Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

The Role of Csf1 During Preimplantation Mouse Development

Bachelor thesis

Marie Jakešová

Supervisor: Alexander W. Bruce, Ph.D.

Supervisor, consultant: Aleksandar Mihajlovic, MSc

České Budějovice, 2014

Jakešová M., 2014: The role of Csf1 during preimplantation mouse development. BSc. Thesis in English, 49 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

The aim of this thesis was to investigate the potential role of one candidate cell fate decision influencing gene, *colony stimulating factor 1 (Csf1),* on the mouse preimplantation development.

Affirmation

I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section.

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Marie Jakešová

Acknowledgments

I am greatly thankful to my supervisor Alexander W. Bruce, Ph.D. for the opportunity to work in his laboratory. I appreciate his never-ending support, encouragement and his valuable comments on my thesis. I would also like to thank Aleksandar Mihajlovic for his great guidance through all the laboratory work, advice, patience, and willingness to help in any occasion. I am also grateful to all the former and current members of our laboratory and the laboratory of RNDr. Alena Krejčí, Ph.D. for their help, advice, enthusiasm and friendship. It was a great pleasure to spend a year with you.

My gratitude goes also to my family and friends for giving me love and support all the way through my studies.

Summary

During development mammalian zygotes give rise not only to embryo itself but also to the supporting extraembryonic tissue layers of the placenta and yolk sac. Therefore a fundamental central question is, how the whole embryo and all the extraembryonic tissues can be formed from just a single cell? During preimplantation embryo development, three distinct cell lineages are set aside during two 'cell fate decisions'. The first of these segregates the differentiating extraembryonic trophectoderm (TE) from the pluripotent inner cell mass (ICM). The second involves the differentiation of primitive endoderm (PE), another extraembryonic layer, from the pluripotent epiblast (EPI) within the ICM. A number of factors have been implicated in the regulation of these two events. These can be summarised as a cell's relative position within an embryo, the establishment of cellular and embryo polarity, differential gene expression, epigenetic modifications and cell-signalling, that all work in concert to establish appropriate and successful early embryonic development. In our group, novel candidate genes hypothesised to function during the early cell fate decisions of preimplantation development are under investigation, using the mouse embryo model. One such candidate gene is the *colony stimulating factor 1* (*Csf1* also known as *macrophage colony-stimulating factor* or *Mcsf*). The known effect of exogenous Csf1 addition on the promotion of trophectodermal development combined with our own lab's unpublished data describing its elevated expression in the inner cells of the embryo, (relative to the outer TE destined cells), indicates that endogenous/ embryo derived Csf1 has potential paracrine activity that in turn promotes TE differentiation of the outer cells. This hypothesis was tested by functional inhibition and knock down of the candidate gene.

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1 Introduction

During evolution a specific characteristic appeared in the eutherian mammals. From a single cell, the fertilized ovum, not only is the embryo proper formed, but also essential extraembryonic tissues that give rise to the embryonic portion of the placenta and the yolk sac required to support *in utero* development.

A central tenant of the mammalian embryo is its remarkably robust and flexible early development during the preimplantation period. Through such regulative properties it is able to overcome major disturbances such as manipulation of the cell number (Tarkowski, 1959; Tarkowski and Wroblewska, 1967). One of the reasons for this flexibility is that the onset of gene expression in mammalian embryo, referred to as zygotic genome activation (ZGA), is started very early (when compared to other models of development, *e.g. Drosophila melanogaster* or *Caenorhabditis elegans*) thereby allowing the embryo to directly respond to environmental cues rather than solely rely on inherited maternal factors. In mouse, a minor ZGA of a select number of genes is initiated at the late 1-cell stage and is followed by major burst of ZGA during the early 2-cell stage. This activation compensates for the degradation of most of the pools of maternal messenger RNAs (mRNAs) and proteins also taking place during this time (mid 2-cell stage) of development (Zernicka-Goetz et al., 2009). Therefore, the early mammalian embryo is remarkably capable of self-regulation in the case of environmental or experimental perturbations.

1.1 Morphology of preimplantation embryo

In preimplantation mouse embryo development, the zygote undergoes at least seven cell cleavage divisions to form a differentiated and highly ordered vesicular structure known as the late blastocyst (Figure 1). During this period the size of the embryo remains approximately the same until the formation of the blastocyst cavity with no attendant increase in overall/ combined cell cytoplasmic volume. The length of the first cell cycle is approximately 18 hours and the duration of the subsequent cycles is 12 hours. These numbers are highly variable among embryos but also among individual blastomeres of the same embryo. The preimplantation period lasts for 4.5 days (also designated in the form of embryonic days – *e.g.* E4.5), after which the late blastocyst hatches out from the *zona pellucida* (the proteinaceous shell originally

surrounding the ovulated oocyte prior to fertilisation) and implants in the uterine endometrium to facilitate its further development (Johnson, 2009).

During the first 3.5 days of development, the embryo undergoes two large morphological changes. Firstly, at the 8-cell stage the cells (often referred to in the preimplantation embryo as 'blastomeres') become polarised along their 'apical-basolateral' axis. The cells develop a polar apical pole enriched in microvilli and other polarity determinants, whilst the basolateral region becomes smooth and non-polar. The blastomeres then compact together forming adherens and tight junctions. The establishment of such polarity represents the generation of the first clearly defined asymmetry of cells in the embryo. The second morphological change occurs during the 32-cell stage and is represented by the formation of a large fluid filled cavity within the embryo (known as the blastocoel) thus establishing the formation of the blastocyst.

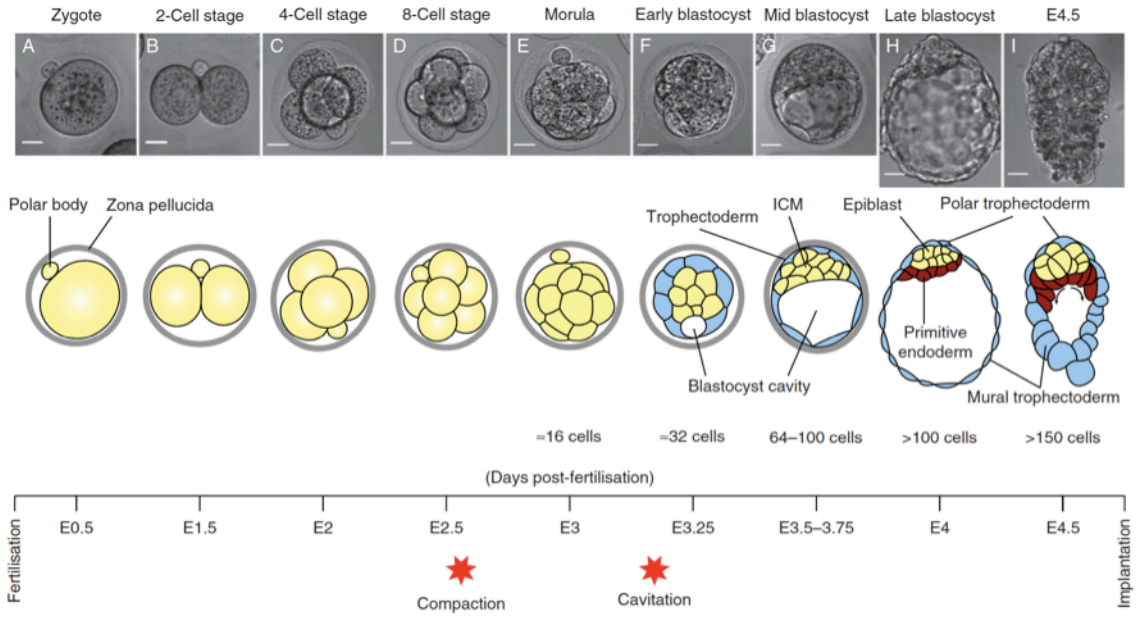

Figure 1: Overview of the mouse preimplantation development. During the first 4.5 days of embryonic development, two main morphological changes occur. Firstly, at the 8-cell stage the embryo compacts and at the 32-cell stage a cavity forms thereby producing a blastocyst. In the late blastocyst three distinct lineages are set. Firstly, during the first cell fate decision, trophectoderm segregates from inner cell mass. Secondly, ICM gives rise to extraembryonic primitive endoderm and pluripotent epiblast. *Taken from Saiz and Plusa (2013).*

1.2 The blastocyst cell lineages

During the preimplantation period of development, the embryo has to undergo two cell fate decisions during which three distinct lineages are derived at the late blastocyst stage. These are characterised by the relative spatial segregation of blastomeres during the transition between the 8- to the 16-cell and the 16- to the 32-cell stages, whereby newly derived inner cells (cells completely encapsulated by other cells inside the embryo) form the pluripotent inner cell mass (ICM), and the outer cells form the trophectoderm (TE) progenitor cells. The ICM further segregates into cells of the epiblast (EPI), residing deep within the ICM that ultimately gives rise to cells of the embryo proper, and cells of the primitive endoderm (PE) on the surface of the ICM in direct contact with the blastocoel (reviewed in Johnson, 2009).

1.3 First cell fate decision

The first cell fate decision segregates two emerging cell lineages during preimplantation development represented by the outer TE destined cells and inner ICM cells that are the progenitors of both EPI and PE cells. This spatial separation of TE and ICM cells occurs from the 8-cell stage and is governed by two kinds of cell division. 'Conservative', or 'symmetric', divisions produce two equal outer cells, whilst 'differentiative', or 'asymmetric' divisions form two different daughter cells, one on the outside and the second on the inside of the embryo. During symmetric divisions, the division plane is perpendicular to the outer surface of the cell; therefore ensuring that both the derived outer daughter cells inherit part of the polarised apical cell surface. Conversely, during asymmetric divisions the division plane is tangential to the outer surface of the cell resulting in one outer-residing daughter cell inheriting the entire apical polarity, whilst the other, inner cell retains only the basal part of the dividing cell, and thus is non-polar. It is these apolar inner cells that populated the ICM whilst the outer cells later form the TE. During mouse preimplantation development there are two rounds of potential asymmetric division occurring in 8-cell stage blastomeres during the fourth cleavage and outer residing 16-cell stage blastomeres at the fifth cleavage (Pedersen et al., 1986). Once the two cell types emerge they become restricted to distinct gene expression patterns. The pluripotency of the ICM is maintained through the activation of *Oct4* and *Nanog,* whilst in the outer cells the transcription factor *Cdx2* becomes upregulated thereby downregulating the pluripotency genes and promoting TE differentiation.

Whilst the importance of cell division plane orientation and the resultant relative spatial position of generated daughter cells is understood, the mechanisms controlling the commitment of cells to either the TE or ICM lineage has not been fully resolved. Historically, three theories trying to explain the causes of the cell lineage derivation and segregation have been proposed and are discussed below.

1.3.1 Mosaic model

This model suggests that there already exist embedded asymmetries in the embryo at the zygote stage that in turn dictate the fate of the blastomeres after later cleavages. This model was based on Dalcq's observations of similarities between rat oocytes and the highly deterministic embryo development of amphibians. He proposed that there exists bilateral symmetry in the mammalian oocyte that would result in the dorsal part of cytoplasm giving rise to the ICM, whilst the ventral part would yield TE progenitors (reviewed by Mulnard, 1992). This hypothesis was soon discredited as many subsequent studies revealed the highly regulative nature of early embryo development in mammals exhibited through the ability of an early preimplantation embryo to compensate for major experimental disturbances such as blastomere removal (Tarkowski, 1959).

1.3.2 Positional model ('inside-outside')

This model was proposed by Tarkowski and Wroblewska (1967), based on their experimental data on the development of disaggregated 4-cell and 8-cell stage blastomeres. They found that even single 8-cell stage blastomeres (1/8) can develop into a blastocyst like structure, although these were mostly "false blastocyst" structures termed trophoblastic vesicles, which were lacking the ICM. The incidence of more appropriate blastocyst formation was much higher when single blastomeres from 4-cell stage embryos were used. Based on these observations Tarkowski and Wroblewska proposed a theory, that any single blastomere derived from an embryo up to and including the 8-cell stage can develop into a morphologically recognisable blastocyst if at least one of the cells becomes completely surrounded by its sister cells during the subsequent cleavage divisions. According to this model it was this microenvironmental change that would trigger the inside cells to segregate to ICM rather than TE cell fate. Conversely, the enclosing cells would be set on the path to acquire the fate of TE. Hillman et al., (1972) tested this so-called 'inside-outside' theory by a series of embryo/ labelled blastomere aggregation experiments. When single labelled blastomeres (derived from 4- to 8-cell stage embryos) were

placed on the outside of unlabelled host embryos (at the 4- to 16-cell stage) they mostly contributed their subsequent progeny within the embryo aggregate to the TE. Alternatively, when a single labelled embryo was completely surrounded by 14 other unlabelled embryos, the labelled cells predominantly formed the ICM. These experiments therefore provided empirical evidence for the validity of the positional hypothesis.

1.3.3 Polarisational model

Even though the described positional model agreed well with multiple experimental observations, it was still unresolved what triggered the internalization of only some cells within the 4th and 5th cleave division of the embryo. It was later reported that concomitant with embryo compaction, 8-cell stage blastomeres become polarised along their 'apical-basolateral axis'. This is manifest by the appearance of a polar, apical membrane covered with microvilli and a smooth non-polar basolateral region through which cell-to-cell contacts are mediated (Ziomek and Johnson, 1980). To pursue this finding further, Johnson and Ziomek, (1981a) performed experiments in which they disaggregated 8-cell stage embryos and cultivated each single 1/8 blastomere separately throughout the following cell division. They observed that the polarised and now isolated 8-cell stage blastomere could divide in one of two manners. In the first, more common case, one larger cell inherited the entirety of the polarised apical pole, distinguishable by the microvilli, and was therefore itself highly polarised, while the other, smaller cell was non-polar. In the second case, the plane of division was directly through the apical pole and therefore both the daughter cells inherited part of the polarity. These observations were the forerunner to identifying the symmetric and asymmetric divisions that occur in the *in vivo* context of the intact embryo at the 4th and 5th cleavages. Accordingly, the authors proved the existence of these two cell division orientation types by disaggregating 16-cell stage embryos into blastomere couplets (2/16) and observing polar and non-polar morphologies consistent with their previous results. Based on these studies Johnson and Ziomek, (1981a) proposed that the differences between inner and outer cells are not caused by their differential position but rather by their differential inheritance. Thus when a blastomere inherits part, or all of the microvillous apical membrane, it becomes polar and forms the TE, whilst if it does not inherit any polarity the blastomere will give rise to the ICM.

1.3.4 Combining the previous models

Even though historically the two latter models have often been portrayed as opposing each other, a more contemporary view is that they in fact work in concert. Ziomek and Johnson, (1982) formed 16-cell stage embryos by aggregating one labelled 16-cell stage blastomere with 15 other unlabelled 16-cell stage blastomeres. They discovered that when smaller, non-polar, inner cells were placed on the outside of the embryo, they were able to induce polarisation and switch their cell fate to TE. In contrast, the bigger and polar outer cells when placed in the inside of the aggregate tended to divide in a differential manner, thereby producing one apolar daughter cell appropriate to its now experimentally redefined spatial location in the embryo. These experiments demonstrated that not only does polarisation influence a cells position within the embryo, but that the reverse is also true. Another important conclusion to come from these experiments was that the blastomeres of a 16-cell stage embryo are not yet fully committed to their cell fate, but remain pluripotent. This conclusion was experimentally substantiated by finding that embryo aggregates derived from either 16 outer or 16 inner blastomeres (taken from multiple 16-cell stage embryos) can successfully develop into functional and appropriately differentiated blastocysts. Although, aggregates composed of outer cells did recompact more slowly, whilst inner cell aggregates cavitated later and exhibited lower implantation rates (Ziomek et al., 1982; Suwinska et al., 2006). Conversely, aggregates of 32 outer or inner 32-cell stage blastomeres (taken from multiple 32-cell stage embryos) do not develop normally; characterised by outer cell aggregates forming trophoblastic vesicles and inner cell aggregates failing to implant consequent to an inability to form the necessary functional trophoblastic giant cells (Suwinska et al., 2006). Therefore whilst cell fate has not yet been irreversibly decided by the 16-cell stage it has been committed to by the 32-cell stage.

1.4 Second cell fate decision

During the second cell fate decision the cells of the ICM segregate into the pluripotent epiblast, that gives rise to embryo proper, and the differentiated primitive endoderm, the second extraembryonic tissue forming the parietal and visceral endoderm layers of the yolk sac. This cell fate decision is executed through the initiation of different transcriptional programs within the emerging lineages. PE specific gene expression is promoted by the Gata4 and Gata6 transcription factors, while in epiblast pluripotency is promoted by the maintenance of Nanog

transcription factor expression. In common with the first cell fate decision, there exist a number of hypothetical models describing how the two lineages are formed.

1.4.1 Positional (induction) model

This hypothesis presumes that the formation of the two distinct PE and EPI cell lineage layers within the blastocyst ICM, described above, is solely dependent on the initial position of ICM blastomeres relative to the forming blastocoel. The model proposes that the ICM cells that are exposed to the blastocoel initiate PE differentiation via an inductive mechanism, whereas cells residing deeper within the ICM are protected from this differentiative cue. The positional (induction) model is supported by the work of Dziadek (1979) who demonstrated that when blastocyst ICM cells were immunosurgically separated from the TE and cultured, a PE cell layer formed on the outer surface of the ICM. The model therefore proposes that all the ICM cells are created equal and bipotent, being able to give rise to both PE and EPI lineages.

1.4.2 Cell sorting model

The cell-sorting model is based on the finding that cells of the E3.5 blastocyst ICM exhibit differential expression of the PE or EPI specific transcription factors. As such the expression pattern of the transcription factors Gata6 (specific to PE) and Nanog (specific to EPI) is mutually exclusive among the nuclei of ICM cells and is distributed in an unbiased "salt and pepper" pattern throughout both the deep and surface layers (Chazaud et al., 2006). Therefore, this model suggests that the ICM cells are already restricted to their fate by the mid-blastocyst stage and are then sorted to their correct positions in the matured E4.5 blastocyst.

1.4.3 Combination model

Two recent time-lapse studies have suggested that the PE/ EPI lineage segregation is not, initially at least, dependent on the differential expression of the specific TFs. Plusa et al., (2008) showed that Nanog and Gata6 proteins are actually co-expressed within the ICM cells of the early blastocyst and only become mutually restricted later on. Moreover, Meilhac et al. (2009) have reported that although Gata6 is crucial for maintaining cells in the outer PE layer of the ICM, its forced overexpression in deeper located ICM cells is not sufficient for relocation to the surface. Based on these studies a new model has been proposed. This combination model integrates both the positional and sorting models, suggesting that a cell's initial position within the ICM has the primary effect on its fate. However, the model also states that cells may also

relocate from inappropriate positions, or switch their fate through changes in gene expression. Moreover, cells unable to achieve lineage specific gene expression appropriate to their position within the ICM die via apoptosis (Plusa et al., 2008; Meilhac et al., 2009; Zernicka-Goetz et al., 2009).

1.4.4 Time inside-time outside hypothesis

A more recent model has its foundation in a described link between the first and the second cell fate decisions that suggests the relative timing of the two rounds of asymmetric cell division that generate the founder cells of the ICM plays an important role in the subsequent differentiation of PE cells. Morris et al., (2010), employing a time-lapse microscopy-based and fully comprehensive cell lineage tracing analysis of all cells from the 8-cell stage onwards, showed that the PE cells of the matured blastocyst are mostly derived from the ICM cells internalised during the second wave of asymmetric cell divisions, whereas EPI cells were most likely to come from the first wave. This hypothesis correlates the time a cell spends in the outer layer of embryo with the potency of its inner localised progeny to form PE (*i.e.* the longer time it spends on the outside, the more likely it will yield PE cells and the less likely it will generate EPI). Accordingly, the more time a cell spends on the outside of the embryo, the more polarised it will become and the more likely it will generate PE. Conversely the sooner the cell is internalised, the more pluripotent it is (Bruce and Zernicka-Goetz, 2010). The study of Yamanaka et al., (2010) challenges the time inside-time outside hypothesis and argues that there is no obvious link between the first and second wave of asymmetric divisions and the segregation of EPI and PE, but rather that the event is stochastic (random) in nature. However, Morris, (2011) postulates that the differences between the findings of the two studies are due to explainable differences in experimental approach.

1.5 Molecular basis of compaction and polarisation

At the 8-cell stage embryos maximize their cell-to-cell contacts and form adherens junctions. This process is calcium-dependent considering that calcium-free medium reverses compaction and disaggregates blastomeres (Ducibella and Anderson, 1975). The Ca^{2+}-dependent molecule responsible for the formation of adherens junctions is E-cadherin (Hyafil et al., 1980). The additional association with catenins (Ozawa et al., 1989), essentially with β-catenin (Pauken and Capco, 1999) was reported later. Upon the onset of compaction, these proteins undergo

post-translational changes (Levy et al., 1986) and localize to the basolateral membrane of individual blastomeres (Sefton et al., 1992). The results of mouse genetic knock out studies, through the maternal germ line, of the *E-cadherin* gene (*Chd1*) and of a N-terminal truncation of *β-catenin* gene have demonstrated that neither protein is required during the very early stages of preimplantation embryo development. However, at the 8-cell stage they become essential for mediating cell-to-cell adhesion during compaction. Accordingly, in the absence of either or both of the maternally provided proteins, compaction is delayed until the 16-cell stage, when the protein is synthesized and post-translationally modified in sufficient amounts from mRNA derived from the paternally inherited allele (de Vries et al., 2004). In contrast, embryos that are genetically null for the *E-cadherin* gene (*Chd1*$^{-/-}$ where both zygotic alleles have been removed) are able to compact at 8-cell stage due to residual maternal protein, but they then fail to cavitate and can not form a functional trophectoderm (Larue et al., 1994). During normal development the establishment of adherens junctions is followed by the formation of tight junctions, thus providing extra cohesion. Moreover, the apical pole of individual blastomeres, free of any cell contacts, becomes enriched in microvilli and in the actin-associated protein ezrin (Louvet et al., 1996). The post-translational phosphorylation of ezrin is crucial for its roles in forming apically localised microvilli in addition to helping to mediate cell adhesion. As such targeted mutation of Thr-567, a key phosphorylation site on the ezrin protein, disturbs the polarised/ apical localisation of ezrin thereby disrupting microfilaments within the microvilli. Secondly, the same mutation also negatively affects the E-cadherin mediated cell adhesions, thus preventing the formation of stable adherens junctions and preventing the onset of compaction (Dard et al., 2004).

Another group of molecules playing key roles during polarisation are the Par-complex family of proteins. The partition–defective (PAR) genes were first described in the nematode *Caenorhabditis elegans* (Kemphues et al., 1988) as critical mutants responsible for the partitioning of cytoplasm during the earliest cell cleavage stages of development. Subsequently, roles in intra-cellular polarisation, spindle positioning and thereby asymmetric division were also reported (Etemad-Moghadam et al., 1995). Homologs of the PAR genes have now been described in a number of other animals. In mouse, during the 8-cell stage, Par proteins exhibit a typically asymmetric distribution. The atypical protein kinase C-zeta (aPKCζ) together with Par6b and Par3 show apical localization as well as at tight junctions, whilst the Par1 protein

(also known as Emk1) is restricted to the baso-lateral domain (Plusa et al., 2005; Vinot et al., 2005). In 2005, Plusa et al. reported that intra-cellular polarity of blastomeres, as demonstrated by the expression and localisation of Par complex proteins, is tightly related to the position and hence fate of their progeny cells following cell division. Accordingly, they demonstrated that the experimental and/ or functional down-regulation of either Par3 or aPKCζ within discreet cell clones of the preimplantation mouse embryo, causes the cells to preferentially divide asymmetrically thus generating more ICM progeny, than their sister cells within the same embryo that remained unperturbed. They also proposed, that such experimentally induced impaired cellular polarity also led any outer residing cells to eventually become internalised by a process of engulfment by their neighbouring and more polarised sister cells (Plusa et al., 2005).

1.6 Molecular basis of the transcriptional control of cell fate decisions

Although they are not thought to directly affect the allocation of cells within the developing preimplantation mouse embryo, some crucially important transcription factors (TFs) have been identified that act to aid the segregation and maintenance of the three blastocyst cell lineages. As such, these TFs do with time become exclusively expressed within the distinct cell lineages. The TFs Oct4 (also known as Pou5f1), Nanog and Sox2 (Nichols et al., 1998; Mitsui et al., 2003; Avilion et al., 2003) are required to maintain the pluripotency of the ICM cells, while the proper segregation and development of TE is dependent on the TFs Tead4, Cdx2, and Gata3 (Nishioka et al., 2009; Jedrusik et al., 2008; Strumpf et al., 2005; Home et al., 2009). During the second cell fate decision, the segregation of the EPI and PE lineages within the ICM is associated with the expression of Nanog (Mitsui et al., 2003) in the EPI and Gata6, Gata4, Sox17, Sox7 and HNF TFs (Koutsourakis et al., 1999; Molkentin et al., 1997; Morris et al., 2010; Artus et al., 2011) in the PE.

1.6.1 Segregation of the trophectoderm and inner cell mass (TE vs. ICM)

The expression of the *Cdx2* gene within the outer, TE destined, cells of the embryo is necessary for their commitment to TE. The fact that *Cdx2* is essential for normal development was shown in genetic knock out studies performed by Strumpf et al., (2005). The *Cdx2*$^{-/-}$ null embryos were shown to initiate cavitation but they failed to implant as a result of low epithelial integrity resulting in a failure to maintain an expanded blastocoel required to hatch from the *zona pellucida*. Furthermore, the outer cells of *Cdx2*$^{-/-}$ null embryos were not able to down-regulate

the ICM marker genes *Oct4* and *Nanog* and eventually underwent apoptosis. Moreover, the transcriptional activation of genes critical to TE functional maintenance within outer cells was also impaired.

In wild-type/ normal embryos the expression of Cdx2 mRNA is first detectable during the 4-cell stage and the protein first appears within a few blastomeres at the 8-cell stage before eventually becoming uniformly expressed in all eight cells. Concomitant with Cdx2 protein expression and coincident with the onset of general apical-basolateral polarisation, Cdx2 mRNA has been shown to localise to the subcortical region and maintains this polarity during the subsequent cell division in resultant outer cells. This polarised localisation of Cdx2 mRNA therefore ensures that no matter how outer cells are generated in the embryo (i.e. two cells via a symmetric division or one cell via asymmetric division) they will always be endowed with more Cdx2 mRNA than their inner neighbours and therefore more able to differentiate into TE, as their relative location within the embryo requires (Jedrusik et al., 2008). Although, the expression of the zygotic copies of the *Cdx2* is not essential for the onset of polarisation (Ralston and Rossant, 2008; Strumpf et al., 2005), Cdx2 does positively influence polarisation by up-regulating the expression of apically localised aPKCζ and Par3 protein suggestive of a positive feedback loop between *Cdx2* expression and general apical-basolateral polarity (Jedrusik et al., 2008).

In addition to the apical polarisation of Cdx2 transcripts, the appropriate expression of the *Cdx2* gene in outer cells is also under the influence of the Hippo signalling pathway components Yap and Lats. Following the first wave of asymmetric divisions (at the 4^{th} cleavage), Hippo signalling inputs permit individual blastomeres to sense cell-to-cell contacts and enable them to distinguish whether they reside on the outside or the inside of the embryo. Such inputs directly influence the post-translational modification and intra-cellular localisation of the yes-associated protein 1 (Yap1), an essential transcriptional co-activator required by the TE promoting TF Tead4. Accordingly, sufficient Hippo signalling inputs received by the completely encapsulated inner cells of the embryo promote the protein kinase Lats to phosphorylate Yap1 that in turn prevents its nuclear localisation. As a consequence, Tead4 protein within the nuclei of inner cells is unable to transcriptionally activate TE specific gene expression. However, in the outer cells of the embryo, insufficient Hippo signalling input, possibly directly antagonised by apically localised polarity factors, results in a pool of unphosphorylated Yap1 that is free to

enter the nucleus and associate with Tead4 to promote appropriate TE specific gene expression. Such TE specific genes include other TE promoting TFs such as *Eomes*, *Cdx2* and *Gata3*, thus further reinforcing TE fate (Nishioka et al., 2009).

Another important TF in TE development is Gata3 (Home et al., 2009, Ralston et al., 2010). The expression patterns of *Gata3* and *Cdx2* are both very similar and both genes are functionally down-stream of and are transcriptional target genes for the earliest known TE promoting TF, Tead4. However, *Gata3* expression is independent of Cdx2, notwithstanding this Gata3 can bind to regulatory elements within the *Cdx2* gene, explaining why the down regulation of *Gata3* expression is also accompanied by a reduction in *Cdx2* expression (Home at al., 2009). Strumpf et al., (2005) described another TF necessary for TE development, the T-box protein eomesodermin (Eomes). *Eomes*$^{-/-}$ null embryos were able to form normal blastocysts but exhibit failures in further TE development (Strumpf et al., 2005).

The crucial transcription factor for the development of ICM is Oct4. Oct4 protein is detectable as early as the 2-cell stage and becomes ICM-specific by the mid-late blastocyst. Genetic knock out studies performed by Nichols et al., (1998) have highlighted the important role of Oct4 during preimplantation development. In *Oct4*$^{-/-}$ null embryos, development proceeds to the blastocyst stage, however embryos present with smaller than normal ICMs that have lost their ability to either differentiate into PE or form *bona fide* EPI. Another illustration of the importance of *Oct4* is provided from studies on embryonic stem (ES) cell lines (themselves derived from isolated mouse blastocyst ICMs) where deletion of the *Oct4* gene is associated with spontaneous differentiation towards TE-like cells (Niwa et al., 2000). *Sox2*$^{-/-}$ deficient embryos exhibit similar a phenotype to *Oct4*$^{-/-}$ null embryos, as they do not maintain epiblast with the cells differentiating into trophectoderm, thereby causing death shortly after implantation (Avilion et al., 2003). *Nanog* is first expressed at the 16-cell stage and becomes restricted to the ICM by the blastocyst stage (Chambers et al., 2003; Hart et al., 2004). The genetic ablation of the *Nanog* gene results in a failure to derive EPI cells in the ICM of late blastocysts (Mitsui et al., 2003). The developmental abilities of such late blastocyst *Nanog*$^{-/-}$ ICM cells were assayed by removing overlaying trophectoderm and culturing on gelatin coated plates *in vitro*. It was discovered that unlike control wild type ICM cells, those derived from *Nanog*$^{-/-}$ ICMs did not persist as undifferentiated cells masses but rather that they differentiated

to parietal endoderm-like cells, which was in contrast to *Oct4*$^{-/-}$ null embryo ICMs that differentiated into trophoblast-like cells.

1.6.2 Segregation of the epiblast and primitive endoderm (EPI vs. PE)

Before reaching the late blastocyst stage (E4.5) the ICM segregates into either differentiating primitive endoderm (PE) or pluripotent epiblast (EPI). This event, as discussed previously (Chazaud et al., 2006, Plusa et al., 2008; Meilhac et al., 2009), is largely dependent upon the expression of certain TFs.

As discussed above, the TF Nanog is crucial for appropriate ICM differentiation and maturation. Moreover, *Nanog*$^{-/-}$ null ES cells lose their pluripotency and express elevated levels of PE marker TF genes such as *Gata4* and *Gata6* thereby differentiating them into PE-like cells (Mitsui et al., 2003). During normal unperturbed preimplantation development, *Gata6* is first expressed ubiquitously in the ICM cells of early blastocysts before becoming restricted to the definitive PE. Genetic ablation of the *Gata6* gene yields blastocysts with small ICM cells, that when kept for 9 days in *in vitro* culture degrade and finally disappear. Characterisation of such blastocysts reveals cells positive for one PE derivative, the parietal endoderm, but demonstrates a complete lack of the other PE derivative, namely the visceral endoderm (Koutsourakis et al., 1999). The phenotype of *Gata4*$^{-/-}$ null embryos presents later in development. At E7.5 *Gata4*$^{-/-}$ null embryos exhibit a size reduction equivalent to a 0.5 day delay in development, when compared to wild type embryos, and are also associated with a later defect in the cohesion of the yolk sac (a derivative of the PE). Additionally, most embryos lacked any detectable signal of visceral endoderm marker alpha-fetoprotein and in cases where alpha-fetoprotein could be detected unusually elevated levels of *Gata6* were observed, suggestive of a compensatory mechanism (Molkentin et al., 1997). Consistently, it is prescient to highlight that *Gata4*$^{-/-}$ deficient ES cells also fail to form visceral endoderm (Soudais et al., 1995). Another important TF in PE differentiation is Sox17. Morris et al., (2010) showed that over-expression of *Sox17* in single 8-cell blastomeres can lead the progeny of that blastomere towards PE formation. Sox17 expression first appears at E3.5, and becomes specific to PE blastomeres. It is crucial for differentiation of ICM and maintenance of the epithelial integrity of PE (Artus et al., 2011; Niakan et al., 2010). Another Sox family member, Sox7, has been shown to be able to functionally substitute for Sox17 (Artus et al., 2011).

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

To investigate gene expression differences between TE and ICM progenitors, an RNA-Seq analysis of the mRNA profiles of inner and outer 16-cell stage mouse blastomeres was previously undertaken (A. W. Bruce – personal communication). It was hypothesised that differentially expressed genes with potentially uncharacterised roles in the first cell fate decision would be contained within this experimental cohort. Accordingly, a few thousand differentially expressed genes were identified. To further rationalise this data set in order to identify the strongest possible candidate cell fate related genes, this list was cross referenced with a pre-existing and published data set describing global gene expression within intact embryos at all stages of preimplantation mouse embryo development (*i.e.* a temporal gene expression analysis from immature oocyte to late blastocyst stages, Wang et al., 2004). Consequently, differentially expressed genes identified from the RNA-Seq analysis that also exhibited peak expression around the 8- to 32-cell stages (concomitant with the timing of the first cell fate decision), or closely modelled the temporal expression profiles of other known and well characterised first cell fate decision related genes were identified and taken forward. The resultant several hundred genes fulfilling both conditions were further analysed for function by gene ontological screening and literature linkages with other developmental processes or genes known to influence cell fate in the preimplantation embryo (using the iHop database; http://www.ihop-net.org/UniPub/iHOP/). Using this strategy, approximately 50 novel candidate genes with a potential to influence the first cell fate decision have been identified (A. W. Bruce, personal communication). One of these candidate genes is the *colony stimulating factor 1* (*Csf1* or *Mcsf*) gene and was the subject of this Bachelors degree dissertation project.

1.8 Colony stimulating factor 1 (Csf1)/ Macrophage colony-stimulating factor (Mcsf)

Colony stimulating factor 1 (Csf1)/ Macrophage colony-stimulating factor (Mcsf), hereafter refereed to as Csf1, is a cytokine that promotes the production, differentiation, proliferation and function of macrophages. Csf1 acts through its receptor Csf1r that is a homo-dimeric tyrosine receptor kinase. A study by Arceci et al., (1992) has shown that the mRNAs for Csf1r are maternally inherited and detected until the early 2-cell stage, when they become degraded.

Following ZGA, new transcripts are expressed at the late 2-cell stage and Csf1r expression is maintained during all the subsequent preimplantation development period. The same authors have also claimed that Csf1 mRNA is not present in the early embryo, but that it is vastly abundant in the cumulus cells, oviducts and uterus (Arceci et al., 1992). However, this result is not in agreement with the above described spatial RNA-Seq and temporal microarray mRNA gene expression analyses (A. W. Bruce, personal communication, Wang et al., 2004), that both describe detectable *Csf1* expression at the 16-cell stage, that is enriched in the inner cell population. This discrepancy most likely reflects technological improvements in sensitivity of mRNA detection assays in the intervening years between the earlier and later studies. There is some precedent for an effect of Csf1 on blastocyst development and cell lineage derivation. Bhatnagar et al. (1995) reported a positive effect of the exogenous addition of Csf1 to mouse embryos in culture on the incidence of blastocyst formation and specifically enhanced trophectoderm development. The authors reported that a Csf1 concentration of 540 U/mL (an approximation of the known *in vivo* concentration, that is derived maternally in the oviduct) was most efficacious. Specifically, they reported that the formation of blastocoel was accelerated, with 94% of treated embryos forming a cavity by day 4 after injection of human chorionic gonadotropin (hCG, used in superovulation, after the injection females are put together with males in order to mate), whilst only 54% of control embryos had cavitated up to that time point. Furthermore the number of TE cells on day five (post hCG) was on average 20 cells higher in the Csf1 treated embryos. The importance of Csf1 in mouse fertility has been demonstrated in a genetic knock out study of the *Csf1* gene (Pollard et al., 1991). The $Csf1^{-/-}$ null mice are characterised with an osteopetrotic phenotype and are hence referred to as $^{op/op}$. The homozygous $^{op/op}$ x $^{op/op}$ crosses are completely infertile. However, if homozygous $^{op/op}$ females are mated with heterozygous $^{op/wt}$ males, live deliveries of pups are possible, albeit at a reduced frequency when compared to wild-type control crosses. This result indicates, that whilst maternally provided Csf1 may be beneficial to pregnancy, it is not absolutely required if the embryo can derive its own Csf1 by transcription, and ultimate translation, from its paternally inherited allele. (Pollard et al., 1991).

The molecular mechanism of Csf1 action is not fully understood. Although it has been shown that Csf1 has a role in the activation of the Ras molecule in murine osteoclasts (Bradley et al., 2008). In an early developmental context Lu et al., (2008) have reported that the activation of

Mapk via Ras promotes ES cells to differentiate towards trophectoderm-like cells. Moreover, that active Ras-MapK signalling enhanced the expression of *Cdx2* thereby driving this differentiation. Conversely, the inhibition of the Ras-MapK pathway in ES cells caused the continuous expression of pluripotency TF *Nanog* gene. Additionally active Ras-MapK signalling has been suggested to have a role in the initiation of *Gata6* expression during PE differentiation (Chazaud et al. 2006). Therefore it is possible that Csf1 mediated activation of Ras/ MapK signalling in the preimplantation mouse embryo may participate in both early cell fate decisions.

Based on the above described evidence, it was hypothesised that zygotically derived Csf1 (*i.e.* Csf1 derived as a direct consequence of the transcription and translation of the embryo's own genetic complement) acts on the outer cells of the preimplantation mouse embryo in a paracrine manner to facilitate TE development. More specifically, that it is the inner cells of the embryo enriched in Csf1 mRNA transcripts when compared to outer cells that provide a Csf1 paracrine signal to outer TE progenitor cells. It was proposed that this embryo-derived signal is outwith and independent of any exogenous/ maternal Csf1 activity provided *in vivo.* To test this hypothesis it was intended to completely eliminate Csf1 activity in *in vitro* cultured and developing preimplantation embryos by chemical inhibition of the Csf1 receptor (Csf1r). In this study, the specific Csf1r inhibitor GW2580 was chosen because it had previously been shown to have potent inhibitory effects on Csf1-induced growth of mouse myeloid cells, human monocytes and bone degradation in human osteoclasts (Conway et al. 2005). According to the hypothesis, if embryo-derived Csf1 was facilitating the formation and differentiation of TE, a reduced number of TE cells (perhaps accompanied by an increased number of ICM cells) in embryos cultured in the presence of GW2580 compared to vehicle treated controls would be expected. Immuno-fluorescence staining of fixed embryo blastocysts, after the *in vitro* culture period, with an anti-Cdx2 antibody (Cdx2 as a well characterised marker gene of the TE cell lineage) was performed in order to assay for *bona fide* TE cells. Unfortunately, the results of the inhibitory experiments proved inconclusive, as it was not possible to test the efficacy of the GW2580 inhibitor. Consequently, it was decided to directly down-regulate *Csf1* expression via an RNA interference (RNAi) mediated strategy using a long double stranded RNA (dsRNA) generated in the lab. Although the results of this second experimental strategy are still preliminary the expected phenotype was observed in one embryo microinjected with anti-Csf1

long dsRNA. However, owing to the low number of embryos in this knock down experiment the results are not conclusive and need to be repeated. Moreover, the efficacy of the anti-Csf1 long dsRNA needs to be confirmed (*e.g.* using RT-PCR).

2 Materials and methods

All chemicals used were of analytical grade and were obtained from Sigma-Aldrich, unless specified otherwise.

2.1 Embryo collection

Mouse embryos were collected from F1 hybrid (CBA x C57BL) females. Females were superovulated by intraperitoneal injection of 7.5 IU of pregnant mare's serum gonadotropin (PMSG) at 4:00 pm. Exactly 48 hours later, the injection of 7.5 IU of human chorionic gonadotropin (hCG) followed. After the hCG injection, females were mated with F1 hybrid males. On the morning after mating, females were separated from males and were checked for the presence of a vaginal sperm plug indicative of a successful mating. 1.5 days post hCG, mice were sacrificed by cervical dislocation and the oviducts were dissected. Embryos at 2-cell stage were collected into M2 medium (used for 2-3 weeks following preparation from frozen stocks according to Nagy et al. 2003) and cultured in KSOM medium (Millipore) in 5% CO_2 atmosphere at 37°C. In the case of zygote microinjections with anti-Csf1 dsRNA, female mice were sacrificed after separation (0.5 days post hCG). The oviducts were collected and the swollen ampulla region was perforated to liberate zygotes together with cumulus cells. After a short treatment with hyaluronidase to remove the cumulus, the resulting zygotes were washed through M2 and KSOM and were prepared for microinjection as described below.

2.2 Mouse embryo *in vitro* culture

35 x 10 mm tissue culture dishes with 11 drops of KSOM based culture media served as culturing plates. The experimental groups were cultured in Csf1 (Gibco) or in inhibitor of its function, GW2580 (LC Laboratories). Csf1 was used in final concentration 540 U/mL (Bhatnagar et al. 1995), which was prepared by 1323x dilution of 25 mg/µl Csf1 in 0.1% BSA stock solution with KSOM. GW2580 was used at a final concentration of 30 µM or 1 µM (Conway et al. 2005) by employing a 3333x or 100000x dilution of 0.1 M stock solution (itself dissolved in 100% DMSO) with KSOM, respectively. The control groups for each condition were prepared in exactly the same dilution manner but using 0.1% BSA solution for Csf1 or 100% DMSO for GW2580. The culturing drops within the plates were covered with 3 mL of mineral oil. In the mineral oil free conditions, the embryos were washed first through plates as

described above and then transferred to 35 x 10 mm tissue culture dishes with 4 bigger (15 µL) drops, which were placed in 60 x 15 mm culturing dishes filled with 6 mL of HPLC water to prevent the embryo culture drops from evaporating. In each experiment, all experimental groups were treated the same way, therefore either all the conditions were cultivated under mineral oil, or all of the groups were cultured in "mineral oil free" conditions. All the media and plates were prepared in laminar hood to avoid contamination and were equilibrated in the incubator (5% CO_2, 37°C) for at least one hour before use.

2.3 Embryo fixation

Embryos were fixed at a point when control embryos cultured in unmodified KSOM had reached the so-called 'half-half' blastocyst stage (a point at which the expanding blastocyst cavity comprises approximately one half of the embryo's volume), if not specified otherwise. Prior to fixation, the zona pellucida was removed by a brief treatment with pre-warmed (37°C) acid Tyrode's solution (the complete removal of the zona pellucida was judged by visual microscopic inspection). The embryos were then transferred to pre-warmed (37°C) 4% para-formaldehyde (Lach-Ner) in phosphate buffered saline solution (PBS) for 20 minutes at 37°C. After fixation embryos were stored at 4°C in PBS in 96-well plates (AMRESCO) coated with 1% agar/ 0.9% NaCl solution for up to one week, prior to immuno-staining procedures (see below).

2.4 Embryo immuno-staining

The staining was performed in 96-well plates coated with the agar solution as above. Embryos were first permeabilised with 0.5% Triton X-100 (Fluka analytical) in PBS for 20 minutes at room temperature (RT), followed by three 10 minute washes in 0.15% Tween-20 in PBS (PBS-T). Then embryos were treated with 2.6 µg/ml NH_4Cl (Lach-Ner) in PBS for 10 minutes and two sets of washes in PBS-T were performed. Embryos were then transferred to a 3% BSA in PBS-T blocking solution and were incubated at 4°C for 4 hours. Embryos were subsequently incubated in primary antibody diluted in blocking solution at 4°C overnight. The primary antibodies and dilutions used were as follows: mouse anti-Cdx2 (1:200, BioGenex) and rabbit anti-Oct4 (1:750, Abcam). On the next day, embryos were washed twice in PBS-T and were further blocked in BSA containing blocking solution for 4 hours at 4°C. An incubation in

fluorescently-conjugated secondary antibodies at 4°C for 1 hour then followed. The secondary antibodies and dilutions employed were as follows: Alexa Fluor 488 goat anti-mouse (1:1000, Invitrogen) to detect epitope bound anti-Cdx2 primary antibody and Alexa Fluor 555 goat anti-rabbit (1:1000, Invitrogen) in the case of anti-Oct4 antibody. The embryos were then washed a further three times in PBS-T and the cell membranes were stained with Texas red-Phalloidin (1:50, Invitrogen) at RT for 20 minutes, to permit visualisation of sub-cortical actin filaments. The embryos were then left in a terminal PBS wash for 30 minutes at RT. Subsequently, embryos were mounted under glass coverslips onto glass bottom dishes pre-treated with 0.1% poly-L-lysine solution. The embryonic cell nuclei were stained by mounting the embryos in Vectashield mounting solution containing 4,6-diamidino-2-phenylindole dilactate (DAPI - Vector Laboratories).

2.5 Anti-Csf1 double stranded RNA (dsRNA) preparation

To generate anti-Csf1 dsRNA, first a DNA template was prepared that would incorporate T_7 bacteriophage RNA polymerase promoter sequences at both the 5' and 3' end of each strand. This template was to be used in an *in vitro* transcription (IVT) reaction to generate the Csf1 specific dsRNA. Accordingly, pre-prepared embryonic stem cell cDNA was used as template in a PCR reaction to generate the DNA template. The sequence of the primers used was as follows (note that underlined text denotes the T_7 RNA polymerase binding sequence and non-underlined sequence is specific to mouse *Csf1* cDNA sequence):

Forward primer: 5'-TAATACGACTCACTATAGGGATTGGGAATGGACACCTGAA-3'

Reverse primer: 5'-TAATACGACTCACTATAGGGGTTGTCTTTAAAGCGCAT-3'

Initially a gradient PCR strategy, using ordinary and relatively inexpensive 'OneTaq' DNA polymerase (New England BioLabs), was used to ascertain the optimum PCR cycling conditions (specifically the optimum primer annealing temperature) to produce the expected 220 bp double stranded DNA template product. This analysis revealed the optimal primer annealing temperature to be 64°C. The PCR was then repeated according to these optimal conditions, using the more expensive 'High Fidelity PCR' DNA polymerase (ROCHE) to produce the desired DNA template for IVT. Thus, the PCR thermocycler was programmed as shown below:

1) initial denaturation at 95°C for 2 min.

2) denaturation at 95°C for 30 sec.

3) primer annealing at 64°C for 30 sec.

4) extension at 72°C for 30 sec.

repeat steps from 2-4 34x

5) final extension at 72°C for 5 min.

6) hold forever at 4°C

After purification using routine phenol-chloroform extraction and ethanol precipitation techniques, the PCR product DNA template (the integrity of which was checked by agarose gel electrophoresis) was used in an IVT reaction to generate the *Csf1* specific dsRNA (MEGAScript T7, Ambion - according to provided instructions). All the described reactions and subsequent purification steps were performed in a laminar hood using dedicated pipettes for RNA preparation to avoid contamination and/ or subsequent dsRNA degradation. The resulting IVT derived dsRNA was purified by phenol-chloroform extraction and then treated with an RNase Cocktail (Ambion – according to provided instructions) to specifically remove any single-stranded RNA without degrading dsRNA. Lastly, a further phenol-chloroform extraction was performed and the dsRNA was precipitated with isopropanol. The final concentration of the dsRNA, following resuspension in nuclease free water, was judged by U.V. spectrophotometry at 2583 ng/µL. The integrity of the dsRNA was checked by agarose gel electrophoresis and the product was stored at -80°C.

2.6 dsRNA microinjection in zygotes

The experimental zygotes were microinjected 20 hours post hCG injection with anti-Csf1 dsRNA diluted to a final concentration of 930 ng/µL along with the fluorescent lineage marker mRNA for dsRED at a final concentration of 75 ng/µL. Such co-injection was to ensure successful delivery of the *Csf1* specific dsRNA (*i.e.* only in red embryos). The control embryos were only injected with dsRED mRNA, of the same concentration. The embryos were fixed individually once they reached the half-half blastocyst stage.

2.7 Confocal microscopy and embryo image acquisition

Embryo images were obtained by laser scanning confocal microscopy using an Olympus FluoView TM1000 IX-80 inverted confocal microscope. A series of 1.05 µm thick confocal z-sections of each embryo were taken throughout individual entire embryos, as to ensure that all

cells would be imaged. The fluorescence signals derived from immunostained embryos were excited with the following lasers and light wavelengths: 405-nm diode laser (DAPI), 488-nm Argon laser (Alexa Fluor 488) and 543-nm HeNe laser (Alexa Fluor 555, Texas red-Phalloidin).

2.8 Image processing and analysis

Images were analysed using the FV10-ASW 3.1 Viewer (Olympus) and IMARIS 6.3.0 softwares (Bitplane AG). In order to find the effect of the experimental conditions on the number of TE/ ICM cells, for each embryo the total cell number (as judge by the presence of DAPI stained cell nuclei – not including the second polar body but noting cells in the mitotic phase of the cell cycle) and the Cdx2/ Oct4 positive cell numbers were recorded. Cdx2 was used as a TE marker. FV10-ASW 3.1 Viewer was used for the counting of each individual embryo. Firstly, using only the blue (DAPI) and red channel (membrane), all nuclei were numbered while gradually moving through all the z-sections. The numbering was checked for ambiguities (e.g. counting one nucleus twice). Therefore the total number of cells was recorded. Secondly, the green channel (Cdx2) was also included and each nucleus was scored either Cdx2 positive or Cdx2 negative based on the presence/ absence of the green signal. Similar approach was used in the case of embryos stained also with Oct4, scoring the nuclei exhibiting the red signal as Oct4 positive/ Cdx2 negative. During the analysis, the mitotic cells were classified by their probability of yielding either Cdx2 positive or Cdx2 negative progeny cells based on their spatial location within the blastocyst and the presence of any cytoplasmic Cdx2 staining (Figure 3). The spatial location was judged based on the membrane staining and was revised using a 3D projection in IMARIS 6.3.0. In case of the anti-Csf1 dsRNA experiment, the membrane was not stained and therefore a 3D projection in IMARIS 6.3.0. served as the main tool to decide the spatial location. Moreover and based on observations originally made by Yamanaka et al. (2010) that outer daughter cells may often be internalized after their division, mitotic cells were always counted as one cell, unless the mitotic cells were in telophase and it was clearly distinguishable where the daughter cells would end up in the embryo.

2.9 Data analysis

The data were analysed in Microsoft Excel (Microsoft). For the statistical analysis, the mitotic cells were included in either Cdx2 positive or Cdx2 negative cells based on the prediction

described above. The subsequent analysis was done in R Studio 0.97.551 using one-way ANOVA. The significant results given by ANOVA were further investigated with Tukey Honest Significant Differences.

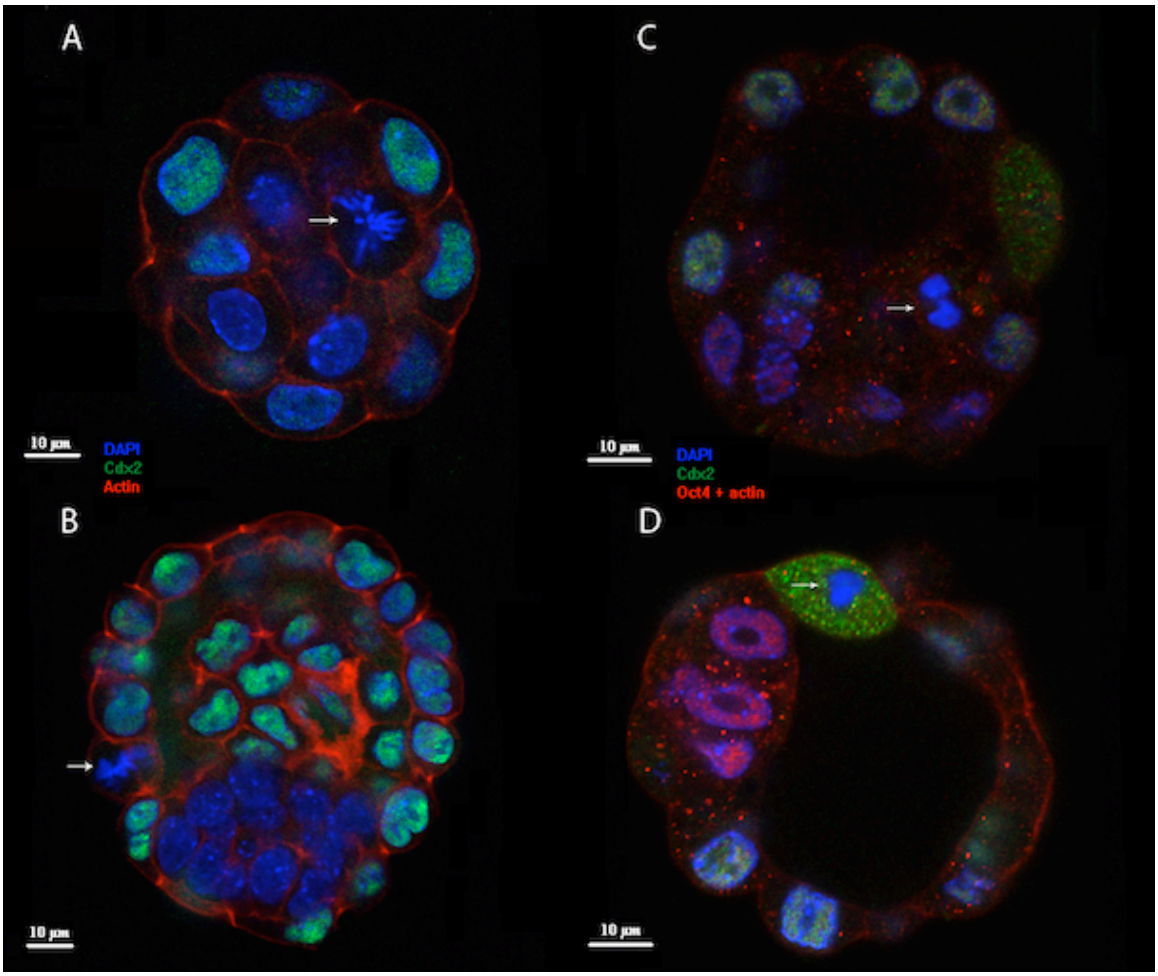

Figure 3: Predicting the allocation of mitotic cells in the blastocyst with respect to Cdx2 expression status. The mitotic cells (white arrows) were assigned as either becoming Cdx2 negative (A and C) or Cdx2 positive (B and D) based on the location within the blastocyst and in some cases (D) on the cytoplasmic Cdx2 staining of outer mitotic cells. The nuclei were stained with DAPI (blue channel), the membrane (actin) was stained with Phalloidin (red channel), Oct4 was labelled with Alexa Fluor 555 (red channel, embryos C and D) and Cdx2 was stained with Alexa Fluor 488 (green channel). The scale bar (10 mm) is different for each embryo image shown.

3 Results and discussion

3.1 Inhibition of Csf1 activity with GW2580

3.1.1 Experiment 1 – 1 µM GW2580

In the first experiments it was attempted to effect inhibition of the Csf1r receptor kinase, thereby blocking the potential cell-fate influencing effects of any endogenously embryo derived Csf1. To this end small, specific and potent chemical inhibitor GW2580 was used. Originally, a 1 µM concentration of the inhibitor was introduced, supplemented directly into the culture media, as suggested by Conway et al., (2005) although it was noted that this concentration had been determined in cell types other than early embryo blastomeres (i.e., mouse myeloid cells, human monocytes and human osteoclasts). It was expected to observe a decreased number of TE cells (Cdx2 positive cells) or an increased number of ICM cells (Cdx2 negative cells) in the experimental group treated with GW2580 in comparison to both the vehicle control group (DMSO) and the culture control group (KSOM). A positive outcome would mean, that the embryo-derived Csf1 has indeed an effect on the formation and the differentiation of TE.

Although all embryos were fixed at approximately the same time, when the KSOM culture control embryos reached the so-called 'half-half' stage, the total number of cells was highly variable both within and between the control and experimental groups. It follows that there was no significant difference in total cell number or Cdx2 positive or Cdx2 negative protein stained cells between the conditions (Table 1). Therefore, in order to obtain more uniform data, in which it would be possible to analyse better the potential differences in Cdx2 positive and negative cells within the experimental groups exhibiting varying numbers of total cells, it was decided to calculate the ratio of Cdx2 positive to Cdx2 negative cells (Cdx2+ve: Cdx2-ve ratio) for every embryo in every group and to then average this ratio from embryos within the same experimental or control groups. Even though a comparison between experimental and control groups revealed that embryos, which had been cultured in the presence of GW2580 exhibited a lower Cdx2+ve: Cdx2-ve ratio to those cultured in the KSOM control group, the difference was not significant ($p = 0.16$). Furthermore the DMSO vehicle control group of embryos had a similar Cdx2+ve: Cdx2-ve ratio as those cultured in the inhibitor, suggesting any effect was most probably attributable to the DMSO used to deliver the inhibitor (Table 1). However, based on these observations it was decided to include a new experimental group in which the

concentration of the inhibitor used in subsequent experiments was increased to 30 µM in an attempt to elicit a phenotype.

Table 1: Summary of experiment 1 (1 µM GW2580). There are given average (SEM) values of the number of Cdx2 positive cells (Cdx2+ve), Cdx2 negative cells (Cdx2-ve), total cells (DAPI) and the ratio of Cdx2 positive and negative cells (Cdx2+ve: Cdx2-ve) of embryos from the first experiment: 1 µM GW2580 group (n = 4), the DMSO vehicle control group (n = 8) and the KSOM culture control group (n = 6). The p-value states if any of the experimental groups differs significantly from the others and was conducted by using a one-way ANOVA test.

	Number of cells			Ratio
	Cdx2	**Cdx2–ve**	**DAPI (total cells)**	**Cdx2+ve: Cdx2-ve**
GW2580 (1 µM)	28.3 (3.9)	14.8 (1.7)	43.0 (5.1)	1.96 (0.21)
DMSO (vehicle control)	25.3 (1.6)	14.6 (1.3)	39.9 (2.4)	1.80 (0.14)
KSOM (culture control)	25.5 (1.3)	11.5 (0.8)	37.0 (1.6)	2.26 (0.15)
p value	0.667	0.218	0.498	0.16

3.1.2 Experiment 2 – 1 µM and 30 µM GW2580

After the first experiment it was decided to increase the concentration of the GW2580 inhibitor to 30 µM in order not to miss any effect of the inhibitor due to a low concentration, which was reported as functional in different tissue types, as mentioned above. Therefore the exact procedure of experiment 1 was repeated with two additional experimental groups with the increased concentration.

Even though all the embryos were again fixed at the same time with regard to the KSOM group, the same problem as in the previous experiment occurred. The embryos again showed a big variability among and between the groups (Table 2). The Cdx2+ve: Cdx2–ve ratio did not reveal any significant differences among the experimental groups ($p = 0.139$). In this experiment, however, the trend changed. The 1 µM GW2580 condition showed a higher ratio than both its vehicle control and the culture control. On the other hand the embryos treated with 30 µM GW2580 showed an opposite effect when compared to its vehicle control, but a slightly higher ratio was observed in comparison to the culture control (Table 2). This ambiguous result brought up the hypothesis, that the inhibitor was not functional under the used conditions.

Table 2: Summary of experiment 2 (1 µM and 30 µM GW2580). There are given average (SEM) values of the Cdx2 positive cells (Cdx2+ve), Cdx2 negative cells (Cdx2-ve), total cells (DAPI) and the ratio of Cdx2 positive and negative cells (Cdx2+ve: Cdx2-ve) of embryos from the experimental 1 µM (n = 6), 30 µM (n = 6) GW2580 group, their DMSO vehicle control groups, i.e., DMSO vehicle control 1 (n = 4), DMSO vehicle control 30 (n = 3) and the KSOM culture control group (n = 3). The p-value states if any of the experimental groups differs significantly from the others and was conducted by using a one-way ANOVA test.

	Number of cells			Ratio
	Cdx2+ve	**Cdx2-ve**	**DAPI (total cells)**	**Cdx2 +ve: Cdx2-ve**
GW2580 (1mM)	30.7 (3.0)	17.8 (2.5)	48.5 (3.9)	1.89 (0.29)
DMSO (vehicle control 1)	30.5 (3.5)	22.5 (2.4)	53.0 (5.9)	1.35 (0.03)
GW2580 (30mM)	20.7 (2.1)	14.3 (1.3)	35.0 (3.3)	1.45 (0.08)
DMSO (vehicle control 30)	24.7 (4.6)	12.7 (3.1)	37.3 (7.5)	2.03 (0.19)
KSOM (culture control)	25.7 (4.2)	19.3 (2.8)	44.3 (6.4)	1.32 (0.06)
p value	0.222	0.155	0.155	0.139

3.1.3 Experiment 3 – 1 µM and 30 µM GW2580 in mineral oil free conditions

Based on the ambiguous results of the previous experiments, it was hypothesised that one possible reason for a lack of effect could be that the inhibitor was diffusing from the KSOM media culture drop into the surrounding mineral oil overlay used in *in vitro* culture of preimplantation embryos. Therefore, to negate this potential problem a standardised mineral oil free culture system was developed (described in the materials and methods section) and the above experiments were repeated.

However, the mineral oil free conditions showed no significant results either (Table 3). The embryos were again not synchronized and varied in the number of cells. The study of the Cdx2+ve: Cdx2-ve ratio revealed different trends to the previous experiments. Even though the 1 µM GW2580 condition showed a slightly lower Cdx2+ve: Cdx2-ve ratio when compared to the culture control, its vehicle control showed an opposing effect. Furthermore the higher concentration of the inhibitor resulted in a much higher Cdx2+ve: Cdx2-ve ratio in comparison to the culture control (Table 3). Thus the results were not conclusive and it was decided to repeat the exact same experiment in order to gain more data and show the reproducibility of the experiment.

Table 3: Summary of experiment 3 (1 µM and 30 µM GW2580 in mineral oil free media). There are given average (SEM) values of the Cdx2 positive cells (Cdx2+ve), Cdx2 negative cells (Cdx2-ve), total cells (DAPI) and the ratio of Cdx2 positive and negative cells (Cdx2+ve: Cdx2-ve) of embryos from the experimental 1 µM (n = 5), 30 µM (n = 6) GW2580 group, their DMSO vehicle control groups, i.e., DMSO vehicle control 1 (n = 5), DMSO vehicle control 30 (n = 6) and the KSOM culture control group (n = 4). The p-value states if any of the experimental groups differs significantly from the others and was conducted by using a one-way ANOVA test.

	Number of cells			Ratio
	Cdx2+ve	**Cdx2-ve**	**DAPI (total cells)**	**Cdx2 +ve: Cdx2-ve**
GW2580 (1mM)	20.4 (0.8)	13.8 (0.6)	34.2 (1.2)	1.49 (0.07)
DMSO (vehicle control 1)	21.4 (2.7)	10.8 (1.1)	32.2 (3.6)	1.98 (0.15)
GW2580 (30mM)	23.7 (2.7)	9.5 (1.6)	33.2 (2.7)	3.13 (0.73)
DMSO (vehicle control 30)	26.2 (2.8)	13.3 (1.7)	39.5 (3.4)	2.31 (0.59)
KSOM (culture control)	20.8 (3.1)	12.8 (1.0)	33.5 (4.0)	1.60 (0.15)
p value	0.57	0.23	0.549	0.236

3.1.4 Experiment 4 - 1 µM and 30 µM GW2580 in mineral oil free conditions

Experiment 4 was performed in exactly the same manner as experiment 3 in order to gather more data for further statistical treatment and also to check the reproducibility of the previous experiment.

According to the results (Table 4) it seemed that the trend of the previous experiment was repeated in the less concentrated (1 µM) GW2580 condition, which is manifest by a slightly lower Cdx2+ve: Cdx2-ve ratio in comparison with the culture control. However, this result is based on the data from a single embryo treated with 1 µM concentration of the inhibitor. Furthermore this embryo was much smaller in size when compared to any other experimental group. On the other hand the Cdx2+ve: Cdx2-ve ratio of its vehicle control (DMSO 1) was again higher than the culture control. In case of the 30 µM inhibitor group, the Cdx2+ve: Cdx2-ve ratio showed an opposing effect to the previous experiment. In this experiment the 30 µM inhibitor group showed the same trend as the less concentrated condition with the ratio being smaller than both the vehicle and culture control groups. As previously, the vehicle control for the 30 µM condition exhibited a higher Cdx2+ve: Cdx2-ve ratio in

comparison to the culture control. The problem with the low reproducibility of the data was probably caused by a small number of embryos in each of the conditions (in total n = 12). Considering the different, non-repetitive outcomes of each of the experiment 1 – 4, in an attempt to receive more uniform and more extended data for statistical analysis, it was decided to merge the data from all the experiments performed.

Table 4: Summary of experiment 4 (1 µM and 30 µM GW2580 in mineral oil free media). There are given average (SEM) values of the Cdx2 positive cells (Cdx2+ve), Cdx2 negative cells (Cdx2-ve), total cells (DAPI) and the ratio of Cdx2 positive and negative cells (Cdx2+ve: Cdx2-ve) of embryos from the experimental 1 µM (n = 1), 30 µM (n = 3) GW2580 group, their DMSO vehicle control groups, i.e., DMSO vehicle control 1 (n = 2), DMSO vehicle control 30 (n = 3) and the KSOM culture control group (n = 3). The p-value shows if any of the experimental groups differs significantly from the others and was conducted by using a one-way ANOVA test.

	Number of cells			Ratio
	Cdx2+ve	**Cdx2-ve**	**DAPI (total cells)**	**Cdx2 +ve: Cdx2-ve**
GW2580 (1mM)	12.0 (0.0)	7.0 (0.0)	21.0 (0.0)	1.63 (0.00)
DMSO (vehicle control 1)	22.0 (1.4)	9.0 (0.7)	31.0 (0.7)	2.50 (0.35)
GW2580 (30mM)	18.7 (0.5)	12.3 (0.7)	31.0 (0.5)	1.53 (0.13)
DMSO (vehicle control 30)	22.3 (1.0)	10.7 (1.1)	33.0 (0.8)	2.19 (0.33)
KSOM (culture control)	22.3 (1.0)	13.0 (2.1)	36.7 (4.7)	1.86 (0.08)
p value	0.155	0.352	0.262	0.308

3.1.5 Statistical analysis of pooled data from experiments 1 - 4

Based on the fact, that the mineral oil free culture system experiments showed no more reproducibility than the original experiments, and that the data was still ambiguous, it was hypothesized that the problem was in small number of embryos in each of the experiment. Consequently, the data of both the normal and the mineral oil free culture condition experiments were pooled. Furthermore, because the variation in the total number of cells within the data was again large and insignificant between experimental and control groups, the analysis was restricted to embryos comprising 29-37 cells in all experimental and control groups (Table 5). The rational for this focus was to observe if control and treated embryos with approximately equivalent numbers of cells exhibited any differences in their Cdx2+ve: Cdx2-ve cell ratio, thus

excluding any biased contribution that could be explained by embryos at different stages of development.

According to the data (Table 5) and its graphical representation (Figure 4), there was a tendency, agreeing with the original hypothesis, that both concentrations of GW2580 showed the expected effect of lower Cdx2 positive cell numbers and lower Cdx2+ve: Cdx2-ve ratio when compared to either vehicle or culture control conditions. However, these differences were not statistically significant.

Table 5: Statistical analysis of pooled data from GW2580 experiments 1 – 4.

There are given average (SEM) values of the Cdx2 positive cells (Cdx2+ve), Cdx2 negative cells (Cdx2-ve), total cells (DAPI) and the ratio of Cdx2 positive and negative cells (Cdx2+ve: Cdx2-ve) of embryos from the experimental 1 μM (n = 6), 30 μM (n = 11) GW2580 group, their DMSO vehicle control groups, i.e., DMSO vehicle control 1 (n = 9), DMSO vehicle control 30 (n = 5) and the KSOM culture control group (n = 9). The p-value states if any of the experimental groups differs significantly from the others and was conducted by using a one-way ANOVA test.

	Number of cells			Ratio
	Cdx2+ve	**Cdx2-ve**	**DAPI (total cells)**	**Cdx2 +ve: Cdx2-ve**
GW2580 (1mM)	19.5 (1.0)	13.5 (1.0)	33.0 (0.4)	1.52 (0.18)
DMSO (vehicle control 1)	22.1 (0.7)	11.8 (0.7)	33.8 (0.8)	1.95 (0.16)
GW2580 (30mM)	19.3 (0.8)	12.5 (0.6)	31.8 (0.6)	1.64 (0.21)
DMSO (vehicle control 30)	20.4 (1.4)	11.4 (0.9)	31.8 (0.9)	1.89 (0.28)
KSOM (culture control)	21.1 (0.6)	12.1 (0.8)	33.2 (0.7)	1.83 (0.15)
p value	0.158	0.599	0.226	0.604

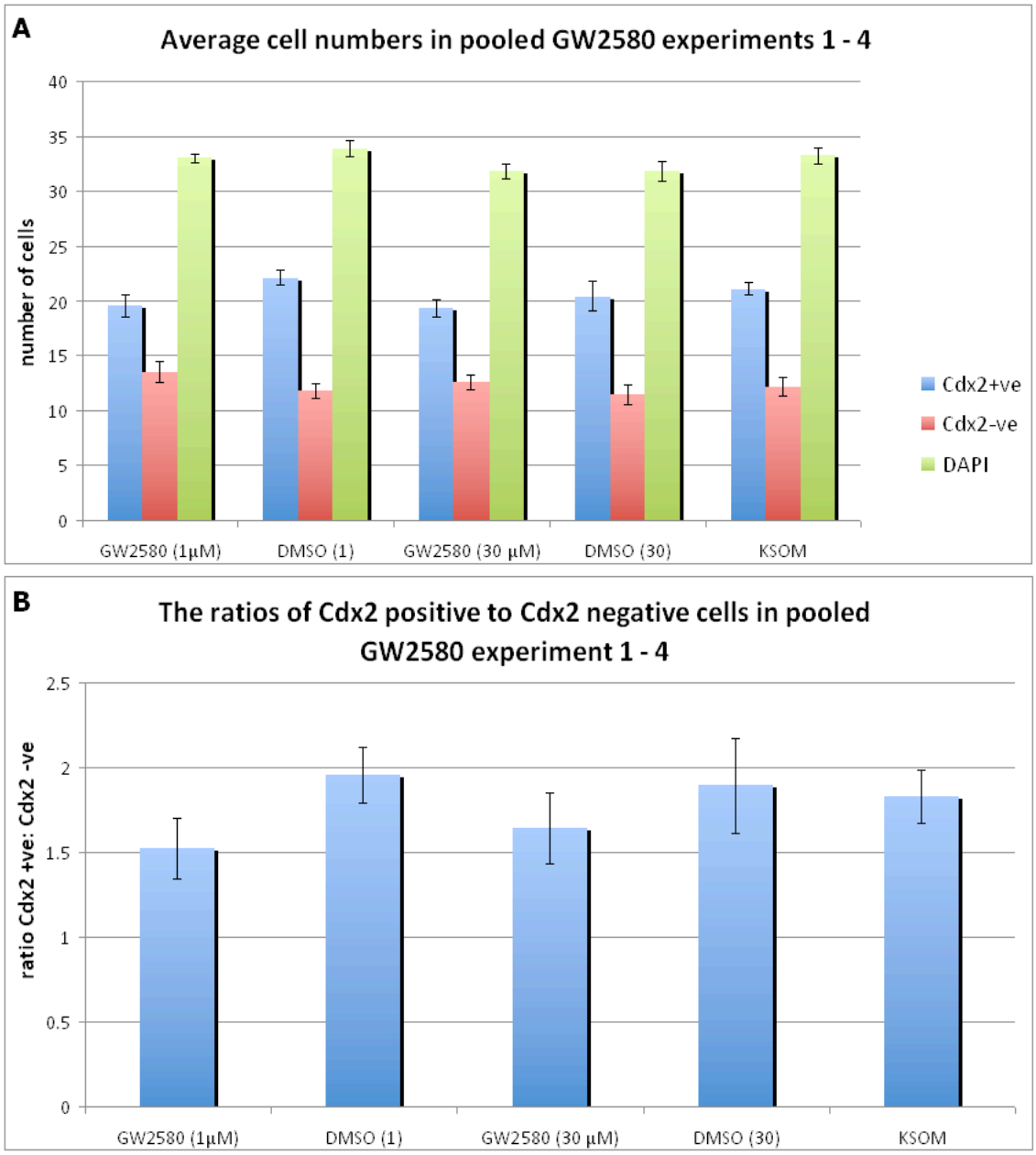

Figure 4: Graphical representation of the pooled data of 29-37 cell stage embryos cultured from the zygote stage in 1 µM and 30 µM GW2580 Csf1r inhibitor (plus vehicle and culture controls).

(A) The average number of Cdx2 positive cells, Cdx2 negative cells, total cells per embryo (after reaching 29-37 cell stage) cultured in either 1 µM GW2580 (1), its vehicle control DMSO (1), 30 µM GW2580 (30), its vehicle control DMSO (30) and unmodified KSOM culture control. Errors are represented as SEM. (B) As in (A) but showing the average Cdx2+ve: Cdx2-ve cell ratio per embryo per experimental or control group.

3.2 Determination of GW2580 activity on exogenous Csf1

3.2.1 Experiment 5 – addition of 540 U/mL GW2580

Considering that culturing embryos in the presence of the Csf1r inhibitor did not provide statistically significant changes in the relative ratio of Cdx2+ve cells per embryo by the early blastocyst stage, it was suspected that the GW2580 being used might be sub-standard and therefore not working optimally. In this scenario, it would be nearly impossible to observe any effects on Cdx2 expression. Therefore, it was decided to test the activity of the GW2580 batch used against exogenous addition of Csf1 protein. Accordingly, the following *in vitro* culture groups were prepared, using the mineral oil free culture system, and the number and ratio of Cdx2+ve: Cdx2-ve cells resulting from the *in vitro* culture of zygotes compared: KSOM culture control, 540 U/mL recombinant Csf1 (Bhatnagar et al., 1995), and four other groups all prepared in the presence of in 540 U/mL recombinant Csf1; 1 μM GW2580 in Csf1, DMSO (1) control in Csf1, 30 μM GW2580 in Csf1 and DMSO (30) in Csf1. According to previously reported experiments (Bhatnagar et al., 1995), the applied dose of recombinant Csf1 results in an observed higher number of total cells, TE cells and thereby a higher TE: ICM ratio. Therefore, it was possible to test the efficacy of the Csf1r inhibitor GW2580 against this backdrop by assessing how effectively the concentrations used could blunt this recombinant Csf1 effect. To rule out any effects of the vehicle itself, the DMSO control groups were included and hypothesised to have no influence on recombinant Csf1 induced increases in Cdx2 positive cells.

As described in Table 6 and shown in Figure 5, the exogenous addition of recombinant Csf1 alone did have the predicted effect (Bhatnagar et al. 1995). Despite the number of embryos in the Csf1 alone group being low (n = 3), there was a significant increase in the number of Cdx2 positive cells when compared to the KSOM culture control group. Surprisingly, there was also an increase in Cdx2+ve cells in the Csf1 alone group compared to both the vehicle control plus Csf1 groups (all $p < 0.05$), indicating an effect of the DMSO vehicle in inhibiting the increase in Cdx2 cells attributable to Csf1. In fact, the number of Cdx2 positive cells in vehicle control plus Csf1 groups is statistically indistinguishable from that observed in the KSOM culture control group. These results indicate that the presence of DMSO alone is sufficient to block the effect of exogenous Csf1 addition on trophectoderm proliferation to basal levels normally observed in *in vitro* culture. Consistently, the experimental conditions in which both the Csf1r inhibitor

GW2580 and Csf1 were added together did not report any statistically significant difference in the number of Cdx2+ve cells (when compared to the Csf1 alone condition) as their corresponding DMSO vehicle controls. When total cell number was assayed across all six experimental groups, the trend was remarkably similar, although no significant differences in the Cdx2+ve: Cdx2-ve cell ratio was observed (suggesting the increased cell numbers observed in the Csf1 alone group are not solely confined to the TE). Collectively, it can be concluded from these results that DMSO alone, surprisingly has the ability to blunt the cell proliferative response of exogenous Csf1 addition to preimplantation mouse embryos. Moreover, that it is accordingly impossible to assay the efficacy of the inhibitor GW2580 at successfully blocking Csf1r using this assay. Moreover, these data are in accord with those presented above in experiments 1-4, where the addition of GW2580 had no statistical effect on either Cdx2+ve or total cell numbers when compared with either KSOM culture controls or DMSO vehicle controls and suggest that an alternative strategy to assaying the effect of inhibiting endogenous Csf1 function is required.

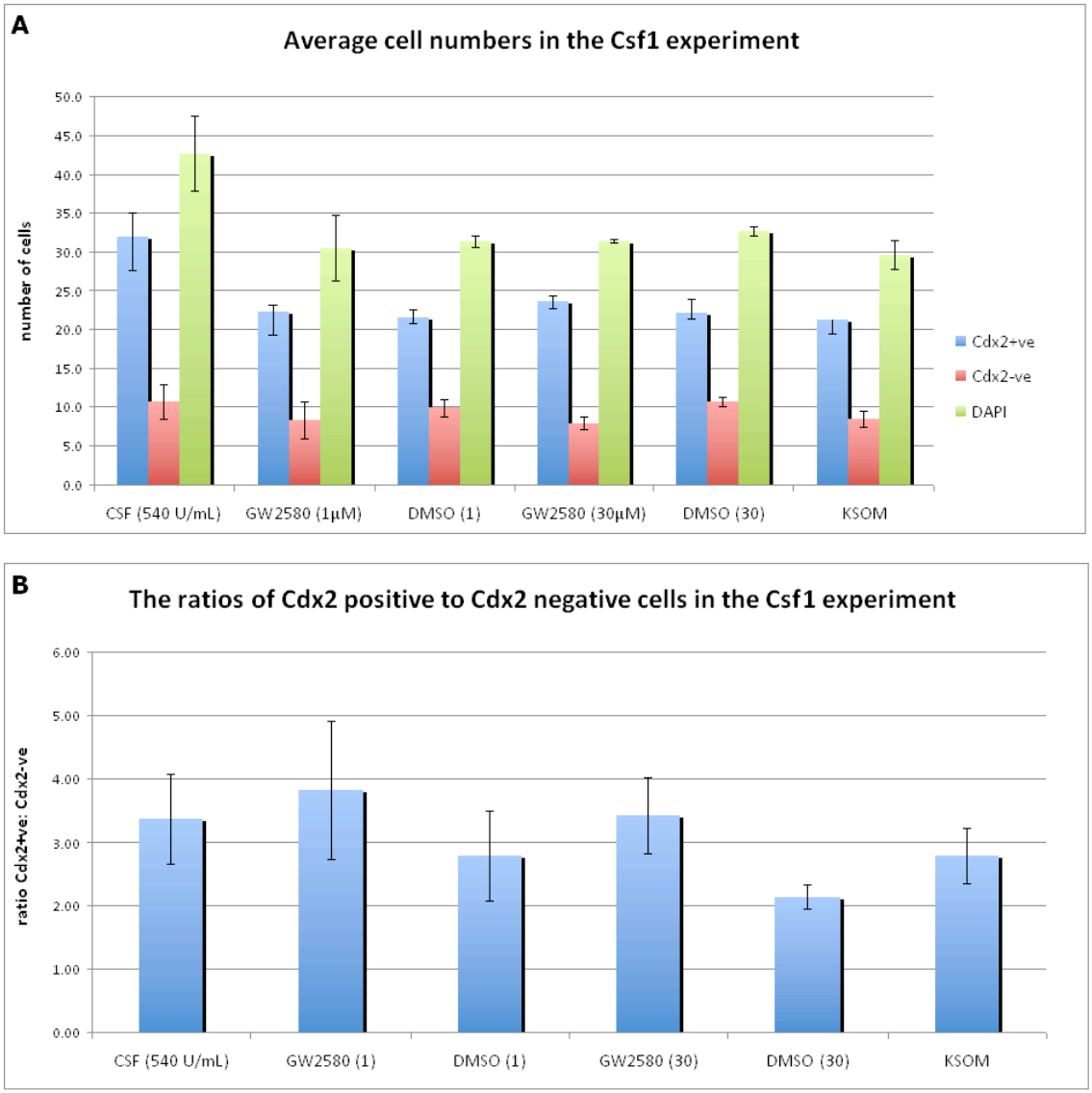

Figure 5: Graphical representation of experiment 5 (Csf1 540 U/ml experiment).
Average number of Cdx2 positive cells, Cdx2 negative cells, total cells (A) and the Cdx2+ve: Cdx2-ve ratio (B) of 540 U/mL Csf1, 1 µM GW2580 in Csf1, its vehicle control DMSO (1) in Csf1, 30 µM GW2580 in Csf1, its vehicle control DMSO (30) in Csf1 and KSOM culture control group.

Table 6: Summary of experiment 5 (Csf1 540 U/ml experiment). There are given average (SEM) values of the Cdx2 positive cells (Cdx2+ve), Cdx2 negative cells (Cdx2-ve), total cells (DAPI) and the ratio of Cdx2 positive and negative cells (Cdx2: Cdx2-ve) of embryos from the experimental Csf1 540 U/mL group (n = 3), the 1 µM (n = 4), 30 µM (n = 7) GW2580 groups in 540 U/mL Csf1, their DMSO vehicle control groups in 540 U/mL Csf1, i.e., DMSO vehicle control 1 (n = 8), DMSO vehicle control 30 (n = 6) and the pure KSOM culture control group (n = 7). The p-value states if any of the experimental groups differs significantly from the others and was conducted by using a one-way ANOVA test. Significant results are given in bold ($p < 0.05$).

	Number of cells			Ratio
	Cdx2+ve	**Cdx2-ve**	**DAPI (total cells)**	**Cdx2 +ve: Cdx2-ve**
Csf1 540 (U/mL)	32.0 (4.5)	10.7 (2.2)	42.7 (4.7)	3.37 (0.72)
GW2580 (1mM) + Csf1	22.3 (3.0)	8.3 (2.4)	30.5 (4.3)	3.82 (1.09)
DMSO (vehicle control 1) + Csf1	21.5 (0.8)	9.9 (1.1)	31.4 (0.8)	2.78 (0.72)
GW2580 (30mM) + Csf1	23.6 (1.0)	7.9 (0.8)	31.4 (0.3)	3.42 (0.61)
DMSO (vehicle control 30) + Csf1	22.0 (0.7)	10.7 (0.6)	32.7 (1.6)	2.13 (0.19)
KSOM (culture control)	21.1 (1.8)	8.4 (1.0)	29.6 (2.0)	2.78 (0.43)
p value	**0.0318**	0.551	**0.0231**	0.662

3.3 Down-regulation of Csf-1

3.3.1 Experiment 6 – RNA interference with anti-Csf1 dsRNA

Given the ambiguous results of the experiments aimed at chemically inhibiting the Csf1r, it was decided to employ an alternative approach to investigate the potential effects of embryo derived Csf1 on early mouse development. Accordingly, an RNA interference (RNAi) strategy was selected to specifically down-regulate the expression of Csf1 mRNA throughout all cells of the developing early mouse embryo. The down-regulation was performed using a large double stranded RNA (dsRNA) with perfect sequence homology to the mouse Csf1 mRNA and no off target mRNA homologies. The dsRNA was microinjected into the cytoplasm of recovered zygote embryos thereby ensuring all subsequent cells would inherit it. Microinjected zygotes were then cultured to the 'half-half' blastocyst stage and analysed as for the above-described experiments.

According to the results summarized in Table 7 and its graphical interpretation in Figure 6, there

were no significant differences between the experimental, anti-Csf1 dsRNA microinjected, embryos and the control microinjected group, albeit that the sample size (in total n = 13) for this initial experiment was small. However, one embryo in the anti-Csf1 dsRNA experimental group did display an effect that was in accord with the original hypothesis (Figure 7). Specifically, from a total of 50 cells, 24 were Cdx2 negative therefore meaning that the Cdx2+ve: Cdx2-ve cell ratio was 1.08 in contrast to the higher ratio observed in the manipulation control embryos (Table 7). One key difference between this embryo and all the other embryos injected with anti-Csf1 dsRNA, was the intensity of the co-injected dsRED (a fluorescent protein gene mRNA, co-injected to confirm successful delivery of the anti-Csf1 dsRNA) signal. The embryo showing the exceptionally low Cdx2+ve: Cdx2-ve ratio had very low dsRED signal indicating a potentially lower dose of delivered anti-Csf1 dsRNA in this embryo (Figure 7). Considering this observation, it was hypothesized that the functional concentration of anti-Csf1 dsRNA may be less than that originally employed (*i.e.* that there might be a titration effect). Therefore, it was decided that in future experiments the concentration of anti-Csf1 dsRNA to be used would be lowered (these efficacy titration experiments are currently on-going by another bachelors student in the laboratory).

Table 7: Summary of experiment 6 (RNAi with anti-Csf1 dsRNA). There are given average (SEM) values of the Cdx2 positive cells (Cdx2+ve), Cdx2 negative cells (Cdx2-ve), total cells (DAPI) and the ratio of Cdx2 positive and negative cells (Cdx2+ve: Cdx2-ve) of embryos from the experimental groups injected with anti-Csf1 dsRNA and showing robust fluorescence – anti-Csf1 dsRNA (n = 4), dsRED-injected manipulation control group (n = 4) and pure KSOM culture control group (n = 3). The p-value states if any of the experimental groups differs significantly from the others and was conducted by using a one-way ANOVA test.

	Number of cells			Ratio
	Cdx2 +ve	**Cdx2 -ve**	**DAPI (total cells)**	**Cdx2 +ve: Cdx2 -ve**
anti-Csf1 dsRNA	20.5 (2.8)	11.8 (3.5)	32.3 (5.6)	2.12 (0.45)
dsRED manipulation control	22.3 (3.5)	13.3 (2.7)	35.5 (6.1)	1.92 (0.32)
KSOM culture control	17.0 (1.6)	7.0 (0.8)	24.0 (2.2)	2.48 (0.28)
p value	0.758	0.589	0.605	0.798

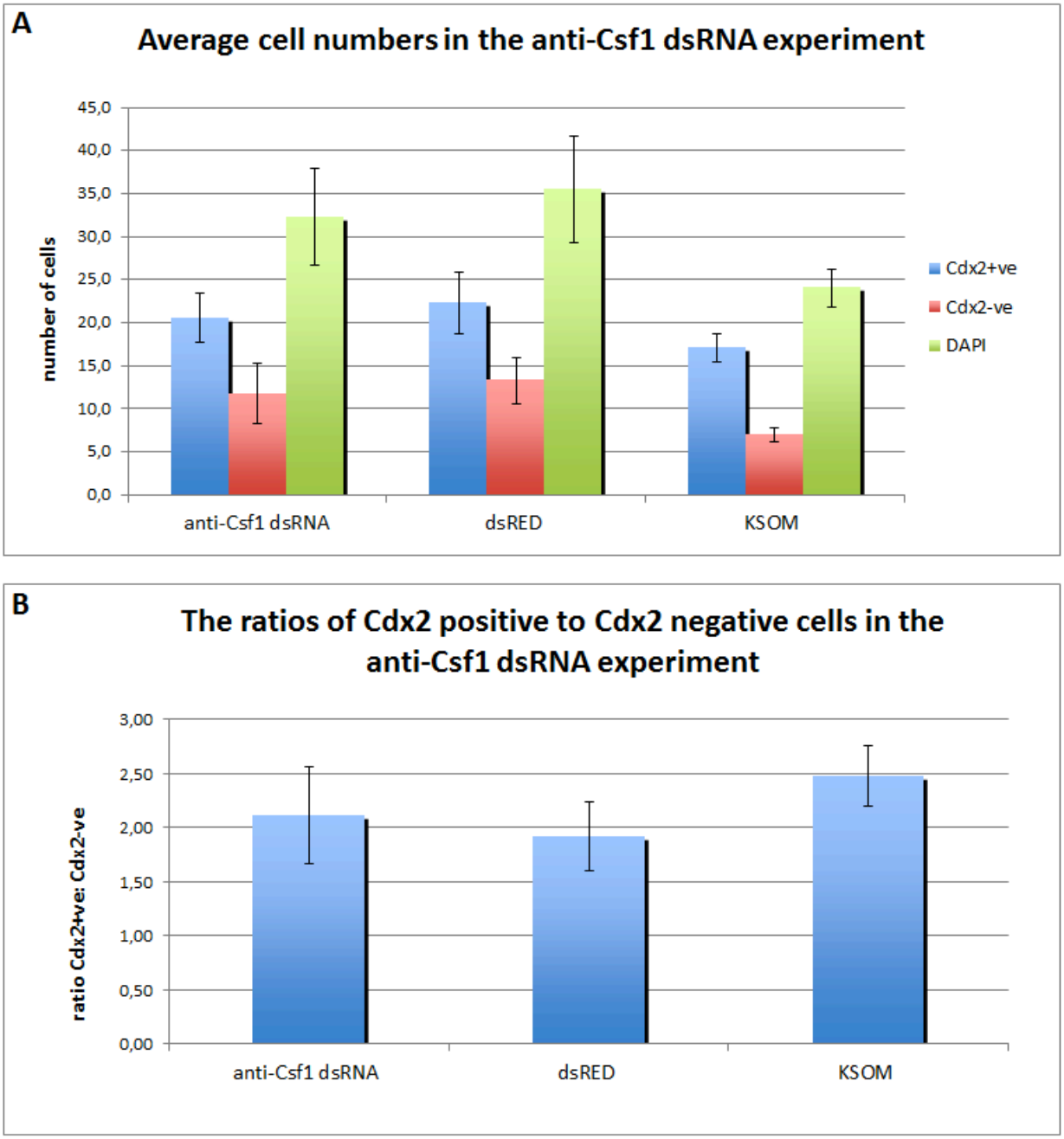

Figure 6: Graphical representation of experiment 6 (RNAi with anti-Csf1 dsRNA).
Average number of Cdx2 positive cells, Cdx2 negative cells, total cells (A) and the Cdx2+ve: Cdx2-ve ratio (B) of anti-Csf1 dsRNA, dsRED manipulation control and KSOM culture control groups.

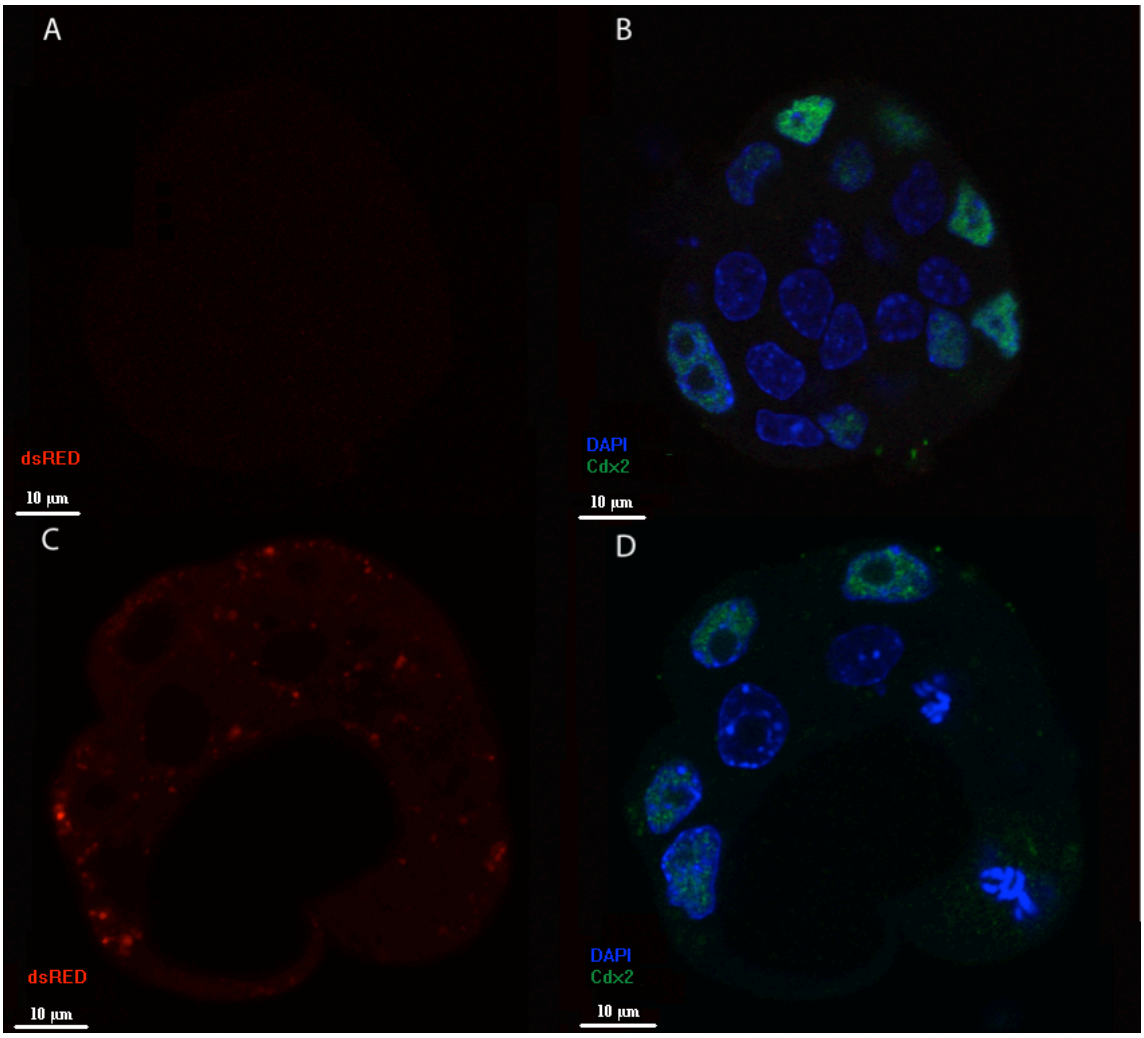

Figure 7: Comparison of two anti-Csf1 dsRNA injected embryos. Panels A and B show the embryo with exceptionally low Cdx2+ve: Cdx2-ve ratio. C and D show a typical representative of the same group, with a more typical Cdx2+ve: Cdx2-ve ratio. Note the lower dsRED signal (red channel) in the low ratio embryo in A compared to that in C– indicating a lower injection volume of anti-Csf1 dsRNA. In panels B and D the representative embryos are stained for DNA (DAPI- blue channel) and Cdx2 protein (Alexa Fluor 555 – green channel). The scale bar (10 mm) is different for each imaged embryo.

4 Conclusions and future perspectives

Unfortunately the results of this study into the potential role of embryo derived Csf1 during the preimplantation stages of mouse embryo development were not conclusive due to a number of factors. One of the biggest problems was the synchronization of the embryos and the fact that the number of Cdx2 positive and negative cells varied largely also among the KSOM control groups. This made it difficult to establish a baseline with which to compare the effect of chemically blocking the Csf1r receptor. Therefore it would be useful in the future to increase the numbers of embryos in all experiments to obtain greater statistical input. Also it was difficult to assess the efficacy of the GW2580 Csf1r inhibitor owing to non-specific effects attributable to the DMSO solvent the drug was dissolved in. The data indicate that DMSO itself has an effect on preimplantation development (at least in relation to the exogenous addition of Csf1 in *in vitro* culture systems). Therefore, a future approach to circumvent this problem would be to utilize a different solvent, that when applied in the vehicle control conditions would not interfere with the biological development of embryos (at least on the level of counting total and Cdx2+ve cells). This approach is currently being investigated, although is limited due to solvent/ chemical compatibility issues. In terms of the RNAi mediated knock down of Csf1 mRNA expression more experimentation is required. The preliminary results suggest a lower concentration of anti-Csf1 dsRNA may elicit the hypothesized phenotype of fewer Cdx2 positive cells by the blastocyst stage. However, this result will need repeating and the efficacy of the anti-Csf1 dsRNA in successfully knocking down the endogenous levels of Csf1 mRNA needs to be confirmed by quantitative real-time PCR (qRT-PCR). Such experiments are on-going in the laboratory. As a future perspective it would also be useful to investigate the causes of infertility in *Csf1* knock out mice (Pollard et al., 1991) and its link to the early preimplantation development. It would be of future interest to find the time point at which the $Csf1^{-/-}$ null embryos start to be abnormal.

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