the potential of insulin to act at all of these stages (Schultz et al., 1992).

Known for its mitogenic effect, insulin has been used for many years as a stimulatory factor in *in vitro* cell culture systems and thus also for *in vitro* embryo production (IVP). *In vitro* concentrations used in the different media are often much higher (1 to 10 mg/ml) compared with those found physiologically in follicular fluid (0.1 to 1 ng/ml) (Laskowski *et al.*, 2016b), and this is due to the different stabilities of insulin *in vitro* and *in vivo* (Hayashi *et al.*, 1978). The reported effects of insulin on early embryonic development vary in the different *in vitro* studies, and often no effects on the blastocyst rate have been observed (Zhang *et al.*, 1991; Shamsuddin *et al.*, 1993; Bowles & Lishman, 1998; Fouladi-Nashta & Campbell, 2006). Possible positive mechanisms of insulin during embryonic development include anti-apoptotic and mitogenic functions (Byrne *et al.*, 2002). However, elevated insulin might increase oxidative stress through its metabolic function in the cells and thus lower embryo survival, and several reported changes in phenotype or gene expression due to insulin-dependent

signalling illustrates the potential role of insulin acting at several different levels during final oocyte maturation and early embryo development.

1.2.8 Comparative aspects

Situations with elevated or decreased levels of circulating insulin exist in both cattle and humans and in both species, the important role of insulin signalling for maintenance of reproductive functions and the link between metabolism and fertility has been described (Schneider, 2004).

The NEB of the dairy cow postpartum is a well-known example where energy deficiency leads to a failure to maintain reproductive functions and shows the important role of insulin in governing fertility functions (Wathes *et al.*, 2003; Pryce *et al.*, 2004; Butler, 2005). The consequences of decreased circulating insulin levels for fertility are late resumption of cyclicity postpartum and delayed ovulation, both of which are poor conditions for early embryonic development and for the maintenance of the pregnancy. This effect is mainly transmitted through the role of insulin in the brain, where GnRH and LH pulse frequency is reduced if insulin levels are low, leading to decreased oestrogen production. The same is observed in humans with severe chronic energy deficiency, such as in anorectic women who develop amenorrhea (De Souza & Metzger, 1991) and who have reduced fertility outcomes for both natural conception and assisted reproductive technologies (ART) (Veleva *et al.*, 2008). Furthermore, it is well known that both obesity in women and overfeeding in cows are detrimental for fertility. Hyperinsulinemia is associated with impaired oocyte quality in over-conditioned cows (Adamiak *et al.*, 2005b) just as women suffering from metabolic syndrome or type 2 diabetes have decreased oocyte quality (Niu *et al.*, 2014) and impaired reproductive functions (Sakumoto *et al.*, 2010; Pantasri & Norman, 2014).

The similarities between the human and bovine species provide an excellent opportunity to elucidate the actions of insulin, and new insights into the relation between metabolic and reproductive disorders could help to improve fertility in both species. Because experiments based on human embryos are ethically controversial, the need for alternative methods to gain further knowledge about underlying mechanisms of impaired fertility due to metabolic imbalance in humans becomes evident. The parallels in human and cattle ovarian reserve, follicular dynamics, and embryo metabolism explain the suitability of the bovine model for human embryonic development (Ménézo & Hérubel, 2002; Campbell *et al.*, 2003a).

1.3 Early embryonic development

1.3.1 Oocyte maturation

Follicular growth until final oocyte maturation takes around three months in the cow *in vivo* (Lussier *et al.*, 1987; Beam & Butler, 1999), with the final maturation occurring inside the follicle after the release of a preovulatory LH peak (Hyttel *et al.,* 1999). This can be simulated for embryo production by a maturation period of 22–24 h in *in vitro* systems (Ward *et al.,* 2002). Oocyte maturation is the first fundamental step in the development of a healthy offspring and is a complexly and highly regulated process where the interaction of the oocyte and its surrounding CCs prepares the oocyte on the cellular and molecular level for successful fertilization (Richards, 2005). The COC is an interacting tissue environment where the surrounding CCs support the oocyte during growth and final maturation by supplying it with metabolites. Pyruvate derived from CC glucose metabolism is the preferred substrate to provide the oocyte with energy (Sutton-McDowall *et al.*, 2010, see also a more complete review in chapter 1.3.4.).

The cells of the COC are connected via gap junctions and communicate through paracrine signalling, and the oocyte is dependent on the surrounding cells for the completion of meiotic maturation (Matzuk *et al.*, 2002; Hyttel *et al.*). The oocyte maturation period is known to be especially sensitive for stressors such as metabolic imbalance, temperature changes, and toxic influences (Moor & Crosby, 1985; Combelles *et al.*, 2009). The early events in life are easily disturbed by such disrupters and the so-called metabolic programming occurs peri-conceptionally (Fowden *et al.*, 2006; Martin-Gronert & Ozanne, 2012) with potential negative effects for the offspring lasting throughout its life and possibly even transmitted to subsequent generations.

In vivo, COCs are exposed to the follicular fluid, the composition of which is closely correlated to the situation in the maternal serum (Spicer & Echternkamp, 1995; Landau *et al.*, 2000). This fact explains the strong link between nutrition, metabolism, and oocyte quality because metabolites and hormones in the circulation will also come in direct contact with the oocyte (Landau *et al.*, 2000; Leroy *et al.*, 2012). The maternal nutritional state can programme the oocyte's metabolism at this early stage of development (O'Callaghan & Boland, 1999; Fleming *et al.*, 2012).

For final maturation and thus being prepared for successful fertilization, the oocyte has to go through several nuclear and cytoplasmic changes (Hyttel *et al.*, 1986; Eppig, 1996; Fulka *et al.*, 1998).

Briefly, cytoplasmic maturation involves a range of metabolic and structural changes, allowing subsequent fertilization, cell cycle progression from meiosis to mitosis, and activation of several pathways for the programming of preimplantation development (Eppig *et al.*, 1994; Trounson *et al.*, 2001).

From having mitochondria distributed in a peripheral pattern, the LH peak induces the formation of mitochondria clusters associated with lipid droplets. At the same time, the formation of the perivitelline space with loss of contact between CCs appears (Kruip *et al.*, 1983).

On the nuclear level, the nuclear envelope ruffles and meiosis resumes, visible by germinal-vesicle breakdown. In the final stage, the polar body is extruded, the mitochondria disperse, and most organelles move to the centre of the oocyte while cortical granules are formed in the periphery (Kruip *et al.*, 1983; Combelles *et al.*, 2002).

1.3.2 Development until the blastocyst stage

The fertilized oocyte is called a zygote and contains all of the materials for initiating the first developmental steps, including new protein synthesis, mRNA activation, protein and RNA degradation, and reorganization of the organelles in the cell (Stitzel & Seydoux, 2007). The first cleavage occurs 25–26 h post fertilization (Hamilton & Laing, 1946; Sakkas, 2001). The first cell divisions are under maternal control and are based on stored mRNA and protein molecules in the oocyte before the embryonic genome takes over transcription (Barnes & Eyestone, 1990) (see more in section 1.3.1). At 42–44 h post fertilization, the embryo should have reached the 4- to 8-cell stage with equal numbers of blastomeres (Betteridge & Fléchon, 1988). Early cleavage dynamics have been reported to be a tool to predict embryo quality and developmental potential (Van Soom *et al.*, 1992; Kubisch *et al.*, 1998; Lonergan *et al.*, 1999), and the fastest-growing embryos seem to have the best viability and potential to reach the morula and blastocyst stages.

The next ultrastructural change is morula formation at the 32-cell stage when compaction occurs (Van Soom *et al.*, 1992). The blastomeres become either part of the embryonic inner cell mass (ICM) or the trophoblast that will form the foetal annexes (Betteridge & Fléchon, 1988). In the bovine, the transition from compacted morula to the blastocyst stage occurs between day 6 and 8 of development (Betteridge & Fléchon, 1988). The most important characteristic of a blastocyst is the formation of the blastocoel cavity and the clearly distinguishable ICM. The embryo is enclosed by the zona pellucida until hatching (Lindner & Wright, 1983).

1.3.3 Embryo morphology

The most accurate evidence for the developmental competence of an embryo is to allow development into a live, healthy offspring. This is, for several reasons, not always applicable for research purposes. Thus, other methods have been established with the aim of predicting oocyte developmental potential and embryo quality (Van Soom *et al.*, 2003).

Basic developmental data at different time points are usually recorded to follow the different developmental steps and to look for signs that the embryo might fail to pass the important thresholds such as first cleavage, embryonic genome activation (EGA), compaction, and blastocyst formation (Andra *et al.*, 1999; Lonergan *et al.*, 2006). On Day 7 and 8, morphological evaluation of blastocyst stages and quality grading of blastocysts is possible at a more advanced level and includes several criteria. The diameter of a Day-8-blastocyst (BC8) is approximately 150 to 190 µm, including a zona pellucida thickness of 12 to 15 µm, and some parameters that are included in the evaluation of embryo quality are shape, colour, cell number, presence of extruded and degenerated cells and size of the perivitelline space (Lindner & Wright, 1983, Crosier *et al.*, 2001). In addition to these, staining for the actin skeleton, mitochondrial pattern (Zijlstra *et al.*, 2008), lipid droplets (Abe *et al.*, 1999, 2002), and apoptosis (Yang *et al.*, 1998; Gjørret *et al.*, 2003) can be used to detect differences in embryo phenotype and allows, together with the developmental rates, conclusions to be drawn about the embryo's viability.

The first important assessment criterion is light microscopy determination of the developmental stage (Shea, 1981) to ensure that the embryo is not retarded in development compared to the other embryos of the same day of development. On Day 8, the blastocyst can be early, blastocyst, expanding, expanded, hatching, or hatched and the different blastocyst stages were described and defined by Lindner and Wright in 1983 (Lindner & Wright, 1983), and the same staging criteria are still used today. In brief, an early blastocyst has already formed a fluid-filled blastocoel and the embryo itself forms around 70 -80% of the volume. The blastocyst stage is characterised by a growing blastocoel that is highly prominent, and compaction of the embryo (differentiation between ICM (darker) and trophoblast) becomes visible. Up to this stage, the diameter of the embryo does not change much from the size of the oocyte. When the embryo is expanding or expanded, the zona pellucida thins and the embryo diameter increases. The next stage is the partly hatched (hatching) or entirely hatched embryo, at which point it has shed the zona pellucida. All of these stages can be found on Day 8 of development.

The next step is to assess the embryo quality grade according to the guidelines that have been developed by the International Embryo Technology

Society (IETS) (Stringfellow DA, 2010). The quality grades are indicated by a descending scale between 1 and 4 where 1 stands for "excellent/good", 2 for "fair", 3 for "poor" and 4 for "dead/degenerated".

Following different types of staining, more characteristics can be evaluated such as cell number, mitochondria distribution, and actin cytoskeleton structure.

A blastocyst on Day 8 contains around 100–200 cells (Byrne *et al.*, 1999; Watson *et al.*, 2000), and larger embryos are often assessed as more viable because they are further advanced in their development. However, there are also other theories that claim that moderate growth is beneficial for the long-term viability of the embryo and health of the offspring (Leese *et al.*, 2008).

Mitochondria are the energy providing organelles in cells and are reported to have important functions in competent oocytes and blastocysts (Lane & Gardner, 1998; Bavister & Squirrell, 2000). The mitochondrial activity and pattern in the embryo varies depending on developmental stage (Tarazona *et al.*, 2006). However, an even distribution between all blastomeres with no accumulations or empty areas within the blastocyst is considered to be beneficial for the viability of the embryo (Båge *et al.*, 2003; González & Sjunnesson, 2013). The same is true for actin distribution in all parts of the embryos where good quality embryos often show an equal distribution pattern, while degraded or low quality embryos show cytoskeleton disintegration and might thus be more fragile and less viable (Zijlstra *et al.*, 2008; González & Sjunnesson, 2013).

1.3.4 Oocyte and embryo metabolism

Oocyte maturation and the first developmental steps such as growth, cell division, and differentiation are energy-consuming processes that require a high availability of energy substrates (Gardner, 1998). The embryo itself or through the CCs is able to metabolize different types of substrates such as glucose, triacylglycerides, and amino acids. Besides these exogenous substrates that are present in oviductal fluid or *in vitro* media, endogenously stored triacylglycerides and glycogen are reported to contribute to energy availability during early development (Brinster, 1971; Ferguson & Leese, 2006). Before elongation, embryos are reliant on oxidative phosphorylation of pyruvate, lactate, and amino acids for ATP production, with increasing glucose consumption in more advanced stages after compaction (Leese, 1995; Thompson *et al.*, 1996; Thompson, 2000). Besides providing energy, carbohydrate and amino acid metabolism generates substrates with functions in the cellular stress response (Gardner, 1998).

Oocyte metabolism is tightly connected to CC metabolism (Figure 4), and in the early stages of development the CCs provide the oocyte with pyruvate, its

most favourable substrate (Sutton-McDowall et al., 2010). Oocyte-CC communication continues even after meiotic resumption when most of the gap junctions are lost (Sutton *et al.*, 2003). Oxygen consumption is a good measure of metabolic activity, and during maturation it is at similar levels as at the blastocyst stage (Houghton & Leese, 2004) and the total ATP content is increased in mature oocytes (Stojkovic *et al.*, 2001; Ferguson & Leese, 2006). It has been shown that LH increases glycolysis in bovine oocytes, and this is assumed to be the mechanism through which LH enhances maturation (Zuelke & Brackett, 1992).

Glucose consumption has been used to predict embryos with high developmental potential in mice (Gardner & Leese, 1987) and cattle (Renard *et al.*, 1980). However, moderate glycolytic activity close to levels that are found *in vivo* seems to be best for viability of the embryo (Lane and Gardner, 1996). Here, elevated insulin levels could contribute to excess glucose consumption during early embryo development and thus have an adverse effect on viability. Glucose metabolized through the pentose phosphate pathway generates ribose that can be used for nucleic acid production and NADPH for glutathione regeneration through reduction, an important pathway against ROS (Wales, 1973; Rieger, 1992; Gardner, 1998).

Lipid metabolism also varies depending on development stage and can be of exogenous or endogenous sources (Ferguson & Leese, 2006; Haggarty *et al.*, 2006). In humans, it has been shown that pre-implantation embryos actively take up fatty acids (Haggarty *et al.*, 2006). Besides triacylglycerides and fatty acids, even cholesterol has important functions during embryogenesis, and impaired cholesterol metabolism might have detrimental consequences for the embryo (Farese & Herz, 1998).

Amino acids are possibly consumed to a different extent depending on embryo developmental stage (Partridge & Leese, 1996; Lane & Gardner, 1998). Alanine and glutamine are precursors for gluconeogenesis (Felig *et al.*, 1970), but their contribution to energy supply is limited. Other functions of amino acids might be of higher relevance during embryo development because they function as substrates for biologically important molecules such as melanin (Korner & Pawelek, 1982), as osmolytes, as buffers, and as regulators of embryo metabolism (reviewed by Gardner, 1998), and this explains their role in embryo development (Takahashi & First, 1992).

In summary, any dysregulation in substrate availability or metabolic functions might lead to disturbances in healthy embryo development. This highlights the importance of developing adequate media for IVP of embryos that is similar to the conditions found *in vivo* because requirements for the different metabolites could change during development, and it also explains why

metabolic disturbances in the mother might lead to conditions that are suboptimal for embryo viability.

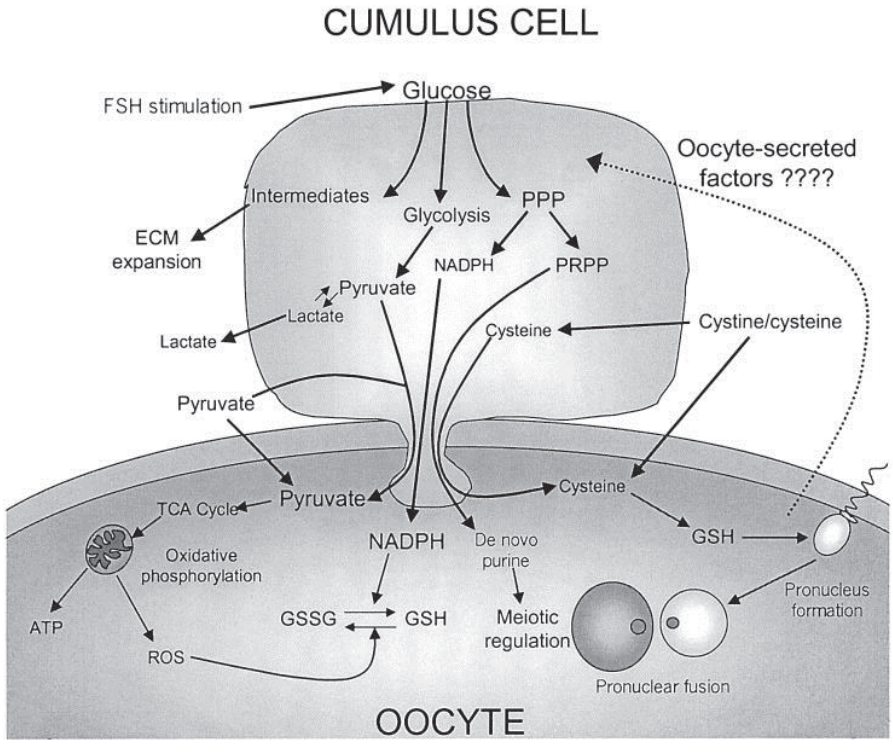

Figure 4. Proposed model of the metabolic interactions and activity of CCs and the oocyte. Numerous energy substrates are supplied to the COC by the surrounding fluid, including glucose, pyruvate, lactate, and amino acids.

Glucose can be utilized via three major pathways: (i) glucose oxidation (the combination of glycolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation); (ii) the pentose phosphate pathway (PPP); or (iii) it can be converted to intermediates and utilized for extracellular matrix (ECM) expansion. FSH stimulates glucose metabolism by cumulus cells. Glucose utilization begins with glycolysis (within cumulus cells) where glucose-6-phosphate is converted to pyruvate, which can then enter the oocyte directly or be converted to lactate. Pyruvate is further oxidized by the TCA cycle within ovum mitochondria, followed by oxidative phosphorylation in the mitochondrial intermembrane where ATP is released by electron transfer. PPP also begins with the oxidation of glucose to glucose-6-phosphate within cumulus cells, with one of the products of the pathway, phosphoribosyl pyrophosphate (PRPP), being used by the oocyte for purine synthesis. Purines are involved in the regulation of nuclear maturation. PPP is also involved in general cytoplasmic homeostasis since NADP+ is reduced to NADPH. Amino acids cystine and cysteine are involved in the production of glutathione (GSH), accumulation of which appears essential for early embryonic development. Although oocyte-secreted factors are known to have major effects on development and differentiation of cumulus cells, there are no data available concerning their effects on the metabolism of cumulus cells. GSSG= oxidized GSH; ROS = reactive oxygen species. Reprinted with permission from (Sutton *et al.*, 2003).

1.3.5 *In vitro* produced embryos

Compared to their *in vivo* counterparts, IVP embryos are in general less viable. Blastocyst development rates are lower *in vitro,* and differences in morphology, metabolism, gene expression, epigenetics, cryotolerance and pregnancy rates after transfer have been studied in order to explain their decreased developmental competence (Wright & Ellington, 1995; Thompson, 1997).

One explanation for these differences is an increased exposure to oxidative stress during *in vitro* culture (Cagnone & Sirard, 2013; de Assis *et al.*, 2015). Many attempts to improve culture conditions and protocols have been made in recent decades resulting in improved IVP, but differences in embryo quality are still observed (Niemann & Wrenzycki, 2000; Galli *et al.*, 2003; Hasler, 2003). For example, Khurana and Niemann (2000b) detected differences in aerobic glycolysis rate and lactate oxidation between IVP and *in vivo* embryos.

Some authors observed differences in morphology in the form of a darker overall appearance with larger blastomeres at early stages and a reduced perivitelline space along with reduced viability of IVP, with fewer IVP embryos surviving the cryopreservation procedures (Khurana & Niemann, 2000a; Rizos *et al.*, 2002). Even if not having a consistently different morphology, a mismatch in timing might exist in IVP embryos that leads to delays in development (Plante & King, 1994).

In vitro, typical developmental rates for bovine embryos are 20–40% (Thompson & Duganzich, 1996; Rizos *et al.*, 2002), and gene expression studies have been used with the aim of discovering the underlying reasons for the impaired developmental potential of IVP embryos. Important genes for development are differentially expressed in IVP embryos, e.g. connexion 43, which plays a role in maintaining compaction (Wrenzycki *et al.*, 1996; Niemann & Wrenzycki, 2000). Also, CCs have a different gene expression pattern depending on whether oocyte maturation occurs *in vitro* or *in vivo* (Tesfaye *et al.*, 2009; Gad *et al.*, 2012)

Current research efforts into gene expression and regulation aim to better understand the epigenetic mechanisms that present a link between the genome and the environment and induce permanent changes in gene expression pattern through metabolic programming (Santos *et al.*, 2003) (see chapter 1.4.2.)

1.3.6 Comparative aspects between human and bovine embryo development

The use of animal models to better understand mammalian embryogenesis is important because such models allow the study of pathophysiological mechanisms in the oocyte and early embryo without evoking ethical

controversies (Leese *et al.*, 1998). Many morphological, metabolic, and gene expression studies require techniques that do not allow the embryo to survive. Still, more knowledge is necessary to improve culture conditions and techniques in both human and bovine IVP (Hasler *et al.*, 1995; Vayena *et al.*, 2002).

There are similarities between cattle and humans in terms of ovarian reserve, follicular dynamics, and embryonic metabolism (Ménézo & Hérubel, 2002; Campbell *et al.*, 2003b) that explain the suitability of the bovine model for human embryonic development. The bovine also has other features as major embryonic genome activation that are more closely related between human and cattle than between human and mice (Telford *et al.*, 1990; Ménézo *et al.*, 2000; Neuber & Powers, 2000). In addition, the durations of pregnancy are very similar between humans and cows.

However, some differences exist in human and bovine IVP, for example the day of transfer is usually on Day 7 or 8 in the bovine (Hasler *et al.*, 1995) and on Day 3 or 5 in the human (Coskun *et al.*, 2000). The reason for this is to avoid the human embryo being outside the natural environment longer than necessary. Moreover, the routines for cryopreservation are different, and most of the human embryos are frozen before transfer because the embryo usually has to be transferred back into the same mother from which the oocyte was derived (Wallach & Trounson, 1986; Mandelbaum *et al.*, 1998). In addition to that, the cryopreservation of embryos allows for the collection of multiple oocytes in only one ovum pick up session and allows those that cannot be used in the first fresh cycle to be saved for later IVP trials. In the cow, IVP embryos are often transferred fresh because the pregnancy results are often better than if using frozen embryos, and the availability of recipients is often not an issue. However, efforts to improve and develop freezing protocols and vitrification of livestock animal embryos have been made, and pregnancy rates after freezing have improved in the bovine species (Niemann, 1991). The combination of IVP and cryopreservation allows the application of new tools such as sexing or genomic selection in dairy cattle breeding programmes because embryos can be biopsied, genotyped, and transferred if the genetic value is high (Chrenek *et al.*, 2001).

1.4 Gene expression in early embryos

1.4.1 Transcriptome

The transcriptome results from the active part of the genome and consists all RNAs, including messenger RNA (mRNA) molecules present at a given time point in a specific tissue (Wang *et al.*, 2009; Schwanhausser *et al.*, 2011).

Because differential mRNA expression reflects the response to intrinsic factors and external influences, transcriptome analysis allows conclusions to be drawn about activated pathways, possible stress mechanisms, and molecular events in tissues, oocytes, and embryos (Katz-Jaffe *et al.*, 2009; Ramsköld *et al.*, 2009; Robert *et al.*, 2011; Sirard, 2012).

During early embryonic development, transcriptome analysis reveals interesting insights into the ongoing developmental steps and possible harmful impacts because certain pathways are characteristic for stress conditions (Leese *et al.,* 2008; Gad *et al.*, 2012). The transcriptome of the embryo is different from other cells because maternal factors dominate the initiation of the first developmental events, and thus the importance of oocyte quality for further development becomes evident (Brevini Gandolfi & Gandolfi, 2001; Sirard *et al.,* 2006). After a short period of common gene expression from both the maternal side and the embryonic genome, maternal products degrade and the embryonic genome takes over transcription (Telford *et al.*, 1990). Embryonic genome activation (EGA) coincides with the first differentiation steps and the expression of genes important for implantation (Misirlioglu *et al.*, 2006). Before EGA, embryonic cells are totipotent until the 4-cell stage (Van de Velde *et al.*, 2008) and are pluripotent until at least the 8-cell or the 16-cell stages (Mitalipova *et al.*, 2001).

EGA is the switch from the maternal, oocyte stored mRNA-translation to embryonic transcriptional activation. Before that, the embryonic genome is transcriptionally silent and the cell divisions are under maternal control (Frei *et al.*, 1989; Kopečný *et al.*, 1989; Vallée *et al.*, 2009), and inhibition of the EGA step blocks further development (Plante *et al.*, 1994). The timing of EGA differs in different species (Watson *et al.*, 1993), and in the bovine, major EGA occurs at the 8–16 cell stage. However, a minor EGA of some genes related to RNA processing is already observed at earlier stages, probably preparing the embryo for major EGA and the subsequent transcription of functional genes (Memili *et al.*, 1998; Kaňka *et al.*, 2009; Graf *et al.*, 2014). These early developmental stages require several activation steps and are very sensitive to disturbances that can lead to unknown consequences for later developmental potential. Kues *et al.* (2008) showed significant differences in gene expression pattern between *in vivo* and *in vitro*-derived embryos that might explain possible differences in developmental potential. The aim of transcriptome studies is to detect possible markers for survival or detrimental events in blastocysts and thus to predict their potential to survive the critical period of preimplantation development and to subsequently be able to implant and develop into a healthy offspring (Ko, 2004). A better understanding of early events at the molecular level could improve the

outcome of ART by allowing the selection of optimal embryos for transfer at an early stage (Niemann & Wrenzycki, 2000; Kues *et al.*, 2008).

1.4.2 Metabolic programming and epigenetics

There is a growing body of evidence showing that influences early in life and especially around the time of fertilization can have a lasting impact on the offspring´s health with long-term and even trans-generational consequences (Calle et al., 2012). These so-called "epigenetic effects" are influenced by environmental factors such as toxins or the nutritional and metabolic state of the mother and can affect gene expression in a permanent manner. The blastocyst is a difficult target for methylation analysis because DNA methylation is progressively reduced during the pre-implantation development and because of high variations in stage-dependent methylation patterns (Santos & Dean, 2006).

There are several epigenetic mechanisms involved in transmitting these effects, such as cytosine-methylation at CpG dinucleotides and histone modifications, both of which can be heritable (Godfrey *et al.*, 2007). CpG methylation is a reversible and dynamic epigenetic mechanism controlling chromatin structure and is involved in regulating short-term and long-term gene expression. In particular, several de-methylation and re-methylation processes during early development have been defined (Chen & Riggs, 2011). While methylation of the oocyte genome is maximal at the germinal vesicle stage (O'Doherty *et al.*, 2012), demethylation occurs in the zygote and during early cleavage. With major embryonic genome activation, methylation increases again and remains until the blastocyst stage (Dobbs *et al.*, 2013). This plasticity of the methylation pattern makes the sensitivity of oocytes and early embryos for epigenetic reprogramming evident (Hackett & Surani, 2012; Marcho *et al.*, 2015). Epigenetic or metabolic programming, potentially leading to alterations in the epigenome of the embryo, occurs through maternal metabolic signals (Portha *et al.*, 2014)

A different DNA methylation pattern leading to changes in chromatin structure at regions controlling transcription of genes can result in different transcriptional activity of these genes and these effects can persist through the entire life of an offspring. Epigenetic dysregulation has been reported to cause metabolic disorders and obesity later in life (Li *et al.*, 2010; Jaeger *et al.*, 2016), and insulin resistance might already be present in oocytes (Turner & Robker, 2015). There is evidence that *in vitro* conditions lead to perturbations in DNA methylation pattern (Chason *et al.*, 2011) that can either be in the form of global hypomethylation (Grace & Sinclair, 2009) or hypermethylation (Deshmukh *et al.*, 2011) compared to the *in vivo* situation.

Efforts to improve *in vitro* culture conditions should pay special attention to this "inheritable" factor of metabolic imbalance that impairs developmental competence and could have long-term consequences on the health of the offspring and subsequent generations (Shi & Haaf, 2002).

2 Aims of the thesis

The overall aim of the thesis was to identify the effects of insulin during oocyte maturation on the developmental potential of bovine blastocysts. The specific objectives were:

- To establish an *in vitro* model for elevated insulin conditions during oocyte maturation by using two different doses and to relate results from the model to *in vivo* situations (**Paper I**).

- To investigate the effect on cleavage, developmental rates, embryo stage, and quality grade and to further define morphological changes in cell count, mitochondrial pattern, and actin structure (**Paper II, III**).

- To investigate the overall effect of insulin exposure on the gene expression pattern (transcriptome) of Day 8 blastocysts (**Paper III**).

- To further define the effects of insulin on the lipid profile of Day 8 blastocysts by analysing gene expression related to lipid metabolism and lipid profile (**Paper IV**).

3 Materials and methods

3.1 Experimental design

To study the effects of insulin on developmental potential of bovine Day 8 blastocysts, several study parts were included (Figure 5).

In **study I**, the *in vitro* model was established by exposing oocytes during maturation to two concentrations of insulin – INS10 (10 µg/ml) and INS0.1 (0.1 µg/ml) – and these were compared to the control group INS0 without any added insulin.

In **study II**, the morphology of the blastocysts was investigated for differences between insulin-treated groups and controls.

In **studies III** and **IV,** gene expression profiles were determined with a special focus on the lipid profile in **study IV** combined with DESI –MS analysis.

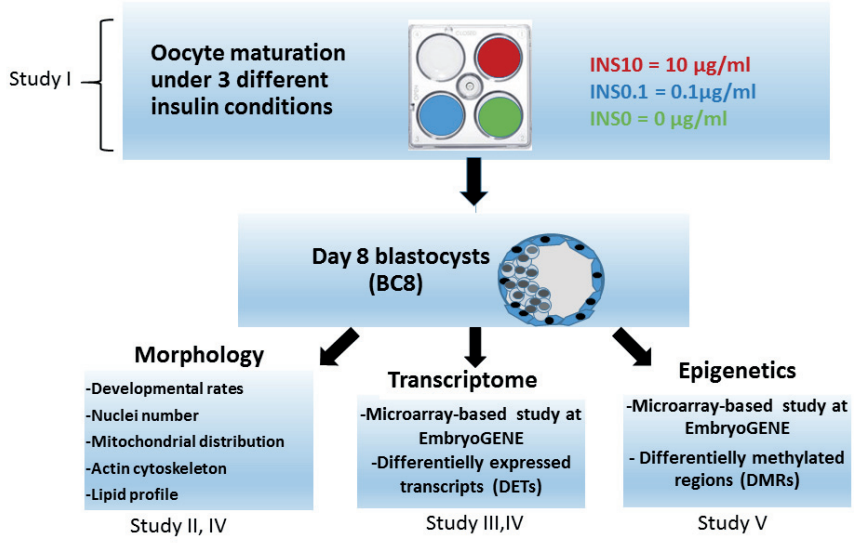

Figure 5. Experimental design and overview of studies I to IV.

3.2 Oocyte origin and *in vitro* production of embryos

For IVP, all media and constituents were obtained from Sigma-Aldrich (Stockholm, Sweden) if not otherwise stated. Media were freshly produced in the laboratory (on a daily or weekly basis depending on proven protocols) according to standard methods described by Gordon and modified according to Abraham (Gordon, 2003; Abraham *et al.*, 2009).

3.2.1 Oocyte collection

As the oocyte source, ovaries were collected at a local abattoir within one hour post mortem and kept at 35°C until arrival at the laboratory. Follicles with diameters of approximately 3–8 mm, were then aspirated, and aspirates containing follicular fluid and oocytes were collected into TCM-199-based search medium. The COCs were stored in search medium for washing, collection, and selection. Only COCs of the best quality-grades 1 or 2 (Gordon, 2003) were selected for maturation. The selected COCs were washed in search medium and randomly allocated to the INS10, INS0.1, or INS0 groups (Figure 5).

3.2.2 *In vitro* maturation and insulin supplementation

Before maturation, the COCs were washed in maturation medium to minimize the risk for contamination of the medium with search medium. Maturation medium consisted of bicarbonate-buffered TCM-199 (M2154) supplemented with 0.68 mM L-glutamine (G8540), 0.156 µg/ml FSH and 0.03 µg/ml LH (Stimufol; PARTNAR Animal Health, Port Huron, Canada), 50 µg/ml gentamicin, and 0.4% BSA (w/v) fraction V. The COCs were matured in groups of 30–45 in a well containing 500 µl medium supplemented either with no (INS0), 0.1 µg/ml (INS0.1), or 10 µg/ml (INS10) bovine insulin (I5500). The groups were incubated for 22 h (24 h after the end of aspiration) at 38.5ºC under an atmosphere of 5% O_2, and 5% CO_2.

3.2.3 *In vitro* fertilization

For the different *in vitro* fertilization steps, the following media were used (for detailed protocols see **Papers II and III**): washing medium, fertilization medium, and capacitation medium (CM). Washing medium consisted of modified Tyrode's albumin lactate pyruvate (mTALP), supplemented with 0.3% fraction V BSA (w/v) and 50 µg/ml gentamicin.
Fertilization medium consisted of mTALP containing 0.6% fatty acid-free BSA (w/v), 50 µg/ml gentamicin, 3 µg/ml heparin (H3149), penicillamine-hypotaurine-adrenaline, and sodium metabisulphite (S9000).

Capacitation medium contained mTALP without $CaCl_2$ and supplemented with 1.25 mg/ml glucose, 50 µg/ml gentamicin, and 0.6% BSA (w/v).

After 22 h of maturation, COCs were transferred to four-well dishes containing 460 µl fertilization medium. For oocyte fertilization, two sperm straws (250 µl each) were thawed and prepared using the swim-up procedure to select the most motile sperm (Ng *et al.*, 1992). The sperm suspension was added to the oocytes in a volume giving a final concentration of 1×10^6 spermatozoa/ml. Groups of 30–45 oocytes per well and spermatozoa were co-incubated at 38.5ºC in a maximally humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 22 h.

3.2.4 *In vitro* culture

In vitro culture was performed in modified synthetic oviductal fluid (mSOF) that consisted of 0.11M sodium chloride (S5886), 7 mM potassium chloride (P5405), 1.19 mM potassium phosphate monobasic (P5655), 25 mM sodium bicarbonate (S5761), 0.33 mM pyruvic acid sodium salt (P4562), 1 mM L-glutamine (G8540), 0.171 mM calcium chloride (C7902), 1.5 mM glucose (G6152), 110

mM sodium lactate (L7900), and 0.49 mM magnesium chloride (M2393), with the addition of minimum essential medium non-essential amino acids solution (100×; M7145), amino acids solution (50×; B6766), 0.4% fatty acid-free BSA (w/v), and 50 µg/ml gentamicin.

After the fertilization period, presumed zygotes were denuded and transferred into culture conditions where they were incubated until Day 8 under a humidified atmosphere of 5% CO_2, 5%O_2, and 90% N_2 at a temperature of 38.5ºC in 500 µl mSOF medium covered with paraffin oil (Ovoil; Vitrolife AB, Gothenburg, Sweden).

3.2.5 Measurement of insulin concentrations by ELISA

Insulin concentrations in the INS0.1 and INS10 groups were analysed at three different time points: before maturation when the medium itself had been pre-incubated and before any COCs were added (BM); after maturation with co-incubation of COCs for 22 h (AM), and a control containing only medium incubated for 22 h without any COCs: "after maturation empty" (AME). A bovine insulin ELISA for quantitative determination of bovine insulin in serum or plasma specially optimized for bovine samples, was used (Mercodia, Nr. 10-1201-01). No advanced statistical analysis was applied because the small study size did not allow for more than a numerical assessment.

3.3 Embryo morphology

3.3.1 Developmental rates, stage, and grade

The proportion of cleaved embryos ("cleavage rate") was checked 44 h after fertilization noting also the proportion of embryos beyond the 2-cell stage. On Day 7 and 8, the blastocyst rate was recorded (number of blastocysts per number of immature oocytes). On Day 8, blastocysts were graded for quality (grades 1 to 4) and developmental stage (blastocyst, expanded blastocyst, hatching blastocyst, or hatched blastocyst) modified according to (Stringfellow DA, 2010; Bo & Mapletoft, 2013). Morphological evaluation is described in detail in chapter 3.3.3 and **Paper II**.

Resulting blastocysts were removed from the culture medium for immediate staining and microscopy or stored frozen at −80°C for future RNA preparation and subsequent gene expression studies.

3.3.2 Embryo staining

In total, 178 Day 8 blastocysts (BC8s) were used for morphological evaluation. Three different dyes were used to stain active mitochondria (Mitotracker Orange, Invitrogen M7510), DNA (Hoechst 33342, Sigma), and actin cytoskeleton (Alexa Fluor 488, A 12379, Invitrogen).

Blastocysts were first stained with 200 nM Mitotracker Orange dissolved in mSOF and put overnight in fixing solution. Actin staining was performed the following day with Alexa Fluor 488 Phalloidin after 10 min of permeabilization with Triton X-100. Hoechst staining was performed with 4.45 µM dye for 20 min. Mounting in black-well plates in Vectashield (Vector Laboratories, Burlingame, CA, USA) was done after three washes in PBS with 0.1% polyvinyl alcohol (PVA) to clean the remaining dye from the embryos. Embryos were placed in the centre of the well and Vectashield was added around the drop until the well was completely filled.

3.3.3 Morphological evaluation of embryos

Images were taken using a confocal laser scanning microscope (LSM 510, Carl Zeiss AB, Jena, Germany). An epifluorescence and a fluorescence microscope camera were used for acquiring the images. Image analysis was performed using ZEN 2008 Light Edition Software (Carl Zeiss; http://www.zeiss.com).

Each BC8 image was captured using standard settings at 20× magnification, and one camera image of the epifluorescence microscope (CAM) was taken with focus in the central area of the blastocyst.

Staging and grading information from the light microscopy analysis was used to identify and evaluate the individual BC8s. During the evaluation process, no information was given about the treatment group or other characteristics (blinded study). Twelve consecutive Z-stack planes were merged into a three-dimensional image in order to assess actin cytoskeleton grade and mitochondria categories in each embryo.

The **number of nuclei** was counted by two independent evaluators from the CAM image (blue Hoechst staining, Figure 6A) that was transformed into a black and white image (Figure 6B). The average of both counts was calculated and if the sums differed by more than 10%, the count was repeated. If the counts still differed by more than 10%, the counts were deleted from the final calculations.

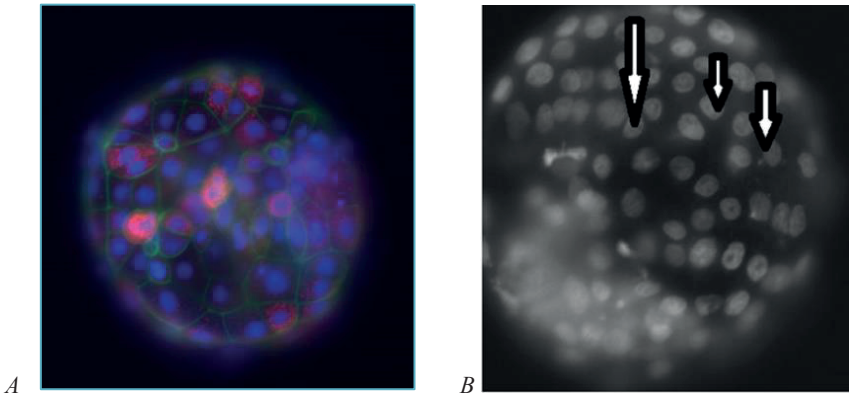

Figure 6 A: Staining of nuclei with Hoechst dye and cell counts in BC8 (confocal 3D image), and 6 *B*: Black and white version with arrows indicating examples for cell nuclei that have been counted.

Actin cytoskeleton structure was evaluated by using three different quality categories, as previously described by (Zijlstra *et al.*, 2008; González & Sjunnesson, 2013) where ACTIN1 was classified as most favourable and ACTIN3 as the lowest quality pattern. All images were evaluated twice by the same person in a blinded procedure. Precise information about the different categories is given in Table 1, and examples are given in Figure 7. Briefly, ACTIN1 indicated a sharp staining of cell borders and an abundant microfilament complement in at least 75% of all cells. ACTIN2 was given to embryos if gross maintenance of the cell outline was observed, but some clumped or scant microfilaments were present in the cytoplasm. If cell integrity was lost and actin filaments were visible as clumps or aggregates, embryos were categorized as ACTIN3.

For classification of **mitochondria staining,** two categories were used to evaluate spatial distribution pattern of active mitochondria.

The first category, MITO, contained three grades according to (González & Sjunnesson, 2013) as described in Table 1 and shown in Figure 5. In brief, MITO1 had an even distribution of active mitochondria in more than 90% of the cells and there were no outlier cells with extreme staining or large accumulations of dye. MITO3 embryos lacked active mitochondria in some cells or areas and had some overstained cells, blurry staining, large accumulations, or unclear cell borders. MITO2 was the intermediate class for embryos that had a few of the characteristics of class MITO3, but only to a small degree.

The second mitochondria category – MitoC ("mitochondria centred") – was used to further describe differences in mitochondria quality based on the spatial distribution of active mitochondria in the cytoplasm. MitoC1 was given to

embryos if there was a clear, even mitochondria distribution pattern in all blastomeres and a clear empty area where the cell nucleus was located. Embryos with a similar pattern in approximately 50–75% of cells were categorized as MitoC2, and all other embryos were graded as MitoC3 (Table 1 and Figure 7; confocal 3D image).

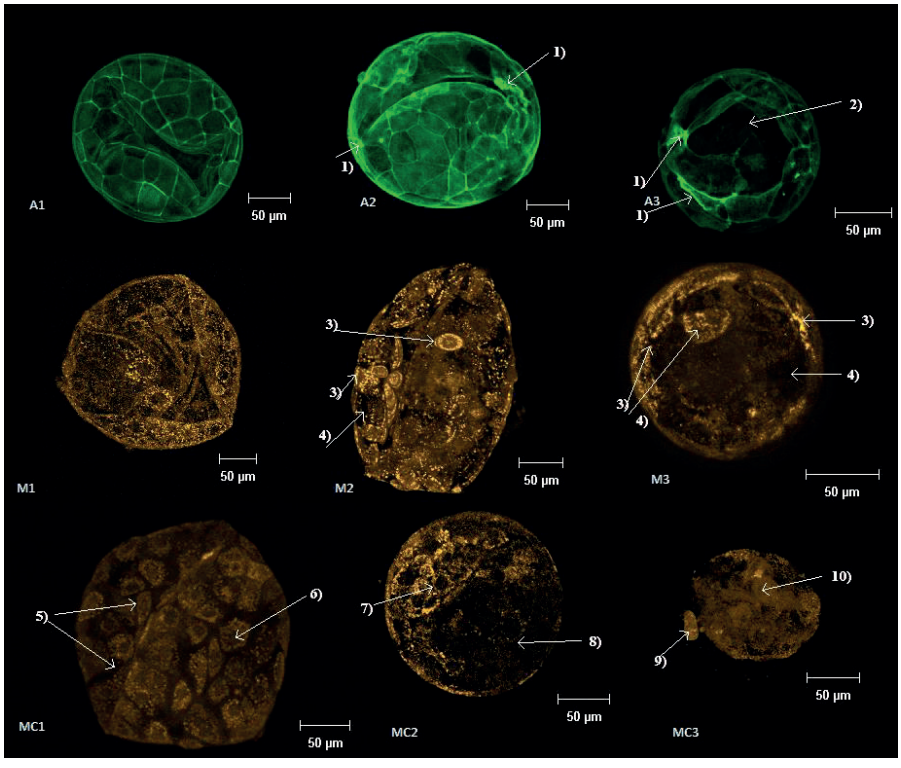

Figure 7. Actin and mitochondria staining and categories. Examples of actin categories in F-actin (Alexa Fluor 488 Phalloidin) stained Day 8 blastocysts (Confocal 3D image).

A1 = ACTIN1; A2 = ACTIN2 and A3 = ACTIN3 (arrows indicating 1) aggregated microfilaments and 2) lost cell integrity). Examples for mitochondria category in active mitochondria (MitoTracker Orange)–stained Day 8 blastocysts: M1 = MITO1; M2 = MITO2, M3 = MITO3 (arrows indicating 3) accumulations/clusters and 4) "empty" areas); MC1 = MitoC1 (arrows indicating 5) clear cell borders and 6) unstained nucleus area); MC2 = MitoC2 (arrows indicating 7) cells with mitochondria distributed around the nucleus and 8) areas with an absence of cells with clear mitochondria distribution around the nucleus); MC3 = MitoC3 (arrows indicating 9) large areas without a distribution pattern around the nucleus and 10) areas with no clear cell borders).

Table 1. *Summary of the morphological categories and characteristics used to asses actin and mitochondria (1= "best" category for all parameters).*

Parameter	Abbreviation	Category	Definition
Actin structure	ACTIN	1	Sharp cell borders, abundant microfilament in cytoplasm
		2	Gross maintenance of cell outline, but some aggregated/clumped microfilaments
		3	Aggregated clumps and loss of cell integrity in the main part of the embryo
Mitochondria	MITO	1	Evenly distributed, distinctly present in almost all cells, distinct points of staining, no or few small accumulations, no extremely stained cells ("cluster/blobs")
		2	1 or 2 deviations from Mito1, but other factors fulfilled
		3	More than 3 serious deviations from class 1
Mitochondria location	MitoC	1	Centered around the nucleus and clear cell borders in all cells
		2	Centered around the nucleus in more than 50% of cells
		3	No centered pattern visible, or less than MitoC2

3.4 Microarray-based transcriptome studies of embryos

Detailed protocols and PCR details are given in **papers II, III, and IV.**

3.4.1 Embryo pooling and freezing

RNAse-free conditions were used for all of the following steps to avoid contamination and degradation of the RNA. Each BC8 was separately put in 2 µl of PBS-PVA and frozen at −80˚C. For RNA extraction, pools of 10 embryos with equally distributed developmental stages and quality grades per pool were generated to provide the minimum amount of RNA for the extraction method. Four replicates of 10 embryos per treatment (INS10, INS0.1, and INS0) were used for the RNA analyses (n = 120 BC8s).

3.4.2 DNA/RNA extraction and RNA amplification and labelling

RNA extraction and the following steps for gene expression analysis were performed according to methods developed by the EmbryoGENE platform Canada (Robert *et al.*, 2011, and **Paper III**). Total RNA and genomic DNA were extracted in parallel using the AllPrep DNA/RNA micro kit (Qiagen, Hilden, Germany), and genomic DNA was kept at −80°C for subsequent epigenetic studies. Total RNA was assessed for quality parameters and quantity and reached RNA integrity numbers above 7.5. The extracted RNA was used for microarray analysis and for validation using reverse transcribed quantitative PCR (RT-qPCR) analysis.

RNA amplification was required because of the limited amount of RNA in the starting material. The RiboAmp®HS-plus RNA-amplification Kit (Life Technologies, ON, Canada) was used for linear amplification based on the Eberwine method (Van Gelder *et al.*, 1990). The purity and concentration of the resulting anti-sense ribonucleic acid (aRNA) was analysed by Nanodrop (Fleige & Pfaffl, 2006). All 12 samples fulfilled the quality criteria. Validation of the amplification step was performed according to methods at EmbryoGENE (Vallée *et al.*, 2009; Gilbert *et al.*, 2010).

For labelling, the Universal Linkage System by Kreatech (Leica Microsystems, ON, Canada) for Agilent microarrays was applied, and 2 µl of CY3-ULS (green) and CY5-ULS (red), respectively, were added to 2 µg of aRNA derived from the treated or control sample. Non-reacted ULS-label was removed using the Picopure RNA Isolation Kit (Life Technologies, ON, Canada). The final aRNA concentration and the efficiency of labelling was checked using Nanodrop before hybridization.

3.4.3 Hybridization of aRNA to Agilent oligo microarray slides

For each of the colours, 825 ng labelled linearly amplified aRNA was used. The microarray hybridization chamber was loaded according to the manufacturer's instructions, and slides were hybridized at 65°C for 17 h. The slides were then washed four times in different buffer solutions.

3.4.4 Microarray data analysis of the transcriptome

The spotted slides were scanned with a PowerScanner (Tecan, Mannedorf, Switzerland), and feature extraction was performed using Array-pro 6.3 (Media Cybernetics, Bethesda, MD, USA). Intensity files were analysed using FlexArray (Genome Quebec, Montreal, Canada) as described by (Blazejczyk *et al.*, 2007). Raw fluorescence intensity data were corrected by background

subtraction, Loess normalisation within arrays, and quantile normalisation between arrays. Data were analysed using Ingenuity Pathway Analysis (IPA) (Qiagen, Redwood City, CA, USA) by constructing pathways and gene interaction patterns affected by insulin treatment.

3.4.5 RT-qPCR validation of candidate genes

Validation of microarray results was performed by reverse transcribed quantitative PCR (RT-qPCR) at the EmbryoGENE platform. RNA was transformed into cDNA using the Qscript Flex cDNA kit (Quanta Biosciences, Gaithersburg, MD, USA) and an oligo-dT according to the manufacturer's instructions. Specific primers for each selected gene were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA).

RT-qPCR was performed using a LightCycler 480 system and SYBR Green I Master mix (Roche Diagnostics, Laval, Canada). To confirm the specificity of each pair of primers, the amplicons were electrophoresed on an agarose gel, purified, and sequenced. A standard curve consisting of five points of the PCR product for each of the primer pairs diluted from 1 pg to 0.1 fg was used for real-time quantification of the PCR output. Data analysis was performed using LightCycler 480 Software 1.5.0 SP4 (version 1.5.0.39) with the second-derivative maximum analysis method. Data normalisation used the GeNORM normalisation factor (Biogazelle, Gent, Belgium) from the expression of the two β-actin (*ACTB*) and beta-2-microglobulin (*B2M*) housekeeping genes. PCR conditions, primer sequences, product sizes, annealing temperatures, and accession numbers are shown in **Papers II and III**.

3.5 Lipid profile by desorption electrospray ionization mass spectroscopy (DESI-MS)

The BC8s were individually frozen in 2 μL of PBS supplemented with 0.1% PVA and shipped on dry ice from the Swedish University of Agricultural Sciences (Uppsala, Sweden) to Purdue University (West Lafayette, IN, USA; USDA permit 126609 Research).

A Thermo Scientific LTQ (San Jose, CA, USA) mass spectrometer was used for the experiments. The DESI spray had 5 kV applied to the stainless steel needle syringe delivering the solvent spray, and nitrogen gas pressure was set at 160 psi. The capillary temperature was set to 275°C. For the detection of positive ions, acetonitrile doped with 10 μg/mL Ag_2NO_3 was used as the solvent system. The solvent flow rate was 8 μL/min. The instrument injection time was 100 ms, with automatic gain control turned on. For the detection of negative ions,

dimethylformamide/acetonitrile (1:1 v/v) was used as the solvent system. The flow rate was 1.5 µL/min, and the instrument injection time was 750 ms, with automatic gain control turned on. The number of averaged microscans was three and the mass range was 200–1000 m/z. the capillary voltage and tube lens potentials were –50 V and –25 V, respectively. MS/MS analysis (by product ion scan) was performed to confirm the lipid identification. Information from previous publications using DESI-MS analysis of bovine embryos with high-resolution MS was also used for lipid identification (González-Serrano *et al.*, 2013; Pirro *et al.*, 2014).

3.6 Statistical analysis

3.6.1 Developmental rates, morphological analysis, and cell counts

The effects of insulin treatment during maturation on developmental rates (cleavage and blastocyst development on Day 7 and 8) were studied by analysis of variance (ANOVA). The PROC GLM procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used, and the results were analysed following the arc sinus $\sqrt{p}$ transformation of the initial percentages. The results were weighted for the number of oocytes per batch. Post-ANOVA multiple comparisons were realised either by using the Scheffé adjustment and/or contrast options available under GLM. In the last case, the contrast option was used to compare the two treated groups together (INS0.1+INS10) to the control (INS0). The significance level was set at $p < 0.05$. For better readability, results are presented as the least squares mean ± s.e.m. of untransformed percentages. Similar procedures were used to analyse the effects of insulin treatment on gross morphology in terms of embryo stage and quality grade. Nonsignificant main effects or interactions were removed, and adjusted estimates from the final model are presented. Morphological response variables (ACTIN, MITO, and MitoC categories) were first analysed by log linear models while using the three initial response categories. In order to obtain sufficient numbers of observations in each box of the contingency tables data, morphological variables belonging to class 2 and 3 responses were merged, and the variables were analysed as a 0/1 response. Percentages of observations belonging to the class 1 response (the best morphological appearance) corresponding to significant effects are presented.

3.6.2 Microarray

An empirical Bayes moderated t-test and the '*limma*' package in R were used to search for differentially expressed genes between the control and INS0.1 and INS10 groups (Smyth Gordon K, 2004). The EmbryoGENE microarray platform's designed pipeline was used to obtain a large-scale comparison between the insulin-treated groups and INS0 controls for transcriptome (Robert *et al.*, 2011) and epigenetics (Shojaei Saadi *et al.*, 2014) analyses. A positive or negative fold-change of >1.5 with $p < 0.05$ was set as the definition for differentially expressed transcripts (DETs).

3.6.3 RT-qPCR

Differences in gene expression between the INS0 and the INS0.1 groups and between the INS0 and the INS10 groups were compared by unpaired t-test (Prism 5; GraphPad Software Inc., La Jolla, CA, USA) after log transformation of the data. Differences in expression were considered as significant at one-tailed $p < 0.05$.

3.6.4 DESI-MS

For each sample, a list of m/z values and ion abundances (after background subtraction) from averaged mass spectra was imported into Matlab (The MathWorks, Inc., Natick, MA), and principal component analysis (PCA) was performed by means of in-house Matlab routines. The full-scan mass spectra over the mass range m/z 650–1400 were structured with 9,000 variables. The first PCA was performed on a data set of 63 rows (i.e. samples) and 9,000 columns (i.e. m/z variables).

All MS spectra were normalized by the standard normal variate transformation, correcting for both baseline shifts and global intensity variations, and then centred according to category column in order to minimize differences due to pooling. Fisher weight was calculated with PCA in order to estimate the discriminative ability, i.e. the ability to separate the two categories. Because lipids in the lower mass region are inherently less important in describing the separation among the samples, which is because the signal intensity of the ions under m/z 850 is much lower, we separately evaluated lower and higher mass ranges to balance their contributions. As an alternative procedure, the mass spectrum of each sample was divided into a low (m/z 650–900) and high (m/z 901–1400) mass range. The two blocks of data were processed by PCA with a mid-level data fusion strategy because they were two independent sources of information collected from the same samples (Pirro *et al.*, 2014). Individual

PCAs were performed on the two blocks of data, after normalization and category column centring, and the first six principal components were fused with a mid-level strategy and PCA was performed again with auto-scaled column data.

4 Main results and discussions

4.1 Method validation study of *in vitro* insulin exposure (Paper I)

The main objective of this work was to investigate the possible effects that an insulin challenge during *in vitro* oocyte maturation has on the developmental potential of the derived embryos. This experimental design was developed with the aim of mimicking the situation during metabolic imbalance such as that which occurs due to hyperinsulinemia and/or insulin resistance.

Reproductive disorders linked to hyper-or hypoinsulinemia are common in dairy cows, and one reason for impaired fertility of overfed or thin animals is decreased oocyte quality and thus impaired embryo developmental potential(Adamiak *et al.*, 2005a; Awasthi *et al.*, 2010). To be able to understand the pathophysiological mechanisms during *in vivo* metabolic challenges, *in vitro* models are good tools to discover possible changes in the early embryo.

To further define the model and experimental set up, literature about measured insulin concentrations in serum and follicular fluid and commonly used insulin concentration in *in vitro* studies was reviewed. This was done in order to identify the differences between the *in vitro* and *in vivo* situation and to be able to design the studies in a way that would respect the special situation of an *in vitro* model while still allowing conclusions with physiological relevance *in vivo*. We discovered a large discrepancy between the *in vivo* and *in vitro* situation, and the applied *in vitro* insulin concentrations were 100 to 1000-fold greater *in vitro* than *in vivo* concentrations (Laskowski *et al.*, 2016a and **Paper I**). One of the reasons for insulin supplementation at supra-physiological levels is the possibility that insulin is unstable *in vitro* due to media containing cysteine (Hayashi *et al.*, 1978). On the other hand, researchers might use insulin for its anti-apoptotic and proliferative actions without taking into account that there

might be a negative influence on the embryo growing under metabolic stress conditions such as hyperinsulinemia. The reason for the elevated doses of insulin might be that this anti-apoptotic and proliferative effect (Byrne *et al.*, 2002) is transmitted through the IGF-1 receptor (Rubin & Baserga, 1995), that also binds insulin but with a lower affinity (Le Roith, 1997; Fürstenberger & Senn, 2002, Figure 8). Thus, in the presence of elevated doses, insulin might mainly act through the IGF-1 receptor. However, the reported results are contradictory and both beneficial as well as detrimental effects of IGF-1 action during early embryo development have been reported (Herrler *et al.*, 1992; Chi *et al.*, 2000; Spanos *et al.*, 2000; Byrne *et al.*, 2002; Makarevich & Markkula, 2002; Block *et al.*, 2008).

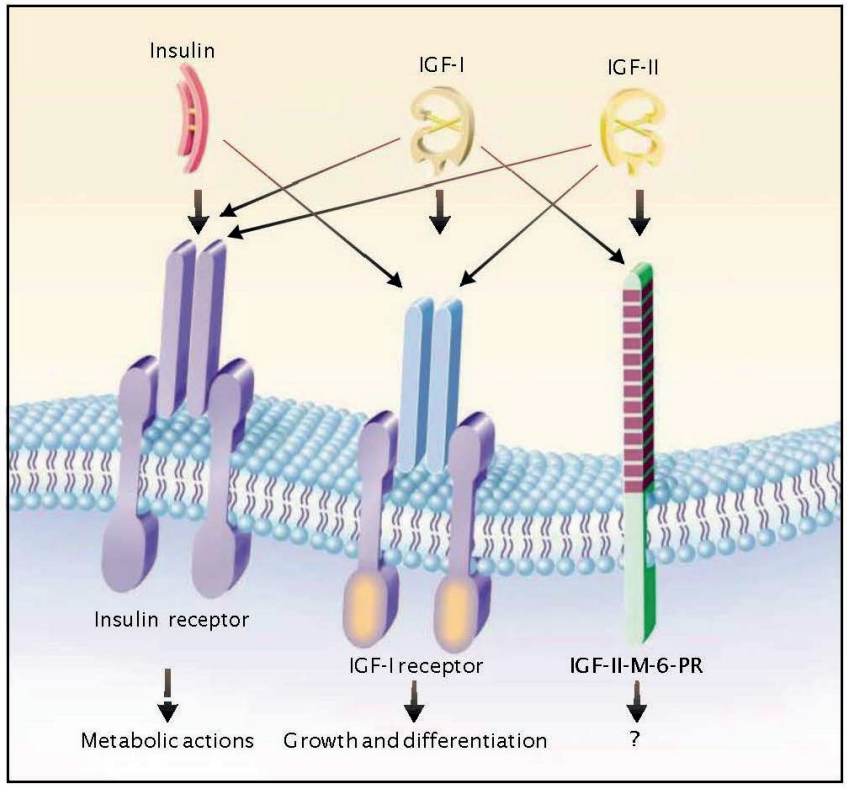

Figure 8. Binding of circulating insulin and IGFs to target cells. Insulin and IGF1 receptors are structurally homologous and are both tyrosine kinases. IGF2R differs structurally from the insulin receptor and IGF1R and is thought to function primarily as a scavenger receptor for IGF2. Insulin binds to its own receptor and to IGF1R. IGF1 and IGF2 bind to the insulin receptor. The relative affinities of ligands are indicated by the width of the arrows in the upper part of the diagram. (Reproduced with permission from Le Roith, 1997, Copyright Massachusetts Medical Society).

Because of their structural similarity, activation of the insulin receptor and IGF-1 receptor lead to similar responses on the cellular level, but their post-receptor pathways differentiate by transmitting metabolic functions through the insulin receptor and growth and differentiation functions through the IGF-1 receptor (Le Roith *et al.*, 1995; Le Roith, 1997).

With our work, we wanted to contribute to a better understanding of insulin-induced actions at the post-receptor level by investigating the gene expression of the embryo and, together with developmental rates and morphology, to present an overall picture of possible mechanisms that change the phenotype of an embryo derived from hyperinsulinemic conditions during oocyte maturation. This might help to better understand pregnancy losses and subfertility in animals suffering from both types of ketosis. Moreover, the programming of metabolism during early development might have consequences for subsequent generations of dairy cows, a fact that illustrates how important fundamental research on embryo quality is. Genetic selection and breeding goals already include health parameters in the cow, and this could also include breeding for improved resistance against metabolic disorders. Nevertheless, the changes that are observed in the embryos originating from elevated insulin during maturation highlight the need to maintain metabolic balance in the maternal organism so as to obtain metabolically healthy offspring.

In our model, we chose to use two different insulin doses, 10 µg/ml and 0.1 µg/ml, to cover both a typical *in vitro* concentration and to reduce one of the doses to a level closer to physiological concentrations but still suitable under *in vitro* conditions. The results of our pilot study showed evidence that insulin is still detectable after 22 h of maturation, and the concentrations did not seem to differ between empty and COC-incubated media, and in none of the samples was the insulin entirely consumed by the COCs (**Paper I**).

The limitations of this study were the small number of samples that did not allow advanced statistics and the need to dilute the samples down to the measurement range of the ELISA (0.05–3.0 µg/L), which could have led to inaccuracies of the analysis. Mercodia Bovine Insulin ELISA (10-1201-01) is a solid-phase two-site enzyme immunoassay based on the sandwich technique, in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule (www.mercodia.com). This does not allow conclusions about the insulin activity itself, but we could validate that our added concentrations were detectable and that the difference between both insulin groups remained throughout maturation. Further dose-dependent studies would be interesting to define a minimum dose *in vitro* in order to avoid the negative effect of hyperinsulinemia during early development.

4.2 Influence of insulin on development rates (Paper II and III)

The objective of recording developmental rates and characteristics of important events during early development was to detect possible differences in developmental potential of the embryos before further studying them through gene expression and morphological analyses.

In all of the different sub-studies, the general tendencies in developmental rates were similar and repeatable (Table 2). There was no difference observed in cleavage rate between the control and insulin groups, but there were significantly lower blastocyst rates on Day 8 in the insulin-treated groups compared to the controls.

Early cleavage is usually reported to be a sign of high developmental competence, and early cleaved embryos have a higher chance to develop into the blastocyst stage (Plante & King, 1992; Plante *et al.*, 1994; Andra *et al.*, 1999; Lonergan *et al.*, 1999). Because we could not observe any difference in cleavage rates between any of the groups, but did see a decreased BC8 rate, the possible negative effect of insulin seems to impair the development at a later stage rather than having an immediate effect. Considering the time of activation of the embryonic genome, early cleavages probably depend on the oocyte, and these mechanisms do not seem to be affected by insulin. On the contrary, disturbances induced by insulin might affect oocyte quality in such a way that EGA is impaired, but these effects are not critical because the differences in the development rates are quite small.

Table 2. *Number of oocytes, cleavage rate, and blastocyst rate on Day 8 (BC8) in the INS0 untreated control group, the INS0.1 oocytes exposed to 0.1 µg/mL insulin during maturation, and the INS10 oocytes exposed to 10 µg/mL insulin during maturation. Table modified from Paper II *and III°.*

Treatment	No. oocytes	Cleavage rate [A] (%)	BC8 [A, B] (%)
INS0°	1079	80.64	23.8 ± 1.3
INS0.1°	1019	78.86	20.4 ± 1.3
INS10°	984	80.91	19.8 ± 1.3
INS0*	695	78.64	20.29 ±0.02
INS0.1*	662	76.92	16.29 ±0.02
INS10*	669	79.82	15.97±0.02

A Cleavage and blastocyst rates show cleaved embryos and blastocysts as a percentage of immature oocytes. B ° $p < 0.03$ and *$p < 0.07$ for the comparison of the INS0.1 and INS10 groups combined versus the INS0 group.

It was evident that blastocyst development was best in the control group and that the insulin-treated groups showed more similarities between each other than

compared to the controls; nevertheless, the highest insulin concentration had the lowest BC8 rates (Table 2). The underlying reasons for the lower survival will be further discussed in chapters 4.3 to 4.5. However, the most plausible hypothesis is that the hyperinsulinemic conditions during oocyte maturation imply metabolic stress at the cell level with an increased presence of oxidative stress-related metabolites that lower embryo viability. (Facchini *et al.*, 2000a; Guerin *et al.*, 2001; Ceriello & Motz, 2004).

The period of oocyte maturation and early development takes place during the most stressful phase of metabolic imbalance because cows usually return to breeding around 60 days postpartum. This means that the oocyte has gone through several metabolic conditions during the time between follicular growth and final maturation with periods of NEB and PEB (Wattiaux, 1996). The *in vitro* model allows for investigations of the embryonic development under stressed conditions, even if it is not the perfect model for the *in vivo* situation where several more metabolites and hormones interact in the system.

A moderate level of embryo metabolism has been suggested to be more beneficial for long-term survival than extremely low or high levels of metabolism (Baumann *et al.*, 2007; Leese *et al.*, 2007), a fact that should be considered when assessing and selecting large and fast developing embryos as the best ones. Hyperinsulinemia during oocyte maturation might lead to insulin resistance in the early embryo and thus to impaired signalling pathways and impaired post-receptor mechanisms and consequently to impaired ability for the embryo to balance its energy homeostasis. It is well known that foetuses from obese mothers can develop insulin resistance in utero (Catalano *et al.*, 2009), and it has become evident that pathological mechanisms could already be set in the preovulatory oocyte and in the preimplantation embryo (Jungheim & Moley, 2008).

In conclusion, the observed developmental rates – together with the results from the gene expression and morphology studies – fit well into the picture that hyperinsulinemia decreases the developmental competence of the embryo. Explanations for the impaired developmental capacity due to hyperinsulinemia will be discussed in the following chapters.

4.3 Insulin and embryo morphology (Paper II and III)

Gross morphology evaluation of developmental stage and embryo quality grade did not show any significant differences between the groups. Nevertheless, when investigating the cytoskeleton and mitochondria quality in detail by confocal microscopy after staining, the phenotypes of the insulin and control groups showed some distinctions. In general, the two insulin groups showed

higher similarity to each other than to the control group in all morphological parameters. The main findings were an increased nuclei number in the insulin groups and that the actin and mitochondria categories that are associated with better quality were more common in the insulin groups compared to controls (Table 3, **Paper II and III**).

Table 3. *Number of nuclei (TN= total number) and actin and mitochondria categories (% of category 1 of all embryos of the same group) in insulin-treated (INS0.1 and INS10) and control (INS0) groups. Table modified from Paper II* and III°*

Group	MITO1 %	MitoC1%	ACTIN1%	NUCLEI TN*	NUCLEI TN°
INS10	52.00 a	50.00 a	32.65 a	101 a	101 a
INS0.1	47.37 a	55.36 a	38.60 a	104 a	100 a
INS0	23.73 b	30.51 b	20.00 b	86 b	93 b

a/b $p<0.05$.

While there is evidence that differences in the phenotypes of embryos between the groups exist on a microscopic level, the interpretation of the findings is more complex. For the number of nuclei, the most logical explanation seems to be the previously described proliferative actions of insulin (Draznin, 2009). The embryos derived from insulin-supplemented conditions grow faster and become larger in the same time period, while the control groups lay "behind" them in development. Because we observed increased BC8 -rates in the control group, it is probable that this enhanced development in the presence of insulin implies stress and thus a higher risk for embryonic death or developmental block, fitting with the "quiet embryo hypothesis" (Leese, 2002). This in contrast to the opinion that the faster-growing embryos have an advantage in developmental competence (Van Soom *et al.*, 1997; Luna *et al.*, 2008). The positive relationship between high cell number and developmental potential is based on the finding that *in vivo* embryos, with a generally better viability, have higher cell numbers compared to their *in vitro* counterparts (Iwasaki *et al.,* 1990). Primarily, this implies that the embryos with higher cell numbers have already advanced to a later stage. Interestingly, we can exclude this "bias" in our studies because no significant difference in terms of embryo stage or grade or in stage-related gene expression could be observed **(Paper III**, Rekik *et al.*, 2011).

One other possibility that has to be considered is that in the insulin-treated groups only the strongest and "best" embryos survived, meaning that the selection mechanism based on the insulin stress condition leads to fewer surviving embryos, but those that do survive are of better average quality. The control group generated more embryos in total, but these included a wider quality range because the weaker ones were able to survive.

In summary, we conclude that the increased cell number of the developing embryos after insulin treatment is not explained by advanced developmental stage, but more probably implies increased proliferation within a respective stage.

The evaluation of the actin cytoskeleton revealed better actin quality in the insulin-treated groups (Table 3 and **Paper II**) with the highest percentage of ACTIN1 in the INS0.1 group. One reason for the obviously more frequent categorizing of the INS0.1 and INS10 embryos in the good actin quality category could be that the mitogenic effects of insulin mean that only embryos able to cope with this metabolic stress survive, and thus better actin quality is observed compared to the non-insulin-treated control embryos. Because the INS0.1 group showed the best actin quality, the proliferative and mitogenic stimuli might dominate in the more moderate insulin concentration compared to the more extreme hyperinsulinemic concentration of INS10, but this observation did not reach significance (Table 3).

Another interesting idea is that the higher proportion of high-quality actin categories in the insulin-treated groups might be explained by the fact that actin plays an important role in insulin signalling in the post-receptor pathways – insulin is reported to have effects on cell morphology by inducing rapid reorganization of the actin cytoskeleton because this is necessary for transmitting the different effects of the insulin signal (Tsakiridis *et al.*, 1994, 1995, 1999). This fits well with our observations that the actin structure in general seems to be better distinguished and thus to be scored higher in the insulin-treated embryos compared to the controls. The insulin-induced modifications that are "activated" by actin are reported to lead to structures beneficial for the association of PI3-K (p85) with GLUT4 vesicles and perhaps also for the transport of GLUT4 to the cell surface (Khayat *et al.*, 2000; Shibasaki *et al.* 1994). This would explain why higher proportions of better-structured embryos are found in the two insulin groups, with the most favourable actin profile in the lower and more moderate insulin concentration.

Interestingly, we could also show that the probability to be assigned the best actin quality category increased with embryo stage, independent of insulin treatment (**Paper II**). This fits with the hypothesis that more advanced stages and more actively proliferating embryos possesses better actin quality. While the reasons for the different actin pattern need to be further elucidated, it is clear that the insulin treatment induces a different actin phenotype than observed in controls and that this phenotype might resemble embryos of further progressed stages. These results also support the hypothesis that only the best-quality embryos were able to survive the insulin challenge, which leads to a narrower distribution of actin quality compared to the controls. This theory is based on the

fact that blastocyst rates are lower in the insulin-treated groups, perhaps due to the fact that embryos with lower developmental potential only survive under less stressful conditions.

For the two classes of mitochondria quality – MITO and MitoC – a similar trend as for the actin assessment could be observed, and the percentages of both categories were higher in the insulin-treated groups and, independently of insulin treatment, for more advanced embryo stages. This confirms the hypothesis that has been set according to the actin and cell number evaluations, postulating that the insulin treatment during *in vitro* maturation leads to a phenotype of accelerated development due to the metabolic and mitogenic actions of insulin (Shepherd *et al.*, 1998; Bevan, 2001).

In the morphological evaluation of the active mitochondria, the best category was characterised as the most distinguished mitochondrial pattern, and here too the prominent, active mitochondria might be a signature of a highly metabolically active embryo, and a more moderately active embryo could be more viable in the long run. Because mitochondria are the energy-providing organelles, their function is important for many processes during early development such as differentiation, mitosis, and molecular transport (Barnett *et al.*, 1996; Båge *et al.*, 2003; Bruce Alberts *et al.* 2002). Active mitochondria and their distribution is a good predictor for embryo viability (Barnett *et al.*, 1996; Tarazona *et al.*, 2006; Van Blerkom, 2008). Because hyperinsulinemic conditions such as those seen in obesity and type 2 diabetes are associated with mitochondrial damage and oxidative stress (Facchini *et al.*, 2000b; Morino *et al.*, 2006), our morphological observations could, together with the gene-expression data, help to better understand the relation between insulin, oxidative stress, and mitochondrial distribution and functions.

To better understand the clinical relevance of these results, it would be interesting to transfer embryos on Day 7 or 8 to a recipient heifer and look at later developmental stages to determine if the changes are transient or if they remain in the growing embryo, foetus, or even in the new-born and adult stages.

4.4 Insulin and embryo gene expression (Paper II, III, IV)

4.4.1 General transcriptome patterns of the embryo and the most relevant pathways activated by insulin during oocyte maturation (Paper III)

Analysis of gene expression in insulin-treated versus control groups resulted in 156 differentially expressed transcripts (DETs) in the INS0.1 group and 229

DETs in the INS10 group with an overlap of 120 DETs (**Paper III**). For most of the genes, a greater fold-change was observed in the INS10 than INS0.1 group, showing a more pronounced effect of insulin on gene expression following maturation in the presence of the higher insulin concentration. The global transcription pattern of embryos developing after insulin treatment during oocyte maturation exhibited an overall increase of gene expression with only four (INS0.1) and five (INS10) genes displaying significantly decreased mRNA levels. The most relevant pathways associated with the DETs were growth/chromatin structure, steroid/cholesterol metabolism, energy supply, NF-E2 p45-related factor-2 (NRF2)-mediated oxidative stress response, cell cycle, cellular compromise, lipid and carbohydrate metabolism, and cellular assembly and organisation.

Representative genes of each pathway were validated by RT-qPCR (**Paper II, III, IV**) with a concordance of 80% with the microarray data. It was obvious that the insulin-treated groups were more similar to each other than to the controls and that in the lower insulin concentration the proliferative actions and influences on cell cycle were more prominent, while in the INS10 group signs of cellular compromise and impaired metabolic functions were detected (Table 4). As previously described by several authors (Cagnone *et al.*, 2012; Cagnone & Sirard, 2013; Van Hoeck *et al.*, 2015), other metabolic stress conditions such as hyperglycaemia and elevated NEFAs and serum lipids can affect the transcriptome of embryos.

The use of two different insulin concentrations and the large scale analysis of transcriptome differences showed that candidate genes involved in apoptosis, differentiation, and metabolism of the BC8 were regulated by insulin. The hypothesis that hyperinsulinemic conditions during maturation increase the growth of the embryo at the expense of decreased viability is further supported by the observation of multiple changes on the transcriptome level. The DETs related to energy metabolism, differentiation, the oxidative stress response, and mitochondrial activity were expected because the functions of their encoded proteins fit well with the described actions of insulin in health and disease (Facchini *et al.*, 2000b; Saltiel & Kahn, 2001; Bloch-Damti & Bashan, 2005).

Table 4. *Influence of 10 µg/ml (IN10) and 0.1 µg/ml insulin (INS0.1) during oocyte maturation on gene expression. The p-value range and the number of molecules with a fold-change >1.5 after insulin treatment are based on microarray analysis and are grouped according to cellular function in Ingenuity Pathway Analysis (Laskowski et al., 2016b).*

Cellular functions	**p-value range**	**Number of molecules**	**Example genes**
INS10:			
Cell Cycle	1.49E-05 – 2.78E-02	23	*LMNA, SOX2, VIM, MAP2K2*
Cellular Compromise	2.59E-04 – 2.78E-02	15	*KEAP1, VIM, LMNA*
Lipid Metabolism	2.83E-04 – 2.78E-02	20	*APOA1, CYP11A1, INSIG1, VIM, DHCR7*
Molecular Transport	2.83E-04 – 2.78E-02	19	*APOA1, CYP11A1*
Small Molecule Biochemistry	2.83E-04 – 2.78E-02	30	*APOA1, CYP11A1, INSIG1, IGF2R*
INS0.1:			
Cell Morphology	8.28E-05 – 2.00E-02	29	*CD81, VIM, LMNA*
Cellular Growth and Proliferation	8.28E-05 – 2.16E-02	63	*ADIPOR2, IGFBP7*
Cell Cycle	1.70E-04 – 1.82E-02	25	*SOX2, LMNA, IGFBP7*
Carbohydrate Metabolism	2.47E-04 – 1.95E-02	9	*APOA1*
Cellular Assembly and Organization	2.47E-04 – 1.82E-02	22	*VIM, LMNA*

ADIPOR2 = Adiponectin receptor 2; APOA1 = Apolipoprotein A 1; CD81 = Cluster of differentiation 81; CYP11A1 = Cytochrome P450 family 11 subfamily A, DHCR7 = 7-Dehydrocholesterol reductase; IGFBP7 = Insulin like growth factor binding protein 7; IGF2R = Insulin like growth factor 2 receptor; INSIG1 = Insulin-induced gene 1; KEAP1 = Kelch like ECH associated protein 1; LMNA = Lamin A/C; MAP2K2 = Mitogen activated protein kinase kinase 2; SOX2 = Sex determining region Y box 2; VIM = Vimentin.

4.4.2 Signatures of an impact of insulin on embryo lipid metabolism (Paper IV)

Downstream analysis of the insulin-induced transcription factors peroxisome proliferator-activated receptor (PPAR) and sterol regulatory element binding protein (SREBP/SREBF) revealed significant fold changes of downstream genes of each of these transcription factors. The effect on genes regulated by PPARα was stronger in INS10, with 14 genes with significant fold changes compared to INS0.1 with 10 genes with significant fold changes, and 10 more DETs regulated by PPARγ were found to be over-expressed in INS0.1. The downstream analysis of SREBF1 and 2 in the INS10 group revealed that transcripts of 7-dehydrocholesterol reductase *(DHCR7)*, fatty acid desaturase 2 *(FADS2),* insulin induced gene 1 *(INSIG1)*, mevalonate diphosphate decarboxylase (*MVD),* and heat shock protein family A (Hsp70) member 1 (*HSPA1A/HSPA1B)* – all of which are crucial for cholesterol and steroid synthesis – were more abundant.

Cholesterol metabolism in the BC8 – with its important function for steroid synthesis – was influenced on multiple levels in both insulin treatments (Table 5).

All observed differences as described in detail in **Paper IV**, show a strong impact of insulin on lipid metabolism in the BC8 and reflect insulin's previously described functions in both physiological and pathological conditions (Saltiel & Kahn, 2001; Kahn *et al.*, 2006). Because cholesterol has important functions during development (Farese & Herz, 1998), dysregulation of related pathways might have detrimental effects on the developmental competence of the embryo. Signatures of lipid accumulation, pathways preventing such accumulation, and increased transcription of genes associated with oxidative stress are other signs of the significant impact of hyperinsulinemic conditions, even several days after exposure.

The impact of insulin on lipid metabolism in the embryo is interesting because this shows similarities to the dysregulation of the metabolism of adult cows where both hyper- and hypoinsulinemia lead to the accumulation of lipids in the circulation, either through alimentary energy excess or increased mobilisation of body fat reserves. Thus metabolic challenges early in life could cause a certain metabolic fragility later in life as well and lead to animals that are less able to cope with metabolic stress.

Table 5. *Cholesterol-related genes with significant p-value ($p<0.05$) and fold change differences in INS10 (a) and INS0.1 (b) compared to INS0, sorted according to their function in lipid metabolism.*

Function/Pathway	p-value	DETs
a) INS10:		
Accumulation of cholesterol	2.83E-03	*ACP5, IGF2R, INSIG1, LAMP1,NR1H2*
Mobilization of cholesterol	5.14E-04	*APOA1, NR1H2*
Metabolism of cholesterol	5.31E-04	*APLP2, APOA1, CYP11A1, DHCR7, INSIG1, NR1H2*
Absorption of cholesterol	5.72E-03	*APOA1, NR1H2, PNLIP*
Homeostasis of cholesterol	6.42E-03	*APOA1, DHCR7, EHD1, NR1H2*
Steroid metabolism	8.21E-03	*ACAA1, APLP2, APOA1, CYP11A1, DHCR7, INSIG1, NR1H2, VIM*
Storage of cholesterol	8.41E-03	*EHD1, NR1H2*
Cleavage of cholesterol	9.34E-03	*CYP11A1*
Recruitment of cholesterol	9.34E-03	*APOA1*
Efflux of cholesterol ester	1.86E-02	*APOA1*
Accumulation of cholesterol ester	2.26E-02	*INSIG1, NR1H2*
Synthesis of cholesterol	2.29E-02	*APOA1, DHCR7, INSIG1*
b) INS0.1:		
Mobilization of cholesterol	4.91E-04	*APOA1, NR1H2*
Steroid metabolism	7.18E-03	*ACAA1, APLP2, APOA1. COMT, DHCR7, NR1H2, NR3C1, VIM*
Recruitment of cholesterol	9.13E-03	*APOA1*
Metabolism of cholesterol	1.72E-02	*APLP2, APOA1, DHCR7, NR1H2*
Depletion of cholesterol ester	1.82E-02	*APOA1*
Efflux of cholesterol ester	1.82E-02	*APOA1*
Accumulation of cholesterol	1.91E-02	*IGF2R, LAMP1, NR1H2*

ACAA1 = Acetyl-CoA acyltransferase 1; ACP5 = Acid phosphatase 5; APLP2 = amyloid precursor-like protein 2; APOA1 = Apolipoprotein A 1; COMT = Catechol-O-methyltransferase; CYP11A1 = Cytochrome P450 family 11 subfamily A member 1; DHCR7 = 7-Dehydrocholesterol reductase; EHD1 = EH domain containing 1; IGF2R = Insulin-like growth factor 2 receptor; INSIG1 = Insulin induced gene 1; NR1H2 = Nuclear receptor subfamily 1 group H member 2; NR3C1 = Nuclear receptor subfamily 3 group C member 1; PNLIP = Pancreatic lipase; VIM = Vimentin.

4.5 Insulin and embryo lipid profile (Paper IV)

Following up on the results of the transcriptome study (4.4), a deeper analysis of the lipid metabolism-related effects on gene expression was performed in **Paper IV**, illustrating the context and possible correlations between DETs.

A chemical lipid profile of BC8 was performed via DESI-MS with the aim of detecting possible differences in the lipid content of the embryos. In summary, the lipid profile analysis revealed slightly downregulated mitochondrial metabolism in response to an insulin challenge (**Paper IV**, as shown by ubiquinone abundance). Moreover, insulin only impacted triacylglycerid and cholesteryl ester abundance to a limited extent. The cholesterol metabolism in embryos derived from insulin-exposed oocytes seems to be influenced to some extent because the control embryos had higher levels of squalene, a cholesterol precursor, along with higher levels of cholesteryl esters of palmitoleic and oleic acids.

Interestingly, the results from the lipid profiling did not show such large differences as the lipid gene expression between insulin-treated embryos compared to controls. However, other studies revealed stronger differences with the same methods, e.g. between *in vitro* and *in vivo* embryos (González-Serrano *et al.*, 2013) and between mature and immature oocytes (Pirro *et al.*, 2014) .

The results of gene expression and lipid profiling led us hypothesise that the embryo might be able to compensate for the insulin challenge by trying to sustain a stable chemical profile, although the lipid-related gene expression reveals signatures of metabolic stress. Some of the gene expression changes were going in both directions – e.g. there were signs of increased lipid accumulation together with signs of countermeasures against such accumulation (Figure 9). Moreover, it has been reported that *in vitro* culture downregulates cholesterol metabolism (González-Serrano *et al.*, 2013). Because this study was *in vitro*-based, the embryos might have been unable to translate the gene expression changes into cholesterol metabolism changes due to limitations imposed by the culture system, or because the chemical lipid profile differences would only be observable at later embryo stages due to the time gap between gene expression observations and phenotypic lipid appearance. However, the lipid profile seems to be more stable compared to the observed transcriptome changes, possibly because in the first days of development a different lipid composition is detrimental for embryo survival.

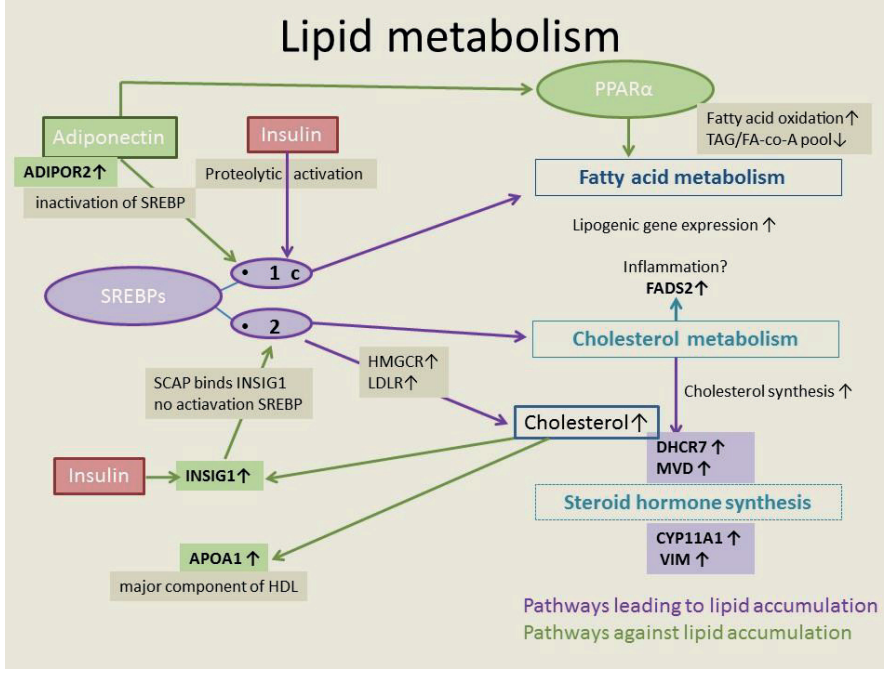

Figure 9. Interactions of insulin-regulated pathways in lipid metabolism. Insulin activates SREBPs that regulate pathways linked to lipid accumulation (purple), and it influences fatty acid metabolism (SREBP1c) and cholesterol and steroid metabolism (SREBP2). In green are pathways and genes acting against lipid accumulation through adiponectin and PPARα- related pathways.

FA = Fatty acid, TAG = Triacylglycerid. Genes upregulated by insulin that protect against lipid accumulation: APOA1 = Apolipoprotein A 1, ADIPOR2 = Adiponectin receptor 2, HDL = High density lipoprotein, HMGCR = 3-Hydroxy-3-methylglutaryl-CoA reductase, INSIG1 = Insulin induced gene 1, LDLR = Low density lipoprotein receptor, PPARα = Peroxisome proliferator-activated receptor alpha. Genes upregulated by insulin that are related to lipid accumulation: CYP11A1 = Cytochrome P450 family 11 subfamily A member 1, DHCR7 = 7-Dehydrocholesterol reductase, FADS2 = Fatty acid desaturase 2, MVD = Mevalonate diphosphate decarboxylase, SCAP = Sterol regulatory element-binding protein cleavage-activating protein, SREBP = sterol regulatory element binding protein, VIM = Vimentin.

5 Concluding remarks

Insulin – a key regulator of energy homeostasis with strong influences on both carbohydrate and lipid metabolism – was chosen to investigate the potential effects of metabolic imbalance during early embryonic development. Through the work performed for this thesis, new insights about and possible explanations for the detrimental effects of hyperinsulinemia could be illustrated.

In summary, this thesis presents a first approach in establishing an *in vitro* model for metabolic imbalance by using elevated insulin conditions during bovine oocyte maturation and investigating the effect on the BC8s.

The results provide a promising base for further research and possible improvement of *in vitro* models of metabolic imbalance. Being a multifactorial problem, it would be interesting to combine several factors associated with hyperinsulinemia such as glucose, IGF-1, IGF-2, and fatty acids in a refined model. Also, it would be interesting to further reduce the insulin levels in *in vitro* studies to avoid the possible negative effects of hyperinsulinemia on the developing embryo, as was seen in our studies.

- An *in vitro* model for metabolic imbalance during early development was developed based on a comparison of insulin concentrations found *in vivo* and used in other *in vitro* studies. The model was established and validated. Both insulin concentrations led to similar effects with a slightly stronger effect on gene expression in the higher insulin dose and a more proliferative effect in the lower dose.

- In the *in vitro* model, there was a clear negative impact of elevated insulin during oocyte maturation on blastocyst developmental rates, while cleavage was not influenced. The mechanisms and underlying reasons for lower blastocyst rates of the insulin groups should be further defined and discussed.

- Evaluation of morphological parameters of the BC8 in terms of total cell count, actin structure, and mitochondria distribution pattern showed differences between embryos originating from the insulin-treated oocytes and the controls. The observed morphological changes could be related to different gene expression patterns of genes encoding proteins related to proliferation, cellular structures, and mitochondrial stress.

- Gene expression patterns in embryos derived from insulin-supplemented oocytes showed an overall increase in gene expression and these genes were coupled to pathways related to growth and proliferation, lipid and energy metabolism, differentiation, the oxidative stress response, and mitochondrial activity.

- There were significant increases in gene expression of pathways linked to lipid metabolism in the insulin-treated groups compared to controls. Lipid profile analyses were performed, and the gene expression relations of cholesterol and steroid metabolism and lipid accumulation as well as mechanisms against such accumulation, were described in detail.

6 Future perspectives

With this work, we aimed to contribute to further knowledge about the different mechanisms induced by an insulin challenge during bovine oocyte maturation. We could answer some questions, but further studies are required to gain increased knowledge concerning the complex actions of insulin during embryonic development. Several key questions still remain to be answered in future studies.

- *Are commonly used doses of insulin during* in vitro *cell and embryo culture too high?*

  There is a large variation between *in vivo* measured and *in vitro* applied insulin concentrations, but no clear explanations have been suggested for this gap. Because several effects of elevated insulin during oocyte maturation are retained and change the phenotype of the embryo, supra-physiological doses of insulin should be used with care. Further studies are necessary to define the minimum and maximum dose that can provide the benefits while avoiding the adverse effects of insulin *in vitro*.

- *How long is insulin active* in vitro*?*

  In our model, we validated the presence of insulin after 22 hours of maturation by measuring insulin with an ELISA. The weakness of this method is that it is a quantitative measurement and does not say anything about the activity of insulin after incubation. A detailed study of insulin activity in culture medium and possible loss of activity due to medium components would be of interest.

- *Can the positive effects of insulin* in vitro *still be achieved using physiological doses?*

  Because insulin has positive effects on proliferation and works as an anti-apoptotic factor, these potential positive effects could be further explored by knowing at what dose the adverse effects in form of increased energy metabolism leading to cellular stress begin to appear. Thus, further studies using different concentrations of insulin would be interesting.

- *How does the morphology and gene expression of the oocyte change if it matures under insulin supplementation?*

  Because multiple changes in phenotype and gene expression in embryos originating from oocytes matured with insulin supplementation could be observed in the present work, another approach would be to investigate the matured oocyte immediately after insulin challenge and compare the results of oocyte gene expression and morphology with the data we have obtained for the blastocyst.

- *Do the changes in gene expression remain in later embryo stages or even after the offspring are born?*

  In our laboratory studies, we were limited to investigating BC8. Because the differences between insulin and control groups were observed 8 days after exposure, it is possible that the induced changes might even remain until later stages and even after offspring are born. To get a complete picture of the possible consequences of hyperinsulinemia in the period around conception, later embryo stages should be analysed as well as the offspring.

- *How does insulin induce epigenetic changes with further consequences for the offspring and subsequent generations?*

  Epigenetic changes are induced by external factors such as the nutritional status of the mother, and these are more stable than changes at the transcriptome level because they interfere with gene regulation and the accessibility of genes for transcription. Epigenetic changes can be transferred to subsequent generations, and this explains their importance and potential risk. Evidence for the possibility of health problems as adults due to metabolic programming early in life has already been shown. Research with human embryos is ethically controversial, and thus the comparative aspects

of such studies should be further explored in order to obtain better knowledge about this potential "inherited factor" of metabolic imbalance.

- *Can this work contribute to the detection of the most viable embryos for transfer?*

  To determine the long-term consequences of the insulin challenge, the embryos should be transferred. Ideally, the embryos would be biopsied before transfer, and the expression of genes related to the changes we have observed in our study should be studied to see if the gene expression profile reliably predicts the later phenotype. However, our present results suggest that excess insulin is unfavourable for embryo production under an IVP programme.

- *Do the embryos that survive the insulin challenge have better promise for future development because they have better morphological parameters?*

  At this point, we cannot be sure about the detrimental or beneficial effects of insulin supplementation. The morphological findings presented here assume a "stronger" phenotype in the insulin-treated groups, while developmental rates are decreased and the gene expression pattern shows signs of stress. Still, it would be interesting to follow up these embryos to be able to assess their future potential.

- *Would genetic selection for insulin sensitivity of cows also improve the metabolic stability of their embryos?*

  A next step would be a genetic screening of animals for a stable response to metabolic challenges because it is already known that differences exist in insulin sensitivity among breeds. Once identified, studies could be performed with the oocytes collected from good and average cows to test their developmental potential under a metabolic challenge *in vitro*.

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Popular science summary

The content of the thesis is about how metabolic disturbances influence the developmental potential of early cattle embryos. Metabolic imbalance is a problem in the lactating cow, who has to solve the challenge of adapting her metabolism to increasing milk yields, which usually leads to decreased fertility. Reproductive health is of large importance in the dairy industry and the aim of this work was to improve the understanding of fertility problems in the dairy cow. Impaired reproductive performance leads to tremendous economic losses for farmers in Sweden and worldwide. Moreover, animal welfare issues due to early culling of infertile cows are a problem.

The early embryo is sensitive to metabolic stress conditions, and decreased embryo survival leads to a delay in getting the cows pregnant in time. This is why more knowledge about improving the management and physical conditions of the cow (the links between fertility, nutrition, milking, and dry period) is needed.

Insulin is an important hormone regulating the body's energy balance in mammals. Insulin concentrations change in situations of energy deficiency or excess, and both conditions are known to decrease fertility in cows and as well as in humans.

In humans, metabolic syndrome, obesity, and type 2 diabetes are common problems that lead to elevated insulin in the body and to insulin resistance. Cows can either get too thin or too fat depending on their energy needs for lactation. Both situations can result in decreased fertility, and insulin is involved in the regulation of the energy balance.

In our experiments, we added two different concentrations of insulin during the period of oocyte maturation in a laboratory study based on slaughterhouse-derived oocytes. Developmental rates, morphologies, and gene expression profiles of 8 day-old embryos deriving from insulin-treated oocytes were investigated.

We found that the embryos originating from insulin treatment had a reduced survival, and we could observe several phenotypic changes in the embryos. After staining and morphological investigations by fluorescence and confocal microscopy, we could observe that the total cell number was increased and cell actin skeleton structures and organelles were different in the insulin-treated groups. This is an indication of differences in the quality of the embryos from the insulin-treated groups.

Moreover, gene expression showed differences, especially in genes related to lipid metabolism, cellular stress responses, and growth, which is in alignment with previously described and known functions of insulin in health and disease. All results taken together showed a clear impact of insulin during early development.

The knowledge obtained through this thesis work contributes to a better understanding of the cellular and molecular background that causes lower embryo potential due to elevated insulin exposure in the dairy cow. Importantly, this work is of comparative value because humans suffering from metabolic imbalance also often have decreased fertility. Moreover, it cannot be excluded that even the surviving embryos of the insulin treatment could potentially have metabolic issues remaining later on in life.

Populärvetenskaplig sammanfattning

Avhandlingen handlar om tidig embryoutveckling hos kor och om hur ämnesomsättning i obalans hos modern påverkar embryots utveckling och överlevnad. Nedsatt fruktsamhet är ett ökande problem i mjölkkobesättningar. Det leder till förlust av djur som har dålig reproduktionsförmåga och ekonomiska förluster för bonden. Både kvigor som är för feta och mjölkkor som inte klarar energiförsörjningen när mjölkproduktionen sätter igång har ibland svårt att bli dräktiga.

På ett liknande sätt finns det ökande problem med infertilitet på människor som ofta också är relaterad till ämnesomsättningssjukdomar som diabetes, fetma och insulinresistens.

Insulin är ett viktig hormon som reglar ämnesomsättningen hos däggdjur. Insulinvärdet i blodet kan vara förhöjt hos djur eller människor som är överviktiga och det kan leda till insulinresistens och diabetes. Dessutom är insulin ett hormon som tillsätts som tillväxtfaktor vid provrörsbefruktning hos människa, utan att det är helt klarlagt vad som är lämplig eller skadlig nivå.

Den tidiga embryoutvecklingen är en väldigt känslig process och embryon blir påverkade av miljön de utvecklas i, det vill säga moderns ämnesomsättning. För att kunna undersöka vilken effekt olika substanser har kan man göra laboratorieförsök med provrörsbefruktning av äggceller. Äggceller samlas från äggstockar från slaktade kor på slakteri och befruktas och odlas utanför livmodern, en modell som gör det möjligt att undersöka embryon närmare under hela utvecklingen. I de studier som avhandlingen är baserad på behandlade vi äggceller med två olika, förhöjda nivåer av insulin under äggcellernas mognad före befruktningen och studerade sedan embryonas överlevnad, utseende och gen-uttryck dag 8 i utvecklingen.

Vi undersökte hur lång tid det tog för embryon att utvecklas och hur många embryon som bildades från äggcellerna, och det visade sig att färre embryon utvecklades normalt vid tillsats av insulin. Vi misstänkte att insulinet under

äggcellsmognaden orsakade stress på cellnivå och undersökte vidare på vilket sätt cellerna påverkades.

Vi färgade embryona och undersökte dem med hjälp av fluorescens- och konfokalmikroskop. Man kunde se att embryonas utseende var annorlunda om äggen mognade vid förhöjd insulinnivå. Cellstrukturer som är kopplade till energiomsättning (mitokondrier) och cellstabilitet (aktin) var förändrade och embryon från de insulinbehandlade grupperna hade fler celler, de hade alltså växt snabbare. Utöver det kunde vi visa många förändringar på gennivå. På detta sätt försökte vi förstå de molekylära mekanismer som ligger bakom den sämre överlevnaden hos embryon som stressats av förändrade förhållanden i moderns ämnesomsättning. Funktioner kopplade till ämnesomsättning, stress, cellväxt och fetma var aktiva i högre grad om äggcellen mognade med insulintillsats och det kan vara förklaringar till varför färre embryon överlevde i insulingrupperna.

Våra resultat bidrar till att bättre förstå processerna bakom att embryon har sämre överlevnad när moderns ämnesomsättning är i obalans. Eftersom så många viktiga kroppsfunktioner anläggs tidigt i utvecklingen finns det en potentiell risk att embryon som överlever ämnesomsättningsstress har en benägenhet att drabbas av ohälsa senare i livet. Mjölksektorn är beroende av friska, fruktsamma kor och det är därför viktigt att förstå sambandet mellan ämnesomsättningsstörningar och dålig fruktsamhet.

Den tidiga embryoutvecklingen hos kor har många likheter med embryoutveckling hos människa, och våra resultat kan också hjälpa till att förstå möjliga orsaker till infertilitet hos insulinresistenta kvinnor. Genom att använda en laboratoriemodell för kor kan man undvika etiska dilemman som annars uppstår när man forskar på humana embryon.

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