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Generating a fluorescently tagged MARK2 fusion protein as a marker for the basolateral membrane of preimplantation stage mouse embryo blastomeres

Bachelor Thesis in Biological Chemistry

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Annotation:

The aim of this thesis was to develop a recombinant MARK2-fluorescent fusion protein construct to serve as a basolateral marker in future studies.

Declaration:

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Stefanie Pezelj

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Abstract

Mouse preimplantation development is initiated by fertilisation of an oocyte by a sperm. To become a full-grown embryo, the zygote initially has to undergo cell divisions to be able to form three different cell lineages. In the first cell fate decision the trophectoderm (TE) and the inner cell mass (ICM) are formed, the latter gives rise to the epiblast (EPI), which will contribute to the future embryo, and the primitive endoderm (PrE), which will contribute to the yolk sac. At the 8-cell stage, blastomeres become polarised with proteins enriched at the contactless apical membrane and at basolateral membranes in contact with other blastomeres. Such blastomeres can either divide symmetrically, whereby two identical outer polar cells are generated, or asymmetrically, where one inner apolar and one outer polar cell are established. Inner cells give rise to ICM, while outer yield TE. Active Hippo signalling is relevant for inner apolar cells because it promotes phosphorylation of the transcriptional co-factor YAP1, whereas in the outer cells YAP1 stays non-phosphorylated, leading to differences in transcriptional regulation and segregation of the two lineages. Since MARK2 is localised to the basolateral part of the membrane and is important for LATS1/2 kinase activity, which regulates phosphorylation of YAP1, it could be used as an effective basolateral membrane marker, or general polarity establishment marker in mouse preimplantation development. Therefore, the aim of this thesis was to generate a fluorescently tagged MARK2-Venus fusion protein through cloning a *Mark2* gene cDNA into a pRN3-vector bearing a fluorescent Venus tag, that could then be used to generate a MARK2-Venus encoding mRNA, via *in vitro transcription* for embryo microinjection. After successful mRNA microinjection into 2-cell stage embryos, confocal microscope imaging was conducted and confirmed the expression of the MARK2-Venus fusion protein and its usefulness as a basolateral membrane marker to support future studies concerning the establishment of cell fate and apico-basolateral polarisation in preimplantation stage mouse embryos.

List of Abbreviations

AMOT	Angiomotin
ATP	Adenosine triphosphate
CDX2	Caudal Type Homeobox 2
DAPI	4′,6-diamidino-2-phenylindole
E-cadherin	Epithelial cadherin
EMK	ELKL Motif Kinase
EOMES	Eomesodermin
EPI	Epiblast
F-actin	Filamentous actin
hCG	Human chorionic gonadotrophin
ICM	Inner cell mass
IVT	In vitro transcription
LATS	Large Tumour Suppressor Kinase
LB Medium	Lysogeny broth medium
MAP	Microtubule-associated proteins
MARK	Microtubule-affinity- regulating-kinase
mRNA	Messenger RNA
MT	Microtubule
Nf2	Neurofibromin 2
PAR	Partitioning defective proteins
PBS(-T)	Phosphate-buffered saline (with Tween20)
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PrE	Primitive endoderm
RNAi	Interference RNA
ROCK	Rho associated coiled-coil containing protein kinase
SOX2	Sex Determining Region Y-Box 2
TAE-Buffer	TRIS-Acetate-EDTA-Puffer
TE	Trophectoderm
UTR	Untranslated Regions
YAP	Yes-Associated Protein
ZGA	Zygotic genome activation
ZP	Zona pellucida

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1 Introduction

1.1 Mouse preimplantation development

The moment of fertilization of an oocyte by a sperm is the starting point of the embryonic development. The fertilization results in a zygote which undergoes a series of cleavage divisions, a process of cell proliferation where no overall volume change of the embryo occurs (Brigid Hogan, 1994; Aiken *et al.*, 2004; Mihajlović and Bruce, 2017). Before implantation into the uterus after 4.5 days, the first two of the seven cell cycles (seen in Figure 1) are about 18 hours long and the others are somewhat shorter, about 12 hours (Brigid Hogan, 1994; Johnson, 2009). Until the major transcriptional activation of zygotic genome (ZGA) occurs during the 2-cell stage, development of the embryo is almost exclusively dependent on maternal transcripts and proteins synthesised during oogenesis and stored in the oocyte (Schultz, 1993; Brigid Hogan, 1994; Aoki *et al.*, 1997; Mihajlović and Bruce, 2017). At the point of major ZGA, relevant embryonic genes become transcriptionally active, while maternal mRNAs are degraded. Nevertheless, maternal proteins and a few mRNAs can persist for longer (Johnson and McConnell, 2004; Mihajlović and Bruce, 2017).

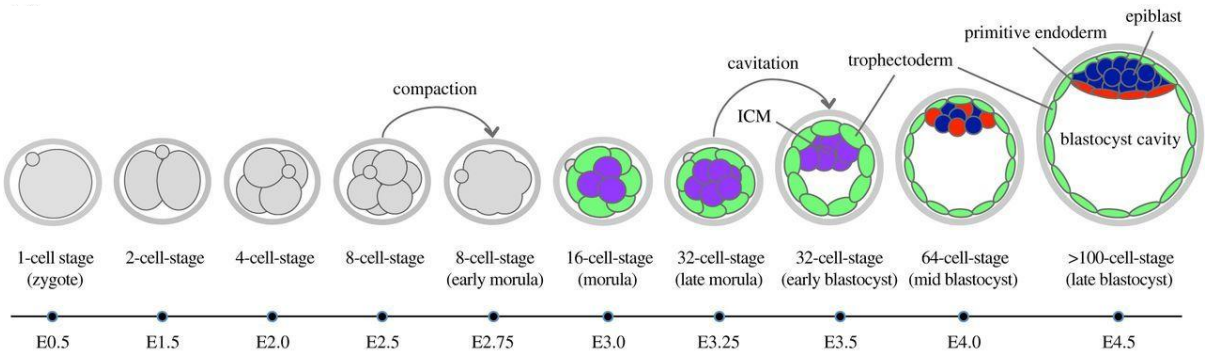

Figure 1: Time period of preimplantation development of mouse embryo with relevant cell cycles and cell lineages implicated by the first and second cell fate decision (taken from Mihajlović and Bruce, 2017).

During the 2-cell stage, if one of the two cells is experimentally destroyed by external forces, the other cell will compensate for this loss and the development will continue, resulting in a normal adult animal. This example shows one of the most distinguishing features of early mammalian development: the plasticity of the early embryo which allows it to adapt to external perturbations, however, with progressing development of the embryo this plasticity is gradually lost (Suwińska *et al.*, 2008; Mihajlović and Bruce, 2017; Posfai *et al.*, 2017).

By embryonic day 4.5 (E4.5, 4.5 days after fertilization), the late blastocyst hatches from its surrounding glycoprotein layer, called *zona pellucida* (ZP), to then implant within the uterine epithelium (Zernicka-Goetz *et al.*, 2009; Fujimori, 2010; Jedrusik, 2015). Such blastocyst consists of three cell lineages, two extraembryonic and one embryonic, essential for successful implantation and further post-implantation embryonic development (seen in Figure 2). On the surface of the embryo, there is an epithelial mono-layer of differentiating trophectoderm (TE) cells which will form the extraembryonic ectoderm being in direct interaction with uterine epithelial cells and therefore ensure contact between the embryo and the mother through the formation of the placenta. Inside the blastocyst embryo, there is another epithelialized differentiating extraembryonic tissue layer in direct contact with a fluid filled cavity, called the primitive endoderm (PrE), that will later develop into the parietal endoderm and visceral endoderm of the yolk sac, and the pluripotent epiblast (EPI) cells that will later differentiate into the all embryonic lineages and cell types (Johnson and Ziomek, 1981; Rossant and Tam, 2009; Zernicka-Goetz *et al.*, 2009; Fujimori, 2010).

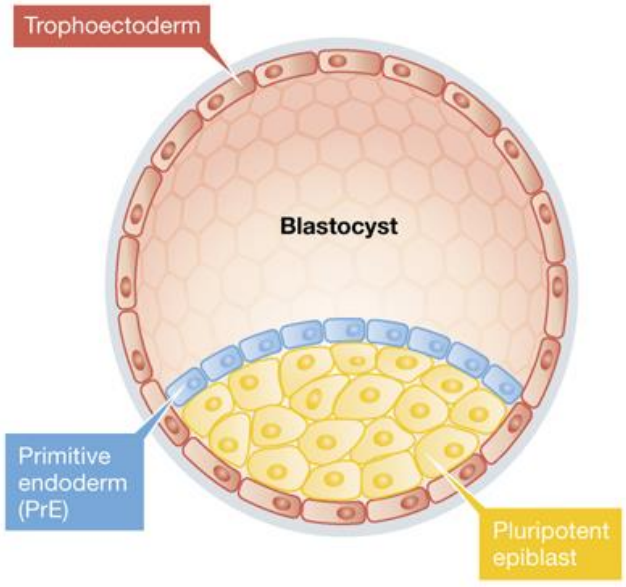

Figure 2: Schematic representation of a blastocyst. Showing the epithelium trophectoderm in red, pluripotent epiblast in yellow and primitive endoderm in blue (taken from AlFatah Mansour and Hanna, 2013).

The segregation of the three cell lineages occurs in the two cell-fate decisions which appear not to be fully functionally independent on each other. Although the molecular mechanisms are not fully understood, the processes playing a role in this segregation involve cell compaction and polarization at 8-cell stage, cell position on the outside or inside the embryo from 16-cell stage, signalling and lineage-specific expression or activity of transcription factors (such as TEAD4, CDX2, NANOG, *etc.*) and co-factors driving the expression of

downstream lineage-specific genes (Johnson, 2009; Fujimori, 2010; Wicklow *et al.*, 2014; Mihajlović *et al.*, 2015).

1.1.1 The first cell fate decision

The first cell fate decision in the mouse preimplantation timeline takes place between the 8- and 32-cell stage and leads to the segregation of two distinct cell populations according to their position within the embryo. The cells on the outside constitute the extraembryonic TE lineage and the cells that are positioned in the inside form the inner cell mass (ICM), which will later contribute to both EPI and PrE lineages (Johnson and Ziomek, 1981; Fleming, 1987; Morris *et al.*, 2010; Anani *et al.*, 2014; Bedzhov *et al.*, 2014). These inner and outer cells are generated during the consecutive 8- to 16-cell and 16- to 32-cell divisions (Dard *et al.*, 2009; Zernicka-Goetz *et al.*, 2009). ICM cells establish pluripotency gene networks regulated by transcription factors such as SOX2 and NANOG, while the outer cells promote the expression of TE promoting transcription factors such as CDX2 and EOMES, which supress the pluripotency (see Figure 3). This leads to differentiation of the outside cells into TE (Jedrusik *et al.*, 2008; Ralston and Rossant, 2008; Nishioka *et al.*, 2009; Zernicka-Goetz *et al.*, 2009).

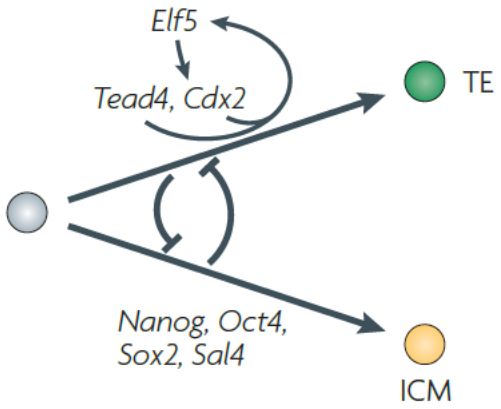

Figure 3: Transcription factors promoting trophectoderm lineage and influencing suppression of inner cell mass and vice versa (taken from Zernicka-Goetz et al., 2009).

Until the early 8-cell stage, individual round-shaped blastomeres are easy to distinguish due to a comparative lack of cell-to-cell adhesions, they are all conceptually on the outside of the embryo (each having a cell contactless domain) and they lack any intra-cellular molecular polarity. Prior to the first cell-fate decision, 8-cell stage embryos have to undergo compaction and polarization, which are the requirements for establishment of differential molecular marker gene expression in inner and outer cells, after cell division (Ziomek, 1980; Johnson, 1981; Sasaki, 2010; Johnson and Ziomek, 1981; Johnson *et al.*, 1979).

1.1.2 Compaction and polarisation

Compaction is the first morphological event in the preimplantation development of the embryo. Increased cell-to-cell adhesion is established and cell boundaries are no longer visible from the outside (Anani *et al.*, 2014; White *et al.*, 2016). At the cell-to-cell contact sites, adherens junctions are formed. The key cell adhesion molecule is epithelial cadherin (E-cadherin), an essential component of adherens junctions (Oda and Takeichi, 2011). It functionally links compaction with the onset of intra-blastomere polarization as changes in its localisation during compaction result in the establishment of so called apico-basolateral polarity (Sefton *et al.*, 1992; Mihajlović and Bruce, 2017).

Intra-cellular polarization results in the organisation of distinct membrane domains; an apical domain on the surface of the embryo without contacts with other cells, and basolateral domain inside the embryo with cell-to-cell contacts. These two distinct membrane domains are a classical feature of the epithelial tissue (Brigid Hogan, 1994; Johnson and McConnell, 2004; Chazaud and Yamanaka, 2016); albeit with the reverse orientation in the embryo (*i.e.* the apical domain facing the outside, rather than internally). The apical domain is enriched for microvilli, structural protein F-actin, an apical protein complex with PAR3, atypical protein kinase C, Rho GTPase activity and microtubule dynamics (Plusa *et al.*, 2005; Yamanaka *et al.*, 2006; Alarcon, 2010; Chazaud and Yamanaka, 2016). For the formation of the apical domain the PAR3 complex plays a crucial role (Houliston *et al.*, 1989; Clayton *et al.*, 1999; Kono *et al.*, 2014). The process of polarisation ends after 3-5 hours when the apico-basolateral axis is established (Johnson and Ziomek, 1981). Importantly, the axis of polarity can be changed experimentally through the change of contact patterns, demonstrating that the establishment of such polarity is not stable but dynamic (Janet Rossant and Roger A. Pedersen, 1986).

The first inner cells are generated during the 8- to 16-cell division. 8-cell stage blastomeres, with their associated apical and basolateral domains, can divide either symmetrically or asymmetrically (seen in Figure 4). Symmetric divisions result in the generation of two outer residing daughter cells, each with an apical and basolateral domain (*i.e.* exhibiting polarity), while inner cells are generated through asymmetric divisions, when only one cell remains on the outside of the embryo, thus inheriting the apical domain and remaining polarised, while the other daughter and inner residing cell inherits only the basolateral domain and becomes apolar (Bischoff *et al.*, 2008; Bedzhov *et al.*, 2014; Krupa *et al.*, 2014; Chazaud and Yamanaka, 2016). Inside cells are generated from outside residing parental cells during the 8-

to 16-cell and 16- to 32-cell divisions, but not *vice versa*. (Graham and Deussen; Johnson and Ziomek, 1981; Pedersen *et al.*, 1986; Fleming, 1987; Sasaki, 2010). In addition, during 16- to 32-cell transition, inner cells can be generated by the division of pre-existing inner cells generated during the previous 8- to 16-cell cleavage (Mihajlović and Bruce, 2017). As a consequence of these cell divisions, the blastomeres acquire either a relative inside or an outside position. The apolar cells that are found inside are completely surrounded by polar outside cells, that remain polar by being in contact with the external environment (Fleming, 1987; Sasaki, 2010).

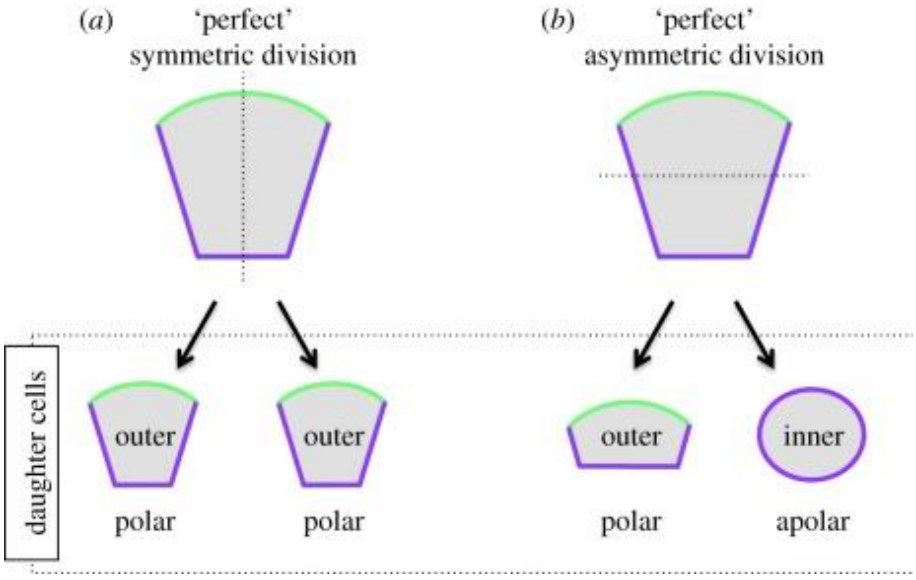

Figure 4: Ideal cell division showing (a) Symmetric cell division having its cleavage plane parallel to the apical–basolateral polarity axis. This cleavage results in two identical polar outer cells. (b) Asymmetric cell division having its cleavage plane orthogonal to the apical–basolateral polarity axis. This cleavage results in one polar outer and one apolar inner cell (taken from Mihajlović and Bruce, 2017).

1.1.3 Hippo signalling and its role in first cell fate decision

First discovered in *Drosophila*, the Hippo signalling pathway plays an important role in regulating many biological processes, including cell proliferation, survival, differentiation, and organ size control (Dong *et al.*, 2007; Zhao *et al.*, 2007; Ota and Sasaki, 2008; Harvey *et al.*, 2013).

In contrast to the outer cells in preimplantation mouse embryos, Hippo signalling is highly active in the inner cells. The activity of the Hippo signalling is regulated by cell–cell contact and in apolar inner cells the Hippo activator Angiomotin (AMOT) localizes at adherens junctions and thus promotes YAP1 phosphorylation by LATS1/2 kinases. However, interactions between AMOT and F-actin, within the intra-cellular apical domain of polarised

outer cells caused suppression of this active Hippo signalling cascade, ensuring YAP1 remains non-phosphorylated (Hirate *et al.*, 2013; Hirate and Sasaki, 2014).

As the phosphorylation status of YAP1 effects its sub-cellular localisation, an important symmetry-breaking event is initiated. This is typified by unphosphorylated YAP1, in polarised outer cells, being able to localise inside the nucleus, whilst phosphorylated YAP1 in apolar inner cells is retained in the cytoplasm. As YAP1 is an essential transcriptional co-activator of the transcription factor TEAD4 (that is expressed in the nuclei of all cells of the embryo, irrespective of their relative spatial location) such differential localisation of YAP1 allows the transcription of TE specific genes in outer polarised cells but prevents their ectopic expression in apolar inner cells, and as such promotes the expression of genes required to maintain pluripotency (Yagi *et al.*, 2007; Zhao *et al.*, 2007; Nishioka *et al.*, 2009; Sasaki, 2010).

1.1.4 Second cell fate decision

As referenced earlier, three different cell types are present in the mouse embryo immediately after implantation. The EPI lineage will give rise to the future embryo/ foetus and the extraembryonic mesoderm. The TE lineage will contribute to the trophoblast cell types that will mostly populate the future placenta and will connect the embryo with the maternal circulation. The ICM cells in contact with the blastocyst cavity form the PrE, from which the yolk sac and some limited endodermal structures of the future embryo/ foetus will form. Importantly, the extraembryonic TE and PrE lineages are absolutely required for the growth of the mammalian foetus in the uterine wall and also act as signalling initiation centres responsible for axial patterning to the EPI (Chazaud *et al.*, 2006; Zernicka-Goetz *et al.*, 2009; Krupa *et al.*, 2014; Lanner, 2014).

It had been classically proposed that differentiation of the PrE at the surface of the ICM in contact with the blastocyst fluid filled cavity was a response to positional signals emanating from the cavity. However, more contemporary models dispute the simplicity of this earlier view. This was typified by the observation of the distribution of the expression of two early molecular protein markers for the emerging PrE (GATA6) and EPI (NANOG) lineages in the maturing blastocyst ICM (Figure 5) (Zernicka-Goetz *et al.*, 2009).

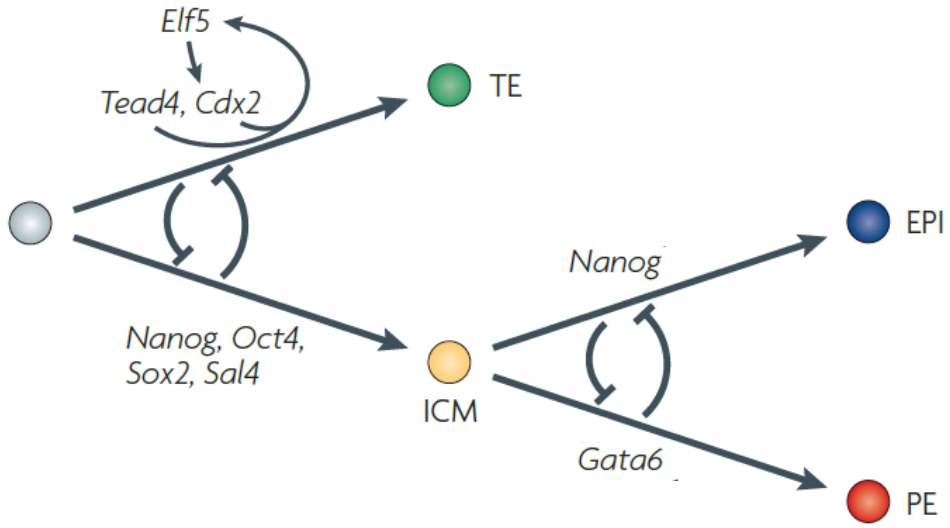

Figure 5: Transcription factor Nanog promoting epiblast lineage and influencing suppression of Gata6 and therefore of primitive endoderm (here referred to as PE), and vice versa (taken from Zernicka-Goetz et al., 2009).

Namely, their essentially random distribution throughout the ICM, in a so called 'salt- and-pepper' pattern, with exclusive PrE-specific GATA6 expression not being limited to the cavity facing surface layer of the ICM (Chazaud et al., 2006; Rossant et al., 2003). Interestingly, individual ICM cells, no matter where they are initially located within the ICM can contribute to PrE or the EPI, but rarely to both lineages, through a process of active cell movement/ sorting to the appropriate tissue layer. These (and other data) suggest that the initial specification of PrE and EPI cell fate within the ICM occur at a stage earlier than blastocyst cavity formation/ expansion (Chazaud et al., 2006).

1.2 Polarity-associated serine/threonine-specific protein kinases

Serine/threonine-specific kinases are enzymes that catalyse the phosphorylation of target proteins containing specific, amino acid sequence context dependent, serine or threonine residues, using ATP as a phosphate donor. Upon phosphorylation, targeted proteins can either be functionally activated or inactivated (Diallo and Prigent, 2011). In this thesis I will focus on the Rho-associated kinases (ROCK1/2) and MARK2/EMK.

1.2.1 Rho-associated kinases (ROCK1/2)

Rho-kinases (ROCK1/2) are regulated by the activity of small Rho-family G-proteins, which are associated with various cellular functions, including cell migration and adhesion, cell polarity and cytoskeletal dynamics (Matsui *et al.*, 1996; Bustelo *et al.*, 2007; Amano *et*

al., 2010). Rho small G-proteins are also known to play a role in compaction and the establishment of intra-cellular apical-basolateral polarity, during preimplantation mouse embryo development; as demonstrated by blastomere flattening and the defective onset of polarisation in 8-cell stage embryos, pharmacologically treated with Rho inhibitory compounds (Clayton *et al.*, 1999). In addition, studies have shown that alternative strategies to inhibit Rho-ROCK1/2 signalling leads to defects in the establishment of apical-basolateral polarity and thus blocks development from the morula to blastocyst stages (Kawagishi *et al.*, 2004; Kono *et al.*, 2014, Mihajlovic and Bruce 2016). Therefore, Rho plays a role in the segregation of the TE and ICM lineages during preimplantation mouse embryo development. Due to its experimental perturbation, specifically in TE cells, Rho also has a recognized role as a suppressor of the Hippo signalling cascade. It has been shown to prevent the phosphorylation of AMOT and thus stabilises its interaction with F-actin (at the apical domain of polarised outer cells) and prevents AMOT from interacting with another upstream activators of the Hippo pathway, at cell-cell contact regions enriched in adherens junctions, such as Nf2/Merlin (Shi *et al.*, 2017).

1.2.2 *Mark2* (microtubule-affinity-regulating kinase 2)

The homologues of the mammalian MARK proteins are the PAR ('partitioning defective') proteins; first discovered in *C. elegans* and which are serine/threonine kinases (Kemphues *et al.*, 1988; Goldstein and Macara, 2007). It has been discovered that PAR proteins regulate cell polarization in different ways and in a wide range of organisms, such as yeast, flies, worms and mammals. Therefore, these proteins most probably arose at a relatively early point in the evolution of animals and as such play fundamental roles in critical intra-cellular polarisation events required during early embryogenesis, *per se*; *i.e.* via the necessary asymmetric positioning of not only proteins and RNAs but also of the mitotic spindle, required to initiate necessary symmetry breaking events (Riechmann, 2004; Goldstein and Macara, 2007). For example, the PAR-1 protein in *Drosophila* regulates not only the stability and the density of microtubules, but also their apicobasal organisation. Cells that lack PAR-1, exhibit an ectopic increase in the concentration of F-actin and spectrin within their lateral domains and therefore expand the density of microtubule (MT) network. Upon over-expression in mammalian tissue cells, MARK proteins have been shown to reduce microtubule density (Doerflinger *et al.*, 2003).

The mammalian *Mark2* gene, also known as *Par1b* or *Emk1* (and other names, but in this thesis simply referred to as *Mark2*), is an equivalent of the *C. elegans Par-1* gene, and belongs

to the serine/threonine kinase family. It should be noted that in mammals there are four *par-1/Mark* kinase homologs/paralogs. These are *Mark1* (*Par1c*), *Mark2* (*Par1b*), *Mark3* (*Par1a*) and *Mark4* (*Par1d*). Since they are collectively associated with many and varied biological processes they are widely expressed in both embryonic and adult tissues. However, in many different examples of polarized tissues, MARK2 has been shown to be crucial for the formation and maintenance of intra-cellular polarity (Wu and Griffin, 2017).

MARK kinases have been shown to functionally alter the stability of MTs (Drewes *et al.*, 1997). MTs are responsible for the tracking of intra-cellular cargos and the influencing cell shape and polarity; the transition between stable and dynamic forms has been shown to be crucially important. Indeed, such transitioning is known to be regulated by many other cellular factors, such as the MT-associated family of proteins (MAPs) and tubulin-associated Tau proteins (Drewes *et al.*, 1997, 1998), that exert their effects by influencing the phosphorylation status of MTs and their associated protein factors. The MT-affinity-regulating kinases (*i.e.* MARKs), are able to phosphorylate the tubulin-binding domain of MAPs and as a result, are able to detach MAPs from MT and thus increase MTs dynamics. Therefore, MARKs have the potential to regulate microtubule stability and thus affect tissue morphogenesis and shape (Drewes *et al.*, 1998); for example in the developing preimplantation mouse embryo.

Together with the PARD6b protein, MARK2 protein has been previously observed on the mitotic spindle apparatus of dividing preimplantation mouse embryo blastomeres. Moreover, during compaction at the 8-cell stage both of these proteins begin to exhibit a differentially polarised intra-cellular redistribution, with MARK2 becoming localized along the basolateral/ cell-cell contact domain (and PARD6b at the contactless apical domain) (Vinot *et al.*, 2005).

It has also been experimentally demonstrated that the stability of MARK proteins is important in the regulation of LATS1/2 kinase activity and hence the phosphorylation of YAP1, during the activation of the Hippo signalling cascade in apolar inner cells (Nguyen *et al.*, 2017). As such, it has been postulated by ourselves and others (Gon *et al.*, 2013) that MARK2 would be an effective basolateral membrane marker in the preimplantation mouse embryo.

2 Aims

The principle aim of this thesis was to create a recombinant MARK2-fluorescent fusion protein construct, that could be expressed in the early/ preimplantation mouse embryo, to facilitate on-going studies relating to the establishment and maintenance of intra-cellular apico-basolateral polarisation and cell-fate.

3 Materials and Methods

All reagents used were sourced from Sigma, unless otherwise stated, and were of analytical grade. If not differently stated, HPLC water was used in all experiments and is simply referred to as water.

3.1 Cloning of *Mark2/Par1* gene cDNA into pRN3-Venus plasmid

3.1.1 Generation of *Mark2/Par1* insert for cloning

To generate a full length *Mark2/Par1* cDNA sequence, PCR was performed with high fidelity KOD polymerase (Millipore) and primers complementary to the start and end of the coding sequence of the *Mark1/Par1* gene, and with extra sequences to incorporate *NheI* restriction sites (at each end) and spacer/ stuffer sequences to assist subsequent restriction enzyme digestion (Table 1). cDNA generated previously in the lab from testis total RNA was used as a template. Therefore, a reaction mixture and a negative control, each having a total volume of 25µL, were prepared (Table 2) and PCR was performed using the cycling conditions listed in Table 3. Cycle steps 2-4 were repeated 37 times.

Table 1: Primers used for PCR reaction (showing the spacer highlighted in turquoise, the NheI restriction site in pink and the start codon in yellow; since the Venus sequence is supposed to be translated together with the Mark2 sequence there is no stop codon)

Primer	Sequence	Company
Primer S	gactatGCTAGCATGTCCAGCGCTCGGACCC	Sigma Aldrich
Primer A	GACTATgctagcAAGCTTCAGCTCATTGGCTATTTTG	Sigma Aldrich

Table 2: PCR reaction mixture

Volume (in µL)	Reagent	Concentration
2.5	KOD Buffer	10x
2.5	$MgSO_4$	2.50mM
2.5	dNTPs	0.25mM
0.75	Primer A	0.30µM
0.75	Primer S	0.30µM
0.25	KOD Hot Start DNA Polymerase (Millipore)	1U
15.25	H_2O	
0.5	cDNA	738ng

Table 3: PCR reaction cycle used for amplification of Mark2 cDNA insert

Step	Temperature (in ^oC)	Time (in seconds)
1.denaturation	95	120
2.denaturation	95	20
3.annealing	60	10
4.elongation	70	120
5.extension	70	600

To check whether the PCR-reaction worked, 2µL of each the reaction and the negative control were electrophoresed on an 0.9% agarose gel. The PCR product, of the confirmed and correct anticipated size, was then purified using conventional phenol-chloroform extraction protocols, as follows. The chilled PCR-product was mixed with 250µL H_2O and combined and then vortexed with 300µL TRIS-saturated phenol-chloroform (pH 8.0), followed by 5 minute centrifugation at 4°C at 16000 rpm (full speed). The aqueous phase was transferred to another tube, to which an equal volume (300µL) of chloroform was added, vortexed and

centrifuged for an additional 5 minutes at 4°C at full speed. The aqueous phase was again carefully transferred into a clean Eppendorf tube and a tenth volume of (30µL) 3M sodium acetate (pH 5.2), 750µL of 100% ethanol and 2µL glycogen solution, for better visibility of the DNA-pellet, were added and then vortexed before being left overnight at -20°C. After centrifuging for 30 minutes at 4°C and full speed the supernatant was removed, and the precipitated DNA pellet was washed twice with 70% Ethanol (500µL) and allowed to air-dry at room temperature, before being dissolved in 20µL H_2O.

3.1.2 Insert and vector restriction enzyme digestion, vector alkaline phosphatase treatment and insert to vector ligation

The purified *Mark2* cDNA PCR product was digested with the restriction enzyme *NheI* (NEBiolabs), whereby a reaction mixture of 50µL total volume was prepared (Table 4). The digestion was then performed for 3 hours at 37°C followed by a 20 minutes incubation at 65°C.

Table 4: Restriction Enzyme digestion of Mark2 cDNA with Nhe1 Enzyme

Volume (in µL)	**Reagent**
20	Insert
24	H_2O
5	Buffer 2.1 (10x)
1	*NheI Enzyme*

Under the same conditions, the pRN3-Venus vector was digested having a total volume of 50µL, as can be seen in Table 5.

Table 5: Restriction Enzyme digestion of pRN3-Venus vector with Nhe1 Enzyme

Volume (in µL)	**Reagent**
3	pRN3-Venus vector (~1.3µg)
41	H_2O
5	Buffer 2.1 (10x)
1	*NheI Enzyme*

This pRN3-Venus vector seen in Figure 6 has a fluorescent tag, the Venus cDNA, already cloned inside (denoted as 'FP' in the figure). Via the *NheI* restriction site, the *Mark2* sequence was designed to be introduced, in-frame, upstream of the Venus cDNA, resulting in a C-terminally fluorescently-tagged *Mark2*-C-terminal-Venus fusion gene. To ensure mRNA construct stability, the vector has 5' and 3' UTR sequences from frog β-globin gene incorporated flanking the multiple cloning site.

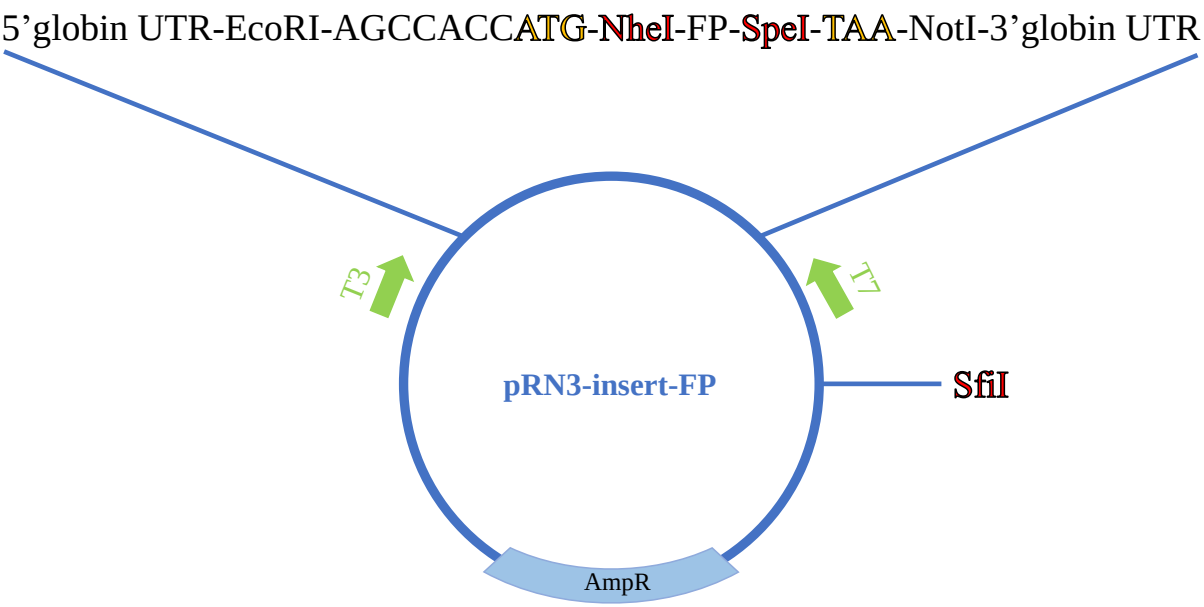

Figure 6: Plasmid map of pRN3 showing start and stop codon in yellow; T3 and T7 promotor in green; NheI, SpeI and SfiI restriction sites in red (where SfiI was used for linearisation); and ampicillin reistant gene denoted as AmpR.

For the purification of the *NheI* digested insert and vector a PCR purification kit (Qiagen) was used. Hence, the ~50 µL of the product from restriction enzyme digestions were each mixed with 250µL Buffer PB, introduced into the column and centrifuged for a minute at room temperature and full speed. After removing the supernatant, 750µL of Buffer PE was added for washing and the column was centrifuged twice at room temperature for a minute at full speed, discarding the flow through each time. Upon addition of 30µL of H_2O on the membrane, the vector was then eluted into a clean Eppendorf tube, by incubating the wetted columns at room temperature for 1 minute and subsequent centrifugation.

To prevent self-ligation of the now linearised *NheI* digested vector the phosphates at the open ends were removed by alkaline phosphatase treatment (Roche), according to manufacturer's instructions; before conducting a phenol-chloroform purification of the vector, as explained above. For the alkaline phosphatase treatment, a 50µL reaction was set up (Table 6) and incubated for 1 hour at 37°C.

Table 6: Alkaline phosphatase treatment of pRN3-Venus vector

Volume (in µL)	Reagent
30	NheI digested pRN3-Venus vector
14	H_2O
5	Buffer 10x
1	Alkaline phosphatase

The *NheI* digested vector/insert ligation reaction was set up as described in Table 7; however the precise concentration of the *NheI* digested insert and vector (pRN3-Venus) was first measured by UV-spectroscopy (using a 'Nanodrop' apparatus). The required molar quantities of prepared insert and vector (3:1 ratio) were then calculated using the online available NEBio calculator tool (https://nebiocalculator.neb.com/#!/ligation). The ligation and negative control (comprising the prepared vector minus the insert) reactions were prepared at room temperature before being placed at 4^{o}C overnight.

Table 7: Ligation reaction mixture

Volume (in µL)	Reagent
1.2	Vector (starting concentration: 40.2 ng/µL)
15.8	Insert (starting concentration: 5.9 ng/µL)
2	10x Ligase Buffer (Roche Diagnostics)
1	T4 DNA Ligase (Roche Diagnostics)

3.1.3 Bacterial transformation

Next, bacterial transformations were conducted by mixing 5µL each of experimental ligation reaction and negative control with 45µL of in-house prepared chemically competent *E. coli* cells (DH5α strain), cooling on ice for 30 minutes before heat-shocking the cells by a 90 second incubation in a water bath set to 42°C. Heat shocked cells were immediately placed back on ice for an additional 10 minutes before 250µL of pre-warmed (37^{o}C) SOC-medium was added and mixed. The cells were then left to recover and initiate antibiotic resistance gene

expression (assuming they had taken up the (un)ligated vector) by being shaken at 600 rpm at 37°C for 30 minutes. The cells were then pelleted by brief centrifugation and the majority of the supernatant removed before the cell pellet was resuspended in residual SOC media. The resulting liquid suspension was then spread over LB agar plates that contain ampicillin (100 µL/mL) and incubated at 37°C overnight. On the following day, a selection of colonies was re-streaked (and numbered) onto a new plate, and this was further incubated at 37°C for 10 hours before being put to 4°C for storage.

3.1.4 Colony PCR (identifying which transformants contain the *Mark2* specific cDNA)

For each eight colonies that were streaked (and numbered) on a replica LB ampicillin containing agar plate (see above), some bacteria were transferred on the end of a pipette tip to 9.5µL of the colony PCR reaction mixture (see Table 8 for master-mix preparation); note a negative control in which no colony derived bacteria were transferred, was also conducted.

Table 8: Reaction mixture of colony PCR

Volume (in µL)	Reagent
50	Ampigene Taq mix
4	Primer A
4	Primer S
40	H_2O

Note that the PCR reaction mixture contained a primer pair recognising the T_3 RNA-polymerase promoter sequence (primer S) in the plasmid vector (5’ to the insert site, the *NheI* sequence – see Figure 6) and the 3’ end of the potentially cloned *Mark2* cDNA fragment (primer A). These insert specific primers (Table 9) only work if the insert of interest is already cloned into the vector and if it is in the correct orientation, *i.e.* with the start codon to the 5’ side and the stop towards the 3’ end on the correct strand. Hence, the PCR reaction identifies those transformants of pRN3-Venus plasmids having the required gene insert incorporated cloned in the appropriate orientation.

Table 9: Primers used for colony PCR reaction

Primer	Sequence	Company
Primer S	GACTATgctagcAAGCTTCAGCTCATTGGCTATTTTG	Sigma Aldrich
Primer A	gactatGCTAGCATGTCCAGCGCTCGGACCC	Sigma Aldrich

The PCR cycling employed conditions are shown in Table 3. Agarose gel electrophoresis was performed to check for the presence of a PCR product of the correct length for each streaked colony that would be indicative of the correctly cloned *Mark2* cDNA sequence insert. Lanes positive for such products were then cross-referenced back to the streaked colony replica plate to identify the original *E-coli* clones that contains the *Mark2* cDNA correctly cloned into the pRN3-Venus vector.

Consequently, two colonies were picked for preparation of bacterial glycerol stock solutions (to store the live cell clones at -80ºC indefinitely) and to inoculate LB ampicillin (100 µL/ml) containing medium (4mL), that was the incubated, with shaking (600 rpm) at 37°C overnight (to allow amplification of the *Mark2* cDNA containing pRN3-Venus plasmid and thus enable subsequent plasmid extraction and purification; permitting the clone to be sequence verified, using conventional Sanger-based sequencing and to be used as template during *in vitro* transcription/IVT – see below).

3.1.5 pRN3-Mark2-Venus plasmid purification

For plasmid DNA purification a small-scale plasmid isolation kit (QIA-Quick Miniprep – Qiagen) was used. Firstly, the 4mL overnight cultures of *Mark2* cDNA containing pRN3-Venus plasmid containing bacteria were pelleted by centrifugation at 16000 rpm (4°C) for 1 minute before removing the supernatant. Secondly, the cell pellet was resuspended in 200µL of buffer P1 before 200µL of cell lysis buffer P2 was added and the mixture was gently mixed by rocking. Successful lysis was indicated by the solution acquiring a blue colour. Next, 300µL of pH neutralising buffer P3 was added, gently mixed and then incubated for 10 minutes on ice (to allow the precipitation of the genomic DNA with other non-DNA cellular components). The neutralised cell extract suspension was then centrifuged (4°C) at 16000 rpm for 10 minutes. The resultant and cleared supernatant was then transferred into a prepared silica matrix containing micro-centrifuge column and spun through at room temperature by centrifugation (8000 rpm, for 1 minute); the eluate was reapplied to the column

and the process repeated before the eluent was discarded. Two times 500µL of provided wash buffer were then similarly washed through the column to remove non-DNA impurities, before the empty column was similarly spun to remove trace amounts of wash buffer. The silica matrix bound purified DNA was eluted from the column by the addition of 25µL of H_2O directly to the matrix surface, followed by a 2 minute room temperature incubation and then 1 minute spin (16000 rpm) in the centrifuge to collect the purified plasmid DNA enriched eluent into a fresh 1.5mL Eppendorf tube. After measuring the concentrations by Nanodrop UV spectroscopy, the insertion of Mark2 cDNA and the insert sequence was confirmed by Sanger sequencing (by an external commercial firm).

3.2 Generation of Mark2-Venus mRNA

3.2.1 Plasmid linearisation

A restriction enzyme digestion was performed on the now confirmed *Mark2* cDNA containing pRN3-Venus plasmid, in order to introduce one double strand break (downstream of the cloned Mark-Venus fusion cDNA – see Figure 6) and to linearise the plasmid in readiness for *in vitro* transcription (IVT - see below). A total reaction volume of 20µL was prepared as described in Table 10 and the digest was incubated at 50°C for 2 hours 30 minutes.

Table 10: Restriction Enzyme digestion of pRN3/pBase with Sfi 1 Enzyme

Volume (in µL)	**Reagent**
6	Plasmid (concentration: 430 ng/µL)
2	Cut Smart Buffer
1	*SfiI Enzyme* (NEBio)
11	H_2O

On completion of the digest, an agarose gel electrophoresis of 1µL of the reaction mixture was performed to confirm the migration of cut plasmid against the same quantity of undigested plasmid, thus confirming successful linearisation. As described above, confirmed linearised plasmid was purified by classical phenol-chloroform extraction and ethanol precipitation and the resulting pure pellet was resupended in 6.5µL of nuclease free H_2O.

3.2.2 *In vitro* transcription

In vitro transcription (IVT) of previously linearised pRN3-Venus plasmid containing *Mark2* cDNA insert (2µg), was performed as instructed by the manual of the Message Machine T3 kit from Ambion. Therefore, a reaction mixture was prepared as described in Table 11 and was incubated at 37°C for 4 hours. The IVT reaction was then pulse spun and 1µL of Turbo DNAseI (from the Ambion DNA-free Kit - Invitrogen) added prior to a further incubation of 20 minutes at 37°C (to remove plasmid derived template DNA).

Table 11: In vitro transcription reaction

Volume (in µL)	Reagent
6	linearised pRN3 plasmid
2	10x T3 Buffer
2	T3 RNA Polymerase
10	2x NTP-cap mix

3.2.3 Poly-A+ tailing (IVT transcript poly-adenylation)

DNAseI digested IVT derived mRNA (see above) was then subject to a further *in vitro* poly-A+ tailing reaction (although an aliquot was retained for later agarose gel electrophoresis prior to preparing the reaction), according to the kit manufacturers protocols (Ambion), as detailed in Table 12. The reaction mixture was prepared at room temperature and then incubated at 37°C for 1 hour. An agarose gel electrophoresis of a sample of the poly-A+ tailing product was performed, alongside the negative control aliquot retained prior to the reaction, to confirm successful poly-adenylation. The *in vitro* derived transcript was then purified by the standard phenol-chloroform extraction and ethanol precipitation methodology and resuspended in a volume of 12µL of nuclease-free H_2O. Note, that IVT products were also derived that were not subject to poly-A+ tailing and both variants were aliquoted and stored for subsequent embryo microinjection.

Table 12: Poly-A+tailing reaction set up

Volume (in µL)	Reagent
20	IVT derived mRNA
36	nuclease-free H_2O
20	5x E-PAP Buffer
10	$MnCl_2$
10	10mM ATP
4	E-PAP

3.3 Embryo cultivation and microinjections

8-9-week old F1 hybrid female (CB57Bl6 x CBA/H) mice were super-ovulated by intra-peritoneal injection of 7.5IU of pregnant mare serum gonadotrophin extract (Sigma Aldrich) and 7.5IU of recombinant human chorionic gonadotrophic (hCG) hormone (Sigma Aldrich) 48 hours later. Following administration of the hCG treatment, the females were immediately placed with F1 stud males for overnight mating. 2-cell stage embryos were recovered from the dissected oviducts of super-ovulated and mated females into in-house prepared M2 medium seen in Table 13, ~42 hours post hCG treatment. Recovered 2-cell stage embryos were then subject to microinjection to introduce the recombinant mRNA encoding MARK2-Venus fusion protein (derived by IVT ± poly-A+tailing – see above) into individual blastomeres, as discussed below. Post-microinjection, embryos were then transferred into commercially available KSOM growth media (Embryo-Max; Millipore) and washed through a series of media drops (~20µL) each, to remove trace amounts of M2 collection media. Washed embryos were then cultured in similar KSOM drops overlaid with mineral oil in 35mm tissue culture plates in a 5% CO_2 containing atmosphere at 37°C until E3.5.

Table 13: M2-medium

Ingredients	Concentration (in g/L)
Bovine serum albumin (BSA)	4.000
Calcium chloride dihydrate	0.252
D-(+)-Glucose	1.000
HEPES	5.004
Magnesium sulphate heptahydrate	0.293
Penicillin G sodium salt	0.060
Potassium Chloride	0.356
Potassium dihydrogen phosphate	0.162
Sodium bicarbonate	0.336
Sodium Chloride	5.552
Sodium DL-lactate solution	2.521
Sodium pyruvate	0.036
Streptomycin sulphate salt	0.050

2-cell stage embryos were subject to the microinjection of mRNA encoding MARK2-Venus fusion protein into individual blastomeres (*i.e.* a single blastomere or both) using an experimental apparatus set up comprised of an inverted fluorescence microscope (OLYMPUS IX71) with manual micromanipulators, a FemtoJet (positive pressure) microinjection machine (Eppendorf) joined to a preformed microinjection capillary needle (by means of a holding electrode), an embryo holding pipette (also mounted to a holding electrode) and a negative capacitance generator/ voltage regulator (WPI) used to pass a membrane depolarising current between the injection needle and the holding pipette (via M2 media on the stage mounted watch-glass/ concaved slide) and designed to allow the microinjection needle to pass the cell plasma membrane with minimal resistance. Hence after collection, recovered 2-cell stage embryos they were transferred to a M2 drop overlaid with mineral oil on a concaved microscope slide that was then placed on the inverted microscope stage. In turn embryos were

held on the holding pipette and then microinjected (under control of the Femptojet and dispensing approx. 2fL volume) with MARK2-Venus encoding recombinant mRNA, transferred into the capillary injection needle, in either one or two blastomeres. For poly-A+ tailed mRNA a concentration of 702.0 ng/µL was used in the capillary needle. Further a concentration of 430.2 ng/µL was obtained for the non-poly-A+tailed mRNA. Successful intra-cellular microinjection was confirmed by visual inspection of the live phase-contrast light image and embryos were then transferred into KSOM media for *in vitro* culture to the correct/ appropriate stage (as described above).

3.4 Embryo fixation, confocal microscopy and image capture

At the required cell/ developmental, embryos were incubated in acid Tyrode's (Sigma) to remove the *zona pellucida*; as visually judged by microscopic inspection. Embryo fixation was then performed by 20 minute incubation (at 37°C) in 4% paraformaldehyde containing PBS (phosphate-buffered saline) solution (PFA) (Santa Cruz Biotechnology). Note that the fixation procedure was performed in 96-well plates coated with 1% agar/ 0.9% NaCl. Following fixation, the embryos were then briefly washed with phosphate-buffered saline containing 0.15% Tween-20 (PBST) and further incubated for 20 minutes at room temperature in fresh PBST. Afterwards, to achieve visualisation of cell nuclei, the fixed embryos were incubated in pure Vectashield (Vector Laboratories) containing 4,6-diamidino-2-phenylindole dilactate DAPI for 20 minutes at room temperature and then terminally washed in PBS.

Specialised confocal plates, comprising glass microscope cover slip bases (Matek), were prepared with fixed and stained embryo containing drops of PBST in order to visualise by inverted fluorescence confocal microscopy (Olympus FLUOVIEW FV10i). The laser excitation and detector emission wave lengths were adjusted to record the fluorescence of the Venus fluorescent tag and the DAPI stain and each assayed embryo was scanned in its entirety as a series of 2µm thick z-sections. Between all visualised embryos the power of the laser and sensitivity of the detector were kept constant. Within the Olympus FluoView V4.1a Viewer (Olympus) software analysis of the images was conducted.

4 Results

4.1 Cloning of *Mark2/Par1* gene cDNA into pRN3-Venus plasmid

A pre-existing preparation of cDNA from mouse derived testis total RNA was used as a template for PCR to generate full length *Mark2* cDNA. This cDNA was amplified to serve as an insert for creation of a recombinant MARK2 protein fused to fluorescent Venus (derived from the plasmid vector pRN3-Venus) at its C-terminus. The correct size of this insert was confirmed by an agarose gel as depicted in Figure 7. Its band size of ~2.75 kbp corresponds to the expected size.

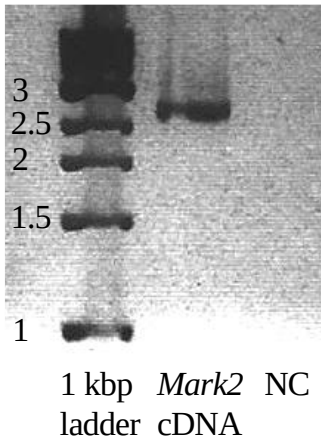

Figure 7: Agarose gel showing 1 kbp ladder, amplified Mark2 cDNA insert and negative control.

Therefore, this insert was used for cloning into pRN3-Venus vector after successful restriction enzyme digestion of both, vector and insert (more details can be found in chapter 3.1).

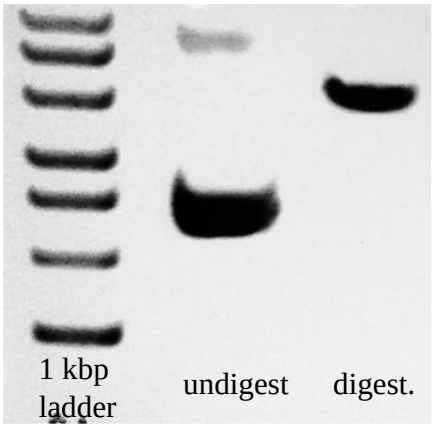

Figure 8: Gel electrophoresis showing 1 kbp ladder (from top to bottom: 6, 5, 4,3, 2.5, 2, 1.5 kbp); undigested pRN3-Venus plasmid and digested pRN3-Venus plasmid.

In Figure 8 the undigested (2 bands at 5.5 and ~2.25 kbp in lane 2) and digested (1 band at 4 kbp in lane 3) pRN3-Venus plasmid can be discriminated. Before successful ligation a concentration of 40.2 ng/µL for the insert and 5.9 ng/µL for the vector were measured. Following a 3:1 ligation reaction ratio, bacterial transformation was performed to choose bacteria that contained the correct insert (whether in correct or wrong orientation); because due to the pRN3-Venus vector encoded ampicillin resistance gene, only bacteria containing pRN3-Venus (hopefully with cloned *Mark2* cDNA derived insert) would be able to successfully grow on the Petri dishes containing the antibiotic ampicillin. Colony PCR was then performed to screen colonies for the correct ligation product.

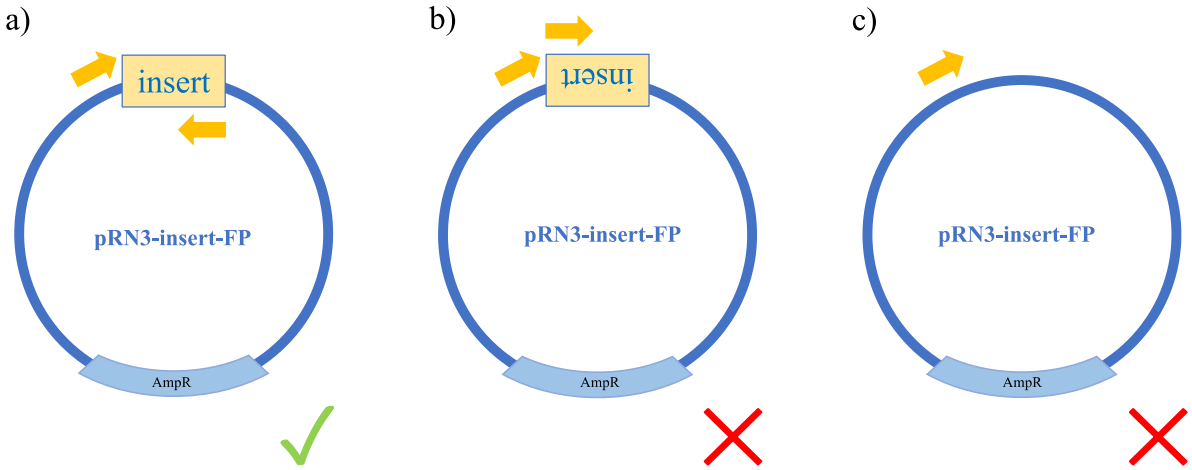

Figure 9: screening of correct insert (ligation product) via colony PCR; a) showing the correct plasmid and possible primer attaching, b) showing the insert in wrong orientation and no possible attachment of primers and c) showing the lack of insert and therefore impossible generation of PCR product.

For the detection of the correctly orientated cloned insert, colony PCR with specific primers was used to not only report if a successful ligation product was present but also if it was in the correct orientation (having the start codon towards the 5' side and the stop codon towards the 3' end on the correct strand). After choosing/designing the correct primers and performing PCR, an agarose gel electrophoresis was conducted. In case of correct incorporation of the ligation product (see panel a) in Figure 9), primers could anneal and create a product, in the case of an insert being cloned with the incorrect orientation (see b) or if there was no cloned product at all (see c), the colony PCR would not lead to the generation of a product. Lanes 4-8 in Figure 10 illustrate colonies were the presence of a correctly orientated insert could be detected, whereas lane 1-3 show colonies without insert or a none specific product, that could be discarded from further consideration. A clearly stated correct product

would show one clear band at the theorised/ desired size/ position on the gel. As is seen on the illustrative gel a wrong product would simply show a band on another unwanted size position or no band at all. In cases were the insert would not be incorporated there would simply be no band on the agarose gel. Since the positive bands on the illustrative gel (Figure 10) are very bright (due to long photographic exposure in the U.V. transilluminator) it is hard to state exactly what their true size is and if they are correct. However, the colony PCR gel belonging to the clones that were ultimately used for further experimentation (not shown), whilst still being very bright, did contain products of the correct band size of ~2.25 kbp. Thus, indicating clones in which the *Mark2* cDNA derived insert had been correctly cloned into the *NheI* site of pRN3-Venus.

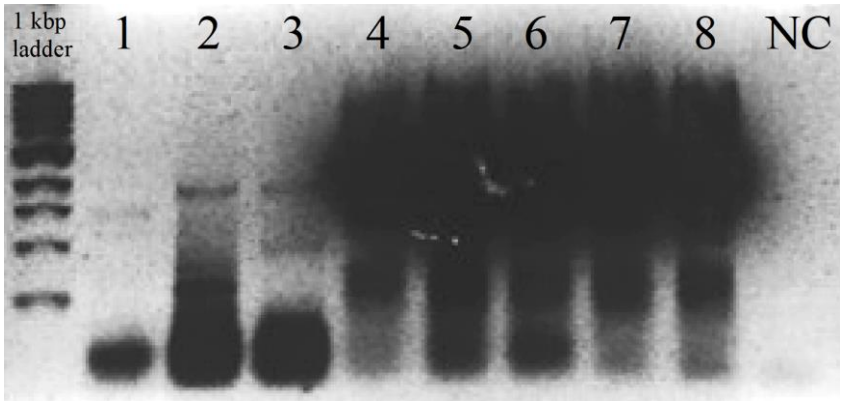

Figure 10: gel electrophoresis of colony PCR with 1-3 incorrect or missing insert, 4-8 positive for correct insert and negative control; 1 kbp ladder (from top to bottom: 10, 8, 6, 5, 4, 3, 2, 1.5, 1, 0.5 kbp).

After the preparation of glycerol stock solutions and Mini-prep isolation, plasmid DNA preparations of such Mark2 cDNA clones were purified (details shown in M&M) and then sent for confirmatory Sanger sequencing of the insert, and subsequent restriction enzyme digestion/ linearization in preparation for *in vitro* transcription/ IVT (see below).

4.2 Generation of recombinant MARK2-Venus fusion protein encoding mRNA

A distinctive restriction site downstream of the insert and Venus component of pRN3-Venus is recognised by the restriction enzyme *SfiI,* that can be used in to linearise the plasmid. The confirmed pRN3-Mark2-Venus plasmid was digested with *SfiI* prior to IVT. As expected, the linearised plasmid showed a band that was larger than the major band of the unrestricted species (that reflects the super-mobile and supercoiled form – see Figure 11); exhibiting a band corresponding to 6 kbp (versus the two bands at 4 kbp and above 10 kbp for the undigested – Figure 11).

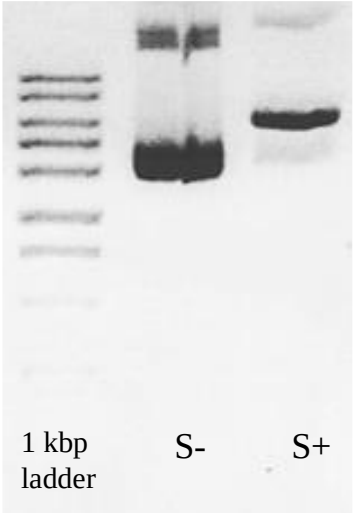

Figure 11: gel electrophoresis after linearisation of plasmid showing S- as undigested plasmid, S+ as digested plasmid with SfiI and 1 kbp ladder (from bottom to top: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 kbp).

Figure 12 shows the agarose electrophoresis gel of the mRNA product derived from the *SfiI* digested plasmid, after IVT was performed; with and without subsequent poly-A+tailing. Such gels illustrate the successfulness of the IVT reactions to generate recombinant MARK2-Venus encoding mRNAs of the expected lengths (note the poly-A+tailing resulting in mRNA species with lower electrophoretic mobility than those without poly-A+tailing). These prepared recombinant mRNAs were measured by UV spectrophotometry, to ascertain their concentration and relative purity, and aliquoted (and stored at -80^{o}C) for later microinjection into mouse embryo blastomeres.

a) b)

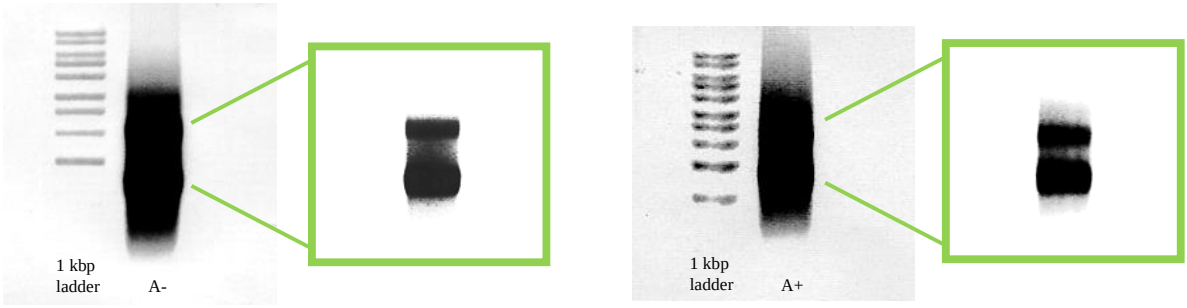

Figure 12: gel electrophoresis of generated mRNA after IVT with a) no poly-A+tailing and b) poly-A+tailing; 1 kbp ladder (from bottom to top: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10).

4.3 Visualisation of fluorescent Venus-tagged MARK2 in preimplantation mouse embryo blastomeres

After successful generation of fluorescent Venus-tagged Mark2 mRNA, the constructs (with and without poly-A+tailing) were microinjected by other lab members (with extensive experience and training) into preimplantation stage mouse embryos; specifically into one blastomere at the 2-cell stage, therefore being present in only half of the embryo. Figure 13 depicts such microinjected embryos (as projected series of z-sections) that were then *in vitro* cultured until the E3.5 stage (*i.e.* 3.5 days after initial oocyte fertilisation). In panels A-B (Figure 13) MARK2-Venus fluorescence/ expression (in grey-scale) can be clearly seen localised to the cell-cell contact regions of the basolateral surface of cells descended from the originally microinjected 2-cell stage blastomere; panels C-D show similarly microinjected embryos whereby the MARK-Venus signal is pseudo-coloured green the nuclear DNA is stained and visualised by DAPI (in blue). In either group of examples, it is possible to observe cells descended from either the microinjected or non-microinjected 2-cell stage blastomeres by the respective presence or absence of MARK2-Venus derived fluorescent signal.

The images show that the MARK2-Venus protein localises to the basolateral parts of the membranes of the blastomeres originating from the injected blastomere and does not localise to the apical membranes. This confirms that the Venus fluorescent tag does not disrupt the expected localisation of the MARK2 protein. However, there is a relatively high background fluorescence in the cytoplasm of the blastomeres of the injected clone, suggesting that the MARK2-Venus protein is also present in the cytoplasm. Nevertheless, a fluorescently tagged MARK2 fusion protein was generated, and we demonstrated that it localises at the basolateral membranes and therefore it can serve as a marker of basolateral membranes and as a reporter for apico-balsolateral polarity in general, in future experiments/ projects.

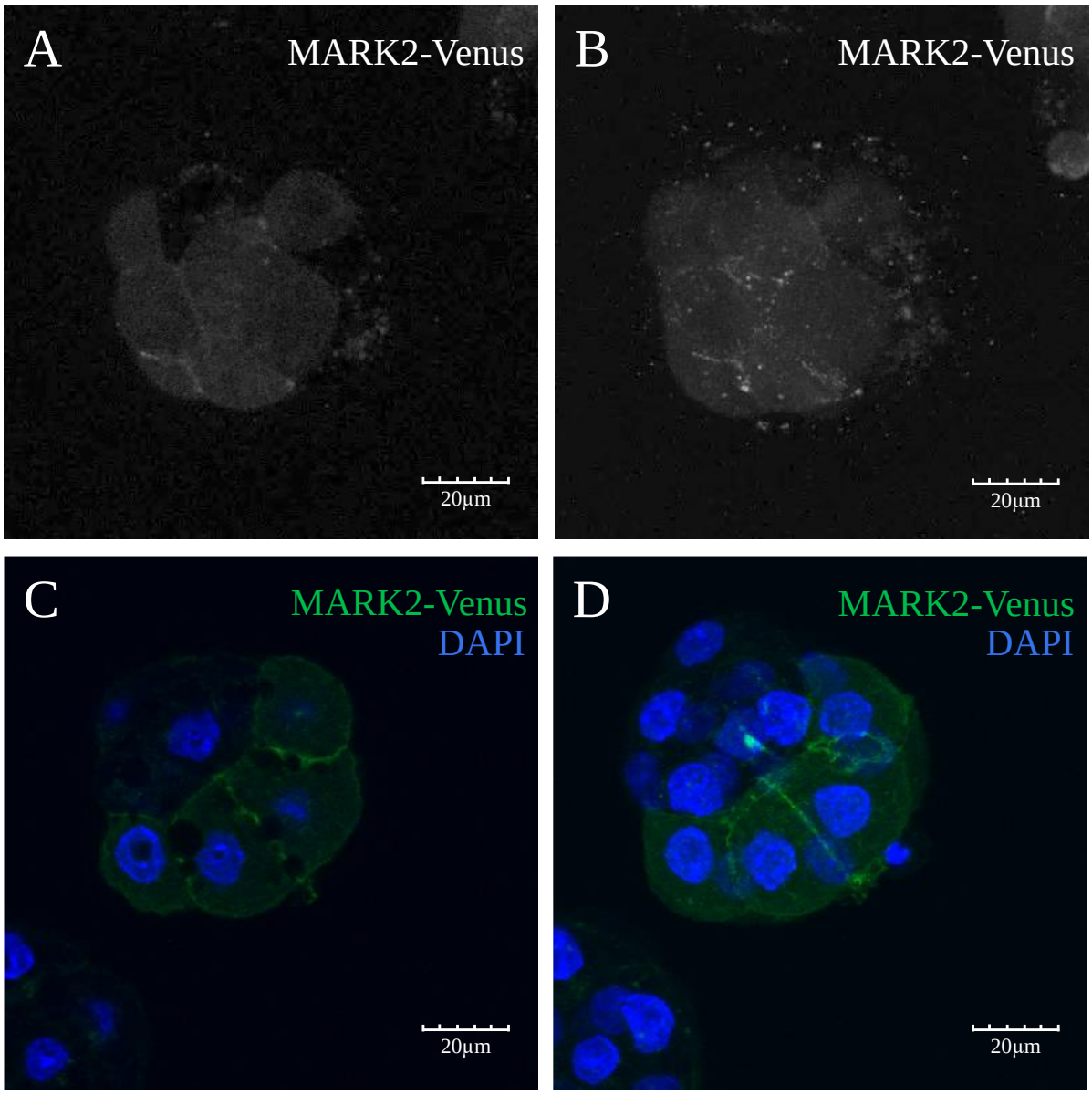

Figure 13: Confocal microscopy of stained embryos showing fluorescently tagged MARK2-Venus fusion protein expression in A and B (in grey-scale) and stained chromosomes of fixed embryos by DAPI staining (in blue) and MARK2-Venus (in green) in C and D.

5 Discussion

The aim of the project described within this thesis was to generate a recombinant fluorescently-tagged *Mark2* mRNA that can be microinjected into individual blastomeres of mouse preimplantation embryos and serve as a marker of polarised basolateral membranes. Therefore, this necessitated the cloning of full length wild-type *Mark2* cDNA into the pRN3 plasmid, that already contained the Venus fluorescent tag coding sequence. Moreover, *in vitro* transcription of the resultant *Mark2*-Venus coding construct was performed, and the derived recombinant mRNA was microinjected into individual blastomeres of 2-cell stage mouse embryos; to confirm/ validate that the overexpression of the MARK2-Venus fusion protein did not affect the developmental progression of preimplantation stage embryos and that the fusion protein localises uniquely to the basolateral membranes, as anticipated/ desired.

As can be seen from the images of E3.5 stage embryos, resulting from individual blastomere microinjections at the 2-cell stage, the fluorescent protein was successfully expressed; thus, confirming that the mRNA has been delivered inside the blastomere during the microinjection procedure. Moreover, the embryos developed as expected, suggesting that MARK2-Venus overexpression does not overtly affect the normal progression of preimplantation development. In addition, the MARK2-Venus fusion protein was visible in most of the basolateral membranes of progeny cells derived from initially microinjected 2-cell stage blastomere, while also being absent from the apical domains/ membranes of such cells; proofing that the fusion of the fluorescent Venus tag/ moiety to the C-terminus of MARK2 does not disturb its normal protein localisation and thus making the derived construct a good candidate as a basolateral membrane marker/ reporter.

Comparing the generated recombinant *Mark2*-Venus mRNA, and its derived fusion protein product, as a basolateral marker to membrane markers used in the studies of Mihajlović et al., 2015; Mihajlović and Bruce, 2016, it can be seen that a more uniform and more confident/reliable staining could/ should be achieved. In order to improve the quality of the MARK2-Venus signal/ visualisation, prior its use in future experiments, the concentration of the injected mRNA should be optimised to reduce/ eliminate the fluorescence in the background/ cytoplasm and for reliable staining in every basolateral domain. In addition, the purity of the mRNA prior to microinjections could be improved, for example by repeated organics extractions and salt based alcohol precipitations. Another factor that should be taken into consideration is reflected in the fact that the mRNA microinjected into embryo blastomeres, and presented in this thesis, lacked an experimentally added poly-A+tail.

Therefore, the microinjection of polyadenylated mRNA could also potentially improve the experimental outcome. Notwithstanding such points related to optimisation of the derived construct's usage, it is quite clear the expressed MARK2-Venus fusion protein could be used as a reporter of the basolateral domains of developing preimplantation mouse embryos. Moreover, it could be utilised to assist functional investigations concerning the effects on the apico-basolateral polarity and cell-fate decisions during the earliest development period of mouse embryogenesis. For example, it could help to confirm or refute the existence/ establishment of polarised basolateral domains (and by inference, counterpart apical domains) under experimental condition where other candidate polarity/ cell-fate gene expression has been dysregulated (for example, using clonal microinjection of specific RNAi constructs).

Since the endogenous MARK2 protein has been reported by Vinot et al., 2005 to be a mastermind for the assembly of the mitotic spindle apparatus of dividing blastomeres, during embryo compaction (at the 8-cell stage) and therefore is known to regulate microtubule stability, the derived *Mark2*-Venus construct may also be of potential use in experiments investigating the effects of Hippo signalling during this time (Drewes et al., 1998; Nguyen et al., 2017). Briefly, the activation of the Hippo signalling pathway in apolar inner cells (defined by plasma membranes in complete cell-to-cell contact with neighbouring cells and enriched in proteins enriched at the basolateral regions of outer cells, such as MARK2) is defined by phosphorylation of the transcriptional co-activator YAP1, which is regulated by the LATS1/2 kinases, whose activity at adherens junctions is also under a degree of regulation by MARK2 (Shi et al., 2017); it is therefore possible that the recombinant MARK2-Venus fluorescent construct derived here could be employed to report changes in apico-basolateral domain/ polarisation that would result from suppression or activation of factors being involved in the activation (in inner cells) or suppression (in outer cells) of the Hippo signalling cascade. For example, it could be used in experiments in conjunction with the chemical inhibition of ROCK kinases, since the small monomeric G-protein Rho is known to play a functional role as a suppressor in Hippo signalling pathway activation in outer cells (Shi et al., 2017). Furthermore, MARK2-Venus could be used in time-lapse confocal microscopy experiments where the timing or pattern of polarity establishment after downregulation of some candidate factors could be monitored in real-time. In addition, MARK2 has shown to be significant for intra-cellular polarity establishment and maintenance in many polarized tissues in addition to the mouse preimplantation embryos, therefore, the fluorescent fusion gene generated here has the potential to be used/ modified other experimental systems and paradigms, in which the establishment of apico-basolateral polarity is important.

Lastly, even though no obvious effects of MARK2-Venus overexpression were observed on the general development of microinjected mouse preimplantation stage embryos, in our hands, it remains possible that there might be subtle changes that would be visible after more detailed inspection, or perhaps later in the development; potentially resulting in potentially non-physiological and irrelevant phenotypes. Therefore, a potential and future experimental refinement could be to fluorescent tag the endogenous/ naturally occurring *Mark2* gene locus in the embryos own genomic DNA (at an early developmental stage – *e.g.* the zygote or 2-cell stages), thus eliminating any potentially unwanted phenotypes that simply arise as a consequence of over-expressing large amounts of recombinant MARK2-Venus protein derived from microinjected IVT mRNA. This is because the expression level of the MARK2-Venus fusion protein would be determined by the normal transcriptional regulatory mechanisms at work in the embryo blastomere nuclei, and thus would result in expression levels of the reporter protein that would be physiologically relevant. The tagging of fluorescent reporter gene coding DNA to endogenous gene loci can now be achieved in the mouse embryo using a robustly optimised variant of the CRISPR-Cas9 genome editing approach (Gu *et al.*, 2018). An additional potential advantage of employing this method would be the fact that no MARK2 protein within manipulated/ transgenic embryos would be without fluorescence and this could therefore improve the fluorescence readout in every basolateral membrane to which MARK2 protein is targeted, at any point in development.

6 Conclusion

Following the aim of generating a fluorescent Venus-tagged *Mark2* mRNA for microinjection into developing preimplantation mouse embryos, amplification of full length *Mark2* cDNA yielding the correct size and therefore allowing ligation of, by *NheI* mediated restriction, pRN3-Venus vector and *Mark2* insert was conducted. After bacterial transformation, colony PCR with specific primers was performed to identify which transformants (*E. coli* clones) had the *Mark2* specific cDNA successfully cloned in the target pRN3-Venus plasmid vector and in the correct orientation. As such two from these positive colonies were picked for infinite storage as glycerol stock solutions. After plasmid extraction, via Mini-prep isolation, the insertion of the *Mark2* cDNA and its correct sequence were positively confirmed by Sanger sequencing. Hence, the generation of MARK2-Venus encoding mRNA was prepared via digestion/ linearisation of the derived recombinant DNA construct and subsequent *in vitro* transcription (IVT). Half of the aliquots of the DNAseI digested IVT mRNA obtained were then subject to further poly-A+tailing, but due to time restraints it was the mRNA without poly-A+tailing that was microinjected into 2-cell stage mouse embryo blastomeres, and presented in this thesis. As can be concluded from the resulting fluorescent confocal micrograph images the recombinant mRNA was successfully delivered to individual blastomeres without disturbing embryo development; illustrating the fact that MARK2 (or more specifically MARK2-Venus) protein over-expression does not have any overtly deleterious effect on normal preimplantation development. Moreover, as the observed MARK2-Venus protein was visible at most basolateral but not at all at apical membranes, the derived construct is a good candidate for basolateral membrane marking.

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