Examining the regulation of the first lineage decision in the bovine pre-implantation embryo

Thesis submitted in partial fulfilment of the requirements for the degree of Master of Biomedical Science

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Abstract

Early embryogenesis is the crucial stage of development that results in the distinction of cell lineages with varying developmental potentials. The first lineage decision involves the transition from totipotent cells into two tissues: the inner cell mass and the trophoblast. Knowledge of how lineage is regulated is important for the agricultural, clinical and therapeutic industries that rely on artificial reproductive technology to progress. However, our current understanding of pre-implantation embryonic development is heavily based on the mouse model. Species-specific regulation of early embryogenesis is an important consideration that has not yet been thoroughly investigated. Cattle provide an opportunity for a new model of embryonic development that will not only serve the agricultural industry but provide new insights that will help establish bovine embryonic stem cells.

In this study, I have established a timeline of protein expression during bovine embryonic development during the first 7 days in *in-vitro* culture. The results show that compaction and polarisation events in cattle are not only delayed compared to the mouse but asynchronous. The establishment of distinct transcription profiles is also delayed in the bovine embryo, suggesting a lack of lineage commitment at the early blastocyst stages. Contrary to the mouse, pluripotency regulator, SOX2, and trophoblast marker GATA3 are not regulated by the YAP-TEAD4 transcriptional complex in cattle, shown by manipulating YAP activity using Verteporfin and LPA. It has also been demonstrated through further functional analyses using Y27632 and Go6983 that the apical domain, responsible for regulating Hippo pathway activity in mice, is not necessary for lineage specification in cattle.

Instead, we propose that transduction of mechanical cues via YAP operates in parallel to the Hippo pathway to establish lineage-specific transcription profiles. The urgency of the mouse blastocyst to implant has meant the species have evolved ways to utilise available signaling machinery to rapidly commit cells to trophoblast lineage. Cattle, who have a much longer pre-implantation period, coordinate information from various signaling inputs to progressively establish lineage commitment. This coordination has created a functional redundancy of the Hippo pathway in the first lineage decision.

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AMOT	Angiomotin
BSA	Bovine serum albumin
CDX2	Caudal type homeobox 2
CI	Confidence interval
ESC	Embryonic stem cells
ESOF	Early synthetic oviduct fluid
F-actin	Filamentous actin
FCS	Fetal calf serum
GATA3	GATA binding protein 3
GTPase	GTP binding protein
HSOF	Hepes synthetic oviduct fluid
ICM	Inner cell mass
IVC	In vitro culture
IVF	In vitro fertilisation
IVM	In vitro maturation
IVP	In vitro production
LATS ½	Large tumor suppressor kinase 1 and 2
LPA	Lysophosphatidic acid
LSOF	Late synthetic oviduct fluid
MFI	Mean fluorescence intensity
NANOG	NANOG homeobox
NF2	Neurofibromatosis type 2
OCT4	Octamer-binding transcription factor 4
PARD6B	Partitioning defective 6 homolog beta
PBS	Phosphate-buffered saline
PKC	Phosphokinase C
PLC	Phospholipase C
RCF	Relative centrifugal force
ROCK	Rho-associated protein kinase

SOX2	SRY-box transcription factor 2
TAZ	Transcriptional co-activator with PDZ binding motif
TB	Trophoblast
TEAD4	TEA Domain transcription factor 4
YAP	Yes1 associated transcriptional regulator
ZO-1a+	Tight junction protein
ZO-2	Tight junction protein

Chapter 1: Introduction

The mammalian embryo begins as a single totipotent cell that assembles itself through continuous cell cleavage events, during which distinct cell lineages emerge from self-directed gene expression programs. I will discuss the regulation of these lineage-specific gene expression profiles within the mouse pre-implantation embryo and compare this to what is so far known about the developmental events in cattle. Early embryogenesis has applications in a variety of fields: agriculture, human fertility and therapeutic medicine. Moreover, increasing our understanding of embryogenesis will enable the progression of the artificial reproductive technology these industries rely on.

1.1 Relevance of early embryogenesis

1.1.1 Agricultural applications

The New Zealand dairy export industry has grown in value over the last 30 years from a $2 billion to a near-$20 billion dollar industry in 2020 (DCANZ, 2020). Reproduction of dairy cows is most commonly carried out by artificial insemination which provides the ability to select genetically tested high-quality sperm. Although this form of reproduction in modern high-producing dairy cows leads to an 88% rate of fertilisation, the percentage of live calves born is only 56% (Diskin, Parr, & Morris, 2011). Embryo mortality rates are mostly attributed to death occurring before implantation, as early as day 7 in several studies that revealed abnormal embryos at this stage of development (Cerri, Juchem, et al., 2009; Cerri, Rutigliano, Chebel, & Santos, 2009; Wiebold, 1988). This loss of viable embryos comes at an estimated cost of $500 million to $1 billion per year in New Zealand (DairyNZ, 2016). To increase calving rates and decrease this financial burden, improvements must be made to the artificial reproduction technology used in the dairy industry. Knowledge of pre-implantation embryonic development is limited, with a lot of what we do “know” being based on the mouse model that has been extensively researched over the years. It is becoming more important to research the species-specific signaling pathways responsible for development, to get a more general and comprehensive understanding of embryonic development as a whole.

1.1.2 Clinical applications

In vitro production (IVP) is a biotechnology procedure that combines three methodologies: *in vitro* maturation (IVM), *in vitro* fertilisation (IVF), and *in vitro* culture (IVC), to produce embryos within a laboratory. The growth of embryos in a culture dish requires specific conditions that have been developed to closely mimic the *in vivo* environment. Media composition has been finely tuned over decades, to establish nutrient-dense environments that support oocyte and embryo development (Brinster, 1965b). The pH of the media must be maintained at physiological conditions using buffers and specific CO_2 concentrations that allow procedures to be carried out both on the bench without fluctuations occurring, and within incubators for extended culture periods (Brinster, 1965a; Swain, 2010). Clinics can also incorporate time-lapse technology to allow continuous observation of embryos during culture.

IVM involves extracting the oocyte from the follicle without having applied exogenous hormone supplements that stimulate maturation *in vivo* (Edwards, 1965; Pincus & Enzmann, 1935). Oocyte culture media was developed to replicate the follicular environment from which the oocytes are derived (Coticchio et al., 2015; R. Li & Albertini, 2013). Cumulus cells remain attached to the oocyte during nuclear and cytoplasmic maturation to ensure the cumulus-oocyte complex maintains its endogenous communication to optimise developmental competency. Bi-directional communication between the oocyte and surrounding cumulus cells ensures cumulus cell expansion and differentiation, both of which contribute to the survival and fertility of the oocyte (Gilchrist, Lane, & Thompson, 2008; Hussein, Thompson, & Gilchrist, 2006). Due to removal of the cumulus-oocyte complex from the follicle, the media must provide not only the nutrients that would compose the fluid within the follicular antrum but also the secretory factors that would be relayed from the granulosa cells and theca cells of the developing follicle (Hussein et al., 2006).

IVF of the matured oocyte by sperm occurs following approximately 24 hr of oocyte maturation culture. Oocytes are transferred into a new medium containing many nutrients, vitamins and growth factors that represent the oviduct fluid in which the oocyte would naturally be fertilised (S. Li & Winuthayanon, 2017). The quality of IVF media has greatly improved since its commercialisation, a process that has increased the standard and batch-batch consistency compared to previously used “in-house” medias (Quinn, 2004). Sperm samples are prepared

using a centrifuge and density gradient to maximise the concentration of live, mobile sperm within the sample (Ricci et al., 2009). This stage of IVP is considered day 0. Culture media, such as early synthetic oviduct fluid (ESOF), is used from culture day 1 until day 5. Human IVP involves selecting and transferring a day 5 blastocyst into the uterus for implantation following its previous culture in the lab. Selection is based either purely on morphological characteristics such as trophoblast cell numbers and inner cell mass density and size, or selection is based on pre-implantation genetic testing results (Verlinsky et al., 2005).

Human artificial reproductive technologies used for intrauterine insemination, IVF and intracytoplasmic sperm injections are becoming increasingly common tools for people addressing infertility. Despite rates of live births having improved to 40-50% per embryo transfer in women up to the age of 35 in recent years (FertilityAssociates, 2018), the technology is limited by what is currently known about embryonic development. Due to ethical restrictions, research into human-specific embryonic development is limited, and therefore, much of the information used for technology and media development is based on domestic animal research, especially the mouse (Harper, Cristina Magli, Lundin, Barratt, & Brison, 2012). The potential to use cattle as a model of human development has arisen in order to explore the conservation of developmental strategies between species. This thesis will provide further detail of cattle pre-implantation embryonic development that could be used to improve the current technology and its rate of success in both cattle and humans.

1.1.3 Therapeutic applications

Stem cells are a valuable biological resource with growing applications in research, therapy, and regenerative medicine (Wobus & Boheler, 2005). Embryonic stem cells (ESCs) are derived from the embryo at a stage when cells are pluripotent and can then be cultured and directed into the desired lineage (Rippon & Bishop, 2004). Successful establishment of ESCs requires knowledge of what regulates pluripotency and differentiation within the embryo of that species naturally, to then be able to manipulate the culture medium in which they are grown (Lorthongpanich, Yang, Piotrowska-Nitsche, Parnpai, & Chan, 2008). It is because of a lack of this knowledge that bovine ESCs have not yet been established in a naïve state. Culture media supplemented with the same differentiation inhibitors and pluripotency promoters, as those used in mouse ESC culture have failed, providing evidence of the differing regulation strategies

between species (Maruotti et al., 2012). Research into the signaling pathways involved in the development of bovine embryos at these very early stages will help shed light on the media supplements needed to maintain the pluripotency required for the successful culture of naïve bovine ESCs.

1.2 Overview of mouse pre-implantation embryonic development

Mouse embryonic development is the main model of early embryogenesis due to having been extensively researched. I will first discuss what is known about the pre-implantation development of the mouse embryo before beginning my research into the similarities and differences of bovine embryonic development. Fertilisation of an oocyte by sperm causes the zygote to initiate mitosis and exclude the extra copy of DNA as a polar body. The following three cleavage divisions take the totipotent zygote from a single cell to the 8 cell embryo, at which stage the blastomeres (individual cells comprising the embryo) are considered morphologically equivalent (Chazaud & Yamanaka, 2016; Kelly, 1977). It is during the 8 cell stage that the mouse embryo is termed a morula and inter- and intra-cellular asymmetries develop (Figure 1).

The 8 cell morula compacts as a result of increased cell-cell adhesions caused by the activation and increase in expression of E-cadherin (Cui et al., 2007; Fleming, Sheth, & Fesenko, 2001). This calcium-dependent event involves blastomeres flattening their basolateral membranes against one another (Shirayoshi, Okada, & Takeichi, 1983) (Figure 1). E-cadherin is the transmembrane component of the adherens junctions linking neighbouring cells and their actin cytoskeletons and is required for the compaction process. Both laser ablation and molecular inhibition of E-cadherin and its intracellular linker, β-catenin, result in the failure of embryos to compact (Fierro-González, White, Silva, & Plachta, 2013). Timing of compaction at the 8 cell stage coincides with progressive polarisation along the radial axis (Reeve & Ziomek, 1981; Zhu, Leung, Shahbazi, & Zernicka-Goetz, 2017).

The next morphological change is the formation of a fluid-filled cavity within the embryo which is now termed a blastocyst. Epithelial differentiation of outer cells and the formation of tight junctions provides a seal for the fluid being pumped into the centre of the embryo with the

help of Na^+/K^+ ion pumps located in the outer cells (Eckert & Fleming, 2008). These outer, epithelial cells are called the trophoblast (TB), and the inner cells offset to one side of the embryo by the blastocyst cavity is called the inner cell mass (ICM) (Figure 1).

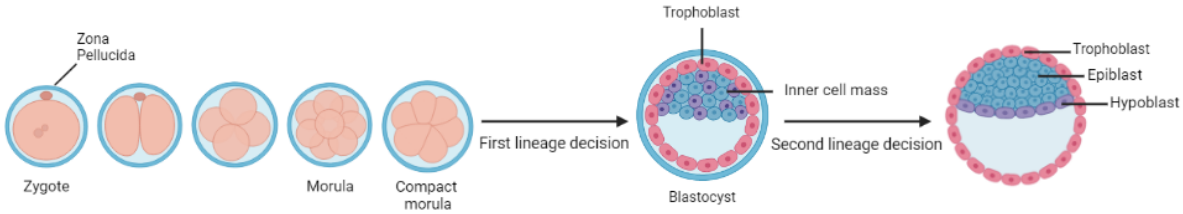

Figure 1 Mouse preimplantation embryonic development. *Developmental series of early mouse embryogenesis. The zone pellucida surrounds the zygote that has resumed mitosis and excluded a polar body. Cleavage stages leading to the morula and its compaction are depicted. The first lineage decision results in the blastocyst structure, consisting of trophoblast cells encasing the inner cell mass and fluid filled blastocyst cavity. The blastocyst then hatches from the zona pellucida and the second lineage decision results in the ICM dividing into the epiblast and hypoblast.*

The mammalian preimplantation embryo undergoes two consecutive lineage divergences during the development of the blastocyst which leads to the trophoblast, hypoblast, and epiblast lineages. The first lineage decision of the embryo determines the contribution of blastomeres to the ICM and the TB (Chazaud & Yamanaka, 2016). The second lineage decision occurs only within the ICM, when a subpopulation of cells differentiate to form the hypoblast which develops the yolk sac, leaving only the epiblast to form embryonic structures (Nichols, Silva, Roode, & Smith, 2009).

1.2.1 The trophoblast lineage

The epithelial differentiation of outer cells and the formation of tight junctions during blastocyst formation, coincides with the formation of the TB lineage (Eckert & Fleming, 2008). This population of TB cells leads to the formation of the cytotrophoblasts and syncytiotrophoblasts needed for the embryo to implant into the maternal endometrium (Malassiné, Frendo, & Evain-Brion, 2003). This need for specification of extraembryonic cells, capable of invading the endometrium, is the motivation behind establishing the TB lineage. Timing of blastocyst implantation varies greatly between species, with mice beginning to implant at approximately day 5, humans day 7, and cattle day 19 (Lonergan & Forde, 2014; Rashid, Lalitkumar, Lalitkumar, & Gemzell-Danielsson, 2011; Wang & Dey, 2006). The urgency

of cells committing to the TB lineage likely varies between different species because of the differing lengths of their pre-implantation periods. Following implantation, eutherian mammals develop a placenta from this same population of TB cells in order to sustain life while in the womb (Carter & Mess, 2007).

1.2.2 The inner cell mass

Unlike the TB, the ICM remains pluripotent, giving its cells the ability to develop into any embryonic structure. Following the second lineage decision, the epiblast remains pluripotent and is capable of undergoing gastrulation. Gastrulation is the re-organisation of the epiblast to form the three germ layers that constitute all embryonic structures: endoderm, mesoderm and ectoderm. Development of the cardiovascular, neurological, skeletal, and musculature systems needed for life continues throughout the species-specific gestation period. The gestation period of mice is approximately 22 days which is immensely shorter than that of humans and cattle who share a similar ~280 day period.

The mechanisms that enable blastomeres to self-organise into the TB or ICM lineage and form these complex embryonic structures have been studied extensively in mice. I will now discuss what is known about the regulation of early mouse embryogenesis during the development of these two distinct cell populations.

1.3 Transcription factors are integral to the first lineage decision

The goal of early embryonic development is to create distinct cell identities that are achieved by establishing lineage-specific transcriptional networks. These transcription profiles regulate the downstream development into the corresponding lineage specific tissue. At the core of these transcription profiles lie key transcription factors such as OCT4, SOX2 and NANOG for the pluripotent ICM; and CDX2 and GATA3 for the TB. Research into the timing of lineage commitment can be carried out using these lineage-specific transcription factors as markers of cell fate. Not only expression levels but the specific location and timing of expression provide insights into the mechanisms operating within blastomeres to differentiate into TB or ICM.

Studies involving gene knock-outs and the manipulation of signaling pathway activity have begun to map out the contribution of these transcription factors to a cell's lineage

commitment. However, whether the role of these transcription factors in regulating lineage decisions is the same in different species, is yet to be seen. In the following sections I will elaborate on these lineage specific transcriptions factors and how their spatial and temporal expression is regulated to form the TB and ICM lineages in the mouse embryo.

1.3.1 TB marker CDX2

CDX2 is a TB-specific transcription factor that is required to maintain TB integrity during blastocyst development and implantation in the mouse (Bou et al., 2017; Strumpf et al., 2005). Knock-out of CDX2 in the mouse embryo results in the blastocyst losing its epithelial integrity and cells undergoing apoptosis (Strumpf et al., 2005). CDX2 is therefore not required for TB formation but is necessary for maintenance. Expression of CDX2 begins at the 8 cell stage due to the activity of Tea Domain 4 (TEAD4), a transcription factor that is expressed ubiquitously within the embryo (Hirate, Cockburn, Rossant, & Sasaki, 2012; Nishioka et al., 2009; Ralston et al., 2010). Regulation of TEAD4 will be discussed in a later section. At the late 16 cell stage, a mosaic pattern of CDX2 expression emerges with increased levels in outer cells (Ralston & Rossant, 2008). This restriction of CDX2 to outer cells can be explained by its asymmetrical distribution within blastomeres at the 8 cell stage. An apical targeting region in the CDX2 mRNA transcript directs enrichment to the polarised, contact-free surface of outer cells. Following an asymmetric division, this anchoring of CDX2 to the apical domain results in the inheritance of the transcription factor by polar, TB-fated daughter cells (Skamagki, Wicher, Jedrusik, Ganguly, & Zernicka-Goetz, 2013).

Localised expression of CDX2 coincides with the establishment and commitment of cells to the TB lineage due to a mutually antagonistic relationship between the lineage-specific transcription factors CDX2 and OCT4 (Niwa et al., 2005). CDX2 down-regulates pluripotent factors OCT4 and Nanog to stabilise the TB lineage, a relationship not seen in cattle (Berg et al., 2011). The discovery of CDX2 binding sites within cis-regulatory elements of OCT4 and Nanog provides evidence of how CDX2 directly binds to repress OCT4 and Nanog transcription in outer cells (Huang et al., 2017). The continued repression of OCT4 by CDX2 is important to maintain the TB lineage, as OCT4 expression has been shown to reprogram differentiated ESCs back to a pluripotent state (T. Wu et al., 2011). Knock-down of CDX2 in mice results in ectopic

OCT4 and Nanog expression in outer cells (Strumpf et al., 2005). It is this ectopic expression, and lack of TB lineage commitment that leads to the loss of TB integrity and failure to implant.

CDX2 expression in the bovine blastocyst is low and not completely restricted to the TB (Berg et al., 2011; Ozawa et al., 2012). Knock-down of CDX2 resulted in normal blastocyst development, indicating its role in cattle, like in mice, does not include the initial direction of cells into the TB lineage (Berg et al., 2011; Goissis & Cibelli, 2014). However, unlike mice whose epithelial integrity is eventually lost without the transcription factor, the bovine embryo develops well into gastrulation stages without CDX2 (Berg et al., 2011). Gene expression analyses also indicate OCT4 is not regulated by CDX2, as it is in mice, due to no changes in OCT4 expression being found in CDX2 knock-down embryos (Goissis & Cibelli, 2014; Sakurai et al., 2016; X. Wu et al., 2016).

1.3.2 TB marker GATA3

GATA3 is a transcription factor that is exclusively expressed alongside CDX2 in the TB of the mouse blastocyst (Ralston et al., 2010). This is because expression of GATA3 is controlled by the same TEAD4 transcription factor as CDX2 (Ralston et al., 2010). Genetic downregulation of GATA3 has severe effects on embryonic development by inhibiting the embryo's ability to transition from the morula to blastocyst stage (Home et al., 2009). This suggests a role for GATA3 in the epithelial differentiation of TB cells needed for blastocyst cavity formation, preceding the need of CDX2 whose role is more closely linked to the maintenance of the TB epithelium. Using a mouse trophoblast stem cell model, GATA3 was shown to play a significant role in the regulation of CDX2 through a direct binding motif in the CDX2 enhancer (Home et al., 2009).

Unlike CDX2, the upregulation of GATA3 gene expression within the TB of the blastocyst is seen in both mice and cattle (Ozawa et al., 2012; Ralston et al., 2010). The regulative GATA3-CDX2 relationship seen in mice may in fact be reciprocal due to siRNA knock-down of CDX2 in the bovine blastocyst causing a significant decrease in GATA3 mRNA (X. Wu et al., 2016).

1.3.3 ICM marker SOX2

While the TB differentiates into a polarised epithelium, the ICM remains pluripotent. SOX2 is one of four transcription factors required to direct somatic fibroblasts into induced pluripotent stem cells (Narayan, Bryant, Shah, Berrozpe, & Ptashne, 2017; Yakubov, Rechavi, Rozenblatt, & Givol, 2010). It then comes as no surprise that SOX2 expression within the mouse blastocyst is exclusive to the ICM. SOX2 is expressed in all cells at the 8 cell stage before becoming enriched in inner cell mass progenitors at the 16 cell stage. This restriction of SOX2 to inner cells at the 16 cell stage, makes it the earliest marker of pluripotency (Gerri et al., 2020; Wicklow et al., 2014). SOX2 is directly down-regulated by the transcription factor TEAD4 in the TB of the mouse blastocyst, in a CDX2-independent manner (Wicklow et al., 2014).

Localised SOX2 expression is not unique to mice, with restriction to the ICM also seen in pigs (Shichao Liu et al., 2015). However, both humans and cattle show some expression in trophoblast cells of the late blastocyst (Gerri et al., 2020). Using various inhibitors and activators of the hippo pathway which controls TEAD4 activity, I aim to analyse whether the regulation of SOX2 by TEAD4 is conserved in cattle.

1.3.4 ICM marker OCT4

OCT4, a member of the POU transcription factor family, is involved in maintaining pluripotency within the ICM of the mouse blastocyst (Narayan et al., 2017). The importance of OCT4 in regulating the pluripotency network is highlighted by its role in producing mouse ESCs (Niwa, Miyazaki, & Smith, 2000; T. Wu et al., 2011). However, OCT4 expression in mice is not restricted to inner cells until after initial blastocyst formation (Dietrich & Hiiragi, 2007). Restriction of OCT4 to the ICM of the blastocyst seems exclusive to the mouse, with ubiquitous expression of OCT4 found in pig, human and cattle embryos (Cauffman, Van de Velde, Liebaers, & Van Steirteghem, 2004; Kirchhof et al., 2000; Shichao Liu et al., 2015). Restriction of OCT4 to the ICM in mice, could be due to the acquisition of a Tcfap2 binding site in the OCT4 enhancer, and the negative regulation by CDX2 within the TB (Berg et al., 2011).

Differences in the temporal and spatial expression of these embryonic lineage markers between species suggests different regulation and differentiation strategies have evolved. To better understand the first lineage decision of mammalian preimplantation embryos, the

timing and localisation of transcription factor expression needs to be better understood in species other than the mouse.

1.4 Morphological features and their involvement in first lineage decision

Polarity, position, and mechanical forces all may influence cellular signaling pathways that control the expression of lineage-specific transcription factors. Two models have been used to describe the first lineage decision in mice: the inside-outside model, and the polarity model (Sasaki, 2010). The inside-outside model hypothesises that the different environments that inside and outside cells of the morula are exposed to dictate the future lineage of the cell (Tarkowski & Wróblewska, 1967). The polarity model posits that polar cells, located at the outside of the morula, have an apical domain that causes heterogeneity of lineage determinants between daughter cells following an asymmetric division (Johnson & Ziomek, 1981). A combination of these two hypotheses may more accurately describe the first lineage decision due to the effect of position on the polarisation of cells (Korotkevich et al., 2017). This idea is supported by the identification of Hippo pathway involvement in the first lineage decision by combining both polarity and positional information (Nishioka et al., 2009). More recently, it is debated if it is at the 8-16 cell stage that cells first determine their fate, or whether genetic biases are generated earlier in development (Torres-Padilla, Parfitt, Kouzarides, & Zernicka-Goetz, 2007).

1.4.1 Establishing polarity

Polarisation of blastomeres creates distinct apical and basolateral domains, a necessary characteristic of epithelial cells (Martin-Belmonte & Perez-Moreno, 2012). Timing of polarisation in mouse blastomeres coincides with compaction at the 8 cell stage due to the basolateral regions becoming enriched in cell adhesion proteins, leaving the apical region contact-free (Fleming et al., 2001). EZRIN, a member of the Ezrin-Radixin-Moesin (ERM) family, is an apically localised scaffold protein that links membrane proteins to the cytoskeleton (Tsukita, Yonemura, & Tsukita, 1997). Recruitment of Ezrin to the apical domain is the first sign of polarisation in mice (Louvet, Aghion, Santa-Maria, Mangeat, & Maro, 1996; Louvet-Vallée, Dard, Santa-Maria, Aghion, & Maro, 2001). Polarisation can also be visualised using fluorescent

surface ligand binding which shows clustering of microvilli to the contact-free region at the 8 cell stage (Reeve & Ziomek, 1981).

1.4.2 Cleavage plane affects the inheritance of polarity

Polarised cells have distinct apical and basolateral regions. Therefore, cleavage plane determines which cells following the 8-16 cell division will inherit the apical domain and the proteins associated with it. Asymmetric, or differentiative, cleavage occurs perpendicular to the apical-basal axis resulting in one polar and one apolar daughter cell (Figure 2). The polar daughter cell remains in the outer position and its apolar sister cell is internalised. This allocation of apolar cells to the inner embryo can be shown in a reduced model. Dissociated blastomeres from an 8 cell embryo undergo an asymmetric division resulting in the polar daughter cell engulfing its apolar sister (Korotkevich et al., 2017). Symmetrical, or conservative, divisions occur along the apical-basal axis and form two polar daughter cells (Figure 2). The orientation of the mitotic spindle and the location of the centrosome control cleavage plane and therefore, influence the polarity of progeny. Regulation of the localisation of the spindle pole to the subapical domain is not well understood, however, it has been found to be recruited by the apical domain (Hebert, DuBoff, Casaletto, Gladden, & McClatchey, 2012; Korotkevich et al., 2017).

If the apical domain of polarised cells was the sole driving force of cleavage plane, all blastomeres at the 8 cell stage would divide asymmetrically. Instead, observations by live imaging show only a small portion of blastomeres divide asymmetrically (Anani, Bhat, Honma-Yamanaka, Krawchuk, & Yamanaka, 2014), indicating a regulatory pathway must enable some cells to divide symmetrically. Hertwig's rule states that cell divisions occur along the longest axis of a cell and therefore, cleavage symmetry depends on cell shape (Dumollard et al., 2017). Following compaction, cell shape asymmetry promotes symmetric divisions (Niwayama et al., 2019), a finding consistent with a higher ratio of polar TB to apolar ICM cells at the 16 cell stage (Anani et al., 2014).

1.4.3 Differences in contractility generated by the apical domain

Mechanical forces have been proposed to influence the first lineage decision by creating biases based on the ability of a cell to contract (Maitre et al., 2016; Samarage et al., 2015). The apical

domain formed during polarisation is thought to reduce cell contractility, therefore, leading polar cells to remain on the outer surface of the embryo (Maitre et al., 2016). This is likely due to contractile proteins: filamentous actin (F-actin) and myosin II, being excluded from the apical domain into the surrounding region where they form F-actin rings (Zhu et al., 2017). Cells that do not inherit the apical domain following an asymmetric division, or inherit only a small portion of the apical domain, have increased contractility and are able to internalise into the centre of the embryo (Maitre et al., 2016) (Figure 2).

In mice, cell sorting has been observed in which some apolar cells end up in the outer position before a correction mechanism internalises them (Anani et al., 2014). Samarage et al. (2015) found that 60.6% of inner cells contributed to the inner cell mass despite resulting from an asymmetric division. This process of cell sorting is likely due to the apolar cells having a higher contractility compared to their polar neighbours (Maitre et al., 2016) and is likely an important regulator of the initial allocation of inner cells.

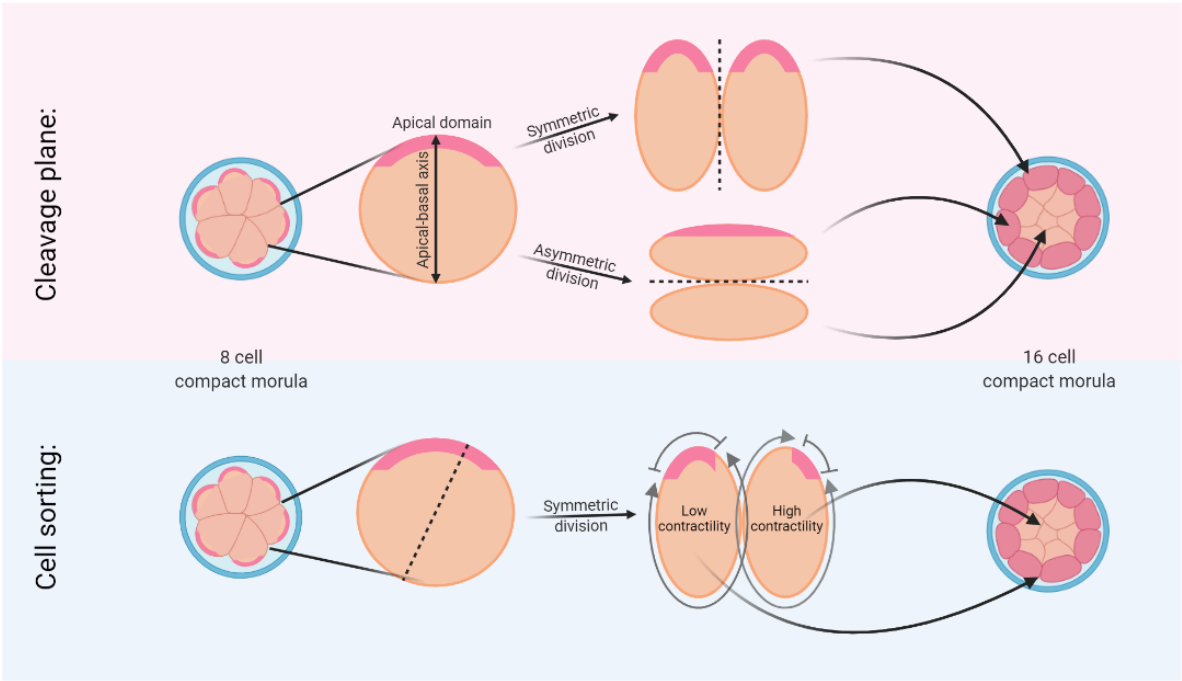

Figure 2 Allocation of cells to inner and outer populations. *Cleavage plane controls the inheritance of polarity. Cell sorting further regulates the position of these cells within the morula. Asymmetric divisions result in one polar daughter cell contributing to the outside of the morula, and one apolar daughter cell that contributes to the inside morula. Symmetric divisions result in two polar daughter cells. However, often both do not inherit the same size portion of the rigid apical domain. The cell that inherits the smaller portion is exposed*

to less of its dampening effects on contractility and is able to internalise into the centre of the morula. The cell with the larger apical domain remains in the outer position due to having low contractility.

1.4.4 Building the apical domain

Polarisation of outer cells results in the cortical enrichment of both polarity regulators and cytoskeletal proteins. The cytoskeleton begins to polarise when myosin II and F-actin accumulate at the contact-free region of outer cells 8 cell stage, in mice (Zhu et al., 2017). This recruitment of myosin II and F-actin is the result of the Phospholipase C (PLC)-dependent Protein kinase C (PKC) activation of Rho GTPase, RhoA (Zhu et al., 2017). The role of Rho-like GTPases in cell adhesion and cytoskeletal organisation has been shown to regulate polarity in a variety of cell types (Angel, Vicente-Manzanares, Tejedor, Serrador, & Sánchez-Madrid, 1999; Clayton, Hall, & Johnson, 1999).

Recruitment of these cytoskeletal components is followed by the restriction of partitioning defective 6 beta (PARD6B) to the apical domain (Zhu et al., 2017). The exclusion of F-actin and myosin II into the surrounding region where they form the well-documented F-actin rings follows PARD6B recruitment (Zenker et al., 2018; Zhu et al., 2017). The apical domain is then considered the portion of the cell within these F-actin rings where polarity regulating networks become established. PKC is consistently located at the apical domain in mice, cattle, and human morula stage blastomeres (Gerri et al., 2020). Indirect inhibition of PKC using a PLC inhibitor prevents PARD6B polarisation and the expression of CDX2 (Zhu et al., 2017). This result indicates that PKC is required for the formation of the apical domain and has downstream effects on lineage marker expression. Inhibition of the PKC isotype, atypical PKC (αPKC), showed the loss of both cortical actin and CDX2 expression (Skamagki et al., 2013). This provides further evidence of the apical domains role in directing the TB lineage.

1.4.5 Cell adhesion is regulated by the apical domain

Tight junctions form during the epithelial differentiation of outer cells and are particularly important in order for the fluid-filled blastocyst cavity to develop (Eckert & Fleming, 2008). αPKC has been shown to regulate the assembly of the tight junction proteins ZO-2 and ZO-1a+ needed for the formation of the blastocyst cavity (Eckert et al., 2004). Use of the αPKC inhibitor, CRT0103390, arrested the mouse embryo at the morula stage due to defective tight

junctions failing to seal fluid inside the blastocyst cavity (Gerri et al., 2020). PARD6B also regulates the formation of cell-cell junctions, particularly tight junctions, downstream of PKC and was shown in a knock-down analysis to also arrest mouse embryos at compaction (Alarcon, 2010; Gerri et al., 2020).

The role of cell adhesion in establishing the epithelial characteristic of the TB lineage is further shown by the model of embryonic development in which the ICM is isolated from the blastocyst and cultured. This isolation of the ICM results in the outer cells spontaneously differentiating to form a polarised epithelial layer (Fleming et al., 2001; Louvet-Vallée et al., 2001). The success of these isolated ICMs in forming tight junctions is shown by their ability to re-cavitate and form mini blastocysts. This method of embryo manipulation shows how important the position and polarity of cells is in the formation of tight junctions and how they are required for blastocyst development.

Compaction and polarisation occur simultaneously at the 8 cell stage in mice, making it difficult to clarify if polarity regulates cell adhesion or vice versa. In pigs, polarisation was shown, using EZRIN, to occur asynchronously, beginning at the morula stage and not completing until the late blastocyst stage (S. Liu et al., 2018). The timing of bovine polarisation also seems to occur later than in mice due to microvilli on the cell surface only beginning to polarise at the 9-15 cell stage (Koyama, Suzuki, Yang, Jiang, & Foote, 1994). Whether this surface polarity corresponds to the timing of cytoplasmic polarisation and apical domain formation in cattle is unknown.

Proteins associated with the apical domain have been shown to regulate the activity of Hippo signaling (Anani et al., 2014; Hirate et al., 2013). The Hippo pathway controls the localisation of YAP, the transcriptional co-activator of TEAD4 which controls the downstream expression of lineage-specific transcription factors (Nishioka et al., 2009). Therefore, the difference in polarity between inner and outer cells regulates the signaling activity that determines the lineage specific transcription profiles.

1.5 Hippo signaling activity regulates lineage-specific gene expression

Hippo signaling is a highly conserved pathway that controls the expression of genes that regulate cell proliferation, apoptosis, and differentiation (Meng, Moroishi, & Guan, 2016). Active Hippo signaling involves the phosphorylation of large tumor suppressor kinases 1 and 2 (LATS 1/2), which in turn phosphorylate the Hippo pathway effectors: YAP and TAZ (Zhao et al., 2007). YAP (and TAZ) are transcriptional co-activators which when phosphorylated remain inactive within the cytoplasm (Zhao et al., 2007). Inactive Hippo signaling results in YAP remaining unphosphorylated and is therefore, able to translocate into the nucleus where it associates with the transcription factor TEAD4 (Chen et al., 2010). The YAP-Tead4 interaction induces expression of genes associated with proliferation and inhibits those involved in apoptosis (Meng et al., 2016; Zhao et al., 2008). The roles of the Hippo pathway, including regulation of organ size and tumorigenesis, have recently been extended to include pre-implantation embryonic development.

1.5.1 Active Hippo signaling directs ICM fate

Angiomotin (AMOT) is an upstream regulator of Hippo signaling in the mouse pre-implantation embryo (Hirate et al., 2013). AMOT localizes to the entire of membrane of inner cells during the 8-16 cell division in mice, where it serves to activate the Hippo pathway and pluripotency gene expression (Leung & Zernicka-Goetz, 2013). Immunostaining of outer cells at this same stage of development show AMOT localized apically. AMOT associates with Neurofibromatosis type 2 (NF2) at the adherens junctions located along the basolateral membrane (Figure 3). It is here at the basolateral domain that AMOT-NF2 serves as the initiator of the Hippo pathway (Hirate et al., 2013). LATS 1/2 kinases stabilize the AMOT-NF2 complex by phosphorylating the S176 residue of AMOT, thereby maintaining its location at the adherens junctions (Hirate et al., 2013). Active Hippo signaling results in the nuclear exclusion of YAP, due to LATS 1/2 phosphorylation (Nishioka et al., 2009). Failure to accumulate nuclear YAP inhibits the YAP-TEAD4 interaction and enables expression of the pluripotency regulator, SOX2 (Wicklow et al., 2014; Zhao et al., 2007; Zhao et al., 2008) (Figure 3). Dominant-negative knock-down of NF2, inactivates the Hippo pathway and causes ectopic expression of CDX2 in inner cells (Cockburn, Biechele, Garner, & Rossant, 2013).

1.5.2 Inactive Hippo signaling enables expression of TB-specific genes

AMOTs actin binding ability is suppressed in inner cells due to the phosphorylation of its S176 residue by LATS 1/2 kinases (Hirate et al., 2013). Rho-associated kinase, ROCK, is an apically located protein that competes with NF2 to bind AMOT. ROCK binding sequesters AMOT to the apical domain where it remains unphosphorylated (Hirate et al., 2013; Shi et al., 2017) (Figure 3). Therefore, unphosphorylated AMOT can bind the cortically enriched F-actin at the apical domain, creating a spatial separation from other Hippo pathway initiators: NF2 and Lats1/2 (Hirate et al., 2013). ROCKs negative regulation of Hippo signaling is demonstrated using the ROCK inhibitor Y27632. Y27632 treatment causes AMOT to dissociate from F-actin and redistribute to the basolateral membrane in the mouse embryo, where it activates the Hippo pathway and reduces CDX2 expression (Kono, Tamashiro, & Alarcon, 2014).

Within outer cells, unphosphorylated YAP can translocate into the nucleus where it acts as a transcriptional co-activator to TEAD4, causing expression of differentiation genes e.g. CDX2 and GATA3 (Nishioka et al., 2009; Ralston et al., 2010; Yagi et al., 2007) (Figure 3). The importance of TEAD4 is highlighted by the more severe phenotype seen when knocked out, compared to CDX2 alone (Nishioka et al., 2009). This is due to the other genes regulated by the TEAD4 transcription factor including: Cellular communication network factor 2 (CCN2) and inhibition of SOX2 (Akizawa et al., 2018; Wicklow et al., 2014).

Thus, the Hippo pathway is a way for cells to coordinate polarity and positional information to regulate the expression of genes involved in the first lineage decision of the mouse embryo. Whether the Hippo pathway is responsible for establishing TB and ICM transcription profiles in cattle remains to be seen.

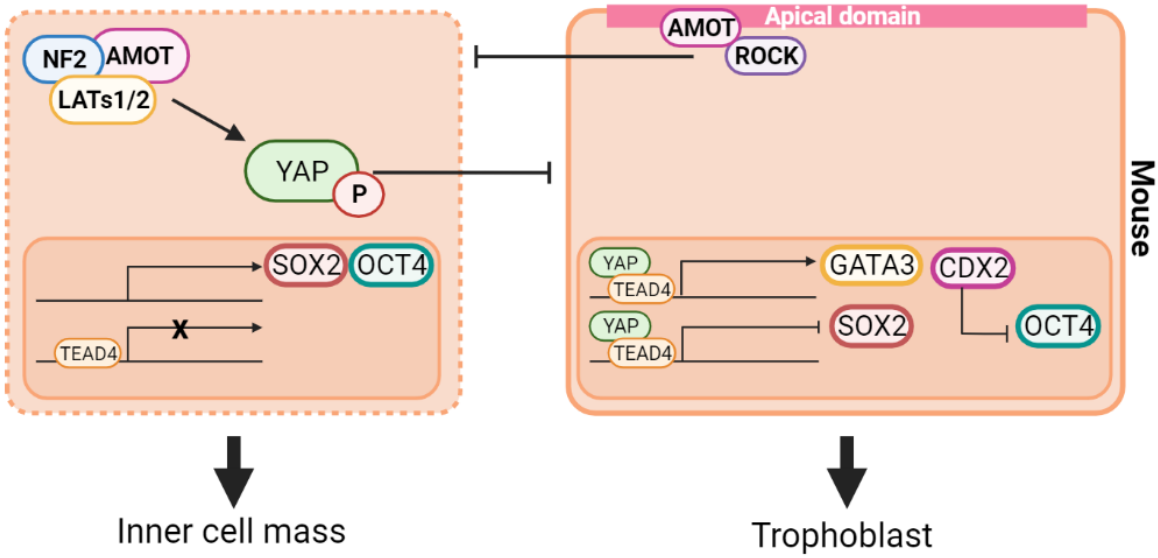

Figure 3 Hippo regulation of lineage-specific transcription profiles in mice. *Active Hippo signaling (left) results in the phosphorylation of YAP, sequestering the transcriptional regulator with the cytoplasm. Active Hippo signaling enables expression of pluripotency regulators SOX2 and OCT4. Inactive hippo signaling (right) in outer cells is due to the apical domain and ROCK sequestering AMOT from fellow Hippo pathway activators. Unphosphorylated YAP translocates into the nucleus where it associates with the transcription factor TEAD4. GATA3 and CDX2 expression are activated while expression of SOX2 is directly inhibited. OCT4 is inhibited by CDX2 binding.*

1.5.3 Species-specific YAP activity

YAP and its association with TEAD transcription factors has been studied during the development of human pluripotent stem cells due to causing expression of genes that promote differentiation and proliferation, while inhibiting apoptosis (Zhao et al., 2008). Lysophosphatidic acid (LPA) is an indirect activator of YAP that works by activating G-protein-coupled receptors G12/13 which inhibit LATS 1/2 kinases (Yu et al., 2012). By inhibiting LATS 1/2 kinases, YAP remains unphosphorylated and is able to translocate into the nucleus, irrespective of cell position or polarity. Therefore, LPA is an indirect activator of YAP that acts by removing its negative regulation by the Hippo pathway (Yu et al., 2012). Surprisingly, this activation of YAP enabled the successful establishment of human naïve pluripotent stem cells, whose pluripotent nature was proven by their ability to form all three germ layers (Qin et al., 2016). This result raises the question of how conserved the role of the Hippo pathway is in determining the first lineage decision and how YAP is involved both differentiation and pluripotency regulation.

LPA has been shown to increase anti-apoptotic factors during porcine embryo culture which lead to an increased blastocyst rate (Zhang et al., 2015). These porcine blastocysts had an increase in both TB and ICM cell numbers, suggesting that increased YAP activity did not have negative effects on the allocation cells to the ICM lineage. The effect of LPA on bovine embryonic development has not yet been explored. Use of LPA in this research will help to further our understanding of the role YAP plays in bovine pre-implantation development.

1.5.4 Alternative signalling involved in lineage specification

Evidence of a Hippo-independent pathway in mice was discovered by Leung and Zernicka-Goetz (2013), who found LATS$^{-/-}$ cells that had overexpression of AMOT, to be better at suppressing CDX2 expression than LATS$^{-/-}$ cells without Amot overexpression. These results suggest AMOT has the ability to bypass Hippo signaling to inhibit YAP-TEAD4 induced gene expression. This Hippo-independent pathway must exist in parallel in order to compensate for LATS 1/2 loss of function. One explanation is AMOT having the ability to directly bind YAP, restricting it to cell junctions and preventing its nuclear accumulation (Zhao et al., 2011).

Notch signaling has been shown to function alongside the Hippo pathway, due to normal embryonic development being able to proceed in the absence of one but not both pathways (Rayon et al., 2014). Strawberry Notch1 (Sbno1) is a component of Notch signaling that when knocked out results in the embryos arresting prior to blastocyst cavity formation. Sbno1 knock-out also results in downregulated CDX2 expression, despite nuclear YAP (Watanabe et al., 2017). These results indicate that both YAP-TEAD4 and Sbno1 enhancer binding are synergistically required to activate CDX2 expression.

How these signaling pathways and their effects on expression correlate to compaction and polarisation events have been well researched in mice, but evidence of their relationship in cattle is limited. Using markers of both morphological events and signaling activity we can begin to clarify the mechanisms controlling the first lineage decision in cattle.

1.6 Research project

The following experiments investigate the expression of lineage-specific proteins in the cattle pre-implantation embryo, to see how conserved their patterns and timing of expression are between the two mammalian species. Immunohistochemistry will be used to analyse spatial and temporal protein expression of key transcription factors during early bovine embryogenesis. From the 8 cell stage until blastocyst expansion, expression will be analysed to see the correlation between lineage decisions and morphological events such as polarisation, compaction and blastocyst cavity formation. Generating a timeline of this information will serve as a baseline to compare functional analyses that manipulate the activity of crucial signaling pathways. Throughout this research, comparisons will be made to the mouse to determine the similarities of mechanisms controlling lineage decisions, while highlighting some key differences.

One focus of this research is analysing polarisation and the formation of the apical domain in cattle and how this affects the localisation of the Hippo effector protein, YAP. Using EZRIN and PARD6B antibodies, the timing polarisation will be analysed. Inhibitors of apical domain regulator αPKC (Go6983) and ROCK (Y27632), will then be used to investigate the role of the apical domain in lineage specification. Analysis of downstream lineage marker expression will clarify the effect of the apical domain on establishing the lineage-specific transcription profiles in the bovine blastocyst.

YAP-TEAD4 transcriptional regulation is the second area of focus for analysing the similarities and potential difference between cattle and mice pre-implantation development. Antibodies against YAP will be used as a marker of Hippo pathway activity. Simultaneous staining of YAP alongside CDX2, GATA3 and SOX2 will provide insights into the Hippo pathways role in regulating each lineage marker. Verteporfin and LPA will enable manipulation of YAP activity in order to investigate its endogenous transcriptional effect. Together, this research will allow us to evaluate how YAP and the apical domain contributes to the formation of the TB and ICM lineages during bovine embryogenesis.

Chapter 2: Methods

2.1 In vitro production of abattoir acquired ovaries

2.1.1 Aspiration of ovarian follicles and IVM

Abattoir-derived ovaries were aspirated using a vacuum pump attached to a needle and bung stopped 15 ml Falcon tube, containing 2 ml of aspiration media at 38°C. The needle was inserted into follicles that have a 3 mm to 6 mm diameter. The follicular fluid and oocyte was removed by vacuum suction. Oocytes were collected and graded in H199 media supplemented with 10% FCS (Table 1). Healthy oocytes (grades 1-4) were transferred through two further H199 + 10% FCS washes before being transferred into a 35 mm petri dish contain B199 media supplemented 10% FCS. B199 + 10% FCS requires equilibration in the 38°C incubator for 2 hr prior to use. 10-15 oocytes were transferred in 10 µl of B199 + 10% FCS into the previously incubated 40 µl drops of maturation media supplemented with a 1% cysteamine (Sigma-Aldrich) (Deleuze & Goudet, 2010) and incubated at 38°C, 5% CO2 for 22-26 hr.

Table 1 Grading of oocytes.

Grade	*Phenotype*
Grade 1	Many layers of tight cumulus cells. Even colouring of cytoplasm.
Grade 2	Cumulus layers are less compact.
Grade 3	Fewer cumulus layers.
Grade 4	Corona layer only, exposing zona pellucida in some areas.
Grade 5	Expanded (dark) cumulus cells, already mature oocyte.
Grade 6	Degenerate. Cytoplasm is dark and speckled.

2.1.2 IVF

Sperm straws were thawed in a 30-35°C water bath for 40 sec before overlaying the sample on a 40/80% BoviPure gradient. The gradient was centrifuged at 300 rcf for 20 min to allow motile sperm to form a pellet. The sperm pellet was then washed in 1 ml Hepes synthetic oviduct fluid (HSOF) while centrifuging at 300 rcf for a further 5 min, before being diluted in 200 µl of IVF media (A). To achieve a concentration of 1.5 million/ml per culture drop, 10 µl of the sperm preparation is diluted in 190 µl water and is counted on a haemocytometer (B) before diluting accordingly with IVF media.

$$Total\ volume\ (C) = \frac{\text{Volume measured (A)}\ x\ \text{Avg. sperm count (B)}}{25}$$

$$Volume\ of\ IVF\ media\ to\ add = Total\ volume\ (C) - Volume\ measured\ (A)$$

Oocytes were removed from IVM drops and washed twice in 500 µl HSOF, during which a gentle agitation by resuspension opens up the cumulus cells. Oocytes were then transferred into 600 µl of IVF media and loaded 5 at time in a 10 µl volume into pre-equilibrated 30 µl IVF drops. 10 µl of the final sperm preparation was then added to each drop and incubated at 38°C, 5% CO2 for 18-24 hrs.

2.1.3 IVC

Fertilized zygotes were washed twice in 500 µl HSOF before being vortexed for 2 minutes in 500 µl HSOF and pelleted by centrifuged at 1800 rpm for 15 sec. Embryos and the cumulus cell debris were transferred through another two 500 µl HSOF washes where all cumulus cells are removed, before adding the zygotes to the first ESOF wash drop in the equilibrated culture dish using a mouth pipette. After transferring all zygotes, in a minimal volume, into the second wash drop of ESOF media, 10 embryos were transferred into each 20 µl ESOF culture drop for incubation. On day 3, a fertilisation check is conducted. All unfertilised and arrested 2 cell embryos were transferred into the wash drops.

On day 5 of culture, embryos were transferred using a mouth pipette through two LSOF wash drops in an equilibrated culture dish. Embryos were then transferred into 20 µl LSOF culture

drops where they remained up until day 7. All dishes were overlaid with mineral oil (Sigma-Aldrich) and made a minimum of 2 hrs ahead of time in order to allow equilibration of the media pH.

2.2 Embryo staging

Embryos were cultured up to day 7 post-fertilisation, where fertilisation is considered day 0. The third cleavage division occurs on day 3 and results in an 8 cell embryo from which point the bulk of our analysis began. An embryo is considered a morula during the fifth cleavage into a 32 cell embryo, until compaction. The next morphological event is cavitation that begins with the formation of micro lumens which eventually coalesce to form a singular dominant blastocoel. This formation of the fluid filled blastocoel marks the beginning of blastocyst development. As fluid is pumped into the cavity TB cells proliferate, causing an increase in diameter of the embryo (Table 2). Gradually the zona pellucida thins due to the expansion of the underlying TE and the blastocyst is termed expanded and begins to hatch from the zona pellucida. Measurements and sizes of blastocysts, observed in this thesis, were obtained in confocal analysis.

Table 2 Blastocyst size in relation to its stage of expansion.

Blastocyst Size (µm)	*Stage*
100 - 109	Early
110 - 119	Mid
120 - 139	Late
140 +	Expanded

2.3 Embryo Fixation

Embryos were washed twice in PBS (OXOID) supplemented with 0.1% Tween-20 (85113; Sigma-Aldrich) (now referred to as PBT) before fixing at room temperature in 4% paraformaldehyde (SCARPA00951000; BioStrategy) in PBS for 20 min. Fixed embryos were washed twice more in PBT then dehydrated gradually in 500 µl 25%, 50%, 70% ethanol solutions for 5 min each and stored at -20°C in 100% ethanol.

2.4 Immunohistochemistry

All steps were carried out at room temperature unless stated otherwise. Rehydration of frozen, fixed embryos through 500 µl 100%, 70%, 50% and 25% ethanol solutions for 5min each before washing twice in PBT. Embryos were permeabilised for 30 min in PBS supplemented with 0.5% Triton X-100. Embryos were incubated for 4 hr in PBT supplemented with 3% BSA and 10% heat-inactivated FBS to block non-specific binding sites. A combination of three primary antibodies at their specific concentration (Table 1) were then added to 200 µl of cold PBT supplemented with 3% BSA along with a random selection of embryos before incubating overnight at 4°C on a gentle rocker. Embryos were rinsed 3 times for 5min each in cold PBT buffer, then washed a further 2 times for 1 hr each in cold PBT supplemented with 3% BSA before being added to the final 1 hr PBT wash supplemented with 3% BSA and the secondary antibodies (Table 3). From this point onwards tubes were kept dark to minimise light contact with sample. Embryos were then washed 3 times for 15 min each in PBT supplemented with 1% BSA, the first containing 200 µl DAPI. Embryos were stored in slow fade diamond (Invitrogen) solution at 4°C until being loaded onto glass slide for confocal imaging analysis.

Table 3 Primary and secondary antibodies

Primary Antibody	*Origin*	*Conc.*	*Secondary Antibody*	*Conc.*
Anti-Cdx2 (MU392A-US; Biogenex)	Mouse	1:2	Alexa Fluor 647 (Life Technologies)	1:500
Anti-Sox2 (sczsc-17320; Santa Cruz)	Goat	1:200	Alexa Fluor 555 (Life Technologies)	1:500
Anti-YAP (4912; Cell Signaling)	Rabbit	1:200	Alexa Fluor 488 (Life Technologies)	1:500
Anti-YAP ((D8H1X) XP(R); Cell Signaling)	Rabbit	1:100	Alexa Fluor 488 (Life Technologies)	1:500
Anti-EZRIN ((3C12) AB4069; Abcam)	Mouse	1:1000	Alexa Fluor 647 (Life Technologies)	1:500
Anti-PARD6B (B10: sc-373910; Santa Cruz)	Mouse	1:200	Alexa Fluor 647 (Life Technologies)	1:500
Anti-Bactin (A2228; Sigma)	Mouse	1:1000	Alexa Fluor 647 (Life Technologies)	1:500
Anti-Gata3 (MA1-028; Pierce)	Mouse	1:100	Alexa Fluor 647 (Life Technologies)	1:500
DAPI (D-3571; Thermo-Fisher)		1:1000		

2.5 Embryo treatments

Embryos at the 8 cell stage were selected on day 3 and transferred to ESOF culture drops containing treatments: Y-27632 (10 µM, 32160801; Sigma-Aldrich), Verteporfin (10 µM , HY-B0146; MCE), LPA (10 µM, 3854; In Vitro Technologies) and Go6983 (5 µM, HY-13689; MCE). On day 5 embryos were transferred to LSOF culture drops containing the same concentration of treatment. Fixation and immunohistochemistry was carried out on the treated embryos as per the previous methodology at day 7. Control embryos were cultured in 20 µl ESOF drops with an equal volume of DMSO to treatments (0.1%).

2.6 Embryo analysis

Images of embryos were obtained from the confocal microscope using the Z-stack function. Embryos were loaded onto a glass slide with a coverslip, separated by ~200 µm gap, and were visualised on a confocal microscope at 20x objective. Analysis was conducted on the images in both confocal viewer and ImageJ in order to obtain cell count information, and mean fluorescent intensity (MFI) data.

Nuclear staining was measured by creating region of interest masks using DAPI staining of cell nuclei. Cytoplasmic staining was measured using a merge image of DAPI and YAP to create a region of interest mask. These masks were then used across all different staining channels to analyse MFI values of the different antibodies. Three slices (with individual masks) that intercept the ICM were measured from each embryo Z-stack. 3 embryos (n = 3) from each treatment group underwent this process. Background staining was taken, and subtracted, from within the blastocoel cavity for all stains except CDX2. High non-specific CDX2 staining within the blastocoel cavity meant a background measurement was instead taken from the area outside of the embryo. In order to compare MFI values between embryos, normalisation was undertaken. MFI of ICM nuclear staining was normalised to the TB nuclear MFI for protein expression analysis and cytoplasmic MFI was normalised to nuclear MFI for analysis of the nuclear to cytoplasmic ratio.

Analysis of the number of cells positive for each antibody were conducted in ImageJ. Three slices for each embryo were combined using the stack function before adjusting the threshold value to accurately represent what is seen in the confocal image. The counting tool was used to manually number the cells strongly (+++) and weakly (+) expressing each antibody with the ICM and TB. This analysis was conducted in addition to the previous, more quantitative, analysis of MFI to support the results on an individual cell basis. Noting, occasionally the classification of the cell into +++ or + could have been subjective.

2.7 Statistics

Statistical methods were indicated for every experiment in the corresponding figure legend. For data showing a normal distribution, unpaired two-tailed students t-test was used to analyse statistical significance of the nuclear to cytoplasmic ratios measured. Fisher's exact test was used to analyse the significance of the contingency table of cell count data. A significance level of 0.05 was used for all statistical analyses.

Chapter 3: Results

3.1 Embryo culture

The average embryonic development rate (now referred to as blastocyst rate) from day 3 to day 7 was 42.4% (42.4 ± 0.79; n = 158). Rate was measured by dividing the number of blastocysts (early, mid, late or expanded) by the number of 8 cell embryos that continued culture following a fertilisation check on day 3.

3.2 Compaction of the bovine morula

Compaction can be visualised due to the flattening of cells against one another caused by an increase in cell junctions (Cui et al., 2007; Fleming et al., 2001). Embryos fixed on days 4 and 5 were subjected to immunofluorescence and confocal microscopy to analyse the timing of bovine compaction. Immuno-fluorescent staining of β-actin is used to visualise the actin filaments that associate with the cell membrane and therefore the morphology of cells as they undergo compaction. In mice, this occurs at the 8 cell stage (Cui et al., 2007). Cattle blastomeres at the 8 and 8-16 cell stage maintained a rounded morphology, with the exception of a few flattened cell borders (Figure 4A).

Compaction can also be seen due to the increase in perivitelline space between the embryos' cell membranes and the zona pellucida (Figure 4A, row 2). This timing of compaction initiation was consistent with that seen using EZRIN, a protein that localises to the entire cell membrane prior to polarisation (Figure 4B). Both β-actin and EZRIN staining showed that bovine compaction begins during the 8-16 cell division. The following 16-32 cell cleavage shows compaction rapidly completing and individual cell borders become difficult to distinguish (Figure 4A, Row 3).

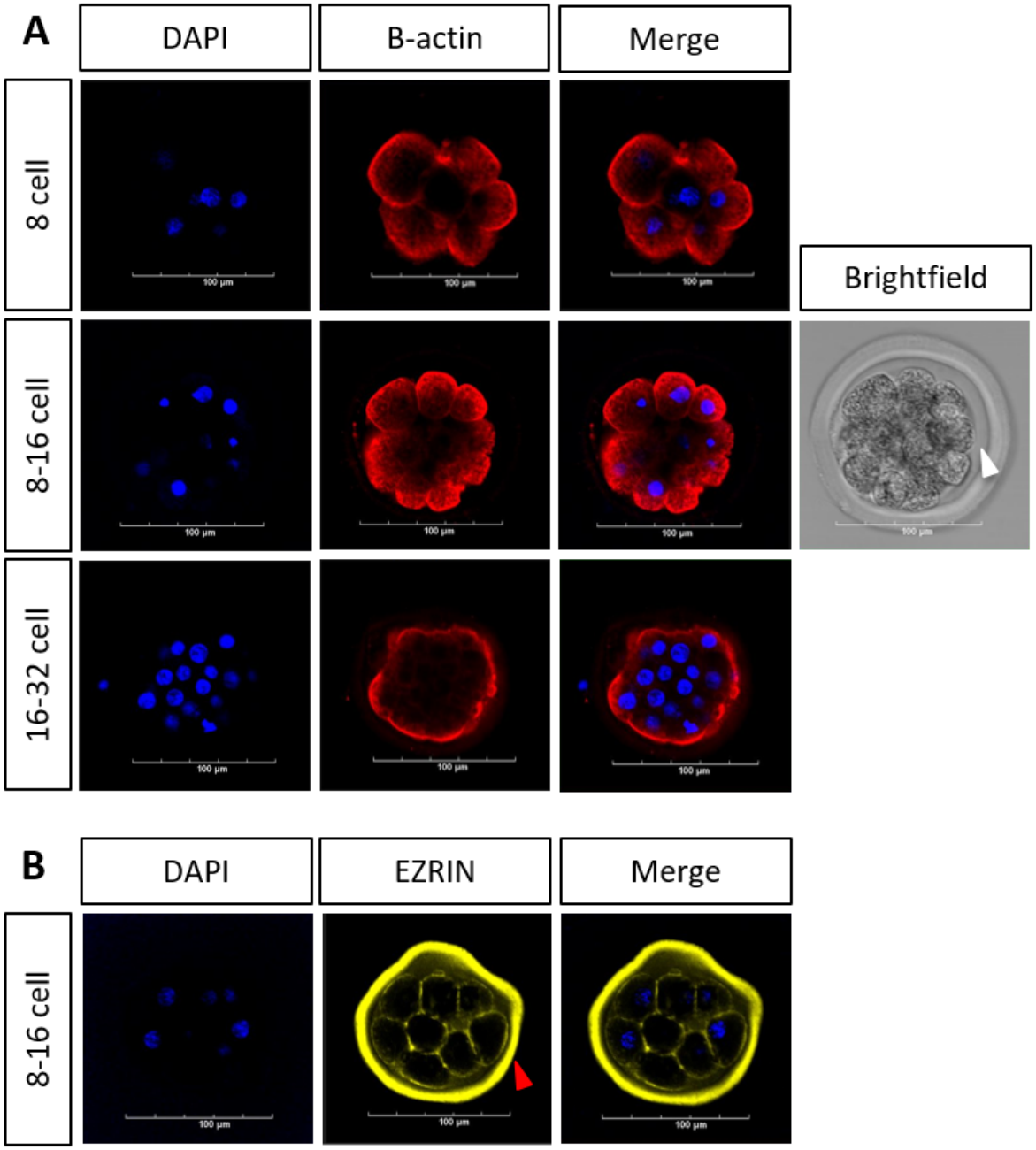

Figure 4 Compaction of the bovine morula. *A) B-actin and DAPI immunostaining at the 8 cell, 8-16 cell and 16-32 cell stage. Brightfield of the 8-16 cell embryo shows the increased perivitelline space (white arrowhead) as a result of compaction initiation. B) EZRIN and DAPI staining at the 8-16 cell stage. Red arrow head points to non-specific staining of zona pellucida seen using EZRIN antibody. 100 µm scale bar in all images.*

3.3 Polarisation of the bovine embryo

Immunofluorescence and confocal microscopy techniques were used to detect the expression of polarity markers EZRIN and PARD6B. Restriction of EZRIN to the contact-free surface of mice blastomeres occurs simultaneously with compaction at the 8 cell stage, marking the polarisation of the outer cells (Louvet et al., 1996). In cattle, EZRIN is localised to the entire cell membrane in the 8 cell embryo (Figure 5, row 1) where it remains until the 16 cell stage. Following compaction completion, EZRIN is exclusively expressed at the entire contact-free region of outer blastomeres (Figure 5, row 3) and remains in this position in TB cells of the blastocyst during its expansion (Figure 5, row 4 and 5). Preferential staining of EZRIN was consistently seen in the polar trophoblast that overlays the ICM (Figure 5, row 5).

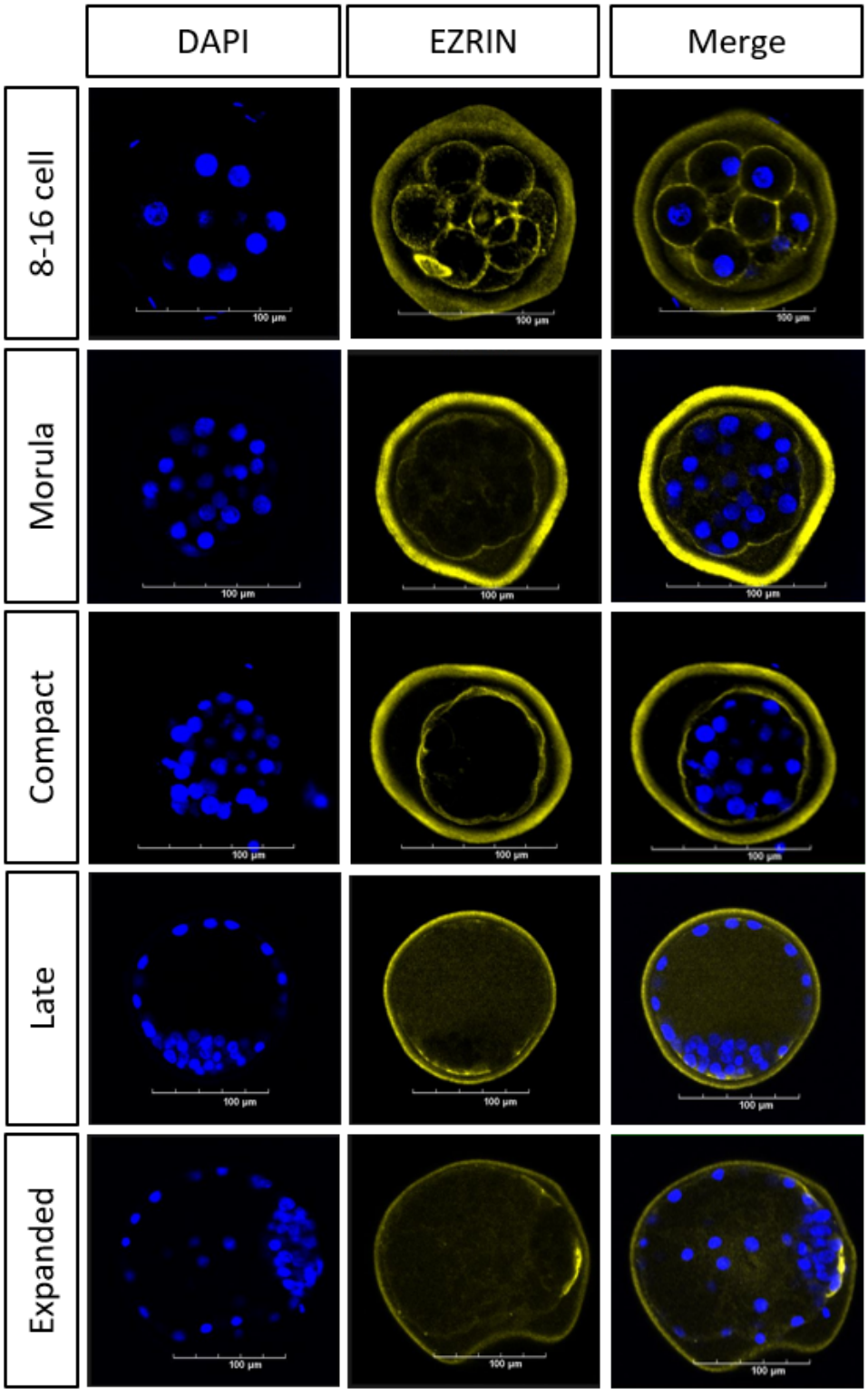

Figure 5 Polarisation of EZRIN in the bovine embryo. *EZRIN and DAPI immunostaining at the 8-16 cell, morula, compact morula, late blastocyst and expanded blastocyst stages of bovine embryonic development. 100 µm scale bar in all images.*

3.3.1 Polarisation of the polarity regulator PARD6B

PARD6B is recruited to the cell-contact-free region by αPKC, marking the formation of the apical domain. This enrichment of PARD6B at the apical domain occurs at the late 8 cell stage in mice (Zhu et al., 2017), something not seen in bovine embryo (Figure 6, row 1). I found expression of PARD6B to be scattered within the cytoplasm up until the compact morula stage when it begins to become restricted to cell junctions (Figure 6, row 2). Only following compaction, during cavitation, does PARD6B localise to the contact-free surface of outer TB cells (Figure 6A, row 3). At the blastocyst stage PARD6B was observed in outer TB cells at cell junctions as well as the apical domain (Figure 6A, row 4 and 5).

Staining of EZRIN and PARD6B at the apical domain was consistently more intense in the polar trophoblast overlying the inner cell mass (Figure 6B, C). To our knowledge, this result is the earliest molecular marker to differentiate between the mural and polar trophoblast.

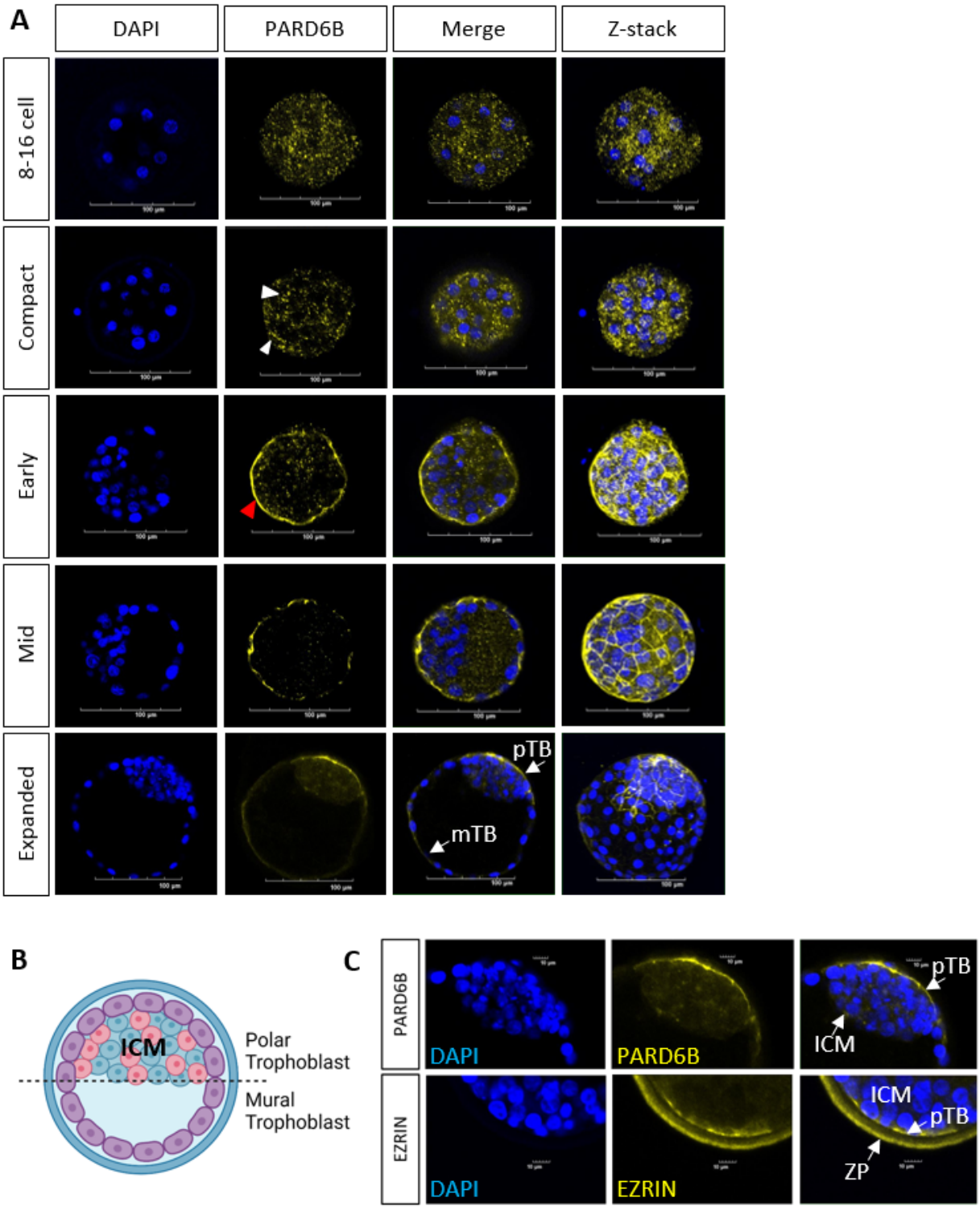

Figure 6 Apical domain formation. *A) PARD6B and DAPI immunostaining at the 8-16 cell, compact morula, early blastocyst, mid blastocyst and expanded blastocyst stage of bovine embryogenesis. White arrowheads point to the initial enreichment of PARD6B at cell junctions, red arrowhead points to apical domain region. 100 µm scale bar in all images. B) Graphical depiction of the polar and mural trophoblast and inner cell mass (ICM) C) PARD6B, EZRIN and DAPI immunostaining, zoom in on polar trophoblast. White arrows point to: inner cell mass (ICM); mural trophoblast (mTB); polar trophoblast (pTB) and zona pellucida (ZP). 10 µm scale bar used.*

3.4 Expression of YAP during bovine blastocyst development

YAP is the effector protein of the Hippo pathway, which when within the nucleus of mouse blastomeres associates with TEAD4 to regulate expression of downstream transcription factors (Nishioka et al., 2009; Ralston et al., 2010; Wicklow et al., 2014). Restriction of YAP to outer blastomeres of the mouse pre-implantation embryo occurs as early as the 16 cell stage (Hirate et al., 2012). Using anti-YAP antibodies (4912; Cell Signaling), cattle embryos at the 8 cell stage show nuclear YAP in all cells (Figure 7A, top row). Following two further cleavage divisions, YAP remains expressed in all cell nuclei (Figure 7A). Once the blastocyst cavity forms, expression becomes restricted to outer cells (Figure 7, row 3). However, even after blastocyst formation, I observed the occasional cell within the ICM maintaining weak nuclear expression.

A second YAP antibody (14074 T; Cell Signaling) was used in order to confirm these initial results regarding YAP expression and localisation. Both antibodies have previously been used, with slightly contradictory results, in early embryology research (Gerri et al., 2020; Negron-Perez & Hansen, 2018). Negron-Perez and Hansen (2018) found YAP (4912; Cell Signaling) in both the cytoplasm and nuclei from the 2 cell stage until the late morula before restricting nuclear expression to TB cells at the blastocyst stage. On the contrary, Gerri et al. (2020) found YAP expression (14074 T; Cell Signaling) restricted to the nuclei of outer cells of the morula. Our results using both antibodies show intense nuclear YAP expression in TB cells with mostly cytoplasmic expression within the ICM (Figure 7B).

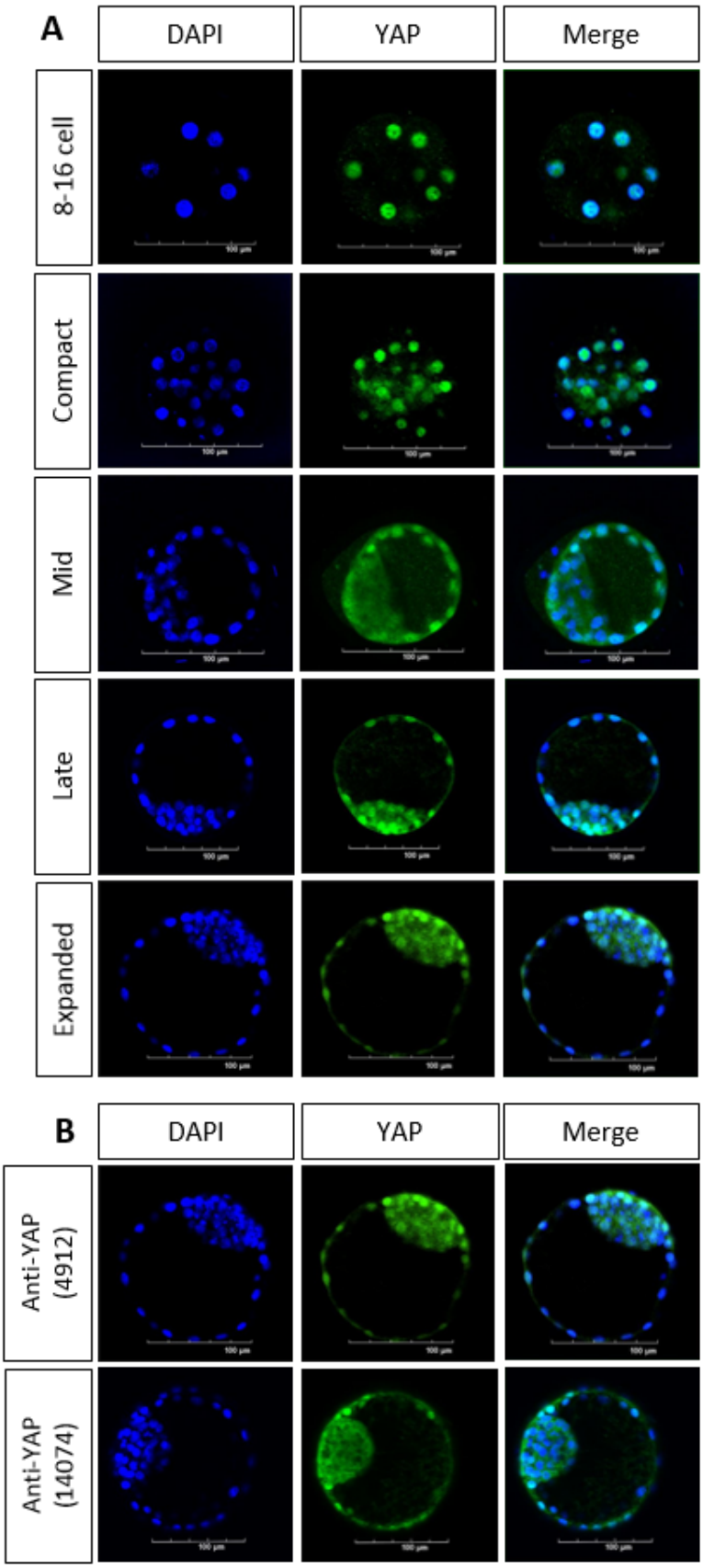

Figure 7 Localisation of YAP during bovine blastocyst development. *A) YAP and DAPI immunostaining at the 8-16 cell, compact morula, mid blastocyst, late blastocyst and expanded blastocyst stages of embryonic development. B) Comparison of 4912 and 14074 YAP primary antibodies. 100 µm scale bar displayed.*

3.5 Expression of the TB-marker CDX2 in bovine embryonic development

In mice, CDX2 expression is directly activated by YAP-TEAD4 (Nishioka et al., 2009). TEAD4 is constitutively nuclear and so the exclusive expression of CDX2 in outer cells of the mouse embryo is controlled by the restriction of nuclear YAP to outer cells (Hirate et al., 2012). Therefore, we would expect CDX2 to be expressed in all cells prior to blastocyst cavity formation, due to nuclear YAP being present is all cells of the bovine morula (Figure 7A, row 2). As expected, CDX2 was expressed in all cells at the 16 cell (Figure 8, row 2) and compact morula stage (Figure 8, row 3) of bovine embryonic development. Post-blastocyst cavity formation, CDX2 expression is restricted to outer, TB cells (Figure 8, row 4 and 5). CDX2 shows co-expression with YAP at all stages of development, including the timing of restriction to the nuclei of TB cells (Figure 8).

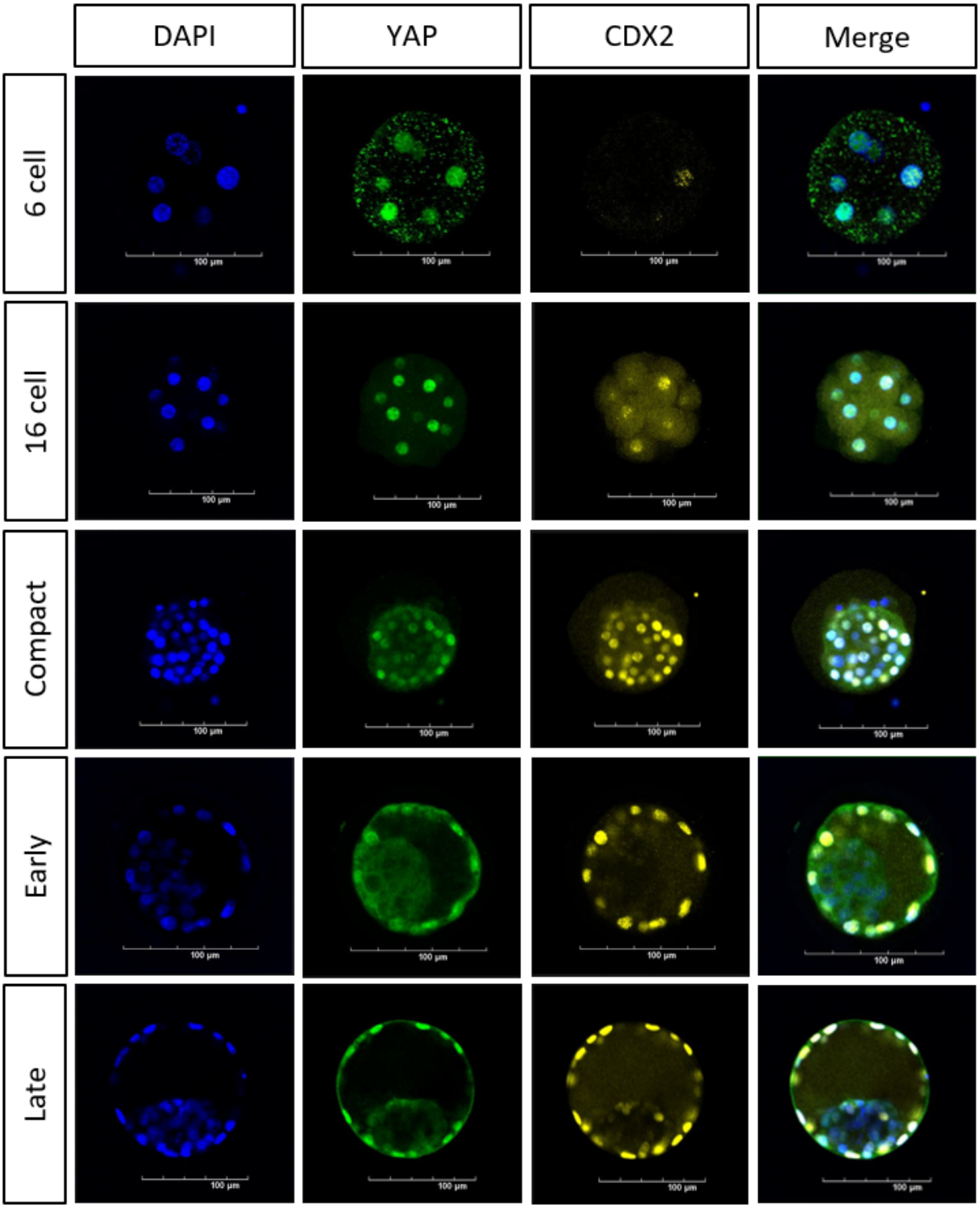

Figure 8 Spatial and temporal CDX2 expression within the developing bovine blastocyst. *CDX2, YAP and DAPI immunostaining at the 6-cell, 16-cell, compact morula, early blastocyst and late blastocyst stage of bovine embryonic development. 100 µm scale bar displayed.*

3.6 Expression of the TB-marker GATA3 in bovine embryonic development

In mice, expression of GATA3 is controlled by the same YAP-TEAD4 transcriptional complex as CDX2 (Ralston et al., 2010) and therefore, follows the same pattern of expression at the 16 cell stage in outer cells. However, in bovine pre-implantation embryos no nuclear expression of GATA3 is seen prior to compaction (Figure 9, row 1, 2 and 3). Upon compaction of the morula, expression can be seen within the nucleus of outer cells (Figure 9, row 4). GATA3 expression remains completely restricted to the TB cells during blastocyst formation and expansion (Figure 9, row 5 and 6).

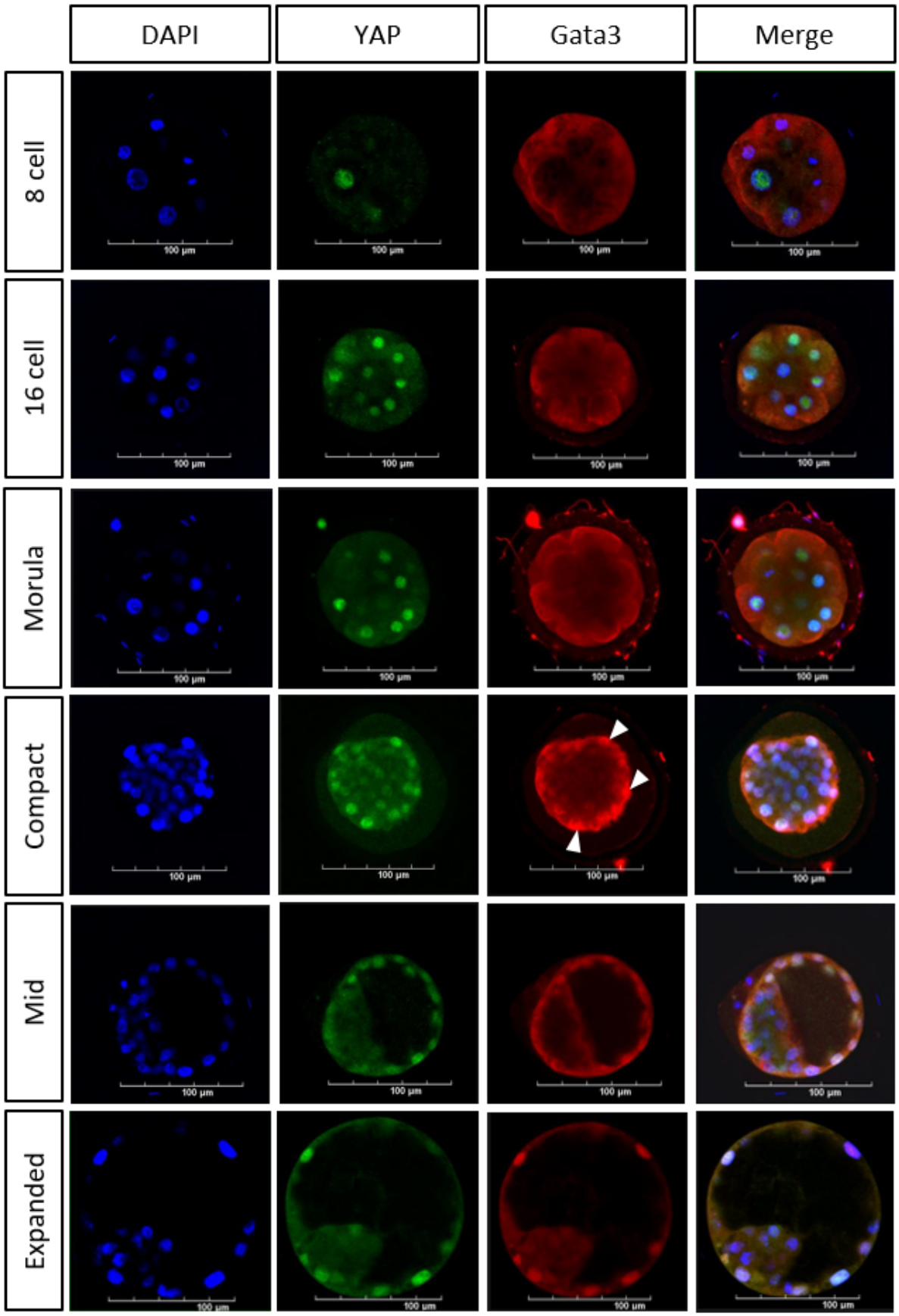

Figure 9 GATA3 expression during bovine blastocyst development. *YAP, GATA3 and DAPI immunostaining at the 8 cell, 16 cell, morula, compact morula, mid blastocyst and expanded blastocyst stage of embryonic development. White arrowheads point to outer cells expressing GATA3. 100 µm scale bar displayed in all images.*

3.7 Expression of SOX2 within the TB and ICM of the bovine blastocyst

SOX2 expression is detected in all cells of the 8 cell mouse embryo, before becoming restricted to inner cells at the 16 cell stage (Wicklow et al., 2014). SOX2 expression was visualised by immunohistochemistry and confocal microscope imaging from the 8 cell stage until the expanded blastocyst. Contrary to SOX2 expression in mice, I found that expression of SOX2 in cattle is maintained in cell nuclei of the TB up until the expanded blastocyst stage (Figure 10A). Although, expression of SOX2 in the TB is present at the expanded blastocyst stage, the intensity is much lower than previous stages of development (Figure 10A, row 6).

Co-expression of SOX2 and CDX2 is not seen in mice due to the inverse regulation TEAD4 has on both of the transcription factors (Wicklow et al., 2014). This creation of a mutually exclusive pattern of SOX2 and CDX2 expression within the ICM and TB was not seen in cattle embryos due to the two proteins being co-expressed during all stages of blastocyst development, until the expanded blastocyst (Figure 10B).

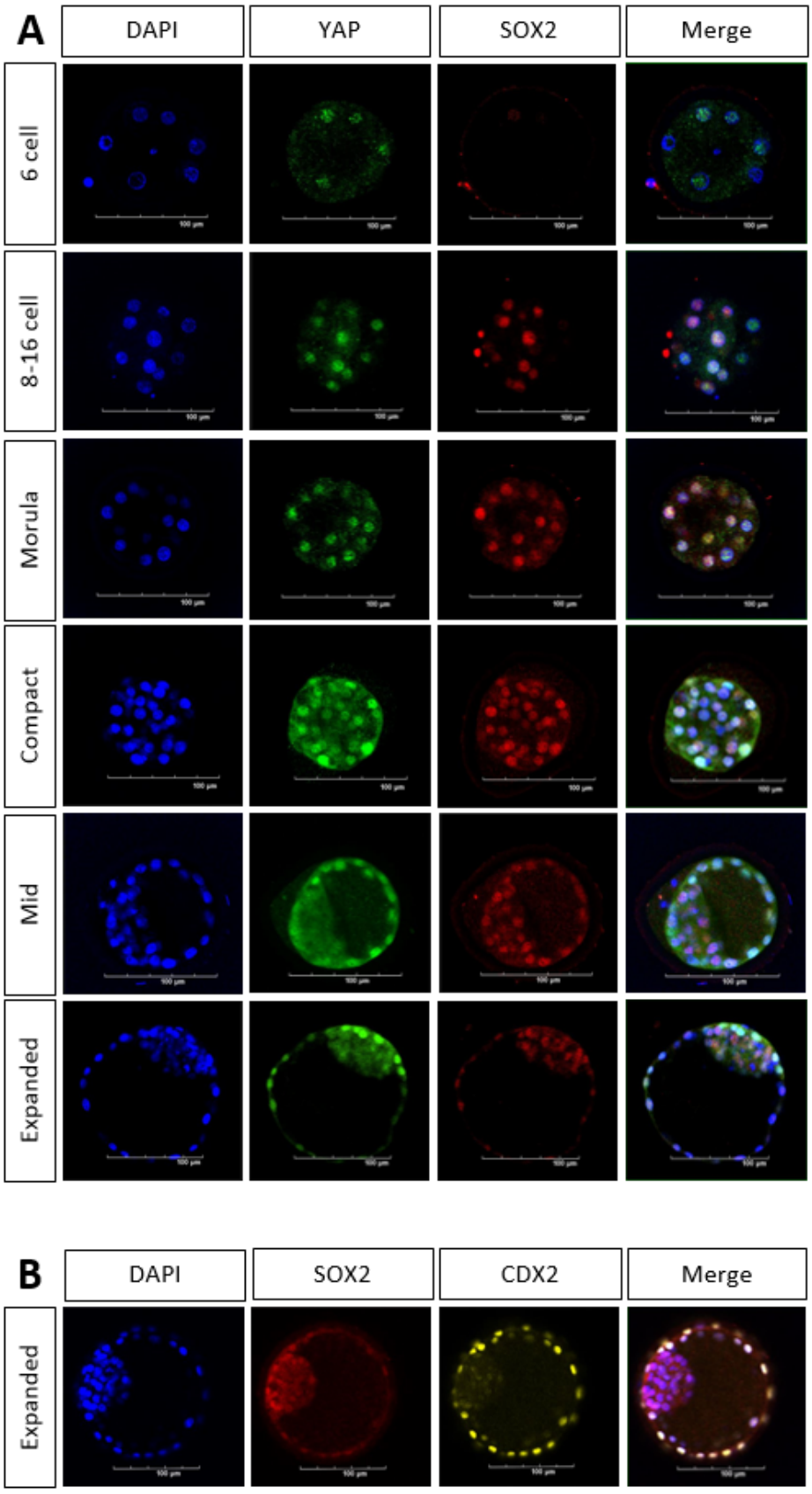

Figure 10 SOX2 expression during bovine blastocyst development. A) SOX2, YAP and DAPI immunostaining at the 6 cell, 8-16 cell, morula, compact morula, mid blastocyst and expanded blastocyst stages of bovine embryonic development. B) Co-expression of SOX2 and CDX2 in the expanded bovine blastocyst. 100 µm scale bar used in all images.

3.8 Effect of αPKC inhibition on polarisation

Go6983 is an inhibitor of αPKC, the kinase that has been found associated with the apical domain, where it is responsible for recruiting myosin and PARD6B (Zhu et al., 2017). In mice, treatment with an αPKC inhibitor results in embryos arresting at the compact morula stage, due to failing to form the tight junctions required for formation of the blastocyst cavity (Eckert & Fleming, 2008; Gerri et al., 2020). To examine the relationship between polarisation events and lineage determination, we interfered with polarisation during embryo culture. The strategy was to treat embryos at the 8 cell stage (Figure 11A), by which point polarisation has not yet occurred (Figure 5, Figure 6). Bovine embryos treated with Go6983 (5 µM) experienced a decreased blastocyst rate of 28.3% (28.3 ± 2.90; n = 152) compared to control DMSO-treated embryos (43.8% ± 2.81; n = 89) (Figure 11B). Go6983-treated embryos that developed to the blastocyst stage still experienced EZRIN only on the apical contact-free surface (Figure 11C). However, PARD6B was no longer restricted to the contact-free surface and instead displayed a punctate expression pattern (Figure 11D).

As apical localisation of the αPKC/PARD6B complex is integral for maintenance of the apical domain and the apical-basal partitioning of many components involved in polarisation maintenance (Gerri et al., 2020; Zhu et al., 2017), we can conclude that αPKC inhibition lead to an abnormal apical domain in cattle embryos, as also reported in mice (Zhu et al., 2017).

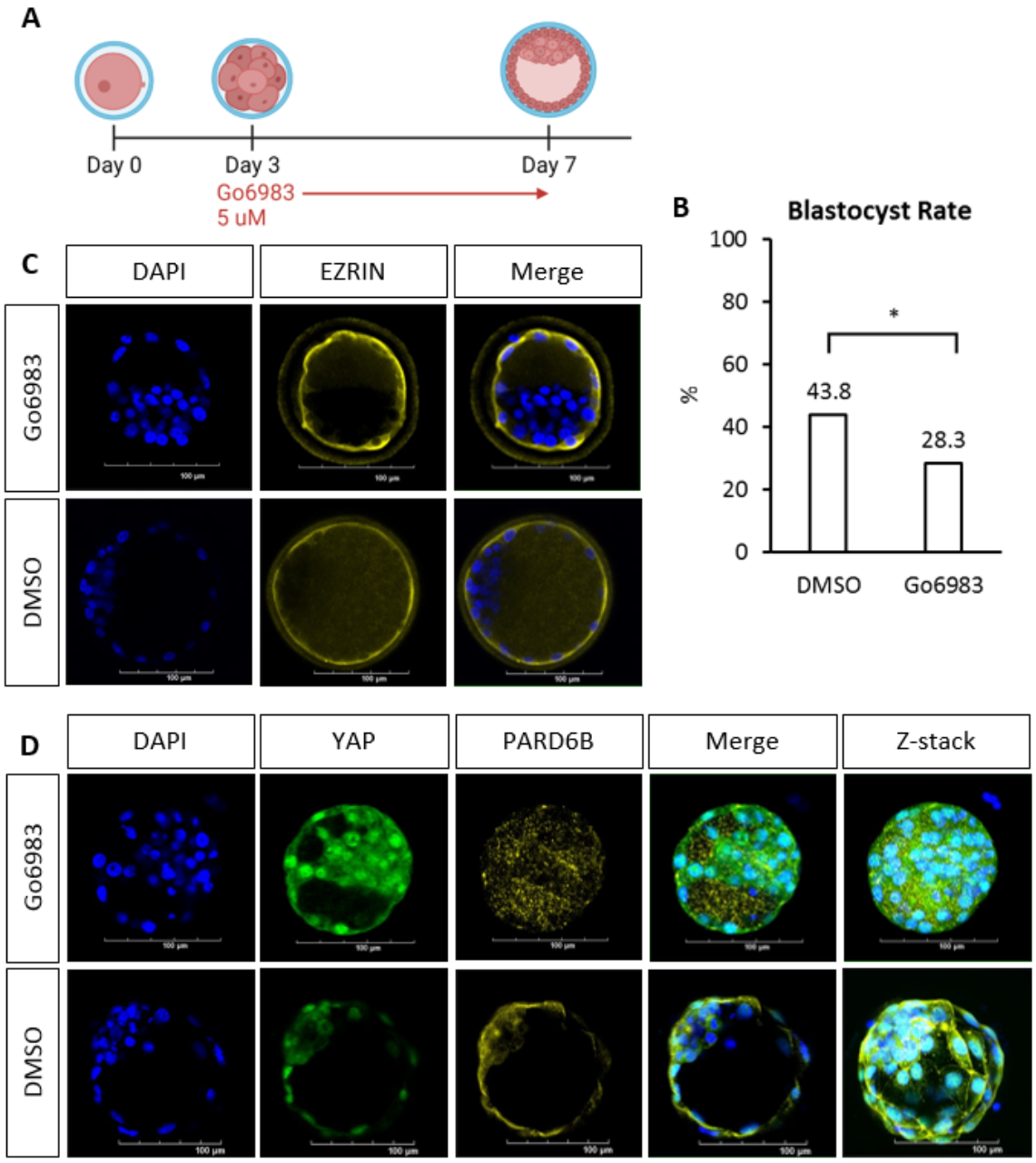

Figure 11 Effect of Go6983 treatment on polarisation and apical domain formation. *A) Treatment plan of Go6983 (5 µM). B) Blastocyst rate of embryos treated with Go6983 and DMSO 43. Significance (<0.05) as per Fisher's exact test with Bonferroni correction. C) EZRIN and DAPI immunostaining of Go6983 and DMSO treated embryos. D) YAP, PARD6B and DAPI immunostaining of Go6983 and DMSO treated embryos. 100 µm scale bar displayed in all images.*

3.9 Effect of αPKC inhibition on lineage determination

Cattle embryos treated with Go6983 (5 µM) were stained using antibodies against the trophoblast marker CDX2, the Hippo marker YAP and the pluripotency marker SOX2. Disruption of the apical domain using Go6983 resulted in the nuclear to cytoplasmic ratio of YAP staining, indicative of YAP activation, not changing within the ICM or TB (Figure 12A,E). However, a significant 4.4% (n = 3) decrease in the number of strongly YAP-positive cells and a 14.4% (n = 3) decrease in weak YAP expression was seen in the ICM (Figure 12C). This result was unexpected as, the apical domain and its associated proteins negatively regulate Hippo signalling in mice embryos (Hirate et al., 2013).

Next we analysed expression of the downstream transcription factor CDX2. The number of CDX2-positive cells within the ICM did not differ significantly between Go6983 and DMSO controls (Figure 12D). The nuclear to cytoplasmic ratio of CDX2 was also not affected and followed the same trend as controls with the TB nuclear expression 2.9-fold (4.62 ± 0.95; n = 3) higher than the ICM (1.59 ± 0.11; n = 3) (Figure 12F). SOX2 expression was completely disrupted by Go6983 treatment in all embryos at the blastocyst stage (n = 15), thus cell number and fluorescent intensity data could not be quantified.

These results using Go6983 show that the apical domain is affected by αPKC inhibition. However, nuclear YAP and downstream CDX2 expression are not dependent on the apical domain within the TB.

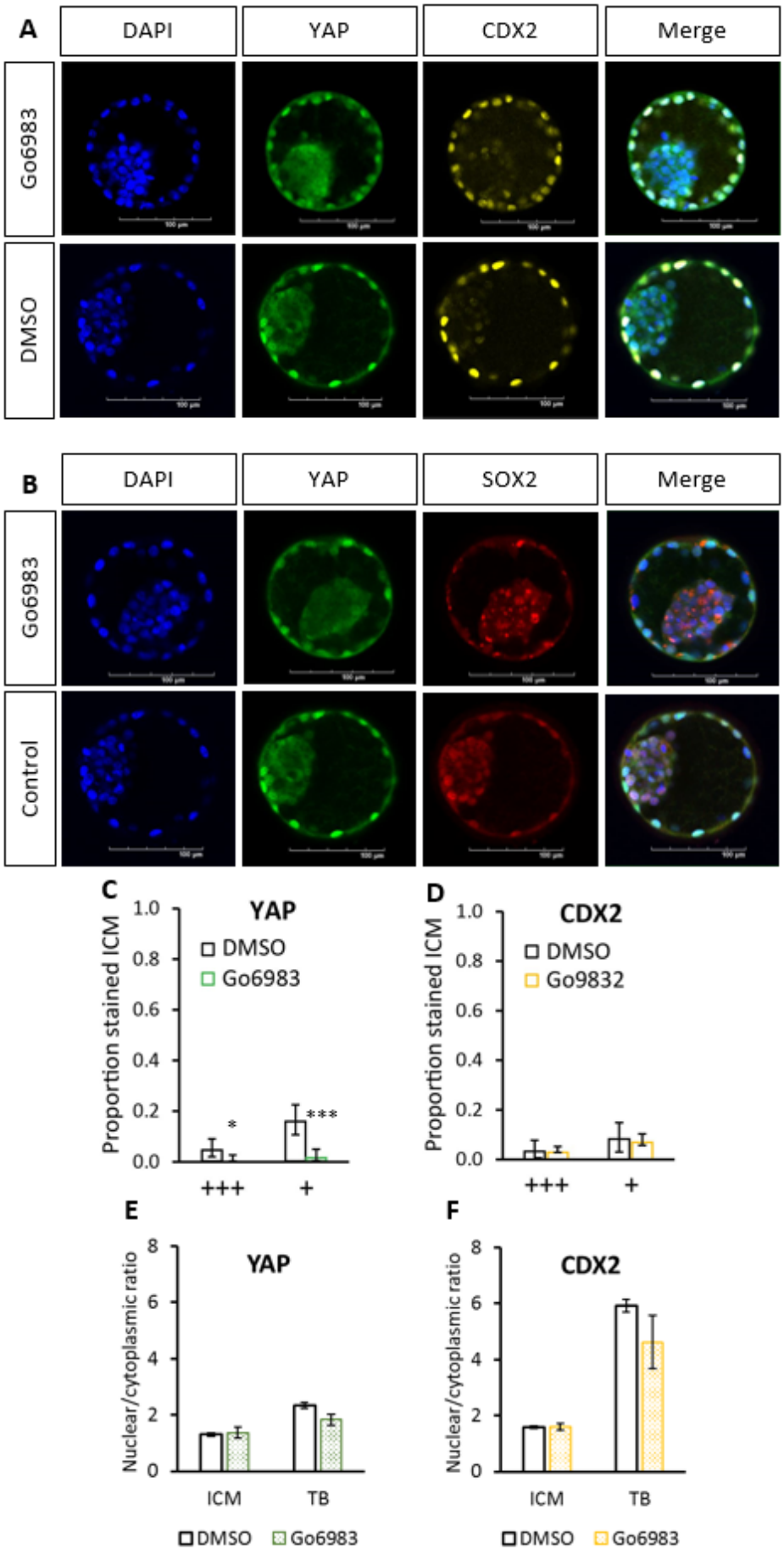
A
DAPI
YAP
CDX2
Merge
Go6983
DMSO
B
DAPI
YAP
SOX2
Merge
Go6983
Control
C
YAP
DMSO
Go6983
Proportion stained ICM
1.0
0.8
0.6
0.4
0.2
0.0
*
***
+++
+
D
CDX2
DMSO
Go9832
Proportion stained ICM
1.0
0.8
0.6
0.4
0.2
0.0
+++
+
E
YAP
Nuclear/cytoplasmic ratio
8
6
4
2
0
ICM
TB
DMSO
Go6983
F
CDX2
Nuclear/cytoplasmic ratio
8
6
4
2
0
ICM
TB
DMSO
Go6983

Figure 12 Effect of aPKC inhibition on CDX2, YAP and SOX2 expression. *A) CDX2, YAP and DAPI immunostaining of Go6983 (5 µM) and DMSO treated embryos. B) SOX2, YAP and DAPI immunostaining of Go6983 (5 µM) and DMSO treated embryos. 100 µm scale bar displayed in all images. C, D) Proportion of strongly positive (+++) and weakly positive (+) YAP and CDX2 nuclei within the ICM of Go6983 and DMSO treated blastocysts. Significance (<0.05) as per Fisher's exact test with Bonferroni correction. *p < 0.05, ***p < 0.001. Error bars, 95% CI. E, F) Nuclear to cytoplasmic staining ratio of YAP and CDX2 mean fluorescence intensity in the ICM and TB of Go6983 and DMSO treated blastocysts. Significance (<0.05) as per Student's two-sample t-test, error bars ± SEM.*

3.11 Effect of ROCK inhibition on lineage specification

I further examined the relationship of the polarisation dependent apical domain to YAP signaling and lineage determination. Y-27632 is a specific inhibitor of Rho-associated kinase ROCK (Narumiya, Ishizaki, & Ufhata, 2000). Rho-ROCK is responsible for restricting AMOT to the apical domain (Narumiya et al., 2000; Shi et al., 2017). When Rho-ROCK is inhibited, AMOT can associate with NF2 and LATS 1/2 kinases to activate Hippo signaling (Cockburn et al., 2013). In mouse embryos, Y27632 causes ectopic Hippo signaling in outer TB cells leading to an increase in SOX2 mRNA expression (Frum, Murphy, & Ralston, 2018; Kono et al., 2014).

Bovine embryos at the 8 cell stage were randomly allocated into treatment and DMSO control groups on day 3 (Figure 13A). At day 7, blastocyst rate of 33.4% (33.4 ± 6.50; n=121) was reached for embryos treated with Y-27632 (10 μM), which is 15% lower (though not significantly so) compared to DMSO controls (43.8% ± 2.80; n=89) (Figure 13B).

By activating Hippo signaling, Y27632 treatment would be expected to lead to a decrease in nuclear YAP (Kono et al., 2014). However, Rock inhibition did not significantly affect the average nuclear to cytoplasmic ratio of YAP in the ICM or TB (Figure 13G). However, there was a 29.9% (n = 3) increase in the number strongly YAP-positive cells within the ICM (Figure 13D). A 24.8% (n = 3) increase in strongly CDX2-positive cells (Figure 13E) was also found within the ICM, providing further evidence of CDX2 regulation downstream of YAP. The number of SOX2 positive cells within the ICM also increased by 30.0% (n = 3) (Figure 13F) following Y27632 treatment.

This result of more YAP and CDX2 positive ICM cells may be due to miss-sorting and will be discussed further in the discussion (Section 4.3.2).

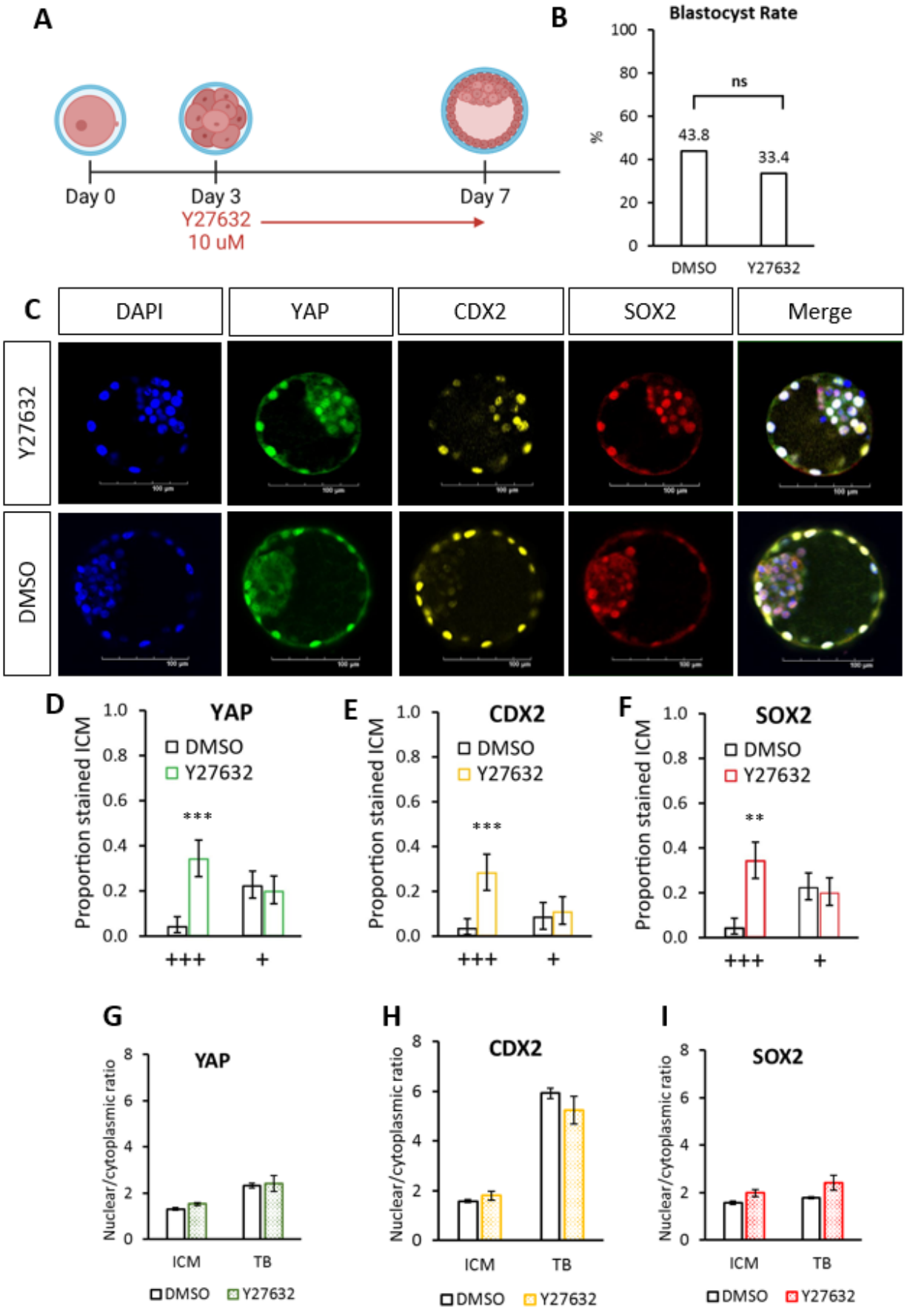
A
Day 0
Day 3
Y27632
10 uM
Day 7
B
Blastocyst Rate
100
80
60
40
20
0
%
ns
43.8
33.4
DMSO
Y27632
C
DAPI
YAP
CDX2
SOX2
Merge
Y27632
DMSO
D
YAP
Proportion stained ICM
DMSO
Y27632
***
+++
+
E
CDX2
Proportion stained ICM
DMSO
Y27632
***
+++
+
F
SOX2
Proportion stained ICM
DMSO
Y27632
**
+++
+
G
YAP
Nuclear/cytoplasmic ratio
ICM
TB
DMSO
Y27632
H
CDX2
Nuclear/cytoplasmic ratio
ICM
TB
DMSO
Y27632
I
SOX2
Nuclear/cytoplasmic ratio
ICM
TB
DMSO
Y27632

Figure 13 Effect of ROCK inhibition on lineage-specific expression in cattle. *A) Treatment of Y27632 (10 µM) from day 3 to day 7. B) Blastocyst rate of Y27632 and DMSO treated embryos at day 7. Significance (< 0.05) used as per Fisher's exact test with Bonferroni correction. C) YAP, CDX2, SOX2 and DAPI immunostaining of Y27632 and DMSO treated bovine blastocysts. 100 µm scale bar displayed. D, E, F) Proportions of strongly positive (+++) and weakly positive (+++) CDX2, YAP and SOX2 nuclear staining in Y27632 and DMSO treated embryos. Significance (<0.05) as per Fisher's exact test with Bonferroni correction. **p < 0.01, ***p < 0.001. Error bars, 95% CI. G, H, I) Nuclear to cytoplasmic ratios of CDX2, YAP and SOX2 staining in the ICM and TB of Y27632 and DMSO treated embryos. Significance (<0.05) as per Student's two-sample t-test, error bars ± SEM.*

3.10 YAP-TEAD4 transcriptional regulation in bovine embryos

Verteporfin directly inhibits the interaction between YAP and TEAD4 within the nucleus (Feng et al., 2016). In mice, inhibition of YAP-TEAD4 leads to down-regulation of CDX2 expression and ectopic expression of pluripotency markers within the TB, indicating an inability of cells to commit to the TB fate without the YAP-TEAD4 transcriptional activity (Nishioka et al., 2009) . Due to TEAD4 directly downregulating SOX2 expression in mice, we would expect an increase in SOX2 expression following Verteporfin treatment (Wicklow et al., 2014).

On day three, embryos were randomly allocated into treatment groups of Verteporfin (10 μM) and DMSO (Figure 14A). Development of all Verteporfin treated embryos arrested prior to cavity formation, with 26.9% (n = 63) arresting at the compaction stage. This suggested that embryos require YAP activity prior to compaction. High sensitivity to Verteporfin treatment prior to compaction was further confirmed by treating embryos with a decreased dose (3 μM) at day 3 (Figure 14A). This lower concentration still did not enable embryos to progress past compaction due to all arresting prior to cavitation. 40.3% (n=62) arrested at compaction, indicating the YAP-TEAD4 transcriptional complex is crucial during early embryogenesis for successful blastocyst development (Figure 14B). I next treated embryos with 10 μM of Verteporfin from day 5, following compaction. Embryos achieved a blastocyst rate of 40.0% (40.0 ± 6.30; n=110), a rate that did not differ significantly from controls (43.8 ± 2.80; n = 89) (Figure 14B).

To analyse the effect of Verteporfin on post-compaction blastocyst development, protein expression of known lineage markers controlled by the YAP-TEAD4 transcriptional complex were analysed in day 7 blastocysts following the 10 μM treatment on day 5. Using immunofluorescence and confocal microscopy YAP, CDX2, GATA3 and SOX2 protein expression were visualised (Figure 14C, D, E). As in control embryos, nuclear YAP was higher in the TB than ICM (Figure 14F). Expression of nuclear YAP was unchanged within the ICM upon Verteporfin treatment, while a non-significant 1.4-fold (3.29 ± 0.36, n = 3) increase in nuclear YAP was seen within the TB (Figure 14F).

GATA3 remained unchanged following Verteporfin treatment (Figure 14D). However, CDX2 showed a significant 0.72-fold (1.13 ± 0.08; n = 3) and 0.67-fold (3.94 ± 0.19; n = 3) decrease in nuclear fluorescence within the ICM and TB, respectively (Figure 14G). The number of SOX2-positive cells within the ICM was not affected by Verteporfin treatment (Figure 14K). Neither was there an effect on the nuclear to cytoplasmic ratio of SOX2 expression in the ICM or TB following Verteporfin treatment (Figure 14H).

This experiment showed that YAP activity is dispensable for maintaining TB and ICM identity from day 5 of development. YAP's role in lineage determination from day 3-5 could not be determined as Verteporfin treatment led to embryo lethality before restriction of lineage markers.

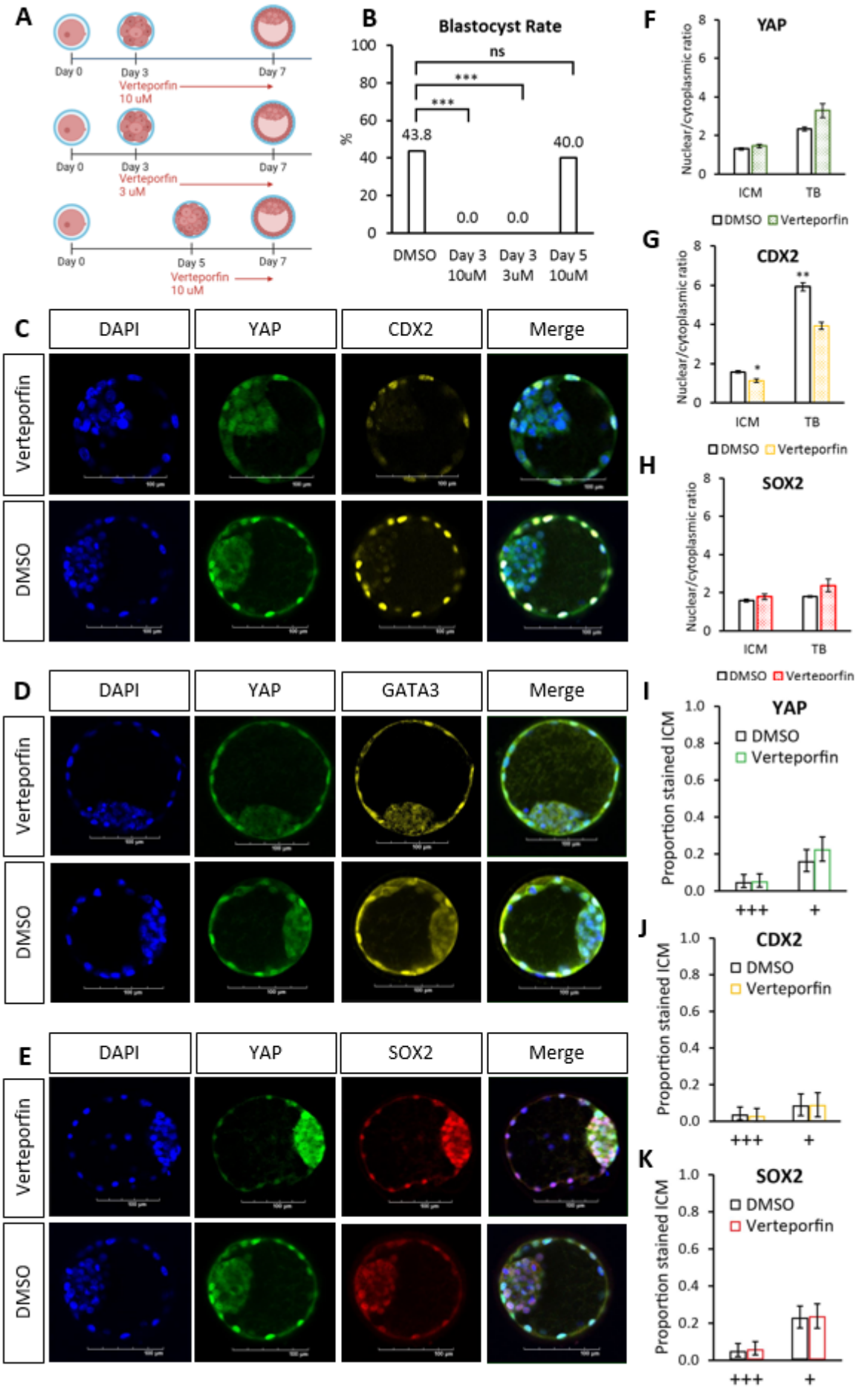
A
Day 0
Day 3
Day 7
Verteporfin
10 uM
Day 0
Day 3
Day 7
Verteporfin
3 uM
Day 0
Day 5
Day 7
Verteporfin
10 uM
B
Blastocyst Rate
%
100
80
60
40
20
0
ns
***
***
43.8
0.0
0.0
40.0
DMSO
Day 3
10uM
Day 3
3uM
Day 5
10uM
C
DAPI
YAP
CDX2
Merge
Verteporfin
DMSO
D
DAPI
YAP
GATA3
Merge
Verteporfin
DMSO
E
DAPI
YAP
SOX2
Merge
Verteporfin
DMSO
F
YAP
Nuclear/cytoplasmic ratio
ICM
TB
DMSO
Verteporfin
G
CDX2
Nuclear/cytoplasmic ratio
ICM
TB
DMSO
Verteporfin
H
SOX2
Nuclear/cytoplasmic ratio
ICM
TB
DMSO
Verteporfin
I
YAP
DMSO
Verteporfin
Proportion stained ICM
+++
+
J
CDX2
DMSO
Verteporfin
Proportion stained ICM
+++
+
K
SOX2
DMSO
Verteporfin
Proportion stained ICM
+++
+

Figure 14 Effect of Verteporfin treatment on lineage-specific expression in cattle. *A) Treatment plans for Verteporfin experiments. B) Blastocyst rate of Verteporfin treated embryos from day 3 (10 µM and 3 µM) and day 5 (10 µM) compared to DMSO controls. Significance (<0.05) used as per Fisher's exact test with Bonferroni correction. C) YAP, CDX2 and DAPI immunostaining of Y27632 (day 5, 10 µM) and DMSO treated blastocysts. D) YAP, GATA3 and DAPI immunostaining of Y27632 (day 5, 10 µM) and DMSO treated blastocysts. E) YAP, SOX2 and DAPI immunostaining of Y27632 (day 5, 10 µM) and DMSO treated blastocysts. 100 µm scale bar displayed in all images. F, G, H) Nuclear to cytoplasmic ratios of YAP, CDX2 and SOX2 staining within the TB and ICM of Y27632 (day 5, 10 µM) and DMSO treated embryos. Significance (<0.05) as per Student's two-sample t-test, error bars ± SEM *p < 0.05, **p<0.01. I, J, K) Proportion of strongly positive (+++) and weakly positive (+) nuclear staining of YAP, CDX2 and SOX2 within the ICM of Y27632 (day 5, 10 µM) and DMSO treated embryos. Significance (<0.05) as per Fisher's exact test with Bonferroni correction. Error bars, 95% CI.*

3.12 Effect of YAP activation using LPA

After examining the effect of inhibiting YAP using Verteporfin, I next aimed to ectopically activate YAP. LPA inhibits the LATS1/2 kinases responsible for YAP phosphorylation by activating their inhibitory G-protein-coupled receptors (Yu et al., 2012). This results in an increase in active, unphosphorylated YAP that we expect to see within the nucleus. Cattle embryos were treated from day 3, before compaction has commenced, with 10 µM LPA (Figure 15A). LPA treated embryos showed an increased rate of development with 17.8% (n = 180) of embryos forming a blastocyst cavity on day 5, a full day earlier than any control embryos (Figure 15B). By day seven 61.5% (61.5 ± 0.22; n = 180) of embryos had formed blastocysts (Figure 15I). This represents a significant 1.5-fold increase in blastocyst rate compared to controls (42.4% ± 0.788; n = 3). This acceleration in development is likely to have effects on the expression of lineage markers within the blastocyst.

As expected, the number of YAP-positive nuclei within the ICM increased by 50% (n = 5) (Figure 15J), corresponding to a 1.8-fold (2.01 ± 0.10; n = 5) increase in the nuclear to cytoplasmic ratio within the ICM (Figure 15F). Changes in the TB were modest presumably because YAP is already nuclear in these cells in untreated embryos (Figure 15F). This increase in nuclear YAP was reflected by CDX2, as shown by the 29.7% (n = 4) increase in CDX2-positive cells within the ICM (Figure 15K) and a 1.7-fold (2.06 ± 0.13; n = 4) and 1.4-fold (4.96 ± 0.25; n = 4) increase in CDX2 fluorescence in LPA treated embryos within the ICM and TB (Figure 15G). Importantly, co-staining of YAP and CDX2 positive cells revealed that YAP acts upstream of CDX2 to direct the TB lineage.

SOX2 is negatively regulated by the YAP-TEAD4 transcriptional complex in mice (Wicklow et al., 2014). No evidence of this negative regulation has been found in cattle (Sections 3.7, 3.11). While there was no significant change in the nuclear to cytoplasmic ratio of SOX2 expression within the ICM or TB (Figure 15H), there was an 11.9% (n = 5) increase in the number of cells strongly expressing SOX2 within the ICM (Figure 15L). This may be related to the increased rate of development of LPA-treated embryos as discussed later (Section 4.2.1).

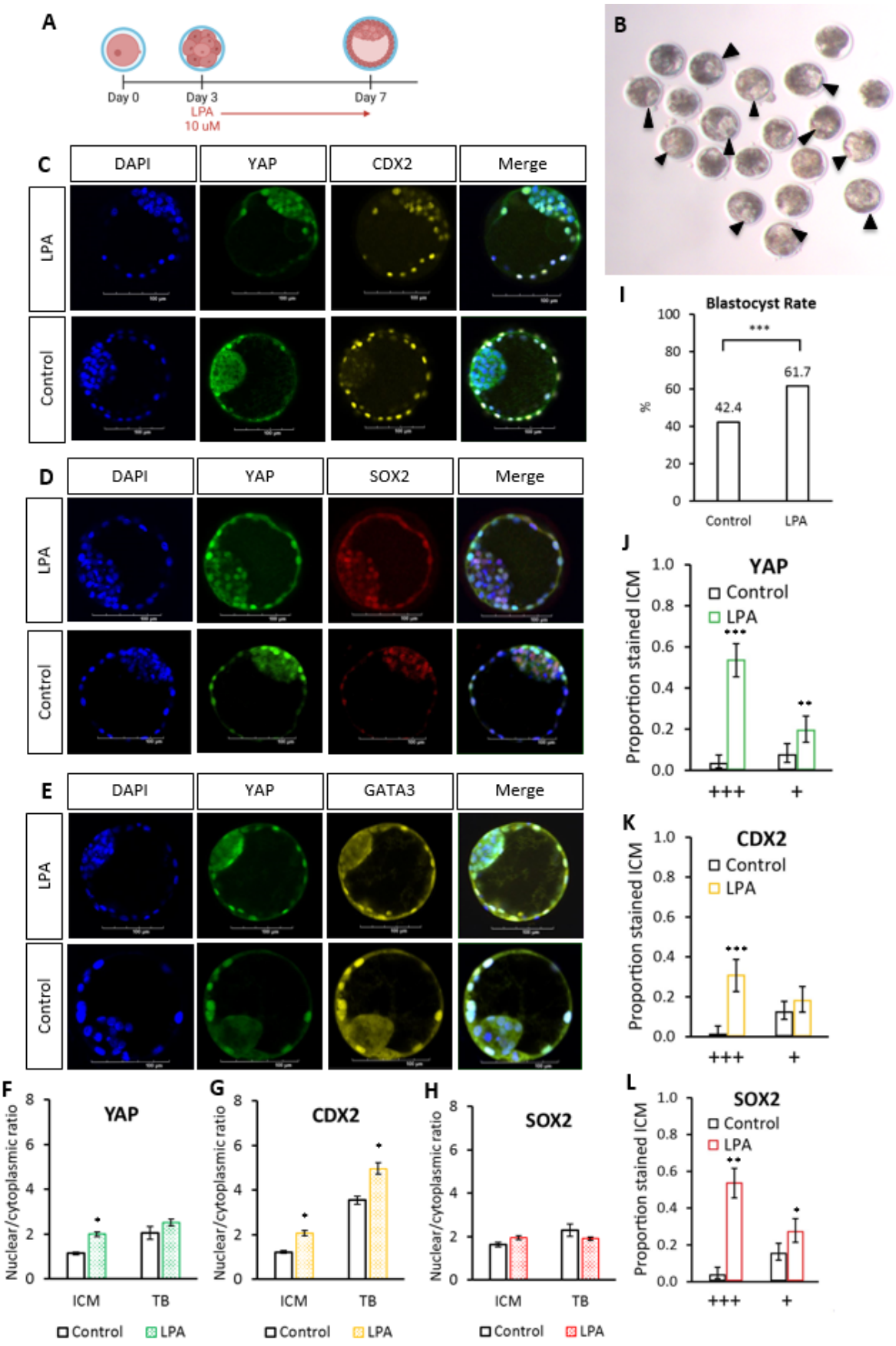
A
Day 0
Day 3
LPA
10 uM
Day 7
B
C
DAPI
YAP
CDX2
Merge
LPA
Control
D
DAPI
YAP
SOX2
Merge
LPA
Control
E
DAPI
YAP
GATA3
Merge
LPA
Control
I
Blastocyst Rate
***
61.7
42.4
%
Control
LPA
J
YAP
Control
LPA
Proportion stained ICM
+++
+
K
CDX2
Control
LPA
Proportion stained ICM
+++
+
F
YAP
Nuclear/cytoplasmic ratio
ICM
TB
Control
LPA
G
CDX2
Nuclear/cytoplasmic ratio
ICM
TB
Control
LPA
H
SOX2
Nuclear/cytoplasmic ratio
ICM
TB
Control
LPA
L
SOX2
Control
LPA
Proportion stained ICM
+++
+

*Figure 15 Effect of LPA on bovine lineage specification. A) LPA (10 µM) treatment plan. B) Image of LPA (10 µM) treated bovine embryos on day 5. C) CDX2, YAP and DAPI immunostaining of LPA and control bovine blastocysts. D) SOX2, YAP and DAPI immunostaining of LPA and control bovine blastocysts. E) GATA3, YAP and DAPI immunostaining of LPA and control bovine blastocysts. 100 µm scare bar displayed in all images. F, G, H) Nuclear to cytoplasmic ratios of YAP, CDX2 and SOX2 staining within the ICM and TB of LPA and control bovine blastocysts. Significance (<0.05) as per Student's two-sample t-test, error bars ± SEM. I) Day 7 blastocyst rate of LPA (10 µM) and control embryos. J, K, L) Proportion of strongly positive (+++) and weakly positive (+++) YAP, CDX2 and SOX2 nuclear staining within the ICM of LPA and control bovine blastocysts. Significance (<0.05) as per Fisher's exact test with Bonferroni correction. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars, 95% CI.*

3.13 Regulation of the apical domain

Restriction of PARD6B to the apical domain by αPKC, coincides with the restriction of YAP and CDX2 to the nuclei of outer cells and SOX2 to the nuclei of inner cells in mice (Gerri et al., 2020; Hirate et al., 2012; Zhu et al., 2017). This correlation between polarity and lineage specification provides evidence that the apical domain regulates lineage. However, a study has shown that PARD6B localisation can be disrupted by Y27632 treatment in mouse embryos (Kono et al., 2014). In order to see whether this relationship between polarity and Hippo signaling is seen in cattle, embryos treated with Y27632, Verteporfin and LPA were stained using an anti-PARD6B antibody.

Firstly, treatment with Y27632 did not cause disruption of PARD6B localisation at the apical domain (Figure 16C), suggesting the relationship between Rho-ROCK and the αPKC-PARD6B complex in mice is not conserved in cattle. In order to see if feedback between Hippo pathway activity (as monitored via YAP) and polarity exists, Verteporfin and LPA treated embryos were imaged using the anti-PARD6B antibody. Neither YAP inhibition using Verteporfin or YAP activation using LPA caused a change in PARD6B spatial expression (Figure 16A, B).

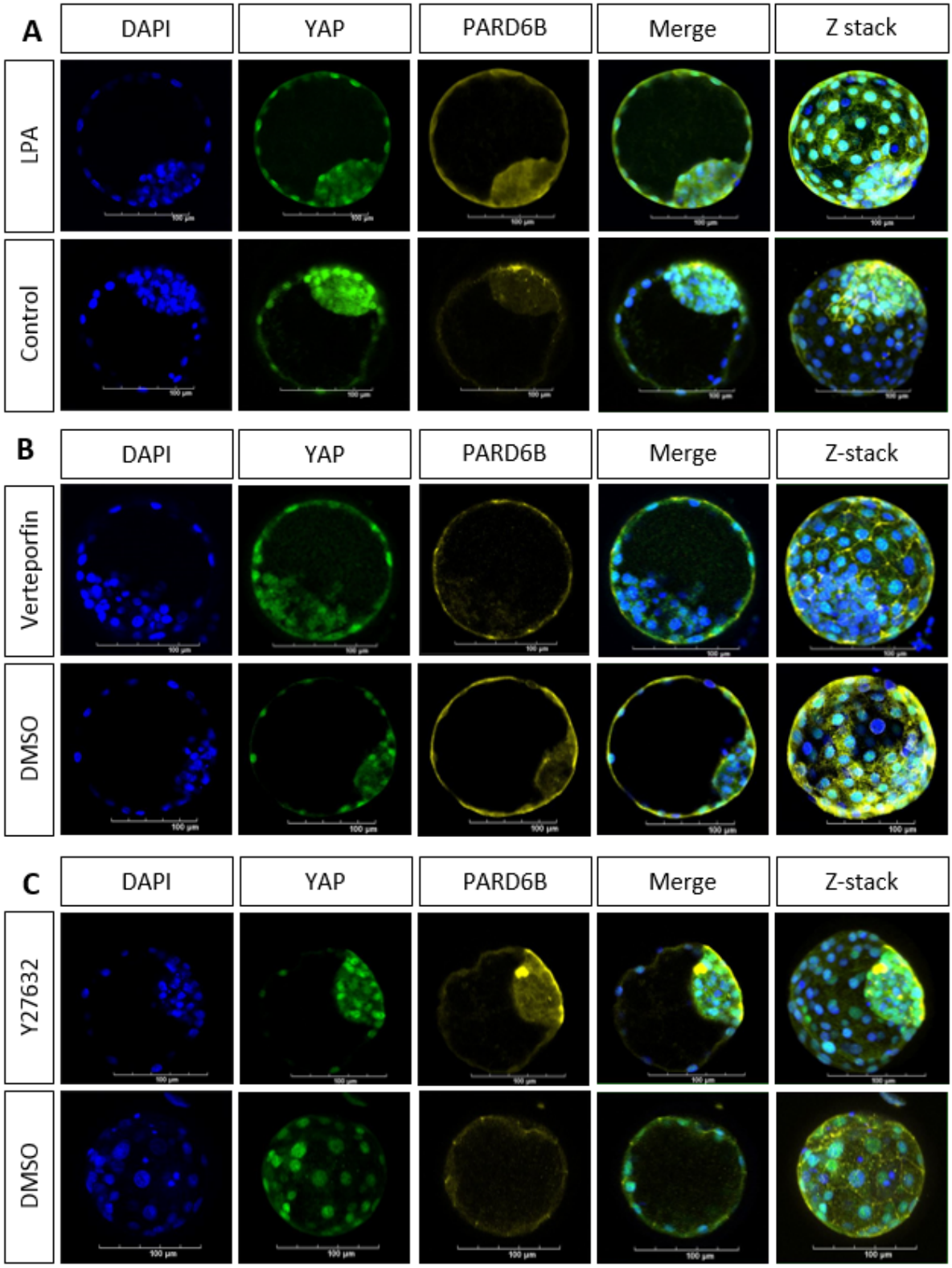

Figure 16 PARD6B expression in response to ROCK and YAP manipulation. *DAPI, YAP and PARD6B staining of bovine blastocysts. Single channels, merge and Z-stack images displayed. A) LPA (10 µM) treated embryos compared to control. B) Verteporfin (10 µM) treated embryos compared to DMSO controls. C) Y27632 (10 µM) treated embryos compared to DMSO controls. 100 µm scale bar is displayed in all images.*

Chapter 4: Discussion

In mice, Hippo pathway regulation of YAP activity is the dominant strategy to control lineage-specific gene expression (Nishioka et al., 2009). The association between YAP and TEAD4 in outer cells, due to an inactive Hippo pathway, is responsible for regulating three important genes involved in both TB development and pluripotent repression. Restriction of AMOT to the apical domain results in active YAP translocating into the nucleus where it activates expression of TB genes: CDX2 and GATA3 (Ralston et al., 2010), while directly inhibiting the pluripotent regulator SOX2 (Wicklow et al., 2014). Inner cells that lack an apical domain allow AMOT to initiate the Hippo pathway, thereby inhibiting YAP and its transcription targets (Negron-Perez & Hansen, 2018; Shi et al., 2017). These cells cannot express CDX2 and GATA3 and instead maintain pluripotency by expressing SOX2 and OCT4. However, localisation of AMOT is not the only method of regulating YAP activity, and is therefore not the only strategy capable of controlling lineage-specific expression.

Mechanical stress can be sensed in cells due to changes in their surface contractility and tension (Jaalouk & Lammerding, 2009). YAP and its partner, TAZ, are known to translate mechanical stimuli into cell signaling activity (Dupont et al., 2011). In mice, it seems this is a secondary method of regulating YAP activity, with the Hippo-AMOT pathway being the main contributor to YAP regulation. In cattle, however, my results suggest mechanotransduction may have a more important role in the organisation and lineage commitment of cells. We have shown that not only does YAP regulate the expression of different genes to the mouse, but the regulation of YAP itself also appears to differ. The commitment of cells to either the TB or ICM involves establishing a lineage-specific transcription profile. I will be discussing some of the different strategy's cattle use for lineage determination.

4.1 Asynchronous compaction and polarisation in the bovine morula

Compaction and polarisation are crucial events in early embryogenesis that establish the cell junctions and protein distributions needed to regulate future lineage decisions. In mice, simultaneous timing of compaction and polarisation at the 8 cell stage contributes to the rapid establishment of the lineage-specific transcription profiles required for blastocyst

development and implantation (Figure 17). The mouse blastocyst implants on day 5 of development, by which point the two distinct TB and ICM lineages have been established (Wang & Dey, 2006). I have shown that compaction of the bovine morula commences at the 8-16 cell stage and is not complete until the 32 cell stage. Due to the bovine blastocyst not implanting until day 19 (Lonergan & Forde, 2014), the need for rapid lineage differentiation is less than in mice. This could explain the slower onset and duration of compaction.

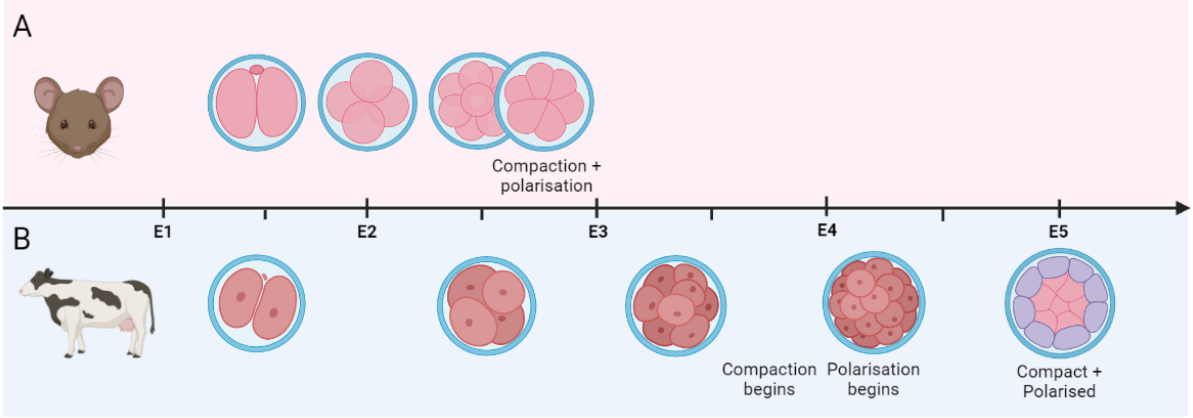

Figure 17 Comparison of compaction and polarisation in the mouse and cattle embryo. *Compaction and polarisation of the mouse morula occurs simultaneously at the 8 cell stage. The bovine morula initiates compaction during the 8-16 cell cleavage division and is complete on day 5 at the 32 cell stage. Polarisation occurs at the 32 cell stage.*

Polarisation results from the cell asymmetries that develop along the apical-basal axis of outer cells and can be visualised by the restriction of EZRIN to the contact-free surfaces (Louvet et al., 1996). Unlike mice, I have shown that polarisation of cattle blastomeres does not initiate simultaneously with compaction. Instead, we see polarisation occurring a full cell division later than in mice, namely at the 16-32 cell division (Day 4), following the beginning of compaction at the 8-16 cell stage (Day 3) (Figure 17). Completion of both compaction and polarisation occurs by the 32 cell stage.

The apical domain is a portion of the contact-free surface that is rich in polarity regulating and signaling proteins. This region can be visualised using PARD6B, which is recruited to the apical domain by a PLC αPKC-dependent mechanism following the initial polarisation of outer cells (Zhu et al., 2017). I found that in cattle, restriction of PARD6B to the apical domain occurs much

later than in mice, during the formation of the initial blastocyst cavity. PARD6B is a polarity protein that has been implicated in the formation of the tight junctions required for the maintenance of the fluid filled cavity within the blastocyst (Alarcon, 2010). The timing of PARD6B restriction to the apical domain during cavity formation suggests the conserved function of the protein in cattle. However, the delay in polarisation suggests cattle experience a redundancy in the role the apical domain plays in the first lineage decision.

4.2 YAP-tead4 transcriptional regulation of lineage specific transcription factors

4.2.1 YAP-TEAD4 does not inhibit SOX2 expression in cattle embryos

YAP is a transcriptional regulator that associates with TEAD4 to activate transcription of CDX2 and GATA3, while simultaneously inhibiting expression of SOX2 to establish the TB lineage in mice (Ralston et al., 2010; Wicklow et al., 2014). This thesis shows that YAP differentially regulates gene expression in bovine embryogenesis, as indicated by the co-expression of YAP, CDX2 and SOX2 within the nucleus TB cells. The co-expression of YAP and SOX2 suggests that YAP is not responsible for the negative regulation of SOX2 in the bovine blastocyst. Further evidence was provided in experiments using Verteporfin, a compound that directly inhibits the interaction between YAP and TEAD4. In mice, use of this inhibitor results in ectopic expression of pluripotency markers within the TB, due to the loss of negative regulation (Nishioka et al., 2009). However, no change in the amount of nuclear staining of SOX2 was seen in the TB or ICM following Verteporfin treatment in cattle.

An indirect activator of YAP (LPA) was used to see if there is a threshold of nuclear YAP required for SOX2 downregulation. As expected, LPA significantly increased the nuclear expression of YAP in both the TB and ICM. However, this increase in nuclear YAP did not reduce SOX2 expression in either the ICM or TB. Instead a small yet significant increase in SOX2 was seen in the ICM. Co-expression of YAP and SOX2, along with the increased expression of SOX2 within the ICM following LPA treatment, has two possible explanations. The first, is that YAP positively regulates SOX2 expression during bovine embryogenesis, while the second explanation is that embryos treated with LPA could show increased SOX2 expression due to being further developed than controls.

I explored the developmental rate of embryos treated with LPA to see if this could explain the increased SOX2 expression within the ICM. Treatment with LPA resulted in an acceleration in blastocyst development, seen with blastocyst cavity formation on day 5, a full day earlier than any control embryos. Restriction of SOX2 to the ICM occurs much later in cattle than mice, with progressive downregulation in the TB occurring during blastocyst expansion (Gerri et al., 2020). Embryos treated with LPA not only formed blastocysts early but had an increased average size on day 7 of 151.1 µm (n = 15) compared to the average control size of 121.7 µm (n = 15). This demonstrated the accelerated development of LPA treated embryos and could explain the increase in SOX2 expression seen within the ICM, independent of increased nuclear YAP.

4.2.2 CDX2 is regulated by YAP in cattle embryos

CDX2 was consistently co-expressed with YAP in the bovine pre-implantation embryo. Evidence that the positive YAP-TEAD4 transcriptional regulation of CDX2, documented in mice, was shown using YAP modulating experiments. Despite Verteporfin not downregulating nuclear YAP, disruption of the YAP-TEAD4 interaction resulted in a significant decrease in the nuclear fluorescent staining of CDX2 protein. This supports previous findings showing CDX2 is downstream of the YAP-TEAD4 transcriptional complex (Nishioka et al., 2009). LPA increases the amount of YAP within the nucleus by removing its inhibition by LATS 1/2 kinases. As expected, an increase in YAP-positive nuclei within the ICM was seen. LPA treatment also resulted in an increased number of CDX2-positive cells. Both Verteporfin and LPA results provide substantial evidence that CDX2 expression is directly regulated by YAP.

Treatment of bovine embryos with the ROCK inhibitor, Y27632, resulted in an increase in the number of YAP-positive and CDX2-positive nuclei within the ICM. These ectopic cells within the ICM that stained positive for YAP co-expressed CDX2, providing further evidence of the conserved function of the YAP transcriptional co-activator in regulating CDX2 transcription.

4.2.3 Effect of YAP-TEAD4 on GATA3

In mice, GATA3 functions downstream of the Hippo pathway and the YAP-TEAD4 complex to promote differentiation of outer cells into the TB lineage (Ralston et al., 2010). GATA3 expression is induced at the 4 cell stage in mice, and is selectively up regulated in outer cells

that express nuclear YAP during morula and blastocyst stages (Gerri et al., 2020; Home et al., 2009). YAP-TEAD4 inhibition using Verteporfin did not causes any visible changes in GATA3 expression within the TB. The reverse experiment, using the YAP activator LPA, also did not show increased GATA3 staining, despite ectopic nuclear YAP, within the ICM. This manipulation of the activity of the YAP-TEAD4 transcriptional complex, suggests GATA3 expression is not regulated by YAP.

Despite our results showing that GATA3 is expressed independently of nuclear YAP in cattle, expression remains restricted to outer cells. However, onset of expression is delayed compared to mice, only occurring in outer cells of the compact morula. This spatial restriction of GATA3 expression to outer cells is maintained throughout blastocyst development and expansion. Therefore, despite the difference in regulation between mice and cattle, GATA3 can still serve as a marker of the TB lineage. Onset of GATA3 expression correlates to the timing of blastocyst cavity formation, suggesting a possible role of GATA3 in the epithelial differentiation of outer cells. Our hypothesis is supported by GATA3 down-regulation studies in the mouse pre-implantation embryo, where embryos failed to transition from the morula to blastocyst structure (Home et al., 2009).

This study has shown that CDX2 and GATA3 remain specific to the TB lineage in cattle. However, their timing of restriction does not occur simultaneously, suggesting that cattle have an alternative mechanism of regulating their expression. GATA3 is the first marker of TB fate due to restricting to outer cells of the compact morula, followed closely by CDX2 restriction occurring during the formation of the early blastocyst cavity. Despite differences in timing of spatially restricted expression, both are exclusively expressed within the TB of the blastocyst. A clear contrast to the mouse pre-implantation embryo is the presence of SOX2 within the TB of the blastocyst, where it is co-expressed with CDX2 and GATA3. Not only does this provide evidence that YAP-TEAD4 is not responsible for SOX2 down-regulation, but the expression of the pluripotency marker, SOX2 in these cells opens up the possibility that at these stages, TB cells are not yet committed to the TB lineage. This is in line with Berg et al. (2011) who found expression of a second pluripotency regulator, OCT4, within the TB.

The SOX2 expression in the TB is not maintained at later stages, with a progressive decrease in intensity seen as the blastocyst expands. Active expression of SOX2 is maintained within the ICM, as seen in the later stage embryos following LPA treatment, where the pluripotency network is being established. TB cells of the bovine blastocyst do not require rapid lineage commitment due to the embryo not implanting into the uterus until day 19. Therefore the progressive depletion of SOX2 is sufficient to ensure that TB cells are fully differentiated by the time of implantation. Mice may actively repress SOX2 in outer cells, using the YAP-TEAD4 transcriptional complex, to enable the rapid lineage commitment required for the differentiated TB cells to implant on day 5. This efficient use of the TB lineage markers promoter likely evolved in mice to ensure the exclusive transcription profiles of the ICM and TB.

4.3 Regulation of YAP by the apical domain

In mice, polarisation results in the cortical enrichment of Rho-ROCK and F-actin that restricts AMOT to the apical domain (Hirate et al., 2013; Shi et al., 2017). Sequestering AMOT to the apical domain results in an inactive Hippo pathway and the nuclear expression of YAP (Hirate et al., 2013). Our hypothesis was that although polarisation is delayed in cattle, the timing of YAP nuclear expression would still coincide with the polarisation of outer cells. However, our results show that the nuclear translocation of YAP is not dependent on the apical domain. Nuclear YAP is expressed in all cells of the bovine embryo prior to polarisation at the 8-16 cell stage. Other mechanisms of YAP regulation must therefore be considered in order for this non-restricted expression to be explained.

YAP is known to be regulated by cell tension and contractility, a quality that is dampened by an apical domain (Dupont et al., 2011; Maitre et al., 2016). Mechanical regulation of cell lineage is of lower importance in the mouse embryo, where the Hippo pathway is considered the main strategy for regulating lineage-specific transcription profiles. However, the nuclear location of YAP in both inner and outer cells pre- and post-polarisation provided the first indication that the apical domain may not be the sole regulator of YAP activity in bovine TB development. Inner cells lacking an apical domain remained capable of nuclear-translocation of YAP prior to

blastocyst formation. These results from investigating bovine embryonic development, highlight the importance of exploring the role of mechanical pathways in lineage specification.

4.3.1 Effect of the apical domain on TB development

αPKC is responsible for regulating the polarity and cytoskeletal proteins involved in the formation of the apical domain and the tight junctions needed for blastocyst development (Eckert et al., 2004; Zhu et al., 2017). Inhibition of αPKC in mice disrupts the cortical accumulation of F-actin (Skamagki et al., 2013). It also results in down-regulation of CDX2 (Skamagki et al., 2013), suggesting that the intact apical domain is required for lineage specification. Indeed, Korotkevich et al. (2017), has shown that the apical domain is sufficient to direct TB lineage in mice, through the surgical manipulation of polar blastomeres. Knock-down of PARD6B, in mice, also results in the downregulated expression of CDX2 (Hirate et al., 2013).

My results show mislocalisation of PARD6B following treatment with the αPKC inhibitor, Go6983. Although, this result is consistent with a disrupted apical domain, maintenance of EZRIN (an actin associated protein) at the contact-free surface of TB cells, shows polarity is maintained to some degree upon αPKC inhibition. Use of Go6983, to disrupt the apical domain and PARD6B localisation, did not result in the expected decrease in the number of CDX2-positive TB cells in cattle. The reason for there being no effect on CDX2 expression within the TB is because nuclear YAP expression was also not affected by Go6983 treatment. Therefore, inhibition of αPKC using Go6983, does not affect YAP-TEAD4 transcriptional activity in the bovine embryo.

This contrasting result, to what has been found in mouse, highlighted that the apical αPKC-PARD6B-complex is not necessary for the TB lineage. Overall this experiment indicated that while αPKC is crucial for the recruitment of PARD6B to the contact-free surface, the role of the apical domain in inhibiting the Hippo pathway and activating YAP, is not conserved between mice and cattle.

4.3.2 Rho-ROCK regulation of lineage

Rho-ROCK is an apically located protein complex that is responsible for restricting the Hippo pathway initiator, AMOT, to its inactive position at the apical domain (Hirate et al., 2013; Shi et al., 2017). The AMOT-F-actin complex is stabilised at the apical domain by ROCK and results in an inactive Hippo pathway and the nuclear translocation of YAP (Hirate et al., 2013). ROCK inhibition, using Y27632, has been used in many functional analyses in mice to explore the role of Rho-ROCK in regulating Hippo pathway activity. ROCK inhibition in mice results in disruption of the apical enrichment of myosin and PARD6B (Zhu et al., 2017), and the failure to accumulate nuclear YAP, resulting in reduced expression of CDX2 (Kono et al., 2014). Therefore, upon ROCK inhibition in cattle, a decrease in YAP and CDX2 positive cells was expected within the TB.

In mice, Rho-ROCK is almost undetectable within the ICM and therefore, inhibition does not have an effect on the already active Hippo pathway (Shi et al., 2017). Surprisingly, a significant increase in the number of YAP-positive and CDX2-positive cells was seen in the ICM following Y27632 treatment. So, why do we see an increase in YAP-TEAD4 transcriptional activity in the ICM following ROCK inhibition? A plausible explanation is that the ROCK inhibitor, Y27632, has affected the sorting of the inner and outer cells, such that outer cells have ectopically landed on the inside.

Our results showed no change in YAP or CDX2 expression within the TB of cattle blastocysts. The maintenance of nuclear, active YAP in the TB suggests that Rho-ROCK is not necessary, or plays a redundant role, in inhibiting the Hippo pathway. Notably, PARD6B staining was normal upon ROCK inhibition, raising the possibility that the apical domain can compensate for the Rho-ROCK-dependent inactivation of Hippo signaling seen in mouse studies. Together, with the results from the αPKC inhibition experiment, we can be confident that the apical domain and the proteins associated with it have a reduced effect on lineage specification in the developing bovine blastocyst.

Cell sorting, or the migration of cells between inner and outer positions, is a process that has been studied in relation to the first lineage decision (Samarage et al., 2015). Polar cells with an apical domain have a reduced capacity to contract due to the exclusion of myosin, from the

apical region into the periphery, creating a rigidity in the cells cortical region (Maitre et al., 2016). Therefore, polar cells and their apical domain-associated proteins remain in the outer position of the embryo and contribute to the TB lineage due to their low contractility. Apolar cells, or cells with only a small apical domain, have higher contractility than their polar neighbours and internalise into the inner embryo.

Rho-ROCK is necessary for maintaining the tension required for cell sorting based on the apical domain-dependent differences in contractility (Dupont et al., 2011). Mechanistically, this is achieved by Rho-ROCK increasing tension by promoting myosin-actin interactions (Jaalouk & Lammerding, 2009). Thus, Y27632, ROCK inhibition allows apical domain containing outer cells, experiencing nuclear YAP and thus CDX2, to erroneously move to the inside.

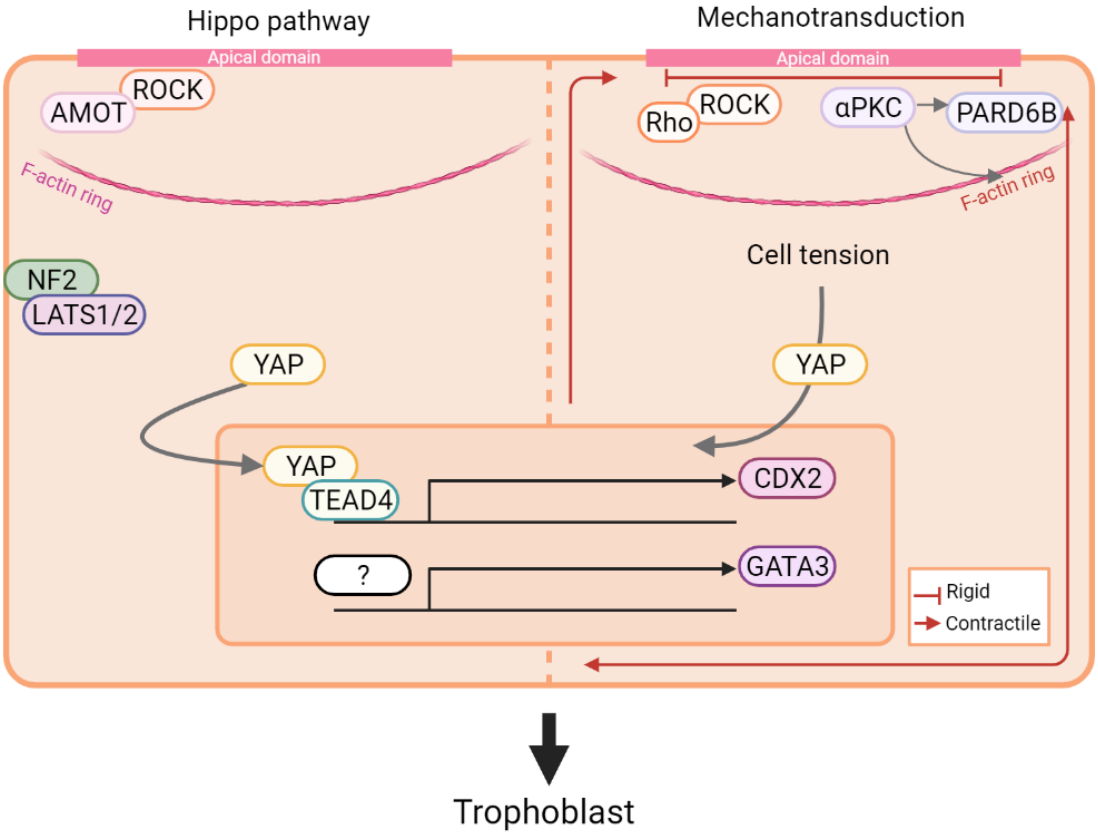

Figure 18 Balance of Hippo pathway and mechanotransduction regulation of the trophoblast lineage in cattle. *Inhibition of the Hippo pathway due to the apical restriction of AMOT by ROCK results in the nuclear translocation of YAP. The rigid apical domain, due to Rho-ROCK regulating actin-myosin interactions causes cell tension. Increased cell tension promotes the nuclear retention of YAP. Rigid and contractile regions displayed in red. CDX2 expression is activated by the YAP-TEAD4 transcriptional complex. GATA3 expression within the trophoblast is regulated by an unknown mechanism.*

4.4 Future directions

This research has allowed us to gain insight into embryogenesis that will be a helpful resource for further research. In mice, the Hippo pathway is credited for being the main regulator of cell fate, along with polarity and cell adhesion. However, this thesis highlights that while its activation and downstream targets appear to differ, YAP is integral to lineage specification. Large differences in the temporal and spatial expression of lineage-specific transcription factors, between mice and cattle, is our first clue that regulatory variation between species exists.

It would be interesting to investigate the expression and localisation of AMOT during bovine blastocyst development. Knock-out studies, similar to those conducted in mice, are required to see if AMOT has a role in Hippo pathway regulation and YAP activity in cattle. This experiment would provide further indication of the level of involvement the Hippo pathway has in establishing the TB and ICM lineages in the bovine blastocyst.

In order to get a comprehensive understanding of contractility and its role in cell lineage, F-actin and myosin will need to be researched. Visualisation of these cytoskeletal components using immunohistochemistry at all stages leading up to blastocyst formation is required. This investigation would need to include further functional analyses using the Y27632 and Go6983 inhibitors, to see whether our hypothesis that changes in contractility are responsible for the ectopic expression of YAP and CDX2 seen within the ICM. An experiment using the ROCK and αPKC inhibitor simultaneously would also shed light on whether there is overlap in these pathways that makes each individually functionally redundant or whether they both are not necessary to establish the TB lineage.

TAZ is a transcriptional co-activator that works in partnership with YAP within the nucleus to cause TEAD4-dependent expression of downstream gene targets (Zhao et al., 2008). Both TAZ and YAP mRNA transcripts have been found in the bovine embryo at all stages of blastocyst development (Sharma & Madan, 2020). The role of TAZ in bovine embryogenesis likely differs to the mouse, after Sharma and Madan (2020) found species-specific differences in the

nuclear-cytoplasmic shuttling of the protein during blastocyst development. In order to make any conclusions about the regulation of lineage specification in cattle, more needs to be known about TAZ. It is possible, that TAZ operates in a parallel, YAP-independent mechanism to establish the TB transcription profile and could be responsible for the differences in regulation of SOX2 and GATA3 seen in cattle.

YAP-TEAD4-independent regulation of SOX2 during bovine blastocyst development was a truly surprising finding during this research. Extended *in-vitro* culture technology is needed, in order to analyse how the progressive down-regulation of SOX2 occurs within the TB. Current research into the culture environment is being conducted to enable culture bovine blastocysts beyond day 8 (Isaac & Pfeffer, 2021). This is important for our understanding of bovine embryonic development due to more accurately representing the longer pre-implantation period. By continuing the research this thesis has investigated beyond the first 7 days of development, a more comprehensive understanding of species-specific regulation and commitment to lineage will be established.

4.5 Concluding remarks

Ultimately, the regulation of pre-implantation mammalian development is extremely complex due to lineage commitment likely being the result of balancing a multitude of signaling inputs. Species variability only adds to this complicated network of regulation. Evolution of species and their pre-implantation development are likely to be heavily influenced by developmental constraints such as the time pressure placed on the embryo to implant into the uterus. Use of already available signaling pathways and genetic programs could be the evolutionary strategy used by mice to enable the rapid differentiation and commitment of cells to the TB lineage. The Hippo pathway and the YAP-TEAD4 complex are an efficient way of regulating the unique, mutually exclusive transcription profiles required for the TB and ICM lineages. This strategy utilises the position and polarity of cells as they undergo early cleavage events in order to generate a distinct transcription profile suitable for implantation.

Cattle have much more time to develop prior to implantation and therefore, in the absence of time pressure have established a more intricate method of lineage commitment. The function of the YAP-TEAD4 transcriptional complex in controlling CDX2 expression is the only conserved mechanism of developing the unique lineage profiles we have found evidence of in this research. Both GATA3 and SOX2 transcription factors are regulated by alternative mechanisms to those used in the mouse. However, despite their delayed restriction to the TB and ICM tissues through alternative regulation, their eventual restriction suggests their roles in regulating lineage at later stages are conserved.

This research has highlighted the importance of species-specific embryonic development studies. The differences in transcriptional regulation highlighted in this research will be extremely important for future studies of bovine pre-implantation development, as well as research into establishing bovine ESCs.

Chapter 5: Appendices

Stocks, solutions & media recipes:

M199 x2 stock	
M199 powder (Sigma M5017)	1 Sachet
dH_2O	500 ml
Kanamycin (Sigma K1377)	63.35 mg

Stock B (250 mM)	
Sodium Bicarbonate (Sigma S576)	525 mg
dH_2O	25 ml

Stock C (33 mM)	
Pyruvic Acid (Sigma P4562)	65 mg
dH_2O	18 ml

Stock H (250 mM Hepes)	
Hepes Free Acid (Sigma H6147)	1500 mg
Hepes Sodium Salt (Sigma H3784)	1625 mg
dH_2O	50 ml

BSA 20%	
BSA (ICPbio low endotoxin ABRE)	4.0 g
0.9% Saline	20 ml

B199 (25 ml)	
2 x M199	12.5 ml
Stock B	2.5 ml
Stock C	150 ul
dH_2O	9.85 ml

H199 (400 ml)	
2 x M199	200 ml
Stock H	24 ml
Stock B	8 ml
dH_2O	168 ml

Aspiration media	
H199	48.9 ml
Heparin	93 ul
BSA (20%)	1 ml

H199 + 10% FCS	
H199	9 ml
FCS	1 ml

B199 + 10% FCS	
B199	9 ml
FCS	1 ml

Heparin 5000 iu/ml	
Heparin	47.17 mg
dH_2O	2 ml

Pyruvate (1 mM)	
Pyruvate (Sigma P4562)	275 mg
dH_2O	2.5 ml

Cysteamine (10 mM)	
Cysteamine (Sigma M6500)	~10 mg
B199 + 10% FCS	mls = weight/1.136

Hypotaurine (1 mM)	
Hypotaurine (Sigma H3506)	~6 mg
0.9% Saline	mls = weight/0.218

Penicillamine (2 mM)	
Penicillamine (Sigma P4875-1G)	~5 mg
0.9% Saline	mls = weight/0.5968

Penicillamine/Hypotaurine stock	
Penicillamine	Equal volumes
Hypotaurine	Equal volumes

Ruakura supplied medias
HSOF
ESOF
LSOF
IVF media
IVM media
Bovipure
Bovidilute

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