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Effects of mTOR and AMPK pathways on specification of inner cell mass (ICM) cell lineages during mouse preimplantation development

Bachelor thesis

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Anotace

Determination of the effects of mTOR and AMPK signalling pathways on specification of epiblast and primitive endoderm lineages between E3.5 and E4.5 and between E3.5 and E5.5 of mouse embryonic development using chemical inhibitors and activators.

Prohlášení

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Abstract

Preimplantation mouse embryonic development starts at oocyte fertilisation followed by a series of cleavage divisions, producing cells called blastomeres, and results in the formation of three cell lineages by the time of uterine implantation (at the late blastocyst stage); trophectoderm, primitive endoderm and epiblast. At this late blastocyst stage, cells of inner cell mass (and trophectoderm) are fully committed to their fate; cells of epiblast express proteins pluripotency markers NANOG and primitive endoderm cells express GATA4 and GATA6 proteins. Compaction and cavitation play a crucial roles, as do a complex network of intrinsic and extrinsic signals and the embryo micro-environment. These mechanisms also underpin adaptive responses to changing conditions (nutritional or other stresses). The AMPK signalling and mTOR pathways regulates metabolic homeostasis. Phosphorylation of AMPK stimulates catabolism, as well as downregulation of mTOR and many drugs act as either AMPK activators or mTOR inhibitors (as treatments for diabetes or female infertility). This thesis is focused on the impact of AMPK activation and mTOR inhibition on the specification of the EPI and PrE lineages during maturing of mouse blastocyst. Results show, that both AMPK activation and mTOR inhibition impact the specification of the inner cell mass, whereby AMPK activation impairs mainly primitive endoderm, mTOR inactivation affects both primitive endoderm and epiblast.

Key words:

preimplantation mouse embryonic development, trophectoderm, inner cell mass, epiblast, primitive endoderm, transcriptional factors, AMPK, mTOR

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Glossary of abbreviations

Adenosine monophosphate	AMP
Adenosine triphosphate	ATP
AMP- activated protein kinase	AMPK
Bovine serum albumin	BSA
Embryonic stem cells	ESCs
Epiblast	EPI
Human chorionic gonadotropin	hCG
Human embryonic stem cells	hESCs
Inner cell mass	ICM
Mammalian target of Rapamycin	mTOR
Paraformaldehyde	PFA
Phosphate buffered saline Tween-20	PBST
Pregnant mare serum gonadotropin	PMSG
Primitive endoderm	PrE
Transcription factors	TFs
Trophectoderm	TE

1. Introduction

1.1 General introduction to mouse preimplantation embryonic development

Mouse embryonic development starts from one single cell, a fertilized egg. Mature oocytes are fertilized by sperm in fallopian tubes shorty after ovulation, forming the zygote. This is followed by a series of cleavage cell divisions, without an overall increasing of embryo size. During these divisions a larger amount of progressively smaller cells are formed, these cells are called blastomeres (Kojima, Tam, & Tam, 2014). The entire embryo is encapsulated within a glycoprotein shell-like layer called the *zona pellucida* and forms a tightly compacted structure called a morula, comprising 16-32-cells (White, Zenker, Bissiere, & Plachta, 2018). Once the morula enters the uterus, a fluid filled cavity develops within the mass of cells. At this stage, the so-called blastocyst stage embryo can hatch from the *zona pellucida* and become implanted into the uterine endometrium to continue development (in the so-called post-implantation stages). The blastocyst comprises three lineages: trophectoderm (TE) and primitive endoderm (PrE), which are extraembryonic tissues, and the pluripotent epiblast (EPI), which forms embryo itself (Fig.1). TE later giving rise to placenta and PrE the yolk sack (Mihajlović & Bruce, 2017).

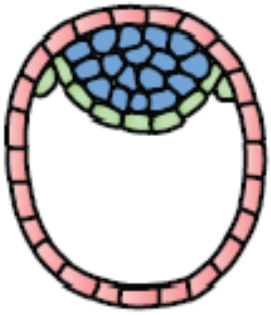

Fig. 1: Schema of blastocyst comprising three distinct lineages: blue EPI, green PrE and red TE (Rossant, 2016)**.**

The developing preimplantation stage embryo has a intrinsic clock that determines the correct timings of key morphological events, such as embryo compaction, polarization of blastomeres and blastocyst formation. Embryonic development can be measured from the commencement of fertilization and the age of embryo can be defined since this point in terms of days (mostly expressed to one decimal place – *e.g.* E1.0). Additionally, the embryonic day of development can be referred to by total cell number or the number of cleavage divisions undertaken but is also sometimes expressed by the time post ovulation, depending on the nature of experimental observations (Kojima, Tam, & Tam, 2014). The division of the zygote into a 2-cell embryo occurs at day E1.0 of embryonic timing. Simultaneously the activation of embryonic genes starts in a minor burst of transcription that is complete by the end of the 2-cell stage (Abe, Funaya, Tsukioka, Kawamu, & al., 2018) and the maternal transcripts inherited from the mature egg are degraded (Zernicka-Goetz, Morris, & Bruce, 2009).

The embryo transits to the stage of 4-cell around E1.5-E2.0 and by E2.5 enters the 8-cells stage (Kojima, Tam, & Tam, 2014). During this stage the blastomeres are still morphologically identical and comprise of cell contacted and contactless membranes (Sutherland AE, 1990). The embryo then undergoes compaction (whereby blastomeres flatten against each other, maximize their contacts and the distinctions between individual cell boundaries become less defined) and individual blastomeres initiate intra-cellular polarization that is concluded by the end of the 8-cell stage (Johnson & Ziomek, 1981). At the fourth cleavage (to generate a 16-cell stage morula), the resulting blastomeres begin to be morphologically distinct, with the creation of two cell types that are either on the surface of the embryo (outer cells) or encapsulated within (inner cells) (Sutherland AE, 1990). These combined stages of compaction, polarization and relative spatial segregation can be considered the first essential events of embryo morphogenesis and are prerequisites for successful development beyond the preimplantation stages (Cockburn & Rossant, 2010).

Embryo compaction happens at the mid- to late 8-cell stage, whereby morphologically identical blastomeres increase their cell contacts by the formation of adherens junctions with neighboring cells, resulting in more intercellular adhesion and embryonic tension (Kojima, Tam, & Tam, 2014). Compaction is shortly followed by intracellular polarization, when the blastomeres become polarized along the apicobasal axis – *i.e.* the radial axis of the embryo (Johnson & Ziomek, 1981) (Sasaki, 2015). Up until to this stage, blastomeres are totipotent cells, which means that they have the ability to generate all extraembryonic (*e.g.* placenta and yolk sac) and all the embryonic cell lineages of the fetus post-implantation; or in reference to the preimplantation development period, the

blastocyst inner cell mass (ICM – comprising both the embryonic and pluripotent epiblast/EPI and the differentiating extraembryonic primitive endoderm/PrE) and the differentiating extraembryonic outer trophectoderm (TE). The cell fate specification of blastocyst stage blastomeres (*i.e.* ICM versus TE) is conferred by the extent of apico-basal polarity, whereby the outer epithelial cells retain polarity and ultimately constitute the TE and the generated inner cells become apolar and resist TE differentiation and retain pluripotency as the blastocyst ICM (Mihajlović & Bruce, 2017).

Blastomere polarization is defined by given intra-cellular localisation of distinct complexes of protein polarity factors to the plasma membrane along the radial axis of the embryo – so-called apico-basolateral polarization of blastomeres. (Johnson & Ziomek, 1981). The apical domain is defined as the part of the blastomere membrane that is on the outside of embryo and is not in contact with any other cells (Korotkevich E., 2017). It is enriched in its own complement of apical polarity factors (PAR3, aPKC, PARD6B) and F-actin and the membrane is characterized by the presence of microvilli (Chazaud & Yamanaka, 2016). The basolateral domain defines the blastomere plasma membranes that are in cell-cell contact and are enriched in different polarity factors (Scribble- Par1 complex) and adherens junctions. However, interestingly polarization can occur without cell-cell contacts and persists from the 8-cell stage in the outer cells of the 16-cell morula and the eventual blastocyst (Chazaud & Yamanaka, 2016). 8-cell stage and outer 16-cell stage blastomeres can divide to produce either two outer residing and hence polarized cells, that are progenitors of the TE, in so-called symmetrical divisions. Alternatively, they can divide to give one outer and polarised TE precursor daughter cell and an apolar (by virtue of the fact it did not inherit the apical domain) inner cell

that will resist TE differentiation and populate the pluripotent ICM; such divisions are referred to as asymmetric (Chazaud & Yamanaka, 2016). In reality, most divisions are oblique versions of asymmetric divisions that generate outer cells with varying degrees polarity and the less polarised, or rarely observed apolar, outer cells become actively internalised into the embryo. Although inner cells are rarely positioned as a direct consequence of a perfectly asymmetric division (Cockburn & Rossant, 2010). Therefore, by E3.5 (32-cell stage) there are already generated two spatially distinct cell lineages with different fates (*i.e.* differentiating outer polarised TE and pluripotent inner apolar ICM), that collectively mark the establishment of the first cell fate decision of mouse embryogenesis (Fig.2) (Cockburn & Rossant, 2010). Note, that the TE has a role during blastocyst hatching and implantation into the uterus and will later generate the embryonic part of the placenta (Ziomek CA, 1982).

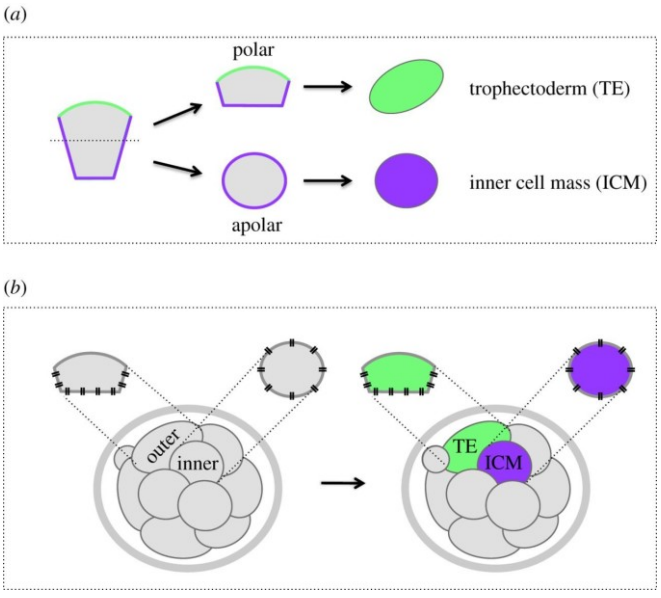

Fig. 2: A schema of the effect of polarization on cell lineage segregation during the first cell fate decision (TE versus ICM) (Mihajlović & Bruce, 2017)**.**

The 32-cell stage (E3.5) represents the point at which the blastocyst fluid-filled cavity is formed, whereby the outside TE cells form a water tight epithelium and the ICM is displaced towards one pole of the embryo. This process is termed blastocoel formation and it is significant for correct ICM development (Sutherland AE, 1990). The blastocoel forms due to the creation of an osmotic gradient across the outer TE cells and is maintained by ATPases and Na^+/H^+ ion pumps that result in the drawing of water, via the trophectoderm, into blastocyst cavity. The part of the TE that surrounds the cavity is known as the mural trophectoderm, whereas that which overlays the ICM is known as the polar trophectoderm (Mihajlović & Bruce, 2017). By around E4.0, at the mid-blastocyst stage, the second cell fate decision is underway and involves the divergence of initially homogenous and unspecified ICM cells into one of two distinct lineages, involving a mechanism of active sorting. These are the differentiating primitive endoderm (PE) and pluripotent epiblast (EPI) (Fig. 3) (Cockburn & Rossant, 2010). Therefore, by late blastocyst (E4.5) stage the EPI cells are situated deep within in the ICM and act as a progenitor pool of cells for the embryonic foetus itself. These EPI cells are separated from blastocyst cavity by monolayer of cell PrE cells, that will give rise to the extraembryonic parietal and visceral endoderm and ultimately the yolk sac membranes (E. E. Morrisey, 1998). Thus, at this developmental time the blastocyst embryo consists of three distinct lineages (the differentiating and extraembryonic TE and PrE and the pluripotent embryonic EPI) and is ready to hatch from the *zona pellucida* in preparation for implantation into the uterus; marking the end of the preimplantation embryonic period of mouse development (Lokken & Ralston, 2016).

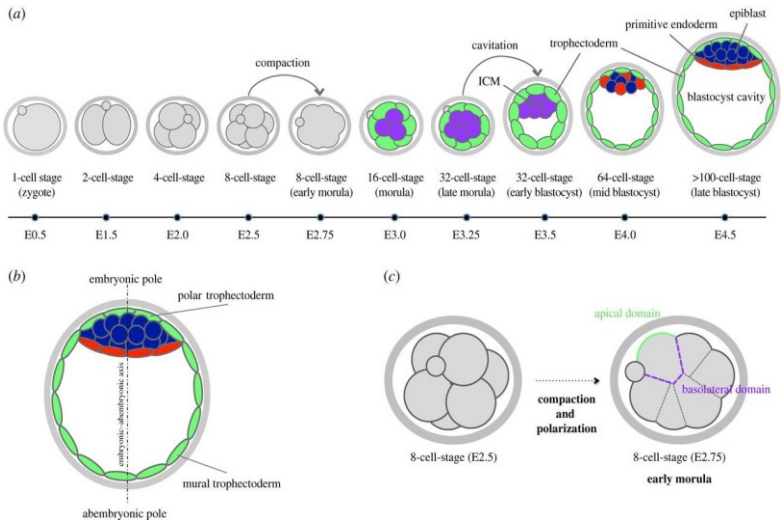

Fig. 3: A schema of mouse preimplantation embryonic developmental timing and blastocyst lineage derivation (Mihajlović & Bruce, 2017)**.**

1.1.1 The first cell fate decision

The first important segregation event during mouse embryonic development marks the loss of cellular totipotency and sets apart the first two cell lineages, where outer cells generate the differentiating trophectoderm (TE) and inner cells become the pluripotent inner cell mass (ICM). This process, in which first two cell lineages are segregated to form either ICM or TE, is referred to as the first cell fate decision (Zernicka-Goetz, Morris, & Bruce, 2009). By the 32-cell (E3.5) stage, the ICM and TE cell fates are segregated. Nevertheless, there are noticeable difference between cells of the emerging two cell lineages that are already observable at the 16-cell stage, where both external and internal cells are each increasingly guided to contribute to one of the two cell lineages (Suwińska, Czołowska, Ożdżeński, & Tarkowski, 2008). There is still not a fully comprehensive and conclusive knowledge of how is the first fate

decision is fully achieved. However, two main models have been proposed (Sasaki, 2015). The first model suggests the importance of cells recognising and responding to relative cell position; namely if they are situated inside or on the outside of the embryo and whether they will respectively specify ICM or TE. Accordingly, due to their position, the differing cells of the embryo are exposed to different extents of cell contacts and are also subject to different environmental impacts, that in turn directs their fate (Saiz & Plusa, 2013). This model is known as the „inside-outside model“ or the „positional model“. The positional model has been supported by experiments manipulating relative cell position within the embryo. It has been demonstrated that inner and outer cells can change their ultimate fate, from ICM or TE respectively, when their relative position in the embryo has been experimentally altered (Tarkowski & Wróblewska, 1967). Later Johnson *et al.* (Johnson, Chisholm, Fleming, & Houliston, 1986) formulated a theory, in which the intracellular apico-basolateral polarity of blastomeres is essential for cell fate decision. This is known as the „polarity“model. This model suggests that cell fate is governed by differential inheritance of apical polarity factors (localised to the contactless apical domain during the initiation of apico-basolateral polarity at the late 8-cell stage - see above), post 8-cell and outer-16-cell cell cleavage division (Saiz & Plusa, 2013). As referenced above, such cleavage divisions can occur along planes that are parallel to the apico-basolateral axis, leading to both daughter cells robustly inheriting apical and basolateral localised polarity factors, and remaining polarised on the outside of the embryo where they differentiate to TE (so called symmetric divisions). Alternatively, cells can divide across the apico-basolateral axis (so-called asymmetric divisions) and result in one outer daughter cell with an intact apical domain (that hence remain polarised and differentiate towards a TE fate) and a second apolar

daughter cell consisting of only basolateral domain material, encapsulated within the embryo, that resists differentiation and remains pluripotent and becomes an ICM founder cell (Fleming, 1987). In practice such asymmetric divisions are rarely perfectly aligned (yet are more prevalent than symmetric divisions) and most often result in daughter cells with unequal polarity, with the least polarised daughter cell most often being internalised (Chazaud, Yamanaka, Pawson, & Rossant, 2006). Therefore, the polarisation models determines that it is the differential inheritance of apical polarity that dictates whether a cell will initiate TE differentiation in an outer position or resist it and remain pluripotent in an inner position (Johnson, Chisholm, Fleming, & Houliston, 1986). It is now generally considered there are aspects of both models that are likely applicable to the situation observed *in/ex vivo*; the so-called "polarity-dependent cell-positioning model" (Fig. 4) (Mihajlović & Bruce, 2017).

The segregation of ICM and TE is mediated through the activity of specific transcription factors (TFs) (Chazaud, Yamanaka, Pawson, & Rossant, 2006). In relation to the specification of the trophectoderm the TF CDX2 plays a major role, while OCT4, NANOG and SOX2 are pluripotency markers and facilitate the formation of the ICM lineage (Ralston, 2008). Consequently, the segregation of the ICM and TE is dependent on the upregulation of the C*dx2* gene in outside cells and conversely the downregulation/transcriptional suppression of *Nanog*, O*ct4* and S*ox2* in the same outer cells. The TF, TEAD4 (TEA domain family member4) and its co-factor YAP1 (Yes-associated protein 1) are responsible for the upregulation of C*dx2* expression in the TE (from the late 8-cell stage onwards). Interestingly, although expressed in all cells of the embryo the YAP1 protein is only found in the nuclei of outer cells (it is cytoplasmic in inner cells), whereas the TEAD4 protein resides

in all cellular nuclei (*i.e.* both in outer and inner blastomeres – although TEAD4 levels are lower in ICM cells - (Nishioka N., 2009)). Thus, only in outer cells can the TEAD4-YAP1 complex activate the appropriate expression of the *Cdx2* gene to promote trophectoderm fate (Nishioka, Yamamoto, Kiyonari, & al., 2008). Equally the lack of nuclear YAP1 protein in inner cells prevents ectopic activation of the *Cdx2* gene and promotes expression of the pluripotency related TF genes; *Nanog*, O*ct4* and S*ox2* (Cockburn & Rossant, 2010). Hence, the establishment of lineage specific TF gene expression establishes a complex network of gene expression regulation underpinning the first cell fate decision (Chunmeng, Wenhao, & Ling, 2019).

The activity of specific cell lineage TFs can be modulated by the differential activation of certain signalling pathways. Specifically, the Hippo-signalling pathway (Yu & Guan, 2013). Hippo-signalling pathway in the developing preimplantation embryo is subject to regulation by relative cell spatial position and polarization dependent mechanisms (Mihajlović & Bruce, 2017). The Hippo-signalling pathway is conserved in mammals and in mice (and also in humans) and is centrally important in regulating the first cell fate decision (Pan, 2010). In the mouse blastocyst, the Hippo-signalling pathway is active in the apolar inner ICM cells but is inactive in the polarised outer TE cells (Chazaud, Yamanaka, Pawson, & Rossant, 2006). The TEAD4 TF (Nishioka, Yamamoto, Kiyonari, & al., 2008) is considered as the transcriptional effector of the Hippo-signalling pathway and when the pathway is active (as in apolar inner cells) the pathway kinases, LATS1 and LATS2, phosphorylate YAP1 (Lorthongpanich, et al., 2013). This phosphorylation prevents nuclear localisation of YAP1 and hence blocks formation of the TEAD4-YAP1 transcriptional complex and induction of the TE specific *Cdx2* gene (Nishioka N., 2009). Simultaneously, the

resulting lack of inhibitory CDX2 TF protein expression ensures the transcription and translation of other TF genes essential for ICM pluripotency and development; such as *Oct4* and *Nanog* (Mitsui, 2003). Conversely, when the Hippo pathway is inactivated, as in polarised outer cells, YAP1 is free to enter the nucleus and complex with TEAD4 to activate genes required for trophectoderm differentiation (and thus supress pluripotency). This process of outer cell Hippo-signalling suppression is dependent on the presence of apico-basolateral polarity and in its absence (either experimentally induced or due to a naturally occurring unbalanced asymmetric distribution) LATS1/2 can become activated and TE specification fails and the outer cells readily internalise to the nascent ICM (Fig.4) (Mihajlović & Bruce, 2017).

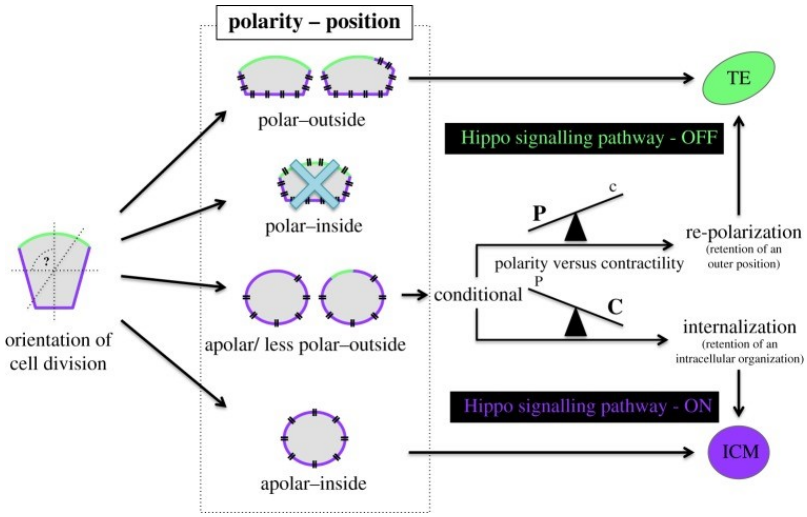

Fig. 4: Schema of the "polarity-dependent cell-positioning" model, which marks that outer polar cells form TE and inner apolar cells form ICM. When polarity (P) prevails over actomyosin contractility (C), cells become outer, suppress the Hippo-signalling pathway and form the TE. In case when contractility (C) prevails over the polarity (P), cells retain intracellular organisation. Moreover, the Hippo-signalling

pathway become active and cells are committed to the ICM fate (Mihajlović & Bruce, 2017).

1.1.2 The second cell fate decision

Following the the first cell fate decision, a further specification and segregation of blastocyst cell lineages occurs in the ICM. This results in the formation of the differentiating primitive endoderm (PrE) and the pluripotent epiblast (EPI), from an initially unspecified population of ICM cells, by the late blastocyst (E4.5) stage. This process is referred to as the second cell fate decision (Eckert J. J., 2004).

At the late blastocyst (E4.5) stage both EPI and PrE cells exclusively express specific TF markers; such as NANOG, SOX2 for the EPI (Artus, 2011) and GATA6, SOX17 and GATA4 in the PrE (Koutsourakis, 1999), although they both express OCT4 (that either dimerises with SOX2 or SOX17 as a requirement for EPI or PrE cell fate, respectively) (Plusa, Piliszek, Frankenberg, Artus, & Hadjantonakis, 2008) (Do, Vinh, & al, 2013). In the early blastocyst (E3.5) ICM, all cells co-express the EPI and PrE markers NANOG and GATA6 (and are hence uncommitted and similar). As development proceeds two populations emerge whereby a mosaic of randomly distributed ICM cells express NANOG or GATA6 in a mutually exclusive manner (~E4.0 – the so-called "salt-and-pepper" pattern), from which the EPI and PrE lineages emerge and actively sort into their correct positions within the ICM (in the case of the PrE, priming the sequential expression of the later markers SOX17 and GATA4) (Chazaud C. Y., 2006). Although the nature of the salt and pepper expression does not necessarily indicate individual cells will form these specific lineages – *i.e.* there is plasticity (Mistri, et al., 2018). At E4.5 the EPI and PrE are

committed to their respective fate (Gardner & Rossant, 1979) . Cells of the PrE are given to create extraembryonic tissues (*e.g.* yolk sac), and in the late blastocyst (E4.5) they occupying the space between blastocoel cavity and the deeper ICM. The EPI cells are found deep inside the ICM (Enders, Given, & Schlafke, 1978) and represent a pool of pluripotent cells, that are progenitors for of the foetal tissues (Rossant, 2016). Thus, the EPI is a source of pluripotent embryonic stem cells (ESCs), which can under the correct laboratory conditions be maintained indefinitely *in vitro* and could be potentially used in medical therapy (Azami, Waku, & Ken Matsumoto, 2017). In contrast to the first cell fate decision, less known about the mechanisms underpinning the second fate decision. However, it has been proposed, that the positional model for the first cell fate could also be applicable to the segregation of EPI and PrE. This would involve an inductive signal acting on ICM cells in contact with the blastocyst cavity that would cause them to differentiate into PrE (a signal not received by cells deeper in the ICM that would form the future EPI) (Cockburn & Rossant, 2010). Although not completely discounted, it is known such a conceptually simple model is not alone sufficient. This is because differentiating PrE cells can actively sort from internal positions of the ICM (and to a lesser extent EPI cells from the surface to deeper positions) and some ICM cells have been observed to yield both PrE and EPI progeny after division (Chazaud, Yamanaka, Pawson, & Rossant, 2006).

As referenced above, by E4.5 the segregation of EPI and PrE cell lineages is complete and PrE cells have activated the expression of latter markers such as GATA4 and SOX17, as well as DAB2 and PDGFRα, in addition to continued expression of GATA6 (Wamaitha, et al., 2015). EPI and PE cells are at this time completely committed to their fate. The GATA6 protein has

an important role in the specification of PrE, however it must require the cooperation of other factors and pathways for correct and complete PrE maturation. For example, despite GATA6 being expressed in all ICM cells of *Nanog*$^{-/-}$ null /mutant embryos the expression of SOX17 and GATA4 is downregulated. Thus, demonstrating the expression of GATA6 alone is not sufficient to direct germane PE maturation (Saiz & Plusa, 2013) (Chazaud, Yamanaka, Pawson, & Rossant, 2006). As with the separation of the TE and ICM lineages in the first cell fate decision, the segregation of EPI and PrE is also accompanied by specific TF gene expression and the induction of gene expression regulatory networks required for the formation of both ICM lineages (Zernicka- Goetz, Morris, & Bruce, 2009) (Saiz & Plusa, 2013). Also, despite early blastocyst (E3.5) stage ICM cells co-expressing the EPI marker NANOG and the PrE marker GATA6, it is known from transcript profiling experiments that such cells already display distinct gene expression patterns that correspond with their ultimate fate, as exemplified by transit through the "salt and pepper" stage (Takaoka & Hamada, 2012).

As stated above, the mid-blastocyst (E4.0) stage "salt-and-pepper" pattern is defined by the spatially randomised and mutually exclusive expression of NANOG and GATA6 across cells of the ICM. The exclusive expression of these TFs is considered the first/earliest marker of EPI and PrE specification (and they are both expressed in all blastomeres as early as the 8-cell stage) (Chazaud C. Y., 2006). Presently, the mechanism by which NANOG and GATA6 (and then later PrE markers – *e.g.* GATA4) distribution becomes mutually exclusive as the "salt and pepper" pattern, from nascent ICM cells with already biased fates, is not entirely clear (Chazaud & Yamanaka, 2016). However, in recent years the importance of signalling through

the FGF (fibroblast grown factor)- extracellular signal-regulated kinase (ERK) pathway has emerged as an important component and is proposed to provide a balance within the ICM between PrE and EPI specification and formation. This is because over-stimulation of the FGF-ERK pathway using exogenous FGF4 during blastocyst maturation causes all ICM cells to adopt a PrE cell fate, defined by GATA6 expression and a lack of NANOG (Krawchuk, 2013). Whereas inhibition of FGF signalling using pharmacological inhibitors of FGFRs (FGF-receptors) and ERK kinases or genetic knockout models of the *Fgf4* or *Grb2* (encoding an intra-cellular signalling adaptor molecule needed to process FGF-based signals) genes cause all ICM cells to convert to an EPI fate (*i.e.* expressing NANOG). It is therefore proposed FGF-signalling from early ICM cells biased to form EPI acts on biased PrE progenitors to cause their differentiation (Yamanaka Y. L., 2010). Moreover, this separation of cell fate is reinforced by the reciprocal repression between GATA6 (GATA4) and NANOG on the expression of each other genes, thus promoting formation of EPI and PrE (Azami, Waku, & Ken Matsumoto, 2017). Additionally, the transcriptional repression and/or downregulation of any induced S*ox17* and G*ata4* gene expression in EPI cells is proposed to be a response to reduced FGF4 signalling processed by these cells, or by would be PrE progenitors in *Fgf4*$^{-/-}$ or *Nanog*$^{-/-}$ mutants in which FGF4 ligand is absent or limiting (Krawchuk, 2013) (Chazaud & Yamanaka, 2016). Therefore, by the late blastocyst stage (E4.5) the ICM is conclusively sorted into PrE and pluripotent EPI (Rossant, 2016). Consequently, the initial heterogenity of early ICM cells, composed of progenitors for either EPI or PrE, is a proposed major feature for later, although still plastic, cell segregation (Zernicka- Goetz, Morris, & Bruce, 2009).

1.1.3 FGF and p38-MAPK signalling during the second cell fate decision

Recent studies have suggested that the activity of the p38 family of mitogen-activated protein kinases (p38-MAPKs) are also important during the second cell fate decision (Thamodaran & Bruce, 2016). Members of the mitogen-activated protein kinase superfamily belong to one of four characterised sub-pathways; the extracellular signal-regulated protein kinases (ERK), Jun N-terminal kinases (JNK), p38-MAPKs or ERK5 kinases. Each of these mitogen-activated protein kinases pathways is characterised by their ability contribute to the transmission of extra-cellular ligand-bound receptor and/or stress signals to specific intra-cellular machinery to obtain specific cellular responses, ranging from cell proliferation/apoptosis to key processes regulating cellular metabolism or developmental differentiation (Zhang, Yang, & Wu, 2007). Pharmacological inhibition of p38-MAPK activity during the second cell fate decision (E3.5-E4.5) has been shown to attenuate PrE specification and strongly block mature PrE formation/differentiation (Thamodaran & Bruce, 2016) (Bora, Thamodaran, Šušor, & Bruce, 2019). Interestingly, blocked PrE formation caused by pharmacological inhibition of FGFRs can be reversed by activating endogenous levels of p38-MAPK. Suggesting p38-MAPK acts downstream of FGF-signalling to promote PrE specification and differentiation (Thamodaran & Bruce, 2016).

1.2 The mTOR signalling pathway

The drug Rapamycin was originally isolated from the bacteria *Streptomyces hygroscopicus*, derived from soil samples

taken on Easter Island in 1964. It has been shown that Rapamycin has prominent antifungal properties (Eng, Sehgal, & Vézina, 1984) and can also act in the clinic as an immunosuppressive and anti-tumor agent (Joungmok & Guan, 2019). In mammals, Rapamycin targets the protein mTOR (standing for mammalian target of Rapamycin – also known as FRAP, RAFT and RAPT (Mirei Murakami, 2004)).

mTOR is highly conserved through evolution and plays a crucial role in regulating cell growth and metabolism by integrating diverse cell signalling and nutritional status inputs (Chung, Kuo, Crabtree, & Blenis, 1992) and appropriately coordinating the activity of cellular catabolic and anabolic processes, such autophagy and protein synthesis (Liu, 2020). mTOR is a protein kinase that belongs to the PI3K-related kinase family and acts to phosphorylate serine/threonine residues on its target proteins. The mTOR protein itself forms the catalytic subunit of two distinct mTOR complexes (mTORC1 and mTORC2), that each significantly differ in their subunit composition, sensitivity to Rapamycin, substrate specificity and hence cellular regulatory roles (Hara, et al., 2002) (Liu, 2020). In the case of mTORC1, there are three typical core components: i) RAPTOR, which is a regulatory protein, ii) mTOR (directly associated with RAPTOR) and iii) mLST8 (standing for, mammalian lethal with Sec13 protein 8). RAPTOR is key for mTORC1 localization and for recruiting mTORC1 substrates via their TOR signalling related protein motifs. It has been suggested, that mLST8 has a stabilising effect on the mTOR kinase domain, although it does not itself lead to phosphorylation of mTORC1 substrates *in vivo* (Yang, et al., 2013). Additionally, mTORC1 also includes two inhibitory subunits: i) DEPTOR (DEP domain containing mTOR interaction protein) (Peterson, et al., 2009) and PRAS40 (proline rich substrate)

(Sancak, et al., 2007) (Saxton & Sabatini, 2017). mTORC1 also dimerizes and forms a lozenge-like structure (as does mTORC2). The mTORC1 complex is largely inactive in isolation, however upon binding of the small GTPase RHEB (in its active form) it becomes catalytic active (Liu, 2020) and Rapamycin displays its inhibitory effect upon mTORC1 by directly binding the active site of mTOR (Yang H. R., 2013).

Similarly, mTORC2 comprises three core subunits: i) RICTOR, ii) mTOR and iii) mLST8. However, unlike mTORC1, mTORC2 is insensitive to Rapamycin due to the substitution of REPTOR for RICTOR. mTORC2 also contains the DEPTOR subunit, as well as PROTOR1/2 and mSIN1 that act as regulatory subunits. Although mTORC2 cannot be directly inhibited by Rapamycin, prolonged Rapamycin treatment can attenuate mTORC2 activity, most probably due to Rapamycin interacting with free mTOR monomers that are eventually incorporated into newly assembled mTORC2 (Sarbassov, et al., 2006) (Saxton & Sabatini, 2017).

The central role of mTOR lies in the regulation of cell growth and metabolism. As cells grow, they must enhance appropriate anabolic processes needed to sustain growth. These include the production of proteins, lipids and nucleotides. Additionally, they must simultaneously suppress catabolic pathways, for example autophagy (Guertin DA, 2006). The activity of such processes are precisely regulated by the output of mTORC1, that acts as a key player in constituting the required homeostatic balance between catabolism and anabolism (Saxton & Sabatini, 2017). Thus, when the cells enter into periods of growth and division, the initiation of specific biosynthetic cascades needed to supply required metabolites is controlled by mTORC1 (and mTORC2) (Liu, 2020).

Accordingly, required induction of protein synthesis and the production of lipids, nucleotides and ATP are promoted by active mTORC1, partly via the phosphorylation of elF4E binding protein (4EBP) and p70-S6 kinase 1 (S6K1) effectors (Holz, Ballif, Gygi, & Blenis, 2005). Additionally, as lipids are essential components of all cellular membranes, as cells increase in size or proliferate, mTORC1 switches on required lipid synthesis (Porstmann, 2008). Moreover, active mTORC1 enhances cell growth through inducing changes in the fate of glucose metabolism. This is achieved by the upregulation/activation of the transcription factor HIF1α (hypoxia inducible factor) to generate increased energy output and enhanced levels of needed carbon units. As such, HIF1α increases the expression of glycolytic enzymes, preferentially shunting energy production in favour of glycolysis over oxidative phosphorylation (Silvera D, 2010). mTORC1 also increases of the activity of the pentose phosphate pathway to stimulate production of NADPH and carbon rich metabolites required for the synthesis of nucleotides and lipids (Saxton & Sabatini, 2017) The ATP producing capacity of the cell is also expanded via mTORC1 mediated enhanced regulation of nuclear encoded mitochondrial transcripts (Liu, 2020). The other important role of active mTORC1 is to suppress cellular catabolism, particularly autophagy. As such it competes with AMPK (adenosine monophosphate activated protein kinase), a key activator of autophagy, to appropriately fine tune this catabolic process. Accordingly, when the cell has enough nutrients, mTORC1 phosphorylates and supresses the kinase ULK1. As ULK1 activity is required to activate AMPK, the formation of autophagosomes required to sustain autophagy is effectively blocked (Komatsu M, 2005). Therefore, the extent of cellular autophagy induction is precisely determined by

relative activities of AMPK and mTORC1 (Liu, 2020). Importantly, both nutrient deprivation or Rapamycin treatment lead to effective mTORC1 inhibition. Thus, under such 'starvation regimes', anabolism in the cell will be blocked and resources will move towards autophagy to derive required metabolites (Saxton & Sabatini, 2017).

mTORC1 is primarily responsible for regulating metabolism in relation to cell growth, while mTORC2 exerts its effects on cell proliferation and survival (Fig. 5) (Saxton & Sabatini, 2017). mTORC2 controls cell proliferation and survival by phosphorylation of PKG/PKA/PKC, the members of AGC family of protein kinases. Indeed, one of mTORC2 typical substrates is PKCα, that like several other members of the PKC family is an actin cytoskeleton regulator (Jacinto, et al., 2004) responsible for coordinating cytoskeletal remodelling and cell migration. (Saxton & Sabatini, 2017). mTORC2 also phosphorylates another member of AGC- kinase family, SGK1, to regulate cell survival via modulation of ion transport (García-Martínez & Alessi, 2008) (Saxton & Sabatini, 2017). Another important role of mTORC2 is phosphorylation of the oncogene protein AKT, to cause its activation. AKT is an early effector of the insulin/PI3K signalling pathway and is responsible for inducing cell proliferation and mediating cellular responses to insulin (Sarbassov, et al., 2006). Amongst other known substrates, mTORC2 has also been shown to phosphorylate NAD kinase and the forkhead-box transcription factor FOXO1/3a, in response to cellular stress (Liu, 2020).

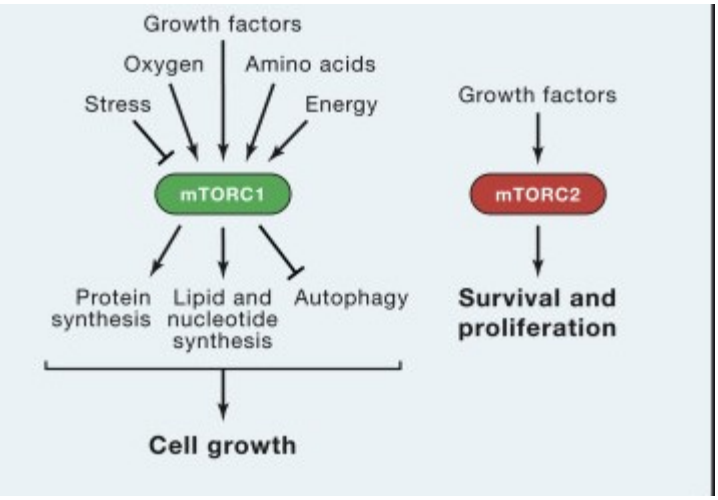

Fig. 5: Summary of the stimulatory and inhibitory inputs to mTOR signalling via mTORC1 and mTORC2 and the cellular outcomes (Saxton & Sabatini, 2017)**.**

In the context of preimplantation embryo development, mTOR activity is well established as a key regulator in human embryonic stem cells (hESCs); especially in regulating long-term undifferentiated hESCs growth. Indeed, it has been shown, that mTOR inhibition in hESCs disrupts pluripotency, impairs cell proliferation and in addition increases differentiation to endoderm and mesoderm derivatives (Zhou, et al., 2009). Moreover, pluripotency supporting factors and signals are integrated by mTOR to supress the transcriptional activity of a several growth-inhibitory and developmentally related genes. Thus, the maintenance of hESC pluripotency and the repression of developmental genes is dependent mTOR activity (Zhou, et al., 2009).

1.2.1 mTOR in mouse preimplantation embryonic development

Restriction of foetal growth is an obstetric complication that can lead to several perinatal risks and can be associated with long-term effects and health risks later in life; for example, metabolic syndrome or neurodevelopmental dysfunction (Dong, et al., 2020). Both embryonic lethality and reduced cell size are phenotypes associated with loss of the *Mtor* gene or inactivation of its substrate, S6 kinase (Murakami, et al., 2004). Thus, active mTOR signalling can be considered as a critical hub of cell growth, survival and metabolism in response to multiple inputs, such as nutrients, energy, stress signals and growth factors (Dong, et al., 2020). Studies shows that mice with mutations in the *Mtor* gene die at E12.5. Moreover, whilst treatment of E4.5 stage mouse blastocysts with Rapamycin does not induce embryonic lethality, it prevents the outgrowth of trophoblast; the precursor of the embryonic component of placenta, that is essential in exchange of nutrients/metabolites (including gas exchange) from mother to the foetus. Thus, mTOR activity is absolutely required for development past earlier post-implantation development (Gangloff YG, 2004).

In preimplantation embryos, mTORC1 is responsible for integrating external and internal signals, including amino acids availability. Indeed, it is the reason that amino acids are often included in *in vitro* embryo culture media and their absence has been described to cause decreased mTORC1 activity in cultured blastocysts. However, the activity of mTORC2 is insensitive to amino acids levels but is reduced by removal of maternal and/or autocrine factors, presumably promoting cell survival (Zamfirescu, Day, & Morris, 2020).

The *ex vivo* culture of mouse blastocysts at E3.5 cannot be sustained beyond 48 hours before embryonic lethality. Interestingly, when certain nutrients, such as amino acids or glucose, are removed from culture media, E3.5 stage blastocyst embryos can survive for several additional days (Bulut-Karslioglu, 2016). These data suggest inhibiting growth pathways can result in a static or dormant state of development (akin to so-called naturally occurring diapaused development). Consistently, pharmacological co-inhibition of mTORC1 and mTORC2 (using the compound INK128) also greatly extends *ex vivo* blastocyst survival. However, sole inhibition of mTORC1 (using Rapamycin) only minimally extends blastocyst survival. Hence, is proposed that inhibition of both mTOR complexes is able to cause developmental diapause of mouse blastocysts, in a manner that mimics nutrient starvation and naturally occurring *in vivo* diapause (Bulut-Karslioglu, 2016).

1.3 AMPK signalling pathway

AMP-activated protein kinase (AMPK) is conserved within all eukaryotes and acts in a signalling pathway that is key to regulating cell growth and reprogramming cellular metabolism (Garcia & Shaw, 2017). It has also been implicated in regulating cell polarity, necessary for embryonic development, and autophagy, as discussed above (Mihaylova & Shaw, 2011). The physiological elevation of AMP/ADP levels represents a typical stress signal that is indicative of low nutrient levels or prolonged exercise/respiration. Such elevated AMP/ADP levels (plus a few known pharmacological compounds) are responsible for activating AMPK (Mihaylova & Shaw, 2011). Thus, as a response to cellular energy stress (manifest by increased level of AMP or/and ADP) induced AMPK activity acts to inhibit

anabolic processes, that consume ATP, and supports catabolic metabolism that generates ATP. Interestingly, the fact, that AMPK is able to so effectively reprogram metabolism is widely used as a therapeutic intervention point to treat many metabolic diseases, including diabetes, cancer, inflammation and obesity (Garcia & Shaw, 2017). However, it is important to note that multiple upstream regulators harbour the potential to control AMPK signalling via induced changes in cellular nutritional and energetic requirements (Gómez & Mariño, 2018).

Structurally, AMPK is a trimeric complex formed by a catalytic α-subunit and one each of a β- and γ- regulatory subunit; the human genome contains two α-subunit genes, two β -subunit genes and three γ-subunit genes (Garcia & Shaw, 2017). The activity of AMPK is directly regulated by phosphorylation (on Thr172) by other upstream kinases. However, it is the γ-subunit that operates as a modulating sensor, that is able to respond to changes in the intra-cellular AMP:ATP or ADP:ATP ratio. Thus, the allosteric binding of AMP confers a conformational change that makes AMPK a better substrate for its activating upstream kinases. Additionally, such AMP binding also inhibits phosphatase directed dephosphorylation of Thr172. The ultimate consequence being the binding of AMP causing increased phosphorylation and decreased dephosphorylation of AMPK, resulting in its enhance activation (Gómez & Mariño, 2018).

The central role of active AMPK signalling is as a metabolic checkpoint capable of inhibiting cell growth, which it achieves via feedback regulation of mTORC1. mTORC1 controls autophagy, a protective process by which a cell engulfs its own organelles in order to provide required metabolites when nutrients levels are low, by supressing formation of autophagosomes when nutrient levels are

sufficient (Mihaylova & Shaw, 2011). Accordingly, active AMPK therefore acts as an antagonist of mTORC1 under starvation conditions (Pezze, a další, 2016).

1.3.1 AMPK in mouse preimplantation development

The influence of AMPK activity on early embryonic development is not very clear. Louden and colleagues find that exposing blastocyst embryos to high levels of insulin or IGF1 leads to insulin-resistance by downregulation of IGFR1. This is manifest as reduced glucose uptake and increased cellular apoptosis, resulting in growth restriction. However, by using specific AMPK activators (that cause increases in the AMP:ATP ratio), these effects could be reversed. Although, active mTORC1 signalling was also observed to increase (Louden E, 2008). Osmotic stress is also known to cause AMPK activation in mouse blastocysts and is associated with reduced expression of potency factors that affect differentiation, for example *Id2* or *Cdx2* (Eckert, Velazquez, & Fleming, 2015). Additionally, it is reported that continuous treatment of 2-cell stage mouse embryos with AICAR, a known AMPK activator, inhibits blastocyst formation and causes reductions in total embryo cell number (associated with changes in the mRNA levels of genes required to form the blastocyst). Moreover, a nine hours AICAR treatment of mouse blastocyst causes blastocoel cavity collapse (implying impaired TE function) (Calder M. D., 2017). Interestingly, it is also reported that AMPK activation mediated by transient (0.5 hours) stress in 2-cell stage embryos, causes a loss of later potency but that this can be reversed by chemical inhibition of AMPK (Xie, 2013).

In our lab, Gahurova *et al*. (*manuscript in preparation*) have shown the AMPK pathway is capable of regulating the

mTOR pathway during 8-cell to 16-cell transition, as AMPK chemical activation replicates spatial cell allocation phenotypes induced by chemical inhibition of mTOR. These data suggests that AMPK is normally inactive at this stage and that its induced chemical activation represses the mTOR pathway. However, it has not been studied whether the AMPK pathway can play a functional role during the later stages of mouse preimplantation development and specifically affect the segregation of EPI and PrE cell fates in the blastocyst ICM (as previously observed by ourselves and other labs in relation to the p38-MAPK (Bora, Thamodaran, Šušor, & Bruce, 2019) (Thamodaran & Bruce, 2016) and mTOR pathways (Bulut-Karslioglu, 2016).

2. Project aims

- To analyse of the impact of AMPK activation, using the chemical GSK621, on specification of EPI and PrE in the maturing mouse blastocyst ICM.
- To analyse of the impact of mTOR downregulation, using chemical inhibitor Torin1, on specification of EPI and PE in the maturing mouse blastocyst ICM.
- To compare the effects of AMPK activation and mTOR inhibition and assess whether AMPK activation is a phenocopy mTOR inhibition (as hypothesised).

3. Materials and methods

3.1 Embryo cultivation

F1 females mice (derived from a CBA/W x C57BL6, male x female cross) at 8-9 weeks old were super-ovulated by intra-peritoneal injection of 7.5IU PMSG (pregnant mare serum gonadotrophin extract, Sigma Aldrich- Merck) and after 48 hours were similarly injected by 7.5IU hCG (human chorionic gonadotrophic hormone, Sigma Aldrich- Merck). Followed by mating with F1 males. 43-44 hours later were females sacrificed by cervical dislocation and the oviducts dissected and immediately placed into in-house prepared M2 medium (composition is shown in Table 1). 2-cell (E1.5) stage embryos were isolated from the oviducts by microdissection and washed in M2 medium (pre-warmed at 37 °C for at least 2 hours). Embryos were then cultured in pre-warmed and gas equilibrated KSOM medium drops (~20µl), under light mineral oil (Irvine Scientific) at 37ºC in a 5% CO_2 atmosphere to the required developmental stages. The KSOM media used was a commercial preparation (Embryo-Max, Milipore) supplemented with amino acids (1X Gibco^{TM} MEM Non-Essential Amino Acids Solution) and 0.5X Gibco^{TM} MEM Amino Acids Solution); abbreviated to KSOM+AA.

For pharmacological activation of the AMPK pathway, embryos were cultured in prepared and equilibrated KSOM+AA drops containing 20µM GSK621 (Selleckchem) E3.5 to E4.5 or from E3.5 to E5.5. KSOM+AA drops/plates prepared by the additional of equal volume of DMSO solvent (Sigma) were used as a vehicle control. For mTOR pharmacological inhibition, KSOM+AA drops/plates were similarly prepared containing 20µM Torin1 (Selleckchem) or DMSO control and transferred embryos cultured from E3.5 to E4.5 or from E3.5 to E5.5. Equal

volume of DMSO (Sigma) was used as a vehicle control. Embryos were also incubated in 5% CO_2 atmosphere at 37 °C incubator. Note, that prior to transfer of embryos at the E3.5 stage to inhibitor/control conditions, such recipient plates/drops were pre-warmed and equilibrated at 37 °C in the 5% CO_2 incubator for at least 3-4 hours. Per experiment, one half of embryos cultured from the 2-cell (E1.5) stage were equally distributed between the AMPK activation/mTOR inhibition and DMSO conditions.

Tab. 1: M2 preparation

STOCK	M2 Media ingred.	g/100ml	TOTAL VOLUME
A (x1O)	NaCl	5.534	10.0ml
	KCl	0.356	
	KH2PO4	0.162	
	MgSO4x7H2O	0.293	
	Na-Lactate 60% syrup	3.2(ml)	
	Glucose	1.000	
	Penicilin	0.060	
	Streptocymycin	0.050	
B (x10)	NaHCO3	2.101	1.6ml
	Phenol Red	0.010	
C (x100)	Na Pyruvate	3.600	1.0ml
D (x100)	CaCl2x2H2O	2.520	1.0ml
E (x10)	HEPES	5.958	8.4ml
F	BSA		400(mg)
G	H20		

3.2 Embryo fixation and immuno-fluorescent staining

When embryos reached the required developmental stage after transfer into pharmacological inhibitors or DMS vehicle control conditions (one day for E4.5 or two days for E5.5) they were fixed in 96 -well plates using 4% PFA (paraformaldehyde) (Santa Cruz Biotechnology) at room temperature (RT) for 20 minutes in the dark. A maximum of 15 embryos were fixed per

well. Embryos were then washed through three wells of 0.15% PBST (phosphate buffered saline plus 0.15% Tween20) and incubated in the third PBST wash for 20 minutes at RT. Thereafter, permeabilization was performed by transferring embryos into 0.5% Triton-X100 (Sigma-Aldrich) in PBS for 20 minutes at RT, followed by another three identical PBST washing steps. Blocking of nonspecific epitopes potentially recognised or non-specifically bound by the primary antibodies used in the experiment was performed by incubating embryos in in 3% BSA (bovine serum albumin - Sigma-Aldrich) containing solution diluted in PBST for 30 minutes at 4°C. Primary antibodies specific for detecting proteins of interest (details in Table 3) were then added at the required dilutions (in BSA-PBST). For NANOG and GATA4 detection the primary antibodies were used as1:200 dilutions in a minimal volume; GATA6 primary antibody was similarly diluted but to a concentration of 1:100. For NANOG detection was used rat anti-NANOG antibody (Abcam, ab80892), for GATA4 detection was used rabbit anti-GATA4 antibody (Santa Cruz, sc-9053) and for GATA6 detection was used goat anti-GATA6 antibody (R&D systems, AF1700). Embryos were incubated in primary antibody in 96-well plates wells covered by light mineral oil at 4°C overnight and then washed the next day in the routine regime of three PBST washes before transfer into a second blocking solution (3% BSA_PBST) for 30 minutes at 4°C. Fluorescently conjugated secondary antibody staining was then performed using donkey anti-rat antibody (Invitrogen, A21208) for NANOG protein detection, donkey anti-rabbit (Invitrogen, A21572) for GATA4 detection and donkey anti-goat (Invitrogen, A21432) for GATA6 detection; each at dilutions of 1:500 in BSA-PBST (Table 2). Secondary antibodies were combinate by mixing donkey anti-rat antibody for NANOG detection to donkey anti-rabbit for GATA4 detection; and donkey anti-rat

antibody for NANOG detection to donkey anti-goat for GATA6 detection. Embryos were incubated with secondary antibody at 4°C for one hour, routinely washed three times in PBST. Embryos were placed in drops of Vectashield mounting media containing DAPI for DNA staining (Vector), ready for fluorescence confocal microscopy imaging (if not immediately imaged, embryo containing drops were stored at 4°C in the dark).

Tab. 2: List of used antibodies.

First antibody	1st Ab: BSA ratio	Secondary antibody	2nd Ab: BSA ratio
Rabbit anti-GATA4	1: 200	Donkey anti- rabbit conjugated to the Alexa Fluor 555	1:500
Rat anti-NANOG	1: 200	Donkey anti-rat conjugated to the Alexa Fluor 488	1: 500
Goat anti-GATA6	1: 100	Donkey anti-goat conjugated to the Alexa Fluor 568	1: 500

3.3. Confocal microscopy

Fixed and immuno-fluorescently stained blastocyst embryos were transferred into specially prepared drops of PBST on glass bottomed 35mm culture dishes, covered by light mineral oil. The embryos were then imaged as a complete series of confocal microscopy z-sections (60-70 sections per embryo, thickness 2μm) using light of the required wavelengths to excite the secondary antibody conjugated fluorescent groups (*i.e.* Alexa-Fluor-555nm, Alexa-Fluor-488nm) allowing visualisation of the desired proteins. Embryos were scanned using an inverted Olympus FLUOVIEW FV10i confocal microscope and control and experimental embryos were scanned with the same non-saturating laser power and photomultiplier gain settings. Acquired micrograph images were then captured processed using

the preparatory Olympus FLUOVIEW FV10i, in readiness for image analysis (see below).

3.4. Blastocyst cell number and lineage quantification and statistics

Total and assay specific cell numbers per embryo, in control and experimental groups, were manually counted from the acquired full confocal z-series micrograph images; based on DNA DAPI nuclei staining (*i.e.* total cell number) and detectable GATA4 or GATA6 and NANOG staining (indicative of PrE or EPI cells within the blastocyst ICM). Additionally, cells located outside of the ICM were categorised as outer cells, as well as cells that did not stain for either NANOG, GATA4/GATA6 (all other cells were classified as ICM cells).Those cells that were positive for both NANOG and GATA4/GATA6 were designated as uncommitted in regard to their cell fate. The collected data in both experimental and control groups were recorded by using Microsoft Excel and statistical analysis was performed by Student's t-test. First, to see if data has a normal data distribution was performed Shapiro- Wilk test. If the data had a normal distribution, then was used Students two- tail t- test. In case of abnormal data distribution was used Mann- Whitney test. P-value was considered as significant if it was less than 0.05 (*< 0.05, **< 0.005, *** < 0.0005.

4. Results

4.1 The effect of AMPK activation, using the chemical GSK621, on specification of EPI (NANOG+) and PrE (GATA4+) in the maturing mouse blastocyst ICM at E4.5

Within maturing mouse blastocyst ICM cells occurs a transition from an initially uncommitted state, defined by co-expression of both GATA6 (PrE) and NANOG (EPI) proteins markers, to a committed state appropriate to their ultimate fate, defined by sole expression of either NANOG (EPI) or GATA4 (PrE) (Kuo, 1997). In this thesis, we investigated the effects of AMPK activation, using the chemical activator GSK621, on ICM cell specification and segregation to the EPI and PrE lineages. Recovered mouse embryos were cultured from 2-cell to the E3.5 stage in KSOM+AA (KSOM supplemented with amino acids) and then moved to KSOM+AA containing either GSK621 or an equal volume of DMSO (as vehicle control) and cultured until to E4.5. Used concentration of GSK621 was previously optimised in the laboratory by Dr. Gahurová to have an effect on embryonic development (at the 8- to 16-cell transition – *unpublished observations*). The embryos were then fixed and double immuno-stained for NANOG and GATA4 (Fig.6), and stained with DAPI (to visualise nuclear chromatin). Stained embryo samples were scanned by fluorescence confocal microscopy and complete z-series obtained, per embryo. The total number of cells per embryo was recorded, as were the numbers of inner cells, outer cells, GATA4 only positive ICM blastomeres, NANOG only positive ICM blastomeres, GATA4 and NANOG double positive ICM blastomeres and apoptotic cells. The average cell numbers, in each category, was then calculated and compared between GSK621-treated and control embryo groups (Fig.7).

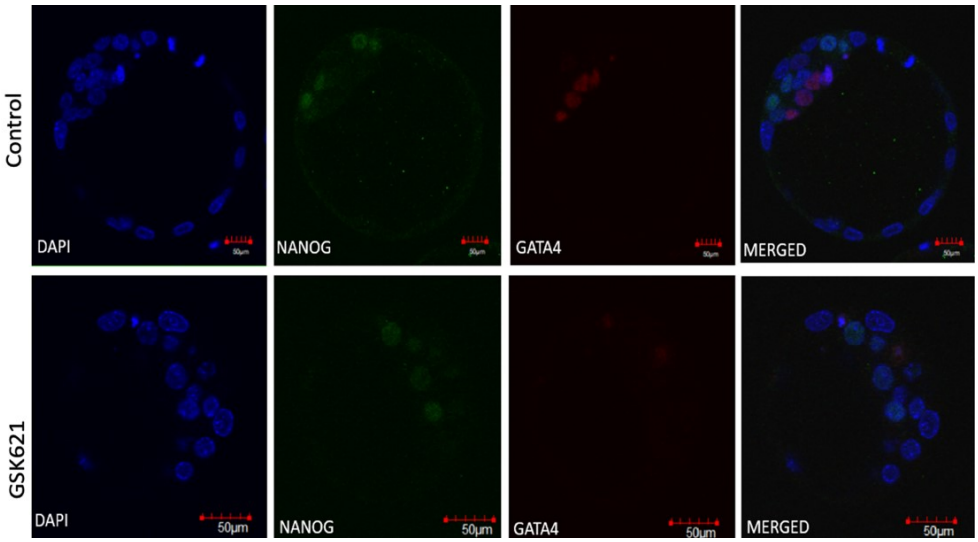

Fig.6: Immuno-fluorescent staining of DMSO control (upper n=26) and GSK621 (n=25) treated (from E3.5-E4.5) embryos (lower) for ICM lineage marker proteins. DAPI (blue), NANOG (green), GATA4 (red) – scale bar = 50µm.

As is obvious from Fig.7, there was a significant reduction in the total number of cells (based on DAPI nuclei staining) in GSK621 treated embryos (an average of 36.8± 7.3 versus 63.2 ± 7.3 in controls). The decreased total cell count was because of significant reductions in both inner and outer cells. Additionally, there was a significant reduction in the number of cells expressing the PrE marker GATA4 in GSK621 treated embryos (an average 1.8± 1.5 versus 8.9 ± 2.8 in control embryos). However, the number of NANOG expressing ICM cells was statistically equal (as was the number of apoptotic cells) between the two groups. Additionally, a small yet statistically significant number (1.6± 2.1) of cells co-expressing NANOG and GATA4 were observed in GSK621 treated embryos that were not observed in control embryos.

The overall (inner and outer) reductions in cell number suggest blastocyst development was delayed/partially arrested

around the point of GSK621 treatment (*i.e.* E3.5/ 32-cell stage). This conclusion is supported by the observation GSK621 treated blastocyst were smaller than controls and had less expanded blastocoel cavities (Fig. 6); although we could not detect any increased incidence of apoptosis that would indicate cytotoxicity associated with AMPK activation by GSK621. Moreover, the fact most ICM cells expressed NANOG alone or in combination with GATA4, in GSK621 treated blastocysts, and the number of GATA4 positive cells is so robustly reduced, strongly indicates a block in PrE specification and differentiation. It is possible that the NANOG alone expressing population of ICM cells in GSK621 treated blastocysts represent; i) cells that have all successfully specified EPI, ii) cells that have failed to specify either EPI or PrE (and thus, remain in the uncommitted state observed in ordinary E3.5 blastocysts and characterised by co-expression of NANOG and GATA6), or, iii) a mixture of specified EPI and uncommitted cells (*i.e.* failed PrE progenitors that are still co-expressing NANOG and GATA6). It should be stressed that although control and AMPK activated blastocysts had equal numbers of NANOG alone expressing ICM cells, the overall size of the ICM was significantly greater in controls (as a function of continued development). However, we concluded that AMPK activation from E3.5 to E4.5 negatively affects development and is associated with aberrant ICM cell-fate specification.

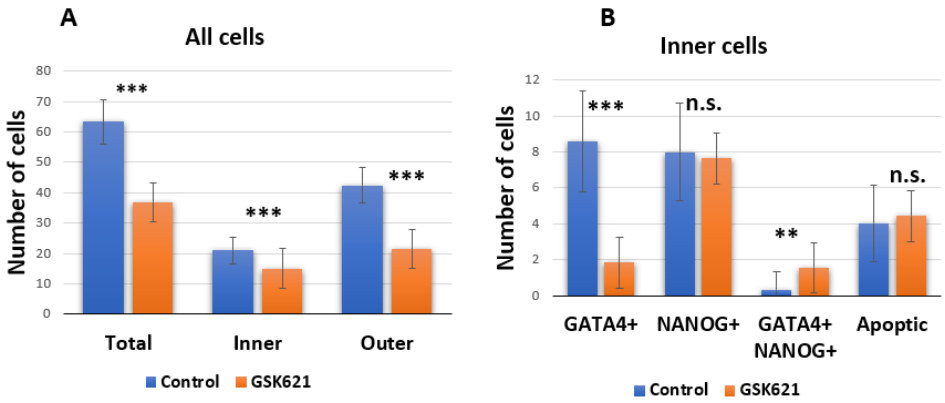

Fig.7: Statistical analysis of cell number and ICM lineage marker expression in GSK621 treated (AMPK activated) embryos versus the control group, between E3.5 and E4.5 (assayed at E4.5).

Chart A: The average number of all cells in control group versus GSK621 treated embryos.

Chart B: Quantification of ICM cells.

4.2 The effect of AMPK activation, using the chemical GSK621, on specification of EPI (NANOG+) and PrE (GATA4+) in the maturing mouse blastocyst ICM by E5.5

To assay if AMPK activation between E3.5 and E4.5 was associated with arrested or delayed/slowed development, we repeated the above experiment exposing cultured embryos to GSK621 between E3.5 and E5.5. As before we immuno-fluorescently stained fixed (E5.5) embryos for NANOG and GATA4 protein markers (Fig.8).

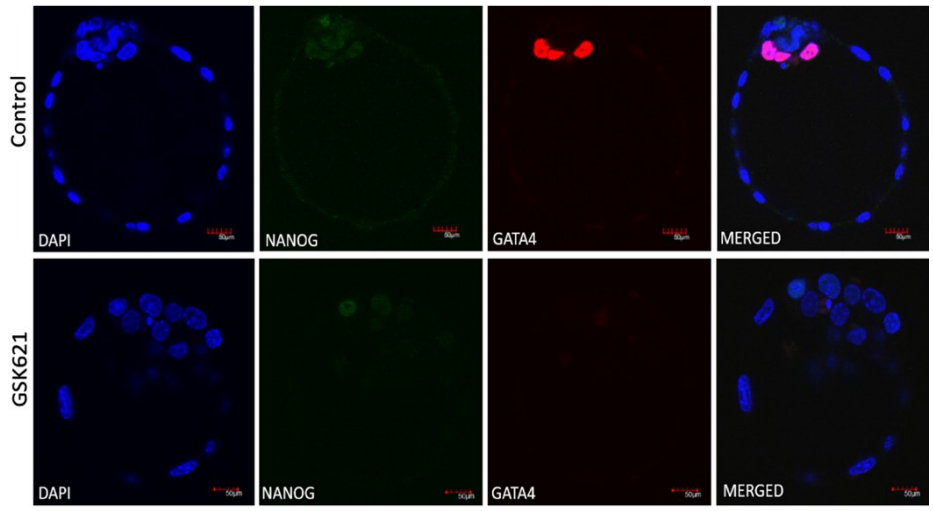

Fig.8: Immuno-fluorescent staining of DMSO control (upper n=18) and GSK621 (n=19) treated (from E3.5-E5.5) embryos (lower) for ICM lineage marker proteins. DAPI (blue), NANOG (green), GATA4 (red) – scale bar = 50μm.

As with the previous experiment, the total number of cells, in both outer and inner populations, was significantly reduced in the GSK621 treated versus control blastocysts (Fig. 9). The GSK621 group comprised an average of 53.3 ± 6.5 total cells versus 93.6 ± 15.1 in the controls. Interestingly, this number is larger than the total number of 36.8 ± 7.3 cells observed in embryos similarly treated with GSK621 assayed at E4.5. Thus, these data indicate that AMPK activation is not associated with completely arrested development (such as may be indicative of induced developmental diapause) but rather slower development/cell proliferation. Moreover, comparing the number of cells in GSK621 treated blastocysts assayed at E4.5 and E5.5, it can be seen majority of the extra cells contributed by E5.5 are within the TE (37.5± 6.4 at E5.5 versus 21.4± 6.6 at E4.5) with only a very modest increase in ICM cell number (15.8±6 at E5.5

versus 15.1± 4.3 at E4.5) – please note, even in control/untreated embryos cultured past the E4.5 stage of normal uterine implantation, cell proliferation in the ICM is not supported, but TE division continues (Johnson, 2009). Indeed, in respect to the expression of ICM lineage markers, the GSK621 induced phenotypes observed at E4.5 and E5.5 are remarkably similar. For example, there are no significant differences in the number of NANOG only expressing cells between GSK621 and control treatments at either assayed developmental time-point. Furthermore, there are similarly significant decreases in the number of GATA4 positive PrE cells and a small but significant population of NANOG and GATA4 co-expressing ICM cells; although the degree of detected apoptotic cell death does reach significance in GSK621 treated blastocysts assayed at E5.5. These data indicate that activation of AMPK during blastocyst maturation does not fully arrest development but does significantly impair cell proliferation. This effect is more profound in the ICM and is associated with defective ICM cell fate specification and derivation, specifically in regard to formation of GATA4 expressing PrE (it is notable that the limited observed GATA4 expression is often associated with atypical, compared to controls, co-expression of NANOG).

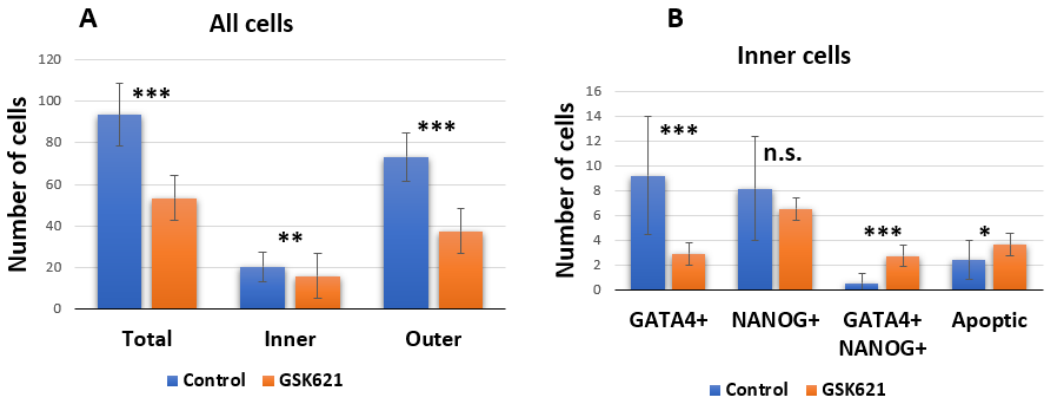

Fig.9: Statistical analysis of cell number and ICM lineage marker expression in GSK621 treated (AMPK activated)

embryos versus the control group, between E3.5 and E5.5 (assayed at E5.5).

Chart A: Average number of all cells in control group versus GSK621 treated embryos.

Chart B: Quantification of ICM cells.

4.3 The impact of AMPK activation by using chemical GSK621 from E3.5 to E4.5 on specification PrE (expression of GATA6) and EPI (expression of NANOG) in the maturing mouse blastocyst

We concluded from the above described experiments that AMPK activation in the maturing mouse blastocyst impairs the expression of the late PrE marker, GATA4 (PrE) but did not seem to significantly affect the number of NANOG (EPI) expressing cells; suggesting at least a block in PrE differentiation. We therefore sought to repeat similar AMPK activation experiments, using GSK621 during blastocyst maturation (between E3.5-E4.5), but performed immuno-fluorescent staining (at E4.5) using an anti-NANOG antibody in combination with an anti-GATA6 anti-sera (note, GATA6 is an early PrE marker, that in unspecified/uncommitted early mouse blastocyst ICM cells is co-expressed with NANOG – Fig.10). We wanted to see what was the proportion of unspecified/uncommitted ICM cells (co-expressing GATA6 and NANOG) in AMPK activated mouse blastocyst, thus helping to answer our original query as to the true nature of NANOG positive expressing cells, and hence overall ICM cell-fate specification, in our previous experiments (see above - *i.e.* options i-iii).

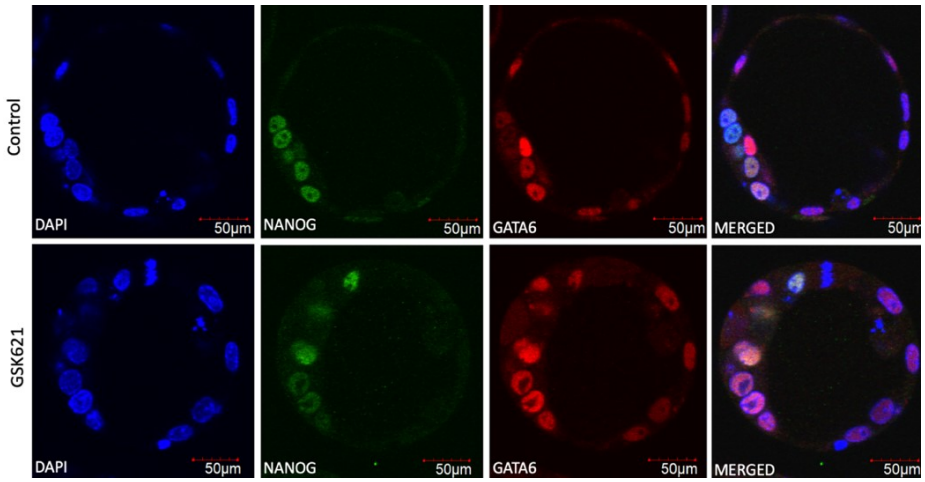

Fig.10: Immuno-fluorescent staining of DMSO control (upper n=15) and GSK621 (n=15) treated (from E3.5- E4.5) embryos (lower) for ICM lineage marker proteins. DAPI (blue), NANOG (green), GATA6 (red)- scale bar = 50µm.

The quantified results of this experiment are shown in Fig.11. Similar to the previous experiments, significant reductions in total cell number, contributed from both inner and outer cell populations, were observed in GSK621 treated embryos. However, the magnitude of this effect was not as great as previously observed, with control embryos consisting of an average of 65.7±7.4 total cells per embryo compared 47.7±5.7 in GSK621 treated embryos (these numbers were respectively 25.4±3.1 and 21.7±2.9 for inner cells and 40.3±5.9 & 26±5.3 for outer cells). It is not clear, why the effect was comparatively weaker in this second experiment. Interestingly, we again observed a highly significant decrease in the number of ICM cells solely expressing the PrE marker, GATA6 in this case, in AMPK activated blastocysts (8.4±1.7 in the GSK621 treatment group and 13.4±1.6 in the controls). This reduction is not as robust as seen using the late PrE marker GATA4 and may reflect the overall less effective GSK621 treatment (as revealed by counting total cell numbers)

in this individual experiment or the fact GATA6 is an earlier PrE marker and such specified PrE cells (solely expressing GATA6) have not yet activated GATA4 protein expression. Nevertheless, these data consistently reveal a significant deficit in PrE cell numbers in AMPK activated blastocysts. Notably, the number of cells solely expressing NANOG was not significantly different between treatment groups in this experiment (the GSK621 treated group had an average of 8.1±1.5 NANOG+ cells per embryo and control had an average of 9.5±2.7 NANOG+ cells); moreover, these numbers were statistically equal with previous GSK621 treatments between E3.5-E4.5 – *see above*); as were the number of apoptotic cells. However, the number of ICM cells co-expressing NANOG and GATA6 was significantly higher in AMPK activated blastocysts than control (respectively, 3.8±1.6 versus 0.4±0.6 cells). Although, a conceptually similar population of NANOG and GATA4 co-expressing cells had been observed in our previous experiments, this typically only represented an isolated, or a couple, cell(s) per embryo, whereas the observed population of NANOG and GATA6 co-expressing cells was much more significant. These combined data suggest AMPK activation in maturing mouse blastocysts (E3.5-E4.5) is associated with a typical specification of NANOG expressing EPI cells but an impairment in PrE specification that is revealed by reduced numbers of ICM cells solely expressing either GATA6 or GATA4 and increased numbers of unspecified/uncommitted cells co-expressing NANOG and GATA6. However, it would have been advantageous to repeat the NANOG and GATA6 expression assay in an experiment using a GSK621 treatment that resulted in the more robust reductions in cell number (as observed when assaying NANOG and GATA4 expression). Accordingly, we may predict an even stronger phenotype on successful PrE specification and differentiation.

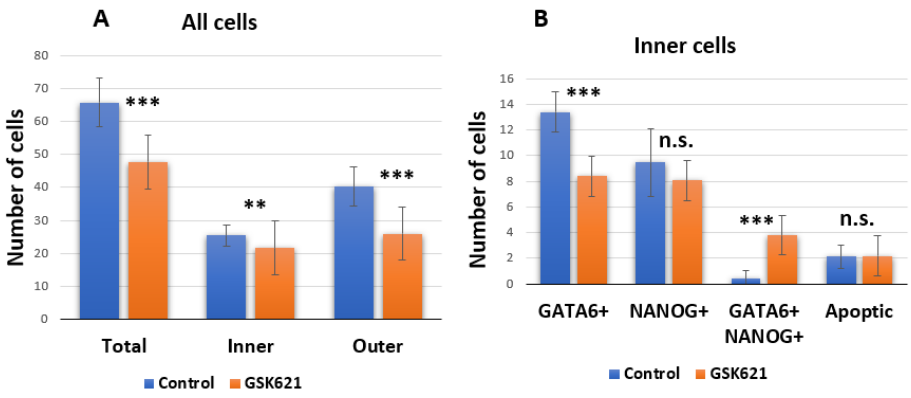

Fig.11: Statistical analysis of cell number and ICM lineage marker expression (NANOG – EPI & GATA6 –PrE) in GSK621 treated (AMPK activated) embryos versus the control group, between E3.5 and E4.5 (assayed at E4.5).

Chart A: The average number of total, inner and outer cells in control group versus GSK621 treated.

Chart B: Quantification of ICM cells.

4.4 The effect of mTOR inhibition, using Torin1 from E3.5 to E4.5, on specification of EPI (NANOG+) and PrE (GATA4+) in maturing mouse blastocyst ICM, focused on GATA4 and NANOG protein detection.

One goal of this thesis was to assay if AMPK activation and mTOR inhibition result in the same phenotype regarding blastocyst ICM lineage specification and derivation (because theoretically AMPK activation should cause downregulation of mTOR, although other regulatory inputs could be at play). To address this question, we used the chemical inhibitor Torin1 to inhibit mTOR activity in experiments similar to those described above using GSK62, and compared the resulting blastocyst ICM

phenotypes. Concentration of Torin1 were prviously optimised in the laboratory by Dr. Gahurová and shown to have an effect on embryonic development (at the 8- to 16-cll transition – *unpublished observations*). Mouse embryos were cultured from the 2-cell stage to E3.5 in KSOM+AA, moved to KSOM+AA supplemented with Torin1 (20µM) or an equal volume of DMSO as vehicle control and further cultured to E4.5 before being fixed and immuno-fluorescently stained for NANOG (EPI) and GATA4 (PrE) blastocyst ICM marker protein expression (Fig.12).

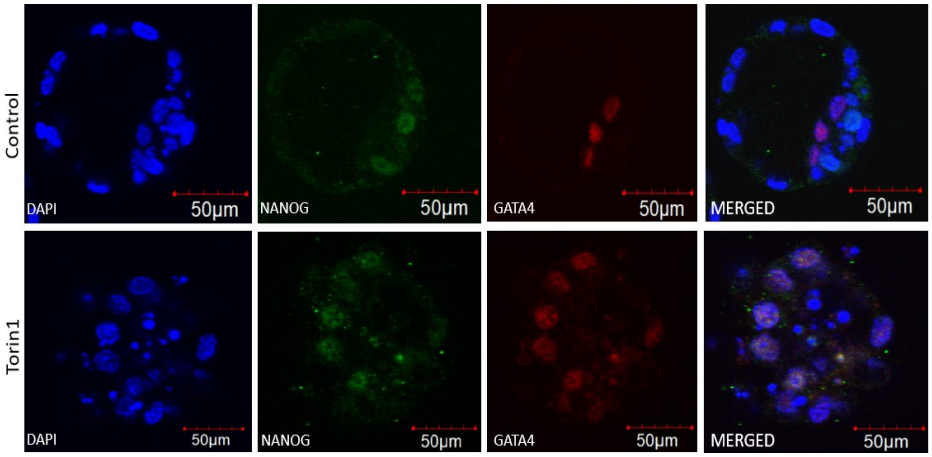

Fig.12: Double immuno-fluorescent staining of late blastocyst at E4.5. DMSO control (upper n=12), Torin1 treated (lower n=11). DAPI (blue), NANOG (green), GATA4 (red)- scale bar = 50µm.

From Figure 13 it is clear that mTOR inhibited blastocysts have significantly fewer total cells than the control group (averaging 41.8±5.2 cells versus 69.1±3.2 cells). Moreover, this is contributed by both fewer outer (averaging 22.6±5.5 cells versus 44.9±3.7 cells) and inner (averaging 19.4±4 cells versus 24.2±2.9 cells) blastomeres, although the effect is most profound in outer cells. Interestingly, just as with GSK621 induced AMPK activation, Torin1 mediated mTOR inhibition similarly causes a reduction in ICM cells expressing GATA4 protein, thus reflecting significantly impaired PrE differentiation (on average

11.7±0.8 cells in the control group and 5.9±1.6 in the Torin1 treated group). There was also a significant reduction in the number of cells only expressing NANOG in Torin1 treated blastocysts (on average 10.4±2.2 cells in the control group versus 6.9±3.5 cells in the Torin1 treated group, p-value= 1.16E-02). Minimal differences in the number of GATA4 and NANOG co-expressing ICM cells were observed (averaging 1.4±1.4 in control versus 3.3± 1.5 cells in Torin1 treated groups). However, unlike the previously described experiments, such cells were observed in control treated embryo ICM as well as those treated with activator/inhibitor. Additionally, Torin1 treatment significantly increased the incidence of apoptotic cells (averaging 3.3±2.1 apoptic cells per embryo versus 0.7±1 in control group – also observable in the immuno-fluorescent staining confocal micrograph images, Fig. 12). Collectively, these data indicate a similar mTOR inhibition ICM phenotype compared to AMPK activation; although it also appears EPI (cells solely expressing NANOG) cell numbers as well as PrE (cells solely expressing GATA4) cells are also reduced.

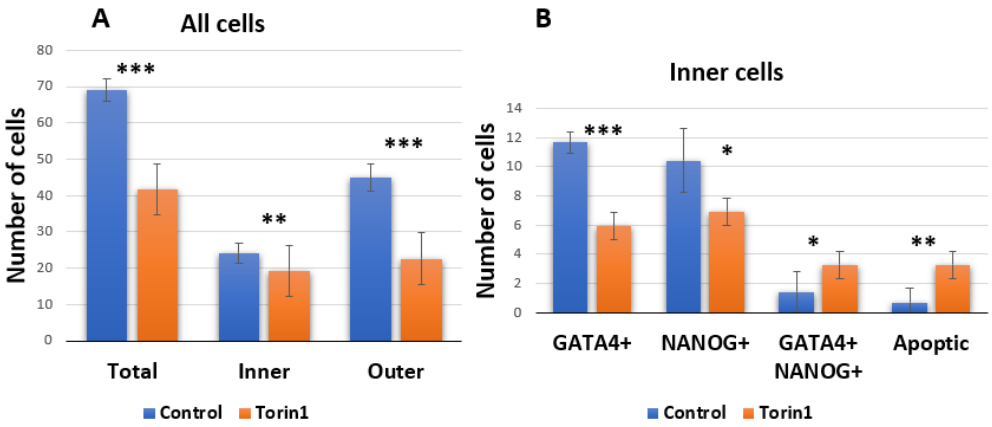

Fig.13: Statistical analysis of cell number and ICM lineage marker expression (NANOG – EPI & GATA4 –PrE) in GSK621 treated (AMPK activated) embryos versus the control group, between E3.5 and E4.5 (assayed at E4.5).

Chart A: The average number of total, inner and outer cells in control group versu Torin1 treated embryos.

Chart B: Quantification of ICM cells.

4.5. The effect of mTOR downregulation using chemical inhibitor Torin1 from E3.5 to E5.5 on specification of EPI and PrE in the maturing mouse blastocyst ICM, focused on GATA4 and NANOG detection

An aim of this thesis was to investigate whether mTOR downregulation could affect specification of EPI and PrE in mouse blastocyst ICM. As shown above, Torin1 treatment from E3.5-E4.5 was associated with a significant block in PrE numbers and also statistically reduced EPI cell numbers. We next assayed the effect of Torin1 mediated mTOR inhibition (note, Torin1 inhibits both mTOR containing complexes, mTORC1 and mTORC2) in mouse blastocysts cultured from E3.5 to E5.5 (followed by fixation and immuno-fluorescent staining against NANOG and GATA4 proteins, with DAPI nuclei stain (Fig.14).

Interestingly, one key difference we observed upon mTOR inhibition between E3.5-E5.5 was the collapse of the blastocyst cavity, whilst control treated blastocysts showed a typical E5.5 phenotype/morphology (Fig.14).

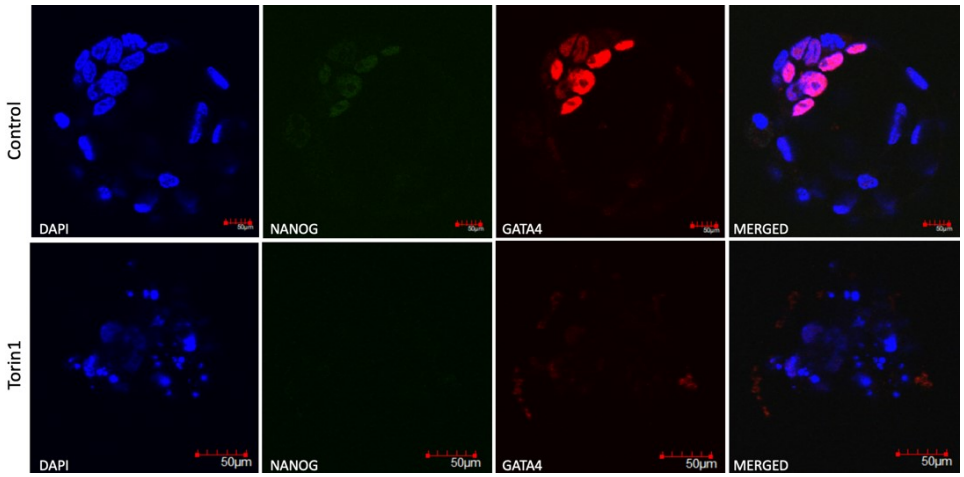

Fig.14: Immuno-fluorescent staining of DMSO control (upper n=12) and Torin1 (n=12) treated (from E3.5-E5.5) embryos (lower) for ICM lineage marker proteins. DAPI (blue), NANOG (green), GATA4 (red) – scale bar = 50µm.

Based on the immuno-fluorescent staining (Fig.14), it is clear that the prolonged Torin1 treatment (E3.5-E5.5) caused apoptosis of many cells compared to controls, suggesting the concentration of Torin1 used in this regime was cytotoxic, therefore precluding accurate cell counting.

4.6. The effect of mTOR downregulation using chemical inhibitor Torin1 from E3.5 to E4.5 on specification of EPI and PE in the maturing mouse blastocyst ICM, focused on GATA6 and NANOG proteins detection

We returned to assaying blastocyst lineage formation in the developmental window of E3.5-E4.5 under mTOR inhibition conditions. However, we assayed the expression of NANOG and GATA6 protein (Fig.15), to determine if Torin1treatment resulted in an increased population of unspecified ICM cells co-expressing both markers.

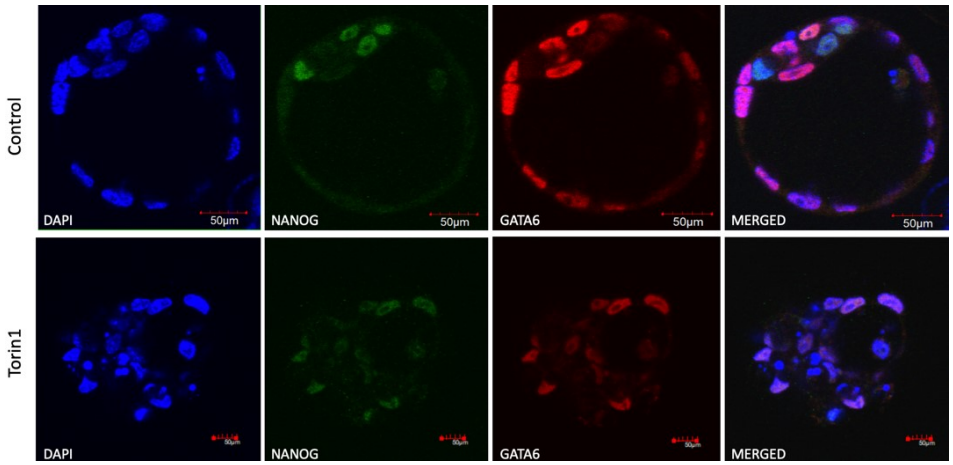

Fig.15: Immuno-fluorescent staining of DMSO control (upper n=15) and Torin1 (n=19) treated (from E3.5-E4.5) embryos(lower) for ICM lineage marker proteins. DAPI (blue), NANOG (green), GATA6 (red) – scale bar = 50µm.

The quantitated results of this experiment are shown in Figure 16. As before, Torin1 treatment caused a highly significant reduction in total cell numbers (averaging 39.3±3.5 cells compared to 64.9±3.1 cells in controls), in both inner (19.5±2.3 in Torin1 treated versus 23.4±2.5 cells in controls) and outer cell (19.8±4.6 in Torin1 treated versus 41.5±3.3 cells in controls) populations. Consistently the effect was more profound for outer cells. Similarly, there were significant reductions in ICM cells solely expressing NANOG (averaging 6.1±1.8 cells compared to 9.2±1.7 ICM cells in controls) and GATA6 (averaging 4.2±2 cells compared to 12.8±1.9 ICM cells in controls) protein, indicating both EPI and PrE derivation were impaired under mTOR inhibited conditions (plus there was a significant increase of apoptotic cells).

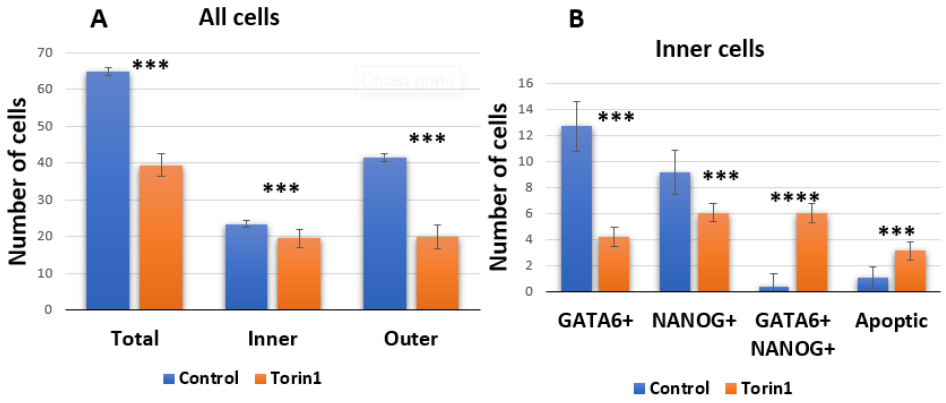

Fig.16: Statistical analysis of impact of mTOR downregulation on specification and segregation of EPI and PrE in the maturing mouse blastocyst.

Chart A: The average number of total, inner and outer cells in control group versus Torin1 treated.

Chart B: Quantification of ICM cells.

Most importantly, Torin1 treatment caused a significantly greater number of ICM cells, that were not fully committed to their fate (based on the co-expression of GATA6 and NANOG). Indeed, in control embryos uncommitted ICM cells were almost absent (0.4±1.1 versus 6.1±2.3 cells in Torin1 treated blastocysts). In addition, in Torin1 treated embryos there were more apoptic cells (3.2±1.1) than in the control group. Thus, whilst the mTOR inhibition phenotype is similar to AMPK activation it is not a complete phenocopy. This is because mTOR inhibition during mouse blastocyst maturation causes deficits in both EPI and PrE populations (with concomitant increases in unspecified cells, indicative of a diapause), but AMPK activation is more specifically associated with blocked PrE specification and differentiation and does not seem to effect EPI specification. However, it must be acknowledged that mTOR inhibition by Torin1 appeared much more cytotoxic (in our hands – possibly related to concentration) than AMPK activation by GSK621.

This is exemplified by the fact that even between E3.5-E4.5, Torin1 treatment could caused a degree of blastocyst cavity collapse (Fig.15).

5. Discussion

In this thesis a main aim was to investigate effect of AMPK activity on the specification of EPI and PrE blastocyst ICM cell lineages by E4.5 and E5.5. Moreover, to compare the observed results with mTOR inhibition to see if AMPK activation would provide a phenocopy (as observed by the expression of NANOG, GATA4, and GATA6). Indeed, Gahurova et al. (from our laboratory - *manuscript in preparation*) have recently discovered, that AMPK chemical activation completely phenocopies mTOR inhibition during 8- to 16-cell division. Indeed, it was also shown that mTOR is regulated by the AMPK pathway during the 8- to 16-cell transition (in regard to regulating the number of inner/founder ICM cells generated). In this work, our data suggest that mTOR activity can be regulated by the AMPK pathway, although it is thought AMPK is normally inactivated at this embryonic stage. However, it is currently not at all clear whether the AMPK pathway can play an active role during the later stages of embryonic preimplantation development and specifically during the segregation of EPI and PrE lineages in the blastocyst ICM. Here, we find that AMPK activation from E3.5 to the late blastocyst (E4.5) stage causes a highly significant decrease in the total number cells, that is reflected in both the ICM and also outer cell populations. In this regard, AMPK activation and mTOR inhibition phenotypes are somewhat similar (averaging approximately 40 total cells). Additionally, both AMPK activation and mTOR inhibition during this time cause significant decreases in the number of PrE cells, as determined by the sole expression of GATA4 or GATA6 protein, confirming impaired PrE differentiation. However, where the AMPK activation and mTOR inhibition phenotypes differ is in the number of cells solely expressing the

EPI marker protein NANOG. Whereas the number of NANOG alone expressing cells is not statistically different after AMPK activation, they are significantly reduced after mTOR inhibition. Thus, our data show a common impairment in PrE differentiation between AMPK activation and mTOR inhibition but a mTOR specific impairment of EPI specification. Interestingly, both treatments result in the increased incidence of unspecified/uncommitted ICM cells (defined by co-expression of NANOG and GATA6 markers) that in the case of AMPK activation most probably reflect impaired PrE progenitors and a mixture of both PrE and EPI progenitors after mTOR inhibition. In addition, AMPK activation and mTOR inhibition caused small but significant increases in cells co-expressing GATA4 and NANOG (also observed at E5.5 in AMPK activated blastocysts, and a cell population rarely observed in control treated blastocysts at E4.5 or E5.5), indicating some PrE progenitors had initiated PrE differentiation but under the pharmacological conditions could not downregulate the pluripotent NANOG marker. Hence, we conclude AMPK activation during mouse blastocyst maturation (E3.5-E4.5) specifically affects PrE cell fate specification and differentiation and is therefore not a simple phenocopy of mTOR inhibition (that has recently been shown to cause developmental diapause when provided to mouse blastocysts at the E3.5 stage (Bulut-Karslioglu, 2016)). Similarly to the reported AMPK activation results, pharmacological inhibition of p38-MAPK activity during mouse blastocyst maturation (E3.5-E4.5) also specifically disrupts ICM differentiation towards to the PrE lineage and has minimal effects on EPI cell differentiation (Thamodaran & Bruce, 2016). The observed PrE differentiation block is marked by a population of unspecified/uncommitted ICM cells (co-expressing NANOG and GATA6) indicating failed PrE

specification (Bora, Thamodaran, Šušor, & Bruce, 2019). These results suggest the p38-MAPK inhibition phenotype may consist of an activated AMPK component (Bora, et al., 2020). It would therefore be interesting to investigate if p38-MAPK inhibition PrE phenotypes could be improved by simultaneous inhibition of the AMPK pathway (*e.g.* using the small chemical compound Dorsomorphin (Zhou G, 2001)) as has recently been shown for similar concomitant mTOR inhibition (Bora, et al., 2020).

Compared to AMPK activation (E3.5-E4.5/E5.5), functional downregulation of mTOR was associated with extensive apoptotic cell death and collapsed blastocyst cavity defects. These phenotypes are further evidence that AMPK activation during mouse blastocyst maturation is not a simple phenocopy of mTOR inhibition. These data also indicate mTOR activity is essential during this embryonic window and that the AMPK pathway is most likely to be inactive during this time in unperturbed development. However, the more severe cell survival and blastocyst morphology phenotypes we observed here are not entirely consistent with other recent reports of mTOR inhibition during mouse blastocyst maturation (E3.5-E4.5) using Torin1 (Bora, et al., 2020) or INK128 (another compound that inactivated both mTORC1 and mTORC2 (Bulut-Karslioglu, 2016)). In these experiments embryos arrested their development with around 40-cells (*i.e.* a so-called developmental diapause) with very little cell death. The reason for this apparent incompatibility is not clear but may reflect the precise stage of embryonic development and timing of Torin1 application in our reported experiments being slightly later. Alternatively, it may reflect differences between Torin1 and INK128 inhibitors and/or concentrations used (although we used the same concentration of Torin1 as reported). If time would

have allowed, we could have revisited these questions in additional experiments. However, despite the cell survival and blastocyst morphology differences it is important to note that mTOR inhibition in our hands (in this thesis) similarly reported both EPI and PrE specification/differentiation phenotypes.

As well as integrating the AMPK pathway, mTOR regulates cell growth and metabolism and is a key player in many important cellular processes, responding to varied environmental inputs (Chung, Kuo, Crabtree, & Blenis, 1992). Indeed, the balance between cellular anabolism and catabolism in response to environmental conditions is controlled by mTOR. Downregulation of the mTOR pathway has characterised roles in cancer (Eng, Sehgal, & Vézina, 1984), diabetes and aging (Laplante & Sabatini, 2012). Thus, it stands to reason it must have crucial roles during embryonic development. Pluripotent stem cells are *in vitro* perpetuated cell line cultures derived from blastocyst ICM that are capable to give rise to all cell types; for this reason they represent a cornerstone of potential regenerative medicine (Nichols J, 2009). Pluripotency itself represents a transient state *in vivo* state, which last only 2-3 days around the time of blastocyst implantation, except in diapause embryos. Embryonic diapause is a reversible state of arrested development that can be induced as a response to changing environmental/nutritional conditions, however it is not really well understood (Fenelon JC, 2014). Recent studies show, that mTOR inhibition induces a reversible diapausing state in maturing mouse blastocysts (Bora, et al., 2020) and embryonic stem cell cultures (categorised by suppressed transcription and retention of pluripotency) (Bulut-Karslioglu, 2016). Furthermore, that the newly identified diapaused pluripotent stem cell state is very similar to the epiblast of diapaused blastocysts. These data implicate mTOR activity as a regulator of

developmental progression at or around the timing of implantation. Thus, these results are important in the context of regenerative medicine, assisted reproduction, cancer and metabolic disorders (Bulut-Karslioglu, 2016). Moreover, Murakami and colleagues have published a study detailing the genetic disruption of the *Mtor* gene by homologous recombination. They show that whilst mice which were heterozygous for mutant *Mtor* develop normally and are fertile, homozygous mutant exhibited disrupted cell proliferation in both embryonic and extraembryonic tissues that resulted in a lethal phenotype shortly after implantation. Additionally, even though homozygous blastocysts appeared normal, the inner cell mass and trophoblast did not proliferate (agreeing with the pharmacological mTOR inhibition data described above and elsewhere (Bora, et al., 2020), (Bulut-Karslioglu, 2016)) (Murakami, et al., 2004). Moreover, it is also not possible to establish embryonic stem cell lines from homozygous *Mtor* mutant blastocysts (Gangloff YG, 2004). Hence, mTOR acts to control proliferation and cell number in the early mouse embryo, thus, underlining its fundamental developmental importance. Additionally, experiments in which preimplantation mouse embryos were treated with Rapamycin (which inhibits only mTORC1) have shown reduced cell size. This study shows early embryonic cell cycle (proliferation) and regulation of cell size are separable processes and moreover, that active mTOR- and PI3K- dependent signals are required for preimplantation embryo blastomeres to grow to appropriate cell size (Fingar, Salama, Tsou, Harlow, & Blenis, 2002). Supported by our results, where mTOR inhibition also caused smaller cell size. The same study also demonstrated mTOR actively signals to downstream S6K1 and 4EBP1/elF4E to regulate cell size (Fingar, Salama, Tsou, Harlow, & Blenis, 2002). We would argue that in general our Torin1 based mTOR inhibition phenotypes (obtained after

inhibition between either E3.5-E4.5 or E3.5-E5.5) are consistent with the relevant published literature.

Rapamycin is often used as an inhibitor of mTOR, however it only inhibits mTORC1. For investigating the additional mTORC2 related role a novel small ATP-competitive molecular inhibitor, Torin1was developed. Torin1 inhibits both the mTORC1 and mTORC2 complexes. However, there is some suggestion that particular mTORC1 functions are more sensitive to Torin1 and could be more resistant to Rapamycin (Carson & Sabatini, 2009). It would therefore be wise to repeat our experiments using both Torin1 (as discussed above) and Rapamycin to take these factors into account when interpreting our data.

Maternal hyperglycaemia in diabetic mothers can cause oxidative and hypoxic stress in embryos, that can thus stimulate activation of the AMPK pathway; leading to disrupted embryonic gene expression and perturbed embryonic development (Lee, Lee, Yoon, Roh, & Kim, 2001). Additionally, there are thought to be many potential AMPK activators present in either the maternal diet or resulting from unintended side effects of various drug treatments (Bolnick, 2017). Hence, an appreciation of the maternal intake of potential AMPK activators, with respect to embryonic developmental stage, may be a future concern worth considering in terms of reproductive health. IFor example, in the context of this study the blastocyst maturation period and the derivation of the functioning extraembryonic PrE required to support post-implantation development (this could equally apply to the *in vitro* culture of human or *in vitro* assisted reproductive technologies, prior to their transfer back to recipient uteri). Alternatively, it has been shown that in mouse oocytes and embryos under stress is, phosphorylation, and hence activation, of AMPK is

beneficial (Wu Y, 2012). Bolnic *et al.* (Bolnick, 2017) have hypothesized that AMPK activation in oocytes and embryos that are not under pathogenic stimuli may be toxic. As referenced above, drugs like Metformin or Aspirin, plus some common dietary supplements, that exert their beneficial therapeutic effects via activation of AMPK activity (Puscheck EE, 2018), have been hypothesised to cause (in high doses) harmful effects on early human preimplantation embryos (Bolnick, 2017). Although primarily a drug for the treatment of diabetes, metformin, is also used as treatment for infertile women with polycystic ovarian syndrome (PCOS) (Palomba S, 2009). Again, given the data present in this thesis, it may be prudent to consider the doses of metformin used in treatment of diabetic women actively seeking pregnancy or PCOS patients, as excessive AMPK activation may be deleterious for any conceived embryos undergoing blastocyst maturation (specifically in terms of PrE specification and differentiation). Notably, the same concerns could be applicable to the use of the AICAR drug that, like Metformin, is frequently employed as a therapeutic AMPK activator (Calder, Edwards, Betts, & Watson, 2017). There is also evidence of arrested *in vitro* development of mouse 2-cell and blastocyst embryos cultured in the presence of various AMPK agonists and dietary supplements. It is thought this is because the stimulated AMPK pathway induces overall catabolism, thus decreasing ATP consuming anabolism which is a key process during preimplantation development (Fryer LG, 2002). Additionally, studies culturing mouse embryos in the presence of Aspirin and Metformin from the 2-cell or blastocyst stages describe decreased expression of OCT4 protein (although this sensitivity was greater from the 2-cell stage) (Bolnick, 2017). Hence collectively, these studies, plus the results of our own blastocyst maturation AMPK activation experiments, highlight the important balance in regulating the AMPK-mTOR pathway to

ensure germane cell growth, proliferation and appropriate lineage specification and differentiation. Moreover, they bring into renewed focus the possible multitude of extrinsic and intrinsic factors that can collectively influence these pathways and require further attention regarding our understanding of preimplantation development under normal, pathological or assisted reproductive conditions.

6. Conclusion

In conclusion, data from the experiments performed in this thesis support the hypothesis that the AMPK pathway is normally inactive during mouse blastocyst maturation and that in its active state, it mainly negatively affects the specification and differentiation of PrE and not EPI cells within the ICM. Furthermore, we found that functional mTOR downregulation also impact proliferation of the ICM, especially affecting the PrE but also causing reduced EPI cell numbers; and was associated with more severe cell survival defects and blastocyst cavity collapse. Despite the similarities of the observed AMPK activation and mTOR inhibition blastocyst maturation phenotypes, we conclude that they are not direct phenocopies of each other. Moreover, that the effect of AMPK activation is focused on PrE specification in a manner similar to previously described p38-MAPK inhibition during the same blastocyst maturation period; hence opening another potential research avenue to study the well described PrE deficits caused by p38-MAPK inhibition in the context of hypothesised activation of AMPK.

7. References

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8. Appendix

Supplementary tables 1

DMSO (CONTROL)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA4+_only	NANOG+_only	GATA4+ NANOG+	Apoptic
1	77	25	52	10	7	0	8
2	68	21	47	9	9	0	3
3	73	27	46	9	10	0	8
4	59	18	41	10	4	0	4
5	53	16	37	3	6	0	7
6	60	14	46	4	6	0	4
7	64	20	44	6	8	3	3
8	78	30	48	15	12	0	3
9	68	24	44	12	4	2	6
10	66	21	45	10	11	0	0
11	69	28	41	13	10	0	5
12	62	24	38	10	10	0	4
13	44	16	28	8	7	0	1
14	54	15	39	11	3	0	1
15	60	29	31	7	15	0	7
16	56	23	33	11	8	0	4
17	64	20	44	9	7	0	4
18	60	19	41	8	8	0	3
19	63	20	43	6	7	0	7
20	58	17	38	7	7	0	3
21	65	21	44	7	9	0	5
22	66	21	45	9	9	0	3
23	67	19	48	11	7	0	1
24	67	18	49	5	5	4	4
25	66	16	50	6	7	0	3
26	62	23	39	7	12	0	4
AVERAGE	**63,22**	**21,22**	**41,87**	**8,91**	**8,00**	**0,22**	**4,09**
SEM	**7,289297**	**4,36789**	**5,830556**	**2,788161788**	**2,727636339**	**1,017538508**	**2,144402**

GSK621 (from E3.5-E4.5)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA4+_only	NANOG+_only	GATA4+ NANOG+	Apoptic
1	26	12	14	0	8	0	4
2	27	15	12	0	9	0	6
3	43	19	10	2	7	4	6
4	44	19	25	0	3	8	8
5	52	22	30	3	9	4	6
6	51	18	33	3	6	6	3
7	38	11	27	3	4	2	2
8	35	13	27	3	5	1	4
9	19	11	8	3	4	0	4
10	45	11	34	0	8	0	3
11	35	13	22	0	9	0	4
12	37	16	21	4	8	2	2
13	38	21	17	1	13	1	6
14	40	23	17	2	16	2	3
15	32	13	19	2	4	2	5
16	28	5	23	0	8	0	4
17	36	9	27	0	7	0	5
18	40	16	24	2	10	0	4
19	33	13	20	3	8	0	2
20	38	16	22	2	9	2	3
21	36	20	16	2	9	2	7
22	40	15	25	1	7	2	5
23	38	19	19	5	10	0	4
24	34	13	21	4	4	1	4
25	36	14	22	1	6	0	7
AVERAGE	**36,84**	**15,08**	**21,4**	**1,84**	**7,64**	**1,56**	**4,44**
SEM	**7,323933**	**4,319722**	**6,557439**	**1,491084616**	**2,942221383**	**2,063169083**	**1,635033**
p-value	1,73E-17	1,37E-05	2,76E-16	2.224E-9	6,52E-01	1.95E-3	4.25E-1

Supplementary tables 1: Quantified cell lineage segregation and specification in individual blastocyst at E4.5 treated with GSK621 (AMPK activator) and control embryos (cultured with DMSO). In both tables are reported total number of cells, inner (ICM) and outer cells (TE) for each individual blastocyst (marked as "Total" in both tables). In second part of table (marked as "Inner") are reported numbers of cells expressing GATA4+ only (later PrE marker), NANOG+ only (EPI marker), GATA4+ NANOG+ co-expressing cells and apoptic cells in each individual blastocyst. The second supplementary table includes a statistical expression of the differences between control group and GSK621 treated embryos,accompanied by p-values results, from two tailed student´s t-test or Mann-Whitney test (based on data distribution). Statistical significance is provide, when p-value is less than 0.05 (*< 0.05, **< 0.005, *** < 0.0005.

Supplementary tables 2

DMSO (CONTROL)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA4+_only	NANOG+_only	GATA4+ NANOG+	Apoptic
1	115	31	84	10	18	0	3
2	84	14	70	4	5	2	3
3	76	7	69	4	2	0	1
4	90	18	72	8	10	0	0
5	120	19	101	14	4	1	0
6	87	20	67	7	10	0	3
7	80	11	69	2	3	2	4
8	95	26	69	13	10	0	3
9	82	14	68	2	6	2	4
10	87	19	68	8	9	0	2
11	111	17	94	10	5	0	2
12	90	24	66	12	10	0	2
13	119	31	88	13	14	0	4
14	72	12	60	2	2	2	6
15	81	26	55	13	11	0	2
16	116	32	84	19	12	0	1
17	93	24	69	14	10	0	0
18	86	20	66	11	6	0	3
AVERAGE	**93,56**	**20,28**	**73,28**	**9,22**	**8,17**	**0,50**	**2,39**
SEM	**15,14**	**7,03**	**11,65**	**4,80**	**4,22**	**0,83**	**1,57**

GSK621 (from E3.5-E5.5)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA4+_only	NANOG+_only	GATA4+ NANOG+	Apoptic
1	51	7	44	0	5	0	2
2	50	12	38	2	6	2	2
3	57	15	42	3	7	2	3
4	54	16	38	3	5	3	5
5	51	11	40	1	9	1	0
6	45	8	37	1	5	0	2
7	48	20	28	5	7	5	3
8	51	13	38	1	9	1	2
9	59	21	38	3	7	6	5
10	49	7	42	0	5	0	2
11	60	13	47	2	4	2	5
12	52	10	42	1	5	0	4
13	65	26	39	8	7	5	6
14	63	18	45	4	6	4	4
15	49	15	34	3	5	3	4
16	42	21	21	3	9	3	6
17	49	23	26	5	10	5	3
18	51	16	35	2	6	2	6
19	66	28	38	8	7	8	5
AVERAGE	**53,26**	**15,79**	**37,47**	**2,89**	**6,53**	**2,74**	**3,63**
SEM	**6,48**	**6,01**	**6,38**	**2,25**	**1,67**	**2,22**	**1,66**
p-value	2.15E-7	5.1E-3	2.08E-7	1.71E-4	2.31E-1	5.42E-4	2.92E-2

Supplementary tables 2: Quantified cell lineage segregation and specification in individual blastocyst at E5.5 treated with GSK621 (AMPK activator) and control embryos (cultured with DMSO). The first part of table report total number of cells, inner (ICM) and outer cells (TE) for each individual blastocyst (marked as "Total" in both tables). In second part of table (marked as "Inner") are reported numbers of cells expressing GATA4+ only (later PrE marker), NANOG+ only (EPI marker), GATA4+ NANOG+ co-expressing cells and apoptic cells in each individual blastocyst. Supplementary table includes a statistical expression of the differences between control group and GSK621 treated embryos, averaged date are accompanied by p-values, resulting from two tailed student´s t-test or Mann-Whitney test (based on data distribution). Statistical significance is provide, when p-value is less than 0.05 (*< 0.05, **< 0.005, *** < 0.0005).

Supplementary tables 3

DMSO (CONTROL)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA6+_only	NANOG+_only	GATA6+ NANOG+	Apoptic
1	51	18	33	13	2	0	3
2	69	25	44	13	10	0	2
3	48	20	28	9	8	0	3
4	67	29	38	16	12	0	1
5	69	29	40	15	11	0	3
6	70	27	43	13	10	1	3
7	60	28	32	13	13	0	2
8	68	28	40	14	12	0	2
9	65	24	41	13	7	1	3
10	70	24	46	15	7	1	1
11	67	29	38	15	12	0	2
12	71	25	46	13	9	0	3
13	76	25	51	13	9	1	2
14	72	26	46	14	10	0	2
15	62	24	38	12	10	2	0
AVERAGE	**65,67**	**25,40**	**40,27**	**13,40**	**9,47**	**0,40**	**2,13**
SEM	**7,38**	**3,12**	**5,90**	**1,58**	**2,65**	**0,61**	**0,88**

GSK621 (from E3.5-E4.5)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA6+_only	NANOG+_only	GATA6+ NANOG+	Apoptic
1	50	22	28	9	9	4	0
2	45	24	21	8	7	5	4
3	45	15	30	9	6	4	0
4	56	25	31	12	11	8	2
5	40	21	19	7	8	2	4
6	44	24	20	9	8	4	3
7	54	21	33	7	8	2	4
8	39	20	19	8	5	6	1
9	39	17	22	5	9	2	1
10	52	20	32	6	8	3	3
11	51	24	27	9	10	4	1
12	53	20	33	10	6	2	2
13	53	26	27	9	9	4	4
14	52	23	29	10	8	3	2
15	42	23	19	8	9	4	2
AVERAGE	**47,67**	**21,67**	**26**	**8,4**	**8,07**	**3,8**	**2,2**
SEM	**5,71**	**2,89**	**5,25**	**1,67**	**1,53**	**1,60**	**1,38**
p-value	5.17E-5	2.73E-3	2.23E-5	8.67E-6	9.82E-2	2.81E-6	9.31E-1

Supplementary tables 3: Quantified cell lineage segregation and specification in individual blastocyst at E4.5 treated with GSK621 (AMPK activator) and control embryos (cultured with DMSO). The first part of tables report number of total cells, inner (ICM) and outer cells (TE) for each individual blastocyst (marked as "Total" in both tables). In second part of tables (marked as "Inner") are reported numbers of cells expressing GATA6+ only (early PrE marker), NANOG+ only (EPI marker), GATA6+ NANOG+ co-expressing cells and

apoptic cells in each individual blastocyst. Supplementary table includes a statistical expression of the differences between control group and GSK621 treated embryos, averaged date are accompanied by p-values, resulting from two tailed student´s t-test or Mann-Whitney test (based on data distribution). Statistical significance is provide, when p-value is less than 0.05 (*< 0.05, **< 0.005, *** < 0.0005).

Supplementary tables 4

DMSO (CONTROL)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA4+_only	NANOG+_only	GATA4+ NANOG+	Apoptic
1	63	25	38	12	10	2	1
2	68	21	47	12	8	1	0
3	74	23	51	11	8	2	2
4	72	26	46	12	11	3	0
5	68	21	47	11	10	0	0
6	70	31	39	13	15	0	3
7	65	24	41	12	10	2	0
8	72	24	48	12	9	3	0
9	70	26	44	10	12	4	0
10	71	26	45	12	14	0	0
11	71	23	48	11	10	0	2
12	65	20	45	12	8	0	0
Average	**69,08**	**24,17**	**44,92**	**11,67**	**10,42**	**1,42**	**0,67**
SEM	**3,20**	**2,85**	**3,71**	**0,75**	**2,18**	**1,38**	**1,03**

Torin1 (from E3.5-E4.5)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA4+_only	NANOG+_only	GATA4+ NANOG+	Apoptic
1	34	23	11	6	8	5	4
2	38	14	24	4	2	2	6
3	43	17	26	4	6	4	3
4	40	22	18	7	9	4	2
5	42	20	22	8	5	4	3
6	48	18	30	5	5	4	4
7	50	22	30	7	9	4	2
8	43	19	24	6	9	2	2
9	35	11	24	3	0	0	8
10	49	25	24	7	11	5	2
11	38	22	16	8	12	2	0
Average	**41,82**	**19,36**	**22,64**	**5,91**	**6,91**	**3,27**	**3,27**
SEM	**5,22**	**3,96**	**5,47**	**1,62**	**3,53**	**1,48**	**2,09**
p-value	1,90E-12	4,23E-03	3,50E-10	4.14E-5	1,16E-02	1.14E-2	1.87E-3

Supplementary tables 4: Quantified cell lineage segregation and specification in individual blastocyst at E4.5 treated with Torin1 (mTOR inhibitor) and control embryos (cultured with DMSO). The first part of table report number of all cells, inner (ICM) and outer cells (TE) for each individual blastocyst

(marked as “Total” in both tables). In second part of table (marked as “Inner”) are reported numbers of cells expressing GATA4+ only (later PrE marker), NANOG+ only (EPI marker), GATA4+ NANOG+ co-expressing cells and apoptic cells in each individual blastocyst. Supplementary table includes a statistical expression of the differences between control group and Torin1 treated embryos, averaged date are accompanied by p-values, resulting from two tailed student´s t-test or Mann-Whitney test (based on data distribution). Statistical significance is provide, when p-value is less than 0.05 (*< 0.05, **< 0.005, *** < 0.0005).

Supplementary tables 5

DMSO (CONTROL)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA6+_only	NANOG+_only	GATA6+ NANOG+	Apoptic
1	64	22	42	12	8	2	0
2	64	26	38	14	10	0	2
3	66	26	40	15	11	0	0
4	65	23	42	10	12	0	1
5	69	24	45	13	9	0	2
5	65	18	47	9	7	0	2
6	67	27	40	14	12	0	1
7	71	24	47	13	9	0	2
8	62	20	42	10	8	0	2
9	69	26	43	16	9	0	1
10	67	21	46	13	7	0	1
11	62	26	36	14	12	0	0
12	60	21	39	12	8	0	1
13	64	24	40	11	9	4	0
14	63	24	39	13	9	0	2
15	60	22,00	38	15	7	0	0
Average	**64,88**	**23,38**	**41,50**	**12,75**	**9,19**	**0,38**	**1,06**
SEM	**3,08**	**2,47**	**3,26**	**1,92**	**1,70**	**1,05**	**0,83**

Torin1 (from E3.5-E4.5)							
TOTAL				INNER			
Embryo	All cells	Inner_all	Outer_all	GATA6+_only	NANOG+_only	GATA6+ NANOG	Apoptic
1	43	23	20	7	5	8	3
2	37	19	18	5	7	4	3
3	35	21	14	4	5	10	2
4	40	19	21	7	3	6	3
5	42	18	24	5	4	6	3
6	40	18	22	6	6	4	2
7	38	15	23	1	7	4	3
8	37	21	16	6	9	2	4
9	35	21	14	2	6	7	6
10	41	22	19	5	7	8	2
11	39	19	20	7	4	4	4
12	35	16	19	2	7	4	3
13	37	20	17	4	8	6	2
14	39	20	19	4	6	8	2
15	35	24	11	3	7	10	4
16	45	19	26	0	5	10	4
17	48	16	32	3	3	5	5
18	40	19	21	4	8	5	2
19	41	21	20	5	9	4	3
Average	**39,32**	**19,53**	**19,79**	**4,21**	**6,11**	**6,05**	**3,16**
SEM	**3,46**	**2,28**	**4,56**	**1,96**	**1,77**	**2,33**	**1,09**
p-value	2,05E-21	5,18E-05	1,12E-16	3,89E-14	1,53E-05	4.26E-7	5.44E-6

Supplementary tables 5: Quantified cell lineage segregation and specification in individual blastocyst at E4.5 treated with Torin1 (mTOR inhibitor) and control embryos (cultured with DMSO). The first part of table report total number of cells, inner (ICM) and outer cells (TE) for each individual blastocyst (marked as "Total" in both tables). In second part of table (marked as "Inner") are reported numbers of cells expressing GATA6+ only (early PrE marker), NANOG+ only (EPI marker), GATA6+ NANOG+ co-expressing cells and apoptic cells in each individual blastocyst. Supplementary table includes a statistical expression of the differences between control group and Torin1 treated embryos, averaged date are accompanied by p-values, resulting from two tailed student´s t-test or Mann-Whitney test (based on data distribution). Statistical significance is provide, when p-value is less than 0.05 (*< 0.05, **< 0.005, *** < 0.0005).