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Author
Schnabellehner Sarah Christina

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Department of Molecular Biology

Thesis Supervisor
Alexander W. Bruce, Ph.D.

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THE ROLE OF CSF1 AND ZFPM1 IN THE PREIMPLANTATION MOUSE DEVELOPMENT

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Branišovská 1645/31a
370 05 České Budějovice

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Annotation

The aim of this thesis was to investigate the potential roles of Csf1 and Zfpm1 during the preimplantation mouse development, as those are considered as potential determinants.

Affirmation

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.

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Schnabellehner Sarah Christina

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Summary

During the preimplantation mouse development three distinctive cell lineages are set aside, that will ultimately give rise to the supporting extraembryonic tissue layers of the placenta and yolk sac as well as the embryo proper. This prompts the fundamental question how the different cell lineages can emerge out of a single cell. Differentiation has been shown to happen during two `cell fate´ decisions. During the first cell fate decision, extraembryonic trophectoderm (TE) cells, segregated from the inner cell mass (ICM) cells, that in turn will either differentiate during the second cell fate decision into primitive endoderm (PrE) cells or remain as pluripotent epiblast (EPI) cells. These two important events are triggered by several factors including an individual cell´s relative position within the embryo, the development of embryonic and cellular polarity, differential gene expression, as well as cell signaling and epigenetic modifications that work together in order to form a successfully developing embryo.

The characterization of novel gene candidates, having hypothetical role in murine preimplantation development, by the laboratory group, has allowed the investigation of two of such genes as part of this thesis; the first being the *Zfpm1* gene, encoding the zinc finger protein multitype 1 protein (Zfpm1, which is also known as friend of Gata-1/ FOG1). As no publications about Zfpm1 protein expression and sub-cellular localization regarding the mouse preimplantation development are reported, the aim was to investigate the protein expression pattern of Zfpm1 protein at distinctive early embryonic stages. The second candidate gene under investigation was the *colony stimulating factor 1* (*Csf1*, which is also known as macrophage colony-stimulating factor/ Mcsf). As it is known that exogenous Csf1 enhances the formation of TE destined cells, and there was evidence that its expression is enriched in ICM cells, it was hypothesized that embryo self-derived Csf1 may, through a paracrine signaling mechanism, promote TE development. This hypothesis was tested using specific knockdown of Csf1, by zygotic anti-Csf1 dsRNA microinjection, but ultimately rejected in its present form.

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1. Introduction

Mammalian preimplantation development is defined by the moment of fertilization to the implantation of the embryo (termed a blastocyst at this stage) in the maternal uterus. During this time, the blastomeres (*i.e.* the name given to cells of the early embryo) gradually lose their totipotency (their ability to contribute equally to all embryonic and extraembryonic developmental lineages), and the resultant blastocyst stage embryos comprise three distinctive cell lineages each defining a distinctive tissue in later development [1]. Therefore, those early developmental decisions that give rise to the three lineages are prerequisites for the successful implantation of the blastocyst and continued development. The mouse embryo model, which is used in preimplantation research, is highly regulative and can overcome even intrusive manipulation like destruction/removal, addition and rearrangement of blastomeres during the first three rounds of cleavage [2][3][4][5]. This flexibility, in common with other mammalian embryos, is largely due to a very early activation of zygotic transcription from the embryos own genome (particularly early in mouse) and the replacing of inherited maternal transcripts, that other simpler organisms (*e.g*. the nematode worm, *C. elegans*) rely upon, well into their embryonic development. Such zygotic genome activation (ZGA) is initiated by the translation of the maternally provided mRNA and takes place in two phases: the minor activation before the first cleavage (late 1-cell stage) and the major activation at the late 2-cell and 8-cell stage [4][6].

1.1. Morphological transitions of the preimplantation embryo

The development from zygote (fertilized egg) to blastocyst includes 7 cell cycles (Fig.1) and with each cleavage division the size of the cell halves, so that the overall size of the early embryo doesn´t change until formation of the blastocyst, fluid filled, cavity. The first two cell cycles take about 18 hours each while the following cell cycles last for 12 hours. Therefore, approximately 3.5 days are needed for an expanded blastocyst with >64 cells to develop and 4.5 days (about 128 cells) for attachment to the uterine endometrium. To be convenient, this time frame is referred to in terms of embryonic days; for example E4.5 denotes four and a half days of embryonic development initiated from the point of sperm-egg fertilization [7].

Two major morphological changes occur during preimplantation mouse development (Fig.1 depicted by blue arrows). At the 8-cell stage compaction occurs, where the blastomeres increase their intercellular contact and lose their distinctive outlines. Accordingly, until the end of the 8-cell stage all blastomeres can be considered to be equivalent, at least in relative space. During, or shortly after, compaction the previous symmetrical 8-cell stage blastomeres become, for the first time, highly polarized along their apical-basal axis. As development continues, the 16-cell stage so-called morula embryo is formed. Subsequently at the 32-cell stage, a fluid filled blastocoelic cavity forms and expands within the embryo. This cavity is surrounded by an epithelialized sheet of outer cells, called the trophectoderm (TE) and is

distinct from a second population of cells, encapsulated within the embryo and residing at the opposite pole of the cavity, known as the inner-cell-mass (ICM) [7][8][9].

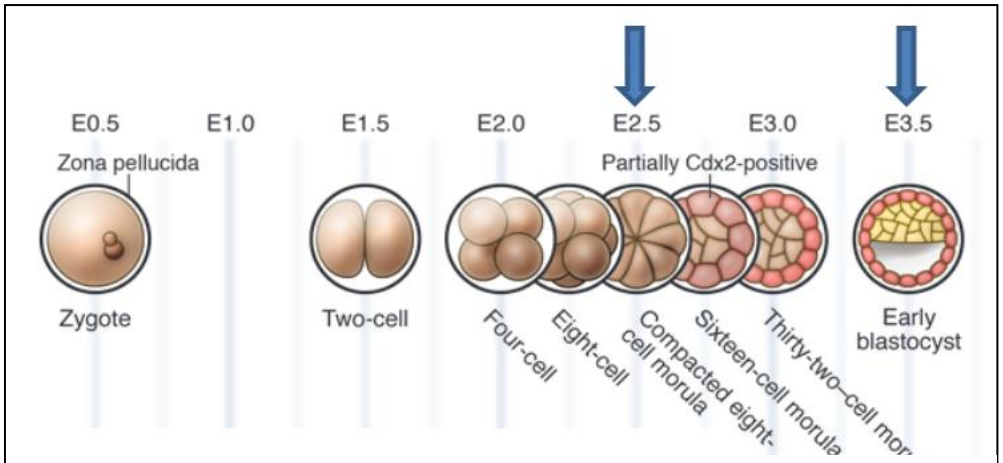


Figure 1: **First 7 cell cycles of the mouse preimplantation development,** depicting the morphological changes, adapted from [5]

1.2. Blastocyst cell lineage

The formation of the three blastocyst cell lineages is essential for correct embryonic development and survival and is typified by two cell fate decisions made before implantation. The first cell fate decision is initiated during the transition from the 8- to 16- cell stage (and is continued during the subsequent 16- to 32- cell transition) and involves the spatial segregation of blastomeres to either the inside- or outside position of the embryo. Outer cells ultimately differentiate into the TE (the first extraembryonic lineage) and the inner cells forming the pluripotent ICM. During the second cell fate decisions, individual ICM cells can differentiate into another extra-embryonic lineage of mono-layered cells in contact with the fluid filled cavity (also called blastocoel), called the primitive endoderm (PE), or retain their pluripotency and form the epiblast (EPI); the cells of which are completely surrounded by TE and PE and reside deep within the ICM, without contact to the cavity. The EPI populations of cells preserve their pluripotency and serve as a progenitor pool for all subsequent embryonic tissues of the foetus [5][10].

1.3. First cell fate decision

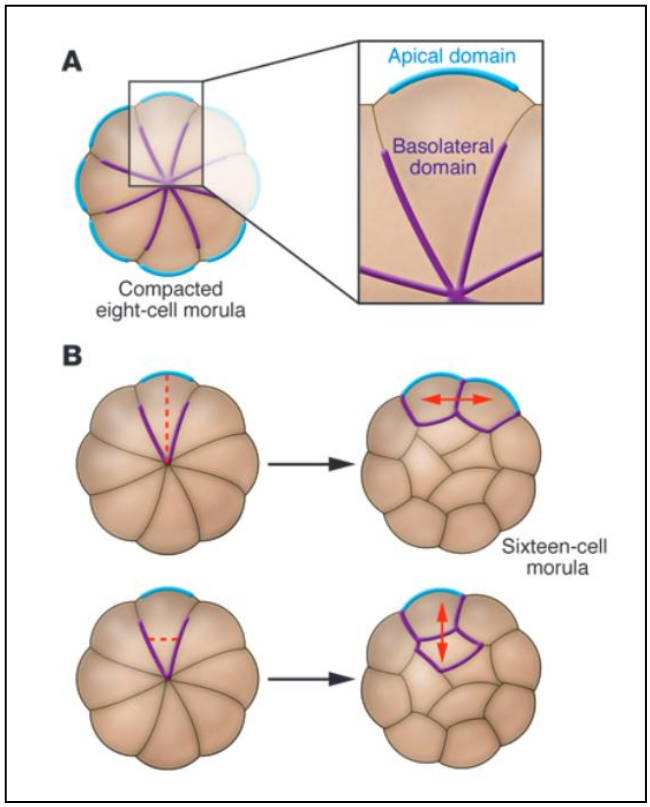


Figure 2: **Symmetric vs. asymmetric cell division** [5]

During compaction at the 8 cell stage, the blastomeres increase their intercellular contacts and establish junctional complexes. Such compacted cells have now a distinctive apical and basolateral membrane domain (Fig.2 A). E-cadherin (also called uvomorulin), which is an important component of adherens junctions (promoting cell-to-cell adhesion), becomes delocalized from the apical (outward facing) domain and localizes at the basolateral (inward facing/ cell-to-cell contact) domain during compaction. Therefore, individual blastomeres gain, for the first time, an apical basolateral polarity whereby their apical membranes differ from the basolateral membranes. Concomitantly, the cytoplasm reorganizes and cell nuclei migrate towards the basolateral domain, whilst the endosomes, actin and most microtubules (with the exception of a few acetylated microtubules that become basolateral localized) accumulate apically, and contributing to a rough outward facing apical surface enriched in microvilli. This compaction/ polarization event is a prerequisite for the later segregation of TE cells and ICM cells, as the now polarized blastomeres are able to either divide symmetrically across the apical-basolateral axis (also known as a conservative division) or asymmetrically along it (also known as a differentiative division) (Fig.2 B). Therefore, symmetric divisions result in two polar daughter cells that both remain on the outside of the embryo, whilst asymmetric divisions produce one polar outside cell and one apolar inside cell. Therefore, the progenitor cells of two distinct blastocyst cell lineages, TE and ICM, are for the first time spatially segregated during the fourth cleavage, depending on the orientation of cell division. Note that (as mentioned above), symmetrical and asymmetrical (outer) cell divisions also occur during the fifth cell division marking the 16- to 32-cell stage transition. Therefore, by the 32-cell stage cells have become committed to either the outer TE or ICM lineages. The mechanisms by which this first cell-fate decision arises are described in three models that are discussed below [5][13][11][12][14][15].

1.3.1. Mosaic model

This model insists that cell-fate (molecular) asymmetry is already present during the earliest stages of the mouse embryonic development and thus influences later call fate. Moreover that such symmetry has its origin in patterning of cell-fate determinants in the egg or early post-fertilization zygote. While this model was considered viable for a long time, the fact that the mammalian mouse model embryo is quite capable regulating its development in response to perturbations, like the removal/ addition or reorganization of cells, to rescue appropriate blastocyst formation, caused it fall from favor [18].

1.3.2. Inside-outside (positional) model

Whether blastomeres will occupy a position within the ICM or TE depends primarily on the relative spatial position occupied within the morula: as outer blastomeres give rise to TE and inner blastomeres to ICM (Fig.3). That's why blastomeres, up to the 8-cell stage, are only able to differentiate towards TE fates, while adoption of ICM fates (*i.e.* EPI or PrE) requires an intracellular environment. This positional (or inside-outside) model was first proposed by Tarkowski and Wroblewska in 1967 and further tested by Hillman et al. by changing the relative position of individual blastomeres within the embryo in order to assay changes in cell fate. The experiment of Hillman and colleagues, took individual blastomeres of 4-cell embryos and placed them peripherally to other recipient 4-cell embryos, where the single blastomeres tended to develop into TE. Therefore, their experiments supported the model of Tarkowski and Wroblewska. As depicted in Fig.3, differences in cell contact can influence the expression of certain and important cell-fate regulating transcription factors, such as the *caudal type homeobox 2* (Cdx2), the mRNA of which is also enriched in the outer apical domain of 8-cell stage blastomeres, which is the reason why it is expressed in the TE but not ICM cells [3][16].

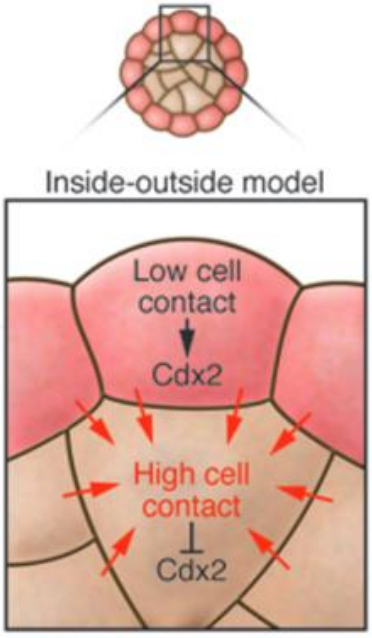


Figure 3: **Visualization of the inside-outside model** [5]

1.3.3. Polarity model

This model of cell-fate determination is solely based on the original observations by Johnson and Ziomek in 1981, that showed compacted cells isolated at the eight cell stage are polarized along their apical-basolateral axis. Specifically, regarding their surface structure including surface microvilli and subcellular components such as endosomes, cytoskeletal elements and microtubule organizing centers. Therefore, division across the apical-basolateral axis results in two outer polarized cells, whilst division along the same axis yields both an apolar inner cell

and a polarized outer cell. This critical event, occurring during the 8- to 16-.cell and 16- to 32-cell stage transition therefore gives rise to a set of two kinds of differentially polarized cells that also differ in their location and ultimately cell-fate. The apolar inner cells contributing to the ICM and the outer cells to the TE, and thus this event and the associated asymmetries in intracellular apical-basolateral polarity, initiate lineage segregation. According to the polarity model, it is the differential inheritance of apical-basolateral polarity and polarity factors that drives the adaption of either TE or ICM cell-fates. This suggests that the cell fate is already established or at least primed at the eight-cell stage and then depending upon the angle of cell division, the relevant cell fate program, in relation to relative cell position, is enacted. It also means that cell polarity must be preserved during the mitosis [13][17][19][20].

1.3.4. Combination of previous models

As it has not been shown that the above described models are mutually exclusive, more recent experiments have shown that there is indeed a degree of validity in both the positional and polarity model. For example, individual blastomeres separated from different 16-cell stage embryos, representing solely inner, solely outer or mixed cell populations, are able to form blastocysts, when reaggregated again, as composite 16-cell stage embryos. Moreover, each of such developing blastocysts displayed TE and ICM characteristics but their developmental timing was slightly different. Aggregates of previously only outer cells recombined slower, but they formed the fluid filled cavity earlier and also divided faster so that they contained more cells at the blastocyst stage when compared to the aggregations of previously only inner cells. Mixed aggregates showed developmental characteristics intermediate to those of the only inner and only outer cells. Importantly, all composite aggregate embryo types were able to develop normally, when transferred back to the uteri of pseudo-pregnant females, *in vivo*! The natural conclusion of such experiments is that blastomeres at the 16-cell stage completely retain their totipotent potential, and are not yet fully committed to one cell-fate or another, although they show morphological and behavioral differences, as the outer cells are larger and polar and the inner cells are smaller and apolar, that during unperturbed development are critical. Moreover, that single blastomeres can adapt to manipulations in their position by adopting a new cell fate. Evidenced by the fact that previously polar outer 16-cell stage blastomeres are able to divide into a polar outer and smaller apolar inner cell, when inserted inside of a composite 16-cell stage embryo and that previously inner blastomeres from a 16-cell stage embryo can spontaneously initiate polarization and form TE cells when transferred to the outside of a 16-cell stage embryo. Hence positional and polarity cues are both crucially important and required for appropriate emergence of cell-fate in the blastocyst stage embryo [16][21][22][23].

1.4. Second cell fate decision

After the first cell fate decision is made, the pluripotent ICM segregates into the primitive endoderm (PrE), and pluripotent epiblast (EPI) lineages. The PrE cell lineage represents a monolayer of cells that develops to cover the surface of the EPI and is in direct contact with the blastocoel. While the TE is segregated from the ICM during the first cell fate decision and gives rise to the embryonic contribution of the placenta, the PrE develops into the parietal and visceral endoderm of the yolk sac. Only the EPI lineage will later develop into the various tissues of the actual embryo/ foetus. Although compared to the first cell fate decision, not as much is known about the second cell-fate decision, different models relating to its formation have been proposed and are discussed below [5].

1.4.1. Inside-outside/ induction model

According to this model the cue for the PrE lineage resides in differences in ICM cell microenvironment, relative to an individual cells position to the forming blastocoel. As such, cells in contact with the blastocoel will adopt PrE fate, while the inner-most cells of the ICM adopt an EPI fate. This model is based on the observations of Dziadek made in 1979, when isolated ICM cells (formed by the selective destruction of blastocyst stage TE cells), from giant blastocysts (formed by a combination/ aggregation of three 8-cell stage embryos to increase the size of the ICM), were able to form a cell layer of PrE cells on the outside of the cell mass. Additionally, new PrE layers were able to be formed on the surface of such isolated ICMs, even after removal of the initial PrE layer. Dziadek proposed that all ICM cells are a homogenous population of bipotent cells, having an equal potential to develop into PrE or EPI cells, based solely on their relative position within the ICM [24].

1.4.2. Cell sorting model

More recent data suggested an alternative model, whereby the majority (although not all) of the blastocyst ICM cells (at E3.5) are able to contribute to either PrE or EPI lineages. This hypothesis comes from data showing that initial cell position doesn´t always define the later fate of that cell (emerging from a bipotent state). For example PrE progenitor cells can initially reside deep within the ICM and then migrate to the surface in contact with the blastocoel to contribute to the PrE. This hypothesis is supported by assaying the protein expression of transcription factors known to correlate with either EPI or PrE cell fate. Also it is known, that the loss of *Nanog* causes all ICM cells to take up parietal endoderm fate, while the embryo completely fails to form any EPI cells. Conversely, the overexpression of another transcription factor gene, *Gata6*, leads to adoption of PrE fate and its loss prevents the formation of a functional visceral endoderm layer. Experiments from Chazaud et al. in 2006 showed that Nanog and Gata6 protein expression between the nuclei of ICM cells are distributed in a mutually exclusive “salt and pepper” like pattern at the 32-cell (E3.5-E3.75) stage, with rarely

an individual cell expressing both markers. These data show whilst ICM cells may remain bipotent it is highly probable that the lineage segregation between PrE and EPI progenitors naturally occurs around the E3.5-E3.75 mark, during unperturbed development. Moreover, cells refine their relative position within the ICM to reflect their specified cell-fate [25][27][28].

1.4.3. Combination model

This model is essentially an amalgam of the two above previously described models. It therefore proposes that EPI and PrE lineage-specific markers are equally expressed in all ICM cells before blastocyst formation and that during further development individual ICM cells progress towards exclusive expression of either PrE or EPI lineage-specific markers, around the 64-cell stage. While the initial position of a cell within the ICM can impact its later fate, a cell is also able to relocate within the ICM in order to reach a spatial position appropriate to the complement of lineage-specific markers it expresses. However, the model also suggests that instead of migrating a cell can also switch its fate by changing its gene expression, or if it is unable to reach an appropriate location, the cell can perform selective apoptosis [29][26].

1.4.4. Time inside – time outside hypothesis

This model is based on the observation from Morris et al. that the timing of internalization determines which ICM cell lineage will be maintained/ specified later. Accordingly, the time ICM progenitor cells spend on the outside of the embryo, where they are polarized and exposed to TE differentiation, reducing their pluripotency and the ability of any of their ICM progeny to develop into the EPI lineage (thus, favoring PrE formation), whilst the early internalization of ICM progenitor cells promotes pluripotency and EPI formation. In the experiment of Morris et al., all cells were traced by time lapse microscopy from the start of internalization (at the 8- to 16- cell transition) until the PrE and EPI became distinguishable at the late blastocyst stage (at E4.5). It was found that cells internalized during the first wave of asymmetric division (8- to 16-cell transition) were statistically more likely to form EPI cells, while cells internalized during the second wave (16- to 32-cell transition) had increased expression of Gata4 and other PrE markers, and were therefore highly biased to form PrE. The very few inner cells generated during a atypical third wave internalization (32- to 64-cell transition) were invariably fated to the PrE lineage. Moreover if atypically high numbers of ICM cells were generated during the first wave of internalization, the “excess” cells were fated towards PrE lineage, meaning that EPI cells segregated by the first wave of internalization are indeed pluripotent. Collectively, these data indicate that the first and second cell fate decisions must be linked in regard to ICM development, as what affects the timing of TE and ICM progenitors also affects the fate of the derived ICM cells [10][30].

1.5. Molecular basis of compaction and polarization

The morphological change of compaction at the 8-cell stage is considered essential for further development of the embryo into a blastocyst comprising the three described cell lineages. The process of compaction can be considered as the increased adhesion of cells to each other and was first considered as the result of differences in cell surface tension and internal gelation. Later it was observed that the formation of microvilli participate in regulating the adherence of blastomeres, beginning at the 4-cell stage. Accordingly, when two cells come into close proximity, it seems as if the microvilli of adjoining cells function in an interdigitation mechanism leading to cell flattening (loss of microvilli from the cell contact regions), increased adhesion and eventual compaction [31][32][33].

Clues as how neighboring cells increased their adhesive properties came from experiments investigating the role of Ca^{2+} cations in *in vitro* growth media. When embryos were cultured in Ca^{2+} free medium, they were shown to be unable to compact. Moreover, already compacted embryos (either cultured *in vivo* or *in vitro*) were unable to maintain their compaction when transferred into Ca^{2+} free medium (for approximately 30 minutes). Lastly, embryos initially cultured in Ca^{2+} free medium were shown to be able to recompact upon transfer into a Ca^{2+} containing medium. It was later shown that the Ca^{2+} dependent molecule, mediating in adhesion, is E-cadherin (also called uvomorulin); a molecule that becomes basolaterally localized during compaction (via a post-translational mechanism). Therefore, E-cadherin is important for the maintenance of cell adhesion of compacted embryos and accordingly compaction can also be similarily and reversibly disturbed by culturing 2-cell-stage embryos with anti-uvomorulin antibodies [34][12][35][36][37][38]. Genetic knockout experiments targeting *E-cadherin* and *β-catenin* (that is associated together with α- and γ-catenin to the cytoplasmic domain of E-cadherin) genes unequivocally demonstrate that E-cadherin and β-catenin (of maternal origin; *i.e*. present as proteins in the egg cytoplasm) are not important for the blastomeres development before compaction but became integral players in mediating cell to cell contact during compaction [39][40][41][42]. As E-cadherin has an extracellular domain permitting hoemophilic interactions with the extracellular domains of E-cadherins from neighboring cells, it is able to form a stable and functional adherens junction. Moreover the intra-cellular, domain of E-cadherin together with its associated catenins (especially β-catenins), interacts with and modifies the underlying actin cytoskeleton [43].

A further membrane protein that becomes restricted to the apical domain is the protein *Ezrin*, a factor that is important for the stabilization of the microvilli. The mRNA of Ezrin is present through the entire preimplantation period but decreases significantly after the 8-cell stage. Before compaction starts Ezrin protein is localized in the cell cortex and migrates during compaction to the apical pole and becomes later limited to in the microvilli, that are also enriched there, by this time. Therefore, Ezrin can be considered as the first cytocortical protein

that is completely restricted to the TE lineage at the 16-cell stage. In its active state Ezrin is phosphorylated at Thr(567) and expression of recombinant mutant Ezrin (when Threonine-567 is mutated to Alanin) causes direct defects in the microvilli formation [44][45][46].

The Par-complex family and *atypical protein kinase C* (aPKC), have a role in the establishment of cell polarity (established during the late 8-cell stage, see earlier, concomitant with compaction) and cell division orientation; also become progressively localized towards the apical membrane domain, from the 8-cell stage, and become localized at the sites where tight-junctions will form between outer TE cells (they also appear on the apical surface of those ICM cells that are in contact to the blastocyst cavity and will form the PrE). Clonal down-regulation of *Par3*, using microinjection techniques, has been shown to cause an increase in the number of asymmetric cell divisions, by modified blastomeres. Hence, demonstrating regulating such polarity factors is important for TE versus ICM cell-fate derivation [47][48].

1.6. Molecular basis of the transcriptional control of cell fate decisions

Which cell lineage will be maintained by a cell, within the developing embryo, depends upon the emergence of key transcriptional programmes, which are under control of specific transcription factors (TFs). Such TFs and transcriptional programmes are able to respond to cues of cell ancestry, cell positional history, cell polarity and division orientation, in order to promote appropriate cell fate. At the 8-cell stage and after the initiation of compaction, individual cells of the embryos can either undergo symmetrical or asymmetrical cell divisions. In the lineage of ICM cells, created by asymmetric cell divisions, TFs such as *Oct4* (also referred to as *Pou5f1*) [49][50], *Sox2* [51], *Sall4* [52] and *Nanog* [27] will be present promoting pluripotency, while in the TE destined cells TFs like *Cdx2* [53], *Eomes* [54], *Tead4* [55][56], *Gata3* [57] are important. Later when the second cell fate decision takes place within the ICM, cells that will differentiate into PrE require expression of the TFs Gata4, Sox17 and Gata6 while cells that will remain pluripotent, and form the EPI, retain the expression of the TF Nanog.

1.6.1. TE vs ICM

By the blastocyst stage the ICM and TE lineages are already established, with each expressing mutually exclusive lineage specific TFs. For the correct inter-cell expression pattern of TFs to be established there is a key interplay with intra-cellular polarity and relative cell positioning within the embryo.

Experimental observations have shown that the development of cell polarity depends upon the conserved partitioning defective (Par) gene family, that includes JAM1, aPKC, Par3 and Par1. The direct influence of aPKC and Par3 upon TE versus ICM cell-fate has been shown via loss of function experiments, whereby clonal down-regulation of these factors causes individual blastomeres to adopt a position within the ICM by either dividing

asymmetrical or by losing their outer position to more highly polarized neighboring outer blastomeres (leading to internalization). Therefore it can be concluded that a tight correlation between cell polarity, cell position and TFs is maintained [6][64][65].

The segregation of the TE and ICM lineages depends on the reciprocal expression of the TF Cdx2. Cdx2 is expressed in the outer cells that will become TE and actively represses there the expression of pluripotency promoting TFs, such as Oct4, Sox2 and Nanog. Cdx2 protein expression is initiated at the late 8-cell stage, however evidence exists that Cdx2 mRNA is not evenly distributed throughout the cytoplasm and enriched at the apical pole, such that following asymmetric cell divisions only the outer cell receives enriched quantities of TE-promoting Cdx2 mRNA. Importantly, interventional strategies aimed at modulating inter-blastomere levels of Cdx2 expression have shown that the concentration of Cdx2 within a blastomere also influences its division symmetry, with higher levels favoring TE-progenitor producing symmetric cell divisions and lower concentrations promoting asymmetric cell division at the 8-cell stage. Cdx2 is also in a positive relationship with the cell polarity gene a-PKC, as increased Cdx2 levels also induce greater levels of aPKC apical polarity, that in itself also promotes increased symmetric cell divisions and generation of TE cells. [66]. The central importance of the *Cdx2* gene was shown by Strumpf et al. using a genetic knock out approach. They observed that whilst *Cdx2*$^{-/-}$ mutants are able to form TE-like cells and also a blastocyst like structure, such embryos failed to implant, as they could not form a viable blastocoel, and consequently died via an apoptotic cell death mechanism. Molecular analysis showed that the TE-like cells of such embryos also expressed genes characteristic for ICM cells (*e.g. Oct3/4* and *Nanog*), that are normally suppressed by Cdx2, and also lack TE specific gene expression [59][53][60]. The Strumpf group was also able to show that mutants of *Eomes*, which is a T-box TF, are able to form and preserve a fully expanded blastocyst but still fail to form trophoblast outgrows *in vitro*. However, they also showed that *Cdx2* is expressed normally in the TE cells and that Oct4 is suppressed in outer cells; thus concluding that Eomes is not initially required for the segregation of the ICM and TE lineages but becomes important in the further differentiation and proliferation of TE, after the late blastocyst stage [53][58][61].It was recently observed that the TEAD/TEF family transcription factor *Tead4* is essential for TE development and is to date the earliest gene identified that is required for proper TE lineage specification, formation and maintenance. *Tead4*$^{-/-}$ embryos do not express any TE specific genes, such as *Cdx2*, but only ICM specific markers. As a consequence the *Tead4*$^{-/-}$ embryos cannot specify a TE lineage or initiate blastocoel formation and are unable to undergo implantation, resulting in a preimplantation lethal phenotype [63]. The TE progenitor *Cdx2* is dependent on the expression of, and transcriptional activation by, *Tead4*. This is evidenced by the lack of Cdx2 protein expression in Tead4$^{-/-}$ null embryos, and ectopic and increased Oct4 protein expression (that is normally down regulated by Cdx2,

independent of Tead4. Accordingly, Tead4 promotes Cdx2 development in the TE lineage that in turn ensures the correct down-regulation of Oct4 and Nanog in those cells [63].

The reason why Cdx2 protein becomes enriched in TE progenitor cells (in addition to the polarity-mediated effects described above) was recently shown to involve the differential activation of the Hippo signaling pathway. In apolar inner cells, activated Hippo-signalling, acting through its mediator kinase *Lats*, excludes an obligate transcriptional co-activation protein of *Tead4*, called *Yap*, from the nucleus. This prevents nuclear localized *Tead4* from activating inappropriate transcription of the *Cdx2* gene. However, in outer TE-progenitor cells, the Hippo-pathway is actively suppressed, allowing Yap access to the nucleus and allowing it to pair up with Tead4 and activate the *Cdx2* gene. As Hippo signaling activity is both dependent upon cell to cell contact and inhibited by the presence of apical polarity factors (as found in the outer cell apical domains), this mechanism restrics Tead4-mediated activation of Cdx2 to outer polarized TE-progenitor cells. Indeed, when cell adhesion is inhibited, for example using an anti-E-cadherin antibody, cell adhesion becomes impaired and active Hippo signaling also decreases, resulting in inner cells containing ectopically localized nuclear Yap and Cdx2 proteins [62][67][68].

The TF *Gata3* is also selectively expressed in the TE lineage and has a direct influence upon *Cdx2* expression. During blastocyst formation, *Gata3* becomes enriched in TE cells and is no longer present in the ICM. Experimental knockdown of *Gata3* expression causes the down-regulation of *Cdx2* levels and is associated with blocked development at the morula to the blastocyst stage. Indeed, it has been discovered using chromatin immunoprecipitation (ChIP) analysis that transcriptional expression of the *Cdx2* gene is directly regulated by *Gata3*, with a conserved *Gata3* motif found in the promoter proximal region [72].

A very important pluripotency promoting TF is the POU domain containing gene *Oct4*. It is crucial in maintaining pluripotency, and it has been shown by the experiments of Nichols et al., that embryos lacking *Oct4*, are unable to develop past the morula stage and cannot form a pluripotent ICM, with all cells differentiating into TE. Interestingly, the relative intra-cellular concentration of *Oct4* is both crucial and highly sensitive in terms of impacting cell fate. Additionally it is known that *Oct4* protein interacts with the *Cdx2* gene in a form of mutual antagonism, that ultimately promotes *Cdx2* expression in the outer cells and *Oct4* expression in inner cells [59][53][69][70][71].

The divergent homeobox transcription factor *Nanog*, named after a mythological land of the “ever-young”, is also expressed in the ICM of the compacted morula and blastocyst. Like *Oct4* it is required for maintenance of pluripotency and also self-renewal of embryonic stem cells. *In vivo*, *Nanog*$^{-/-}$ mutant/ null embryos comprise ICMs in which the inner cells have uniformly adopted a PrE fate and are not pluripotent; hence highlighting *the important* role of *Nanog* during the second cell fate decision (see below) [73][52][74][27][75].

1.6.2. PrE vs EPI

After internalization, the derived ICM cells, will either differentiate into PrE (the monolayer of cells in contact with the blastocoel) or remain pluripotent and populate the EPI lineage (found deep within the ICM). Successful differentiation of the PrE lineage requires up-regulation of the TFs Gata6 and Gata4, that in turn functionally antagonize the pluripotency TFs, Sall4, Nanog, Oct4, whereas continued and prolonged expression of Nanog destines ICM cells towards the EPI lineage [6].

The TF Sox17 was recently identified as a novel PrE marker and is initially randomly expressed in subset of blastocyst ICM cells, in a manner comparable to a “salt and pepper” pattern, before becoming restricted with other PrE TFs, to the emerging PrE. Niakan et al. have shown that Sox17 actively down-regulates TFs that are correlated with EPI lineage specification (and would promote pluripotency), whilst it promotes the expression of genes that support PrE formation. In so doing, Sox17 competes with Nanog for individual chromatin binding sites, in order to regulate the transcription of proximal target genes. For example Sox17 stimulates the transcriptional expression of the PE-promoting TF genes *Gata6* and *Gata4*, whilst simultaneously antagonizing the EPI–related TF genes *Nanog*, *Sox2* and *Oct4* [6][76][30][87].

The expression of the PrE-associated TFs Gata6 and the closely related Gata4, requires an activated intra-cellular signaling cascade involving the adaptor protein Grb2; genetic knockouts of the *Gbr2* gene causes inhibition of the formation of PrE cells and instead promotes all ICM cells to express the EPI specific TF Nanog. When recombinant *Gata6* is overexpressed in embryonic stem cells (ESC) it causes PrE transformation/ differentiation and it is hypothesized that both Gata6 and Gata4 functionally antagonize Nanog, thus permitting PrE differentiation. Interestingly, *Gata6* and *Gata4* genetic mutant embryos are able to develop a recognizable PrE, when cultured *in vitro,* but die at a time-point shortly after implantation (into pseudo-pregnant mice) at E5.5 because of a failure to form a functional visceral endoderm (a derivative of the PrE, needed for yolk sac formation) [80][81][82][83][20][25].
Another important factor in PrE formation is fibroblast growth factor (Fgf4). Genetic knockouts of *Fgf4* (ligand), and its receptor (*Fgfr2*) plus its downstream effector *Grb2* all result in blocked PrE formation (*n.b*. embryos are able to implant but are not able to develop further due to lack of PrE derivatives). Consistent with the important role of Fgf4 in PrE formation, the phenotypic PrE blocks associated with $Fgf4^{-/-}$ $^{-/-}$ embryos can be compensated and reversed by supplementing the culture medium with exogenous recombinant Fgf4 protein. Experiments conducted by Yamanaka et al. have shown that modulation of the Fgf4 signal during blastocyst formation is able to alter ICM cell fate as measured by the induced expression of Gata6. Thus leading to the suggestion that cell fate is not yet irreversibly commited and can

still be altered during the early- to mid- stages of blastocyst maturation despite the already separated expression of PrE and EPI markers between cells of the ICM [6][77][79].

Important TFs necessary to maintain pluripotency and inhibit differentiation are required to form the EPI and include Sall4, Nanog, Oct4 and Sox2. However, genetic ablation of *Sall4*, *Oct4* and *Nanog* (including the removal of maternally inherited transcript and proteins) reveals arrested development phenotypes at the 4-cell stage and uncompacted 8-cell stages; indicating an additional and essential early role in preimplantation development (genetic ablation of only the zygotically derived expression of these factors, results in ICM and specific EPI-related phenotypes). The expression of *Sall4*, *Nanog*, *Oct4* and *Sox2* are important in establishing an auto-regulating network that not only enhances the expression of each factor within the network, but also resists differentiation, promotes pluripotency and leads to the emergence of the EPI lineage [69][27][84].

The spalt gene family member *Sall4*, is required for pluripotency in embryonic stem cells (ESC), themselves derived from blastocyst ICMs, and reduction of Sall4 levels promotes differentiation into TE-like cells. Sall4, acts as a transcriptional activator and initiates the expression of the *Oct4* gene by binding to a highly conserved regulatory region of the *Oct4* gene distal enhancer, thus directing to its expression *in vitro* as well *in vivo* [85].

Oct4 (a mammalian POU-domain containing transcription factor that is centrally important during the first cell fate decision, by down-regulating *Cdx2* gene transcription/ expression) is also important during the second cell fate decision, as it promotes pluripotency. As such, it first becomes expressed in blastomeres at the 8-cell stage and then is enriched in EPI cells during later development of the blastocyst [49][86][52].

1.7. Identification of novel candidate genes in the segregation of the trophectoderm and inner cell mass

In order to determine, within the Bruce lab, novel candidate genes influencing the first cell fate decision, TE and ICM lineage progenitors were analyzed. Accordingly, mRNA sequence analysis of the inner and outer cells, from the 16-cell stage embryo, was conducted in order to determine genes that are differently expressed in the two presumptive lineages. It was anticipated that such differential expressions could indicate potential roles in the segregation of TE from ICM. About 1000 differentially expressed genes were obtained from this sequence analysis. Their number was narrowed down by comparison with another reference list containing the global gene expression of mRNA from intact embryos at all stages of preimplantation mouse embryo development [88]. Further restriction of the obtained range was achieved by selecting those genes whose mRNA expression analysis also showed their maximum expression at the 8- to 32-cell stage, as during this time the specification between ICM and TE becomes complete. Also by comparison with the temporal expression profiles of

already well known genes participating in the first cell fate decision, further selection was achieved. Only those genes that fulfilled the above criteria were further analyzed and numbered around 100 shortlisted genes. These were further narrowed by gene ontological screenings and analyzing the literature for linkages to other developmental processes or their connection to other well established lineage specific genes. These selection processes yielded about 50 novel candidate genes that harbor great potential to influence TE and ICM cell lineage separation. Contained within this list are the genes encoding the *colony stimulating factor 1* (*Csf1*) and *zinc finger protein 1* (*Zfpm1*); it was these genes that were investigated in the present study.

1.8. Zinc finger protein 1 - Zfpm1

The zinc finger protein multitype 1 (Zfpm1, also known as friend of Gata-1/ FOG 1) protein, encoded by the *Zfpm1* gene, is a transcriptional cofactor that has an important and characterized role in the differentiation of erythroid and megakaryotic blood cell lineages. Zfpm1 forms heterodimers with transcription factors of the Gata family including: Gata1, Gata2 and Gata3. Such heterodimers have the ability to either activate or repress transcriptional activity, depending on cellular and target gene promoter context. For example, some Gata protein-Zfpm1 heterodimers have been shown to activate the transcription of genes such as *Nfe2*, *Itga2b*, *alpha*- and *beta-globin* while the same heterodimers suppress the expression of Klf1 derived transcripts. Zfpm1 was first identified by Tsang et al. as a binding partner of the TF Gata1. It is coexpressed with Gata1 in erythroid and megakaryotic cells but also during embryonic development; Zfpm1 transcripts are detectable in two extraembryonic mesodermal derivatives, the yolk sac and the allantois. Gata1 and Zfpm1 closely cooperate during the differentiation of erythroid and megakaryocytic cells from pluripotent hematopietic stem cells, as they synergistically activate the transcription of key target genes from a hematopietic-specific regulatory enhancer containing regions [95][96]. Tanaka and colleagues have found that Zfpm1 promotes proliferation of primitive and definitive erythroid cells throughout the differentiation period and also suppresses magakaryopoiesis, but only in the early stages of differentiation, after which it is required to enhance it during later stages. Another important observation is that Zfpm1 can also block the proliferation of ESCs, but via mechanisms distinctive from those observed in erythroid and megakaryopoiesis differentiation. Therefore, it has been concluded that Zfpm1 functions as a suppressor of cell proliferation, but this depends upon cell context of extent of existing differentiation [97]. Zfpm1 is also important in the development and differentiation of mast-cells, although it is not present in mature mast-cells. Indeed, the expression of Zfpm1 in the mast-cell progenitor cells inhibits their differentiation, instead favoring their development into neutrophils. Accordingly, Zfpm1 inhibits an important interaction between a TF called PU.1 and Gata1, that is considered a prerequisite for the mast-cell differentiation. Moreover if Zfpm1 is ectopically expressed in the

mature mast-cell lineage, a significant loss of mast-cell characteristic ensues, that is attributed to the down-regulation of MITF, another TF required for mast-cell differentiation and maturation. Therefore, Zfpm1 can also inhibit cell differentiation in another context, namely mast-cell differentiation [98].

In order to characterize the protein expression pattern of the Zfpm1 protein during preimplantation mouse development, embryos were fixed at developmental stages and immuno-fluorescently stained using anti-Zfpm1 antibodies. To be able to compare the staining results, the embryos were divided into two groups and each was stained with a different anti-Zfpm1 antibody, raised in a different animal (*i.e.* goat or rabbit – see results section).

1.9. Colony stimulating factor 1 - Csf1

The *colony stimulating factor 1* (*Csf1* or *M-CSF*) is a protein coding gene, that acts as a cytokine and is best characterized as controlling cell survival, proliferation, differentiation and chemokine release in haematopoietic precursor cells, such as macrophages. The receptor for this cytokine is called colony stimulating factor receptor 1 (Csf1r or cFMS) and conducts the Csf1 signal by ligand-induced dimerization and tyrosine autophosphorylation. Therefore the receptor protein belongs to the Csf1/Pdgf receptor family [89].

In 1992 Arceci et al. investigated the expression of Csf1 and the closely related steel factor (SF) during preimplantation mouse development. They observed that the concentration of Csf1r mRNA increases to peak expression during mouse oocyte maturation but drops significantly after fertilization, becoming no longer detectable at the 2-cell stage. At the late 2-cell stage, following ZGA, Csf1r mRNA levels become detectable again but are maintained at comparatively lower levels throughout the whole preimplantation period. The same authors reported that Csf1 transcript levels were not detectable during the preimplantation period, but that they were found in cumulus cells, oviducts and the uterus, leading to the suggestion that Csf1 has a maternally provided paracrine function during the preimplantation development. [90] Contradictory to such published results are the observations from the above describe spatial mRNA-sequencing and temporal microarray mRNA sequence analysis, designed to identify novel cell-fate influencing genes, that demonstrate that Csf1 mRNA is expressed in the mouse preimplantation embryo. The most probable explanation for the discrepancy between these two results, in relation to *Csf1* expression, lies in the technical improvements, and specifically sensitivity, associated with the later study compared with the earlier 1992 report [88][*A.W.Bruce, personal communication*]. The first evidence that Csf1 can actually influence cell lineage differentiation in the developing mouse preimplantation embryo, was first demonstrated by Bhatnagar et al., whose team was able to show that the addition of Csf1 to *in vitro* culturing medium significantly enhanced TE cell formation (by about 20 percent without significantly lowering the number of ICM cells). Moreover they reported blastocyst formation that was significantly accelerated by day E4.0 and ascertained that the appropriate/ minimal

concentration of Csf1, to attain the observed TE phenotypes, was 540U ml^{-1} (*n.b.* higher concentrations inhibited the blastocyst formation) [91]. In order to determine the dependence of fertility upon the *Csf1* gene, crosses between osteopetrotic Csf1 mutant mice (op/op) have been performed. Crosses between $females^{op/op}$ and $males^{op/op}$ exhibited persistent infertility (*i.e*. failure in embryogenesis), while crosses between heterozygous $males^{+/op}$ and homozygous $females^{op/op}$, as well as crosses between heterozygous $females^{+/op}$ and homozygous $males^{op/op}$ were able to produce viable offspring. These observations suggest that a maternal Csf1 source (*i.e*. provided in a paracrine manner from, for example, culumnus or tubial fluid) is not required, for successful development, as long as a paternal Csf1 allele is provided to the embryo and thus is able to successful compensate for the lack of maternally provided Csf1 [92]. Therefore, these data suggest an autocrine role for the embryo derived Csf1, in regulating the embryos own development, potentially during the preimplantation period.Csf1 has also been shown to be important for osteoclast survival, by activating Ras, that in turn coordinates the activation of PI3K and Raf, thus mediating MEK/ERK pathway activation. Nevertheless the exact molecular mechanism of the Csf1 mediated effect remains unclear. [93] It is important to note that the Ras-MEK/ERK pathway is also known to influence the early preimplantation mouse development, as the activation of MEK/ERK promotes the differentiation of ES towards the TE lineage by enhancing *Cdx2* expression. [94]

Therefore, due to the above observations it was hypothesized that zygotically/ self embryo derived Csf1 may act autocrinally/ paracrinally on the outside cells of the developing mouse embryo to enhance TE differentiation/ development. Moreover, that the inner cells, having a higher concentration of *Csf1*-derived mRNA, in comparison to the outer cells (as determined from the spatial mRNA sequence screen at the 16-cell stage (see above), are the sources of this localized cytokine based signaling. In order to test the dependence of zygotically provided Csf1 upon TE development, it was hypothesized that the elimination/ removal of embryo-derived Csf1 (in defined *in vitro* culture conditions, lacking any external source of Csf1) would lead to a decrease in TE cell formation while the number of ICM cells would likely remain the same or increase. Therefore, an experimental schema was designed to directly down-regulate *Csf1* expression throughout the embryo using a RNA interference (RNAi) based method. Accordingly, anti-Csf1 double stranded RNA (dsRNA) was microinjected, together with *discosoma* red (DsRed) mRNA (that produces a red fluorescent protein to control for a successful injection and also provides an convenient microinjection control) into mouse zygotes, followed by *in vitro* culturing and fixation at the early-blastocyst/ 32-cell (E3.5) stage. The fixed embryos were then immuno-fluorescencently stained using an anti-Cdx2 antibody (as a reliable TE marker) and the total number of TE cells (and overall cells) calculated and compared with controls (in which Csf1 expression had not been targeted). As only a low number of embryos were analyzed it was not possible to

unequivocally confirm a reliable effect on the TE cell number after RNAi-based knockdown of zygotic *Csf1* expression; however, the anticipated effect was not observed.

2. Materials and Methods

All chemicals used in the following experiments were of analytical grade and obtained by the company Sigma-Aldrich, unless otherwise stated.

2.1. Embryo collection

Female 8-week old F1 hybrid mice (derived from C57BL/6 x CBA/W crosses), were superovulated by intraperitoneal injection of pregnant mare´s serum gonadotropin (PMSG, 7.5IU) at 16:00 and injected again with human chrorionic gonadotropin (hCG, 7.5IU) exactly 48hours later. Immediately after hCG injection females were transferred (a maximum of two) to cages containing one F1 stud male, in order for mating. The next morning the females were separated and successful mating was determined by the presence or absence of a vaginal sperm plug. Females were sacrificed 1.5 days post hCG injection by cervical dislocation and following dissection the oviducts were removed into pre-warmed (37°C) M2 medium [18]. 2-cell stage embryos were liberated from the oviducts into M2 media by micro-dissection and then transferred, through a series of washing drops, into KSOM *in vitro* embryo culture media (Merck Millipore) plates (see below).

In cases were zygotic microinjections were to be performed, the females were sacrificed at E0.5 (around midday on the first day post hCG injection). The zygotes were released into pre-warmed (37°C) M2 media, from the swelled ampulla region of dissected oviducts, together with accompanying cumulus cells that were then removed by treatment with hyaloronidase (diluted in 37°C pre-warmed M2 medium). The obtained denuded zygotes were further washed through M2 [18] and transferred into KSOM (obtained by Merck Millipore) for *in vitro* culture, before being prepared for the microinjection as described below.

2.2. Anti-Csf1 double stranded RNA (dsRNA) preparation

For the zygote microinjections, anti-Csf1 double stranded RNA (dsRNA) had to be generated. Therefore, a DNA template incorporating the T_7 bacteriophage RNA polymerase promoter sequence at the 5´ end of each strand was prepared by PCR for further use in an *in vitro* transcription (IVT) reaction, where the Csf1 specific dsRNA will be created. As a template, pre-prepared embryonic stem cell complementary DNA (cDNA) was used in the PCR reaction to generate the DNA template. The following Csf1 specific primer sequences were used (underlined sequence represents the T_7 RNA polymerase primer sequence while the non-underlined stands for the specific *Csf1* cDNA sequence):

Forward primer: 5`-<u>TAATACGACTCACTATAGGG</u>ATTGGGAATGGACACCTGAA-3´.

Reverse primer: 5´-TAATACGACTCACTATAGGGGTTGTCTTTAAAGCGCAT-3`.

In order to determine the ideal PCR cycling conditions (most important was to investigate the ideal primer annealing temperature), a relatively cheap `OneTaq´ DNA polymerase (New England BioLabs) was first used to create the 220 bp double stranded DNA template, revealing the ideal temperature for the primer annealing step to be at 64°C. The PCR was repeated under ideal conditions, previously determined, with the `High fidelity PCR´ DNA polymerase (Roche; expensive) generating the wanted DNA template for the subsequent IVT. The PCR thermocycler settings were programmed as listed below:

- 95°C for 2 minutes, initial denaturation
- 95°C for 30 seconds, denaturation
- 64°C for 30 seconds, primer annealing
- 72°C for 30 seconds, extension
- 72°C for 5 minutes, final extension
- 4°C, hold

For purification standard phenol-chloroform extraction and ethanol precipitation techniques were applied, giving the pure DNA template, which was checked for integrity by agarose gel electrophoresis. Using the purified *Csf1* specific DNA template, dsRNA was generated in an IVT reaction (MEGAscript T7 by Ambion, following the provided instructions). The obtained IVT derived dsRNA was further purified by extraction with phenol chloroform, followed by treatment with an RNAse cocktail (from Ambion following the provided instructions) in order to remove any remaining single-stranded RNA. A further phenol/ chloroform extraction was performed, and the purified dsRNA was precipitated with isopropanol. The final concentration and purity of the obtained anti-Csf1 dsRNA, suspended in nuclease free water, was determined by UV spectrophotometry and the dsRNA integrity was checked by agarose gel electrophoresis. Finally the anti-Csf1 dsRNA was stored at -80°C. In order to avoid contamination of the dsRNA, the above described reactions were performed in a laminar hood using special pipettes that are dedicated towards RNA preparation.

2.3. Anti-Csf1 dsRNA microinjection

The zygotic microinjections with anti-Csf1 dsRNA (diluted to final concentration of 930 ng/µl) together with the fluorescent specific lineage marker mRNA for DsRed (final concentration of 75 ng/µl; used to trace successful microinjection) were performed 20 hours post hCG injection. The control group embryos were solely microinjected with the DsRed mRNA (at an equal concentration).

2.4. Embryo *in vitro* culture

Culturing plates, 35x10 mm, were prepared; each containing 11 drops (~15µl) of KSOM medium overlaid with 3ml sterile mineral oil. In order to equilibrate to the *in vitro* culturing

conditions (37°C, 5% CO_2), the plates were stored for at least one hour in the incubator before transferring the embryos, washing through the drops, into the culturing plates.

2.5. Embryo fixation

Embryos were fixed at the uncompacted and compacted 8-cell, mid-16-cell, 32-cell and late blastocyst stages. Whilst the uncompacted 8-cell stage embryos were selected by counting the number of the blastomeres under the microscope, the later stages were collected according to the standardized time-points, expressed as embryonic days [*e.g*. mid-16-cell stage as embryonic day 3.0 (E3.0); 3 days after the assumed fertilization, taken as the mid-point of the dark cycle on the night of mating]. Firstly the *zona pellucida* was removed by transferring embryos into drops of prewarmed (37°C) acid Tyrode´s solution and visually confirming its removal. Next embryos were washed through prewarmed KSOM drops before being transferred to the prewarmed paraformaldehyde (4% in a phosphate buffered saline solution, obtained from Lach-Ner) and incubated for 20 minutes at 37°C. Finally, the fixed embryos were stored in 96-well, flat bottomed, polystyrene plates (obtained by Amresco), that were pre-coated with agar (1% agar, 0.9% NaCl) to prevent sticking, and filled with a phosphate buffered saline solution (PBS). In this way the embryos could be stored at 4°C for up to one week before being further used processed (*e.g*. immune-fluorescent staining).

2.6. Embryo immuno-staining

Similar agar coated 96-well plates, to those described above, were prepared and the following reagents placed in successive wells (for each individual immuno-fluorescent staining regime), PFA fixed embryos were then passed through the successive treatments, for the designated incubation times/ conditions:

- 0.5% Triton X-100 (Fluka Analytical) in PBS for 20 minutes (for cell permeabilization) at room temperature.
- PBS-Tween 20 (0.15% Tween in PBS, referred to as PBS-T) for 10 minutes (x3 wells) at room temperature.
- NH_4Cl (about 2.6 µg/ml in PBS, prepared fresh, obtained from Lach-Ner) for 10 minutes (removed residual PFA) at room temperature.
- PBS-T for 10 minutes (2-wells) at room temperature.
- BSA blocking solution (3% BSA in PBS-T), incubated for 4hours at 4°C.
- primary antibody solution (primary antibody diluted in 3% BSA in PBS-T), incubated at 4°C overnight.
- PBS-T for 10 minutes (x2 wells) at room temperature.
- BSA blocking solution for 1 hour at 4°C.
- secondary antibody solution (secondary antibody diluted in 3% BSA in PBS-T), incubated for 1h at 4°C

- PBS-T for 10 minutes, (x3 wells) at room temperature.
- Texas red-phalloidin staining (1:50 dilution in PBS, obtained from Invitrogen) for 30 minutes at room temperature.
- mounting of immuno-stained embryos on glass bottom dishes

The following dilutions of the antibodies were used:

Zfpm1 experiment:

- primary antibodies:
    - rabbit anti-Zfpm1 (LS-C30123, Lifespan Biosciences), 1:300 in 3% BSA in PBS-T
    - goat anti-Zfpm1 (sc-9361, Santa Cruz Biotechnologies), 1:200 in 3% BSA in PBS-T
- fluorescently-conjugated secondary antibodies:
    - Goat-derived Alexa Fluor 488 conjugated anti-rabbit lgG antibody (A11043, Invitrogen), 1:500 in 3% BSA in PBS-T, used for the detection of epitope bound rabbit anti-Zfpm1 primary antibody
    - Donkey derived Alexa Fluor 488 conjugated anti-goat lgG antibody (A11055, Invitrogen), 1:500 in 3% BSA in PBS-T, used for the detection of epitope bound goat anti-Zfpm1 primary antibody
    - Donkey derived Cy 3 conjugated anti-goat lgG antibody (705-165-003, Jackson ImmunoResearch Europe), 1:1000 in 3% BSA in PBS-T, used for the detection of epitope bound goat anti-Zfpm1 primary antibody, when simultaneous detection of rabbit anti-Zfpm1 primary antibody was performed

Csf1 experiment:

- primary antibody: mouse anti-Cdx2 (MU392A-UC, BioGenex), 1:200 in 3% BSA in PBS-T
- fluorescently-conjugated secondary antibody: goat-derived Alexa Fluor 488 conjugated anti-rabbit lgG antibody (A11043, Invitrogen), 1:1000 in 3% BSA in PBS-T, used for the detection of the mouse anti-Cdx2 primary antibody.

Finally, stained embryos were mounted on glass bottom dishes, covered by Vectashield (Vector Laboratories) mounting solution containing 4,6-diamidino-2-phenylindole dilactate (DAPI – to stain the DNA/ cell nuclei) and protected by glass cover slips.

2.7. Confocal microscopy image acquisation

The embryos were imaged using Olympus FluoView TM1000IX-80 inverted laser scanning confocal microscopy. In order to ensure that all embryo cells were imaged, a z-series comprising individual 2 μm stacks was made. Laser output settings were minimalized and images were taken with one saturated pixel, to ensure a maximum dynamic range over which

to image the samples. Individual embryos were imaged using the same acquisition settings to allow side-by-side comparisons. The following lasers and excitation wavelengths were used to image in the stated channel:

- 543nm Helium-Neon laser, excitation of Texas red-phalloidin, Cy3 and DsRed
- 488nm Argon laser, excitation of Alexa Fluor 488
- 405nm diode laser, excitation of DAPI

2.8. Confocal microscopy image analysis

The recorded images where analyzed with the FV10-ASW3.1 software provided by Olympus. In the case of the Zfpm1 experiments, cell membranes were stained with Texas red-phalloidin (red), DNA with DAPI (blue) and Zfpm1 with Alexa Fluor 488 (green) or Cy3 (red) to ascertain the subcellular localization of any detected Zfpm1 protein (*e.g*. on the membrane or in the nuclei). For the Csf1 experiments the cells were imaged in the DsRed specific channel (to verify successful zygote microinjection; red), DNA with DAPI (blue) and Cdx2 with Alexa Fluor 488 (green) in order to localize TE progenitor cells.

2.9. Data Analysis

For all calculations the open-source statistical program package R (Version 0.97.551.) under Windows 7 was used. Note that all statistical tests were used in an explorative way, therefore no correction of the type 1 error (two sided, 5%) was made, meaning that the results are only descriptive. Normal-distribution-tests for continuous variables, using the Kolmogorov-Smirnov-Test with Lilliefors Correction at a type-I error-rate of 5% were used. As test of variance homogeneity for continuous variables, the F-test was used at a type-I error-rate of 5%. If normality and variance homogeneity for two independent groups were assumed the two sample-independent t-test was used. In the case of variance heterogeneity, the Welch's t-test was used. Moreover, in the case of non normality the exact Mann-Whitney-U test was used.

<u>Zfpm1 experiment:</u>

Individual embryos were grouped according to their developmental stages (the uncompacted 8-cell and compacted 8-cell stage group displayed exactly the same staining pattern and most of the embryos from the uncompacted 8-cell group had already started with compaction, therefore the two groups were combined in one 8-cell stage´ group, together with the mid-16-cell and blastocyst stage groups) and divided into two further subgroups. Each subgroup was stained with either rabbit anti-Zfpm1 or goat anti-Zfpm1, following comparison between the two groups regarding their mean number of Zfpm1 stained cells (distinguished by the location of Zfpm1 protein: membrane & nuclei, membrane only, nuclei only or no staining). The total number of cells in an embryo was determined by counting the number of DAPI positive cell nuclei, apoptotic cells were neglected in the counting (discernible by the unique DAPI staining

of the apoptotic nuclei) and mitotic cells were counted as one cell (the mitotic cells make up the group of cells that have Zfpm1 protein localized in the membrane but not in the nucleus). The ratio of the different staining patterns to the total cell number, per embryo in each group, was determined in order to be able to compare the ratios of those patterns between the 8-cell stage group and the mid-16-cell group (*e.g*. ratio of membrane and nuclei localized Zfpm1 protein of the 8-cell stage embryo compared with the ratio of the mid-16-cell stage). This analysis would determine if the localization of the Zfpm1 protein become differently localized during the preimplantation development. Accordingly, the whole dataset of each group was first tested for a normal distribution, by applying the `Kolmogorov-Smirnov normality test´. If the compared datasets were both normally distributed, they were tested against each other using the `F-test´. Depending on its outcome, the statistical significance was determined with either the ` Two sample t-test´ or the `Welch's t-test´. In cases in which one or both datasets were not normally distributed (as tested by the Kolmogorov-Smirnov normality test), the statistical significance was queried by the `unpaired Wilcoxon-Test (which is also known as the `Wilcoxon-Mann-Whitney-Test´).

Csf1 experiment:

Each cell, per embryo, of the control and experimental embryo groups was described by its cell position (*i.e*. inner or outer) and Cdx2 protein expression status (*i.e*. Cdx2 positive, meaning that Cdx2 is expressed in the particular cell, or Cdx2 negative, when the cell does not express it). A cells spatial position was determined by its position within the embryo. Note that all individual cells were discernible by counting the number of DAPI positive cell nuclei. Whether the microinjection had been successfully performed was determined by controlling if, in all cells, microinjected DsRed mRNA derived fluorescence was detectable. Accordingly any statistical significant differences between the control group (microinjection of DsRed) and the experimental group (microinjection of DsRed and anti-Csf1 dsRNA) where then identified; this involved first testing if the whole datasets of each group were normally distributed, by applying the `Kolmogorov-Smirnov normality test´. In the cases where the compared datasets were both normally distributed, they were tested against each other using the `F-test´ and depending on its outcome, the statistical significance determined with either the ` Two sample t-test´ or the `Welch's t-test´. If one or both datasets are not normally distributed (tested by the Kolmogorov-Smirnov normality test) their statistically significance assessed using the `unpaired Wilcoxon-Test´ (which is also known as the `Wilcoxon-Mann-Whitney-Test´).

3. Zfpm1 results

As the aim of this thesis was to characterize the protein expression pattern of the Zfpm1 during preimplantation mouse development, embryos were fixed at different developmental stages and immuno-fluorescently stained by using anti-Zfpm1 antibodies. To be able to

compare the staining results, the embryos were divided into two groups and each was stained with a different anti-Zfpm1 antibody, raised in a different animal (*i.e.* goat or rabbit). It was hypothesized that Zfpm1 protein expression would be lineage specific, with expression being restricted to the blastocyst EPI layer, based on the general Zfpm1 related literature indicating that Zfpm1 protein is involved in the inhibition of cell differentiation in other cellular contexts. Therefore it was also predicted that Zfpm1 protein may be expressed within the ICM progenitor/ inner cells during earlier developmental stages. The following experiments were designed to indicate whether such hypotheses were correct or if Zfpm1 protein becomes localized within other early embryo cell lineages and therefore could influence the preimplantation embryo in a different manner to that proposed.

3.1. Experiment 1

The specific aim of this first experiment was to assay the expression of Zfpm1, at the protein level, within blastocyst stage embryos and to determine whether detectable Zfpm1 expression would be limited to a specific lineage and if it shows any remarkable intra-cellular localization. The lineage specific hypothesis was based upon the fact that the general Zfpm1 related literature (see section 1.8) indicates an important role for Zfpm1 in suppressing cell differentiation and it was anticipated that expression may be restricted to the pluripotent EPI lineage, deep within the ICM. Therefore, to achieve this aim, two anti-Zfpm1 anti-sera (one raised on rabbit and the second one raised in goat) were employed in immuno-fluorescent staining of *in vitro* cultured and fixed mouse blastocysts at both the mid- and late-blastocyst stages, and imaged by confocal microscopy. Specifically, blastocysts at either stage were either immuno-stained using one or the other of the anti-Zfpm1 antibodies, or a combination of both (each detectable using a specific secondary antibody conjugated to distinct fluorophores). The reason for this third group was to ascertain if the Zfpm1 protein pattern revealed by each antibody separately would consistently co-localize, thus providing extra confidence in the specificity of the results. The confocal microscopy image analysis (as summarized in Table 1) revealed a lack of detectable anti-Zfpm1 immuno-fluorescent staining at either blastocyst stage, with any of the three combinations of specific primary antibodies used. Possible explanations for these data include a lack of Zfpm1 protein expression at this stage or a deficiency in the immuno-staining protocol used.

	nuclear & membrane	only membrane	only nuclear	no staining	total cell number	cell stage
goat anti-Zfpm1 (n=6)	0	0	0	54.7	54.7	~64
rabbit anti-Zfpm1 (n=7)	0	0	0	49.9	49.9	
goat and rabbit anti-Zfpm1 (n=1)	0	0	0	71.0	71.0	

goat anti-Zfpm1 (n=3)	0	0	0	116.7	116.7	~100
rabbit anti-Zfpm1 (n=5)	0	0	0	91.0	91.0	
goat and rabbit anti-Zfpm1 (n=5)	0	0	0	107.8	107.8	

Table 1: **Mean values of the anti-Zfpm1 blastocyst immuno-staining results**. Table 1 indicates the mean number of cells expressing Zfpm1 protein at the specified intra cellular locations (*i.e*. nuclei & membrane, just membrane/ nuclear, no Zfpm1 protein expression) within *in vitro* cultured mouse mid- (>64 cells) and late-blastocyst (>100 cells) embryos. Moreover, the table also indicates the mean total cell number, as well as the average cell stage of the three differently stained groups (i.e. the embryos immuno-stained using rabbit or goat raised anti-Zfpm1 antibodies, or a combination of both).

Therefore, it was decided to again perform embryo fixation and repeat the immuno-fluorescent staining at earlier developmental stages, where one could be more confident (based on the mRNA expression screens described in the introduction) that Zfpm1 protein would be expressed. Accordingly, fixation of *in vitro* cultured embryos was performed at the 8- and mid 16-cell stages; it was hypothesized that Zfpm1 protein would exhibit enriched expression within the ICM cells of 16-cell stage embryos (for the same reasons as given above) and furthermore there could be differential intercellular expression in 8-cell stage embryos (that possibly contributes to ICM enrichment in the 16-cell stage). However, in order to simplify the experiment it was decided to just focus on one anti-Zfpm1 antibody and the variant raised in rabbit was selected. The localization of Zfpm1 protein was again observed by confocal image microscopy and overview of the results are summarized in Table 2 (see also Figure 4 for a representative confocal image)

.	nuclear & membrane	only membrane	only nuclear	no staining	total cell number	cell stage
rabbit anti-Zfpm1 (n=4)	7.8	0.5	0	0	8.3	8
rabbit anti-Zfpm1 (n=7)	14.4	0	1.6	0,1	16,1	~16

Table 2: **Mean values of the anti Zfpm1 immuno staining results in 8- and mid 16-cell stage embryos, using the anti-sera raised in rabbit.** Table 2 indicates the mean number of cells expressing Zfpm1 protein at the specified intra-cellular locations (*i.e*. nuclei & membrane, just membrane/ nuclear, or no detectable Zfpm1 protein expression) within *in vitro* cultured 8-cell and mid-16-cell stage mouse embryos. Also, the table indicates the mean total cell number, as well as the approximate cell stages of embryos immuno-stained using rabbit raised anti-Zfpm1 antibodies.

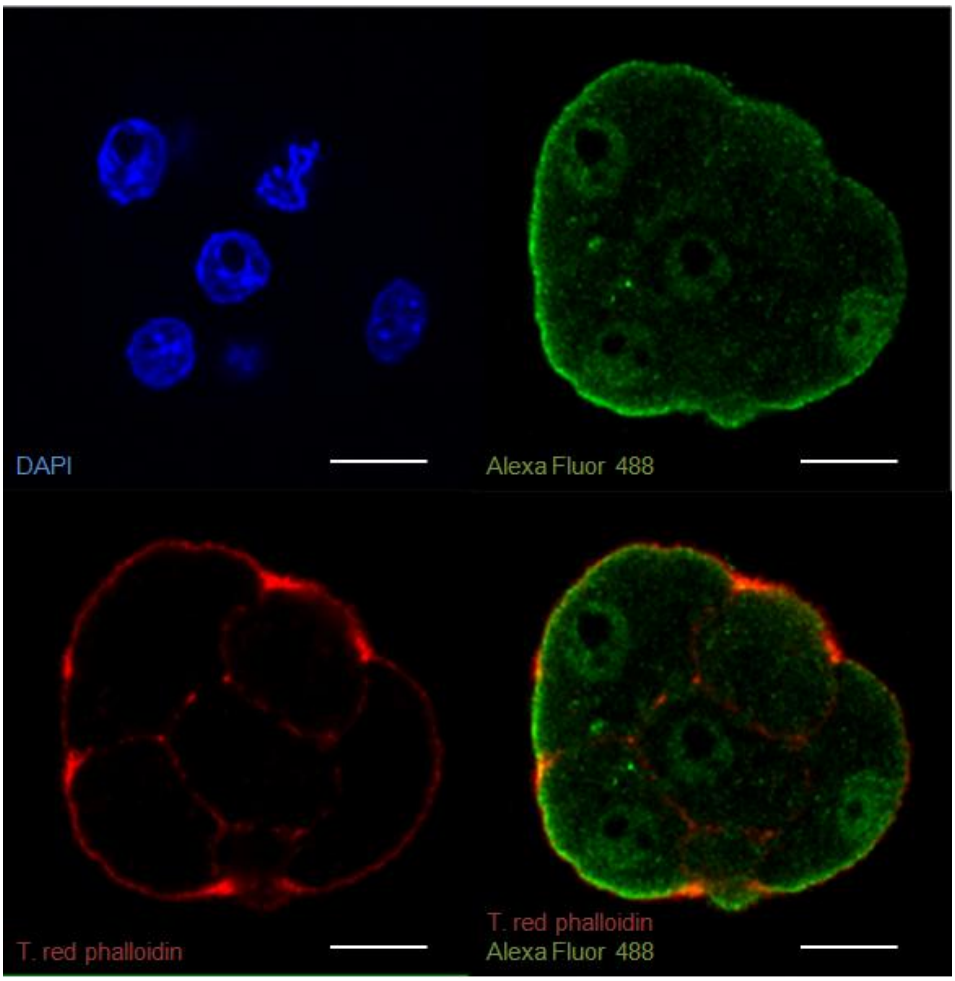


Figure 4: **Staining of a representative 16-cell stage embryo stained with rabbit-anti-Zfpm1**. First quadrant (top right) depicts the localization of Zfpm1 protein at the apical membrane and the nuclei of outer cells, but just the nucleus of the inner cells (green, Alexa Fluor 488). Second quadrant (top left) indicates position of individual nuclei (blue, DAPI) and the third quadrant (bottom left) displays the membrane of the individual cells (red, Texas red phalloidin) while the fourth quadrant (bottom right) shows the merge of the Alexa Fluor 488 and Texas red phalloidin channel. Scale bar = 20μm, a single confocal z-series stack is shown.

As shown in Figure 4 (above; detailing a single z-series slice confocal micrograph of a representative example 16-cell stage embryo), the immuno-staining revealed a detectable signal (see green, Alexa Fluor 488 channel) that was intracellular localized to the apical membrane (in outer cells) and the nucleus (in inner cells, only the nucleus). This anti-Zfpm1 derived membrane staining did not result from cross excitation of the Texas red-phalloidin actin staining, as anti-Zfpm1 derived membrane signal is restricted to the outer cells apical membranes (not in cell to cell contact with neighboring cells) whereas the Texas red-phalloidin signal is also present in basolateral (*i.e*. cell contact) regions, devoid of anti-Zfpm1 signal. Therefore it would seem that the rabbit anti-Zfpm1 antisera reports, somewhat unexpected, target protein expression at the apical membrane (only in 8- and outer 16-cell stage blastomeres) in addition the nuclei of all cells (that are not in mitosis).

A similar experiment and analysis, to that described above, was then repeated using the anti-Zfpm1 antibody raised in goat, to see if the two antibodies would give equivalent immuno-fluorescent staining at the 8- and 16-cell stages. However, confocal microscopy image analysis showed that, using the goat derived anti-Zfpm1 antibody, Zfpm1 protein was neither detected at either membrane or within cell nuclei in 8-, 16- and post-16-cell stage embryos (see summary in Table 3).

	nuclear & membrane	only membrane	only nuclear	no staining	total cell number	cell stage
goat anti-Zfpm1 (n=4)	0	0	0	8	8	8
goat anti-Zfpm1 (n=3)	0	0	0	15.7	15.7	~16
goat anti-Zfpm1 (n=3)	0	0	0	24.5	24.5	>24

Table 3: **Mean values of the anti-Zfpm1 immuno staining results in 8-and mid-16 cell stage embryos, using anti-sera raised in rabbit.** Table 3 indicates the mean number of cells expressing Zfpm1 protein at certain locations (*i.e*. nuclei & membrane, just membrane/ nuclear, no Zfpm1 protein expression) within *in vitro* cultured 8-cell, mid-16-cell and 25-cell (between mid-16 cell stage and early blastocyst stage) stage mouse embryos. Also, the table indicates the mean total cell number, as well as the approximate cell stages of embryos immuno-stained using goat raised anti-Zfpm1 antibodies.

The reason for the contradictory results, using rabbit and goat derived primary anti-Zfpm1 antibodies, is not clear. It is possible that it reflected an unintentional methodological omission in preparing the goat derived anti-Zfpm1 samples. Equally it may have reflected unspecific immuno-fluorescent staining using the rabbit anti-Zfpm1 reagent. However, using the latter reagent, detectable signal/ expression was observed in cell nuclei that would be consistent with its characterized role as a transcriptional co-factor, however, the observed membrane staining had not been previously reported and was unexpected, potentially reflecting a non-specific protein-antibody interaction.

3.2. Experiment 2

The aims of this experiment were to try and to replicate the previous results obtained by using the rabbit derived primary anti-Zfpm1 antibodiy (*i.e*. to see if the observed Zfpm1 immuno-staining pattern would be consistent), but also repeat the immuno-staining using the goat derived primary anti-Zfpm1 antibodies, in order to see if the previously obtained results may have been caused by a methodological omission in preparing the samples (*i.e*. checking for consistency). Therefore, immuno-staining utilizing the rabbit anti-Zfpm1 and goat anti-Zfpm1 anti-sera at the 8-cell and mid-16-cell stage was performed (but regrettably, the mid 16-cell stage embryo group, that were to be characterized using rabbit anti-Zfpm1 antibody, were lost

during the immuno-staining procedure, as sometimes embryos become stuck in the mouth pipette).

The confocal image analysis (as summarized in Table 4) revealed that those 8-cell stage embryos immuno-stained with rabbit anti-Zfpm1 antibodies displayed the exactly same staining pattern as in the previous experiment (*i.e*. nuclear and membrane localized Zfpm1 protein; depicted in the representative 8-cell stage image in Figure 5). This result demonstrates the immuno-staining pattern observed using the rabbit derived anti-sera is consistent, at least at the 8-cell stage. Moreover, the goat raised anti-Zfpm1 antibody also consistently reported a lack of detectable immuno-fluorescent anti-Zfpm1 staining at both the 8- and mid-16 cell stages. Therefore, the results from this experimental repetition were consistent, using both antibodies, with the previously acquired data. Of course, it is possible that the lack of immuno-staining using the goat-derived antibody could still have been caused by some unintentional methodological omission or that the staining using the rabbit anti-Zfpm1 reagent reflected a non-specific antibody interaction. However, the combined data do demonstrate consistency in the manner the experiment was conducted and in the case of goat derived reagent data suggest the lack of detectable expression was not likely caused by a sporadic human error.

	nuclear & membrane	only membrane	only nuclear	no staining	total cell number	cell stage
goat anti-Zfpm1 (n=5)	0	0	0	7.8	7.8	~8
rabbit anti-Zfpm1 (n=5)	8	0	0	0	8	
goat anti-Zfpm1 (n=1)	0	0	0	12	12	~16

Table 4: **Mean values of the staining results**. Table 4 indicates the mean number of cells expressing Zfpm1 protein at the specified locations (nuclei & membrane, just membrane/ nuclear, no Zfpm1 protein expression) within *in vitro* cultured 8-cell and mid-16-cell stage mouse embryos. Also, the table indicates the total cell number, as well as the cell stages of embryos immuno-stained using either rabbit or goat raised anti-Zfpm1 antibodies.

As depicted in Figure 5, this time showing a representative 8-cell stage embryo immuno-stained for Zfpm1 using the rabbit anti-sera reagent, consistent nuclear and apical membrane localization/ signal was observed. As before, the anti-Zfpm1 derived membrane staining is not the result of cross excitation of the Texas red-phalloidin actin staining, as the anti-Zfpm1 derived membrane signal is restricted to the apical membrane while the Texas red-phalloidin signal is also present in basolateral regions, which are absent of anti-Zfpm1 signal. Therefore, in overall summary of this experiment, it was concluded that the rabbit-derived anti-Zfpm1 immuno-staining, having given consecutively the same staining results in the 8-cell embryos (and probably also in the mid-16-cell stages), is consistent. However, given the same results were not observed using the goat-derived anti-Zfpm1 reagent there was still a question mark

about specificity. The most likely reason for the lack of consistent results using the Zfpm1 antibody raised in goat is an incompatibility of this reagent with the standardized immuno-fluorescent staining protocol used in the laboratory.

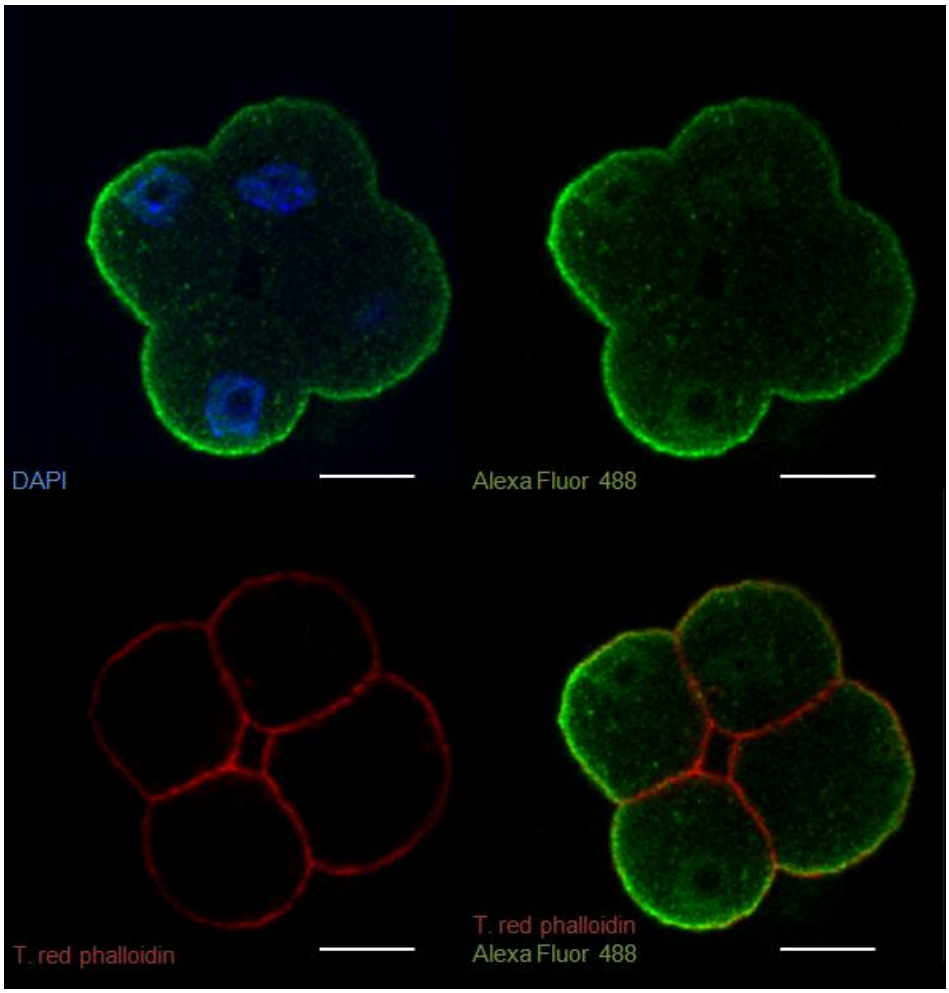


Figure 5: **Immuno-fluorescent anti-Zfpm1 staining of a representative 8-cell stage embryo stained with the anti-sera raised in rabbit.** First quadrant (top right) depicts the localization of Zfpm1 protein at the apical membrane and the nuclei (green, Alexa Fluor 488). Second quadrant (top left) indicates position of individual nuclei (blue, DAPI), and the third quadrant (lower left) displays the membrane of the individual cells (red, Texas red phalloidin) while the fourth quadrant (lower right) shows a merged image of all the other three channels. Scale bar = 20 μm and a single confocal z-series stack is shown.

3.3. Experiment 3

The aim of this experiment was to further test if the previously described lack of immuno-staining in the goat derived anti-Zfpm1 antibody group was consistent (therefore virtually eliminating the prospect that errors made during the sample preparation were the cause of the lack of signal) and to further confirm that the immuno-staining using the rabbit anti-Zfpm1, is potentially indicative of that Zfpm1 protein expression that is located in both the cell nuclei (congruent with its role as a transcriptional co-factor) and the apical membrane (completely unexpected), in 8- and mid-16- cell stage preimplantation mouse embryos. Accordingly, the

experiment was repeated, as described above in experiment 2 and consistently embryos immuno-stained with rabbit anti-Zfpm1 antibody exhibited both nuclear and membrane localized Zfpm1 protein/ signal, in all 8-cell and outer mid-16 cell blastomeres, plus solely nuclear expression in inner cells (note the embryo had begun to transit from the 16-cell stage and consequently had more inner cells with respect to those described in experiment 2. With regard to embryos immuno-stained with goat derived anti-Zfpm1 reagents, a lack of immuno-staining was again observed, however, upon increasing the laser excitation power and photomultiplier tube gain settings on the confocal microscope, some very weak membrane and nuclear Zfpm1 protein localization was observable (see Figure 6 for a representative 8-cell stage embryo immuno-stained with goat raised anti-Zfpm1). This was most readily seen in embryos immuno-stained at the 8-cell stage and was much less prominent at the 16-cell stage (or in embryos that had begun to transit towards the 32-cell stage – see the summary Table 5). It was not possible to determine if the goat raised reagent signal was truly indicative of Zfpm1 protein localization or the result of an image artefact caused by the cross excitation of the Texas red-phalloidin actin staining (in regard to the membrane) caused by enhanced image acquisition settings. Indeed when compared with the membrane staining obtained with the rabbit raised anti-sera, the membrane staining was not restricted to the apical domain and was present at all cortical regions/ membranes, in a manner similar to that of phalloidin detected actin; thus substantiating the artefact-based conclusion.

	nuclear & membrane	only membrane	only nuclear	no staining	total cell number	cell stage
goat anti-Zfpm1 (n=7)	*6,1*	*1,4*	0	0,3	7,9	~8
rabbit anti-Zfpm1 (n=6)	8.5	0.3	0	0	8.8	
goat anti-Zfpm1 (n=9)	*2,3*	*2,6*	0	11,1	16	~16
rabbit anti-Zfpm1 (n=11)	13.6	0.1	4.7	0.2	18.6	
goat anti-Zfpm1 (n=5)	0	0	0	24,6	24,6	~26

Table 5: **Mean values of the anti-Zfpm1 immuno staining results in 8-cell and mid-16-cell stage embryos, using the anti-sera raised in rabbit**. Table 5 indicates the mean number of cells expressing Zfpm1 protein at specified intra-cellular locations (*i.e*. nuclei & membrane, just membrane/ nuclear, no Zfpm1 protein expression) within *in vitro* cultured 8-cell, mid-16-cell and 16-to 32-cell transitional stage mouse embryos. Also, the table indicates the total cell number, as well as the cell stages of embryos immuno-stained using rabbit or goat raised anti-Zfpm1 antibodies. The presumably artefact results, obtained using the goat-raised antisera and enhanced confocal microscopy settings, are indicated by italic numbers.

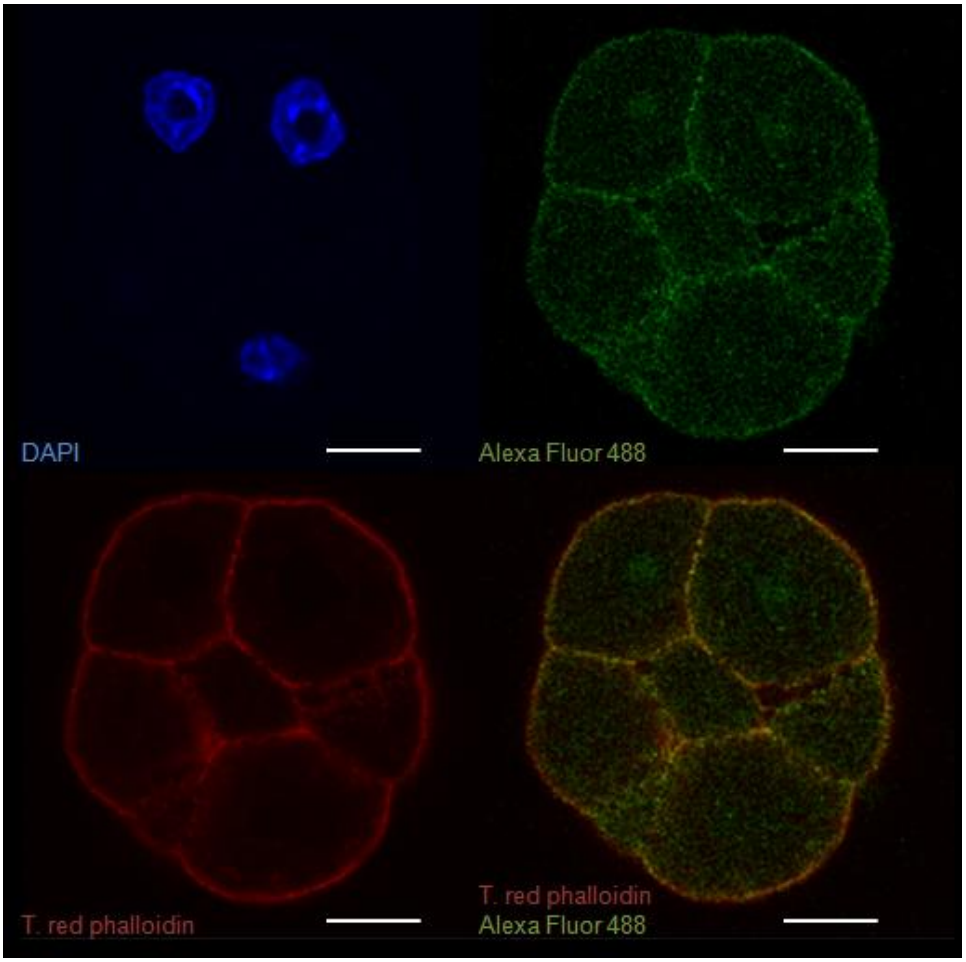


Figure 6: **Immuno-fluorescent anti-Zfpm1 staining of a representative 8-cell stage embryo stained with the anti-sera raised in goat.** First quadrant (top right) depicts the localization of Zfpm1 protein at the apical membrane and the nuclei (green, Alexa Fluor 488). Second quadrant (top left) indicates position of individual nuclei (blue, DAPI) and the third quadrant (bottom left) displays the membrane of the individual cells (red, Texas red phalloidin), while the fourth quadrant (bottom right) shows the overlay of the Alexa Fluor 488 and Texas red phalloidin channel). Scale bar = 20μm and a single confocal z-series stack is shown.

In the Figure 6 (above), it is clearly visible that the Zfpm1 protein signal appears “localized” where there is also Texas red-phalloidin staining. Therefore, in relation to the results of the experiment 3, it can be concluded that the lack of immuno-staining in the goat derived anti-Zfpm1 antibody group, when imaged under normal conditions, most likely reflects inappropriateness of the reagent for immuno-fluorescent staining in the preimplantation mouse embryos, at the 8- and 16-cell stage. It is possible that by using the enhanced confocal image acquisition settings, some specific signal is observable but this is not objective enough in nature to make any conclusions on Zfpm1 protein expression, using this reagent alone. Furthermore the immuno-staining with rabbit anti-Zfpm1 indicated that a robust and consistently present immuno-staining signal is detectable in the cell nuclei and at the apical membrane of 8- and mid-16-cell stage embryos, that most probably reflects Zfpm1 expression and sub-cellular localization. It can be concluded that the lack of immuno-staining in the goat

derived anti-Zfpm1 antibody group is again either based on errors made during preparing the samples or other undefined deficiencies. Furthermore the immuno-staining with rabbit anti-Zfpm1 indicates that Zfpm1 protein is located/ expressed in the cell nuclei, as well as at the apical membrane.

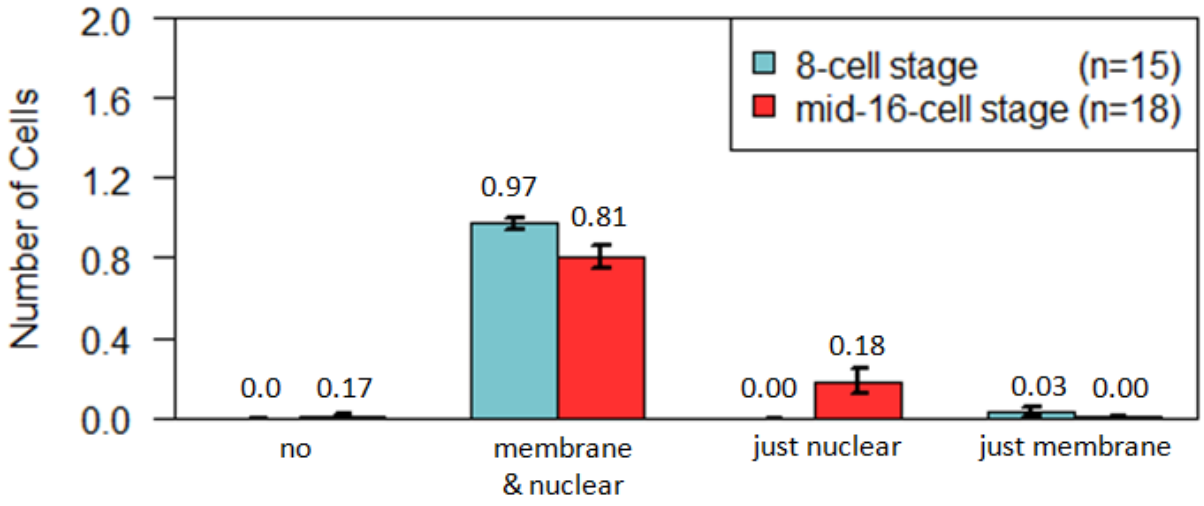
3.4. Zfpm1 discussion and further perspectives

It was initially hypothesized that the Zfpm1 protein would be lineage specifically expressed and likely to become restricted within the blastocyst stage embryo EPI populations or their progenitors at earlier developmental stages. However, it was found (based on the most reliable results obtained using the rabbit-derived anti-Zfpm1 antibody) that detectable levels of Zfpm1 protein expression were not present in blastocyst stage embryos. Therefore, the expression pattern within earlier stages was assayed, namely the 8-cell and mid-16-cell stages. It was observed that the expression of Zfpm1 protein appears to be localized to the apical membrane and the nucleus of all 8- and outer 16-cell stage blastomeres, with inner 16-cell stage embryos only displaying nuclear localized protein.

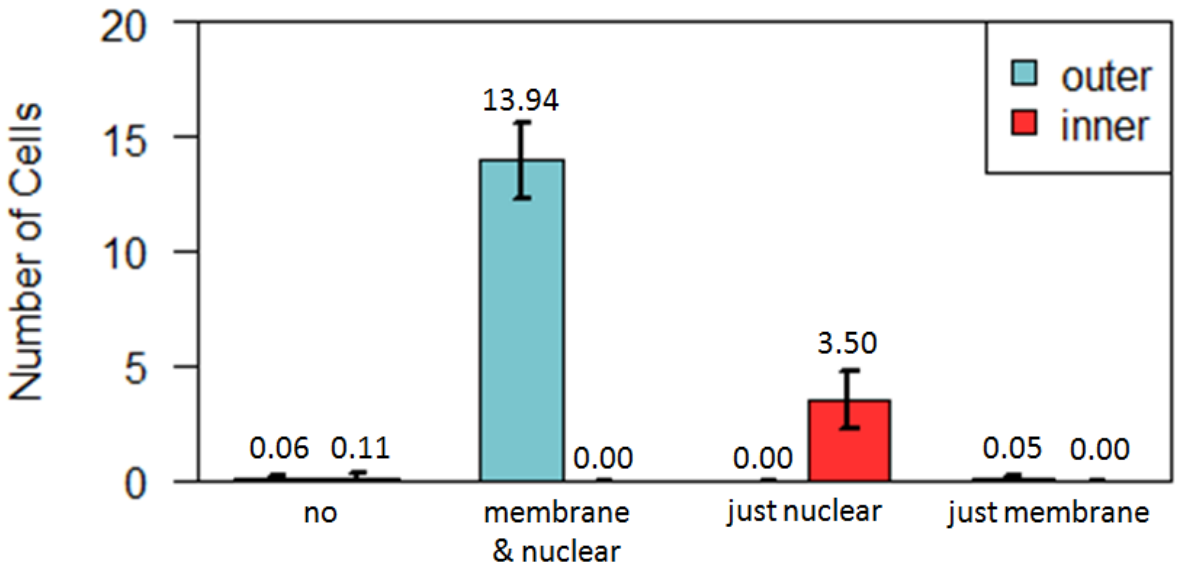
The ratio of each of the different immuno-staining patterns (*i.e*. membrane and nuclear, just membrane/ nuclear or no localized Zfpm1 protein) to total cell number was comapred between the 8- and mid-16-cell stage embryo datasets (*i.e*. combining all previously obtained data from the rabbit-derived anti-Zfpm1 antibody) in order to identify any statistically significant changes. Therefore each immuno-staining pattern group was first independently tested for a normal distribution of their values, using the Kolmogorov-Smirnov Normality test (employing the Lilliefors significance correction). As neither the different immuno-staining patterns of the 8-, nor mid-16-cell stage dataset were normal distributed (*n.b.* normal distribution would be very unlikely as the cells divide in a semi-synchronous wave of cleavages) the unpaired Wilcoxon test used to evaluate of the significance p-value related to any difference in the ratios of the immuno-staining patterns. Accordingly, it was found that there were no significant differences comparing the ratios of those blastomeres lacking membrane as well as nuclear localized Zfpm1 protein. Moreover, there was no statistical significant difference between the ratios of the cells that have just membrane localized Zfpm1 protein. However, there was an apparent heterogeneity between the expression of Zfpm1 protein of the inner and outer blastomeres observed at the mid-16-cell stage (see Graph 2 and compare with Graph 3). All outer 16-cell-stage blastomeres (as well as all cells at the 8-cell stage) have apical membrane localized Zfpm1 protein/ signal, while the inner cells just display nuclear localized Zfpm1 protein. Therefore, the ratios of the membrane and nuclear localized Zfpm1 protein differed significantly, as well as the ratios of the nuclear localized Zfpm1 comparing the 8-cell stage and mid-16-cell stage groups. The comparisons of the different staining patterns are depicted below in Graph 2 and the average numbers of cells with exhibiting the different staining patterns in the 8-cell and mid-16-cell stage group are given in Table 6. Moreover, the

individual immuno-staining patterns of the inner and outer cells of the mid-16-cell group were also analyzed separately (see Graph 3), showing a clear and apparent heterogeneity between the immuno-staining pattern of the inner and outer blastomeres, as regards the nuclear and membrane expression of Zfpm1 (i.e. nuclear and membrane Zfpm1 protein was nearly exclusively found in the outer cells, whilst just the nuclear pattern was observed in the inner cells).

It is tempting to speculate, based on the similar localization of other characterized polarity factors, that such polarized apical membrane localization of Zfpm1 might be important in cell lineage derivation as any cells dividing asymmetrically would only contribute the apically localized Zfpm1 protein to the outer, and not inner daughter cells; as both of these cells then have different fates in later development, it might be possible that Zfpm1 is involved in the first cell-fate decision. Also, as the apical membrane localization of Zfpm1 was unexpected, it is possible that the results may indicate a novel mechanism by which Zfpm1-regulated transcription may be controlled. Indeed, the sequestration of transcription factors and cofactors to the plasma membrane, only to be released and translocated to the nucleus after an appropriate and often extracellular cue is received, is known for other proteins; *e.g.* Smads/ Stats and Notch intracellular domain. Therefore, it would be of high interest to perform further experiments with Zfpm1 in order to determine its specific influence upon preimplantation mouse development.



Graph 2: **Ratios of the anti-Zfpm1 immuno staining patterns, comparing 8-cell and mid-16-cell stage embryos, using the anti-sera raised in rabbit**. Graph 1 indicates the mean number of cells expressing Zfpm1 protein at specified intra-cellular locations [*i.e*. nuclei & membrane (in graph named `membrane and nuclear´), just membrane/ nuclear (equally named in the graph), no Zfpm1 (named `no´) protein expression) within *in vitro* cultured 8-cell and mid-16-cell mouse embryos. The error bar displays represents the variability of data the range of total cell numbers, with a confidence interval of 95% and `n´ indicating the number of embryos observed.



Graph 3: **Number of the anti-Zfpm1 immuno staining patterns, within the mid-16-cell stage embryos, using the anti-sera raised in rabbit**. Graph 2 indicates the mean number of cells expressing Zfpm1 protein at specified intra-cellular locations [*i.e*. nuclei & membrane (in graph named `membrane and nuclear´), just membrane/ nuclear (equally named in the graph), no Zfpm1 (is named `no´) protein expression) compared to their spatial position within the embryo (*i.e*. ìnner´, `outer´) within *in vitro* cultured mid-16-cell mouse embryos. The error bar displays represents the variability of data the range of total cell numbers, with a confidence interval of 95% and n=18.

	mean number of cells	
	8-cell stage (n=15)	mid-16-cell stage (n=18)
nuclear and membrane localized Zfpm1 protein	8.13	13.94
p-value	<0.001	
just nuclear localized Zfpm1 protein	0.00	3.50
p-value	<0.001	
just membrane localized Zfpm1 protein	0.27	0.06
p-value	0.082	
no localized Zfpm1 protein	0.00	0.17
p-value	0.111	

Table 6: **Mean values of the anti-Zfpm1 immuno staining results in 8-cell and mid-16-cell stage embryos, using the anti-sera raised in rabbit**. Table 6 indicates the mean number of cells expressing Zfpm1 protein at specified intra-cellular locations (*i.e*. nuclei & membrane, just membrane/ nuclear, no Zfpm1 protein expression) within *in vitro* cultured 8-cell and mid-16-cell stage mouse embryos. Also, the table indicates the significance p-values obtained by comparing the ratios of each specific immuno-staining pattern group with the same group of the opposite developmental stage.

4. Csf1 results

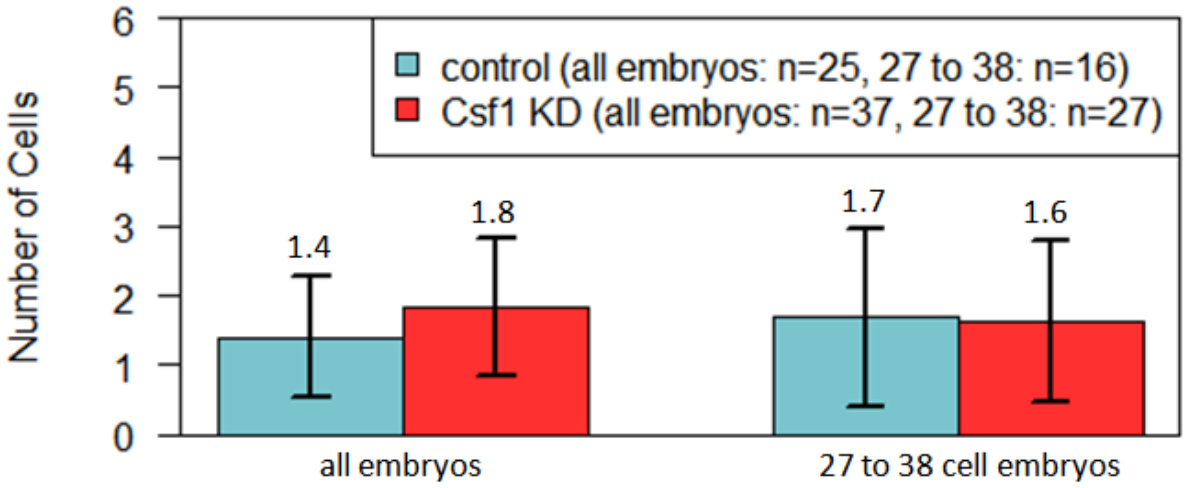
In order to determine any potential effects of embryo derived Csf1 upon TE and ICM development, anti-Csf1 dsRNA was microinjected into 1-cell/ zygote stage embryos, to down-regulate the endogenous mRNA in all cells during the preimplantation development period (*i.e.* an RNA interference-based method). Accordingly, immuno-fluorescently stained embryos, fixed at the desired developmental stage (*i.e.* E3.5/ early blastocyst), were imaged using laser scanning confocal microscopy and the total cell number (derived by counting all DAPI stained cell nuclei), number of fragmented cells (counting of the DAPI stained fragmented cell nuclei), number of Cdx2 positive and negative cells (used as specific TE marker and detected using immuno-fluorescent staining), as well as their relative spatial position (*i.e*. inner or outer) within the embryo was evaluated. According to this experimental design, a captured z-series of confocal micrographs for each studied embryo was analyzed, by which sequential overlays of the different imaged channels were annotated to reveal the number of cells in each category. As DsRed mRNA was microinjected in both control (*i.e.* alone) or experimental (*i.e.* together with the Csf1 dsRNA) embryo groups, to confirm successful microinjection, the resulting red fluorescence did not permit the use of Texas red phalloidin for actin/ membrane staining. Therefore a combination of the phase contrast light microscope channel image and nuclear shape were used in order to determine the correct spatial position of individual cells within the embryo (note, TE cell nuclei are typically flattened in morphology versus their counterparts in the ICM). Furthermore, any mitotic cells were counted as a single cell. Moreover, statistical analysis was performed on the quantification of all observed characteristic categories (*i.e.* total cell number, inner/ outer position, Cdx2 positive/ negative, apoptosis *etc.*) in order to determine if significant differences were between the experimental, Csf1 depleted, and control group blastocysts; and used to confirm or reject the hypothesis that down-regulation of embryonic derived Csf1 would cause a decreased number of TE cells, by the blastocyst stage.

4.1. Comparison of the mean total cell number of the control and Csf1 knockdown blastocysts

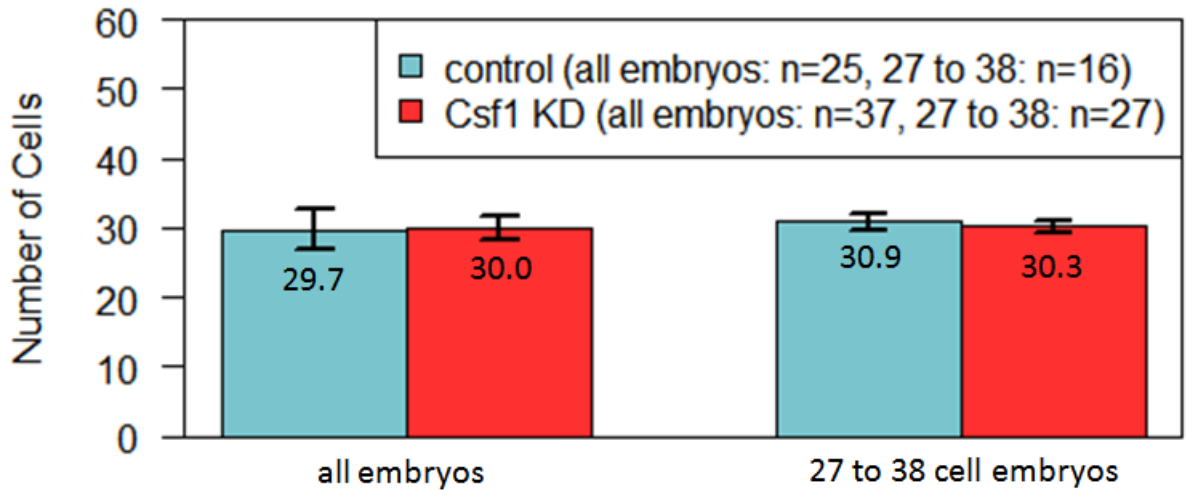
The total number of cells in the control (microinjection of DsRed) and the Csf1 knock-down groups (microinjection of DsRed mRNA and anti-Csf1 dsRNA) of microinjected zygotes, that were subsequently *in vitro* cultured to the 32-cell/ early blastocyst (E3.5) cell stage, was determined and compared, for any statistically significant difference. In order to choose the correct statistical analysis method, each group was first independently tested for a normal distribution of their values, using the Kolmogorov-Smirnov Normality test, employing the Lilliefors significance correction. As neither the control dataset, nor the Csf1 knock-down group was normal distributed (*n.b.* normal distribution would be very unlikely as the cells divide in a semi-synchronous wave of cleavages, for example the transition from 16- to 32-cell stage) an

unpaired Wilcoxon test was chosen to evaluate the significance p-value related to any difference in the mean number of total cells. Prior to determining the statistical significance in total cell number between the control and experimental Csf1 knockdown embryo groups, it was decided to split the data in a number of ways. Firstly, to additionally consider only embryos with a defined range of cells (namely, <27 - >38; in addition to all embryos from each group) and to represent the data with fragmented cell nuclei, respectively excluded or included in the analyses. The reasoning for the former distinction was to see if embryos (from either group) with approximately equivalent numbers of cells, exhibited any preferential lineage biases (e.g. Csf1 knockdown embryos having the same number of total cells but less TE and more ICM); as well as observing any differences in total cell number, *per se*. The reasoning for analyzing the data with or without the inclusion of fragmented cell nuclei, was to Csf1 knockdown was associated with increased or decreased frequency of apoptotic cell death (see Graph 4). Although it is important to note that cell nuclei fragmentation was clearly detected at this development time-point, equating to approximately one cell per embryo (in the control group condition), there was no significant difference detectable in the mean total number, as well as in the position occupied by the fragmented nuclei.

It was originally hypothesized that the Csf1 knockdown embryos posses the same mean number of total cells, but less TE cells and more ICM cells in comparison to the control group. Accordingly, it was found that there were no significant differences in the total number of cells between the control and experimental Csf1 knockdown groups and this was irrespective of the exclusion/ inclusion of fragment cell nuclei from the quantification (see Graph 4); meaning Csf1 knockdown/ Csf1-specific dsRNA microinjection had no effect on apparent apoptosis rates in the *in vitro* cultured embryos. Unsurprising, equivocal results, reporting non-statistically significant differences in total cell number, were also obtained when the quantification was restricted to embryos within the <27 to >38 cell range (as the outliers from the above analysis were removed; data not shown). Therefore, in conclusion, the attempted knockdown of embryo derived Csf1, using a Csf1-specific dsRNA microinjection based strategy, did not cause any significant changes in the total number of cells by the 32-cell/ early blastocyst stage (see Graph 5 indicating the mean number of total cells in the control and Csf1 knockdown group, including and excluding embryos with less than 27 or more than 38 cells) was the same.



Graph 4: **Graphical representation of the mean number of fragmented nuclei in the control group (blue) and Csf1 knock-down group (Csf1 KD; red)**, `all embryos.´ indicates that all embryos were included in that dataset tested, while `27 to 38´ indicates that just a certain range of embryos, with a total cell number corresponding to that range, were included in the analysis. The error bars represents the variability of the data, with a confidence interval of 95% and `n´ indicating the number of embryos observed.

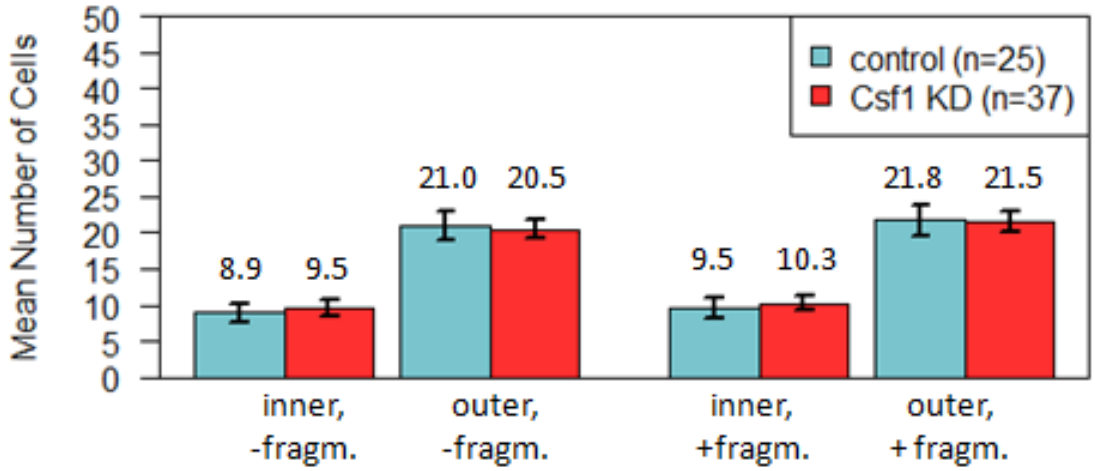


Graph 5: **Graphical representation of the mean number of total cells in the control group (blue) and Csf1 knockdown group (Csf1 KD; red)**, `all embryos.´ indicates that all embryos were included in that dataset tested, while `27 to 38´ indicates that just a certain range of embryos, with a total cell number corresponding to that range, were included in the analysis. Also, the fragmented nuclei were excluded. The error bars represents the variability of data the range of total cell numbers, with a confidence interval of 95% and `n´ indicating the number of embryos observed.

4.2. Comparison of cell position in the control and Csf1 knockdown blastocysts

It was next determined if the mean number of inner and outer cells in the Csf1 knockdown group differed significantly from those in the control group (again the same subgroups were analyzed, in addition to the complete dataset, as described above). Once again no significant difference in the number of outer and inner cells was uncovered between the control and Csf1

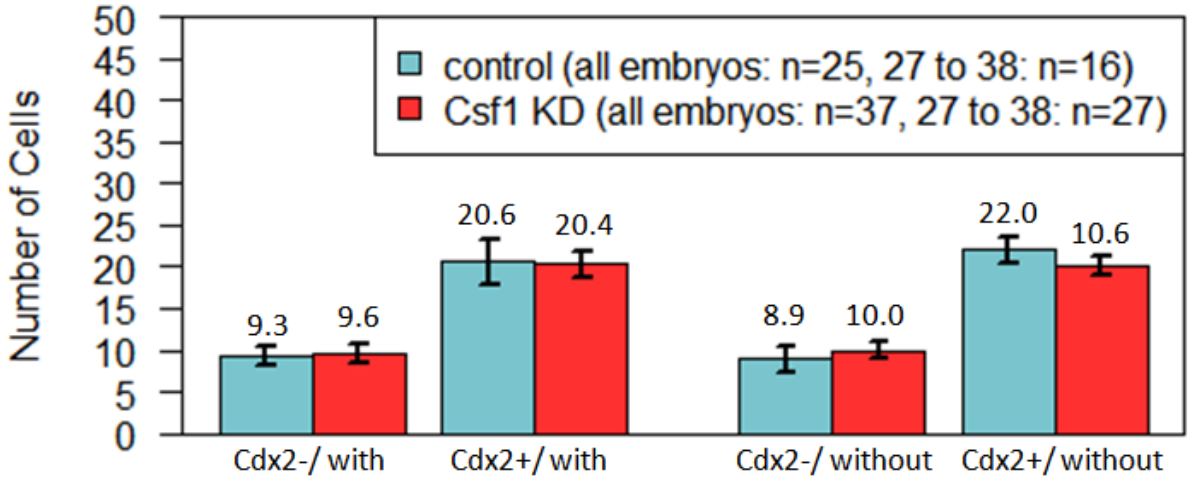
knockdown groups (see Graph 6 comparing the mean number of outer and inner cells between the control and the Csf1 knockdown groups, including all embryo data points; note the data subgroups in which fragmented nuclei were either excluded, “-fragm” on left, or included on the right, “+fragm”, are shown.



Graph 1: **Graphical representation of the mean number of inner and outer cells in the control group (blue) and Csf1 knockdown group (Csf1 KD; red)**, `+ fragm.´ indicates that the cells with fragmented nuclei were included in the datasets, while `- fragm.´ indicates that cells without fragmented nuclei were excluded, while `inner´ denotes the group of cells localized inside and `outer´ the group of cells localized the outside the embryo. Also, no embryos were excluded in this graphical representation (*i.e.* the limited range filter, 27 to 38 was not employed). The error bars represents the variability of data the range of total cell numbers, with a confidence interval of 95% and `n´ indicating the number of embryos observed.

4.3. Comparison of Cdx2 staining (indicative of TE cells) in the control and Csf1 knockdown blastocysts

As described above the analysis of cell position in Csf1 knockdown and control early blastocysts revealed no statistical significant difference in spatial allocation. Therefore, the number of Cdx2 positive (usually indicative of outer TE) and Cdx2 negative (usually indicative of inner ICM) cells, was also determined, as misplaced spatially allocated cells either positive or negative for Cdx2 may have arisen as a consequence of Csf1 knockdown. However, no statistically significant differences in Cdx2 positive or negative cells were observed (amongst all the subgroups assayed, including embryos of limited total cell number range, >27 to <38), nor were there any misplaced cells (*i.e.* according to Cdx2 expression status; see Graph 7). Note the data subgroups in which embryos with <27 or >38 cells were either included, (indicated in Graph 7 by “with” on left), or excluded (indicated by “without” on the right), are shown.



Graph 2: **Graphical representation of the mean number of Cdx2 positive (Cdx2+) and Cdx2 negative (Cdx2) cells in the control group (blue) and Csf1 knock-down group (Csf1 KD; red),** fragmented nuclei were excluded and mitotic nuclei were treated as Cdx2+ if they contained cytoplasmic Cdx2+ staining. Also, `with´ indicates the dataset in which all embryos were considered, while `without´ means that just embryos within the 27 to 38 range of total cell numbers were included in the datasets. The error bar displays represents the variability of data the range with a confidence interval of 95% and `n´ indicating the number of embryos observed.

4.4. Analysis of Cdx2 staining (indicative of TE cells) in regard to the control and Csf1 knockdown blastocysts

As one of the previously described analysis showed no statistical significant difference in the mean number of inner and outer localized cells between control and Csf1 knockdown group, those Cdx2+ and Cdx2- cells were further analyzed in relation to their position, as it would be possible that such TE specified cells (indicated by Cdx2+ immuno-staining) become misplaced within the ICM as a result of the Csf1 knockdown. Therefore, outer localized Cdx2+/ - as well as inner Cdx2+/ - cells were compared in the same subgroups as above. However, there were again no significant differences between the control and Csf1 knockdown group examined.

4.5. Csf1 conclusion and further perspectives

The derived data suggests that, contrary to the hypothesis, there is no major role for embryo derived Csf1 in promoting TE cell proliferation or survival during preimplantation mouse embryo development. However, two important caveat remains, in that the efficacy of the Csf1-specific dsRNA in causing a successful, RNAi mediated, knockdown in Csf1 mRNA/ protein expression was not determined, because of the time limitations of the project. Therefore, such verification should be performed, using real-time Q-RTPCR with Csf1 specific oligonucleotide primers, to assess the degree of knockdown in blastocysts derived from Csf1 dsRNA microinjected zygotes, versus the control condition. A demonstration that the normalized levels (calculated by comparison to results obtained using primers that detect an unaffected yet abundant gene mRNA *e.g.* Gapdh or actin) of Csf1 mRNA transcript were indeed knocked

down would provide added confidence of a lack of an important embryo derived Csf1 role, whereas the opposite would suggest the Csf1-specific dsRNA reagent was not efficient enough to reveal any potential phenotype. The second caveat would be discrepancies in the correct time of fixation, as errors regarding proper synchronized fixation could also lead to misleading assumptions upon the impact of Csf1 knockdown on the preimplantation mouse development. Therefore, it would be necessary to perform RNAi mediated, knockdown in Csf1 mRNA/ protein expression, with a reasonable larger quantity of immuno-stained embryos, that had been more precisely fixed according to developmental stage, in any further experiments.

5. Overall discussion/ conclusion and further perspectives

The experiments regarding localizing the expression of the Zfpm1 protein revealed that the hypothesis previously formed (*i.e*. that the Zfpm1 protein is lineage specifically expressed and becomes restricted within the blastocyst stage embryo EPI populations or their progenitors at earlier developmental stages) was wrong. Instead it was surprising to observe the Zfpm1 protein expressed in the apical membrane, as well as in the nuclei of 8- and outer 16-cell stage blastomeres with inner 16-cell stage embryos only displaying nuclear localized protein. Still, the expression of the Zfpm1 protein has to be compared with differently raised anti-Zfpm1 antibodies in order to provide extra confidence in the specificity of the observed interactions. Alternatively, recombinant Zfpm1 mRNA, into which a detectable tag had been incorporated, could be expressed in the embryo and it's subcellular localisation assayed to see if it agrees with that detected using the rabbit raise anti-Zfpm1 antibody in the described immuno-fluorescent staining. Also it would be useful to repeat immuno-staining of more developmental stages so that the Zfpm1 protein expressions could be traced during the preimplantation development (although the immuno-staining of early and late blastocyst stage embryo was performed, but only once). Concluding, as mentioned in 3.4., it is possible that the Zfpm1 protein localization display a novel mechanism by which Zfpm1-regulated transcription may be controlled. Therefore, it would be of high interest to perform further experiments with the Zfpm1 protein in order to determine its specific influence and molecular mechanisms upon the preimplantation mouse development.

The effect of embryo derived Csf1 upon the preimplantation development was inconclusive, as the lack of influence upon the TE cell development could be caused by inefficiency of the Csf1-specific dsRNA. Therefore, it is suggested to confirm successful knockdown of the Csf1 mRNA by quantitative real-time PCR with Csf1 specific oligonucleotide primers, to assess the degree of knockdown in blastocysts derived from Csf1 dsRNA microinjected zygotes, versus the control condition in further experiments. Moreover, the embryo fixation, of a reasonable large quantity, has to be correctly timed, as errors regarding proper synchronized fixation could also lead to misleading presumption upon the impact of Csf1 knockdown on the preimplantation mouse development.

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