Stem Cell Technologies for Generating Cells and Organs for Regenerative Medicine

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ABSTRACT

The rapid progress in the fields of stem cell biology and interspecies chimerism over the past few years has shown promise towards being able to generate transplantable human organs. Since the discovery of induced pluripotent stem cells (iPSCs), efforts have been focused towards being able to develop human tissue-specific cells as well as whole organs. The ultimate goal in the field of interspecies chimerism and blastocyst complementation is to develop transplantable human organs in relevant host animals such as pigs. In spite of the massive progress in these fields, there are still major hurdles, both technological as well as ethical, that need to be tackled.

The current study focuses on developing an optimum 3D *ex-vivo* system for culturing mouse embryos until the implantation stage. This ex-vivo system can be used to study the development of wild-type and chimeric embryos. This study also demonstrates the strength of single-cell RNA sequence (sc-RNA seq) analysis techniques in predicting the most similar early embryonic developmental stages across species by using sc-RNA seq data of early embryonic development. The predicted results from the *in-silico* studies can then be tested in the *in vitro* and *in vivo* interspecies chimerism and blastocyst complementation studies. Lastly, this study discusses combining approaches of sc-RNA seq analysis with optimal *ex-vivo* embryonic culture systems to optimize and perform precise stage-matching experiments, closely observe and analyze grafted cells and their progeny in the host embryos, and to discover crucial gene networks for interspecies chimerism.

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Section I- Introduction to Stem Cells

The ever-growing demand for organs required for transplantation therapy cannot be met by the severe shortage of donors and there is a need for an alternate method of developing whole functional organs. The reprogramming of somatic cells into induced pluripotent stem cells (iPSCs)- cells that can differentiate into any cell type in the body, was a breakthrough in the field of regenerative medicine (Takahashi & Yamanaka, 2006) and demonstrates immense potential as an alternate method for generating whole transplantable organs.

The following sections are going to provide an overview of stem cells and their potential for *in vitro* organ generation, as well as the hurdles yet to be tackled to make stem cell-derived organs a reality in transplantation therapy.

A. Embryonic Stem Cells

Embryonic stem cells (ESCs) are undifferentiated pluripotent cells that are derived from the inner cell mass of a pre-implantation or peri-implantation blastocyst. The first embryonic stem cells were established in the early 1980s from mouse blastocysts (Kaufman & Evans, 1981). Previously, it had been observed that the mouse blastocyst contained pluripotent stem cells that were capable of chimera formation and giving rise to teratocarcinomas (Stevens, 1970). This led towards the effort to develop a pluripotent cell line derived from the mouse blastocyst. The mouse ESCs (mESCs) have normal karyotype, can be cultured indefinitely, and can differentiate into cells derived from all three germ layers- the ectoderm, mesoderm and endoderm. Further, mESCs are able to form chimeras when injected into a mouse blastocyst and also give rise to teratomas when injected subcutaneously into immunocompromised mice (Kaufman & Evans, 1981). After the establishment of mESCs, ESCs were derived from other species such as rats (Li et al., 2008), pigs (Notarianni et al., 1990), rabbits (Schoonjans et al., 1996), non-human primates (J A Thomson et al., 1995) and humans (Li et al., 2008) (James A. Thomson, 1998). Human ESCs (hESCs) were derived from the inner cell mass of late stage human blastocysts and correspond to mouse Epiblast stem cells (EpiSCs) (Nichols & Smith, 2009). The hESCs display indefinite proliferation, capacity to differentiate into cells of all three germ layers and also form teratomas

on injection into immunocompromised mice (James A. Thomson, 1998). Previously, stem cells have been successfully used in the clinic for generating skin layers for skin grafting (Bianco & Robey, 2001) and for replacing damaged cornea surfaces with cornea epithelium (Pellegrini et al., 1997). Since the derivation of hESCs, efforts have been focused towards differentiating these cells into relevant cell lineages that could be translated into the clinic for cell therapy. hESCs have been differentiated into many cell types including neural cells, pancreatic islets, cardiomyocytes (Wu, 2015), and functional endoderm cells that can give rise to endoderm derived lineages on transplantation into mouse kidney capsules (Kubo et al., 2004).

While the progress in the stem cell field has increased the hope of being able to develop organs for transplantation therapy, there are some drawbacks to the use of ESCs. The establishment of human ESC lines raises ethical issues as this process requires destruction of human embryos. Also, long-term cultures of ESCs leads to impairment of their ability to self-renew and differentiate (Łos et al., 2018). Further, with hESC-derived cells used in cell therapy, there is always the increased risk of immune rejection and tumor formation (Damdimopoulou et al., 2016) (Wu, 2015).

B. Induced Pluripotent Stem Cells

The ethical concerns and risk of immune rejection associated with ESCs were overcome with the discovery of induced pluripotent stem cells (iPSCs). iPSCs are ESC-like cells that are derived from somatic cells and have multi-lineage potential. iPSCs were derived for the first time from mouse fibroblasts through retrovirus-mediated introduction of four reprogramming factors- Oct3/4, Sox2, Klf4, c-Myc (Takahashi & Yamanaka, 2006). Mouse-derived iPSCs can also generate adult mice using tetraploid complementation- the most stringent test for pluripotency (Boland et al., 2012). Soon after the discovery of mouse iPSCs, human fibroblasts and other somatic tissues were reprogrammed into human iPSCs (Takahashi et al., 2007). iPSCs show normal karyotype, increased telomerase activity, indefinite proliferation, and can differentiate into cells of all embryonic lineages. iPSCs also form teratomas on injection into immunocompromised mice and can contribute to adult tissues in mouse chimeras (Okita et al., 2007). The potential of developing

whole organs for transplantation increased drastically with the discovery of human iPSCs. This meant limitless generation of human pluripotent stem cells that could be derived from any human somatic cell and did not require access to human embryos. Further, patient-specific iPSCs hold potential for generating autologous cells and organs for therapy which reduces the risk of immune rejection.

Human iPSCs are currently being tested for regeneration of neural cells in diseases such as Parkinson's and Alzheimer's. Other stem-cell derived cell types being tested for cell therapy include cornea limbal cells, hepatocytes, alveolar epithelial cells (Łos et al., 2019). Cell therapy using hPSC-derived cardiomyocytes have shown to improve the function of infarcted hearts in animal models of mouse and guinea pigs (Mou et al., 2015). Currently, human clinical trials are testing the efficiency of stem cell-derived pancreatic beta cells for islet replacement in case of type I diabetes patients (https://viacyte.com/clinical-trials/).

Other than ESCs and iPSCs, the body contains adult tissue-specific stem cells that are multipotent in nature and are responsible for repair and homeostasis of the tissues. The most common types of adult stem cells used in clinical cell therapies include mesenchymal stem cell (MSCs) and hematopoietic stem cells (HSCs) (Łos et al., 2018). Autologous MSCs are widely used for bone reconstruction (Shao et al., 2015). Transplantation of MSCs into patients with advanced heart failure has shown improvement of cardiac function after myocardial infarction (Segers & Lee, 2008). HSCs are extracted from their niche in the bone marrow and have been used for a long time to treat aggressive leukemia (Lagasse et al., 2001) (Frändberg et al., 2015).

C. Hurdles in generating cells and organs from ESCs and iPSCs

The function of stem cells to repair, replenish and maintain tissues in the body has been widely studied and demonstrated (Łos et al., 2018). Stem cells have also been used in cell therapies and many clinical trials using stem cell-derived somatic cells are currently ongoing. While stem cells have been effective to promote repair of tissues, the ability to generate solid organs using stem cells has not been demonstrated so far. Tissue engineering technique using decellularized scaffolds have been successfully used to generate a functional trachea that was used to replace the patient's left bronchus (Macchiarini et al., 2008).While decellularized

scaffolds greatly reduce the risk of immune rejection, they are limited by the availability of donors. Artificial scaffolds that recapitulate the 3D morphology of the organs and can be populated with stem cell-derived organ-specific cells are being developed. However, the development of a vasculature through the organs as well as recapitulation of the complex interactions and cell lineages of *in vivo* developed organs poses many difficulties (Taylor, 2009). Bioprinting is an effective technique to construct layers of cells on top of each other but the development of solid organs is still a hurdle (Mou et al., 2015).

Other than the technical issues of generating whole organs *in vitro*, there are also concerns regarding the nature of stem cells and the vectors and genes used for reprogramming somatic cells into iPSCs (Menon et al., 2016). Residual stem cells increase the risk of teratoma formation *in vivo* and hence, safety precautions need to be taken to ensure that all the stem cells used in the system have fully matured into the organ-specific somatic cells (De Los Angeles et al., 2018).

Stem cells hold massive potential to overcome the current shortage of transplantable organs by generating personalized organs using autologous patient-derived cells, hence, reducing the risk of immune rejection. However, there are still many limitations that must be addressed to make *in vitro* stem cell-derived transplantable organs a reality.

Sections II- Blastocyst Complementation for Generating Cells and Organs via Chimeras

With the discovery of induced pluripotent stem cells, the hope of being able to generate personalized organs suitable for transplantation has drastically increased. However, the *in vitro* techniques used for generating functional organs have lots of limitations that need to be addressed with regards to 1) replicating the 3D morphology of the organs, 2) ensuring the organs contain the desired differentiated cell types without contamination by residual stem cells, 3) vascularization of the organs so that they are functional post-transplantation, 4) culturing organs in conditions that replicate the complex interactions between the different tissues in the body as well as between the cells within the tissue (Kobayashi et al., 2010) (Usui et al., 2012).

Previously, it has been demonstrated that a single stem cell implanted into cleared mouse fat-pads could give rise to the whole mammary gland (Shackleton et al., 2006). Also, implantation of a single stem cell under the renal capsule of athymic mouse hosts could generate the prostate gland (Leong et al., 2008). These studies demonstrate that under the right conditions, stem cells are capable of whole organs generation. There is a need for a system that can not only optimize the pluripotency of the stem cells for generating organs, but can also recapitulate the *in vivo* environment, hence overcoming the drawbacks of the *in vitro* culture systems.

The ability of mouse pluripotent stem cells (mPSCs) to generate chimeras and contribute to multiple adult tissues when introduced into mouse embryos had been previously demonstrated (Mintz, 1964) (Kaufman & Evans, 1981). Further, using blastocyst complementation for the first time, it had been observed that RAG2 -/- mice deficient in T and B lymphocytes could be complemented with mouse ESCs to restore normal T and B lymphocytes (J. Chen et al., 1993). The T and B lymphocytes were derived from the donor mouse ESCs that were injected into the host RAG2 -/- mouse blastocysts and filled up the ‘developmental niche’ created by the knockout of the RAG2 gene in the host embryo.

Using the technique of blastocyst complementation, the ultimate goal is to generate functional human organs for transplantation therapy derived from human

pluripotent stem cells (hPSCs) and grown in relevant host animals such as pigs, sheep.

The following sections are going to provide an overview of the development in the field of intraspecies and interspecies chimerism, blastocyst complementation, and the hurdles that are yet to be tackled to achieve the goal of regenerative medicine- to produce transplantable human organs.

A. Intraspecies Chimeras

In intraspecies chimerism, donor stem cells are introduced into a host embryo of the same species to generate chimeras in which the donor cells contribute to multiple organs of the host animal. Intraspecies chimerism has been successfully demonstrated in rodents (Tarkowski, 1961) (Mintz, 1964), pigs (Nagashima et al., 2004), and other relevant animals used for chimeric studies. Using blastocyst complementation, it has been shown that mESCs injected into a Pdx1 -/- mouse blastocysts can form functional pancreas with a chimerism efficiency of 50% (Kobayashi et al., 2010). Similarly, functional kidneys largely made of donor mPSCs (mouse ESCs and iPSCs) were generated using host Sall1 -/- mouse blastocysts (Usui et al., 2012). In each of these studies, knockout of the relevant genes, either Pdx1 or Sall1, created a 'developmental niche' that was complemented by the injected donor mouse stem cells. Blastocyst complementation was also used to generate functional lungs in mouse-mouse chimeras (Mori et al., 2019). Injection of mPSCs into Flk1 -/- mouse blastocysts gave rise to chimeras with the vascular endothelial and hematopoietic cells from the donor mPSCs (Hamanaka et al., 2018). In 2013, pig-pig chimerism was demonstrated by injecting pig blastomeres (pluripotent cells) into Pdx1-Hes1 cloned pig embryos which rescued the apancreatic phenotype seen in Pdx1-Hes1 expressing pigs (Matsunari et al., 2013). The same technique was used to rescue ocular development in an ophthalmic pig model (H. Zhang et al., 2018). The generation of intraspecies chimerism is important to understand the factors involved in successful blastocyst complementation and to improve the efficiency of chimerism. However, in order to achieve the ultimate goal of developing human organs, more relevant

studies exploring interspecies chimerism and the difficulties associated with overcoming the xenogenic barriers need to be carried out.

B. Interspecies Chimeras

Interspecies chimerism involves integrating donor stem cells of one species with the embryo of another species such that the donor species' cells contribute during development of the host animal. One of the earliest interspecies chimerism was between cattle and was known as 'geep', a chimera between a goat and sheep (Fehilly et al., 1984). Since then, multiple interspecies chimerism studies on evolutionarily close rodent species such as mice and rats have been done. Live mouse and rat interspecies chimeras were developed by injection of mouse and rat PSCs into rat and mouse blastocysts respectively with an efficiency of 20% which is less than the 50% efficiency of mouse intraspecies chimerism that was observed in the same study (Kobayashi et al., 2010). In this study, the authors also used interspecies blastocyst complementation to generate rat pancreas in Pdx1 -/- mice hosts (Kobayashi et al., 2010). Due to the small number of rat islets developed in the mouse model, the functionality of the generated rat pancreas could not be tested in a rat model induced with diabetes. Hence, in 2017, the experiment was repeated to generate mouse islets in Pdx1 -/- rats (Yamaguchi et al., 2017). The mouse islets that developed in the mouse-rat chimera were functional and could maintain blood glucose levels when implanted into a diabetes-induced mouse model. Blastocyst complementation has been successfully used for generating other organs in rodent interspecies chimeras- rat thymus in an athymic mouse model (Isotani et al., 2011), functional mouse kidney in Sal1 -/- rat model (Goto et al., 2019). The efficiency of interspecies chimerism decreases as the evolutionary distance between the donor and host species increases (Rashid et al., 2014). A mouse-rat chimera would have higher chimerism efficiency than a human-mouse or human-pig chimera. However, in order to generate human transplantable organs, more studies have focused efforts towards using human or non-human primate PSCs in animal hosts to generate chimeras. A previous study has shown that the orthotopic transplantation of hPSC-derived beta cells into a neonatal mouse could give rise to functional human beta cells in the adult

mouse (Ma et al., 2018). This demonstrates the potential of human cells to grow and develop into functional tissues in an animal host. Since the contribution of human cells to animal hosts such as rodents and pigs in interspecies chimerism is low due to the evolutionary distance, efforts have been made to overcome this barrier by matching the developmental times of the donor cells with the cells in the host embryo which plays an important role in successful chimerism (Cohen et al., 2018). In studies highlighting the importance of matching developmental times in chimerism, successful chimerism was observed when 1) mESCs were injected into early E3.5 blastocysts and when 2) multipotent somatic mouse neural crest cells (mNCCs) were injected into later stage E8.5 gastrulating mouse blastocysts (Cohen et al., 2016). However, when the developmental times were mismatched- 1) mESCs injected into E8.5 mouse blastocyst or 2) mNCCs injected into E3.5 blastocysts, chimerism failed (Cohen et al., 2018). By matching the developmental times of the donor cells and cells from the host embryo, neural crest cells derived from mouse, rat and human iPSCs were each injected *in utero* into E8.5 mouse blastocysts and could successfully form mouse-mouse intraspecies chimeras as well as rat-mouse, human-mouse interspecies chimeras (Cohen et al., 2016). Human PSCs injected into gastrulating mouse embryos (E6-E7.5) also formed successful chimeras (Mascetti & Pedersen, 2016a). Other studies have demonstrated that the overexpression of anti-apoptotic genes such as Bcl2, BMI1 in the donor hPSC cells injected into the host animal embryos can increase the efficiency of chimerism and overcome the mismatch in developmental times between the donor and host cells. Human iPSCs overexpressing either Bcl2 (Masaki et al., 2016) (X. Wang et al., 2018) or BMI1 (K. Huang et al., 2018) could successfully integrate into mouse blastocysts and form human-mouse chimeras. BCL2-overexpressing hiPSCs could also complement ETV2-null pig embryos and differentiate into the endothelial lineages in the human-pig chimera (Das et al., 2020). Although successful chimerism has been observed in interspecies chimeras between human and non-human hosts, the efficiency of generating chimeras as well as the contribution of the human cells in the chimeras is still extremely low. Future studies in the field would be focused on addressing these

drawbacks and understanding the mechanisms of optimal chimerism.

C. Hurdles in generating organs via Blastocyst Complementation

Although the field of blastocyst complementation has progressed rapidly in the past few years, there are still concerns to be addressed and hurdles that need to be tackled. In order to develop human organs of suitable sizes for transplantation, relevant host animals such as pigs are needed. The somatic cell nuclear transfer technology (SCNT) has been well established in pigs which can be used to generate large number of pig embryos *in vitro* that have the relevant gene knockouts for blastocyst complementation (Rashid et al., 2014). Other than a relevant host animal, there are many other factors that need to be optimized- the type of donor cells, the genes specific to organ generation that need to be knocked out of the host embryos, the developmental times of the donor cells and the host embryos, and the techniques to increase survival of donor cells and contribution to organ generation (Rashid et al., 2014) (Yamaguchi, 2019). Optimization of these factors would involve comparing the early embryonic development of the different species, looking into proteins and transcription factors that are common and differentially expressed between species and using this information to optimize the process of interspecies chimerism (Boroviak et al., 2018). Other issues that need to be addressed include making sure that the human organs developed in the animal hosts have a hPSC-derived vasculature and also taking into consideration the risk of immune rejection after transplantation into patients (Rashid et al., 2014). Contribution of hPSCs to the endothelium needs to be optimized by knocking out the relevant genes during blastocyst complementation (Das et al., 2020). A better understanding of the chimeric animal immune system and how the cells in the host animal develop tolerance towards the human donor cell antigens is required (Cohen et al., 2020). Other than the technical issues of blastocyst complementation and chimera formation, there are also ethical issues and concerns that need to be addressed. In previous studies, transplantation of human glial progenitor cells into the mouse brain resulted in enhanced long-term potentiation and an increase in the learning ability of the mice (Han et al., 2013). In rat-mouse interspecies chimeras, rat ESCs-derived

spermatozoa was formed from the injected rat ESCs (Isotani et al., 2011). This raises concerns with regards to the contribution of the donor hiPSCs to the animals' brains resulting in the development of a 'human' consciousness and speech ability in the host animals. Also, ethical concerns exist regarding the human cells' contribution to the gonads of the human-animal chimeras resulting in their ability to give birth to progeny with human-specific phenotypes and abilities (Yamaguchi, 2019) (Rashid et al., 2014). Although these results are highly unlikely due to the low efficiency of chimerism, they need to be addressed for the field to progress forward. Some solutions to these issues could be 1) to use hiPSCs with conditionally expressed factors that differentiate only into specific cell lineages, 2) to use committed progenitors that are injected into late stage embryos, 3) to integrate suicide genes under the control of brain or gonad cell specific progenitors (Rashid et al., 2014) (Kobayashi et al., 2015). For example, it has been observed that forced expression of Mixl1 in mouse donor ESCs injected into a host mouse blastocyst results in intraspecific chimeras with donor cells contributing preferentially only to the endodermal organs (Kobayashi et al., 2015). More studies along the same direction are required to address the ethical controversies and update current policies regarding research in the chimerism field. In 2019, Japan updated their human-non-human chimerism guidelines to allow transplantation of hiPSC-containing embryos into the uterus of host non-human animals (Sawai et al., 2019). While this is a step forward towards the ultimate goal of regenerative medicine, the functionality and safety of the hPSC-derived organs grown in the non-human chimeras would have to be rigorously tested before they are ready for transplantation into patients (Yamaguchi, 2019) (Nagashima et al., 2004).

Section III – Early Embryonic Development

Studying the early human embryonic development is important for providing key insights into the normal human developmental processes, improving the existing assisted reproductive technologies, developing stem cell based therapies including tissue regeneration and repair, and for increasing the success of blastocyst complementation studies (Niakan et al., 2012). However, studying healthy human embryonic development is difficult due to several technological limitations and ethical concerns (Niu et al., 2019). Most of the studies involving human embryos are performed using *in vitro* fertilization (IVF) embryos that are difficult to obtain. Further, no human embryo can be cultured *in vitro* for more than 14 days post fertilization (Wamaitha & Niakan, 2018) (Hyun et al., 2016). Since the basic early embryonic development is well-conserved among different mammalian species, other animal models such as mice, non-human primates, and pigs have been studied to provide information into normal human early development (Reijo Pera & Prezzoto, 2016). Aristotle, the father of embryology, was among the first to use experimental approaches to study early embryonic development using different species of birds (DDA Thompson, 1910). He concluded that the developmental processes are conserved across species, but the time of development varied between the species. He further hypothesized that the basic early embryonic development is also conserved among other species including humans (DDA Thompson, 1910). Over the years, numerous studies have supported this hypothesis and insight has been gained into human embryonic development using animal models. Studying the early embryonic development of relevant animal models such as mouse, non-human primates, cattle, and pigs has also helped progress the field of interspecies chimerism and blastocyst complementation (Nakamura et al., 2016) (De Los Angeles et al., 2018). These studies have helped to match the developmental stages of the host embryos with the grafted cells, improve the success rates of blastocyst complementation studies, bringing the field closer to the ultimate goal of generating human tissues in relevant animal hosts.

The following sections will discuss the early embryonic development in mouse- an extensively studied animal model. Subsequently, the early embryonic development in other relevant species- humans, non-human primates and pigs will be discussed, highlighting the similarities and differences between each species. The last section will

discuss the importance of transcriptome analysis in discovering similarities and differences in early embryonic development across species.

A. Early Embryonic Development in Mouse- Zygote until Implantation stage

Over the decades, the early embryonic development in the mouse has been extensively studied to gain more information into the embryonic development in other species. Using the mouse as a model holds the following advantages- they have a short developmental time period, easy to manage colonies, and multiple mutant models of the mouse and genetic tools exist which makes studying the different processes involved in development easier (Reijo Pera & Prezzoto, 2016).

Following fertilization, the mouse oocyte activates to form the totipotent zygote (E1). The mouse zygote undergoes rapid cell division to form 2 cell (E1.5), 4 cell (E2) and 8 cell (E3) embryo (Figure 1). The overall size of the embryo remains the same throughout the division while the cell number increases. Until the early 2-cell stage, the cells in the embryo do not undergo active transcription and the embryo use the maternally inherited RNA and proteins from the oocyte for functioning (Flach et al., 1982). Around the early 2-cell stage, the embryo undergoes zygote gene activation (ZGA) after which the cells in the embryo actively transcribe their own RNA (Bensaude et al., 1983) (Gao et al., 2017). At the 8-cell stage (E3), a symmetry breaking event is observed, and the embryo undergoes polarization. The cells on the outside of the embryo develop an apical layer on their outer contact-free surface while the cells on the inside develop a basolateral domain (Ziomek & Johnson, 1980). Along with polarization, the embryo also undergoes compaction during which the cell-cell contact increases and the cells develop a flattened morphology. Until the 16-cell stage, all the blastomeres in the embryo can give rise to all three lineages of the blastocyst. Around E3.5, at the 32-cell stage, the cells in the embryo undergo lineage segregation giving rise to 2 cell lineages- the pluripotent inner cell mass (ICM) and the trophectoderm (TE) (Figure 1) (Brinster, 1963). During this time (E3.5), the blastocoel cavity begins to form and grow in size pushing the ICM to one side of the blastocyst. An increase in the tight junctions between the cells of the TE is observed to support the blastocoel formation (H. Wang et al., 2008) . After

expansion of the cavity and multiple cell rearrangements, the ICM further divides into 2 cell lineages- the epiblast (EPI) and the primitive endoderm (PrE) around E4 (Figure 1). The EPI gives rise to the embryo proper and the extraembryonic mesoderm, the PrE contributes to extra-embryonic tissues and is required for nutrient supply, and the TE contributes to the placenta (Gardner & Rossant, 1979) (Guo et al., 2010). The embryo has now completed its pre-implantation development.

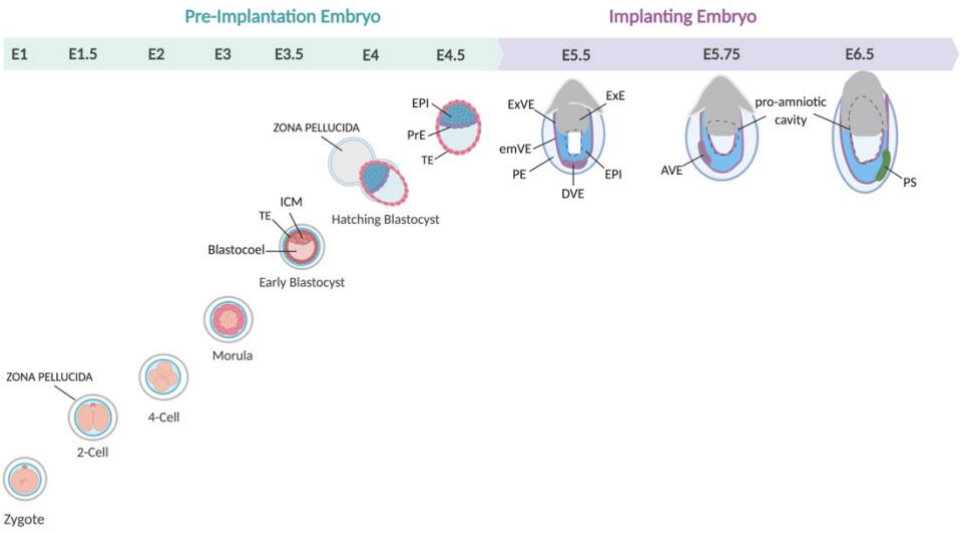

Figure 1: Early Embryonic Development in Mouse *(Created with BioRender.com)*

The embryo hatches out of its zona pellucida due to a combination of signaling, proteolytic lysis, and pressure from the growing embryo (Seshagiri et al., 2009), and then implants into the uterus around E4- E4.5. The walls of the uterus begin to thicken and undergo decidualization as the stromal cells of the uterus wall rapidly proliferate to support the implanting embryo (Gellersen & Brosens, 2014). The epithelial cells on the uterus wall undergo invagination and engulf the embryo as it continues to grow (Petropoulos et al., 2016). Around E5.5, the embryo develops an egg-cylinder shaped morphology, unique to the mouse embryo, as depicted in Figure 1. From E4.5- E6.5, the TE and the PrE encapsulate the EPI and the PrE gives rise to two main lineages- the visceral endoderm (VE) surrounding the EPI and the distal visceral endoderm (DVE)

emerging at the distal tip of the embryo (embryo attaches to the uterus at the proximal side) (Nowotschin & Hadjantonakis, 2010). The TE gives rise to the extraembryonic ectoderm (ExE). The VE and the DVE are surrounded by the parietal endoderm (PE). Around E5.75, the DVE actively migrates to the embryonic-extraembryonic epiblast junction and forms the anterior visceral endoderm (AVE) (Figure 1). The size of the pro-amniotic cavity continues to grow in size from E5.5 to E6.5. Around E6.5, the development of the primitive streak marks the start of gastrulation after which the embryo continues to undergo morphological changes giving rise to the body plan, and mesoderm and endoderm lineages arise (Nowotschin & Hadjantonakis, 2010).

Over the years, a lot has been learnt about early embryonic development in mice. However, some key questions still remain to be answered with respect to the external cues and local interactions that drive the patterning of the embryo, and processes such as ZGA, polarization, and compaction (White et al., 2018). Further, the upstream processes that regulate the gene expression in the embryonic cells starting from the totipotent identical blastomeres to the specific cell lineages (EPI, TE, PrE) still needs to be understood. Some studies have analyzed the polarization and compaction stage of the embryo to determine the upstream mechanical cues that can lead to the symmetry break in the embryo (Motosugi et al., 2005). Others have studied the blastomeres at the early 2-cell and 4-cell stages and demonstrated differential histone arginine methylation in the blastomeres which could bias them towards particular cell fates (Torres-Padilla et al., 2007). A study analyzing epigenetic profiles found differential expression of the chromatin modifier PRDM14 between blastomeres at the 4-cell stage (Burton et al., 2013). Forced expression of the PRDM14 at the 2-cell stage increased histone arginine methylation and promoted the blastomeres towards the pluripotent ICM fate. Analysis of the transcriptome in combination with genetic techniques have also helped to identify transcription factors that play crucial roles in ZGA at 2 cell stage in mouse. Maternal transcription factors such as Nfya (Lu et al., 2016), Dux (Z. Chen & Zhang, 2019), and Yap1 (Yu et al., 2016), and maternally derived chromatin remodeling factors such as Brg1 (Bultman et al., 2006) and Snf2h (Alda-Catalinas et al., 2020) are crucial for

ZGA. Knockout models of these factors lead to impaired ZGA or developmental arrest at the 2-cell stage.

B. Early Embryonic Development in Humans and Non-Human Primates

Most of the early human embryonic development studies are done using IVF embryos. Much of what is known about the human implanting embryo is from studies using non-human primate models such as the rhesus and cynomolgus macaques (Enders et al., 1986). The pre-implantation embryonic development is well-conserved across the human, non-human primate and mouse species. However, unlike the short mouse pre-implantation development (around 4 days), the human and non-human primates have a prolonged pre-implantation developmental time of 6-7 days (Niakan et al., 2012). Further, in humans, the ZGA takes place around the 4-8 cell stage embryo (Braude et al., 1988), and in monkeys around 8-16 cell stage (Schramm & Bavister, 1999), unlike the 2-cell stage embryo in mice (Bensaude et al., 1983).

After fertilization, the human zygote undergoes cell division to form 2-cell (E1), 4-cell (E2), and 8-cell stage embryo (E3), undergoes polarization and compaction at the morula stage (E4), and gives rise to the ICM and the TE (E5). The blastocoel cavity continues to grow in size and after multiple cell rearrangements (E6), the ICM generates the EPI and PrE cell lineages (E7) (Niakan et al., 2012). This is followed by hatching of the embryo from the zona pellucida, and then implantation of the embryo into the uterus wall from E8 to E12 before the start of gastrulation (Finn & McLaren, 1967)(Cockburn & Rossant, 2010). During the implantation stages, there are distinct differences in the morphology of the mouse and primate embryos. Unlike the egg-cylinder shaped morphology of the implanting mouse embryo (Figure 1), the human and non-human primate embryos continue to grow in size while maintaining their spherical morphology (Figure 2). Further, unlike the mouse, where invagination of the uterus wall and engulfment of the embryo is observed, the primate embryos attach to the uterus wall and the TE lineage cells of the embryo invade into the uterus wall (Carter et al., 2015). Around E8 – E12, cavities begin to form within the EPI and the PrE/Hypoblast lineages, giving rise to the amniotic cavity and the yolk sac respectively (Figure 2). A bilaminar disc is formed at E12 consisting of two layers

formed by the EPI and PrE cells (Figure 2). During gastrulation, the primitive streak arises at the bilaminar disc and gives rise to the ectoderm, mesoderm and endoderm lineages (Enders et al., 1986).

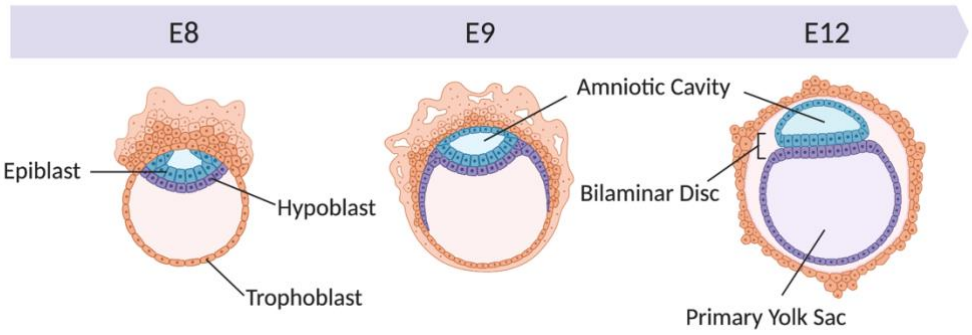

Figure 2: Implanting Human Embryo *(Created with BioRender.com)*

C. Early Embryonic Development in Pigs

Pigs have been used as animal models for studying many human diseases due to the similarities in their anatomy and physiology to humans. Further, many transgenic pig models and genetic tools exist which makes studying early embryonic development in pigs feasible (Bassols et al., 2014). Understanding early pig embryonic development is especially important in chimerism studies. The ultimate goal of interspecies chimerism and blastocyst complementation is to be able to develop transplantable human tissues and organs in relevant host organisms such as pigs. Studying early pig embryonic development will enable researches in stage matching the grafted cells with the host embryo and also gain insight into relevant genes and pathways that can be manipulated to increase the success of chimerism.

The timeline for the early embryonic development in pigs is similar to human and non-human primates. In pigs, ZGA takes place at the 4-cell stage (S. Cao et al., 2014). The embryo undergoes cell divisions and forms the morula around E4-E5. Polarization and compaction are also observed during the morula stage. The embryo then give rise to the ICM and TE cell lineages around E5-E6 and the ICM further segregates into the EPI and PrE lineages around E7-E8 (Ramos-Ibeas et al., 2019). The embryo undergo implantation around E10-E11. Similar to the human embryo, the pig embryo maintains the spherical morphology as it

continues to grow and implant. During implantation, the pig embryo TE fuses with the uterus wall and TE cells do not invade or undergo invagination as observed in primate and mouse embryos respectively (Lee & DeMayo, 2004). A unique feature in pigs is the crucial role that fatty acid metabolism plays throughout early embryonic development (S. Cao et al., 2014). Studies looking into the metabolic and epigenetic profiles of the developing embryos have demonstrated how the metabolism switches from oxidative phosphorylation in naïve ICM cells to more glycolysis in the primed EPI state of the pig embryo (Ramos-Ibeas et al., 2019).

D. Transcriptome Analysis of Early Embryonic Development across Species

Most of the key stage-specific transcription factors (TFs) currently known are co-expressed in early progenitor cells during development before their expression is specified to particular lineages (EPI, TE, PrE) at the later stages (Petropoulos et al., 2016). Although the mouse model has been well studied, many questions remain about the upstream processes regulating the patterning seen in the embryo such as how do identical totipotent embryos lead to the symmetry break in the embryo, how are the genes regulated through development, what are the inter- and intra-cellular interactions and mechanical forces involved that give rise to the different cell lineages? Also, very little is known about how conserved the transcriptome of the early embryonic development is across species such as mice, humans, non-human primates, pigs etc. This is especially relevant to blastocyst complementation studies where the graft cells from species A need to integrate into the developmental niche in the host embryo of species B to form species A specific tissues/organs. The low success rate in blastocyst complementation between distant species has led to the hypothesis that although homologous genes of key transcriptional factors exist across species, their level of expression, and the developmental time at which they are expressed might vary, given the variable developmental timelines between the species. Studies using traditional methods such as staining of the TFs and knockout models to compare pre-implantation embryos (zygotes to blastocysts) across species such as mice and humans have provided evidence for the same (Blakeley, Fogarty, Del Valle, et al., 2015). For example, CDX2 expression which is specific to the TE lineage in late blastocysts, first appears at the morula stage in mice but is not

observed in the human embryos until the blastocyst stage (Blakeley, Fogarty, Del Valle, et al., 2015). Laminin plays a crucial role in mouse PE development (Niakan et al., 2010) but is not present during human PE specification (Niakan & Eggan, 2013). TGF-beta signaling was found to be important for human EPI development but dispensable in mouse EPI formation (Blakeley, Fogarty, Valle, et al., 2015). FGF/Erk signaling is crucial for mouse EPI and PrE specification but is dispensable in humans (Roode et al., 2012) (Niakan & Eggan, 2013).
Development of techniques for analysis of the genome, transcriptome and epigenetics of the cells have helped overcome the limitations of staining and knockout models and proved useful to get a complete picture of the transcriptome landscape during early embryonic development within each species and across species. Some of the earliest studies using microarray technology in human pre-implantation embryos proved that it was possible to analyze global gene expression in single oocytes and embryos with very few cells (Dobson et al., 2004). They observed two main waves of transcription- first wave from the oocyte to the 4-cell stage due to the maternal inherited RNAs, and the second wave from the 4-cell to the 8-cell stage due to the ZGA in the embryos (P. Zhang et al., 2009). Another study analyzed the global gene expression across 7 stages of pre-implantation development in 3 species- human, mice and bovine, and demonstrated that around 40% of the orthologous genes across these 3 species were differentially expressed during the embryonic development (Xie et al., 2010). To overcome the limitations of whole genome analysis techniques, some studies used single-cell RNA analysis with PCR (Guo et al., 2010) and microfluidics single-cell approach for epigenetic profiling (Burton et al., 2013) to analyze the cells in the embryos at a single-cell level.
The dramatic improvement in single-cell RNA sequencing (sc-RNA seq) technologies has enabled high-throughput sequencing of embryos across developmental times and across species. Sc-RNA seq technique has been used to sequence and analyze transcriptomes in mouse pre-implantation, peri-implantation (E3.5- E6.5) (Mohammed et al., 2017), and post-gastrulation embryos (E9.5- E13.5) (J. Cao et al., 2019). Comparative analysis of sc-RNA seq data between human and mouse pre-implantation embryos across 6 stages (Niakan & Eggan, 2013) has provided information about transcripts that are

conserved and distinct among the species (Blakeley, Fogarty, Valle, et al., 2015). The study demonstrated that although many genes are conserved between the two species, they may be expressed at different developmental times (Xue et al., 2013), and their levels of expression and function may vary between the species (Niakan & Eggan, 2013) (Blakeley, Fogarty, Valle, et al., 2015). These studies also concluded that based on the gene expression, the human mid- to late-blastocyst is more similar to the mouse peri-implantation embryo. These studies also compared human embryonic stem cells (hESCs) to different mouse embryonic developmental stages and verified highest similarity of hESCs with the mouse EPI (Tesar et al., 2007) (Niakan & Eggan, 2013).

Sc-RNA sequencing has also been used to look into the transcriptomes of non-human primates such as cynomolgus monkeys (Nakamura et al., 2016) and marmosets (Boroviak et al., 2018). The transcriptomes, metabolic and epigenetic profiles of other relevant human models such as pigs have also been explored (S. Cao et al., 2014) (Ramos-Ibeas et al., 2019). Studies comparing the transcriptome of sequentially developing pre-implantation embryos across 3 species - mice, humans, marmosets (Boroviak et al., 2018), mice, humans, cynomolgus monkeys (Nakamura et al., 2016), and pigs, humans, mice (S. Cao et al., 2014) (Ramos-Ibeas et al., 2019) have provided deep insight into the similarities and differences in the regulation of gene expression across the 3 species. The cynomolgus monkey late post-implantation EPI transcriptome was found to be similar to the transcriptome of human iPSCs, both of which matched with the mouse pre-gastrulation EPI (Nakamura et al., 2016). The pig EPI matched closely with the human late-ICM and human EPI transcriptome as well as the hESC transcriptome (Ramos-Ibeas et al., 2019). The results from the sc-RNA seq studies described above can be used for stage matching the host embryos with grafted cells during interspecies chimerism and blastocyst complementation studies. A few studies have demonstrated the importance of stage matching in intraspecies chimerism. Injection of ‘primed’ mouse epiblast stem cells (mEpiSCs) into the mouse pre-implantation embryos do not form chimeras (Brons et al., 2007) (James et al., 2006). However, the injection of the mEpiSCs into gastrulating mouse embryos lead to successful chimerism (Y. Huang et al., 2012). With relevance to interspecies chimerism, studies have

demonstrated that by stage matching the mouse embryos and the human grafted cells, that is, by injecting hPSCs and hESCs into late stage gastrulating mouse embryos, successful chimeras are formed overcoming the interspecies barrier (Mascetti & Pedersen, 2016b) (Cohen et al., 2016). Combining these studies with sc-RNA seq technology will help gain insight into the key gene networks that are crucial for human-mouse chimeras. Similar approaches can then be used to identify key gene networks required for other interspecies chimeras such as human-pig chimeras.

Conclusions- Over the years, much been learnt about early embryonic development in different species including human, non-human primates, mice, pigs, etc. Multiple scRNA seq studies performed over the years have generated, analyzed, and compared multiple datasets of embryos across many species. These scRNA seq datasets contain a wealth of information with the potential to answer many relevant questions. However, there is still a need for better data analysis techniques and strategies to make full use of this potential. Further, for comparisons across species, the accuracy of the developmental time points chosen for scRNA seq dataset generation and analysis is crucial (Freour & Vassena, 2017). This is especially true in case of mouse embryos as they have a relatively short early embryonic developmental timeline with the transcriptome undergoing rapid changes. In case of chimerism and blastocyst complementation studies, there is a need for systems that will enable clear observation and tracing of the grafted cells in the host embryo throughout early embryonic development, and also enable easy analysis of their molecular and cellular interactions to answer many questions in the field (Nowotschin & Hadjantonakis, 2010). Optimized 3D ex-vivo culture systems have been developed for this purpose to support humans (Xiang et al., 2020) and non-human primates (Niu et al., 2019) developing embryos. These ex-vivo systems in combination with high-throughput data analysis will help us gain insight into the early development and also device strategies to optimize the chimerism and blastocyst complementation studies.

Section IV- *Ex Vivo* System to Study Mouse Early Embryo Development and Chimeric Embryos

Most of the current chimerism/ blastocysts complementation (BC) studies make use of *in vivo* models to analyze and optimize the process. These *in vivo* models involve chimeric/ BC embryos growing *in utero* in the host animal. While *in vivo* models precisely recapitulate the developmental processes, the limitations in the existing imaging technologies do not allow us to closely monitor and track the grafted cells in the host embryo at each developmental stage. Early embryonic development is a rapid process where changes take place every few hours and the *in vivo* BC models limit precise stage-matching of the grafted cells with the host embryos. The difference in developmental stages between the grafted and the host cells can have major effects on the success rates and reproducibility of the studies. Further, the host uterus which supports the chimeric embryos is a complex environment where multiple factors come into play, such as, signaling molecules, hormones, mechanical forces, to name a few. Figuring out the major interspecies barriers and other factors that inhibit BC, or conversely, the factors that are conducive to BC is difficult to assess with high throughput using *in vivo* models.

To overcome the drawbacks of the *in vivo* BC studies, many 2D and 3D *ex-vivo* culture systems have been developed that recapitulate the *in vivo* environment and are able to support early embryonic development. These cultures have been used to study the patterning of the embryos, the transitions in cell lineages, and changes in gene expression through time using single-cell RNA sequencing (scRNA seq). Over the past few years, many 3D ex-vivo systems for different species such as mouse, human, non-human primate, and pig have been developed. Based on the species, different combinations of medium and gels have been tested to optimize the culture systems. Human blastocysts are able to develop up to the pre-gastrulation stage (E14) when cultured in a combination of *in vitro* culture (IVC1 + IVC2) mediums and 10% Matrigel on a low attachment plate (Xiang et al., 2020). This human blastocyst culture system has been modified to support cynomolgus monkey blastocysts up to the early post-implantation stage (E20) (Niu et al., 2019). In both these systems, scRNA seq has been performed at different time points to delineate the cell lineages and track transcriptomic changes through development.

Several culture media have been optimized to support the early pre-implantation development of the mouse from the oocyte to the blastocyst stage (E4) in 2D. Many of these culture media such as HTF, KSOM, M2, M16 have been used over the years to extract blastocysts from zygotes, and to support mouse embryos during genetic manipulations (Mohd-Fazirul et al., 2015) (Perin et al., 2008). Hence, the early pre-implantation development of the mouse has been extensively studied. However, beyond E4, the blastocysts do not continue to develop, enter into diapause, and need to be transferred *in utero* for continued development (Reijo Pera & Prezzoto, 2016). Limited studies have been able to efficiently support the blastocysts beyond E4, during the peri-implantation stage (E4.5-E5.5), when the blastocyst forms an egg-cylinder morphology (Morris et al., 2012).

The **aim of this study** is to test different combinations of medium and gel to find the optimal *ex-vivo* culture condition that can support the growth of the mouse blastocysts up to the implanting stage. Since the host uterus that supports *in vivo* embryonic growth is a complex environment where the embryo is exposed to multiple factors, it will be difficult to replicate this complex environment using gels with defined compositions. The *ex-vivo* systems for culturing human and primate embryos until the pre-gastrulation and gastrulation stages, respectively, have used Matrigel at different concentrations in combination with appropriate medium as the 3D gel for embedding the embryos. Matrigel is a complex mixture of proteins derived from mouse tumor cells (Hughes et al., 2010). The composition of Matrigel is not well-defined but multiple studies using stem cells rely on Matrigel as the substrate for the stem cells to attach and proliferate while maintaining their undifferentiated state. In this study, we test two different commonly used mouse embryo mediums, HTF and KSOM, in combination with different gels, hydrogel and Matrigel, to develop the optimum environment to support mouse embryos until the implantation stage.

Other than the optimum nutrient supply, the *ex-vivo* system should also be able to provide the right mechanical support for the rapidly growing mouse embryos and hence, the concentration of the 3D gel used for embedding the mouse embryos needs to be optimized. A higher than optimal concentration can inhibit growth while a lower than optimal concentration will not provide the right resistance required for the embryonic cells to attach to the matrix and continue development.

A. Methods for *Ex-Vivo* System to study Mouse Early Embryonic Development

1. Madin-Darby Canine Kidney (MDCK) Cells in 3D Cultures

MDCK cells were expanded in 2D culture and used for the 3D culture. These preliminary experiments were performed to optimize the gel compositions that support formation of 3D cyst-like structures from MDCK cells. Two different kinds of gels were tested- a) a combination of Hydrogel and Matrigel, and b) Matrigel.

1.1. 3D MDCK cell culture in 60% Mebiol Mixture (Wei Shen Lab Protocol)

MDCK cells cultured in T25 flasks with MEM I (**Table 1**) were used for the 3D culture. Mebiol and Matrigel used for gel preparation were defrosted at 4°C overnight. All the steps for the 3D culture were performed on ice using pre-chilled tubes and pipets to prevent solidification of gels. A MDCK cell suspension in MEM II (**Table 1**) was prepared. The concentration of the cells in the cell suspension was 4x the final concentration of cells/gel. The optimal final concentration for 3D cysts formation was 50,000 MDCK cells/100uL gel. The cell suspension was carefully mixed with the 60% Mebiol mixture (**Table 2**), avoiding formation of bubbles. First, the cell suspension and Matrigel were thoroughly mixed on ice followed by the addition of Mebiol. To a pre-heated 48-well plate, 100uL of the final gel mixture containing cells was added to the center of each well. Subsequently, 200uL of the MEM I was added per well. The gels were incubated for 7 days at 37°C, 5% CO2 during which 3D cysts formation could be observed. Medium change was carried out every two days with MEM I.

Added to basal Minimal Essential Medium	*MEM I*	*MEM II*
Fetal Bovine Serum	10%	40%
L-Glutamine	1%	4%
Penicillin/Streptomycin	1%	4%

Table 1: Medium for MDCK Culture

Components of the 60% Mebiol Mixture	*Concentrations*
Mebiol (Advanced Biomatrix #5180)	60%
Matrigel (BD 35623 #47743-722)	15%
Cell Suspension (MDCK + MEM II)	25%

Table 2: Composition of 60% Mebiol Mixture

1.2. 3D MDCK cell culture in 75% Matrigel

The protocol for 3D MDCK cell culture in 75% Matrigel is similar to the protocol described in **1.1**. All the steps were performed on ice using pre-chilled pipettes and tubes to prevent solidification of the gels. MDCK cell suspension in MEM II (**Table 1**) was prepared in 75% Matrigel (**Table 3**). To each well of the pre-heated 48-well plate, 100uL of the final gel mixture followed by 200uL of MEM I (**Table1**) was added. The 48-well plate was incubated for 7 days at 37°C, 5% CO2. Medium change was carried out every two days with MEM I.

Components of the 75% Matrigel Mixture	*Concentrations*
Matrigel (BD 35623 #47743-722)	75%
Cell Suspension (MDCK + MEM II)	25%

Table 3:Composition of 75% Matrigel Mixture

2. *Ex-vivo* 3D Mouse Blastocyst Cultures

Embryonic day 3.5 early mouse blastocysts from C57 mice were used for the *ex-vivo* 3D cultures. These E3.5 blastocysts were either extracted at the zygote stage (0.5 days *post coitum*) from the oviduct of pregnant mice and then cultured *in vitro* until the blastocyst stage or were directly extracted as blastocysts from the pregnant mice's oviduct at E3.5. For the 3D *ex-vivo* culture, the early blastocysts were cultured in 48 well plates with a gel volume of 300uL/well. About 10-15 blastocysts were cultured per 300uL gel. Mebiol and/or Matrigel to be used for preparation of the gels was defrosted at 4°C overnight. All the steps for gel preparation were performed on ice and pre-chilled pipettes and tubes were used. The medium used for the 3D embryonic cultures was equilibrated at 37°C, 5% CO2 for at least 6 hours before

use. 300uL of gel mixture per well was prepared using a combination of culture medium and stock gel. The composition of the different gels and mediums tested in this study were listed in **Table 4** and **Table 5**.

Gel Mixture (Total= 300uL)	*Composition*	*Medium change/ addition per gel*
60% Mebiol Mixture (100uL) + 10% Matrigel (200uL)	**Table 2** (cell suspension replaced by medium) + 10% Matrigel in 90% Medium	Every day, 150uL of the gel is replaced with freshly prepared 10% Matrigel mixture.
Base (100uL) + 10% Matrigel (200uL)	Base - 100% Matrigel + 10% Matrigel in 90% Medium	
10% Matrigel Mixture	10% Matrigel in 90% Medium	
20%, 30%, 40%, 50% Matrigel Mixtures	20%, 30%, 40% and 50% Matrigel prepared in 80%, 70%, 60% and 50% Medium respectively	Every day, 150uL of freshly prepared 20%, 30%, 40%, or 50% Matrigel mixtures are added to the respective gels.

Table 4: Composition of Gel Mixtures tested for 3D Cultures

S. NO.	*Mediums used for Gel Mixture Preparation*
1.	HTF + 10uM ROCK inhibitor
2.	KSOM Mouse Embryo Medium (Sigma-Aldrich #MR-020P-5F) + 10uM ROCK inhibitor

Table 5: Mediums used for preparation of Gel Mixtures

2.1. 3D blastocyst culture- 60% Mebiol Mixture (100uL) with 10% Matrigel (200uL)

50uL of 60% Mebiol Mixture was added to the center of each well of the 48-well plate. The 48-well plate was then incubated at 37°C, 5% CO2 for 10 minutes to allow the gel to solidify. Using a 2uL pipette, the blastocysts were pulled out of their *in vitro* 2D cultures and carefully pipetted onto the center of the solidified gels in the 48-well plate. The blastocysts were then topped with 50uL of 60% Mebiol Mixture and once again incubated at 37°C, 5% CO2 for 10 minutes to allow the gel to solidify. Subsequently, 200uL of 10% Matrigel prepared in 90% Medium was added per well.

2.2. 3D blastocyst culture- Base Matrigel (100uL) with 10% Matrigel (200uL)

The base Matrigel was first prepared by adding 100uL of 100% Matrigel to the center of each well of the 48-well plate. The 48-well plate was then incubated at 37°C, 5% CO2 for 10 minutes to allow the gel to solidify. Using a 2uL pipette, the blastocysts were pulled out of their *in vitro* 2D cultures and carefully pipetted onto the solidified base Matrigels in the 48-well plate. The blastocysts were then topped with 200uL of 10% Matrigel prepared in 90% Medium per well.

2.3. 3D blastocyst culture- 10%, 20%, 30%, 40%, 50% Matrigel (300uL each)

After preparation of the gel mixtures, half of the gel volume per well, that is 150uL, was added to the center of each well. The 48-well plate was then incubated at 37°C, 5% CO2 for 10 minutes to allow the gel to solidify. Using a 2uL pipette, the blastocysts were pulled out of their *in vitro* 2D cultures and carefully pipetted onto the solidified gels in the 48-well plate. The blastocysts were then topped with the remaining half of each gel mixture (150uL) to form a total gel volume of 300uL.

All the gels were incubated at 37°C, 5% CO2 and the blastocysts were observed until E7-E7.5. Medium changes/ additions were carried out based on the gel mixtures used (**Table 4**).

3. **Immunofluorescence Staining of Mouse Embryos**

Immunofluorescence staining was performed to observe the different proteins and their localization in the mouse embryos. The main steps included fixation followed by permeabilization, blocking, primary antibody staining, secondary antibody staining, DAPI staining and then preparation of the samples for imaging. The staining protocol for the *ex-vivo* cultured embryos embedded in the 3D gels slightly differed from the protocol used for non-embedded free early mouse blastocysts (cultured *in vitro* from the zygote stage in only HTF medium). These differences are highlighted in each step below.

Preparation steps for staining protocol-

A. **Non-embedded free early mouse blastocysts**- The free mouse blastocysts were pipetted from their *in vitro* 2D HTF medium culture and subsequently stained by transferring them into 100uL droplets of the reagents at each step (Figure 3). The 100uL reagent droplets at each step were pipetted into circles drawn with a PAP pen on a glass slide as demonstrated in (Figure 3).

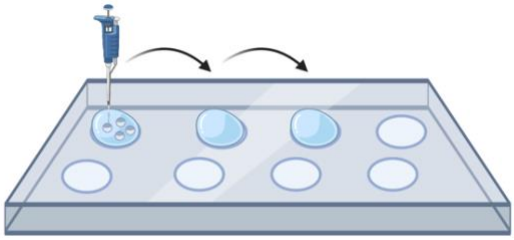

Figure 3: Setup for Staining Non-Embedded Mouse Blastocysts. 100uL of appropriate reagent drops were pipetted into circles drawn with a PAP pen on glass slides. The blastocysts were transferred between the drops at each stage of the staining protocol. *(Created with BioRender.com)*

B. ***Ex-vivo* cultured gel-embedded embryos**- For better visualization during immunofluorescence, 48-well/ 8-well plates with cover-slip bottoms were used for the *ex-vivo* 3D culture. The *ex-vivo* gel-embedded mouse embryos were then stained in their respective wells. This helped to conserve the

morphology of the embedded embryos within the gels. The reagents at each step were added into the wells on top of the gels. Between each step, the reagents were pipetted out carefully so as to not disturb the gels.

The following steps were performed at room temperature unless otherwise specified.

I. **Fixation**- Fixing the embryos helps preserve their morphology and protein expression and prevents the samples from deteriorating outside the normal culture conditions.

(a) **Non-embedded free early mouse blastocysts**- The embryos were fixed by transferring them into 100uL drops of 2% PFA solution (Paraformaldehyde solution (SC-281692)) prepared in 0.1% PVA/PBS (0.1% Polyvinyl Alcohol in Phosphate Buffered Saline) for 10 minutes at room temperature. The 0.1% PVA/PBS solution prevents the embryos from sticking to the glass surface of the slides. After fixation, the embryos were washed 3 times in 100uL drops of 0.1% PVA/PBS solution, 5 minutes per wash, at room temperature.

(b) ***Ex-vivo* cultured gel-embedded embryos**- To each well of the culture plate, 400uL of the fixing solution (1% Glutaraldehyde (Sigma-G6257) + 4%PFA in PBS) was added on top of the gels and incubated for 25 minutes at room temperature. The fixation solution was then carefully pipetted out keeping the gels intact. This was followed by 3 wash steps, each with 400uL of 0.1% PVA/PBS solution for 15 minutes at room temperature. Since the embryos were embedded in Matrigel, an **extra quenching step** was performed by adding 400uL/gel of 0.1% Sodium Borohydride (CAS 16940-66-2) in PBS for 10 minutes at room temperature. This helps to reduce the autofluorescence observed due to the Matrigel during fluorescence imaging. Quenching was followed by 3 wash steps, each with 400uL of 0.1% PVA/PBS solution for 15 minutes at room temperature.

II. **Permeabilization and Blocking**- Permeabilization allows the entry of antibodies into the intracellular compartments of the cells of the fixed embryos. The blocking step helps to reduce non-specific binding of the antibodies in the embryos.

(a) **Non-embedded free early mouse blastocysts**- The fixed embryos were transferred to 100uL drops of permeabilization solution (1% Triton X in 0.1% PVA/PBS) and incubated for 10 minutes at room temperature. This was followed by 1 washing step in 100uL drops of 0.1% PVA/PBS solution, 5 minutes, at room temperature. The embryos were then transferred into 100uL drops of the blocking solution (10% Normal Donkey Serum (Jackson Immuno Code- 017-000-121) in 0.1% PVA/PBS) and incubated for 60 minutes at room temperature.

(b) ***Ex-vivo* cultured gel-embedded embryos**- To each well of the culture plate, 400uL of the permeabilization solution (1% Triton X in 0.1% PVA/PBS) was added on top of the gels and incubated for 15 minutes at room temperature. The gels were then washed in 0.1% PVA/PBS for 15 minutes at room temperature. Subsequently, 400uL/gel of blocking solution (10% Normal Donkey Serum in 0.1% PVA/PBS) was added and incubated overnight at 4°C.

III. **Primary Antibody Staining**- The embryos were stained with primary antibodies specific to the proteins of interest. Primary antibody solutions are prepared using the recommended concentrations of primary antibodies in the blocking solution (10% Normal Donkey Serum in 0.1% PVA/PBS). The primary antibodies used in this study are listed in Table 6.

(a) **Non-embedded free early mouse blastocysts**- After permeabilization and blocking, the embryos were transferred into 100uL drops of appropriate primary antibody solutions (Table 6) prepared in the blocking solution. The embryos were incubated in the

primary antibody solutions overnight at 4°C. After the overnight primary antibody staining, the embryos were washed 3 times, 5 minutes per wash, by transferring them into 100uL drops of 0.1% PVA/PBS at room temperature to remove unbound primary antibodies from the samples.

(b) ***Ex-vivo*** **cultured gel-embedded embryos**- To each well of the culture plate, 200uL of the appropriate primary antibody solutions (Table 6) were added on top of the gels and incubated overnight at 4°C. The gels were then washed 3 times, each with 400uL of 0.1% PVA/PBS solution for 15 minutes at room temperature to remove any unbound antibodies from the samples.

IV. **Secondary Antibody Staining**- The secondary antibodies attached with fluorophore tags recognize specific primary antibodies bound to proteins in the embryos and help to visualize these proteins of interest. Since the secondary antibodies contain fluorophores, the **subsequent steps were performed in the dark** to prevent photobleaching. The secondary antibodies used in this study are listed in Table 7.

(a) **Non-embedded free early mouse blastocysts**- After primary antibody staining, the embryos were transferred into 100uL drops of secondary antibody solutions prepared in 0.1% PVA/PBS (Table 7) and incubated in the dark for 90 minutes at room temperature. The embryos were then washed 3 times, 5 minutes per wash, by transferring them into 100uL drops of 0.1% PVA/PBS at room temperature to remove unbound secondary antibodies from the samples.

(b) ***Ex-vivo*** **cultured gel-embedded embryos**- To each well of the culture plate, 250uL of the secondary antibody solution (Table 7) was added on top of the gels and incubated for 2 hours at room temperature. The gels were then washed 3 times, each with 400uL of

0.1% PVA/PBS solution for 15 minutes at room temperature to remove any unbound secondary antibodies from the samples.

V. **DAPI staining**- DAPI is a blue fluorescent dye that stains the DNA in the nucleus of cells and can be visualized using the fluorescence microscope.

(a) **Non-embedded free early mouse blastocysts**- After secondary antibody staining, the embryos were transferred into 100uL drops of the DAPI working solution (DAPI in 0.1% PVA/PBS at 1:1000) and incubated for 10 minutes at room temperature. The embryos were then washed 3 times, 5 minutes per wash, by transferring them into 100uL drops of 0.1% PVA/PBS at room temperature.

(b) ***Ex-vivo* cultured gel-embedded embryos**- To each well of the culture plate, 400uL of the DAPI working solution (DAPI in 0.1% PVA/PBS at 1:1000) was added on top of the gels and incubated for 20 minutes at room temperature. The gels were then washed 2 times, each with 400uL of 0.1% PVA/PBS solution for 15 minutes at room temperature.

VI. **Preparing the embryos for imaging**-

(a) **Non-embedded free early mouse blastocysts**- After the washing steps, the embryos were ready to be mounted onto concave glass slides for imaging. The final mounting medium used was 20% fluoroshield (Immu-Mount 9990402) in 0.1% PVA/PBS. Direct introduction of the embryos from the wash solution (0.1% PVA/PBS) into the 20% fluoroshield medium can distort their morphology due to the drastic change in viscosity. Hence, the embryos were gradually introduced into more viscous solutions by incubating them for 5 minutes each in 100uL drops of 5%, 10% and finally 20% fluoroshield in 0.1% PVA/PBS. The embryos were then mounted onto concave

glass slides with 20% fluoroshield in 0.1% PVA/PBS. Coverslips were carefully laid on top of the slides to avoid the formation of bubbles.

(b) ***Ex-vivo* cultured gel-embedded embryos**- Since the embryos were cultured in plates with cover-slip bottoms, they could be imaged in their own culture wells. To each well, 200uL of PBS was added and the plates were sealed with parafilm to prevent drying of the gels.

1. **Imaging the mouse embryos-** Brightfield images of the mouse embryos were captured using the Leica Inverted Epifluorescence Microscope System (Leica DMi8) which is suited for both brightfield and fluorescence imaging. Immunofluorescence images of the early mouse blastocysts and the gel-embedded *ex-vivo* cultured late mouse embryos were captured using the Nikon A1R Flim Confocal Microscope using the laser intensities 405 nm, 488 nm, 561 nm and 640 nm.

2. **Analysis of the brightfield and immunofluorescence images-** The acquired images were processed using the ImageJ software version 2.1.0/1.53c.

S. NO.	*Primary Antibodies*	*Host Species/Ig*
1.	Oct-4 (sc-8628)	Goat pAb
2.	SOX2 (ab97959)	Rabbit mAb
3.	Nanog (ab97959)	Mouse mAb
4.	E-Cadherin (ab2076672)	Mouse IgG
5.	CDX2 (sc-19478)	Goat pAb

Table 6: Primary Antibodies used for Immunofluorescence Staining

S. NO.	*Secondary Antibodies*	*Fluorescent Wavelength*
1.	Donkey anti-Mouse (Abcam 150107)	647
2.	Donkey anti-Goat (Abcam 150129)	488
3.	Donkey anti-Rabbit (Abcam 150074)	555

Table 7: Secondary Antibodies used in Immunofluorescence Staining

B. Results for *Ex-Vivo* System to Study Mouse Early Embryonic Development and Chimeric Embryos

The experiments towards optimizing the 3D ex-vivo system to culture early mouse blastocysts until the implanting stage are detailed in this section. First, preliminary experiments using MDCK cells in 3D gels were performed to establish a 3D culture system that can be translated for culturing mouse blastocysts. Wild type (WT) mouse blastocysts were then cultured in different combinations of medium and gel to find the optimal condition that can support the *ex-vivo* growth of the embryos until the implanting stage. The optimal conditions were then used to culture Hhex KO mouse blastocysts to test if the results can be translated to blastocyst complementation studies. WT mouse embryos in the optimal 3D *ex-vivo* culture were also stained for relevant proteins.

1. Preliminary experiments with MDCK cells in 3D gels

The Madin-Darby Canine Kidney (MDCK) cells are a well-established mammalian epithelial cell line. In this study, these cells were cultured in different 3D gels using appropriate medium and their ability to form 3D cyst-like structures was determined.

1.1. 3D MDCK cell culture in 60% Mebiol Mixture (Wei Shen Lab Protocol)

According to the protocol in **Methods Section IV A 1.1**, the MDCK cells were cultured in 60% Mebiol Mixture and their ability to form 3D cyst-like structures was analyzed until day 7 in culture. Spherical cyst-like structures with varying sizes were observed at day 5 and day 7 in culture (Figure 4, A-B). The 60% Mebiol Mixture was conducive to the growth of 3D cyst-like structures from the MDCK cells.

Since the Mebiol hydrogel is difficult to work with and pipetting exact volumes of the hydrogel is difficult due to its highly viscous nature, the next experiment tested the ability of MDCK cells to give rise to cyst-like structures in 3D gels composed of only Matrigel, which is comparatively easier to work with.

1.2. 3D MDCK cell culture in 75% Matrigel

According to the protocol in **Methods Section IV A 1.2**, the MDCK cells were cultured in 75% Matrigel for up to 7 days. Cyst-like structures were observed in

the culture by day 5 (Figure 4, C-D). Further, the cysts were larger and varied in morphology compared to the cyst-like structures from the 60% Mebiol Mixture condition. In the 75% Matrigel condition, smaller round cysts aggregated together to form larger cysts as observed in Figure 4, C-D. The cysts from the 60% Mebiol Mixture were single spherical structures and no aggregation of cysts was observed (Figure 4, A-B).

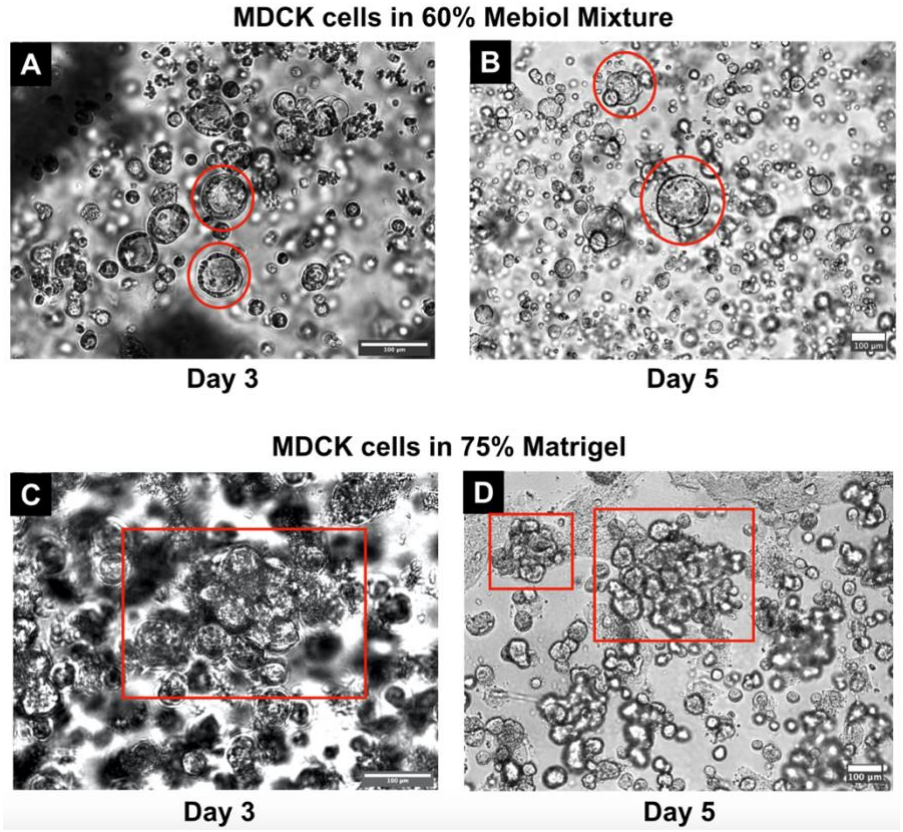

Figure 4: MDCK Cells in 3D Gels. *(A-B) MDCK cells were cultured in 60% Mebiol Mixture (60% Mebiol hydrogel + 15% Matrigel + 25% cell suspension) and observed till day 5. In the 60% Mebiol Mixture, spherical cyst-like structures were formed. (C-D) MDCK cells were cultured in 75% Matrigel (75% Matrigel + 25% cell suspension) and observed till day 5. In the 75% Matrigel condition, smaller cysts aggregated together to form larger cysts and varied in morphology compared to the 60% Mebiol condition cysts. Scale bar in all figures= 100 µm.*

Conclusions: The MDCK cells cultured in the 3D gel conditions, 60% Mebiol Mixture and the 75% Matrigel, were able to give rise to cyst-like structures. The morphology and the size of the cysts varied between the conditions as observed in Figure 4. The subsequent experiments focused on using these 3D gel systems and optimizing them to be able to culture early mouse blastocysts up to the implanting stage.

2. Optimizing the 3D *Ex-Vivo* Culture Conditions for Mouse Blastocysts

From the preliminary experiments, it was observed that the 60% Mebiol Mixture and the 75% Matrigel *ex-vivo* cultures enabled formation of 3D cyst-like structures from MDCK cells. The following experiments focused on optimizing the medium and gels used in the 3D culture to enable the *ex-vivo* development of early mouse blastocysts until the implanting stage. The goal is to be able to find the optimum conditions that allow the cultured E3.5 mouse blastocysts to hatch out of their zona pellucida and continue to grow in size to form the characteristic egg-cylinder morphology usually observed in *in vivo* implanting mouse embryos (E5.5-E7).

2.1. 3D Blastocyst cultures in HTF Medium + 3 different gels

Wild type mouse blastocysts at E3.5 were cultured in 3 different gels with HTF medium as detailed in **Methods Section IV A 2.1-2.3** -

1) 60% Mebiol Mixture (100uL) with 10% Matrigel (200uL)
2) Base Matrigel (100uL) with 10% Matrigel (200uL)
3) 10% Matrigel (300uL)

The gel conditions 2 and 3, Base Matrigel + 10% Matrigel and just 10% Matrigel, were previously tested for optimizing *ex-vivo* cultures of human and primate blastocysts (Xiang et al., 2020) (Niu et al., 2019).

In this set of experiments, the blastocysts in each condition were observed until E9 and images were taken at regular time points- E5, E7 and E9 (Figure 5). In all three conditions, the blastocysts did not hatch out of their zona pellucida and disintegrated by E7 and E9 (Figure 5). HTF medium is a commonly used medium for supporting early mouse blastocysts up to the pre-implantation stage. Since it is possible that the HTF medium used in all 3 gels does not support the growth of the blastocysts beyond the pre-implantation stage, in the next set of experiments, the HTF medium is substituted with KSOM medium (contains EDTA which is absent in HTF) and the growth of the blastocysts is observed. Several studies have compared the efficiency of the different mediums for mouse pre-implantation development but none have been tested in the context of post-implantation development (Mohd-Fazirul et al., 2015) (Perin et al., 2008).

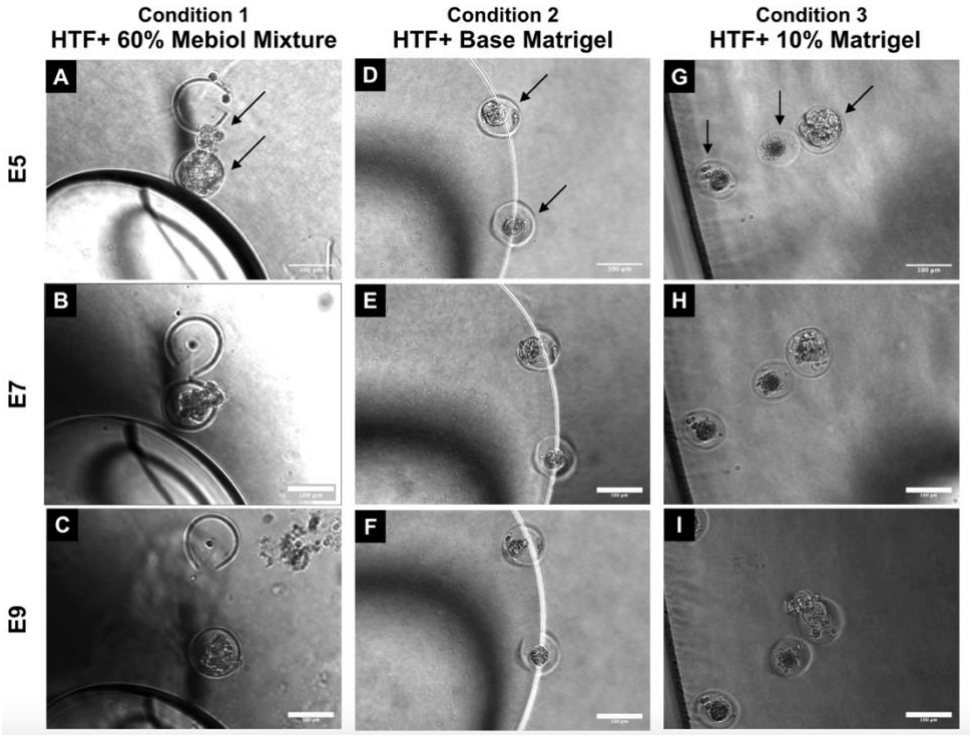

Figure 5: Mouse Embryos in HTF Medium + 3 Gels. (A-C) WT E3.5 mouse blastocysts were cultured in HTF medium + 60% Mebiol Mixture. (D-F) WT E3.5 mouse blastocysts were cultured in HTF medium + Base Matrigel with 10% Matrigel (Base layer of 100% Matrigel + 10% Matrigel in HTF Medium). (G-I) WT E3.5 mouse blastocysts are cultured in HTF medium + 10% Matrigel. In all three conditions, the blastocysts did not hatch out of the zona pellucida and undergo disintegration by E9. Scale bar in all figures= 100 µm.

2.2. 3D Blastocyst cultures in KSOM Medium + 3 different gels

Wild type mouse blastocysts at E3.5 were cultured in 3 different gels with KSOM medium as detailed in **Methods Section IV A 2.1-2.3**. A control condition with blastocysts cultured in just the KSOM medium was also included.

1) 60% Mebiol Mixture (100uL) with 10% Matrigel (200uL)

2) Base Matrigel (100uL) with 10% Matrigel (200uL)

3) 10% Matrigel (300uL)

4) Control Condition- KSOM medium (300uL)

The blastocysts in each condition were observed until E7-E7.5.

In the first gel condition, 60% Mebiol Mixture with 10% Matrigel, no growth of the blastocysts was observed. The blastocysts did not hatch out of their zona pellucida and disintegrated by E7 (Figure 6**,** A). In the second gel condition, Base Matrigel with 10% Matrigel, the blastocysts did not hatch out of their zona

pellucida and showed limited growth. Unlike the first gel condition, the cells in the blastocysts looked healthier and intact at E7 (Figure 6**,** B-D) but disintegrated by E10 (Figure 6**,** E-F). Further, for the same gel composition of Base Matrigel with 10% Matrigel condition, the blastocysts are healthier when cultured in KSOM medium (Figure 6, B-D) when compared to the HTF medium (Figure 5, D-F).

In the third condition, where the gel is composed of 10% Matrigel with KSOM medium, healthy blastocysts successfully hatched out of their zona pellucida on E5.5 (Figure 6, G-I) and grew in size till E6.5 (Figure 6, J-L). However, by E7.5, most of the blastocysts did not continue to grow but began to disintegrate (Figure 6, M-O). The cells from the blastocysts' outgrowth appeared to undergo differentiation at E7.5 (increase in the processes extending from the cells is observed) (Figure 6, N-O), but further staining experiments would be necessary to confirm this. The blastocysts cultured in KSOM medium + 10% Matrigel were healthier, hatched out of their zona pellucida, and grew in size until E6.5 (Figure 6, G-L), unlike the blastocysts cultured in the HTF medium + 10% Matrigel where they disintegrated (Figure 5, G-I). The blastocysts cultured in only the KSOM medium remained intact up to E7.5 but no growth was observed and they appeared to have entered diapause (Figure 6, P-R) as observed in previous studies (Reijo Pera & Prezzoto, 2016).

Conclusions: Between the KSOM medium (Figure 6) and the HTF medium tested in the previous section (Figure 5), KSOM is more conducive to maintenance and growth of the blastocysts in the different 3D gel conditions. Blastocysts cultured in only the KSOM medium without gel entered diapause (Figure 6, P-R). For the same medium (KSOM), when comparing the 3 gel conditions- 60% Mebiol Mixture with 10% Matrigel, Base Matrigel with 10% Matrigel, and 10% Matrigel, the third condition with 10% Matrigel was the most favorable to the growth of the blastocysts. In the 10% Matrigel + KSOM medium combination, healthy blastocysts successfully hatched out of their zona pellucida and continued to grow in size until E6.5 (Figure 6, G-O). However, after E6.5, the blastocysts began to disintegrate. It is possible that while the 10% Matrigel is suitable for the blastocysts to hatch out of their zona pellucida, the gel might not be strong enough to support the growing blastocysts and hence leads to their

disintegration by E7.5. Therefore, a higher concentration of 3D gel might be required to provide increased support to the rapidly growing embryos. In the subsequent experiments, higher concentrations of Matrigel- 20%, 30% and 40% Matrigel with KSOM medium were tested for their ability to support the growing blastocysts.

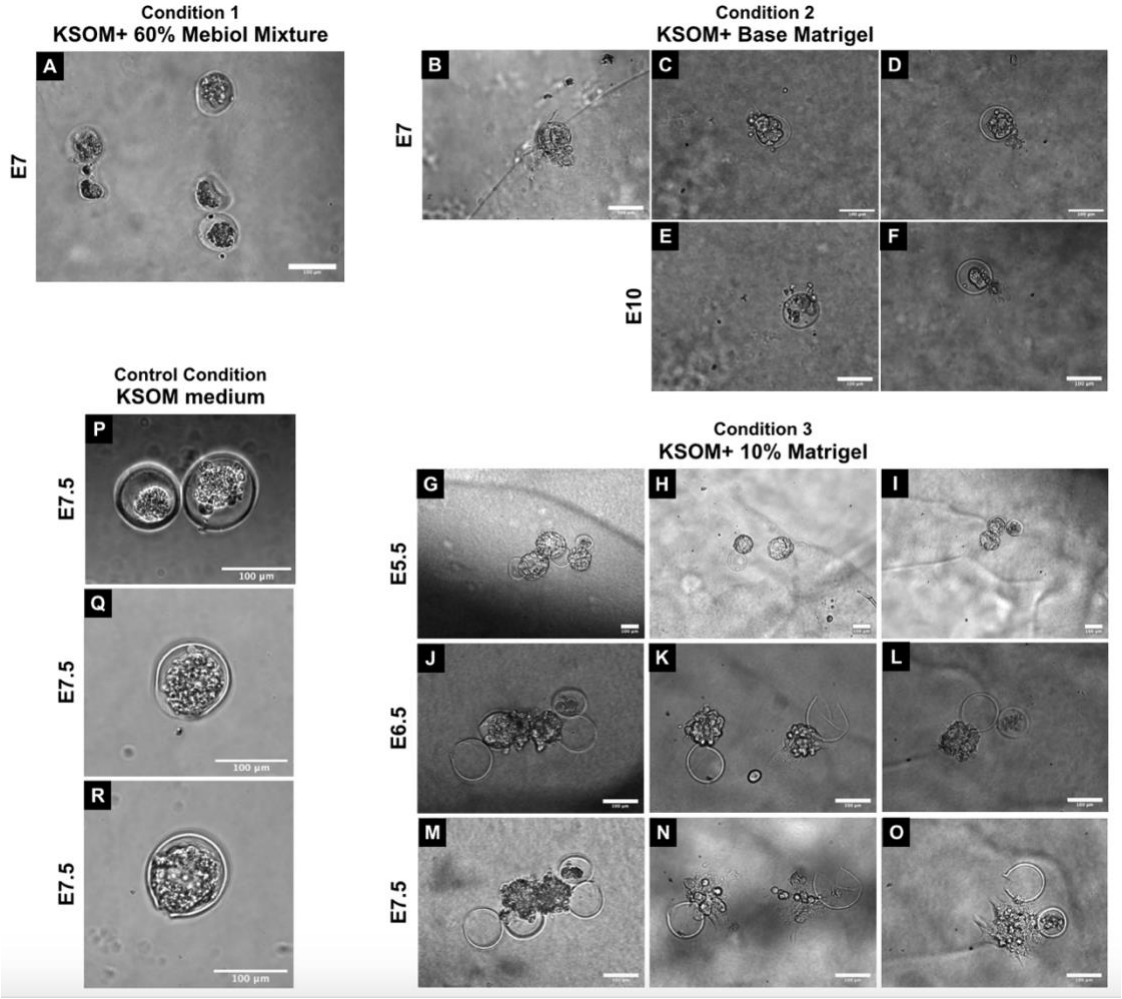

Figure 6: Mouse Embryos in KSOM Medium + 3 Gels. (A) WT E3.5 mouse blastocysts were cultured in KSOM medium + 60% Mebiol Mixture. The blastocysts underwent disintegration by E7. (B-F) WT E3.5 mouse blastocysts were cultured in KSOM medium + Base Matrigel with 10% Matrigel. The blastocysts were limited in growth and did not hatch out of their zona pellucida. (G-0) WT E3.5 mouse blastocysts were cultured in KSOM medium + 10% Matrigel. The blastocysts hatched out of the zona pellucida by E5.5, grew in size till E6.5 after which they disintegrated by E7.5. (P-R) WT E3.5 mouse blastocysts were cultured in KSOM medium. The blastocysts entered diapause and did not continue to grow. Scale bar in all figures= 100 µm.

2.3. 3D Blastocyst cultures in KSOM Medium + 20%, 30% and 40% Matrigel

Wild type mouse blastocysts at E3.5 were cultured in 3 different concentrations of Matrigel with KSOM medium as detailed in **Methods Section IV A 2.3** –

1) 20% Matrigel (300uL)
2) 30% Matrigel (300uL)
3) 40% Matrigel (300uL)

In each condition, the blastocysts were observed until E9.

In the 20% Matrigel condition, healthy blastocysts were able to grow in size until E7 (Figure 7, D-F). However, after E6, their growth was limited and most of the blastocysts disintegrated by E9 (Figure 7, G-I), similar to the 10% Matrigel condition (Figure 6, G-O). In the 30% Matrigel condition, healthy blastocysts hatched out of their zona pellucida around E5.5 (Figure 7, J-M) and continued to grow in size until E6.5 (Figure 7, N-P). The blastocysts in the 30% Matrigel condition were healthier and formed larger structures compared to most of the blastocysts cultured in the 20% Matrigel. However, they did not continue to grow in size beyond E6.5 (Figure 7, Q-T). In the 40% Matrigel condition, blastocysts hatched out of their zona pellucida around E5 (Figure 8, A-C). These blastocysts grew in size until E7 and formed egg-cylinder like structures (Figure 8, D-I), similar to *in vivo* implanting mouse embryos. By E8, the growth of the embryos declined, they started to shrink in size, and began to disintegrate by E9 (Figure 8, J-O). Hence, the 40% Matrigel + KSOM condition is able to support the growth of the blastocysts until E7 when they form the long egg-cylinder like structures, similar to the *in vivo* implanting mouse embryos at the same developmental time.

Conclusions- The 20% Matrigel condition allowed the blastocysts to hatch out of their zona pellucida and grow until E6 after which the blastocysts disintegrated, similar to the 10% Matrigel condition (Figure 6, G-O). In a higher concentration of 30% Matrigel, the blastocysts were healthier and grew to a bigger size until E6.5 when compared to the blastocysts in the 20% Matrigel (Figure 7). The 40% Matrigel condition was the optimal condition for allowing blastocysts to hatch out of their zona pellucida, grow in size, and develop the egg-cylinder morphology (Figure 8) observed in *in vivo* implanting mouse embryos. These embryos could be maintained in culture until E7.5 after which they began to shrink in size and disintegrate. It is possible that the 40% Matrigel cannot support the rapidly

growing mouse embryos beyond E7 and a higher concentration of Matrigel might be needed for higher support. An optimal gel density is required that is dense enough to support the growing blastocysts but not too dense to prevent the flow of medium and nutrients into the blastocysts. The subsequent experiments will compare the 40% Matrigel with a higher concentration of 50% Matrigel in KSOM.

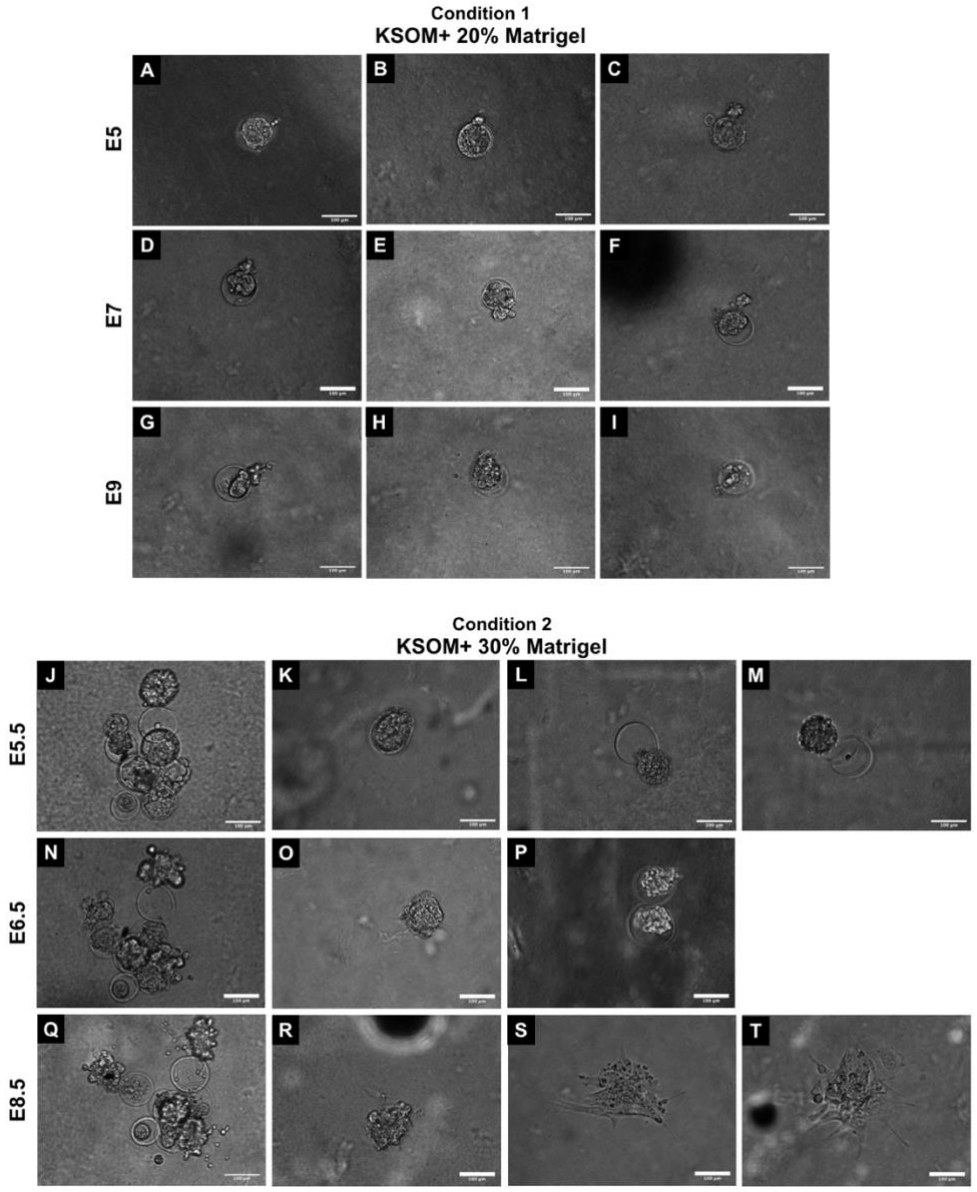

Figure 7: Mouse Embryos in 20% and 30% Matrigel (KSOM medium). (A-I) WT E3.5 mouse blastocysts were cultured in KSOM medium + 20% Matrigel. The blastocysts were able to grow in size up to E7 but disintegrated by E9. (J-T) WT E3.5 mouse blastocysts were cultured in KSOM medium + 30% Matrigel. The blastocysts hatched out of the zona pellucida by E5.5, grew in size till E6.5 after which they disintegrated by E8.5. Scale bar in all figures= 100 µm.

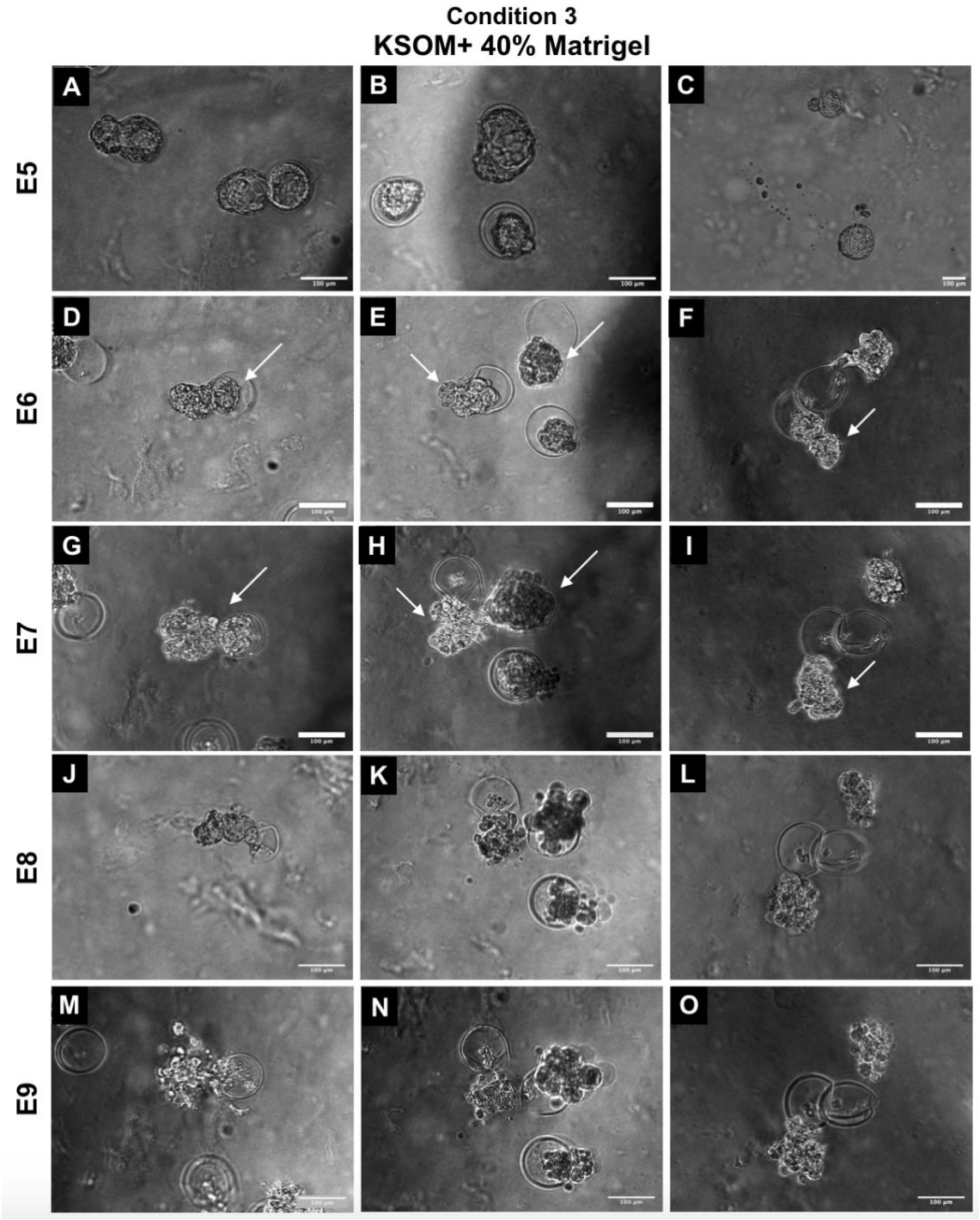

Figure 8: Mouse Embryos in 40% Matrigel (KSOM medium). (A-O) WT E3.5 mouse blastocysts were cultured in KSOM medium + 40% Matrigel. The blastocysts hatched out of their zona pellucida, grew in size till E7, and formed the egg-cylinder shaped morphology similar to the *in vivo* implanting mouse embryos. By E8, the blastocysts began to disintegrate. Scale bar in all figures= 100 µm.

2.4. 3D Blastocyst cultures in KSOM Medium + 40% and 50% Matrigel

Wild type mouse blastocysts at E3.5 were cultured in 2 different concentrations of Matrigel with KSOM medium as detailed in **Methods Section IV A 2.3** –

1) 50% Matrigel (300uL)
2) Control condition- 40% Matrigel (300uL)

The blastocysts were observed until E6.5-E7.5. In the 50% Matrigel condition, most of the blastocysts grew in size up to E5.5. However, the blastocysts seemed to be restricted in their capacity to hatch out of the zona pellucida and by E6.5, most of the blastocysts began to disintegrate (Figure 9, A-I). In the 40% Matrigel condition, the embryos continued to grow in size and form egg-cylinder like morphology structures (Figure 9, J-R) as observed in the previous experiment (Figure 8).

Conclusions- On increasing the concentration of the gel to 50% Matrigel, the blastocysts grew in size till E5.5. However, they did not form the egg-cylinder shaped morphology observed in the 40% Matrigel condition, had limited growth, and began to disintegrate by E6.5.

Optimal 3D *Ex-Vivo* system for WT mouse embryos- From the different combinations of medium and gels tested in this study, the **40% Matrigel + KSOM medium** combination was the optimal 3D *ex-vivo* culture condition for WT mouse embryos. The efficiency of this optimum condition to give rise to egg-cylinder shaped structures from healthy E3.5 blastocysts was ~70% (67.06% ± 2%, refer Appendix for details). This condition enabled E3.5 mouse embryos to grow in size up to E7 and form the egg-cylinder shaped morphology similar to *in vivo* implanting mouse embryos. The length of *in vivo* developing E6.5 embryos was around 150µm (Morris et al., 2012) and the *ex-vivo* 40% Matrigel cultured mouse embryos seemed to be of a similar length based on the bright field images. However, since the embryos are embedded in 3D gels, exact length measurements could not be performed based on the 2D bright field images. The subsequent experiments will test the capacity of the optimal *ex-vivo* culture condition- 40% Matrigel + KSOM medium, to support the growth of hematopoietically-expressed homeobox protein (Hhex) gene knockout (KO) mouse embryos that have been developed in the lab. The ultimate goal is to enable blastocyst complemented embryos,

such as, Hhex KO mouse embryos complemented with human stem cells, to be studied closely in this *ex-vivo* system until the implantation stage.

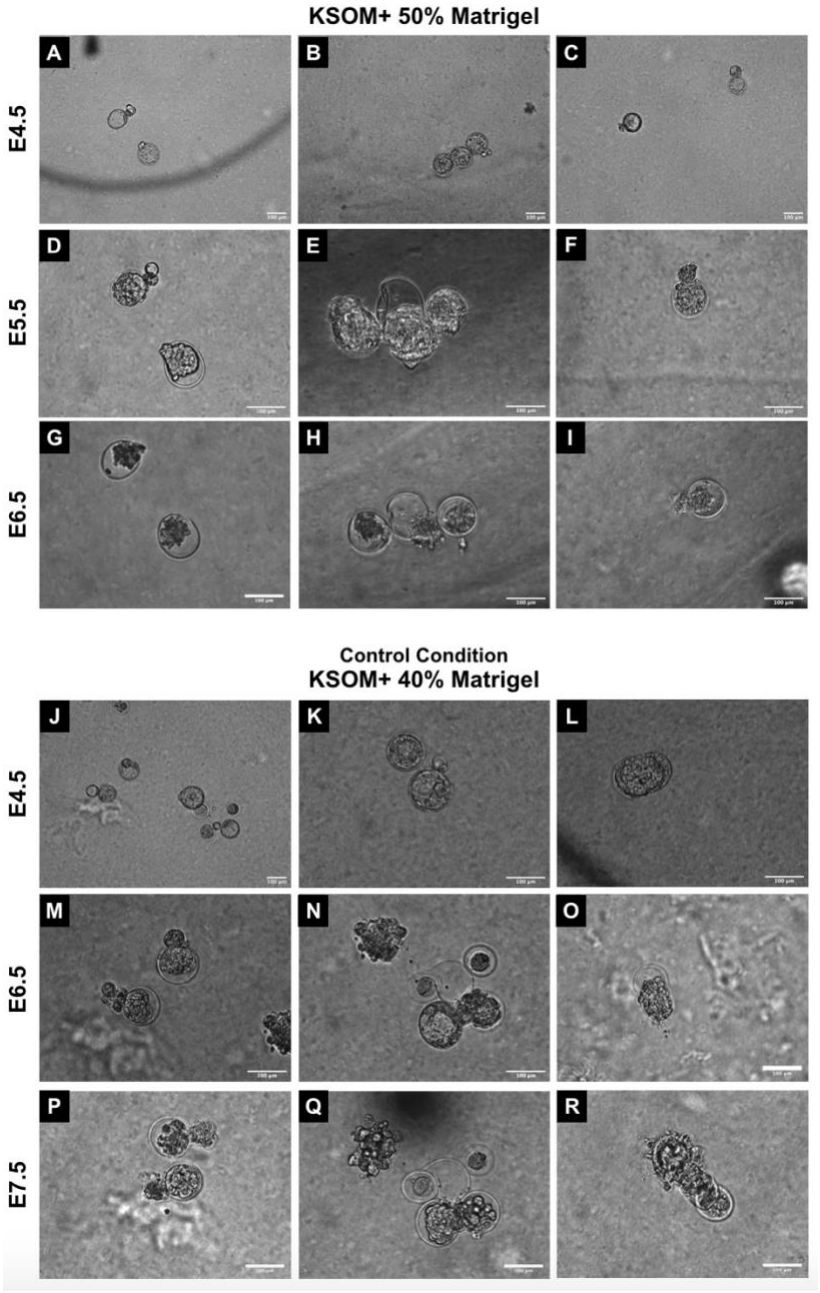

Figure 9: Mouse Embryo in 50% and 40% Matrigel (KSOM medium). (A-I) WT E3.5 mouse blastocysts were cultured in KSOM medium + 50% Matrigel. The blastocysts hatched out of their zona pellucida, grew in size till E5.5 after which they began to disintegrate. (J-R) WT E3.5 mouse blastocysts were cultured in KSOM medium + 40% Matrigel. The blastocysts hatched out of their zona pellucida, grew in size till E7, and formed the egg-cylinder shaped morphology similar to the *in vivo* implanting mouse embryos. By E8, the blastocysts began to disintegrate. Scale bar in all figures= 100 µm.

3. **Hhex KO Blastocysts cultured in the 40% Matrigel + KSOM medium**
   Hematopoietically-expressed homeobox protein (Hhex) gene KO mouse embryos generated in the Low lab for blastocyst complementation studies were used in this experiment. Among other gene regulatory functions, Hhex acts as a crucial transcription factor in the development of liver, forebrain and thyroid. The Hhex gene was knocked out using electroporation of relevant CRISPR-Cas9 sgRNAs at the zygote stage. The mouse embryos were then cultured in appropriate medium up to the blastocyst stage and the Hhex KO was verified by genotyping the embryos sampled after electroporation. Figure 10 depicts a genotyping experiment to detect WT (+/+), KO (-/-) and heterozygous KO (+/-) blastocysts after electroporation. Electroporated E3.5 mouse blastocysts were cultured in 40% Matrigel (300uL) with KSOM medium as detailed in **Methods Section IV A 2.3**. The blastocysts were observed until E7.5. Similar to the WT blastocysts, the Hhex KO blastocysts hatched out of their zona pellucida and continued to grow up to E7.5 forming the egg-cylinder shaped morphology also observed in *in vivo* implanting mouse embryos (Figure 11). After E7.5, no further growth was observed, and the blastocysts underwent disintegration.

Conclusions- The electroporated mouse embryos were able to grow in size and give rise to the egg-cylinder shaped morphology observed in *in vivo* mouse implanting embryos (Figure 11). The **optimal *ex-vivo* culture condition of 40% Matrigel + KSOM medium** was able to support the growth of both WT and electroporated mouse embryos. Since the efficiency of obtaining complete Hhex gene KO following electroporation is as high as 70% (as previously calculated from multiple experiments), it can be assumed that most of the blastocysts cultured in this system are Hhex complete KO or heterozygous KO embryos. However, in the future, genotyping of the E7 electroporated mouse embryos post-culture will be necessary to verify complete Hhex KO. Future experiments would also involve complementing the Hhex KO embryos by injection of human stem cells and observing the contribution of the human grafted cells to the Hhex developmental niche of the mouse embryos.

In the subsequent experiments, WT E4 free blastocysts and WT E7 Matrigel-embedded mouse embryos will be stained with protein specific-fluorescent dyes to observe the distribution of the cells inside the structures and the expression of key transcription factors.

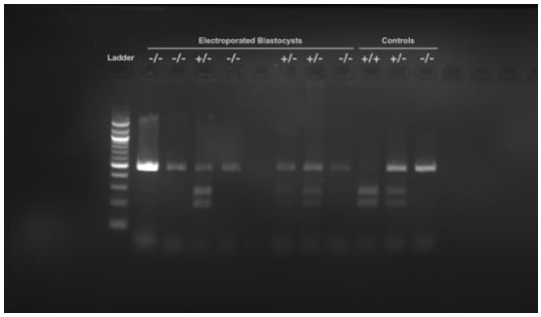

Figure 10: Genotyping of Electroporated E3.5 Blastocysts. WT E1 zygotes were electroporated with relevant CRISPR-Cas9 sgRNA system to target the Hhex gene for complete knockout (KO). The electroporated blastocysts were cultured in HTF medium until E3.5 after which genotyping was performed to determine the extent of Hhex KO. The controls included WT Hhex DNA (+/+), heterozygous Hhex KO DNA (+/-), and homozygous Hhex KO DNA (-/-).

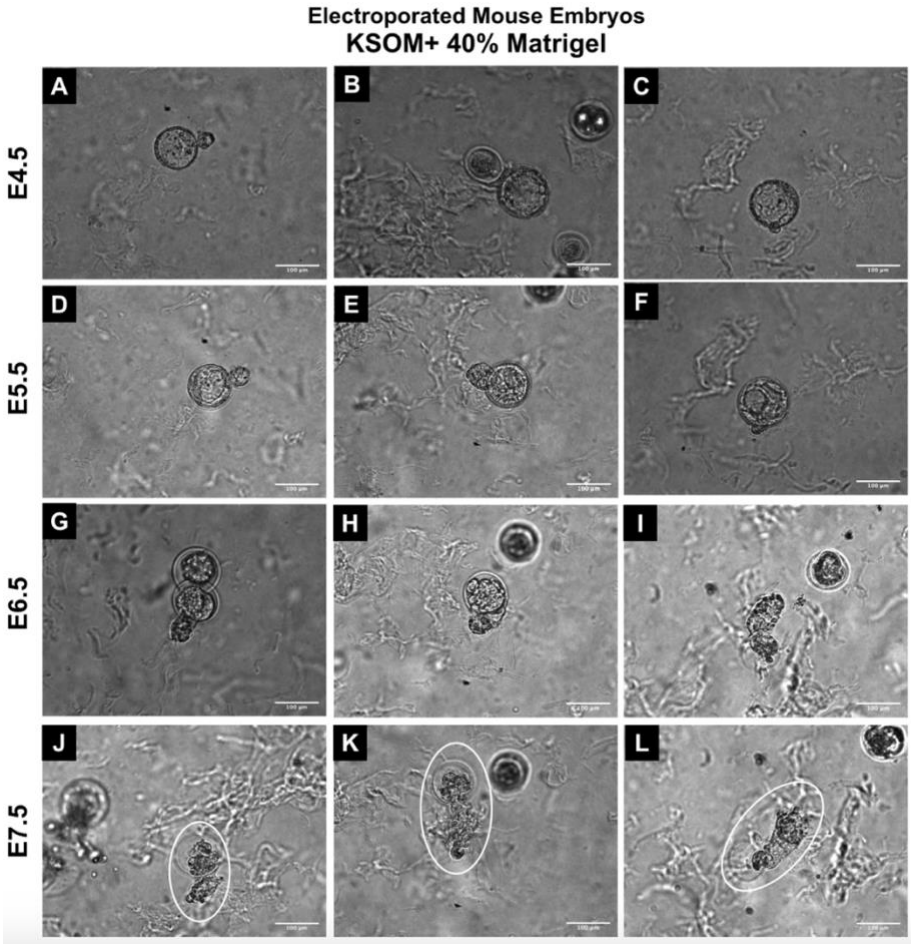

Figure 11: Electroporated Mouse Embryos in 40% Matrigel (KSOM Medium) WT E3.5 mouse blastocysts were cultured in KSOM medium + 40% Matrigel. The blastocysts hatched out of their zona pellucida, grew in size till E7, and formed the egg-cylinder shaped morphology similar to the *in vivo* implanting mouse embryos. By E8, the blastocysts began to disintegrate. Scale bar in all figures= 100 µm.

4. **Immunofluorescence Staining of WT Mouse Embryos**

After optimization of the *ex-vivo* 3D culture system, the next goal was to delineate the cell lineages in the gel embedded E7 WT mouse embryos. The primary and secondary antibodies used for this purpose are listed in Table 6, Table 7. Before staining the *ex-vivo* cultured embryos, the specificity of the antibodies was first tested in E4 mouse pre-implantation WT blastocysts that have been extracted from the mouse uterus at the zygote stage and cultured in HTF medium up to the blastocyst stage.

4.1 Immunofluorescence staining of the E4 WT mouse pre-implantation blastocysts

Wild-type E4 mouse blastocysts were stained for the key pluripotency transcription factors- Oct4, Sox2, and Nanog, the cell-cell adhesion molecule E-Cadherin, the trophectoderm (TE) specific factor Cdx2, and nuclear specific DAPI. These antibodies were chosen based on their availability in the lab and the blastocysts were stained as described in the **Methods Section IV A 3**. As seen in Figure 12, the E4 pre-implantation blastocysts expressed the key pluripotency factors Oct4, Sox2 and Nanog in the nuclei (co-localization with DAPI). A higher expression was observed in the inner cell mass (ICM) when compared to the TE of the blastocysts as expected (White et al., 2018). Expression of the pluripotency factors was not ICM-restricted and was observed in the TE as previously reported (Szczepańska et al., 2011). The E-Cadherin expression was observed at the cell-cell junctions in the blastocysts Figure 12, E-F) as expected. Non-specific expression of the TE factor Cdx2 was observed for several blastocysts which could be due to the poor quality of the antibody. Based on these results, the expression of the transcription factors Oct4, Sox2, Nanog, and the cell-cell adhesion molecule E-Cadherin were subsequently tested in the gel-embedded E7 mouse embryos cultured in the optimum *ex-vivo* condition of 40% Matrigel + KSOM medium.

4.2 Immunofluorescence staining of the WT *ex-vivo* cultured E7 Matrigel-embedded mouse embryos

Mouse embryos cultured in the optimal *ex-vivo* system- 40% Matrigel + KSOM medium were stained for expression of pluripotency factors Oct4, Sox2, Nanog, and the cell-cell adhesion molecule E-Cadherin as described in the **Methods Section IV**

A 3. The embryos were also stained for nuclear specific DAPI. From Figure 13, the morphology of the E7 mouse embryos and their nuclei could be observed. Since the structures were cultured and embedded in the 3D Matrigel, not all the cells depicted were in the same plane of imaging. The nuclei of the cells in the E7 mouse embryos were larger than the nuclei of the cells in the E4 pre-implantation blastocysts (Figure 12). Further, the morphology of the nuclei varied between cells in the same embryo and some nuclei had an elongated morphology and may represent mitotic cells (Figure 13). Highly compacted DNA could be clearly observed as the bright specks within each nucleus with the punctate staining pattern typical of mouse nuclei (Figure 13). From Figure 14, Oct-4 and Sox2 expression, possibly nuclear, could be observed. Co-localization of Oct-4 and Sox2 was observed (Figure 14, C-D). Very low expression of Nanog and non-specific expression of E-cadherin was observed for the embryos embedded in the 3D Matrigel (data not shown).

Conclusions from the staining experiments- The E4 mouse pre-implantation blastocysts expressed the pluripotency factors Oct-4, Sox-2, and Nanog as well as the cell-cell adhesion molecule E-Cadherin. After culturing the mouse blastocysts in the optimal *ex-vivo* condition with 40% Matrigel + KSOM medium, the embryos were stained at E7. At E7, the mouse embryos expressed the pluripotency factors Oct-4 and Sox2 and they seemed to co-localize (Figure 14, C-D). Expression of Nanog and E-cadherin across multiple samples were very low and not observed. Downregulation of Nanog is observed in the *in vivo* developing mouse embryos after E4.5 (Chambers et al., 2003) and it is possible that the current staining protocol developed for the 3D Matrigel-embedded mouse embryos is not optimal for detecting the low levels of Nanog and the protocol would need to be optimized. Further, based on the current images, it is difficult to decipher the exact location of the pluripotency factors Oct-4 and Sox2, that is, if they were expressed in the nucleus or in the cytoplasm of the cells. The cells in the mouse embryo possess stem cell-like high nuclear to cytoplasmic ratio and the Oct-4 and Sox2 that we observe may be nuclear localized. This is supported by the empty spaces that could be observed between the nuclei in the confocal images (Figure 14, C-D) which may suggest the presence of cytoplasm around the enlarged nuclei (as observed in the brightfield image in Figure 14, A), devoid of DAPI, Oct-4 or Sox2 expression.

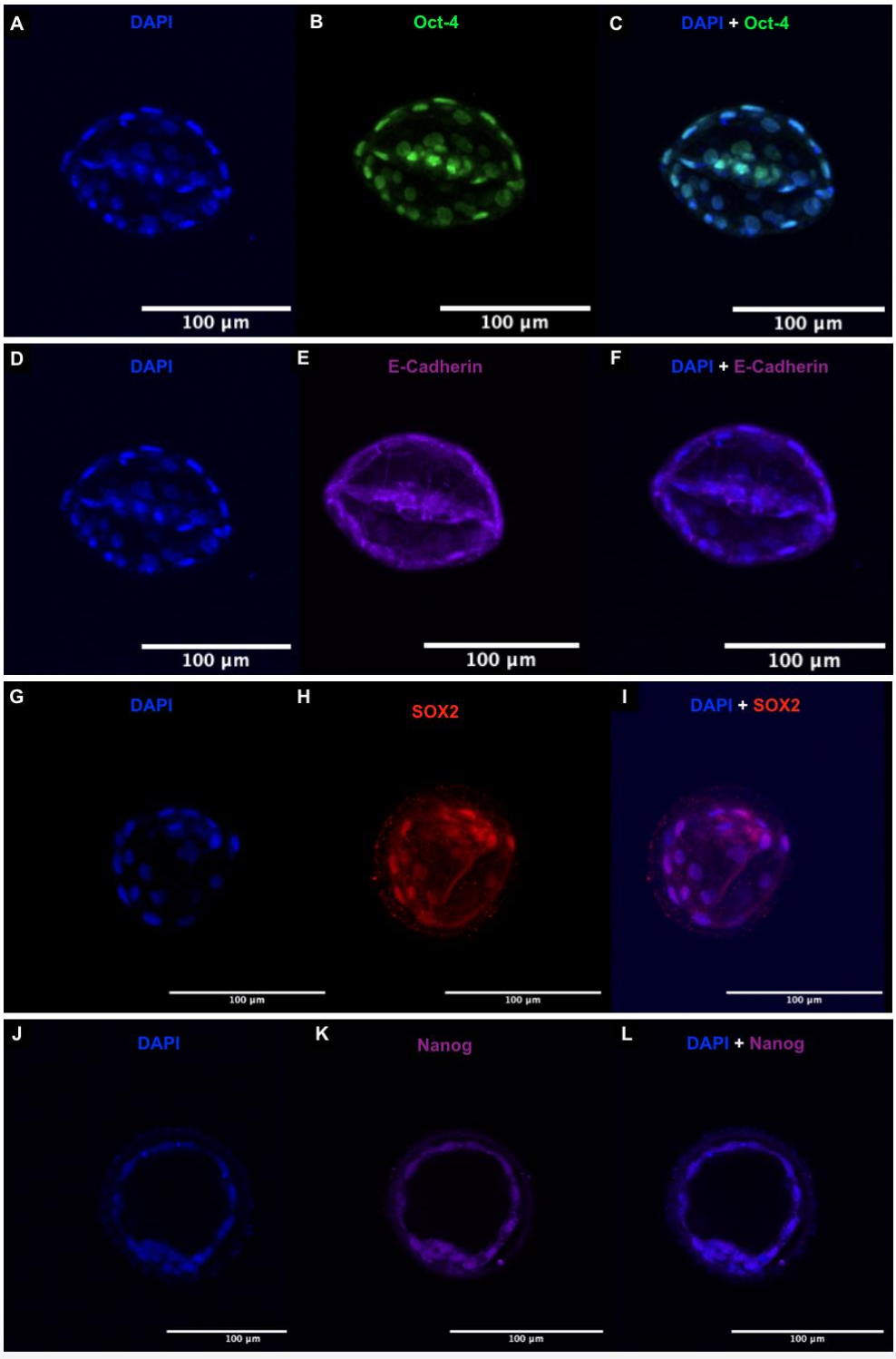

Figure 12: WT E4 Mouse Pre-Implantation Blastocysts. WT E4 mouse blastocysts cultured in HTF medium were stained for key pluripotency factors Oct-4, SOX2, and Nanog and the cell-cell adhesion molecule E-Cadherin. Nuclei specific expression of DAPI, Oct-4, SOX2 and Nanog were observed. E-Cadherin was expressed in the junctions between the cells of the blastocyst. Confocal microscopy was used for all images. Scale bar in all figures= 100 µm.

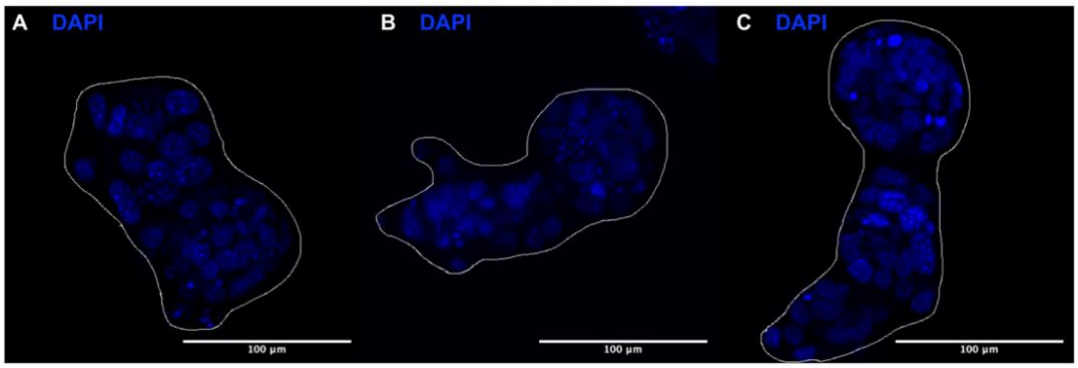

Figure 13: DAPI staining of WT E7 Mouse Embryos embedded in KSOM + 40% Matrigel. WT E7 Matrigel-embedded mouse embryos were stained with nuclear-specific DAPI. Highly compacted DNA within each nucleus could be observed as bright specks. The punctate staining pattern typical in mouse nuclei was also observed. Scale bar in all figures= 100 µm.

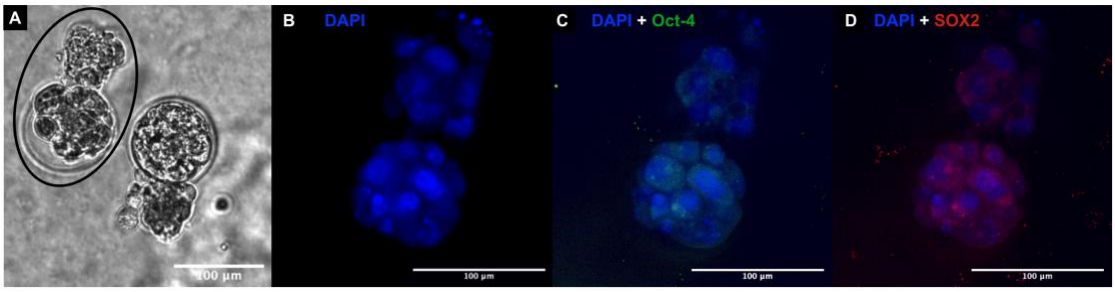

Figure 14: Immunofluorescence staining of WT E7 Mouse Embryos embedded in KSOM + 40% Matrigel. WT E7 Matrigel-embedded mouse embryos were stained for pluripotency factors Oct-4 and SOX2. (A) Bright field image of the structure stained in (B-D). (B) DAPI staining specific to nuclei. (C-D) Cell specific Oct-4 and SOX2 expression was observed. Confocal microscopy was used for B-D. Scale bar in all figures= 100 µm.

Many studies have also demonstrated that Oct-4 and Sox2 shuttle between the nucleus and the cytoplasm and both these proteins are regulated by active export between the nucleus and the cytoplasm in embryonic stem cells and early embryos (Nakamura et al., 2016) (Plachta et al., 2011) (Baltus et al., 2009). However, in order to draw conclusions about the signaling mechanisms in the E7 embryos of the *ex-vivo* system, expression of more relevant proteins would need to be tested and further optimized staining protocols would be necessary.

C. Discussion for *Ex Vivo* System to Study Mouse Early Embryo Development and Chimeric Embryos

In this section, different combinations of gel and medium were tested with the goal to develop an optimized *ex-vivo* system that can support the growth of E3.5 mouse blastocysts until the implantation stage. The optimal ex-vivo culture condition from this study is 40% Matrigel + KSOM medium. Mouse E3.5 blastocysts cultured in this *ex-vivo* system continued to grow in size until E7 and formed the egg-cylinder shaped morphology observed in *in vivo* implanting mouse epiblasts. The efficiency of developing egg-cylinder shaped embryos at E7 in this system was as high as ~ 70% and the system supported the growth of both WT and Hhex KO blastocysts. The 40% Matrigel is the optimum density to support the growing mouse embryos. An increase or decrease in the gel concentration led to early dissociation of the embryos. This demonstrates the importance of an optimum external matrix that is strong enough to mechanically support the rapidly growing mouse embryo while also allowing the free flow of nutrients into the embryos through the gel. There are multiple explanations for the disintegration of the embryos beyond E8. It is possible that the elongated E7 mouse embryos in the 40% Matrigel did not get sufficient supply of nutrients on the interior of the embryos leading to disintegration. It is also possible that the *ex-vivo* culture developed in this study is able to support the blastocysts only up to E7 after which the embryos need to be transferred *in utero* or into a more suitable culture that can support implantation-stage mouse embryos. Future studies will be focused towards further optimization of the *ex-vivo* culture conditions as well as characterization of the developing mouse embryos.

Several groups have focused efforts towards developing *ex-vivo* systems that enable the growth of pre-implantation embryos until the implanting or gastrulating stage. An *ex-vivo* system for culturing human embryos enables the growth of the embryos until the pre-gastrulation stage, E14- the time point beyond which culturing of human embryos outside the human host is currently not allowed due to ethical concerns (Xiang et al., 2020). Using a similar protocol, primate embryos have been cultured *ex-vivo* until the late gastrulating stage (E20) (Niu et al., 2019). In both these studies, sc-RNA sequencing analysis was used to confirm the similarities between the *ex-vivo* cultured and the *in vivo* developing human and primate early embryos. An *ex-*

vivo 3D culture for pig pre-implantation embryos allows development until the late blastocyst stage up to E9 (Laughlin et al., 2017). The current published *ex-vivo* system for mouse embryos gives rise to the egg-cylinder shaped embryos at E8 (Morris et al., 2012). In this system, the embryos were cultured in 2D on collagen-coated polyacrylamide gels. The E8 embryos expressed the key pluripotency markers, as well as the primitive streak protein Brachyury, and an anterior visceral endoderm protein Lefty1. The E8 embryos grown in the 2D *ex-vivo* system had a more flattened egg-cylinder morphology unlike the E7 Matrigel-embedded embryos developed in this study. Interestingly, in the 2D *ex-vivo* system, the blastocysts that were cultured at E3 completely disintegrated and lost their morphology around E6 before the cells came together and reformed the egg-cylinder morphology around E7.5-E8 (Herriges & Morrisey, 2014). The 3D Matrigel-embedded mouse embryos developed in this study demonstrated a continuous growth in size from E3.5 to E7. The embryos remained intact until E7 after which they began to disintegrate around E8. Based on the results from the 2D *ex-vivo* system (Morris et al., 2012), it would be interesting to see if the cells of the 3D Matrigel-embedded disintegrated embryos at E8 are able to regain the egg-cylinder morphology and continue growth beyond E8 if left in culture.

For immunofluorescence staining studies, preliminary experiments with WT E4 blastocysts were performed to confirm the specificity of the available antibodies. Subsequently, KSOM+ 40% Matrigel-embedded E7 mouse embryos were stained and expression of Oct-4 and Sox2 was observed. The nuclear DAPI stain enabled observation of the arrangement and morphology of the cells inside the egg-cylinder shaped E7 mouse embryos. The Matrigel-embedded E7 embryos also expressed the pluripotency transcription factors- Oct-4 and Sox2. In order to conclude that the embryos cultured in the optimized *ex-vivo* system are similar to the *in vivo* implanting mouse embryos, more studies need to be performed looking into the expression of other late-stage mouse embryo-specific proteins, such as, Gata6, Oct6, Sox17, and brachyury. Future studies would also involve performing time lapse experiments from the start of the culture at E3.5 to the end at E7. The morphological and transcriptomic data collected at the different timepoints can then be compared with the *in vivo* implanting mouse embryos at the same developmental time points. Ultimately, the goal would be to use this system to closely study and gain insight into

the factors that either limit or improve the efficiency of interspecies chimerism and blastocyst complementation. Studies involving the injection of human stem cells into the *ex-vivo* cultured mouse embryos at different developmental timepoints for precise stage-matching between the human grafted cells and the host mouse embryos need to be performed. The results obtained from *in silico* stage-matching experiments using existing sc-RNA sequencing datasets can be used to predict the optimum developmental time at which the grafted cells should be injected into the *ex-vivo* cultured host embryos. Translation of the results and methodology used in this system to more relevant animal host embryos such as pig will bring the field of interspecies chimerism and blastocyst complementation closer to the ultimate goal of developing transplantable human organs in host animals.

Section V- Single Cell RNA sequencing to Study the Transcriptome of Early Embryonic Development Across Species

The improvement in single-cell RNA sequencing (sc-RNA seq) technologies over the last decade has enabled high-throughput sequencing of developing embryos across multiple species including human, non-human primates, mouse, pig etc. These sc-RNA seq datasets have also been useful for analysis of species-specific differences in the expression of key genes involved in important developmental pathways. Analysis of the sc-RNA seq datasets has also enabled comparison of embryonic developmental stages and pluripotent stem cells across species and prediction of developmental stages that match across species. The stage-matching results predicted from these *in silico* studies can then be tested in *in vivo* and *in vitro* interspecies chimerism and blastocyst complementation studies. This combined approach will help save time and money and help progress the field of interspecies chimerism and blastocyst complementation towards the ultimate goal of developing transplantable human organs in animal hosts.

Multiple studies have compared the early embryonic development across species such as human and mouse (Niakan & Eggan, 2013), human, marmoset, and mouse (Boroviak et al., 2018), mouse, and pigs (whole genome sequencing) ((S. Cao et al., 2014). Multiple studies have also compared pluripotent stem cells (PSCs) of one species with the embryonic developmental stages of another host species. For example, hPSCs were compared with pig embryonic stages (Ramos-Ibeas et al., 2019), as well as cynomolgus monkey embryonic development stages (Niu et al., 2019). All of these studies have been significant in analyzing the changes in gene expression within each species and also the transcripts that are conserved and distinct between species. In this study, sc-RNA seq analysis of early embryonic development across four species relevant to interspecies chimerism studies, that is, human, marmoset, mouse and pig is performed. The sc-RNA seq datasets for each species is compiled together from previous studies. The aim of this study is to demonstrate the strength of sc-RNA seq analysis in predicting and matching developmental stages across species. The predicted results from the sc-RNA seq analysis were validated by comparison with previous *in vitro* and *in vivo* stage-matching experiments where successful interspecies chimerism was demonstrated.

A. Methods for Single Cell RNA sequencing to Study the Transcriptome of Early Embryonic Development Across Species

1. Compiling publicly available datasets of embryonic development across species

The sc-RNA sequencing datasets used for analysis in this study were compiled from multiple studies. The human datasets were compiled from three studies ((Yan et al., 2013), (Blakeley, Fogarty, Del Valle, et al., 2015), (Petropoulos et al., 2016)) with accession numbers- GSE36552, GSE66507 and E-MTAB-3929. The marmoset datasets were obtained from a single study (Boroviak et al., 2018) with accession number E-MTAB-7078. The mouse datasets were extracted from two studies ((Deng et al., 2014a), (Mohammed et al., 2017)) with accession numbers GSE45719 and GSE100597. The pig datasets were obtained from one study (Ramos-Ibeas et al., 2019) with accession number GSE112380.

The raw-count matrices are filtered for mitochondrial RNA contamination and normalized using negative binomial regression method that is implemented using the SCTransform wrapper in Seurat. The top 3000 most variable genes across all the datasets were identified using the SelectIntegrationFeatures function in Seurat. Anchors were identified from the top variable genes using the FindIntegrationAnchors function and the datasets for each species were integrated together using the IntegrateData function in Seurat.

2. Generation of PCA (Principal Component Analysis) plots and UMAPs (Uniform Manifold Approximation and Projection)

PCA and UMAPs are techniques of dimension reduction for visualizing datasets. PCA takes into account the maximum variance between the datasets for clustering the datasets. The principal components (PCs) were calculated and plotted using the RunPCA function in Seurat.

In a UMAP, similarities and differences between multiple classes of datasets can be visualized based on the distances between the datasets. Generation of a UMAP uses 2 main theorems and 2 main assumptions (McInnes et al., 2018). The details of the process are described below-

1. Theorem 1- By building a simplicial complex out of the cover of a topological space, it is possible to recover all of the important topology information of the space.
2. Theorem 2- It is possible to turn the datasets into fuzzy simplicial sets and join them together by taking a fuzzy union in which the weights of edges of the simplicial sets are combined together.
3. Assumption 1- All the datasets are uniformly distributed on the manifold. Each dataset is mapped to a Euclidean space on the manifold. To make sure that assumption 1 holds true, each dataset is mapped to the Euclidean space on different scales. A different notion of distance exists (more compact or further away) depending on the cluster the dataset belongs to. For each datapoint on the manifold, a topological cover is then applied that varies in size based on the actual distance scale, but these covers are at equal distances from each other on the manifold (McInnes et al., 2018).
4. Assumption 2- The manifest is locally connected.
5. For the topological covers in the manifold, the algebraic theorem 1 is applied and a simplicial complex is built from which all of the topological information can be recovered. Each point on the manifold has a different weight.
6. From the manifold, a low dimension layout is optimized using a force directed graph layout formula that optimizes the clusters of datasets as well as the gaps between the clusters. Hence, UMAP has a good understanding of not only the similar datasets but also the different classes of datasets in the global space.

The weighted k-means clustering algorithm works on steps 1 through 5 and this is followed by optimization of the low dimensional representation of the datasets in a UMAP. In this study, UMAPs were developed using the UMAP algorithm in the RunUMAP function in Seurat.

3. Clustering of genes into 20 clusters based on similarities in gene expression

The top 20 PCs were embedded into two dimensions in the UMAPs. The Shared Nearest Neighbor (SNN) graph was created for the top 20 PCs using the FindNeighbors function in Seurat with the resolution parameter set at 0.7.

4. **Generation of heatmaps for interspecies and intraspecies datasets**

   The datasets were filtered and the genes with read counts below the minimum threshold set by the filterByExpr function in edgeR were filtered. The datasets were then normalized using TMM (Trimmed Mean of M-values) normalization technique which was implemented using the calcNormFactors function in the edgeR package. The heatmaps with hierarchical clustering were generated using the average common value of each species in the pheatmap package in R.

B. Results for Single Cell RNA sequencing to Study the Transcriptome of Early Embryonic Development Across Species

The overall *in silico* analysis approach for this study is summarized in Figure 15. Existing single cell RNA sequencing (sc-RNA seq) datasets for early embryonic development were compiled from multiple studies. The species selected for this study are relevant to interspecies chimerism and blastocyst complementation studies. Sc-RNA seq datasets for human (Yan et al., 2013) (Blakeley, Fogarty, Del Valle, et al., 2015) (Petropoulos et al., 2016), marmoset (Boroviak et al., 2018), mouse (Deng et al., 2014b) (Mohammed et al., 2017), and pig (Ramos-Ibeas et al., 2019) were extracted. The embryonic stages analyzed in each species are listed in Figure 15. In some species, multiple datasets representing a similar stage of development was present. The stages for all the species were selected based on the availability of sc-RNA datasets. First, the early embryonic development datasets across all the species were visualized using PCA plots and UMAPs. Clustering of datasets across the species based on similarities in gene expression was then performed and similar developmental stages between all four stages were predicted based on the clustering results. Subsequently, heatmaps were generated to observe the patterns of gene expression across species and across developmental stages within each species. Lastly, sc-RNA seq data of human pluripotent stem cells (PSCs) were clustered and compared with mouse embryonic stages to predict the optimum stages for introduction of the human cells into the mouse host embryos for interspecies chimerism and blastocyst complementation studies.

1. Interspecies comparisons of gene expression- between species and across all embryonic stages

The early embryonic sc-RNA seq datasets for the species were obtained and processed as detailed in **Methods Section V A 1**.

1.1 Visualization of embryonic stages in each species- PCA plots and UMAPs

For each of the species, PCA plots and UMAPs were generated across all the embryonic developmental stages as detailed in **Methods Section V A 2-3**. The PCA plots for human, marmosets and mouse formed the typical U shape

observed when sequential embryonic developmental stages are plotted (Figure 16).

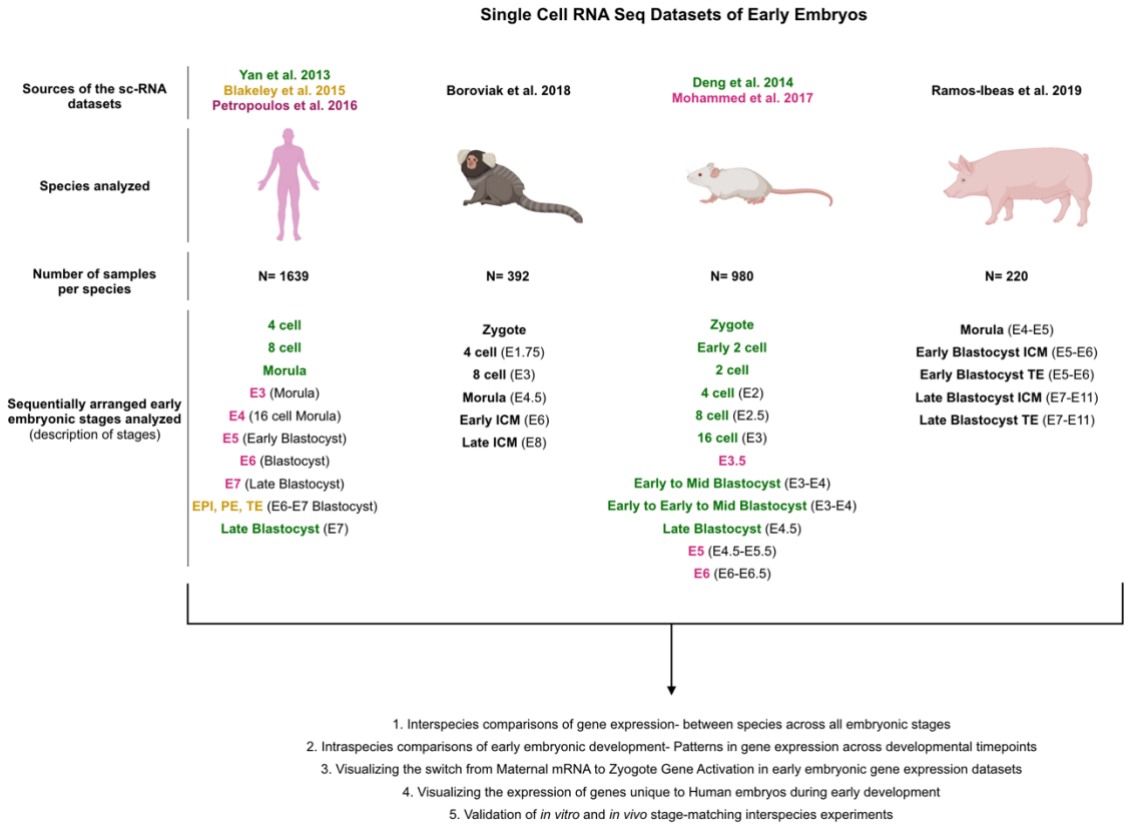

***Figure 15: Summary of the sc-RNA sequence analysis performed**. Sc-RNA seq datasets for four species- human, marmoset, mouse and pig were extracted from publicly available databases. For each species, the stages analyzed are indicated. (Created with Biorender.com)*

In case of the pig datasets, this pattern was not observed due to availability of data only from the morula to the late blastocyst stages. The sc-RNA seq data for the earlier stages from zygote to 4-cell stage could not be found from the publicly available datasets. The UMAP for each species with the embryonic stages is depicted in Figure 17. The embryonic stages similar to each other clustered closely together and the general trend of cell state transitions between the different embryonic stages could be observed. For example, in the UMAP for the human species, the gradual transition from E3 to E4, E5, E6 and E7, as depicted by the direction of the arrow in Figure 17 A could be observed. A similar trend was seen in other species (Figure 17 B, C, D).

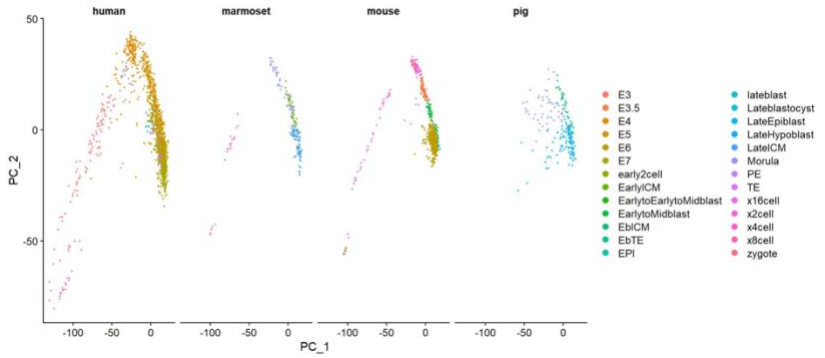

Figure 16: PCA plots for human, marmoset, mouse and pig early embryonic datasets. *PCA plots for human, marmoset and mouse have the typical U shape observed while plotting early embryonic developmental time points. Zygote to 4-cell stages were missing for the pig species which explains the lack of the U pattern in the pig PCA plot.*

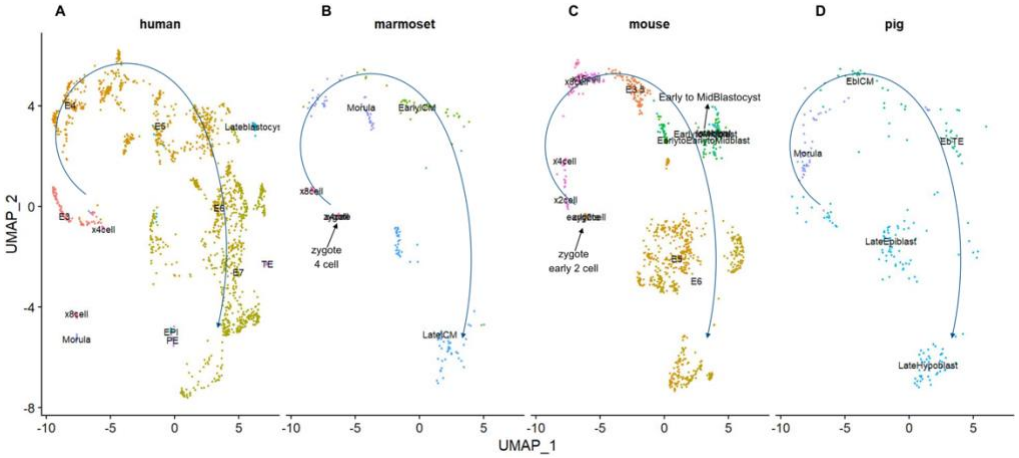

Figure 17: UMAPs were developed for human, marmoset, mouse and pig species. *A clear transition could be observed from the earlier to the latter stages of development in each species (direction of development is indicated by the arrow).*

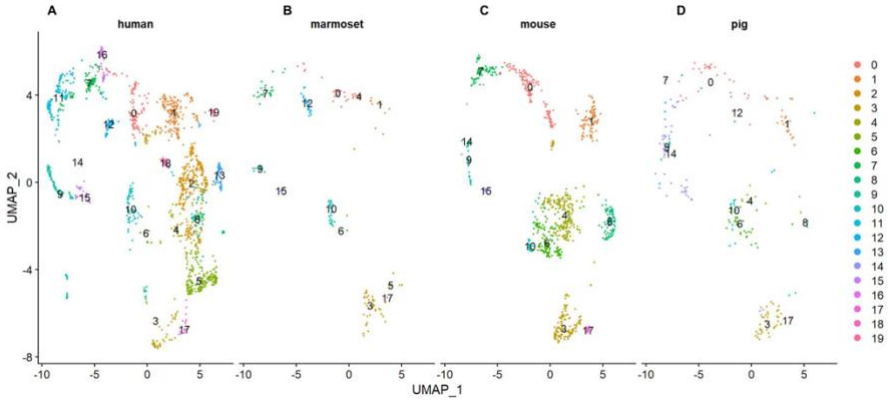

Figure 18: UMAPs were grouped into 20 clusters based on similarities in gene expression across the four species. *The clusters were numbered 0-19.*

Conclusions- A similar trend in embryonic development was conserved across the human, marmoset, mouse and pig species as observed from the PCA and UMAP plots. The UMAPs enabled visualization of cell state transitions between the embryonic stages based on gene expression.

1.2 Clustering of datasets across species based on gene expression

In order to compare the similarities in gene expression during embryonic development across all the species, the datasets for all developmental stages in each species was divided into 20 clusters as detailed in **Methods Section V 4**. The 20 clusters are depicted in the UMAP for each species in Figure 18. Each cluster contained datasets based on similarities in gene expression. For examples, cluster 1 in humans contained datasets with similar gene expression to cluster 1 in marmoset, mouse and pigs. For each cluster, the embryonic stages represented by each species were analyzed. The clusters are numbered from 0-19 as shown in Figure 18.

1.2.1 Gene expression unique to human embryos was observed from clustering analysis

From the 20 clusters, it was observed that clusters 2, 11, 13, 16, 18 and 19 contained datasets specific to only the human developing embryo as seen in Figure 19. The other species analyzed, that is, marmoset, mouse and pig were not present in these clusters. Further, it could be observed that the stages represented in these clusters are E4 (compacted 16 cell morula) (Figure 19 B), E5 (early blastocyst) (Figure 19 D), E6 (mid blastocyst) (Figure 19 A, E), E7 (late blastocyst) (Figure 19 A, C), and late blastocyst (Figure 19 F). Most of these stages corresponded to the human early to mid to late blastocyst stages. Hence, these unique subsets of human-specific genes were upregulated during the blastocyst stages of embryonic development.

Conclusions- Using the clustering analysis method, it was possible to isolate datasets containing subsets of genes uniquely upregulated in embryos of a particular species, in this case, humans. Future studies

would involve using bioinformatics techniques like Gene Ontology and KEGG pathway analysis to analyze each of the genes upregulated, their functions in the cells, and the various signaling pathways that they contribute to.

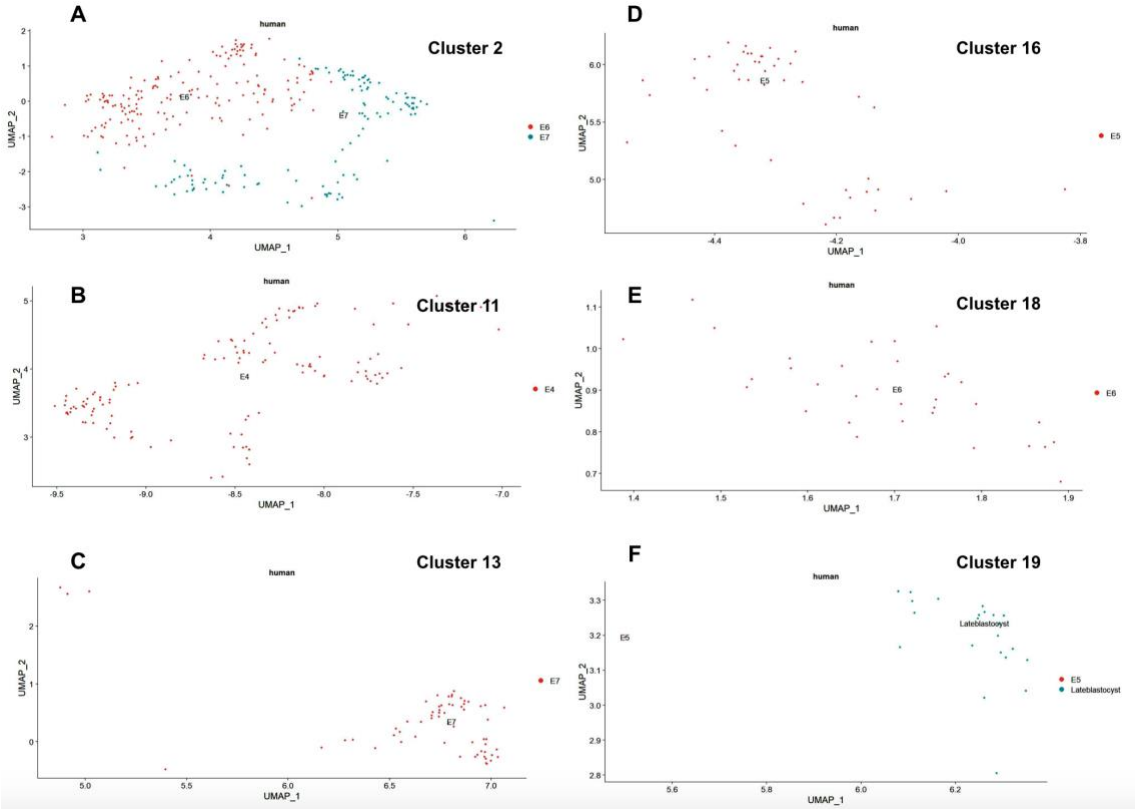

Figure 19: Gene clusters unique to human embryos. *Out of the 20 clusters across the four species, clusters 2, 11, 13, 16, 18 and 19 contained datasets with genes uniquely upregulated in early human embryos. From the clusters, it could be observed that the human E5, E6, E7 and Late blastocyst stages (early to mid to late blastocyst stages) demonstrated upregulation of these human-specific genes.*

1.2.2 Prediction of *in vitro* and *in vivo* validated stage-matching chimeric studies from clustering analysis

Multiple studies to date have demonstrated the importance of stage-matching in interspecies chimerism (Cohen et al., 2018). Matching the graft human PSCs with the right stage of the host animal embryo can help overcome the interspecies barriers and give rise to successful interspecies chimerism. In human-mouse chimeras, *in vitro* and *in vivo*

studies have demonstrated that human PSCs (hiPSCs and hESCs) injected into gastrulating mouse embryos (E6 to E7) successfully integrate into the embryos and differentiate into tissue-specific lineages (Mascetti & Pedersen, 2016a) (Cohen et al., 2016). The human ESCs used in these chimeric studies were extracted from human blastocysts (E6-E7) and are transcriptionally similar to human blastocyst stages of development. Further, sc-RNA seq experiments have demonstrated significant similarities between the hiPSCs and hESCs (Narsinh et al., 2011).

Here, the goal is to predict the stage-matching results from the existing *in vitro* and *in vivo* chimeric studies using clustering analysis. Clusters containing gastrulating mouse embryo stage E6 (E6-E6.5) datasets were extracted. The human embryonic stages that are present in these clusters and hence similar to the E6 mouse stages were then noted. According to the results from the previous successful *in vitro* and *in vivo* stage-matching chimerism studies, the mouse E6 datasets should closely resemble the human E6-E7 blastocyst stages (mid to late blastocyst) from which hESCs are extracted.

From the clusters containing the mouse E6 datasets, that is, clusters 1, 4, 8, and 17, it could be observed that human datasets specific to E6 (Figure 20, A-D), E7 (Figure 20, B-D), and Late blastocyst (E7) (Figure 20, A), were largely present. Human datasets specific to EPI, TE and PE, which represent the epiblast, trophectoderm and primitive endoderm of E6-E7 blastocysts were also present in the clusters (Figure 20, A, C). These results are in agreement with the results from the *in vitro* and *in vivo* stage-matching studies. Interestingly, the marmoset Late ICM (Figure 20, A, B, D), and the pig Late epiblast (Figure 20, B-C), and Late Hypoblast (Figure 20, A, D), were also present in these clusters which demonstrates similarities between these datasets and the human E6-E7 blastocysts. Cluster 10 (Figure 20, E) further highlights the similarities between the human E6, E7 and Late Blastocyst stages with the marmoset Late ICM and the pig Late Epiblast stage. The mouse embryonic stage in cluster 10

is E5, which represents the stage of development right before the onset of gastrulation. In previous studies, the marmoset late ICM has been shown

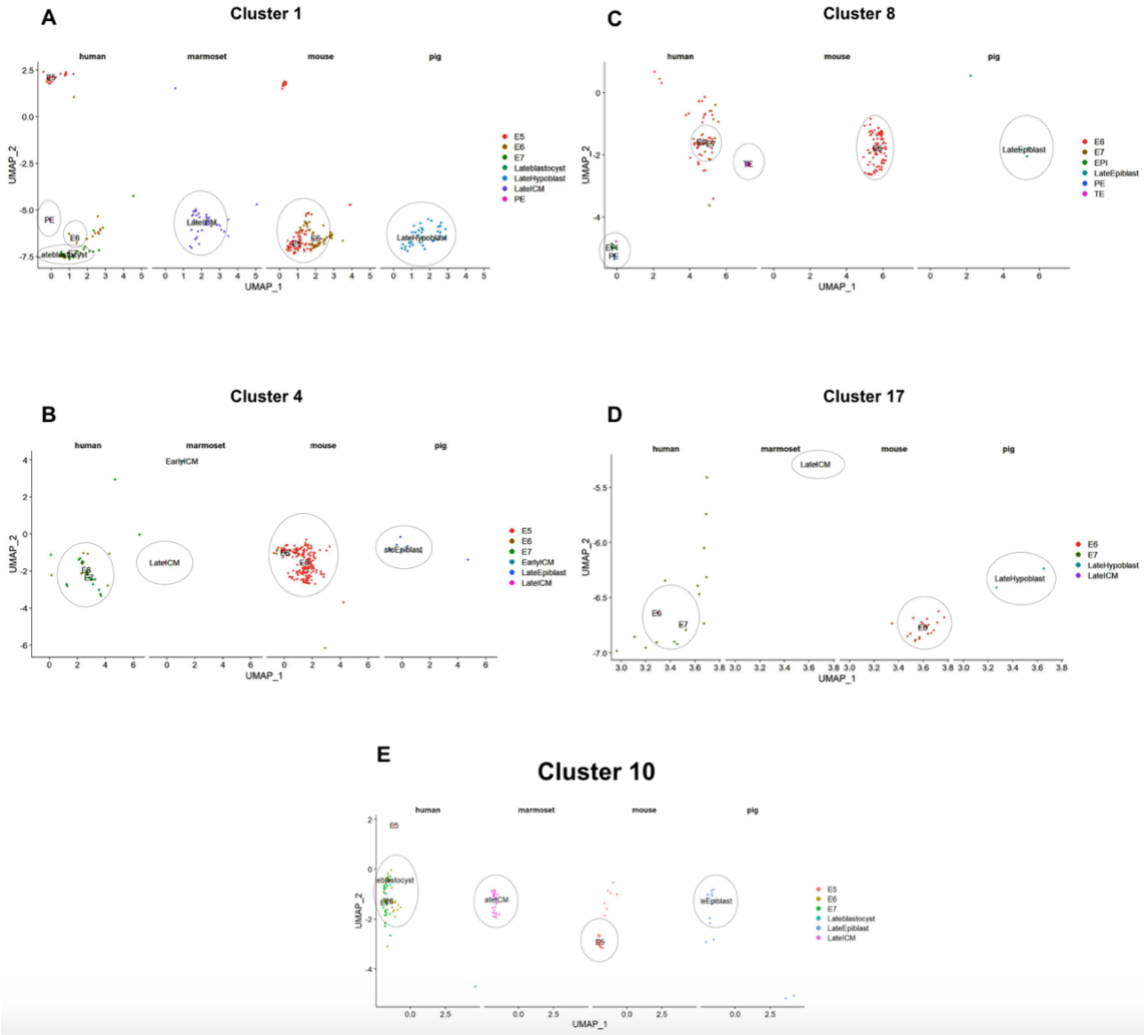

Figure 20: Interspecies comparisons of gene expression. *(A-D) Clusters containing mouse E6 stages were extracted. The human stages in these clusters were then analyzed to predict the human developmental stage that is most similar to the mouse E6 stage. (A-E) Similarities between the human and the marmoset and pig stages were also analyzed. The analysis results indicated that the human blastocyst stages resembles the mouse E6 implantation stage as well as the marmoset late ICM and the pig Late Epiblast and Late Hypoblast.*

to have similar gene expression to human ICM (Boroviak et al., 2018). Further, successful integration of primate ESCs (similar to hESCs and derived from primate blastocyst stages) into late stage pig blastocysts has been demonstrated in *in vivo* studies (Fu et al., 2020). A previous study analyzing sc-RNA seq data has demonstrated the highest similarities

between hESCs and the pig late blastocyst stage when comparing the other pig pre-implantation stages and has proposed this combination for blastocyst complementation studies (Ramos-Ibeas et al., 2019). Hence, the similarities between human E6-E7 blastocysts, the gastrulating mouse embryos, primate late ICM, and the pig late blastocyst stages (epiblasts and hypoblasts) obtained from this clustering analysis are in agreement with previous stage-matching studies.

Conclusions- Clustering analysis of sc-RNA seq datasets across the four species was used to predict embryonic stages that are most similar to each other transcriptomically. These predicted results can be tested in stage-matching *in vitro* and *in vivo* experiments to develop successful interspecies chimeras. Future studies would involve analyzing the remaining clusters and predicting other similar developmental stages across the four species.

1.3 Generation of heatmaps across all species for all embryonic stages

In order to visualize the patterns of gene expression (upregulation and downregulation) in all the developmental stages across the four species, heatmaps of the top 300 and top 500 most differentially expressed genes across all the datasets were generated as detailed in **Methods Section V 5** and shown in Figure 21, A-B. From both the heatmaps, it could be observed that most of the embryonic developmental stages clustered together based on the species they belonged to which denotes significant species-specific differences. However, these species-specific differences were not as pronounced for some of the earlier developmental stages (shaded in red) in Figure 21, A-B, that is, the mouse zygote to 2-cell stages, human 4-cell stage, and marmoset zygote to 4-cell stages. All of these stages are pre-zygote gene activation stages. The zygote gene activation (ZGA) begins at the 2-cell stage in mouse, 4-8 cell stage in human and 8-cell stage in marmoset. During the embryonic stages prior to the ZGA, the oocyte-derived maternal mRNAs and proteins are used by the embryo and no transcription of embryonic genes takes place. However, after the ZGA, the embryo rapidly undergoes transcription and begins to translate its own

proteins. It is possible that the pre-ZGA stages in each species that clustered together (shaded in red in Figure 21, A-B) did not show the species-specific differences that are much more pronounced in the post-ZGA embryonic developmental stages. Since only the post-ZGA stages for the pig species were present (morula to late blastocyst) in the pig datasets being analyzed, no pig specific dataset was observed in the pre-ZGA cluster (pig ZGA occurs at the 4-cell stage) (Figure 21, A-B).

Conclusions- In each species, the embryonic stages after ZGA demonstrated significant species-specific differences and clustered together. The pre-ZGA stages in all the species did not show pronounced species-specific differences, were more similar to each other compared to post-ZGA stages and clustered together. In the subsequent experiments, heatmaps of the top 1000 differentially expressed genes across all the developmental stages within each species were generated. These intraspecies heatmaps allow better visualization of the pre-ZGA versus post-ZGA changes in gene expression though embryonic stages.

2. **Intraspecies comparisons of gene expression- Generation of species-specific heatmaps for all the early embryonic developmental stages**

   For each of the four species, heatmaps of the top 1000 differentially expressed genes across all the developmental stages were generated as detailed in **Methods Section V 5**. In the heatmaps for the human (Figure 22, A), marmoset (Figure 22, B) and mouse (Figure 22, C), the pre-ZGA stages (shaded in red) clustered together and showed significant differences in the overall gene expression compared to post-ZGA stages. In case of the human embryonic developmental stages, all the post-ZGA stages had similar trends in gene expression when compared to the pre-ZGA except for the E4 stage (shaded in red) (Figure 22, A). The E4 stage in humans is the compacted morula stage which follows the 8-cell stage (ZGA takes place at the 4-8 cell stage). At the E4 stage, the gene expression may still be in the process of transition, that is, more maternal mRNAs are downregulated, and embryonic genes are being transcribed and upregulated. By the E5 stage, the pattern of gene expression more closely resembled the other post-ZGA (Figure 22, A). In case of the heatmap generated from the pig datasets, the sharp difference in gene expressions

could not be observed due to absence of the pre-ZGA datasets (zygote to 4-cell stages) (Figure 22, D). However, it could be observed that the morula and the early ICM and TE clustered closely together when compared to the later blastocyst stages, that is, the Late Epiblast and the Late Hypoblast.

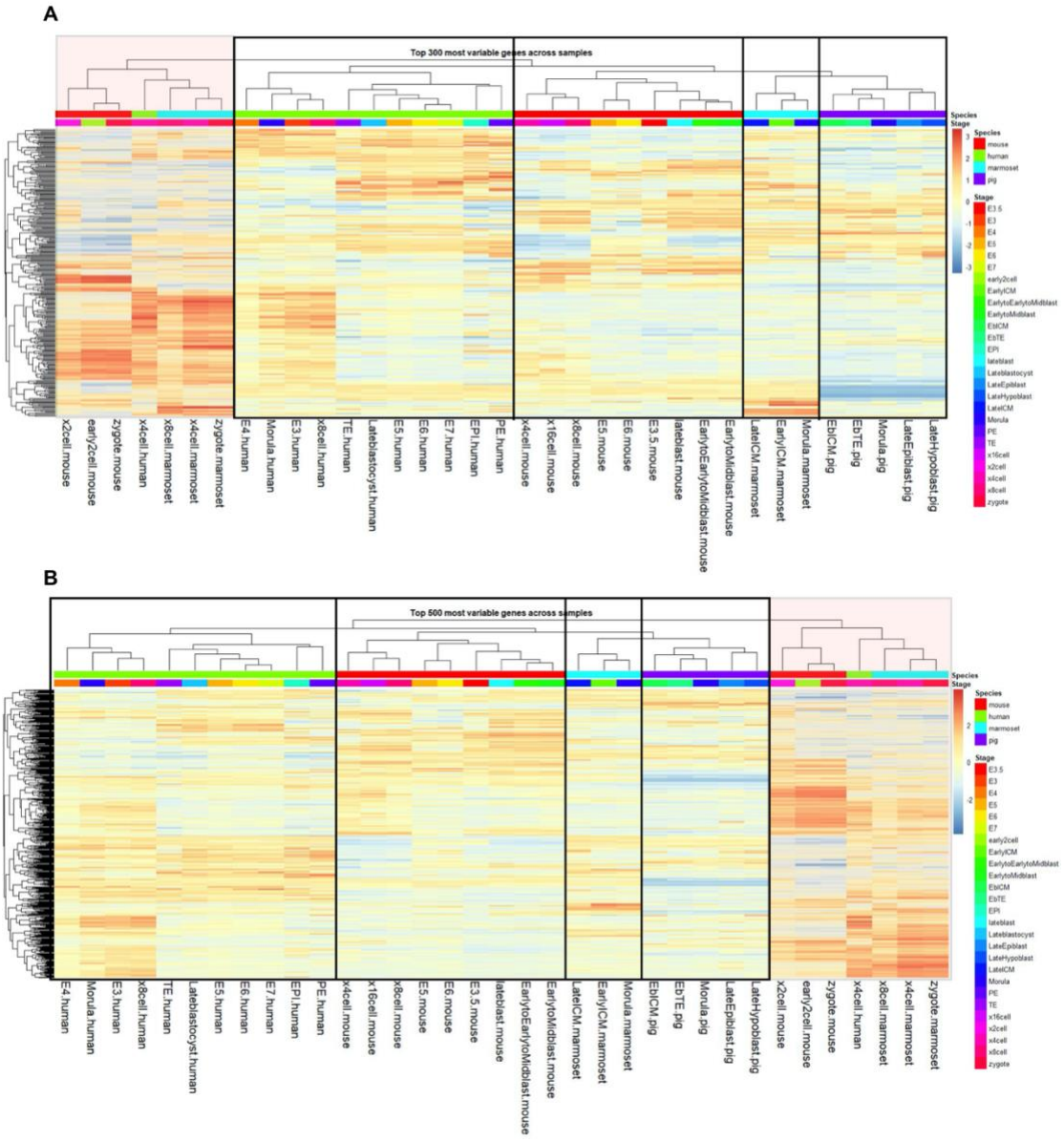

Figure 21: Heatmaps of the top 300 and 500 differentially expressed genes across four species- (A) Human, (B) Marmoset, (C) Mouse, and (D) Pig. *All the post-ZGA stages showed species-specific differences and clustered together. However, the pre-ZGA stages for all the species demonstrated less pronounced species-specific differences and clustered together (shaded in red).*

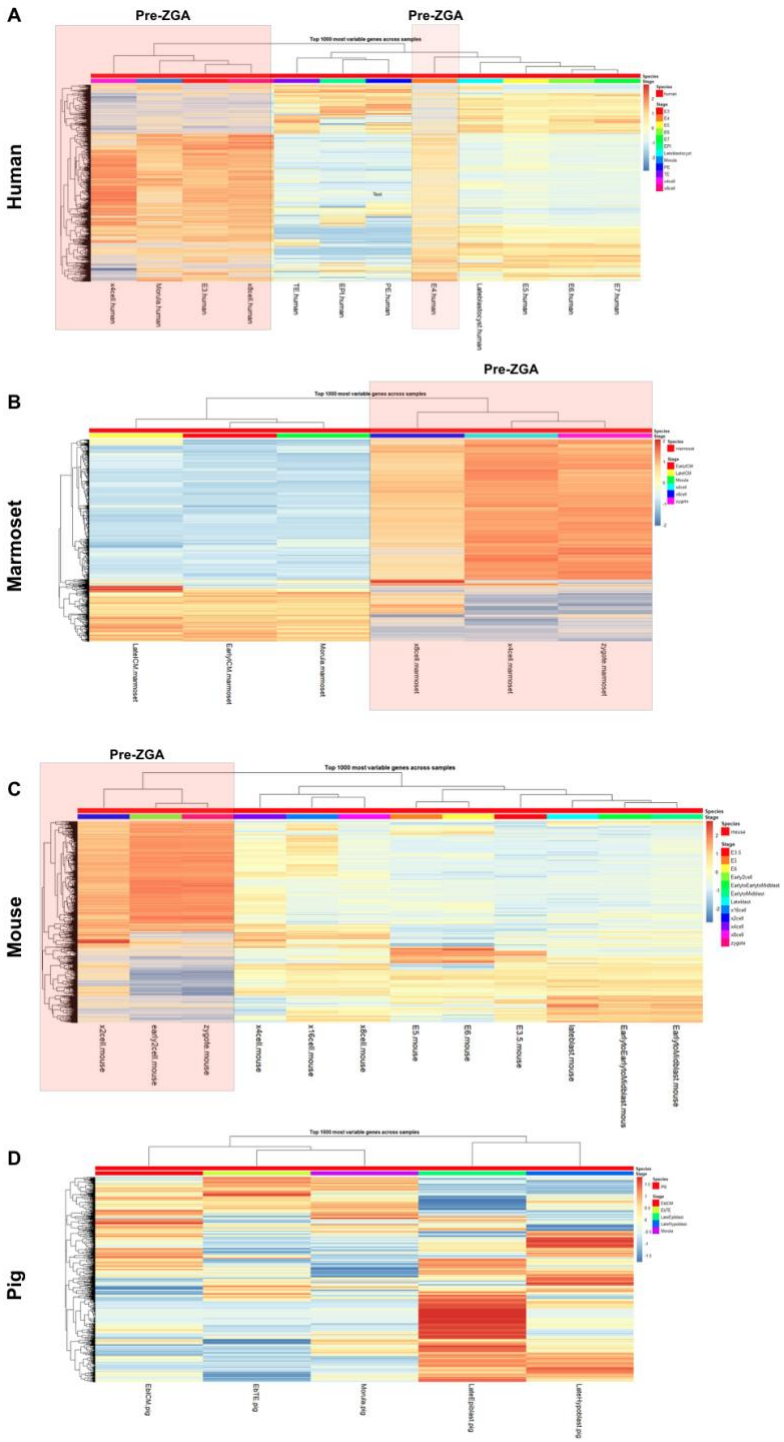

Figure 22: Heatmap of top 1000 differentially expressed genes across all early embryonic stages for each species. *(A-C) The heatmaps for human, marmoset and mouse showed a distinct change in gene expression between the pre-ZGA (shaded in red) and the post-ZGA (unshaded) stages. (D) Since pre-ZGA stages datasets were not available for the pig species, the distinct changes observed in other species was not observed.*

Conclusions- By generating heatmaps across developmental stages in each species, the changes in gene expression between the pre-ZGA and post-ZGA stages could be observed. From the heatmaps, the expression of other genes involved in key developmental pathways could also be observed. Future experiments would involve isolating these key genes and observing how they change between embryonic stages. The pattern of expression of these genes can provide insights into how oscillations in gene expression can contribute to the different developmental speed of each species.

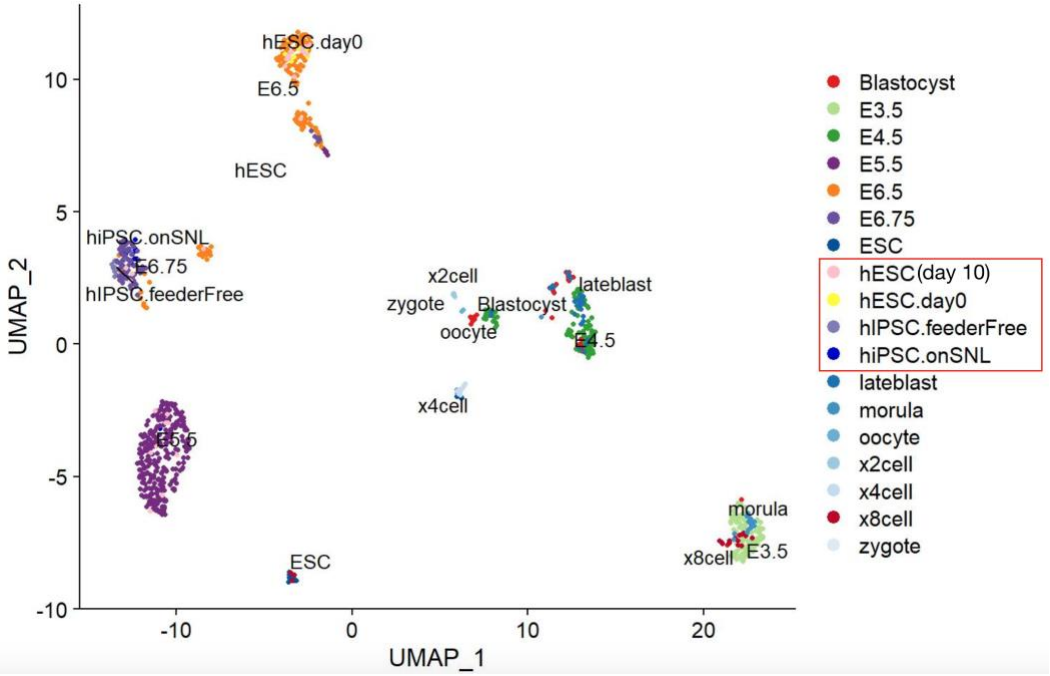

Figure 23: Clustering of hPSCs with Mouse Embryonic Stages. *The hPSCs which include the hESCs and hiPSCs clustered with mouse post-implantation stages from E5.5 to E6.75.*

3. Clustering of human hPSCs with Mouse Early Embryonic Stages

In this study, UMAP was used to cluster hPSCs (hESCs and hiPSCs) with mouse early embryonic developmental stages to predict the stages of the mouse embryo that are most similar to the hPSCs. Two sets of hiPSCs were used for this analysis, a feeder layer-free hiPSCs line and hiPSCs grown on SNL fibroblasts. Datasets for the hESCs were extracted from a study where they compared hESCs at day 0 and at day 10 in culture (Yan et al., 2013). The hiPSCs and hESCs were clustered with mouse early embryonic stages as shown in Figure 23. The hiPSCs clustered closely with the post-implantation E6.75 mouse embryo stages (Figure 23). The hESCs at

day 0 clustered closely with the post-implantation E6.5 mouse embryo stages. The hESCs at day 10 clustered with multiple post-implantation stages of the mouse embryo from E 5.5- E 6.75 (Figure 23**)**. In all the cases, the hPSCs clustered with mouse post-implantation embryonic stages and is in agreement with results from previous studies. No clustering of the hPSCs with mouse pre-implantation stages was observed. The variation in clustering seen between the hiPSCs and the hESCs may be due to differences in their culture conditions and highlights the impact of culture conditions on the transcriptome of stem cells.

C. Discussions for Single Cell RNA sequencing to Study the Transcriptome of Early Embryonic Development Across Species

In this study, sc-RNA seq analysis of early embryonic development across four species- human, marmoset, mouse and pig was performed. Clustering analysis and visualization techniques such as PCA plots, UMAPs, and heatmaps were used to predict similarities in developmental stages between species. These predicted results were validated by comparing them to previous *in vitro* and *in vivo* stage-matching studies where successful interspecies chimerism was observed. Using sc-RNA seq analysis, datasets containing genes unique to human embryonic development that are not upregulated in the early embryos of marmoset, mouse or pig could be extracted. Further, the changes in gene expression before and after the zygote gene activation process could be clearly observed from the heatmaps. Clustering of the hPSCs with mouse post-implantation stages and not pre-implantation stages could be observed. This suggests that hPSCs injected into mouse post-implantation stages are more likely to form successful chimeras. These results are in agreement with previous sc-RNA seq studies as well as *in vitro* and *in vivo* experiments.

Previous studies comparing human and mouse embryos have provided insight into transcripts that are conserved and distinct among the two species (Niakan & Eggan, 2013). The study comparing human, marmoset, and mouse, generated stage-specific gene expression modules across the three species (Boroviak et al., 2018). The results demonstrated that although most of the key genes are expressed in all three species, the embryonic stages at which they are expressed varies and their functions might also vary between species. The whole genome sequencing study comparing mouse and pig pre-implantation embryonic development highlighted the distinct features in pig early development (S. Cao et al., 2014). The results from other *in vivo* and sc-RNA seq analysis studies comparing hPSCs with pig stages (Ramos-Ibeas et al., 2019) and primate ESCs with pig stages (Fu et al., 2020) demonstrated that hPSCs and primate ESCs are most similar to the pig late blastocyst stage and the results are in agreement with the predictions from this study.

In this study, sc-RNA seq analysis across four species, human, marmoset, mouse and pig was performed. The stage-matching results predicted using an *in silico* approach in this study are in agreement with previous stage-matching studies performed *in vitro* or *in*

vivo where successful interspecies chimerism was observed. Further, datasets containing genes uniquely expressed in human developing embryos, and pre-ZGA and post-ZGA changes in gene expression could be visualized for individual species and across the four species. Some of the limitations of the study are the lack of the earlier developmental time points in pigs, that is, the zygote to the 4-cell stages. Further, the difference in the number of datasets between the species is significant and might impact the robustness of the conclusions drawn. Nevertheless, this study demonstrates the strength of sc-RNA seq analysis in predicting and matching embryonic stages across species. This approach used in combination with *in vitro*, *in vivo* as well as the *ex-vivo* culture systems being developed will enable precise stage-matching experiments. Further, this combined approach will also help to study and define the gene networks that exemplify different combinations of interspecies chimeras and bring the field closer to the ultimate goal of developing transplantable human organs in relevant animal hosts like pigs.

Section VI- Application of Single Cell RNA sequencing for Assessment of *Ex-Vivo* Embryo Development of Chimeras

Over the years, considerable progress has been made in the field of blastocyst complementation. However, major hurdles still need to be tackled to achieve the ultimate goal of developing transplantable human organs in relevant host animals. The current protocols for interspecies chimerism have been successful in developing human-mouse chimeras. However, the efficiency of chimerism is still very low and blastocyst complementation studies have been less successful. Further, very little progress has been made in interspecies chimerism studies involving relevant host animals such as pig. Most of the *in vitro* and *in vivo* studies to date have used trial and error to stage-match the graft cells with the host animal embryo. In addition to this, the imaging technologies used in these studies do not allow real-time tracing of the grafted cells or their progeny in the *in vivo* studies. In the human-mouse chimerism studies, significant difficulty has been encountered in identifying and extracting the peri-implantation mouse embryo (E4.5-E5.5) from the uterus. The mouse embryo undergoes rapid proliferation with changes in morphology and gene expression taking place every few hours. This increases the difficulty of performing precise *in vivo* stage-matching experiments for human-mouse interspecies chimerism. Other than these technical difficulties, the existing NIH policies due to ethical concerns do not allow injection of human stem cells into the blastocyst stage of animal embryos.

In order to overcome these hurdles, in this study, an *ex-vivo* 3D gel system was developed that enabled mouse embryos to grow until E7 and form the egg-cylinder morphology observed in *in vivo* implanting mouse embryos. This *ex-vivo* system can be used to closely study the early embryonic developmental stages including the peri-implantation stages. Further, injection of human stem cells into the E5.5-E7 *ex-vivo* cultured mouse embryos bypasses the current NIH policies that do not allow injection of human cells into the mouse blastocyst stage (E3.5 to E4.5) and allows stage-matching experiments for successful human-mouse chimerism. This *ex-vivo* system, as described in **Section V**, in combination with sc-RNA seq analysis techniques described in **Section VI**, will greatly help to optimize human-mouse interspecies chimerism and blastocyst complementation studies (Figure 24). The results and experimental approaches used for

optimizing human-mouse chimeras can then be translated to other interspecies chimeras such as human-pig chimeras.

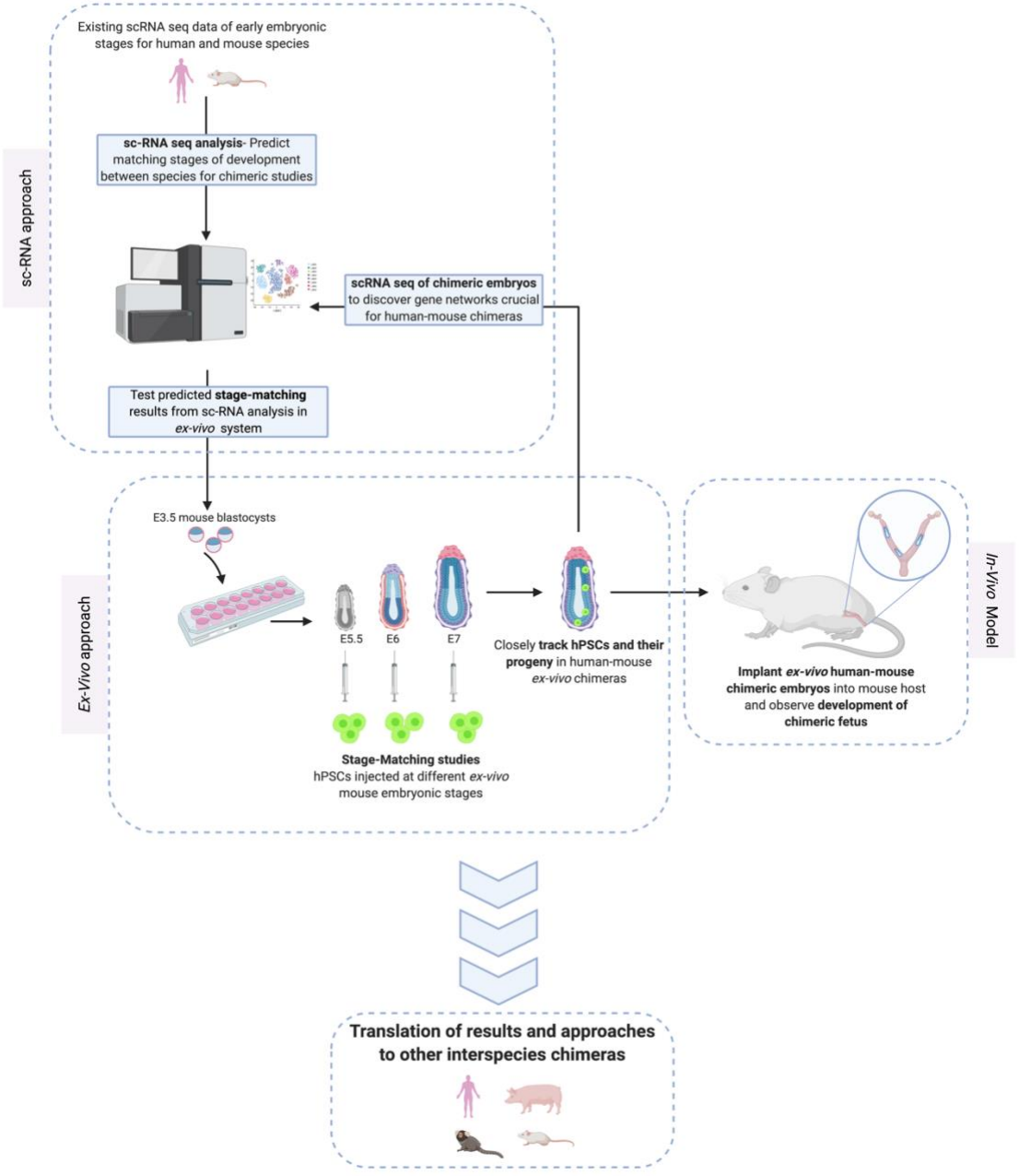

Figure 24: Combining approaches of sc-RNA seq Analysis and Ex-Vivo Embryonic Culture to optimize Interspecies Chimerism. *(Created with BioRender.com)*

Similar to the approach in **Section VI**, publicly available sc-RNA sequencing datasets for early embryonic development across human and mouse species can be analyzed using bioinformatic techniques to predict exact stages of development that are most similar between the human and mouse species. sc-RNA seq data of the human pluripotent stem cells (hPSCs) can also be matched with stages of mouse early embryos. The results predicted from these studies can then be tested in the optimized *ex-vivo* system developed in **Section V**. The hPSCs can be injected at precise embryonic stages (predicted using sc-RNA seq analysis) of the *ex-vivo* gel-embedded mouse embryos as demonstrated in Figure 24 to optimize the efficiency of human-mouse chimerism. The *ex-vivo* system allows real-time tracking of the hPSCs and their progeny in the mouse embryos. The *ex-vivo* system can also be translated into microarrays where high-throughput screening of precise stage-matching experiments can be performed. Multiple timepoints at which hPSCs are injected into mouse embryonic stages of development can be tested simultaneously, increasing the efficiency and reproducibility of human-mouse interspecies chimerism. Further, the transcriptome and proteome of the injected human grafted cells, their progeny and the cells in the mouse embryos can be extracted from the *ex-vivo* system and the interactions between the grafted cells and the host embryos can be studied. Successful human-mouse chimeras can also be implanted *in utero* in pseudo-pregnant mice and the development of the human-mouse chimeras can be observed. From the microarray *ex-vivo* studies, successful human-mouse chimeras can be used for sc-RNA sequencing. From the analysis of the sc-RNA seq data, gene networks that exemplify successful human-mouse chimeras can be discovered. This combined approach of using sc-RNA seq analysis techniques with the *ex-vivo* embryonic culture system can be translated to other interspecies chimeras such as human-pig chimeras bringing the field of interspecies chimerism and blastocyst complementation closer to the ultimate goal of developing transplantable human organs in animal hosts.

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Appendix: Efficiency of Mouse Embryonic Culture with KSOM + 40% Matrigel

The efficiency of the *ex-vivo* culture condition with KSOM medium + 40% Matrigel was calculated as described below. 4 biological replicates, that is C57 mice, were used. Each well with multiple blastocysts was treated as a technical replicate. Within each well, the number of E7 egg-cylinder shaped mouse embryos that developed from the E3.5 blastocysts cultured was calculated and used to determine the mean (E7 embryos/ healthy E3.5 blastocysts).

Biological replicates (C57 Mice)	Well 1	Well 2
1	8/11	5/8
2	8/10	7/12
3	4/7	4/5
4	2/3	3/5

Mean ± SD =67.06% ± 2%