

Developing a Stem-Cell Based System of Embryonic Development Organized by a Morphogen Signaling Centre



By

Simoné Rossouw

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Supervisor: Dr Mubeen Goolam

Co-Supervisor: Professor Neil Davies

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Abbreviations

2D	Two-dimensional
2i	Two molecule inhibitor
3D	Three-dimensional
Ac	Acrylate
ANOVA	Analysis of variance
AP	Anterior-posterior
AVE	Anterior visceral endoderm
B27	B-27 supplement
BMP4	Bone-morphogenic protein-4
Bp	Base pair
Bra	Brachyury
cDNA	Complementary DNA
CDX2	Caudal type homeobox2
Cer-1	Cerberus-like protein 1 (Cer-I)
CHIR99021	Chiron/CHIR
CK1γ	casein kinase 1γ
CNS	Central nervous system
CO₂	Carbon dioxide
Ct	Cycle threshold/Crossover threshold
Dkk1	Dkkopf1
DMEM	Dulbecco's Modified Eagle's Medium
DMEM-F12	Dulbecco's Modified Eagle's Medium/Ham's factor twelve
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DVE	Distal visceral endoderm
DVL	Dishevelled
EB	Embryoid body

ECAD	E-cadherin
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal-transition
EN1/2	Engrailed homeobox 1/2
EOMES	Eomesdermin
EPC	Ectoplacental cone
Epi	Epiblast
ERK	Extracellular signal-regulated kinase
ExE	Extraembryonic ectoderm
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOXA2	Forkhead box transcription factor A2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gp130	Glycoprotein 130
GSK3β	Glycogen synthase kinase 3 β
hr/hrs	Hour/Hours
ICM	Inner cell mass
IgG	Immunoglobulin G
iMEFs	Inactive mouse embryonic fibroblasts
IRX6	Iroquois homeobox gene 6
IVF	<i>in vitro</i> fertilization
iXEN	Inducible extraembryonic endoderm cells
JAK	Janus kinase
Lefty1	Left-right determining factor 1
IHX1	LIM homeobox protein 1
LIF	Leukaemia inhibitory factor
LRP	Receptor-related protein
MAPK	Mitogen activated protein kinase
mESCs	Mouse embryonic stem cells
MESP	Mesoderm posterior

MET	Mesenchymal-to-epithelial transition
min	Minute
MIXL1	MIX1 homeobox-like 1
ml	Millilitre
mRNA	Messenger RNA
mTSCs	Mouse trophoblast stem cells
N2	N-2 supplement
NaCl	Sodium Chloride
NE	Neuroectoderm
OCT4	Octamer binding transcription factor 4
Oligo dT	Oligonucleotide deoxythymine
OTX2	Orthodenticle Homeobox 2
ParE	Parietal Endoderm
PBS	Phosphate buffered saline
P-D	Proximal-distal
PD0325091	MEK inhibitor
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PGRN	pluripotency gene regulatory network
PI3K	Phosphatidylinositol-3phosphate kinases
POU	Pit-Oct-Unc
PrE	Primitive endoderm
PSCs	Pluripotent stem cells
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
RPM	Rates per minute
RTK	Receptor tyrosine kinase
SC	Signalling centre
SE	Surface ectoderm
SEM	Standard Error of Mean
Ser/Th3	Serine/threonine kinase 3

SH	Sulfhydryl
SMAD	Suppressor of Mothers against Decapentaplegic
SNAIL-1	Snail Family Transcriptional Repressor 1
SOX	SRY box
STAT3	Signal transducer and activate of transcription 3
TE	Trophectoderm
Tead4	Transcriptional enhancer activator domain family 4
TGF-β	Transforming growth factor β
TS	Trophoblast stem
VE	Visceral Endoderm
Wd	Dry weight
WNT	Wingless-INT
Ws	Swollen weight
XEN	Extraembryonic endoderm cells
YAP	Yes Associated Protein

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Abstract

Successful mammalian development is dependent on the precise communication and coordination from both the embryonic and extraembryonic tissues. The interactions ensure that the tissues become spatially localized within the embryo for the process of gastrulation, a pivotal event whereby the three germ layers: mesoderm, endoderm, and ectoderm are specified. Bone morphogenetic protein 4 (BMP4), secreted from the extraembryonic ectoderm is required for gastrulation to start whereby it activates downstream signaling molecules wingless inhibitor 3 (WNT3) and Nodal in a concentration dependent manner to specify both the mesoderm and endoderm. The ectoderm is formed in the absence of these signaling molecules. Together, these signaling molecules and mechanical constraints from the extracellular matrix (ECM), shape the developing embryo, although less is known about the precise mechanisms by which mechanical constraints function during gastrulation. As a result, defects within the coordination of cells and tissues during gastrulation can lead to congenital malformations and even embryonic death. The mechanisms underlying gastrulation are difficult to study *in vivo*, as gastrulation occurs post-implantation rendering the embryo inaccessible for direct *in vivo* experimentation. *In vitro* stem cell derived embryo-like structures called gastruloids are able to circumvent these impediments posed by *in vivo* research. Gastruloids form derivatives of all three germ layers in a spatially coordinated manner and resemble key *in vivo* aspects of gastrulation. In addition, gastruloids are formed solely from mouse embryonic stem cells, thereby providing an excellent model system to study the effects of BMP4 during gastrulation. This model is however restricted in that they exhibit limited morphogenesis and have no anterior neural structures present.

Here, we incorporated bone morphogenetic protein-4 as a pulse and a spatially restricted signaling center into gastruloid culture to investigate the extent to which they can recapitulate gastrulation-like events *in vitro*. To investigate this, our laboratory has successfully grown mouse embryonic stem cells in a pluripotent state for extended periods of culture in defined cultured conditions. Pluripotency was assessed and confirmed by light microscopy, qPCR, and immunocytochemistry by the presence of pluripotency factors OCT4 and NANOG. Culture conditions were changed to disrupt pluripotency and direct differentiation into gastruloids following established protocols. Gastruloid formation was assessed by light microscopy to investigate morphogenesis while mRNA and protein levels of several genes required during gastrulation was evaluated by qPCR and immunocytochemistry, respectively.

All gastruloids illustrated an exit in pluripotency and expressed both WNT3 and Nodal, required for the formation of the mesoderm and endoderm. Markers for the mesoderm and endoderm: SOX17, GATA6, Bra, and MIXL1 was expressed in varying levels while only gastruloids formed with a bone

morphogenetic protein-4 signaling center, expressed markers for anterior neural lineages as seen by an upregulation in POU3F1, EN1, OTX2, IRX6, and SCUBE1. Interestingly, gastruloids cultured in polyethylene glycol-acrylate-sulfhydryl hydrogels had no change in morphology. Confocal imaging confirmed the localization of brachyury, a primitive streak marker in some gastruloids.

Collectively, the results show that the gastruloids have the capability to undergo self-organization in a spatially coordinated manner and that bone morphogenetic protein-4 could replace the Chiron pulse in conventional gastruloid protocols to induce gastruloid formation. The incorporation of a spatially restricted signaling center illustrates the importance of concentration gradients for anterior neural induction. Finally, gastruloids grown in hydrogels illustrate that gastrulation most likely occurs from a combination of factors and events occurring together and that mechanical constraints alone are insufficient to drive morphogenesis.

CHAPTER 1

Introduction

Embryonic development has always been at the centre of fascination; how a single cell can differentiate and generate enough diversity to develop into a complex multicellular organism. In Lewis Wolpert's words: "It is not birth, marriage, or death, but gastrulation which is truly the most important time in your life". Rightfully so, as gastrulation, a critical post-implantation event, ensures that all cells in the embryo end up in the correct spatial location and subsequently transforms these cells into all the tissues that will constitute the entire adult organism. The misregulation of these mechanisms during embryonic development may result in congenital malformations and embryonic death [1] accounting for up to 50 % of pregnancy failures [2, 3]. Successful development is therefore reliant on ensuring the precise and intricate regulation of these mechanisms and developmental events including cell movement and organization, tissue growth, and cellular specification to ensure the formation of a fully functional body plan. It is therefore fundamentally important to understand this highly dynamic process.

1. The process of mouse development

1.1 Pre- and peri-implantation development

Mammalian development has been best characterised in mice owing to their rapid reproduction cycle of 19 – 21 days [4] and relative ease with which they can be bred whilst overcoming the challenges of experimenting with human embryos [5]. Mice are also the most intensively studied surrogate for humans as they share a similar genomic organization and conserved gene orders as 99 % of mice genes have human homologues and mutations in the mouse genes produce similar effects in their human homologues [6, 7].

Following fertilization, the totipotent unicellular zygote undergoes a series of mitotic divisions, without increasing in overall size resulting in smaller and smaller cells termed 'blastomeres' [8], as it travels to the uterus [9-12]. By E2.5, the cleavage division yields a morula comprised of eight totipotent blastomeres and undergoes its first major morphogenetic event known as compaction. Critical during this process is a calcium-dependent adhesion molecule, E-cadherin (ECAD), which flattens the blastomeres by increasing intracellular cell adhesion [13, 14]. As a result, individual blastomeres undergo polarization, thereby establishing the first apical-basal domain of the embryo. This process

sets the stage during which the embryo will specify three major cell types: the trophectoderm (TE), primitive endoderm (PrE), and the epiblast (Epi) in two cell-fate decisions [14-17].

1.1.1 *The first cell fate decision*

The first cell decision occurs within the morula as a result of the inside-outside polarity and symmetric cell divisions which triggers the generation of two distinct subpopulations: the inner cells mass (ICM) and the trophectoderm (TE). The TE is an extraembryonic tissue that gives rise to supporting tissues of the embryo such as the embryonic placenta while the ICM gives rise to all the tissues of the embryo proper [18]. Enforcement of the first cell fate decision has been shown to be critically regulated by the Hippo signalling cascade, which has first been identified in *Drosophila* as a growth control pathway [19]. Inside-outside cell polarity within the morula activates differential Hippo signalling thereby committing cell fate towards the TE lineage or the ICM [15, 20-24]. The involvement of Hippo signalling in enforcing the first cell fate decision was first observed in embryos lacking Transcriptional enhancer activator domain family 4 (Tead4). This study by Yagi *et al* examined the role of Tead4 during mouse embryogenesis by creating a Tead4 knockout mouse line [25]. The results presented revealed that Tead4 mutant embryos did not express TE-specific genes, such as Caudal type homeobox2 (CDX2), but did express ICM-specific genes, such as Octamer binding transcription factor 4 (OCT4). Tead4 mutant embryos underwent preimplantation lethality as they failed to generate trophoblast stem (TS) cells *in vitro*, and to express CDX2. These CDX2 mutant blastocysts had no blastocoel cavity which may account for why these CDX2 mutant blastocysts did not implant into the endometrium. The Tead4 mutant embryos prevented the expression of CDX2, however, not OCT4 as its expression was restricted to the ICM of wild-type and heterozygous blastocysts and expressed in every blastomere of Tead4 mutant embryos. The results from this study illustrates the requirement of Tead4 for specification of the TE cell lineage.

Serine/threonine kinase three (Ser/Thr3) regulates proliferation through inhibiting cell mediated contacts and is required for Hippo signalling, which is only activated within the apolar inner blastomeres [26]. Hippo signalling activates Yes Associated Protein (Yap1) phosphorylation which prevents Tead4 from functioning in the nucleus (Figure 1). Consequently, the apolar inner blastomeres retain expression of pluripotency genes NANOG [27] and OCT4 [28], thereby specifying the ICM [16, 29, 30]. In contrast, Hippo signalling is inactive in the polar outer blastomeres as their apical side is not in contact with other cells. As a result, Yap1 remains unphosphorylated and travels to the nucleus where it binds to and activates Tead4. Consequently, Tead4 promotes the expression of CDX2 whose activation results in the commitment of the outer blastomeres towards the TE lineage, which

surrounds the ICM and will give rise to all the trophoblast cells types that make up the majority of the foetal part of the placenta [21, 24, 31] (Figure 1).

Previous research by Strumpf *et al* [32] has illustrated that OCT4 is expressed in all blastomeres of the early morulae, however, as the polar outer blastomeres start to differentiate into the TE lineage, OCT4 expression becomes repressed as CDX2 expression increases. This has been confirmed in another study by Niwa *et al* [33] using an epitomal expression vector system to overexpress CDX2 in mouse embryonic stem cells (mESCs). The resulting cells had enlarged nuclei that expressed the surface TE marker, Cadherin3, and transfecting these cells with Fibroblast Growth Factor 4 (FGF4) gave rise to TS-like epithelial cells, indicating that CDX2 is sufficient to trigger TE lineage differentiation. Altogether this study suggested that OCT4 and CDX2 reciprocally inhibits one another and that their ratios in ES cells is crucial in determining cell fate. OCT4 repression led to the upregulation of CDX2, and this high CDX2:OCT4 ratio triggered mESC differentiation towards the TE. In contrast, the upregulation of OCT4 suppressed CDX2, where the high OCT4:CDX2 ratio resulted in commitment towards the ICM [33]. However, the previous study by Yagi *et al* suggested that it may be the OCT4:TEAD4 ratio that specifies the TE lineage as the Tead4 mutant embryos precented CDX2 expression but not OCT4.

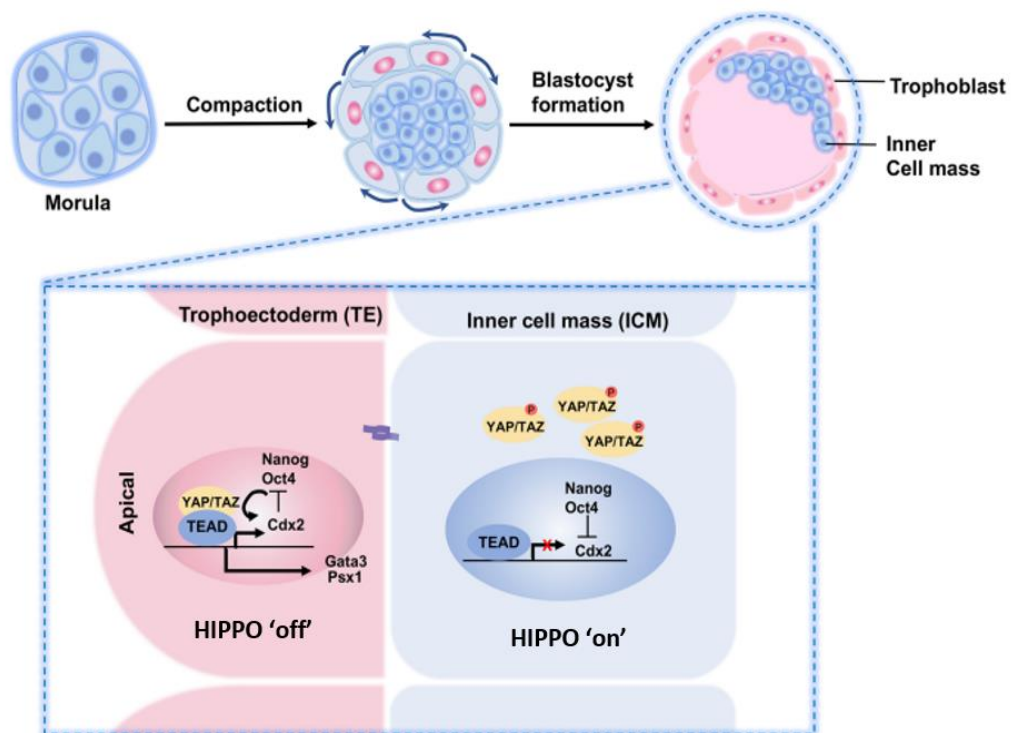


Figure 1: Illustration of YAP regulation for the specification of the TE and ICM in the early mouse embryo. During pre-implantation, all cells express NANOG, CDX2, and OCT4. Compaction result in the establishment of inner and outer cell polarity. In the polarized outer cells, the HIPPO pathway is inactive, therefore allowing the presence of Yap1 in the nucleus. TEAD4 binds to Yap1 to promote TE

specifications through the activation of TE-specific genes CDX2, GATA3, and PSX1. In contrast, the HIPPO pathway is active in the apolar inner cells resulting the phosphorylation of Yap1 which is subsequently sequestered in the cytoplasm thereby inhibiting its interaction with TEAD4. This inhibition prevents to activation of TE-specific genes thereby giving rise to the ICM through maintaining OCT4 and NANOG expression. Figure obtained and adapted from Chen et al, 2019 [34].

Correct segregation of individual blastomeres into TE and ICM is also dependent on cell adhesion, involving ECAD [14, 35, 36]. One study evaluated ECAD mutant embryos that lacked both maternal and zygotic ECAD and found that while the majority was able to divide normally till E3.5, some never developed past the 16-cell stage. In addition, a large portion of the ECAD mutant embryos expressed CDX2 in significantly higher levels than wild-type embryos, thereby suggesting the role of ECAD in restricting CDX2 expression and TE fate. These ECAD mutant embryos also failed to normally segregate the TE-ICM-like cells, concluding that ECAD plays a central role in ensuring the normal allocation of cells during the first cell fate decision [36]. This observation was confirmed through another study when embryos lacking the ECAD encoding gene, *Cdh1*, was able to specify the TE lineage, but could not form TE epithelium of the ICM [14]. These studies conclusively showed the requirement of ECAD in successful segregation into the ICM and TE cell lineage.

1.1.2 The second cell fate decision

Much less is known about the second cell fate decision within the ICM, where the cells either segregate into the epiblast (Epi), that will give rise to the entire foetus and extraembryonic mesoderm [37-45], and the PrE, which form the extraembryonic endoderm layers of the visceral and parietal yolk sac [29, 42, 46]. Genetic evidence has implicated OCT4 and homeodomain NANOG in specifying the Epi [27], and Gata family transcriptional factors, specifically GATA binding protein 6 (GATA6) in specifying the PrE. Chazaud et al [29] illustrated that the individual cells of the ICM exhibit cell heterogeneity for NANOG or GATA6 by E3.5. Through chimera and lineage tracing analysis, no consistent spatial expression pattern was observed as both genes were expressed in a characteristic 'salt and pepper' manner.

In the same study, the mitogen-activate protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) abbreviated as the MAPK/ERK pathway, has been shown to be crucial in specification of the Epi and PrE during the second cell fate decision. The adaptor molecule, Grb2, is a crucial molecule in signal transduction of several receptor tyrosine kinases [47]. Chazaud et al [29] generated Grb2 mutant embryos that showed no GATA6 expression at E4.5 by in situ hybridization. The Grb2 mutant embryos prevented the activation of the MAPK/ERK pathway causing all ICM cells to become NANOG and OCT4

positive with no PrE formation observed. This observation is different to wild-type embryos that express OCT4/NANOG and GATA6 in a salt-and-pepper distribution within the ICM, thereby suggesting the importance of the MAPK/ERK pathway during PrE and Epi lineage specification [29].

In addition, FGF signalling is thought to be the major MAPK/ERK pathway involved upstream of Grb2 as homozygous mutants of FGF4 and FGFR2 was unable to form the PE as a result of expressing similar phenotypes to Grb2 mutants [48]. FGF4 is the only ligand produced by the mouse ICM [42] and comprise four receptors, of which only FGF receptor one and two (FGFR1/FGFR2) is essential for PrE development [38, 39]. FGFR1 is expressed within all cells of the preimplantation embryo and its ablation results in reduced PrE cells and peri-implantation lethality, whereas FGFR2 is expressed in PrE and TE cells but not in Epi cells [38, 39] and its ablation triggers abnormal placental development resulting in embryonic lethality [39]. This study treated cells with FGF treatment containing FGFR1 and FGFR2 knockout compounds resulting in the loss of PrE fate within ICM cells [39]. In a separate study, Ohnishi *et al* [49] collected live single cells from the ICM of mouse blastocysts to examine whether these cells were dependent on FGF signalling during Epi and PrE lineage segregation. The results from single-cell gene expression data on FGF4 mutant embryos revealed the developmental arrest of the PrE and Epi cell lineage thereby indicating the requirement of FGF4 in segregating the two lineages. The loss of FGF4 also resulted in suppressed PrE markers as opposed to Epi markers, indicating its role in specification of the PrE [49].

After the first cell fate decision in the embryo, NANOG is expressed throughout the ICM [32] and in collaboration with OCT4-SRY box 2 (SOX2) upregulates and activates FGF4 expression. The FGF4 ligands bind to FGFR1 and FGFR2 triggering the activation of the MAPK/ERK pathway which, in turn, represses NANOG expression (Figure 2). The MAPK/ERK pathway activation by FGF4 promotes the expression of GATA6 which cooperatively activates downstream genes such SRY-Box Transcription Factor 17 (SOX17) and GATA4 transcription factors [42, 50] through the inhibition of NANOG. The remaining NANOG cell population will become precursors for the Epi [37-39] and forms a repressive auto-regulatory loop of GATA6 (Figure 2) [29, 37, 43, 49, 50]. With time, the cells expressing GATA6/SOX17 migrate by active cell movement and apoptosis to line the blastocoel cavity while preventing further cell mixing as they form around the clustered Epi [40, 51]. By this stage, the Epi and PrE cells are specified and no experimental manipulation that decreases PrE cell numbers can increase Epi cell numbers, and vice versa.

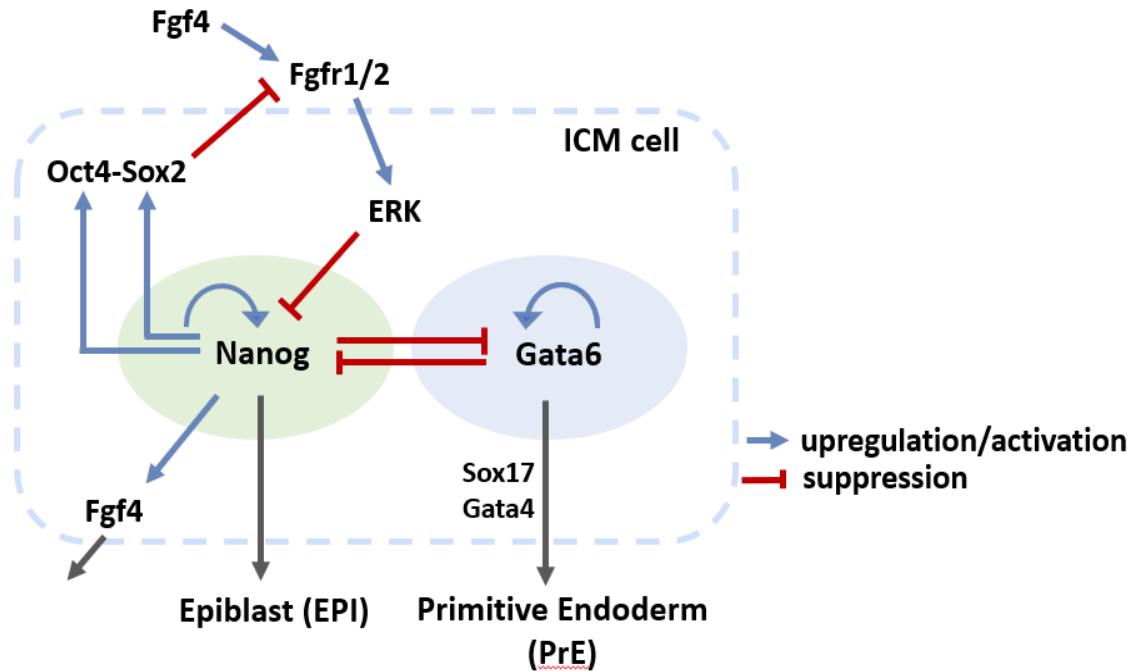


Figure 2: Schematic of the molecular interactions specifying the PrE and EPI during the second cell fate decision. *NANOG and GATA6 repress each other through auto-regulatory loops. NANOG activates OCT4, SOX2, as well as FGF4. Upregulation of FGF4 by NANOG activates the MAPK/ERK pathway via FGFR1 and FGFR2 resulting in NANOG suppression and GATA6 upregulation. The OCT4-SOX2 complex in turn upregulates FGF4 while inhibiting FGFR2. Illustration adapted from Toyooka, 2021 [52].*

1.1.3 Regulation of pluripotency within the Epi

Pluripotency is a transient property during mammalian embryogenesis where cells progressively differentiate from a pluripotent state into distinct cell lineages and, as a result, lose their developmental potential. The ICM of the embryo is pluripotent, however this property becomes restricted to the Epi during the second cell fate decision. The pluripotent Epi cells therefore possess the ability to give rise to the entire foetus, except for extraembryonic lineages. Pluripotency has been shown to be regulated by a highly interconnected pluripotency gene regulatory network (PGRN) that is anchored by a set of three core pluripotency factors: OCT4, NANOG, and SOX2 that prevent differentiation by promoting proliferation [20, 53, 54]. The foundation of the PGRN relies on the precise regulation and balanced expression of the pluripotency genes that inhibit differentiation by promoting proliferation where the disruption of the PGRN promotes differentiation of the ICM into the TE [27, 28].

OCT4 plays a key role in both maintaining and re-establishing pluripotency. OCT4 is a transcription factor that comprises three domains: a central Pit-Oct-Unc (POU) domain for DNA binding, an N-

terminal transactivation domain, and a cell-type specific C-terminal domain. OCT4 belongs to the POU transcription factor and is encoded by POU5F1 which control the expression of its target genes through binding an octameric sequence motif [55]. During development, OCT4 is first expressed at the four-cell stage and continues to be expressed in the ICM, germ cells, and Epi cells whereupon its expression rapidly decreases upon differentiation. It has been observed that the targeted deletion of OCT4 in embryos revealed its absolute requirement in formation of the pluripotent ICM lineage [28]. In this study, the sequences encoding the two DNA-binding domains and the C-terminal domain was deleted and Exon 2-5 of the OCT4 gene was replaced by an internal ribosome entry site (IRES)- β geo cassette to generate OCT4 mutant alleles. Male and female animals heterozygous for the OCT4 deletion transmitted OCT4 mutant alleles to half their progeny. These embryos were able to develop until the blastocyst stage but died at the time of implantation because of a lack of pluripotent ICM cells and instead differentiated into the extraembryonic trophoblast lineage [28]. In addition, proliferation of the trophoblast was not maintained in the OCT4 mutant embryos, but was restored by FGF4, an OCT4 gene product, thereby suggesting the role of OCT4 in determining paracrine FGF signalling from stem cell to the TE. It has been shown conclusively by this study and supporting studies [56] that the absence of OCT4 result in Epi cells reverting to the trophoblast lineage, thereby signifying OCT4 as an important regulator in cell fate decisions through its influence of FGF4 as well as its requirement for the specification of the ICM and maintaining pluripotency within the ICM and Epi.

SOX2 is a sex-determining region-Y-related transcription factors belonging to the DNA binding protein family. SOX2 is expressed in all blastomeres at the 4-cell stage and becomes restricted to the ICM and Epi of the blastocyst [57]. OCT4 shares a common DNA binding site with SOX2 allowing the two transcription factors to heterodimerize and synergistically activate the expression of their target genes such OCT4 [58], SOX2 [58], NANOG [59], FGF4 [60] and UTF1 [61] (Figure 3). SOX2 has also been found to form heterodimers with OCT4 in the absence of DNA through interacting with the POU domain of OCT4 [62]. Figure 3 was constructed by Akberdin *et al* [63] by evaluating numerical stimulation *in silico* on recent experimental data to identify patterns of ESC states whose existence depends on the strength of SOX2, OCT4, and NANOG. The homeobox-containing protein, NANOG, is only expressed and although the precise mechanisms whereby NANOG maintains pluripotency is not yet fully understood, NANOG is thought to form an interconnected regulatory circuit with OCT4:SOX2 as the core PGRN as they co-occupy a large number of their target genes [58, 64].

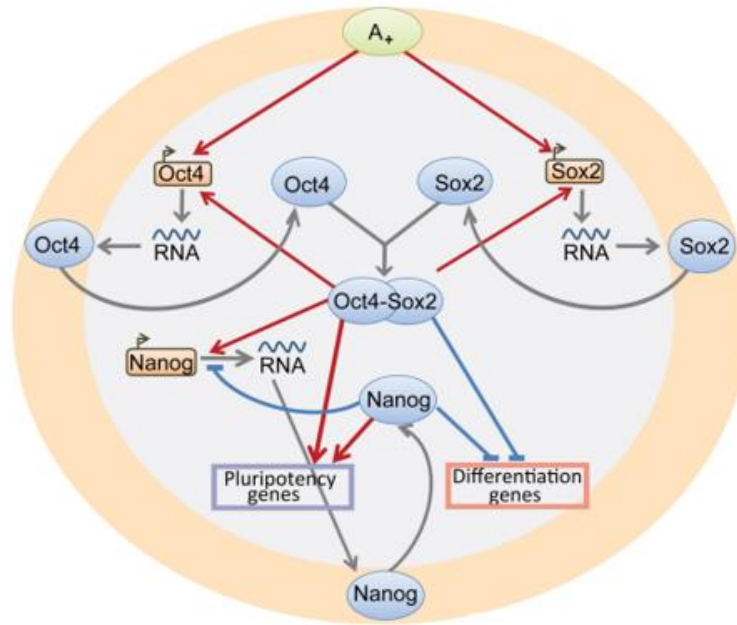


Figure 3: Schematic of the core pluripotency gene regulatory network. External positive signals (A^+) trigger the transcription and translation of OCT4 and SOX2 triggering their activation. OCT4 and SOX2 form a heterodimer: OCT4-SOX2 which positively regulates pluripotency genes NANOG, OCT4, and SOX2. NANOG represses its own transcription. The PRGN of OCT4, NANOG, and SOX2 simultaneously prevent the activation of differentiation genes thereby reinforcing pluripotency within the cells. Image taken from Akberdin *et al* [63].

However, proposed by Akberdin *et al* [63], the experimental model illustrated that high NANOG levels result in its autorepression. This corresponds to studies confirming that high NANOG is required during ground state/naïve pluripotency, but becomes downregulated in the immediate post-implantation Epi as the Epi undergoes a change into primed pluripotency [65, 66] and this downregulation is required for the initiation of lineage specification [67, 68].

1.2 Implantation

By E4.5, the late-stage blastocyst hatches out of the zona pellucida, thereby rendering the blastocyst active for attachment and adhesion to the maternal endometrium. Upon implantation, the mammalian embryo dramatically changes shape from a spherical shaped blastocyst into the so-called egg-cylinder [69]. The molecular mechanisms governing this transition is largely unknown but has been thought to be as a result of changes in the TE and PrE [70]. Prior to attachment, the TE can be distinguished by two subpopulations; the mural TE localized at the abembryonic pole encompassing the blastocoel cavity, and the polar TE which is in direct contact with the ICM at the embryonic pole of the embryo [71, 72]. Christodoulou *et al* [70] filmed 20 developing preimplantation mouse embryos

from Cdx2-GFP transgenic or CD1 females mated with either Cdx2-GFP transgenic, F1, MF1, or CD1 males to determine whether polar TE cell flow must be terminated to allow for the formation of the extraembryonic ectoderm (ExE). The TE at two developmental stages in developing blastocysts expressing CDX2-GFP were visualized and results indicated that as the polar TE to mural TE flow stopped, a CDX2 gradient indicative of TE potency [32], was established with higher CDX2 levels on the polar side than on the mural TE. This CDX2 gradient was only evident upon termination of the flow and not prior to implantation suggesting that differential properties of mural and polar TE cell flow regulate polar TE cell flow during implantation [70]. In the same study, the role of FGF in maintaining TE multipotency was confirmed by culturing E3.5 recovered blastocysts and cultured them till E4.5 whereupon they were treated with FGF4 for 24 hours. The presence of FGF prevented mural TE differentiation as indicated by CDX2 expression levels suggesting that positional information from the Epi, by regulating FGF expression, is responsible for maintenance of polar TE multipotency due to the gradient of CDX2 expression.

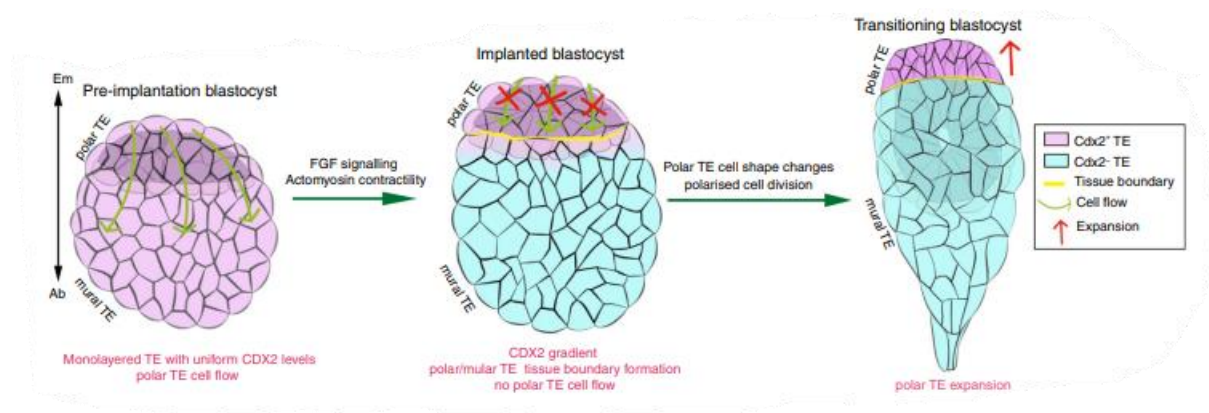


Figure 4: Molecular events required for the expansion of the polar TE during implantation. *CDX2 becomes downregulated in the mural TE and maintains its expression in the polar TE thereby creating an CDX2 gradient. As a result, a tissue-boundary is formed between the mural TE and polar TE. The mural TE initiates attachment to the endometrium while the polar TE will give rise to the ExE. Image obtained from Christodoulou et al [70].*

It was also found that FGF signalling along with high actomyosin contractility at the interface of the mural/polar TE results in the formation of a tissue boundary between the mural and polar TE (Figure 4). This conclusion was achieved by culturing E4.5 blastocysts for 20 hours in the presence of ROCK inhibitor to inhibit actomyosin contractility resulting in significantly narrowed angle of the cell-cell border at the polar/mural TE interface indicating defective boundary formation whereas FGF inhibition also resulted in defective tissue boundary formation. Subsequently the role of the tissue boundary was established by inhibiting FGF signalling or actomyosin contractility in implanting

embryos and assessing their further development. It was shown that FGF inhibition and actomyosin contractility inhibition led to the failure of the polar TE to differentiate forming the ExE and undergoing the characteristic egg-cylinder morphology change thus demonstrating the importance of the tissue boundary in initiating the morphology change in the embryo [70].

Due to the established CDX2 gradient, CDX2 is downregulated in the mural TE triggering an epithelial to mesenchymal-like transition (EMT) [73] in the mural TE that transforms the cells into trophoblast giant cells that mediate adhesion of the blastocyst onto the endometrium [70-73]. The mural TE will eventually give rise to the parietal yolk sac.

1.3 Post-implantation development

1.3.1 *Peri-implantation development*

The polar TE does not play a role during implantation as its multipotency is maintained through upregulated CDX2 [70]. Instead, the polar TE proliferates and change morphology by becoming columnar due to the actomyosin contractility imposed by the tissue boundary. As a result, the polar TE gives rise to the ExE and ectoplacental cone (EPC) which will become the placenta [70, 72]. The ExE forms a discrete cup-shaped layer of cells at the proximal axes of the embryo. During the final stage of blastocyst to egg-cylinder transformation, the PrE that directly lines the Epi differentiates into the visceral endoderm (VE), which will cover both the Epi and the newly formed ExE, and the parietal endoderm (ParE). Insights into the stage of development found, by analysing the PrE at consecutive implantation stages, the PrE is first cuboidal in shape and localize at the distal tip of the Epi whereupon implantation they become columnar [70]. However, as development progressed and the polar TE expands, the PrE at the distal tip change shape again into cuboidal cells as they spread over the Epi forming the VE layer, and the ParE that migrates toward the mural TE [70]. By E5.0, the morphological transformation into the egg-cylinder is completed comprising the Epi and ExE, which is lined by the VE (Figure 5) [74, 75].

At E5.0, the naïve pluripotent Epi initiates epithelialization by forming an epithelium with defined polarity. The cells in the middle of the Epi that has no direct contact with the extracellular matrix (ECM) undergoes apoptosis giving rise to the Epi cavity [74, 75]. A second cavity emerges through apical constriction driven invagination of the ExE, forming an ExE cavity, that will eventually unite with the Epi cavity forming a single pro-amniotic cavity [70] and the conceptus starts to elongate along the proximal-distal (P-D) axis (figure 5). By this stage, the Epi enters a state of primed pluripotency.

1.3.2 *Pre-gastrulation patterning*

Reciprocal signalling between the Epi, VE, and ExE by secreted growth factors of the transforming growth factor β (TGF- β) superfamily, including Nodal [76] and bone morphogenetic protein-4 (BMP4), along with Wingless-INT-3 (WNT3) and FGF families results in regionalized gene-expression patterns. Signalling from these molecules are required for the successful establishment and specification of the anterior-posterior (AP) axis. Signalling during this period is crucial as the AP axes establishment sets the stage for cell patterning and is essential for all subsequent processes during embryogenesis, including cell lineage allocation and tissue differentiation [77].

The mechanisms whereby the AP axes is established, and cells are patterned is not yet fully understood, but one classical explanation for this has been built around the notion of Positional Information. Introduced by Lewis Wolpert in 1969 [78], positional information states that cells acquire fates by reading and interpreting molecular instructions encoded in signalling molecules termed morphogens. These morphogens diffuse from a fixed source thereby creating a concentration gradient and cells are then transformed into various fates depending on their position relative to the morphogen [79-81]. In mouse embryogenesis, BMP4 is crucial for initiating AP patterning and gastrulation.

1.3.1 *Anterior-Posterior axis establishment and Primitive Streak formation*

At E5.0 a specialised group of VE cells form at the distal portion of the embryo, termed the distal VE (DVE) and is morphologically distinct from the neighbouring VE as they differentiate from squamous epithelium to columnar epithelium [82] (Figure 5). Nodal is initially expressed within the Epi and is maintained by VE signalling [83]. Nodal is secreted from the Epi as a precursor whereupon pro-Nodal expressing factors cleave nodal into mature Nodal. The Nodal co-receptor crypto and mature Nodal activates the type I-II receptor complexes resulting in phosphorylation of downstream intracellular effectors Suppressor of Mothers against Decapentaplegic 2 (SMAD2) and SMAD3 in the overlying VE (Figure 6) which translocate to the nucleus where they bind to co-factors, Co-SMAD and SMAD4 to target the activation of downstream genes such as Forkhead box transcription factor 2 (FOXA2) and LIM homeobox protein 1 (LHX1), which, together with SMAD2, signals the production of extracellular Nodal antagonists Left-right determining factor 1 (Lefty1), and Cerberus-like protein 1 (Cer-I) within the DVE [77, 82, 84-86]. Lefty1 and Cer-I forms a negative feedback loop with Nodal by restricting

Nodal signalling to the most proximal part of the Epi (Figure 5) thereby creating a signalling gradient of Nodal within the Epi [77].

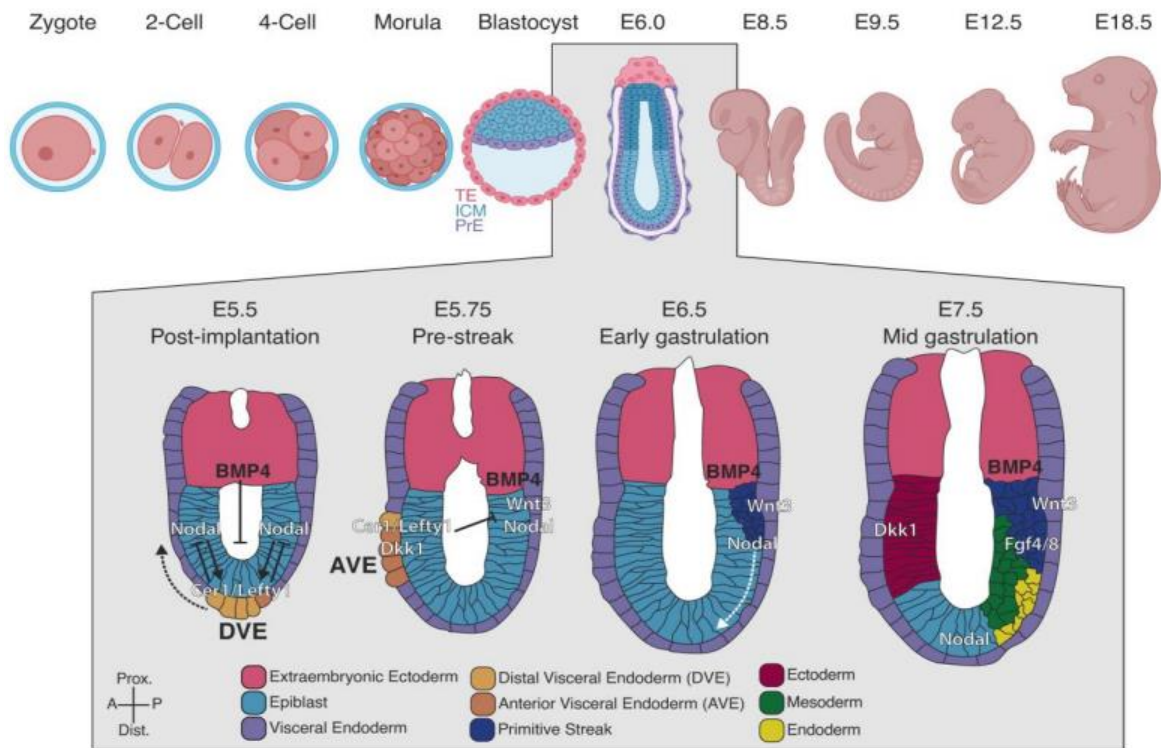


Figure 5: Stages of embryonic development illustrating anterior-posterior axis patterning prior and during gastrulation. The ExE secretes BMP4 by E5.0 inducing the uniform expression activation of Nodal and WNT3 in the Epi. At the same time, the DVE cells become columnar in shape and start to express Nodal antagonists; Cer1 and Lefty1. The DVE breaks symmetry as it undergoes active cell migration to the anterior of the embryo forming the AVE which secretes Cer1, Lefty, and a WNT3 antagonist Dkk1. As a result, WNT3 and Nodal is concentrated in the posterior of the Epi resulting the formation of the PS. The PS elongates and initiates gastrulation as Epi ingress through the PS forming the mesoderm and endoderm germ layer. On the opposite side, where the concentration of WNT3 and Nodal is absent, the ectoderm is formed by E7.5. Figure adapted from Bardot and Hadjantonakis, 2020.

Nodal signalling is essential for AP establishment, as it has been observed in a study by Robertson *et al* [87] that the elimination of FoxH1, a Nodal enhancer required for regulating Nodal activity, from the mouse germ line resulted in subsequent Nodal mutant embryos undergoing developmental arrest prior to gastrulation, indicating its role in Epi and ExE patterning [87]. Nodal maintains interactions with the proximal ExE whose signalling is crucial for proximal Epi patterning. The ExE secretes the BMP4 morphogen whose ligands signal through SMAD1, SMAD5, and SMAD8 [88] to activate WNT3

expression uniformly within the Epi while maintaining Nodal expression (Figure 5) thereby establishing a feedback system with Nodal and WNT3 [77, 83, 88, 89].

Shortly after, at E5.75, the DVE breaks radial symmetry by undergoing active cell migration towards the anterior of the embryo until it reaches the boundary between the Epi and ExE, forming the anterior VE (AVE) [90, 91]. This rapid migration of the DVE towards the prospective anterior of the embryo establishes the AP axis of the embryo [82, 92]. The AVE continues to act as a signalling centre by secreting Nodal antagonists, however, starts to secrete a WNT3 antagonist, Dkk1 (Dkk1) thereby further restricting both Nodal and WNT3 activity to the proximo-posterior of the Epi [77, 93, 94]. As a result, an AP gradient of WNT3 and Nodal is established within the Epi (Figure 5). The AVE will pattern the future anterior side of the embryo by imparting neuroectodermal character [94], whereas Nodal and WNT3 signals in the posterior of the embryo will instruct cell fate specification into mesodermal and endodermal fates.

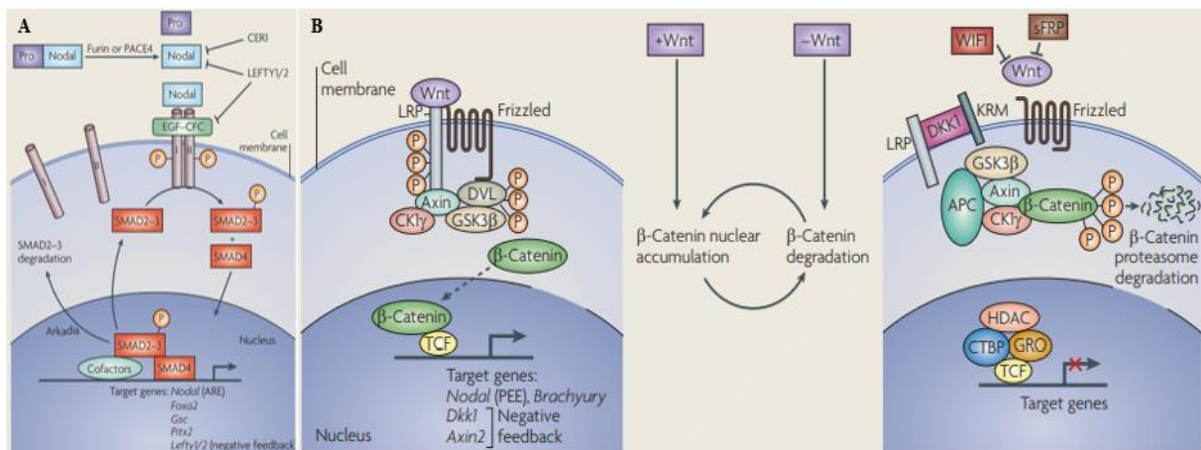


Figure 6: Representation of the Nodal and Wnt3 pathway. A: Pro-Nodal is cleaved into mature Nodal which binds to and phosphorylates EGF-CFC receptors thereby activating SMAD2 and SMAD3 signaling. Upon translocation to the nucleus, SMAD2 and SMAD3 binds to co-factors and SMAD4 to activate target genes such as Nodal, FOXA2, and Lefty1. B: Activated WNT binds to LRP and frizzled receptors which recruit GSK3 β allowing β -catenin to translocate to the nucleus where it binds to TCF to upregulate Nodal, Bra, and Dkk1. In contrast, during WNT inhibition, β -catenin is degraded in the cytoplasm as GSK3 β binds to β -catenin forming a destruction complex and, as a result, no target genes are activated. Figure obtained from Arnold and Robertson [77].

1.4 Gastrulation

At E6.0, the Epi is patterned by regional differences in gene expression setting the stage for the process of gastrulation. During gastrulation, molecular patterns within the Epi occurs followed by morphological changes giving rise to the three germ layers: ectoderm, mesoderm, and definitive endoderm. The germ layers comprise all progenitor cells from which all foetal tissues will develop [95]. The successful orchestration of the mechanisms leading to gastrulation is pivotal for successful embryonic development, and therefore, highly sensitive to genetic and mechanical perturbations.

1.4.1 *Initiation of the Primitive Streak*

Formation of the PS, a groove in the proximal Epi (Figure 5), is dependent on high WNT3 and Nodal signalling at the proximo-posterior of the embryo [86, 96, 97]. Liu *et al* [96] generated a targeted mutation in mESCs to inactivate WNT3 and found that WNT3 mutant mice were able to form an egg cylinder but lacked Bra and FGF8 expression which are markers of the PS. As a result, these mice lacked the PS and could not undergo gastrulation thereby highlighting the absolute requirement of WNT3 for PS formation in mice. Upon WNT3 activation, WNT ligands bind to frizzled low-density-lipoprotein receptor-related protein (LRP) co-receptors triggering its phosphorylation by casein kinase 1 γ (CK1 γ) and glycogen synthase kinase 3 β (GSK3 β) [77] (Figure 6). The LRP and frizzled receptors recruits Dishevelled (DVL) and axin proteins which prevents the formation of a β -catenin phosphorylating 'destruction complex'. As a result, β -catenin is free to translocate from the cytoplasm to the nucleus where it interacts with T-cell-specific factor (TCF) transcription factors to regulate target gene transcription of genes required for the formation of the PS. In contrast, the removal of WNT3 renders the β -catenin 'destruction complex' active resulting in the degradation of phosphorylated β -catenin by proteasomes in the cytoplasm. Consequently, β -catenin is depleted in the nucleus and cannot bind to TCF, thereby target gene activation is inhibited (Figure 5) [77].

It has been conclusively shown that canonical WNT signalling is required for the initiation of gastrulation. Previous work by Liu *et al* [96] generated a targeted mutation in mESCs to inactivate WNT. The results found that while the wild-type mice were able to gastrulate and form organized structures, WNT3 null mutants lacked the PS and the mesoderm germ layer whilst subsequently dying by E10.5 of embryogenesis as no WNT3 null mutants could be recovered at this stage [96]. Barrow *et al* [98] also established the requirement of WNT3 during gastrulation. In this study, mESCs lacking a functional WNT allele were generated and marked by ubiquitous β -galactosidase expression. These WNT $^{-/-}$ mESCs were then aggregated with wild-type morulae to generate chimeric embryos where the extraembryonic tissues were derived uniquely from wild type host cells. Subsequent WNT3 null embryos resulted in defects in axial realignment. Examination of conditional WNT3 activity removal

by chimeric analysis revealed the requirement of WNT3 expression for PS formation and subsequently mesoderm formation [98].

The PS continues to elongate for the next 36 hours. During this period, BMP4 gives rise to extraembryonic tissues, including the mesodermal layer of the chorion and the visceral yolk sac mesoderm and blood islands. By E6.0, the T-box transcription factor Brachyury (Bra), a downstream target of β -catenin, is expressed throughout the entire PS [99].

1.4.2 *Formation of the nascent mesoderm*

The nascent mesoderm is the first germ layer to emerge during gastrulation and forms between the Epi and overlaying VE [100]. BMP4 signals from the ExE and high canonical WNT3, NODAL and FGF signals are required for mesoderm induction. This was confirmed in a study by Dunn *et al* [101] by manipulating the expression ratios of SMAD2 and SMAD3 *in vivo* and evaluating the changes by in situ hybridization and quantitative reverse transcriptase polymerase chain reactions (qRT-PCR). SMAD2; SMAD3 double heterozygous animals are fully viable and fertile in contrast to loss of SMAD3 in one wild-type copy of SMAD2 animals which resulted in embryonic lethality around EE9.5. These SMAD3 mutant embryos illustrated defective anterior patterning resulting in their developmental arrest. However, SMAD2:SMAD3 double homozygous mutants entirely lacked the mesoderm and failed to gastrulate, thereby showing the importance of Nodal signalling through SMAD2 and SMAD3 in dose-dependent behaviours initiate both AP patterning and subsequent mesoderm formation [101].

Epi cells near the PS becomes downregulated in ECAD resulting in the loosening of epithelial adherence junctions rendering the Epi cells detached from the basement membrane [102]. ECAD becomes downregulated as a result of FGF8 signalling through FGFR1 which induces the expression of Snail Family Transcriptional Repressor 1 (SNAIL1) [103, 104] which binds directly to the promoter of ECAD thereby inhibiting its activity allowing epithelial cells in the Epi to break away from the tightly ordered columnar epithelium.

The phenotypes of FGF8 and FGF4 Epi mutant embryos were analysed and showed that Epi cells move into the PS and underwent an EMT where they failed to migrate away from the PS to form the mesoderm and definitive endoderm [105]. In addition, in embryos where SNAIL1 is eliminated the mesoderm is able to form, however, these cells retain epithelial morphology as they fail to efficiently downregulate ECAD expression [104]. As a result, the EMT program is activated and the Epi cells ingressing through the PS undergoes morphological and cytoskeletal rearrangements.

EOMES plays a crucial role in the EMT and mesodermal cell migration [106, 107]. EOMES is first expressed in the TE where it becomes restricted to the ExE following implantation and subsequently

being induced during PS formation with genetic evidence suggesting that it is a target of Nodal [106]. A well-described SOX2.Cre strain [108] was used to delete EOMES in Epi derivatives [106]. The subsequent EOMES mutant embryos expressed markers of the nascent mesoderm through the upregulation of Bra and WNT3, but form a thickened PS, thereby failing to migrate. It was found that EOMES mutant embryos failed to effectively downregulate ECAD while FGF8 and its targets SNAIL1 expression was unaffected. These EOMES mutant embryos also entirely lacked the definitive endoderm thereby indicating that EOMES is implicated in the EMT and definitive endoderm formation [106].

The basic helix-loop-helix transcription factors mesoderm posterior 1 (MESP1) and MESP2 also plays a role in the EMT during gastrulation and further on during development by serving as markers for cardiac progenitors [109, 110]. Another transcription factor, MIX1 homeobox-like 1 (MIXL1), is expressed in the intermediate PS [111] and plays a role in the EMT during gastrulation. The cells from the very posterior Epi ingress through the PS first giving rise to the extra-embryonic mesoderm, followed by the migration of cells from the mid PS contributing to the lateral plate, cardiac and paraxial mesoderm, and finally the anterior PS cells giving rise the axial mesoderm including the notochord [103, 104, 112-114]. As the nascent mesoderm ingress below the Epi and migrate to extend towards the anterior side, the embryo promptly increases in size and cell number.

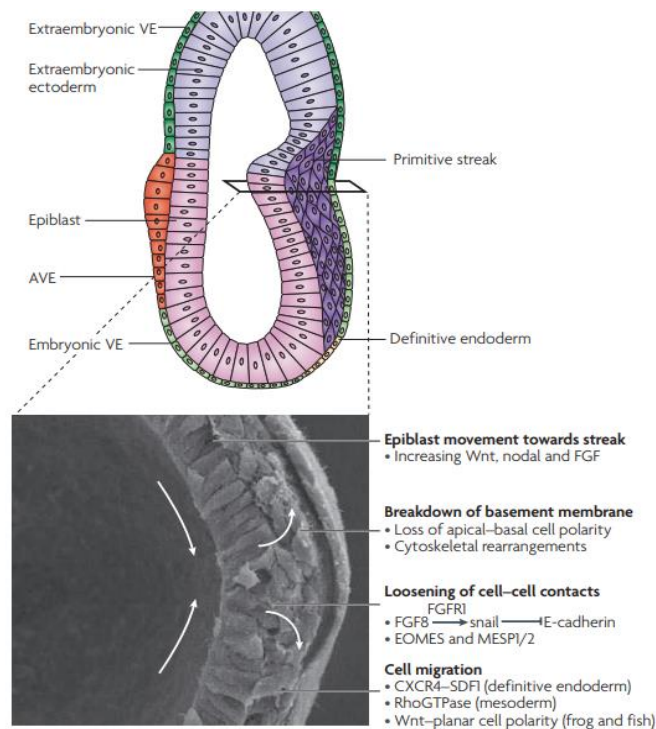


Figure 7: Representation of the EMT process at the PS during gastrulation. Cells from the Epi travel towards the PS where they become influenced by high levels of WNT3, FGF8, and Nodal. As a result,

the Epi cells near the PS lose their apical-basal polarity and detaches from the basement membrane to undergo cytoskeletal rearrangements enabling them to migrate. Signaling events involving FGF8 and SNAIL1 downregulates ECAD thereby allowing cells to migrate through the PS. EMT is initiated through CDH1 downregulation by EOMES, MESP1, and MESP2. The nascent mesoderm germ layer is formed between the Epi and VE. Figure from Arnold and Robertson [77].

1.4.3 Formation of the definitive endoderm

The second germ layer to form is the definitive endoderm at E6.5 as Epi cells migrate anteriorly between the Epi and VE [115]. The endoderm progenitors prevent the activation of EMT transcription factors and retain ECAD expression indicating a partial EMT followed by a rapid mesenchymal-to-epithelial transition (MET) in the region of the PS that lack SNAIL1 expression [116]. EOMES acts downstream of Nodal to regulate the formation of the definitive endoderm. Additional transcription factors such as SOX17 [117], GATA4, GATA6, MIXL1, and FOXA2 [118] are crucial in the specification of the definitive endoderm. By E7.5 the definitive endoderm successfully segregated from the mesoderm progenitors as a result of tight gene junction activation and changes in protein expression levels [77, 92, 93] and will give rise to all internal tissues, organs, digestive-, and respiratory systems.

1.4.4 Formation of the ectoderm

The cells in the anterior of the Epi fail to migrate through the PS, and instead give rise to the neuroectoderm (NE) from which the central nervous system (CNS) and epidermis originates. The loss of pluripotency in the Epi and subsequent differentiation into the NE has been suggested to be as a result of loss of Nodal [119]. Embryos deficient in Nodal were investigated and found to adopt anterior identity with gene expression analysis illustrating that Nodal deficient Epi cells prematurely acquired neuroepithelial characteristics despite the Nodal mutant embryos lacking AVE and mesendoderm derivatives [119]. This study argued the possible role of Nodal in preventing the emergence of anterior neural fates prior to gastrulation. In addition, the continued expression of Cer1 and Lefty1 by the AVE ensures the maintenance of NE precursors at the anterior side of the embryo as double mutant embryos lacking Cer1 and Lefty1 illustrates expansion of the mesoderm with no NE formation [97].

By E7.0, the BMP4 gradient is dampened by Noggin and Chordin, BMP4 antagonists, such that the portion Epi cells closest the node, where the BMP4 concentration is low, differentiation into the NE express SOX1 [120] in the absence of SMAD signalling. In addition, AVE signalling and signalling from the emerged mesoderm and definitive endoderm germ layers facilitates cell fate specification of the NE by inducing the expression of neural specific genes such as Engrailed homeobox 1/2 (EN1/2) and Orthodenticle Homeobox 2 (OTX2) whose activity is required for development of the future head,

brain, and CNS after gastrulation [121, 122]. In contrast, high BMP4 signalling, mediated by SMAD1/5/8, will form the surface ectoderm (SE) which will form the epidermis by E7.5 [123-125]. BMP4 can no longer influence lineage commitment by E7.5 as cells have lost competence to form the opposing tissue [126]. BMP4 is thus an important regulator of the events that occur during gastrulation and anterior neural induction and its range of action should be tightly controlled for these events to proceed normally.

Gastrulation is completed by E7.5 with cell fates determined along the AP axis depending on the cell's exposure, concentration, and duration of Nodal, FGF, BMP4, and WNT3 [76, 77, 127, 128]. The embryo inverts following the completion of gastrulation bringing the definitive ectoderm to the outside, the definitive endoderm to the inside, and the definitive mesoderm remains as the middle layer. The failure to gastrulate results in embryonic death shortly after implantation [129, 130].

1.5 The role of the extracellular matrix during embryogenesis

The ECM is a complex heterogeneous and dynamic network of macromolecules forming both the chemical and physical environment surrounding all cell types [131]. The ECM was once considered only as a supportive scaffold but has been found to play a critical role during development, due to its ability to regulate adhesion, cell movement, and communication [132-134]. The proteins that comprise the ECM confers instructive signals to the surrounding cells during morphogenesis [135-137] while the physical properties of the ECM, such as stiffness and porosity, change throughout development to aid in propagating and resisting forces that influence cell behaviour such as migration, polarisation, survival and differentiation [138]. However, due to its heterogeneity in composition, the precise role of its constituents has remained a challenging area to study. One major discovery was the modularity of the ECM components, whereby different assemblies of the ECM components yield specialized functions during development [139].

While some ECM components are present during preimplantation, the basement membrane is the first ECM structure to form shortly before implantation [140]. The basement membrane comprises collagens and laminins that form independent networks linked by nidogens [140, 141]. While mice carrying null mutations in genes encoding nidogen and chains of collagen are able to successfully develop into blastocysts and implant [142, 143] suggests that these ECM components may not be required to drive blastocyst formation.

In contrast, gastrulation has shown to be dependent on active signalling from the ECM to ensure extensive cellular reorganization and coordination of morphogenesis, differentiation, and proliferation [144]. Specifically, the fibronectin family of ECM proteins has been suggested to play a

vital role during gastrulation when fibronectin null mouse embryos could initiate gastrulation normally but showed a shortened AP axes and severe defects in mesoderm derived tissues [145]. Due to the mesoderm defects, the variable deformation in heart formation was observed [145]. This may suggest that the structural support provided by the molecules within the ECM is important for elongation. Laminin has also been suggested to restrict migration into narrow streams during the development of the neural crest whose formation is dependent on the directed migration of neural crest cells along the ECM [146]. Laminin alpha5 mutant mice show expanded neural crest migratory streams compared to wild type mice suggesting its requirement for proper migration and differentiation of neural crest cells [146].

Furthermore, ECM degradation during embryogenesis by collagen cleavage has been shown to produce biologically active fragments such as endostatin that regulates migration and proliferations [147]. In addition, the degradation of the ECM can also release important signalling molecules such as WNT and FGF which play critical roles during gastrulation [148]. Finally, the spatial-temporal regulation of the ECM has been shown to be critical for differentiation. Myogenic differentiation in the mouse limb bud occurs as collagen and laminin expression increases while fibronectin expression decreases [149] suggesting that ECM remodelling throughout embryogenesis aid in cell fate specification of tissues [150]. While it is clear that the ECM plays a critical role in normal development and morphogenesis, a growing area of interest comprise the mechanical contributions of ECM architecture to these processes during embryonic development.

1.6 *In vitro* models of mouse embryogenesis

Insights into mammalian embryology largely stem from research on animals, specifically the use of embryos derived from mice. However, studying embryo development is particularly challenging. First, accessing *in vivo* embryos is made difficult during embryogenesis. During preimplantation development, the embryo is located within the oviduct and uterine flushing cannot always remove the embryo thereby having to resort to time-consuming methods such as surgically removing the embryos. During implantation development, as soon as the embryo attaches to the endometrium it becomes inaccessible for direct *in vivo* experimentation with this period being referred to as the 'black box of development' [3] while also becoming technically difficult to remove. Second, obtaining human embryos for research and experimentation is very difficult due to costs and legal implications. In addition, human embryos cannot be cultured past 14 days, which is before the process of gastrulation. There is also the consideration of *in vitro* fertilization (IVF) embryo validity for research as 'IVF rejects' might be considered inferior to those obtained *in vivo* [151]. Given that the crucial period of post-implantation development occurs *in utero*, the complete representation of the signalling mechanisms

driving cell fate specification and morphogenesis during embryogenesis still remains unclear. Therefore, the discovery that pluripotent ESCs can be derived from the ICM of the pre-implantation blastocyst and directed *in vitro* into various embryo-like structures established a new field of biology to gain insights into the mechanisms that drive development that was not possible with *in vivo* embryo research while also being easy to access, track, manipulate, and scale [152, 153].

1.6.1 *Maintaining embryonic stem cell pluripotency in culture*

As development progresses, all cells within the embryo gradually lose their potency; from a totipotent zygote that will give rise to the whole organism, to fully differentiated adult cell types that make up each lineage in the body. As discussed earlier, the Epi represents two stages of pluripotency where the preimplantation Epi cells are considered to be in a naïve state of pluripotency capable of contributing to all embryonic tissues of the foetus and do not express differentiation genes. In contrast, the post-implantation Epi is considered to be in a primed state of pluripotency referring to the ability to contribute to any embryonic lineage of the foetus, but are expressing some differentiation genes [46]. Each of these states can be captured *in vitro* using embryonic and extra-embryonic stem cells. mESCs derived from the pre-implantation blastocyst were the first stem cell line to be derived and isolated in 1981 [154] and have the potential to self-renew in culture while maintaining a normal karyotype, and serve as a tool in the study of developmental biology [155, 156].

mESCs are transcriptionally equivalent to the E4.5 Epi of the mouse embryo [65]. mESCs were historically maintained in co-culture with mitotically inactive mouse embryonic fibroblasts (iMEFs) in serum containing medium [154] and was later discovered that the active components maintaining pluripotency within the medium was the BMP signalling pathway [157] and leukaemia inhibitory factor (LIF) pathway [158] which could support mESC self-renewal in the absence of feeder cells. These molecule components stimulate the expression of OCT4, SOX2, and NANOG [156] which is characteristic of the pluripotent Epi, while BMP can also inhibit signalling via the MAPK/ERK pathway thereby preventing cell differentiation [159].

LIF belongs to an interleukin-6 family of cytokines that mediate inflammation, neural regeneration and embryonic development [160]. A signalling cascade is initiated when LIF binds to a low-affinity LIF receptor (LIFR) which is associated with co-receptor subunit glycoprotein-130 (gp130) on mESCs [161]. LIFR and gp130 forms heterodimers and activate three signalling pathways; the tyrosine Janus kinase (JAK/STAT3) pathway, phosphatidylinositol-3phosphate kinases (PI3K/AKT) signalling, and MAPK/ERK signalling [162]. The two former pathways promote self-renewal while the latter promotes differentiation. JAKs phosphorylates the tyrosine residues thereby recruiting cytoplasmic STAT3 which in turn becomes substrates for JAK-mediated phosphorylation [163]. As a result, STAT3 dimerized and

translocated to the nucleus, where the homodimers activate gene transcription of OCT4, SOX2, and NANOG while simultaneously inhibiting differentiation [164-166]. The LIFR and gp130 receptor complex dimerization leads to the phosphorylation of p85 and SPH2 thereby activating the PI3K and MAPK/ERK pathways, respectively. However, it has been demonstrated that only the JAK/STAT3 pathway is essential for LIF-mediated self-renewal of mESCs [161]. In this study, Niwa *et al* observed that a dominant-negative mutant of STAT3 blocked endogenous STAT3 activation which abrogated mESC self-renewal even in the presence of LIF [161].

In contrast, LIF-mediated MAPK/ERK signalling downregulates NANOG, OCT4, and SOX2 and prepare mESCs for differentiation when conditions are favourable [167-169]. Therefore, inhibition of the LIF-mediated MAPK/ERK pathway is essential to promote continued mESC self-renewal in culture. This advancement in understanding how this pathway affects pluripotency has led to the establishment of completely defined culture conditions for mESCs that do not rely on feeder layers of iMEFs or serum containing medium. This culture condition consists of LIF, and two small molecule inhibitors (2i) referred 2iLIF medium. One of the inhibitors is PD0325091 (MEK inhibitor) and functions by blocking MAPK/ERK signalling thereby inhibiting differentiation, whilst the other inhibitor molecule is CHIR99021 (Chiron/CHIR) which is a glycogen synthase kinase (GSK)-3 β inhibitor, a known repressor of WNT3 signalling. When Chiron is added along with LIF and the MEK-inhibitor, Chiron promotes active WNT3 signalling which makes the cells more resistant to other pro-differentiation signals such as FGF4 [170-173]. The 2iLIF defined conditions allow mESCs to be maintained in a homogenous ground state of naïve pluripotency, however, some heterogeneity has been reported in this culture [174]. In contrast, removing 2iLIF from the culture medium disrupts the pluripotency pathways thereby signalling mESCs to exit their pluripotency state by undergoing differentiation. By changing the culture conditions, various embryo-like structures can be formed *in vitro*, serving as a powerful tool for elucidating key aspects of embryogenesis while being followed in real-time, offering a level of experimentation not possible *in vivo*.

1.6.2 Embryoid bodies

The field of *in vitro* stem cell models of embryogenesis originated in the 1960's and 1970's as a result of a coincidental observation when the analysis of neonatal mouse testes teratocarcinomas exhibited a structural similarity to the day 5 developing mouse embryo [175]. The teratocarcinoma cells were able to aggregate and spontaneously differentiate when left in culture, albeit in a spatially disorganised manner. [176-178]. These tumour aggregates were given the term embryoid bodies (EBs) and, although disorganized, illustrated reproducible axial morphogenesis potential *in vitro* [106]. In addition, EBs formed from embryonal carcinoma (EC) were found to be transplantable [177, 179], and

when EC were isolated from tumours and aggregated into EBs, they differentiated into mesoderm and endoderm layers in a spatially organized manner resembling the mouse embryo at day 5 of development [178, 180]. EC cells were soon replaced by mESCs following their isolation in 1981 [154] to form EBs *in vitro* that differentiated into blood islands and myocardium [181].

In the following 30 years, EBs have been broadly used for the differentiation of cell types originating from all three germ layers [182, 183]. Unfortunately, EBs still remain inadequate to fully recapitulate the embryonic environment faithfully. Firstly, there is no defined scale-up platform for EBs. The methods for forming EBs are based on preventing mESC attachment to the surface of culture plates, thereby allowing aggregate formation. The two main methods of EB formation are either by hanging drop culture, which is inherently incapable of large-scale production and labour-intensive or static suspension culture where there is no control of cell aggregation and EBs often fuse together forming large aggregates. This negatively impacts cell proliferation and differentiation while also contributing to extensive cell death [184]. And since the precise number and spatial coordination of various cell interactions influence mESC differentiation [185, 186], it is crucial to be able to control the quality, number, and size of EBs *in vitro*. Traditional EBs also lack the embryonic architecture of the embryo during gastrulation while exhibiting unorganized tissue differentiation and therefore does not faithfully mimic the *in vivo* embryo.

More recently, a pioneering study by ten Berge *et al* shown that large EBs formed from mESCs, in which the WNT pathway is activated, could self-organize and result in some level of axial organization which was demonstrated by asymmetric WNT3 signalling and Bra expression with some cells undergoing an EMT (Figure 8A) [187]. While this study provides an improvement to traditional EB formation, these polarised EBs only recapitulate gastrulation-like events and AP determination to a limited extent [187, 188]. Interestingly, when EBs were cultured in serum, they developed to resemble exogastrulae and formed an extension signified by a Bra expression domain in a WNT dependent manner. However, despite the occasional occurrence of spatial organisation, these regions are still not structured as in the embryo. These advances in EB culture have led to further optimization of culture methods to form more advanced *in vitro* stem cell models that recapitulate various aspects of pre- and post-implantation development [10, 189-192].

1.6.3 Pre- and peri-implantation models

Both pre- and peri-implantation models aim to recapitulate the developmental events leading to blastocyst formation. By combining ESCs and extraembryonic stem cells *in vitro*, a tractable model system, referred to as hybrid/whole-conceptus models can be formed to study early embryogenesis. As discussed earlier, contributions from extraembryonic tissues are required within the Epi as its

absence results in developmental arrest [193, 194]. When combining mESCs with mouse trophoblast stem cells (mTSCs) and/or extraembryonic endoderm cells (XENs) different *in vitro* structures can be formed in culture [195-197].

The earliest whole conceptus model is the 'blastoid' developed by Rivron *et al* [195]. To form blastoids, mESCs of defined numbers were aggregated into EB-like structures in microwell arrays whereafter a defined number of mTSCs are seeded onto the mESC EB-like structures (Figure 8B). In the presence of WNT and adenosine monophosphate, the mESCs and mTSCs starts to interact with one another whereupon they self-organize and cavitated with the mTSCs forming a cyst-like structure containing a cluster of mESCs on the inside with a blastocoel cavity. This morphology characteristic is similar to the cellular organization of the developing mouse E3.5 blastocyst [195]. In addition, blastoids are similar in size, shape, expression pluripotency markers in the ICM-like compartment and TE-like markers in the mTSCs derived cyst, and also have the ability to implant in utero. Unfortunately, although blastoids induce decidualization when transplanted into the mouse uterus, they fail to develop further [195]. Blastoids revealed the importance of morphogen gradients and their interactions with transcription factors involved in the architecture of the embryo while also emphasizing the role of BMP and Nodal in regulating TE proliferation and epithelialization [195]. Recent attempts have utilised expanded potential mESCs to form a PrE-like layer within the blastoids [198, 199] while other efforts aimed at prolonging blastoid culture until the gastrulation stage by incorporating extraembryonic endoderm cells in blastoid culture [200].

ETS embryos are hybrid models aimed to model post-implantation development. To form ETS embryos, mESCs and mTSCs are grown in medium supplemented with FGF4 to maintain TSCs in a proliferative state and aggregated in a three-dimensional (3D) extracellular matrix known as Matrigel [196, 201]. Matrigel is a reconstituted commercial basement membrane matrix derived from Engelbrecht-Holm-Swarm mouse sarcoma tumour cells and is thought to recapitulate aspects of the ECM that is produced by the PrE [202]. By 120 hours of culture, ETS-embryos embedded in Matrigel mimic the events of the peri-implantation embryo from E5.0 to E6.5 as this model induces Epi-like rosettes in mESCs, undergo pro-amniotic cavity formation, symmetry breaking, and exhibit morphogen gradients (Figure 8C) [75, 196, 201]. The addition of FGF4 exhibited the importance of the ECM in inducing self-organization of the stem cells while Matrigel mimicking the PrE layer provided structural and chemical support. Under BMP stimulation, primordial germ cell-like cells were formed within ETS embryos [196]. Unfortunately, the cell interactions between the mTSCs and mESCs occur only by chance, and as a result, the occurrence frequency of forming ETS embryos are very low.

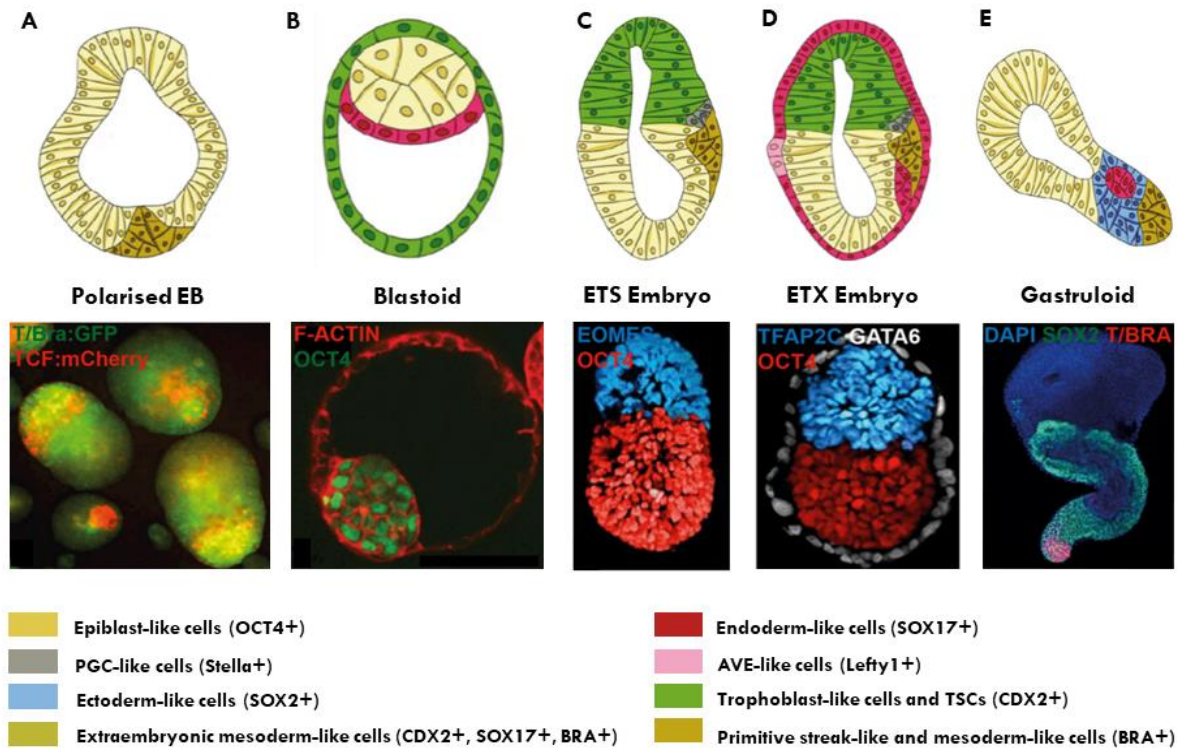


Figure 8: Representation of the main *in vitro* models described. A: Polarised EBs formed solely from mESCs illustrate polarised Bra expression upon WNT signalling. B and C: Blastoids and ETS embryos are both formed by combining mESCs and mTSCs, however, blastoids resemble the blastocyst stage whereas ETS embryos recapitulate the peri-implantation stage while undergoing pro-amniotic cavity formation. D: ETX embryos combine mESCs, mTSCs, and XEN cells and also resemble the peri-implantation stage while exhibiting signs of initiating gastrulation. E: Gastruloids are formed solely from mESCs and illustrate AP elongation and gastrulation. Figure adapted from [203] with images in the lower panel reproduced from A [187], B [195], C [196], D [204], E [205].

The derivation of extraembryonic endoderm stem cells (XEN) from the PrE layer of the embryo led to the substitution of Matrigel as an PrE ECM surrogate in ETS embryos where XEN cells mimic the presence of the PrE and VE *in vitro* [204, 206, 207]. Combining mESCs, mTSCs, and XEN cells in culture led to the formation of ETX embryos (Figure 8D) [204] with XEN cells forming an outer layer surrounding the TE-like cyst structure that encapsulates the Epi-like cluster. ETX embryos undergo symmetry breaking, cavitation, and EMT events without the need for external signalling molecules thereby signifying the role of the extraembryonic endoderm to instruct the Epi as the interactions between the mESCs and XEN cells resulted in Epi-like polarization and epithelialization [204]. Confirmed by transcriptomic analysis, ETX embryos are very similar to E6.5 natural embryos and are able to undergo gastrulation-like events and axial specification of the nascent mesoderm and definitive endoderm layer [204]. In a recent study by Amadei *et al* published in 2022 combined mESCs

with mTSCs and inducible XEN (iXEN) cells to form ETiX embryoids equivalent to E8.5 embryos [208]. ETiX embryoids completed gastrulation and underwent neurulation, as observed by head fold with defined forebrain and midbrain regions, and organogenesis as seen by a beating heart-like structure, a trunk comprising a neural tube, and somites, a gut tube, and primordial germ cells. In addition, the entire ETiX embryoid models develop within an extraembryonic yolk sac that forms blood islands [208]. This model also illustrates the self-organising capability of the stem cells as no additional external signalling was added. Although this model is one of the most advanced *in vitro* models of embryogenesis to date, ETiX embryoids illustrates great variability in size and the efficiency rate of forming these structures are significantly low as the efficiency of ETiX embryoid development from day 4 to 5 was on average 21 % [208]. This may be as a result of mTSCs and mESCs requiring different culture medium which necessitated the use of undefined culture conditions that may have contributed to the difficulty of developing ETiX embryoids *in vitro*. In an attempt to overcome this limitation, a study published in 2022 by Lau *et al* developed an *in vitro* model that reconstitutes the three cell lineages of the post-implantation embryo through transcription mediated induction of stem cells [209]. In this study mTSCs were substituted with mESCs treated with doxycycline to overexpress CDX2. XEN cells were also replaced by inducing GATA4 expression (iGATA4 mESCs) in mESCs, which generated XEN cells at an earlier stage of development. The resulting structures, termed EiTiX embryoids, underwent gastrulation and some neurulation stages through the development of headfolds and brain, as well as organogenesis through the development of beating heart-like structures and the development of extraembryonic tissues such as the yolk sac and chorion. Single-cell RNA analysis revealed a similarity to E5.5 to E8.5 natural embryo [209]. The addition of FGF4 and heparin in culture medium increased the efficiency formation rate, although this rate of 15.5% by day 4 was lower than that of ETiX embryoid formation [209]. In conclusion, in all approaches, formation of ETX embryos is infrequent.

While these hybrid models provide great insights into the mechanisms of embryogenesis, none of these models, to date, have the potential to directly mimic the *in vivo* embryo and have a great limitation in that their formation rates are significantly low. This necessitated research into the development of models that mimic certain aspects of embryogenesis in more detail.

1.6.4 Gastruloids

Research building upon EBs have led to the establishment of gastruloids, an *in vitro* model system formed solely from mESCs that demonstrate the self-organising potential of mESCs by undergoing axial morphogenesis [210, 211]. In this model, developed in 2014, a defined number of mESCs (150 – 300 mESCs) are aggregated in suspension and exposed to specified signalling conditions allowing the

aggregates to self-organise and elongate with structures resembling the post-occipital embryo (Figure 8D) [210-212]. In the original protocol [210-212] mESCs are left to aggregate for 48 hrs allowing for lineage priming and allowing mESCs to enter the Epi-like stage. Chiron is added as a 24-hour pulse at 48 hrs and provides WNT signalling to induce the formation of a PS-like domain with localised Bra expression required for gastrulation-like events to take place [205, 210]. Withdrawal of Chiron results in symmetry breaking, self-organization, and elongation until 120 hrs where the aggregates reached a molecular complexity of an E8.5 – E9.5 developing mouse embryo [213]. Gastruloids are able to undergo germ layer specification along the three embryonic axes and form spatially discrete gene expression domains [213]. An interesting observation from the gastruloid model is the occurrence of self-organization and symmetry breaking in the absence of extraembryonic tissues or signalling provided from these tissues such as BMP4, thereby suggesting their dispensability to capture gastrulation-like events [212]. Time-resolved RNA-sequencing revealed that gastruloid formation mimic the transcriptional changes that occur in the post-occipital embryo [210].

During gastruloid formation, Bra expression is first observed in 48h aggregates upon Chiron treatment, however, in an undefined region. Following the Chiron pulse, a Bra expression pole appears on one side of the gastruloid, and as time progresses, this Bra expression pole extends as the aggregate elongates, a manner similar to what is observed in the mouse tailbud [211]. The Bra expression pole is situated opposite the Gata6 positive domain, corresponding to the cardiac crescent which, in the embryo, marks the post-occipital region [214]. In addition, the expression of SOX2, a neuroectoderm marker, is located centrally as well as at the rostral extremity of the gastruloid, further indicating that Bra is expressed in a region similar to the PS of the embryo. RNA-sequencing on pooled gastruloids confirmed that gastruloids mimic the transcriptional stages taking place in the post-occipital mouse embryo as they share a similar temporal evolution of gene expression with natural embryos [205]. Strikingly Nodal is expressed throughout the gastruloid at 72hrs after which its expression is restricted to a region in the ventral embryo by 120hrs which is similar to the embryo where Nodal is expressed throughout at E5.5 and becomes restricted to the PS at E6.5 where it continues to be expressed until it concentrates at the node when this structure appears [215]. The extrusion of cells from the Bra expression region within the gastruloid indicates that the cells are actively exiting the gastruloid thereby undergoing an EMT-like process.

RNA sequencing on individual gastruloids further confirmed that sequential gene expression occurs reproducibly in a spatial-temporal pattern similar to the genes in the embryo, however, this gene expression patterns do not correlate with morphogenesis thereby suggesting that there might not be enough metabolic activity within the gastruloids to drive morphogenesis [205]. In the same study, Beccari *et al* linked multi-axial patterning in the gastruloids to the spatiotemporal activation of Hox

gene clusters along the AP axis [205], a phenomenon which is conserved across many species [216]. Concomitantly, it was observed that gastruloids with no Chiron treatment grew in size and differentiated into neuroectoderm-like tissue without elongating thereby suggesting that gastruloid elongation is a product of both proliferation and cell rearrangement indicating that Chiron treated gastruloids, hereafter referred to as Chiron gastruloids, are likely undergoing morphogenetic movements as observed by polarised Bra expression [205, 210, 211].

All of this evidence demonstrates the ability of gastruloids to reproducibly mimic early developmental events *in vitro*. Unfortunately, gastruloids are limited in that they lack anterior neural lineages. It was shown in a study by Turner *et al* [211] that Chiron gastruloids resemble Dkk mutants for which anterior domains are posteriorized as a result of excess WNT signalling thereby reporting why Chiron gastruloids fail to form anterior neural lineages. It may also be as a result of the lack of extraembryonic tissues which has a known role in induction of brain development thereby suggesting why gastruloids fail to develop tissues corresponding to brain regions. As a result, Chiron gastruloids are considered a good model system resembling the post-occipital embryo. In addition, Chiron gastruloids do not resemble the architecture of the natural embryo but are able to mimic gastrulation *in vitro*, at least at gene expression levels.

In recent years, various laboratories have built upon the initial gastruloid protocol to reveal the developmental plasticity of gastruloids to induce different cell fates by simply changing the culture conditions. In one study [217], the removal of Chiron and supplementation with XAV939, a small molecule inhibitor of the WNT Pathway which has been shown to stabilize the Epi identity [218, 219], formed Epi-like aggregates undergoing elongation and development of an anterior neural domain as demonstrated by localized expression of OTX2, a marker for indicating forebrain and midbrain identity [121]. As a result, the aggregates illustrated a more complete AP development as early WNT signalling inhibition led to the expression of neural-like markers. In contrast, culturing gastruloids with as FGF, ascorbic acid, and vascular endothelial growth factor which are factors known to promote cardiogenesis upregulates cardiac-specific gene expression patterns within the gastruloids [220]. The gastruloids developed contractile heart-like structures with a self-organised primitive heart tube. The heart-like domain contracted at the same pace as within the embryo thereby indicating the physiological relevance of this model [220]. A preprint article has also demonstrated the ability of gastruloids to form gut-like structures, undergo epithelialization during gut-tube formation, and spatial activation of the gene network governing endoderm formation and maturation [221].

The ability of gastruloids to recapitulate somitogenesis, a critical process that give rise to vertebrae, skeletal muscle, as well as to some dermis [222], has also been investigated. When compared to E8.5

natural embryos, they similarly downregulated FGF and WNT signalling while upregulating somite differentiation markers. Unfortunately, they were unable to form somites themselves [223], however, by embedding the gastruloids in 10 % Matrigel, resulted in the formation of a string of somite-like structures along the AP axis [224]. This observation suggests that Matrigel, which mimics some aspects of the ECM, might be essential for somite and neural-tube formation [202], however, as neural-tube like structures can also be induced by replacing Matrigel with XEN cells [225], it still remains unclear whether the observed effects is as a result of mechanical forces exerted onto gastruloids or chemical components within Matrigel or XEN cells. However, while the tractability of the gastruloid system offers an opportunity to gain insights through testing the effect of ECM stiffness or individual components of Matrigel/XEN cells on gastruloid formation, this has remained a poorly studied area. Synthetic hydrogels offer a promising alternative to complex animal-derived hydrogels such as Matrigel and collagen [226] to test the effect mechanical stiffness on gastruloid architecture. One such study investigated the effect of varying concentrations of a synthetic base hydrogel known as polyethylene glycol (PEG) on gastruloids growth [227]. Factor XIII, a transglutaminase enzyme involved in blood clotting [228], was activated by mixing PEG-vinylsulphone (VS) conjugates to form stable hydrogels. This study found that gastruloid elongation was dependent on the stiffness of the PEG-VS hydrogels used and could lead to the development of tissues resembling the E9.5 mouse embryo [227]. Compared to Matrigel, the gastruloids cultured in PEG-VS hydrogels illustrated that cell behaviour is likely dependent on the properties of the ECM environment and not constituents in Matrigel. While these results need to be reproduced, the study highlights that the ECM microenvironment plays a role in driving morphogenesis during embryogenesis [227]. Work on two-dimensional (2D) geometrically confined laminin-coated substrates demonstrated radial patterning resulting in germ layer formation [155] with a similar observation seen in mESCs differentiated in 3D fibrin gels in a stiffness dependent manner [229].

The use of Matrigel has brought up several concerns as they show significant batch-batch variability while its biochemical and physical properties may not be optimally matched to the developing tissues. Taken together with various gastruloid protocols obtaining vastly different results with gastruloids cultured in different percentages of Matrigel, raises the concern of the exact properties ensuing these variability [230]. As a result, Matrigel does not accurately mimic the *in vivo* ECM environment that drives embryogenesis [230]. In another attempt to substitute Matrigel, a study used a more biometric but less defined Matrigel as an ECM component in gastruloids and formed epithelialized and cavitated cysts that resemble the post-implantation Epi morphologically [75, 231]. However, these structures failed to undergo axial morphogenesis, suggesting that culture conditions allowing Epi-like cyst formation were not permissive for continued development. In a recent study, Girgin *et al* attempted

to combine features from ETS embryos with gastruloids to recapitulate peri-gastrulation by coaxing pluripotent stem cells (PSCs) into uniform Epi-like aggregates in a matrix-free manner [232]. Both epithelialized and non-epithelialized Epi-like cells were merged with mTSCs forming EpiTS embryoids that could undergo symmetry breaking with polarised *Bra* expression, axial morphogenesis, and anterior development [232]. To date, despite several studies illustrating the importance of the ECM environment during embryogenesis, the majority of the gastruloid protocols to date still do not take this observation into account [233-235].

In 2021, Xu *et al* drastically modified the gastruloid protocol to achieve a more complex embryo-like organization by simultaneously forming a posterior and anterior domain [236]. This study built on a previous study in 2014, by the same author, in zebrafish [237] which illustrated the importance of morphogen signalling gradients in controlling symmetry breaking and cell fate patterning. It has been demonstrated in the study that opposing BMP4 and NODAL gradients was sufficient to induce the formation of a complete zebrafish embryonic axis *in vitro* [237]. By implementing the findings of this study with the gastruloid protocol, the authors developed a new protocol based on fusing two distinct aggregates of different sizes in which the smaller aggregate is exposed to BMP4 for an 8-hour period to induce BMP4 expression within the mESC aggregate. The small BMP4 treated aggregate induce expression of WNT3 and NODAL thereby serving as the instructing centre once fused with naïve aggregate allowing a controlled internal morphogenetic gradient. The merged structures are termed embryoids and they develop AP and dorsal-ventral patterning observed by both a posterior PS-like domain and an anterior brain-like structure, similar to neural-stage mouse embryo [236]. These embryoids form the three germ layers through a gastrulation-like process and form a concrete axial mesoderm structure, notochord, which is absent in Chiron gastruloids [236]. While this model indicated the importance of morphogen signalling during gastrulation by overcoming one of the limitations from Chiron gastruloids, they fail to examine the effect of the mechanical environment from the ECM on embryonic development.

In conclusion, a critical requirement of any *in vitro* model system is that it presents an accurate reconstruction of the *in vivo* events. *In vitro* models of mouse embryogenesis within themselves are still novel, and while providing excellent opportunities to gain insights into post-implantation development, there is still a need to develop models that fully recapitulate the complexities that occur during post-implantation development. If this can be achieved, such a model has the potential for high-throughput investigations such as drug screening and disease modelling which will greatly reduce experimentation on animals and serve as a greater alternative to test drugs prior to clinical studies.

1.7 Aims and Objectives

This project aimed to address the limitations of the Chiron gastruloid model by investigating the significance of BMP4 morphogens in initiating gastrulation-like events *in vitro* towards developing a more complete embryo-like model. Another limitation to address will be examining the effect of ECM mechanical stiffness during gastrulation-like events, a gap that has not yet been fully examined. To achieve this, the following specific objectives of this project were:

Objective 1: Culture mESCs while maintaining their pluripotency

Objective 2: Investigate the self-organizing properties of mESCs into Chiron gastruloids

Objective 3: Examine the effect of a BMP4 as a pulse and as a signalling centre on gastruloid formation

Objective 4: Assess the morphogenetic changes that occur in gastruloids in the presence mechanical forces

CHAPTER 2

Materials and Methods

2.1 Cell culture preparations

2.1.1. *Cell lines*

For all cell culture experiments carried out during this research, mESCs derived from the 129/Ola mouse sub-strain were used. iMEFs were harvested from E13.5 mouse embryos of C57BL/6 mice.

2.1.2. *Medium for cell culture*

Table 1: Medium compositions used during all cell culture experiments.

Medium	Composition
Inactivated mouse embryonic fibroblast (iMEF) medium	Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM-F12) supplemented with 15% Fetal Bovine Serum (FBS), 1% Glutamax and Penicillin-Streptomycin, and 0.1% 50mM β -mercaptoethanol
2iLIF medium	iMEF medium supplemented with 1% nonessential amino acids (NEAA), 1% Sodium Pyruvate, 10ng/50ml LIF, 3 μ M CHIR99021 (Chiron) and 2 μ M PDO325901 (MEK-inhibitor)
mESC freezing medium	2iLIF medium containing 20% dimethyl sulfoxide (DMSO) and 10% FBS
N2B27 medium	50% iMEF medium and 50% neurobasal A supplemented with 100X N-2 Supplement, 100X B-27 supplement, and 0.05% β -mercaptoethanol
Secondary medium	N2B27 supplemented with 3 μ M CHIR99021 (Chiron)
Iso-Osmotic PBS for Hydrogels	20.7g/litre Solution A (0.15M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, MW 138), 53.7g/litre Solution B (0.15M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ MW 358.14) and 9g/litre NaCl (0.15M) with a pH +/- 7.7

2.2 Co-culture of Adherent cells

2.2.1 *Preparation of iMEF feeder layers*

6-well adherent culture plates were freshly coated with 0.1% gelatin on the day of plating iMEFs. 1ml 0.1% gelatin was added to each well and incubated for 30 min whereafter excess gelatin was aspirated

and left to dry in the tissue-culture (TC) hood. MEFs were inactivated through mitomycin C treatment, which inhibits the cells from replicating without affecting gene transcription and protein synthesis of the cells, during a MEF factory workshop at UCT. iMEFs were retrieved from the liquid nitrogen tank, thawed in a 37°C water bath and resuspended in a 15ml centrifuge tube containing iMEF medium with centrifugation at 2000 Revolutions Per Minute (RPM) for 5 min. iMEF medium was aspirated and replaced with fresh pre-warmed iMEF medium and 1.5×10^5 iMEFs were plated per well containing 2ml iMEF medium. The presence of cells was confirmed by light microscopy and left to settle for 48 hrs in a humidified incubator set to 37°C, 5% CO₂.

2.2.2 Co-culture of mESCs on iMEFs

iMEF feeder plates were checked, by light microscopy, to ensure attachment by 48 hrs. iMEF medium was aspirated and replaced with 2ml fresh pre-warmed 2iLIF medium and incubated for at least 2hrs. mESCs were retrieved from the liquid nitrogen tank and thawed in a 37°C water. Thawed mESCs were resuspended in a 15 ml centrifuge tube containing pre-warmed 2iLIF medium and centrifuged at 2000 RPM for 5 min. Following aspiration of the supernatant, the mESC pellet was resuspended in 2iLIF medium and transferred unto the wells containing the iMEF feeder layers. The co-cultures were maintained in a humidified incubator at 37°C, 5% CO₂ with daily medium changes. The growth and maintenance of mESCs were assessed by light microscopy. Newly thawed mESCs were passaged twice before subsequent experimental use.

mESCs were routinely passaged once they reached a confluency between 60 and 70%. During passaging, 2iLIF medium was aspirated and washed twice with PBS. The plates were then incubated for 20 min with 200µl Dispase (5mg/ml) per well at 37°C, 5% CO₂. Dispase is a gentle enzyme that lifts mESCs without disturbing the iMEF feeder layer. mESC detachment was confirmed by light microscopy and 800 µl 2iLIF medium was added to each well to neutralize the Dispase. The resulting 1ml cell suspension was transferred to a 15ml centrifuge tube for centrifugation at 2000 RPM for 5 min. Following aspiration of the supernatant, the pellet was resuspended in fresh 2iLIF and seeded using a 1:2 - 1:4 ratio, depending on the confluency of the starting culture.

Frozen mESC stocks were prepared by resuspending the mESC pellet in mESC freezing medium as 1ml aliquots in cryotubes for three days at -80°C whereafter they were transferred to a liquid nitrogen tank for long term storage at -150°C. However, for Ribonucleic acid (RNA) collection, the resulting mESC pellet was flash-frozen in liquid nitrogen and stored at -80°C until use.

2.3 Differentiation treatments in suspension cell culture

mESCs were differentiated *in vitro* utilising 3D suspension culture and by changing the culture medium and conditions to allow for differentiation into EBs and gastruloids. All differentiation protocols mentioned below were carried out after mESCs were lifted with Dispase.

2.3.1 Embryoid Body (EB) formation

Dispase was neutralized with iMEF medium and a cut p1000 tip was used to transfer the mESC colonies to a 15ml centrifuge tube, being careful to avoid disrupting the mESC colonies, and were left for 10 minutes to settle at the bottom of the centrifuge tube. The excess iMEF medium was aspirated and replaced with 2ml fresh pre-warmed iMEF medium while slowly triturating using a cut p1000 tip to break the mESC colonies into smaller clusters, being careful not to dissociate them into single cells. thereafter, the mESCs were transferred to a 6cm bacteriological dish containing 4ml pre-warmed iMEF medium and left overnight at 37°C and 5% CO₂. The presence of EBs were confirmed by light microscopy and medium was changed daily. In order to avoid losing EBs during medium changes, the 6cm bacteriological dish was gently swirled in circular motions allowing the EBs to collect at the centre of the plate thereby allowing the aspiration of iMEF medium from the periphery. Care was taken not to remove too much medium that would dry the EBs out. EBs were grown until 144hrs whereafter they were collected for RNA extraction by transferring EBs, with a cut p1000 tip, to a 15ml centrifuge tube and centrifuging at 2000 RPM for 5 minutes. The iMEF medium was subsequently aspirated and the resulting EB pellet were 'flash-frozen' by putting the 15ml centrifuge tube containing the EB pellet directly in liquid nitrogen to kill the cells. The samples were stored at -80°C until use.

2.3.2 Chiron Gastruloid Formation

Chiron gastruloids were formed using the original protocol described in [212], with slight modifications. mESCs were grown to 40 – 60% confluency, rinsed twice with 1ml pre-warmed PBS, and treated with Dispase as per section 2.2, however Dispase was neutralised with iMEF medium instead of 2iLIF medium. mESC colonies were dissociated into a single cell suspension by forcefully ejecting the suspension against the wall of the plate and the suspension was subsequently transferred to a 15ml centrifuge tube for centrifugation at 1300 RPM for 3 minutes. Following aspiration of the supernatant, the mESCs were resuspended in 2 ml iMEF medium and dissociated into single cell colonies by triturating forcefully. In order to ensure that only mESCs are used for differentiation experiments, iMEF depletion was carried out to remove any iMEFs that may have been transferred during Dispase treatment. During this process, the single cell mESC cell suspension was plated onto a freshly coated gelatin-coated plate, in iMEF medium and incubated for 25 min in a humidified incubator. The cell suspension was carefully removed, avoiding disturbing up iMEFs that attached to

the plate, and transferred to a new well. The suspension was triturated again to ensure a single cell suspension, followed by a 20 min incubation in a humidified incubator. The cell suspension was once again carefully removed and transferred to a new well, triturated, and incubated for 15 min in a humidified incubator. Following the final incubation step, the cell suspension was carefully transferred to a 15-ml centrifuge tube for centrifugation at 1300 RPM for 3 minutes. The resulting mESC pellet were resuspended in 1ml pre-warmed N2B27 medium. mESCs were counted via a haematocytometer to determine the density of the suspension and the concentration was then adjusted accordingly to generate 4×10^4 cells/4ml N2B27.

The mESC suspension were subsequently transferred to a sterile boat. A 200 μ l multichannel pipette, set to 40 μ l, was used to transfer the cell suspension to a non-tissue-culture-treated, low-adherence 'U'-bottomed 96-well plate (referred to as 96-well plates from now on) so that each well would have 400 cells/40ul. The presence of cells in each well was confirmed using an EVOS microscope and transferred to a humidified incubator at 37°C, 5% CO₂ to allow the mESCs to settle and aggregate for 48 hrs. Following the confirmation of spherical formation at 48 hrs, fresh pre-warmed secondary medium is prepared by adding 3 μ M Chiron to an appropriate amount of N2B27 in order to add 100 μ l secondary medium to each well at a 45° angle using a multichannel pipette. The aggregates were incubated for 24 hrs whereafter 100 μ l medium was removed by slowly aspirating at a 45° angle, ensuring that 40 μ l is always left within each well to avoid drying out the aggregates. 100 μ l fresh N2B27 medium was added to each well with sufficient force in order to move the aggregates within the well, thereby avoiding adhesion to the plate. Thereafter, medium was changed daily until 120 hrs or 144 hrs.

2.3.3 *BMP4-pulse gastruloid formation*

BMP4-pulse gastruloids were formed in the same manner as with the Chiron gastruloids describes in section 2.3.2, however, with the addition of a BMP4 pulse. 24hrs after mESCs were plated onto the 96-well plates, 100 μ l 1ng/ml BMP4 was added to each well at a 45° angle using a multichannel pipette. Following a 24-hr incubation 100 μ l of the medium was removed and replaced with fresh secondary medium until 72hrs, whereafter medium was changed back to N2B27 and changed daily as per the protocol described in section 2.3.2.

2.3.4 *BMP4 signalling centre gastruloid formation*

To form BMP4 signalling centre (SC) gastruloids, an optimised protocol was formed by combining steps from the Chiron gastruloid protocol and the embryoid protocol developed by Xu *et al* [236]. mESCs were grown until 60% – 70% confluency and lifted with Dispase as per section 2.2. Following

reconstitution in 2iLIF medium, the cell suspension was plated onto freshly made 0.1% gelatin coated plates without an iMEF feeder layer. mESCs were left to settle for 48 hrs and lifted with Dispase which was neutralised in iMEF medium. Subsequently, iMEF depletion was carried out as per section 2.3.2. Following the centrifugation step, the cells were counted via a haematocytometer to determine the density of the suspension which was then adjusted accordingly to generate two cell suspensions, one at a concentration of 2.5×10^4 cells/4ml N2B27 and another at a concentration of 1.5×10^4 cells/4ml N2B27. Each cell suspension was transferred to a different weigh boat and a p200 multichannel pipette was used to transfer the cells to two 96-well plates. The 2.5×10^4 cells/4ml N2B27 cell suspension generated 250 mESCs/40 μ l N2B27 in each well which was left to settle for 48 hrs and labelled 'naïve aggregates'.

The 1.5×10^4 cells/4ml N2B27 cell suspension generated 150 mESCs/40 μ l N2B27 in each well and was left to settle for 40 hrs at which 1ng/ml BMP4 was added to each well as an 8 hr treatment and labelled 'BMP4-treated aggregates'. At 48 hrs, the BMP4-treated aggregates were washed with N2B27 to remove traces of BMP4. 100 μ l Fresh N2B27 medium was added to the naïve aggregates at a 45° angle with enough force ensuring that the aggregates did not adhere to the bottom of the plate. The BMP4-treated aggregates were then transferred to the naïve aggregate plate using a p200 multichannel pipette. After 1 hr, the EVOS microscope was used to confirm that the two-aggregate merged into one aggregate at the bottom of the plate. Aggregates that did not merge at all was marked and excluded from downstream analysis. Medium was changed daily with fresh N2B27 up to 120hrs or 144hrs.

2.3.5 BMP4 signalling centre-gastruloids in PEG-Ac-SH hydrogel scaffold

BMP4 SC gastruloids in a hydrogel scaffold was formed as described in section 2.3.4, however, at 96hrs 40 μ l 1.5% PEG-Ac-SH was added directly to each well by forcefully ejecting to allow the aggregate to move freely within the plate.

2.3.6 Morphological quantification of gastruloids

Gastruloids and EBs were viewed daily using an EVOS microscope after medium changes. Calibrated images were taken of selected gastruloids/EBs, and ImageJ software was used to process the images as well as to measure the length and area of the gastruloids/EBs. The measurements were quantified in Microsoft Excel, and the axial ratio was calculated using the following formula: major axis length/minor axis length.

2.4 Hydrogel scaffold formation

2.4.1 Swelling ratio

Polyethylene glycol (PEG)-Acrylate-Sulfhydryl (SH) hydrogels was constructed at a range of concentrations: 7%, 5%, 2.5%, 2%, 1.875%, and 1.5% to determine to optimal hydrogel composite percentage. Performing swelling ratios on the hydrogel composites gives an indication of the mesh size which may influence nutrient transport throughout the hydrogels and thus influence the proliferation and differentiation of encapsulated cells. The weights required to make up each percentage gel were calculated using stoichiometry of 10-PEG-4-Acrylate and 10-PEG-4-SH. To make a 100µl PEG-Ac-SH gel, the appropriate weight of 10-PEG-4-Ac/50µl and 10-PEG-4-SH/50µl required was measured using a 4 decimal scale and dissolved in Iso-Osmotic PBS by vortexing for 15 seconds. The two constructs were then combined and vortexed again making the 100µl PEG-Ac-SH gel. Two 50µl drops were then placed on a piece of parafilm paper within a tissue culture plate and allowed to completely gel before placing them in a 37°C incubator for 1 hour. Wet tissue paper was placed around the gels to prevent drying out of the hydrogel constructs. The weight of the dry gels (Wd) was measured on the 4 decimal scale by gently removing it from the parafilm with a scalpel. Thereafter the hydrogels were placed in individual 35mm dishes filled with enough Iso-Osmotic PBS to cover the hydrogels. The weight of the swollen hydrogels (Ws) was measured every 24 hrs by blotting off excess PBS prior to weighing until 168 hrs. The swelling ratio of each hydrogel construct was calculated using the formula $(Ws - Wd)/Wd \times 100$ in Microsoft Excel.

2.5 Quantitative real-time polymerase chain reaction (qPCR)

The levels of specific protein-coding genes present during embryogenesis was assessed by qPCR in mESCs, EBs, and the gastruloid variations by quantifying the mRNA levels. In summary, RNA was extracted from the different samples, purified, and converted into complementary deoxyribonucleic acid (cDNA). The resulting cDNA was amplified by PCR using various forward and reverse primers that are specific to a particular gene within the cDNA sequence. The crossover threshold (CT) values were used to calculate the relative fold expression of each gene within the sample.

2.5.1 RNA extraction

mESC and EB RNA sample collection was described in their respective sections. Sample collection for the different gastruloids at 120 hrs and 144 hrs was carried out by removing the medium from the aggregated in the 96-well plates using a p200 multichannel pipette and transferring the medium to a sterile boat. Each 96-well plate is then checked using an EVOS microscope to ensure no gastruloids were left behind. The medium with the gastruloids in the sterile boat was then transferred to a 15ml

centrifuge tube and centrifuged 3000RPM for 5minutes. Following removal of the supernatant, the cell pellet was flash frozen in liquid nitrogen and stored at -80°C or immediately used for RNA extraction.

Total RNA extraction was performed using the High Pure RNA isolation kit (Roche, Germany) according to the manufacturer's instructions. Briefly, cell samples, stored at -80°C, were retrieved and put on ice to thaw. Once completely thawed, samples were resuspended in 200µl PBS followed by 400µl of High Pure RNA Isolation Kit Lysis Binding Buffer. This was followed by a 15 second vortex allowing the Lysis Binding Buffer to permeabilize the cell membranes and denature proteins. The entire sample was then transferred to a High Filter tube that is placed within a collection tube and centrifuged at 9200 RPM for 15 seconds allowing the binding of RNA to the membrane of the spin column. A master-mix of DNase I in DNase Incubation Buffer was made and added directly onto the glass filter fleece. The samples were then incubated for 15 minutes at 15°C - 25°C to remove genomic DNA. Thereafter, 500µl Wash Buffer I was added to each sample and centrifuged at 9200 RPM for 15 seconds. This was followed by two consecutive washes of 200 µl Wash Buffer II per sample which was centrifuged at 13000 RPM for 2 minutes to hydrolytically cleave the DNA backbone and subsequently remove the degraded DNA so that only RNA is left behind. The second wash step ensures that residual impurities is removed from the RNA. Following the washes, the spin column was then transferred to a labelled 1.5 ml microcentrifuge tube and allowed to air-dry for 5 minutes. The RNA was eluted in 50 - 100µl Elution Buffer and centrifuged at 9200 RPM for 1 minute.

2.5.2 Quantification of extracted RNA

The concentration and purity of the eluted RNA was determined by spectrophotometry using a Nanodrop ND-1000 spectrophotometer (Agilent Technologies, Boeblingen, Germany). Elution buffer was used to blank the spectrophotometer. RNA purity was measured using the absorbance maxima of nucleic acids (260 nm) to the absorbance at 280 nm ($A_{260/280}$) ratio where 1.8 is accepted as pure for RNA. Only samples with a ratio of 1.8 and higher was used for further analysis. Eluted RNA samples were stored at -80°C till further use. Purity of PEG-Ac-SH samples at 144 hrs is shown in Appendix Figure 38.

2.5.3 Generation of cDNA from extracted RNA

cDNA was generated by adding 1µg of purified RNA, per sample, to a PCR tube containing 1µl Oligonucleotide deoxythymine (Oligo dT) primer to make final volume of 5 µl and allowed to denature at 70°C for 5 minutes followed by 4°C for 5 minutes in the Bio-Rad C1000 Touch Thermal Cycler. Thereafter, 15 µl reverse transcription master-mix consisting of Nuclease free water, 5X ImProm-IITM

reaction buffer, 1.5-8mM magnesium chloride, 0.5mM deoxyribonucleotide triphosphates (dNTPs), 2500 units RNasinR ribonuclease inhibitor, and ImProm-IITM reverse transcriptase was added to each sample. Annealing occurred at 25°C for 5 minutes, reverse transcription at 42°C for 1hr, and inactivation of reverse transcriptase at 70°C for 5 minutes. The synthesized cDNA was stored at -20°C until use.

2.5.4 qPCR of cDNA

For all samples, qPCR was performed on a minimum of two biological replicates. For each sample 8µl master-mix was prepared containing the following reagents: 5µl Power SYBR Green PCR master mix (Applied Biosystems), 0.2µl of 10µM forward primer, 0.2µl of 10 µM reverse primer, and 2.6µl sabax water. SYBR Green is a fluorescent dye that binds to the minor groove of double stranded DNA (dsDNA), emitting fluorescence when bound allowing the levels of amplified product to be monitored. The cDNA was diluted 1:1 in sabax water. 96-well PCR plates was used in which 2µl diluted cDNA or sabax water (for the non-template control) was added to 8µl of the master-mix. The plate was subsequently sealed, centrifuged at 3000 rpm for 1 minute, and placed in the Applied Biosystems StepOne Plus thermal cycler (Applied Biosystems, Carlsbad, CA, USA).

StepOne Plus software was used to design the layout of 96-well plate and the thermal cycling conditions were set as followed for each qPCR run: denaturation for 15 minutes at 95°C, 35 cycles of annealing and amplification for 5 seconds at 95°C, 3 seconds at 55°C, and 5 seconds at 72°C, followed by melting for 15 seconds at 65°C and a cooling step of 30 seconds at 40°C. Each sample reaction was quantified in triplicate and a non-template control was run with every primer set to assess specificity and monitoring of contamination. The list of primer sets used for qPCR are shown in Table 2. Product specificity, amplification of a single PCR product, was confirmed through melting curve analysis (Appendix, Figure 39) and data was subsequently analysed using the 2- $\Delta\Delta C_t$ method [238] in Microsoft Excel with GAPDH used as the internal control against which the relative mRNA gene expression levels of the samples were normalised. GraphPad Prism version 9.0 software was used to run statistical analysis, which is described in more detail in section 2.8.

Table 2: Sequences of primers used for qPCR.

Gene	Forward Primer	Reverse Primer
GAPDH	AGGTCGGTGTGAACGGATTC	TGTAGACCATGTAGTTGAGGTCA
OCT4	CAATGCCGTGAAGTTGGAGAAG	GGCTGAACACCTTTCCAAAGAGA
NANOG	TCTTCCTGGTCCCCACAGTTT	GCAAGAATAGTTCTCGGGATGAA
WNT3	CTCGCTGGCTACCCAATTG	CTTCACACCTTCTGCTACGCT

BMP4	TTCCTGGTAACCGAATGCTGA	CCTGAATCTCGGCGACTTTTT
NODAL	CCTGGAGCGCATTTGGATG	ACTTTTCTGCTCGACTGGACA
SOX2	GCGGAGTGGAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
SOX17	GATGCGGGATACGCCAGTG	CCACCACCTCGCCTTTCAC
GATA6	TTGCTCCGGTAACAGCAGTG	GTGGTCGCTTGTGTAGAAGGA
MIXL1	ACGCAGTGCTTTCCAAACC	CCCGCAAGTGGATGTCTGG
BRACHYURY	GCTGGATTACATGGTCCCAAG	GGCACTTCAGAAATCGGAGGG
ECAD	CAGGTCTCCTCATGGCTTTGC	CTTCCGAAAAGAAGGCTGTCC
EOMES	TCGCTGTGACGGCCTACCAA	AGGGGAATCCGTGGGAGATGGA
POU3F1	TTCAAGCAACGACGCATCAA	TGCGAGAACACGTTACCGTAGA
SCUBE2	CCATGCTGATGCACTGTGTCA	AGTGTGTTGTCACATTCATCCAT
EN1	ACACAACCCTGCGATCCTACT	GGACGGTCCGAATAGCGTG
IRX6	TGGTGTCTGCAAGTTCCAGTG	AGCCGACTGTCATAGGGTGT
OTX2	GAATCCAGGGTGCAGGTATGG	CTGAACTCACTTCCCGAGCTG
β-CATENIN (CTNNB1)	ATGGAGCCGGACAGAAAAGC	CTTGCCACTCAGGGAAGGA
FGF8	AGGGGAAGCTAATTGCCAAGA	CCTTGCGGGTAAAGGCCAT
CDX2	AGTGAGCTGGCTGCCACACT	GCTGCTGCTGCTTCTTCTGA
SNAIL-1	CTTGTGTCTGCACGACCTGT	ACATCCGAGTGGGTTTGGAG

2.6 Agarose gel electrophoresis

PCR products were separated on a 1% w/v agarose gel to confirm the amplicon size produced by the primers. 1X TBE buffer was prepared and poured onto the casting tray containing the 1% Agarose gel. 2µl SmartGlow Loading dye 6x concentration (Accuris, USA) was mixed 8µl sample and loaded onto the gel and 2µl SmartGlow Loading dye 6x concentration was added to 10µl 100bp DNA ladder (ThermoFisher Scientific). Electrophoresis was performed at 100 V for 30 minutes and visualised under ultraviolet light. Representative gel run for the NANOG primer set is shown in the Appendix, Figure 40.

2.7 Immunocytochemistry

2.7.1 Staining of mESCs and iMEFs from adherent cell cultures

iMEFs were plated on 18mm autoclaved coverslips within 12-well tissue culture plates containing iMEF medium. After 48hrs, attachment of the iMEFs were confirmed and the medium was aspirated and replaced with 2iLIF medium for at least two hours. mESCs between 60% and 70% confluency was lifted with Dispase treatment as per section 2.2 and plated onto the iMEF coverslips in 2iLIF medium. Once

the mESCs reached 60 – 70% confluency, the cells were washed twice with PBS for 3 min and fixed in ice cold 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). PFA was removed by three consecutive 5-min PBS washes. Cells were then permeabilized with ice cold methanol for 15 minutes and washed twice with PBS for 10-min incubations. Thereafter, cells were blocked for two hours in 3% Bovine Serum Albumin (BSA)/0.01% Triton-X-PBS at RT and incubated with Rabbit polyclonal to NANOG antibody (1:25) or Rabbit polyclonal to OCT4 antibody (1:200), diluted in 1% BSA overnight at 4°C. A detailed list of antibodies and dyes used are summarised in Table 3. Cells were swashed in PBS and incubated in the dark with Donkey-anti-Rabbit Cy3 secondary antibody, diluted 1:500 in 1% BSA, for 2 hours. Cells were subsequently washed with PBS twice and stained with nuclear dye Hoechst33342, diluted 1:1000 in 1% BSA, in the dark for 15 minutes at RT. Following another two washes in PBS, the cells were mounted onto glass slides using Mowiol mounting medium and left to dry overnight in the dark. Samples are stored at 4°C until visualization.

2.7.2 Staining of structures from suspension cell cultures

Gastruloids were viewed at 120 hrs and 144 hrs using an EVOS microscope and selected based on their morphology for immunostaining analysis. The medium in selected gastruloids was removed from each well and fixed in 4% PFA for 1 hour at 4°C on a rotating belly dancer shaker (IBI scientific) set to a low RPM speed. Fixed gastruloids were subsequently washed three times with PBST (0.05% Tween20/PBS) for 5 – 10 minutes. Gastruloids were then permeabilised for 1 hour at RT in 0.5% Triton-X/PBS while gently rocking on a rotating belly dancer shaker and subsequently washed three times in PBST for 5 – 10 minutes. Thereafter, gastruloids were blocked for 1 hour at RT in 10% FBS/0.2% Triton-X/PBS while gently rocking on a rotating belly dancer shaker. Gastruloids were then incubated overnight at 4°C with the appropriate primary antibody (listed in table 3) diluted in block while gently rocking on a rotating belly dancer shaker. Following primary Ab incubation, gastruloids were washed three times in PSB and incubated overnight at 4°C with the respective secondary antibody (Donkey-anti-Mouse Alexa 488 for anti-Ecadherin and Donkey-anti-Goat Cy3 for anti-h/mBrachyury and anti-hSox17) in the dark. The secondary antibody was removed by three 5-min PBS washes followed by three 20-min Hoechst diluted 1:1000 in PBS washes. Gastruloids were then incubated with Hoechst, diluted 1:1000 in PBS, overnight in the dark at 4°C while gently rocking on a rotating belly dancer shaker and subsequently mounted. A drop of Mowiol was placed on a glass dish and the gastruloid was gently removed using a p200 pipette. The pipette with the gastruloid was held vertically and was gently taped to allow the gastruloid to move to the tip until it hanged at the edge during which it is gently placed on the drop of Mowiol. Gastruloids were left to dry for at least 72 hrs before visualization.

Table 3: Antibodies used for immunocytochemistry.

Primary Antibody	Catalogue Number and Supplier	Source	Species	Dilution	Secondary Antibody	Dilution
anti-Oct4	AB19857 Abcam	Polyclonal Rabbit IgG	Mouse/ Human	1:200	Donkey-anti- Rabbit Alexa 488	1:500
anti-Nanog	AB80892 Abcam	Polyclonal Rabbit IgG	Mouse	1:25	Donkey-anti- Rabbit Alexa 488	1:500
anti- h/mBrachyury	AF2085 R&D systems	Polyclonal Goat IgG	Mouse/ Human	1:200	Donkey-anti- Goat Cy3	1:500
anti-hSox17	AF1924 R&D systems	Polyclonal Goat IgG	Human	1:100	Donkey-anti- Goat Cy3	1:500
anti- Ecadherin	M108 Takara Bio Inc.	Monoclonal Mouse (Clone ECCD-2)	Mouse	1:500	Donkey-anti- Mouse Alexa 488	1:500

2.7.3 Confocal Microscopy

All immunochemistry-stained samples were visualised in the dark by confocal microscopy (Zeiss LSM 510 Meta with NLO) using ZEN 2009 Software and the following lasers: Argon 488nm green laser, solid state 561 nm red laser, and MaiTai 2 photon 750 nm blue laser for Hoechst/DAPI. Images were taken as individual snapshots or z-stacks and imaged using ImageJ software.

2.8 Statistical and Data analysis

GraphPad Prim version 9.0 was used to perform statistical analysis and generating figures. Data is expressed as the mean $\pm$ standard error of mean (SEM) if two or more independent experiments (n=2) were performed. For all experiments, data were processed by checking for outliers and normality to decide prior to the statistical test. Ordinary One-Way ANOVA with Dunnett's Multiple Comparisons post-test was used when comparing each treatment to the control. Two-way ANOVA with Dunnett's Multiple Comparisons post-test was used to compare each condition to the respective time-point. Statistical significance was denoted by (*) if $p < 0.05$, very significant (**) if $p < 0.001$, or highly significant (***) if $p < 0.0001$. When extracting RNA from gastruloids one 96-well plate was representative of one biological replicate. Two independent biological replicates, each performed in triplicate for the indicated gene, was used for qPCR analysis as done by various studies referenced here [205, 211, 220, 239, 240] due to limited biological material.

CHAPTER 3:

Propagation and maintenance of mESC pluripotency

mESCs are derived from the blastocyst of the pre-implantation mouse embryo and are capable of retaining all the unique characteristics of this stage of development when grown under the correct conditions in culture. The most important feature of the pre-implantation blastocyst is its pluripotent nature. As mentioned in section 1.6.1, the Epi can either be in a naïve state of pluripotency, a characteristic of the preimplantation embryo, or in a primed pluripotent state where the Epi cells become primed for differentiation during gastrulation [46]. OCT4 is the master transcriptional regulator of pluripotency and works in conjunction with NANOG and SOX2 forming the PGRN. While other genes form part of the PGRN, the three abovementioned genes are indispensable for the maintenance of pluripotency and inhibition of differentiation cues [241]. These transcription factors have also been shown to play critical roles in initiating cell fate decisions [68]. In order to utilise mESCs for the development of *in vitro* embryonic models, it is essential to ensure that mESCs are maintained in a state of naïve pluripotency in order to prevent biased differentiation in subsequent experimental analysis. Thus, the first objective of this study focuses on establishing pluripotent mESCs in culture for extended periods of time.

3.1 The regulation and maintenance of mESC pluripotency in culture

To date, the canonical LIF-STAT3 pathway has been well documented to induce and maintain mESC self-renewal and pluripotency by activating the core pluripotent factors OCT4 and NANOG [161]. However, some cells start to differentiate during continued culture in LIF medium. In this regard, recent work displayed an improvement in maintaining mESC pluripotency through the addition of two inhibitor molecules (2i) which inhibits the MAPK/ERK and GSK-3 β pathways in LIF medium [170-173] thereby ensuring the propagation of mESCs while simultaneously inhibiting differentiation [173].

3.1.1. *2iLIF culture condition support mESC self-renewal and pluripotency*

To culture mESCs while maintaining their pluripotency for extended periods of time, mESCs were grown in specific culture conditions known to establish pluripotency and self-renewal. Briefly, mESCs were grown on iMEF feeder layers cultured on freshly made 0.1% gelatin coated 6-well plates. mESCs were grown in 2iLIF medium with daily medium changes and passaged when they reached a confluency between 60% and 70%. Using light microscopy, morphological characteristics of the mESCs were assessed first to examine the state of the mESCs. The iMEF feeder layer presented as flat elongated spindle-shaped cells with thin nuclei in iMEF medium (Figure 9 A). mESCs grown in 2iLIF

medium on iMEF feeder layers formed healthy pluripotent mESCs colonies as indicated by their uniform size, defined borders, and attachment to the iMEF feeder layer (Figure 9 B-D). Interestingly, mESCs started to differentiate at passage 15 and above even though they were still grown in 2iLIF medium on iMEF feeder layers. Morphologically, this was observed by mESC colonies growing too close to one another and emerge as large differentiating cells that lost their defined borders, shape, and detached from the iMEF feeder layer (Figure 9 E-F). Based on this observation, only mESCs cultured until passage 12 were used for subsequent experimental analysis.

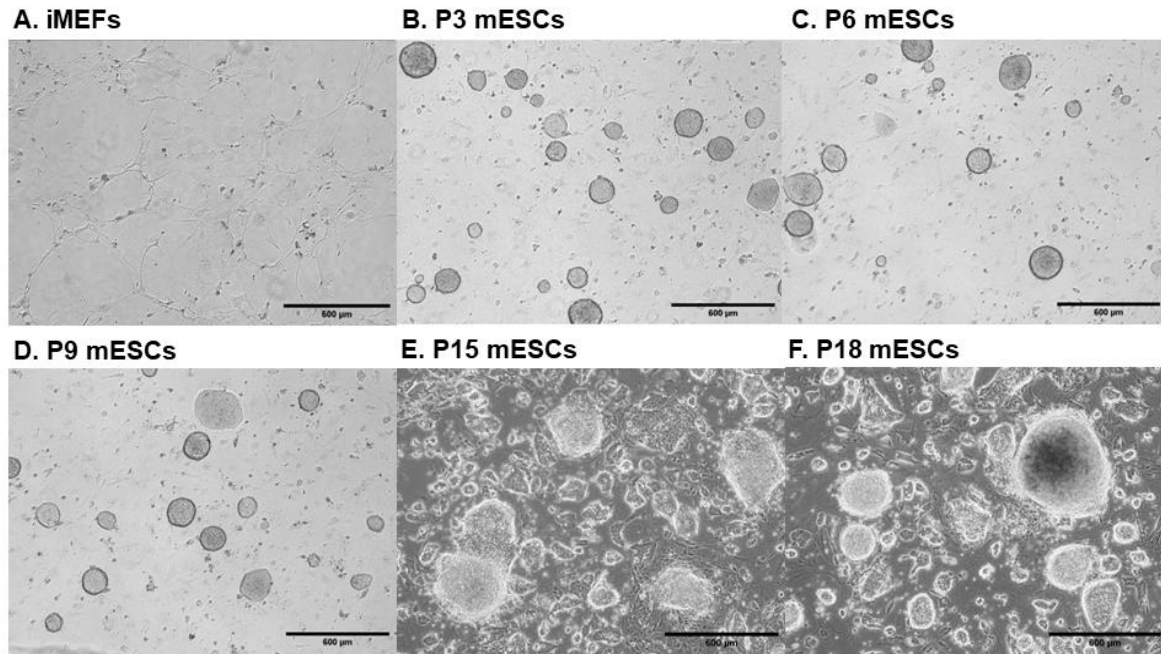


Figure 9: Morphology of mESCs cultured in 2iLIF medium. A: 0.1% gelatin coated well of a 6-well plate with an iMEF feeder layer seeded at a density of 2.5×10^4 grown in MEF medium and imaged four days post plating. B - F: 129/Ola mESCs grown on iMEF feeder layers in 2iLIF medium and imaged at passage 3, 6, 9, 15, and 18. Representative phase contrast microscopic images of the cells and colonies at 10X magnification with scale bar = 600µm.

3.2 Gene and protein expression of pluripotency genes in mESCs

While morphological characteristics serves as good indicator of pluripotency, they cannot effectively confirm the presence of activated pluripotency pathways within the mESCs. As a result, to investigate whether the mESCs were in effect pluripotent, the presence of pluripotency transcription factors OCT4 and NANOG was evaluated by both qPCR and immunocytochemistry to determine whether the culture conditions could maintain the PGRN within the mESCs.

3.2.1. OCT4 and NANOG is present at gene expression level within mESCs

Briefly, for qPCR analysis, RNA was extracted from iMEFs, grown in the absence of mESCs, and from mESCs at passages P2-P12 and subsequently converted into cDNA to measure the relative fold expression levels of OCT4 and NANOG by qPCR. iMEFs were used as a reference for cells that are not pluripotent and do not express OCT4 or NANOG. Compared to iMEFs, both OCT4 and NANOG was highly expressed in mESCs (Figure 10). OCT4 gene expression was significantly upregulated compared to NANOG gene expression in mESCs. In addition, OCT4 gene expression levels fluctuated between the passage groups, with the highest expression observed in the middle passage group while NANOG expression levels remained largely consistent between the passage groups (Figure 10).

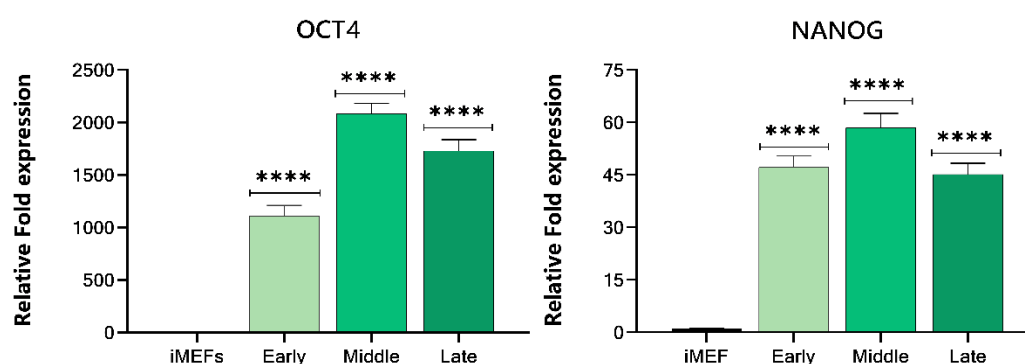


Figure 10: Relative gene expression levels of OCT4 and NANOG in adherent mESCs. *qPCR was performed on reverse transcribed RNA using primers specific to OCT4 and NANOG. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing mESCs to the iMEF control. mESCs passages were grouped into three categories: Early (P2-P4), Middle (P5 – P8), and Late (P9-12). GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the standard error of the mean (SEM). This Figure represents pooled data from two biological experiments performed in triplicate.*

3.2.2. OCT4 and NANOG is present at protein level within mESCs

Fluorescent immunocytochemistry was carried out to visualise both OCT4 and NANOG antigens at protein level within mESCs. Visualization through confocal imaging showed that OCT4 was not expressed in iMEFs, however, was significantly expressed within the mESC colony (Figure 11A). As expected, iMEFs did not express NANOG when observed by confocal imaging while the mESC colony highly expressed NANOG as seen in the lower right panel of Figure 12 B.

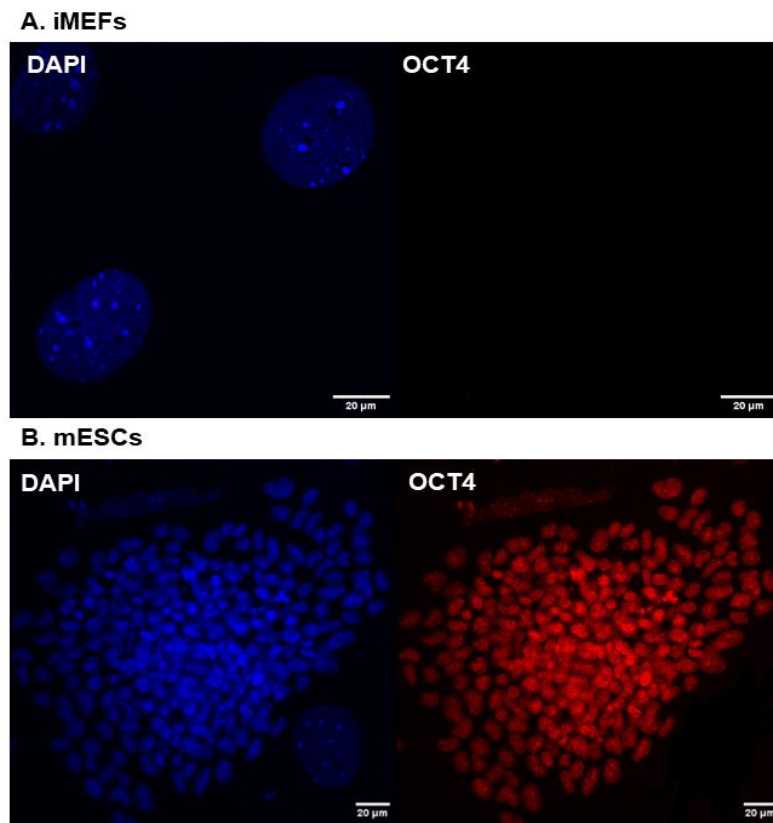


Figure 11: Immunofluorescence detection of OCT4 in mESCs. *iMEFs and mESCs were seeded on coverslips and processed for immunocytochemistry using Rabbit Polyclonal OCT4 antibody (1:200) and a donkey anti-Rabbit Cy3 secondary antibody (1:500). Cells were stained with Hoechst and viewed using the DAPI filter to determine the location of the nuclei. Cells were viewed using confocal microscopy and processed in ImageJ. Representative images depict iMEFs and a mESC colony (scale bars, 20μm).*

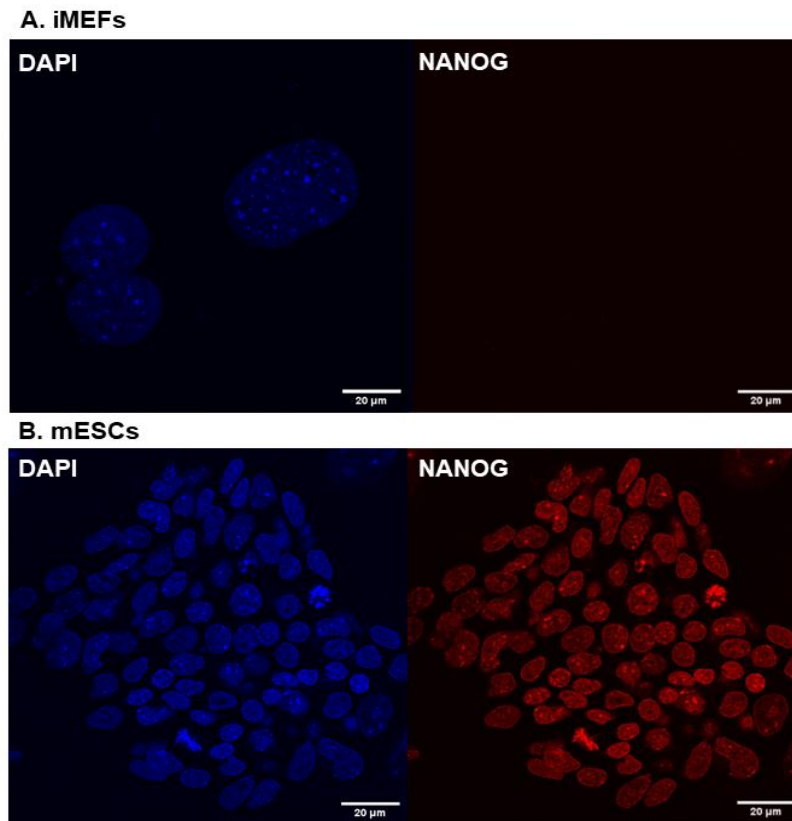


Figure 12: Immunofluorescence detection of NANOG in mESCs. *iMEFs and mESCs were seeded on coverslips and processed for immunocytochemistry using Rabbit Polyclonal NANOG antibody (1:25) and a donkey-anti-Rabbit Cy3 secondary antibody (1:500). Cells were stained with Hoechst and viewed using the DAPI filter to determine the location of the nuclei. Cells were viewed using confocal microscopy and processed in ImageJ. Representative images depict iMEFs and a mESC colony (scale bars, 20µm).*

3.3 Discussion

Culturing mESCs in an undifferentiated state for extended periods of time has been thoroughly examined in several previous studies investigating the addition or inhibition of molecules involved in the pluripotency signalling pathway as well as regulating the culture environment through mESC culture on iMEF feeder layers. The results depicted in chapter 3 collectively demonstrated the ability to maintain mESC pluripotency on iMEF feeder layers in 2iLIF culture conditions.

In vivo, the ICM cells undergo proliferation and development in a microenvironment which consists of supporting cells that secrete soluble factors and insoluble ECM proteins forming a 3D network [242]. The combined chemical and physical factors encoded by this stem cell microenvironment regulates self-renewal, differentiation, and stem cell migration [243-245]. A critical factor of this microenvironment is providing anchorage to stem cells through membrane bound proteins of supporting cells [246, 247] and ECM proteins [248]. In order to mimic this microenvironment *in vitro*, mESCs are typically cultured on a monolayer of iMEFs which provide a matrix that produce growth factors, deliver adhesion molecules and ECM compounds that support mESC attachment and propagation [249]. It has also been suggested that differentiation in feeder-dependent mESCs takes longer as they express lower expression levels of differentiation markers [250]. As per the results in Figure 8 B-D, mESC colonies firmly attached to the iMEF feeder layer throughout culture up until P15. It was observed that the use of 0.1% gelatin facilitated the attachment of iMEF feeder layers in MEF medium thereby allowing mESCs to be plated after 48hrs.

mESCs are typically cultured in serum supplemented with LIF, which activates JAK/STAT3 signalling thereby keeping mESCs in a pluripotent state [161]. In this culture method, mESCs are able to self-renew in the absence of an iMEF feeder layer [158, 251]. However, serum supplemented with LIF generates a heterogenous population of mESCs as a result of auto-inductive stimulation of the MAPK/ERK pathway by FGF4 [170]. As a result, two small inhibitor molecules, CHIR99021 and PD0325901, was introduced to inhibit WNT signalling and the MAPK/ERK pathway respectively. The use of 2iLIF, that blocks both the MAPK/ERK pathway and GSK3 β was confirmed to be sufficient to maintain mESCs in a naïve state of pluripotency [252]. FBS was added to 2iLIF medium as it provides essential nutrients and hormones as well as altering the physiochemical properties of the medium [250].

As seen in Figure 9 B-D, mESCs expressed all the morphological hallmarks of a pluripotent stem cell population by forming round colonies with defined borders while being nicely spaced and attached to the iMEF feeder layers. Optimal mESC growth was observed with daily medium changes instead of every second day and the replenishment of LIF every 7 days. As discussed in Chapter 1, the canonical

LIF-STAT3 pathway has been well documented to induce and maintain mESC self-renewal and pluripotency by activating the core pluripotent factors OCT4 and NANOG [161]. The use of 2iLIF medium has also been shown to express NANOG homogenously, which potentiates naive pluripotent gene transcription [172]. This was confirmed in this study with the results depicted in Figure 10 which illustrate relative homogenous NANOG levels throughout the various mESC passages P2 – P15. mESCs cultured in 2iLIF medium also exhibited significantly elevated expression of OCT4 compared to NANOG (Figure 10). Evidence has suggested that Chiron operates through β -catenin to maintain pluripotency [253] which has illustrated heterogeneities in NANOG expression [253, 254]. In addition, studies have found that under pluripotency promoting conditions, β -catenin forms a complex with ECAD and OCT4 thereby enhancing its pluripotency activity [255, 256]. While β -catenin can also bind to NANOG, it does so to a lesser extent [256]. Therefore, it may be the introduced activity of β -catenin by Chiron accounting for the significant upregulation of OCT4 in the mESCs. Furthermore, it has also been shown that NANOG is homogenously expressed in 2iLIF medium [257] similar to what was observed in this study throughout passages.

Interestingly, in Figure 9 E and F, mESCs at passages 15 and above differentiated despite being cultured on iMEF feeder layers and in 2iLIF medium. Differentiating colonies have a distinct morphology in that they start to grow too close to one another, lose their shape and defined borders, and detach from the iMEF feeder layer. As a result of this observation, only mESCs grown until passage 12 was used for subsequent experiment in this study. mESCs are extremely sensitive to their culture conditions which can affect their health, proliferation, and gene expression levels. As a result, optimum culture conditions are required to maintain healthy mESC growth *in vitro*. Continued dissociation of mESCs into a single cell suspension during passaging may have resulted in cell death while simultaneously promoting differentiation. In addition, the use of Dispase, a gentle enzyme used to lift mESCs without damaging cell membrane and preventing the carryover of iMEFs, has been shown to generate genetic instability over long periods in culture [258]. Although Dispase generates less genetic instability compared to other enzymes, its use may have contributed to the observed differentiation of mESCs at passages 15 and higher. The use of serum and gelatin also have implications in promoting differentiation of mESCs. FBS is an animal product that is known to contains various growth factors and undefined components that promote mESC growth, however, FBS has been suggested to contain potential differentiation factors that may affect the plating efficiency, growth, and differentiation of mESCs [259].

Gelatin is used to enhance mESC growth by increasing the proliferation rates, however, when used to grow mESCs in 2iLIF in the absence of an iMEF feeder layer culture, gelatin has been shown to exhibit some expression of differentiation markers [250]. This study grew iMEFs on gelatin before introducing

mESCs as mESCs did not attach when gelatin was used in the absence of an iMEF feeder layer. It was also observed that mESCs grown in FBS-free 2iLIF medium exhibited lower growth rates and decreased attachment. In addition, FBS has also been shown to protect cells from enzymatic treatment thereby signifying the indispensability of FBS and gelatin for efficient mESC growth.

In conclusion, this chapter has collectively shown that 2iLIF medium was sufficient to prevent spontaneous differentiation while simultaneously promoting mESC self-renewal and pluripotency for up to 15 passages. The expression of OCT4 and NANOG, genes specific for undifferentiated mESCs, were examined in mESCs up until passage 12 by both qPCR and immunocytochemistry. The establishment of pluripotency ensures that subsequent analysis is performed using a mESC population that is not biased towards differentiation into a specific lineage. As a result, pluripotent mESCs provide a tractable, scalable, and manipulable platform for subsequent experimental analysis *in vitro* [260].

CHAPTER 4

Self-organization of mESCs into gastruloids

Pluripotent mESCs have been used to model embryonic development *in vitro* for several years. The demand for such *in vitro* models has increased exponentially as it presents a powerful tool to elucidate the mechanisms of cell fate decisions that occur during embryogenesis while circumventing the impediments posed by *in vivo* research such as the ethical and technical limitations. While mESCs are capable of giving rise to all the cell types of the embryo proper, they do so in a spatially disorganised manner when grown as 2D cultures or as EBs [261, 262]. However as mentioned in Chapter 1, the results of culturing EC cells as small aggregates that demonstrated axial elongation morphogenesis [188], paved the way for the development of similar 3D cultures, specifically the gastruloid model, that can reveal the morphogenetic potential of mESCs. 3D *in vitro* differentiation of mESCs into gastruloids is achieved by changing the culture conditions to remove the molecules that maintain the pluripotency pathway allowing cells to exit pluripotency and undergo directed differentiation. Conventional gastruloid formation involves the addition of a WNT agonist, Chiron, which allows mESC aggregates to break symmetry and undergo elongation [205, 210-212, 261, 263]. However, the *in vivo* embryo relies on BMP4 to generate WNT3 and NODAL gradients resulting in a morphology change into an egg-cylinder shape during gastrulation [1, 77, 264]. BMP4 has also been shown to be critical for gastrulation-like events in the embryoid model [236]. In order to evaluate the effectiveness of Chiron and BMP4 to induce gastrulation-like events *in vitro*, this chapter will examine the morphological changes that arise within mESCs when introduced to Chiron and BMP4 using the gastruloid model.

4.1 *In vitro* differentiation of mESCs into gastruloids is dependent on defined culture conditions

Although growing mESCs in adherent culture is useful to study various gene networks *in vitro*, development requires the 3D organisation of tissues for cell interactions to take place correctly in order to gain insights into cell dynamics and gene expression changes. The first 3D *in vitro* model developed was EBs which illustrate reproducible axial morphogenesis potential *in vitro* [106]. Although EB formation occurs in a spatially disorganized manner, they were used as a reference control for all downstream results. The culture conditions to form EBs and the different gastruloid formations are summarised in Figure 13. Briefly, EBs were formed by plating mESC colonies in bacteriological suspension plates in MEF medium. Condition B depicts Chiron gastruloid formation

according to the original protocol with slight modifications [210-212]. 400 mESCs were aggregated in 96-well plates in N2B27 medium and Chiron was introduced as a 24-hr pulse between 48 hrs and 72 hrs. To investigate the effect BMP4 may have on gastruloid formation, 400 mESCs were treated with BMP4 as a pre-treatment for 24 hrs prior to the addition of the Chiron pulse at 48hrs. Condition B culture conditions is based upon the gastruloid protocol and the embryoid protocol [236]. Briefly, 150 mESCs were aggregated and treated with BMP4 for 8hrs prior to merging the aggregates with naïve 250 cell mESC aggregates. Finally, to examine the effect of mechanical stiffness on gastruloid formation, Condition D was repeated and grown in a 1.5% PEG-Ac-SH hydrogel from 72 hrs until 144 hrs.

4.1.1. *Morphogenetic changes ensue in vitro differentiation of mESCs*

Morphology serves as an effective indicator of aggregate evaluation and when combined with image analysis, the size, shape, and area of aggregates can be quantified. In order to evaluate the aggregates formed from each protocol summarised in Figure 13, all aggregates were imaged every 24 hrs until 144 hrs using the EVOS phase contrast microscope. ImageJ software was used to analyse the data and GraphPad Prism was used to summarise the results. Variation in morphology was evaluated by categorising the aggregates into the different shapes they illustrated by 144 hrs. The growth of the aggregates over the course of the experiment was determined by measuring the major axis of each aggregate. Finally, the axial ratio (major/minor axis) was measured for each aggregate.

The results show that EBs grown in bacteriological dishes in MEF medium did not attach to the bottom of the plate throughout culturing (Figure 14 A). The representative images in Figure 14 A illustrate variation in morphology where EBs presented as either round/circular or elongated slightly from one axis. Quantification of the morphological variation illustrated that the majority of EBs have rounded shapes ($n = 12$) compared to elongated shapes ($n = 8$) at 144 hrs (Figure 14 C). When measuring the major axis lengths, EBs illustrated variations in size within each time point, however, EB growth between the time points was non-significant with an average size between 60 μm and 120 μm in length at each time point (Figure 14 D). At the 96-hr time point, some EBs illustrated a spike in length ($n = 150 \mu\text{m}$) after which the EBs seemed lose their length with the next highest length observed at 120 hrs being around 130 μm . There was no significant difference in the axial ratios of the EBs grown between 24 hrs and 144 hrs (Figure 14 E) which ranged from 1.01 – 1.04.

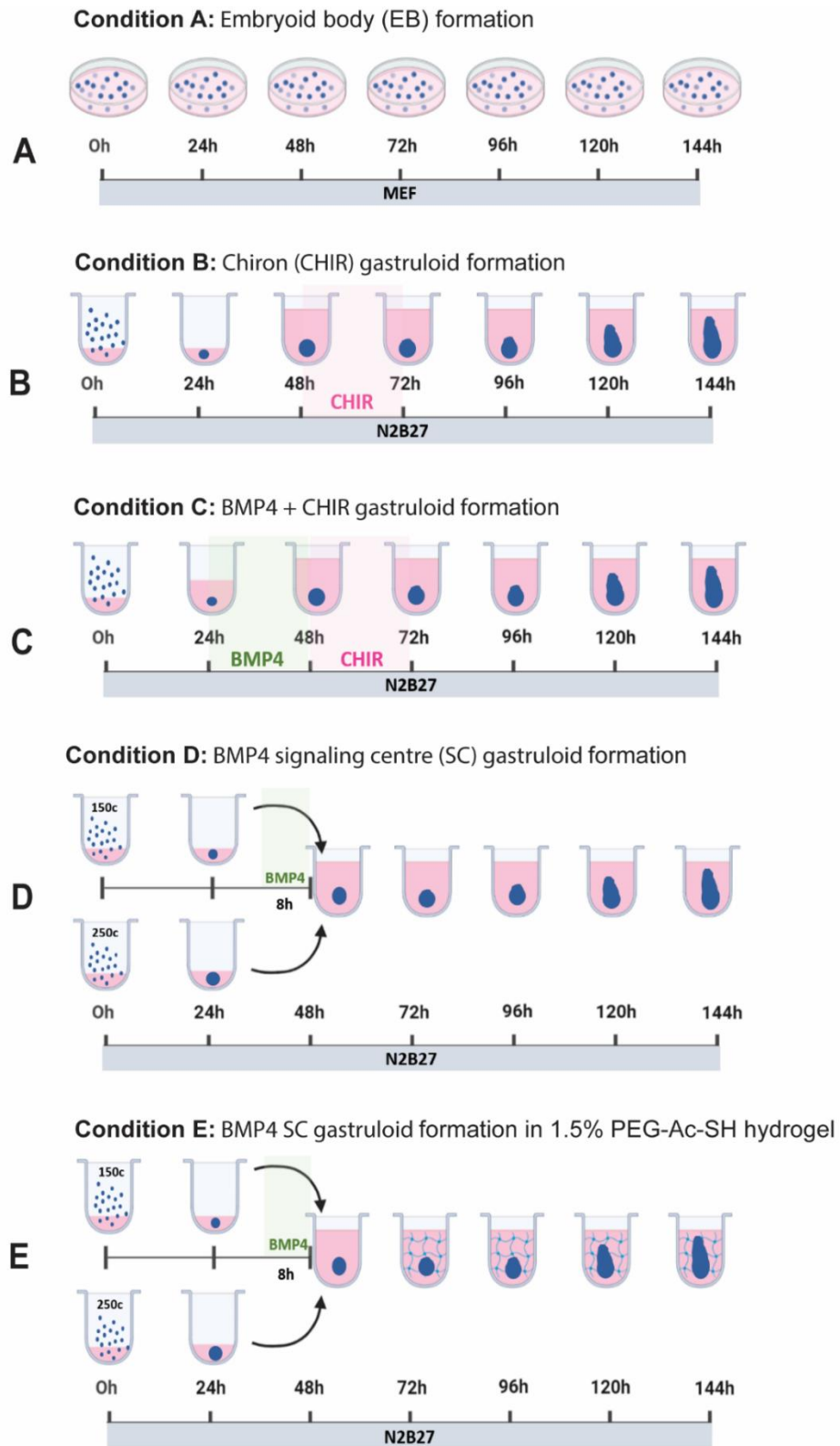


Figure 13: Schematic of the culture protocols for mESC differentiation. *Condition A: mESCs are left to differentiate in bacteriological dishes in MEF medium for 144h. Condition B: 400 mESCs were allowed to aggregate in N2B27 medium and Chiron was added between 48 hrs and 72 hrs. Aggregates were cultured in suspension until 144 hrs. Condition C: 400 mESCs were allowed to aggregate in N2B27*

medium followed by a BMP4 pulse between 24 hrs and 48 hrs and a Chiron pulse between 48 hrs and 72 hrs. Aggregates were grown in suspension until 144 hrs. Condition E: 150 cell aggregates were treated with BMP4 for 8 hrs between 40 hrs and 48 hrs and subsequently merged with 250 cell naïve aggregates and grown in suspension until 144 hrs in N2B27 medium. Condition E: Follows the same protocol as in Condition D, however, 1.5% PEG-Ac-SH is added at 72 hrs and aggregates were grown until 144 hrs. hrs: hours, CHIR: Chiron, BMP4: Bone morphogenic protein-4.

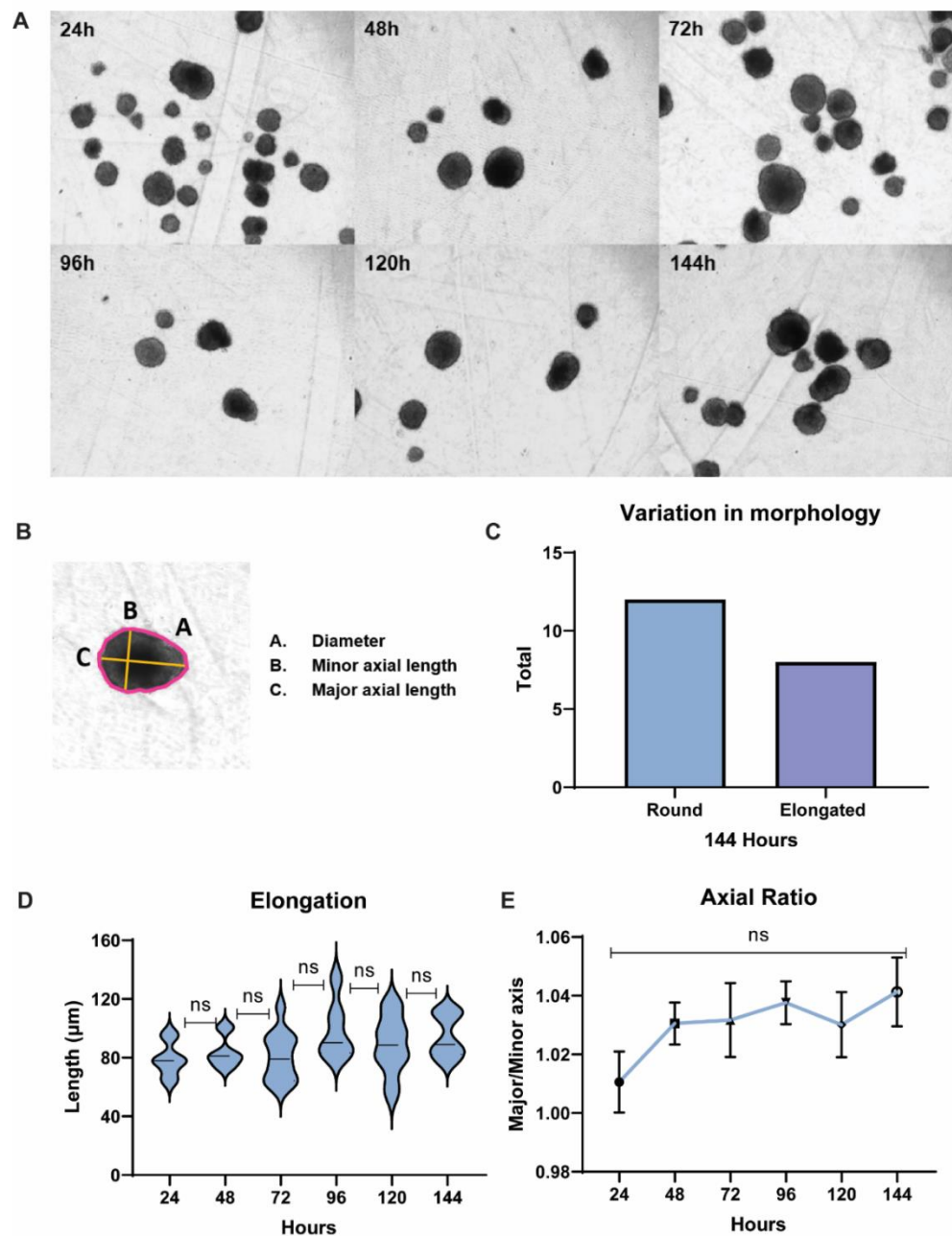


Figure 14: Morphology of Condition A - Embryoid bodies. A: mESCs at 60% confluency were lifted and allowed to settle to the bottom of a conical tube where they were collected and placed in

*bacteriological plates and grown in iMEF medium. Representative images of EB formation were taken when observed by phase contrast microscopy every 24 hrs until 144 hrs. B: Schematic illustration of the measurements taken for quantification. C: Aggregates were categorized according to their variations in morphology at 144 hrs. D: EB elongation was calculated by measuring the major axis length of each aggregate every 24 hrs until 144 hrs. E: Axial ratios was determined using the following formula: major axis length/minor axis length. One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing time points. Statistical significance is indicated on top of each measurement where *****: $p < 0.0001$, ***: $p < 0.001$, **: $P < 0.05$, ns: non-significant, while error bars represent the SEM. N = 20 EBs, 4 independent experiments. Scale bar: 600 μm .*

Representative images of Chiron gastruloids are depicted in Figure 15 A. By 48 hrs, the aggregates were spherical in shape and have doubled in size. Chiron was added to the culture medium at 48 hrs as a 24 hr pulse. Observation at 72 hrs showed a slight break in symmetry within the aggregates which can be observed more clearly at 96 hrs, where the aggregate starts to elongate from one axis. As time progresses, the aggregates continued to elongate until 144 hrs. It was also observed that while the majority of aggregates elongated at 144 hrs ($n = 13$), some aggregates did not break symmetry and instead remained circular in shape ($n = 7$) as illustrated in Figure 15 C. A significant change in growth rates were observed within Chiron gastruloids (Figure 15 D). 24 hr aggregates ranged in size between 70 μm and 140 μm and continued to elongate from one axis with maximum elongation between 180 μm – 280 μm was observed at 144 hrs. When looking at the results from the axial ratios within Chiron gastruloids, there was no significant difference between 24 hrs and 48 hrs (Figure 15 D). Although, an increase in axial ratio is observed at 72 hrs, the difference was still non-significant, but does illustrate that symmetry has been broken. However, between 72 hrs and 120 hrs, a significant change in the axial ratio was observed changing from around 1.4 to 1.8. The change from 120 hrs to 144 hrs was non-significant, however the highest axial ratio was observed at 144 hrs with a ratio of 1.9.

To examine the effect of BMP4 on Chiron gastruloid formation, BMP4 was added as a pulse between 24 hrs and 48 hrs. The results show that the application of 1 ng/ml BMP4 in N2B27 medium leads to aggregates rapidly doubling in size by 48 hrs (Figure 16 A). Immediately following the 24-hr BMP4 pulse, Chiron was added between 48 hrs and 72 hrs with symmetry breaking observed as early as 72 hrs. At 96 hrs, elongation from one axis could be observed and the aggregates continued to elongate until 120 hrs. Interestingly, at 144 hrs, the aggregates seemed to lose their elongated shape and become more circular in shape ($n = 17$) with only a few maintaining their elongated shapes ($n = 3$) (Figure 16 C). When looking at the quantification data in Figure 16 D, a significant increase in aggregate size was observed between 24 hrs and 48 hrs, the time period where BMP4 was introduced in culture.

Between 48 hrs and 96 hrs, the aggregates continued to elongate slowly with a significant increase in length observed at 120 hrs where aggregates were between 180 μm and 220 μm in size. At 144 hrs, the aggregate size varied between 190 μm and 210 μm indicating a loss in major axial length. The axial ratios between 24 hrs and 48 hrs were non-significant with a dramatic change in axial ratio observed from 48 hrs (1.0) to 72 hrs (1.2) indicating a break in radial symmetry. The axial ratio continued to increase until 120 hrs (1.6) whereafter the axial ratio decreases to 1.5 at 144 hrs.

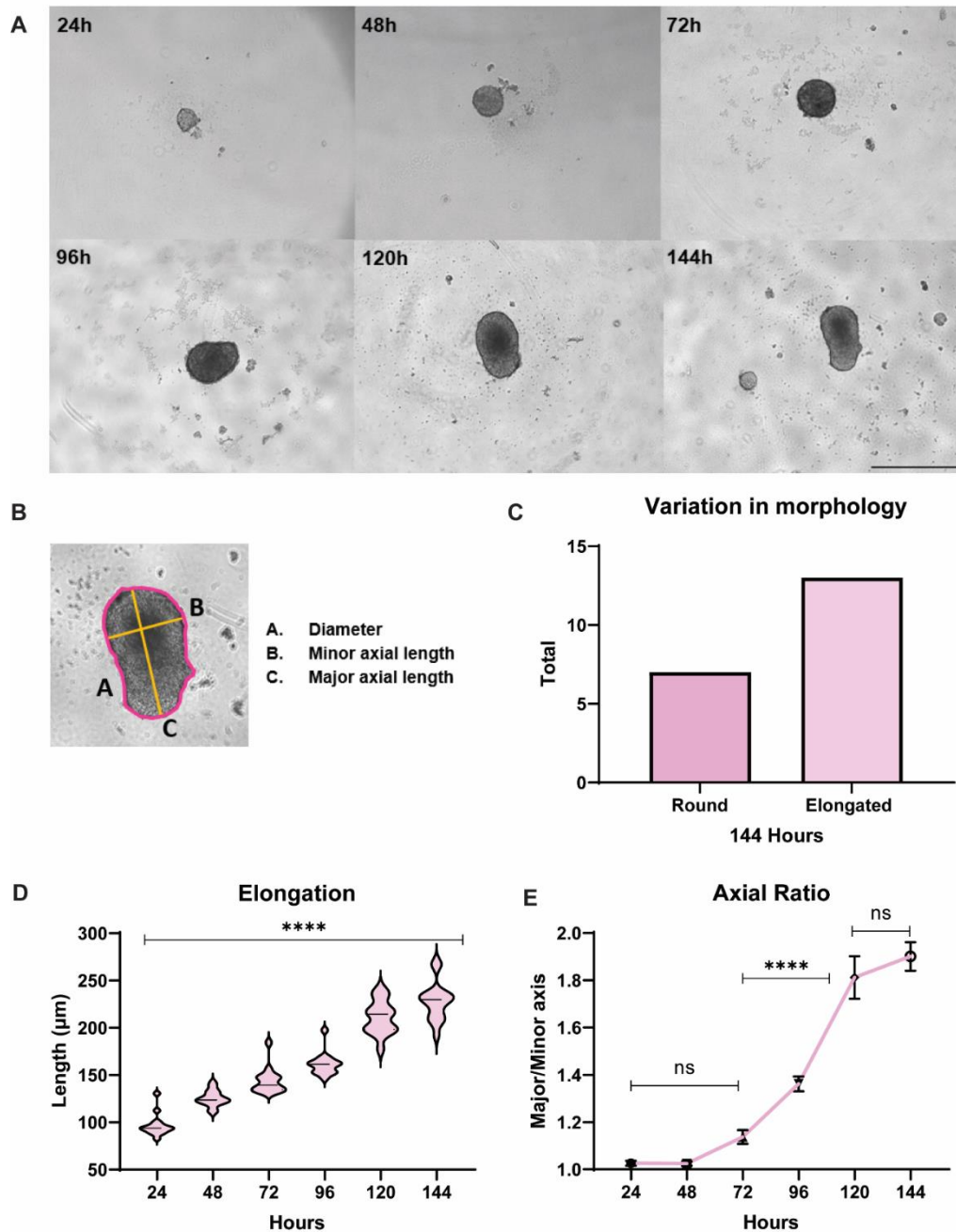


Figure 15: Morphology of Condition B – Chiron gastruloids. A: 400 mESCs were allowed to aggregate in 96-well plates in N2B27 medium. Chiron was added between 48 hrs and 72 hrs. Representative images of Chiron gastruloid formation were taken when observed by phase contrast microscopy every

24 hrs until 144 hrs. B: Schematic illustration of the measurements taken for quantification. C: Aggregates were categorized according to their variation in morphology at 144 hrs. D: Aggregate elongation was calculated by measuring the major axis length of each aggregate every 24 hrs until 144 hrs. E: Axial ratios was determined using the following formula: major axis length/minor axis length. One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing time points. Statistical significance is indicated on top of each measurement where ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.05$, ns: non-significant, while error bars represent the SEM. N = 20 EBs, 4 independent experiments. Scale bar: 600 μm .

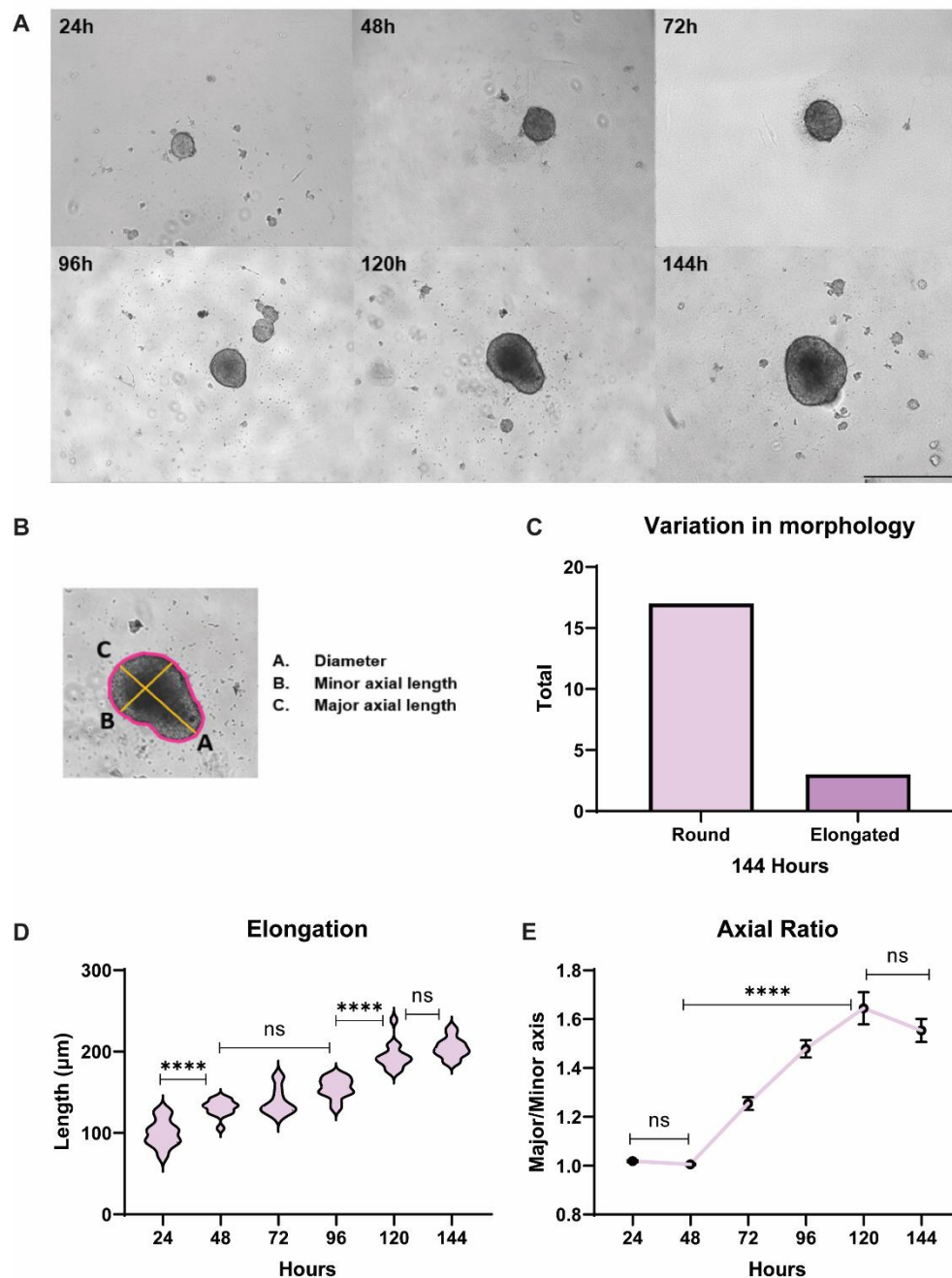


Figure 16: Morphology of Condition C: BMP4 + Chiron gastruloids. 400 mESCs were allowed to aggregate in 96-well plates in N2B27 medium. BMP4 was added between 24 hrs and 48 hrs followed by the addition of Chiron at 48 hrs and 72 hrs. Representative images of BMP4 + Chiron gastruloid formation were taken when observed by phase contrast microscopy every 24 hrs until 144 hrs. B: Schematic illustration of the measurements taken for quantification. C: Aggregates were categorized according to their variation in morphology at 144 hrs. D: Aggregate elongation was calculated by measuring the major axis length of each aggregate every 24 hrs until 144 hrs. E: Axial ratios was determined using the following formula: major axis length/minor axis length. One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing time points. Statistical significance is indicated on top of each measurement where ****: $p < 0.0001$, ***: $p < 0.001$, **: $P < 0.05$, ns: non-significant, while error bars represent the SEM. N = 20 EBs, 4 independent experiments. Scale bar: 600 μm .

In order to investigate the effects of BMP4 to initiate gastrulation-like events, BMP4 was introduced as a signalling centre (SC) as depicted in Figure 13. The typical morphology of BMP4 SC gastruloids is depicted in Figure 17 A. The aggregates in the 24-hr window are naïve 250 mESC aggregates. The image at 48 hrs represents a naïve 250 cell aggregate that merged with a 150 cell BMP4-treated aggregate. A break in symmetry was observed at 72 hrs where the aggregate started to elongate from one axis. Interestingly, two distinct changes in aggregate shape could be observed between 96 hrs and 144 hrs. At 96 hrs, the aggregates either elongated from one axis but subsequently bent into a bean shape by 120 hrs, or the aggregates continued to elongate from one axis until 144 hrs. A total of 10 aggregates bent into a bean shape, where the other aggregates elongate ($n = 5$). As with the other results described, some aggregates did not undergo any morphological change and remained circular in shape ($n = 5$). When looking at the elongation of BMP4 SC gastruloids, there was no significant difference between 24 hrs and 48 hrs, however a significant change in length was observed from 72 hrs until 120 hrs. There was no significant difference in the length of BMP4 SC gastruloids between 120 hrs and 144 hrs, with maximum elongation being between 160 μm and 250 μm . There was no significant change in axial ratios between 24 hr and 48 hr BMP4 SC gastruloids (Figure 17 E). However, at 48 hrs, the axial ratio of 1.0 increased to 1.25 at 72 hrs thereby indicating a break in radial symmetry within the BMP4 SC gastruloids. The axial ratio continued to increase until it reached 1.62 at 144 hrs.

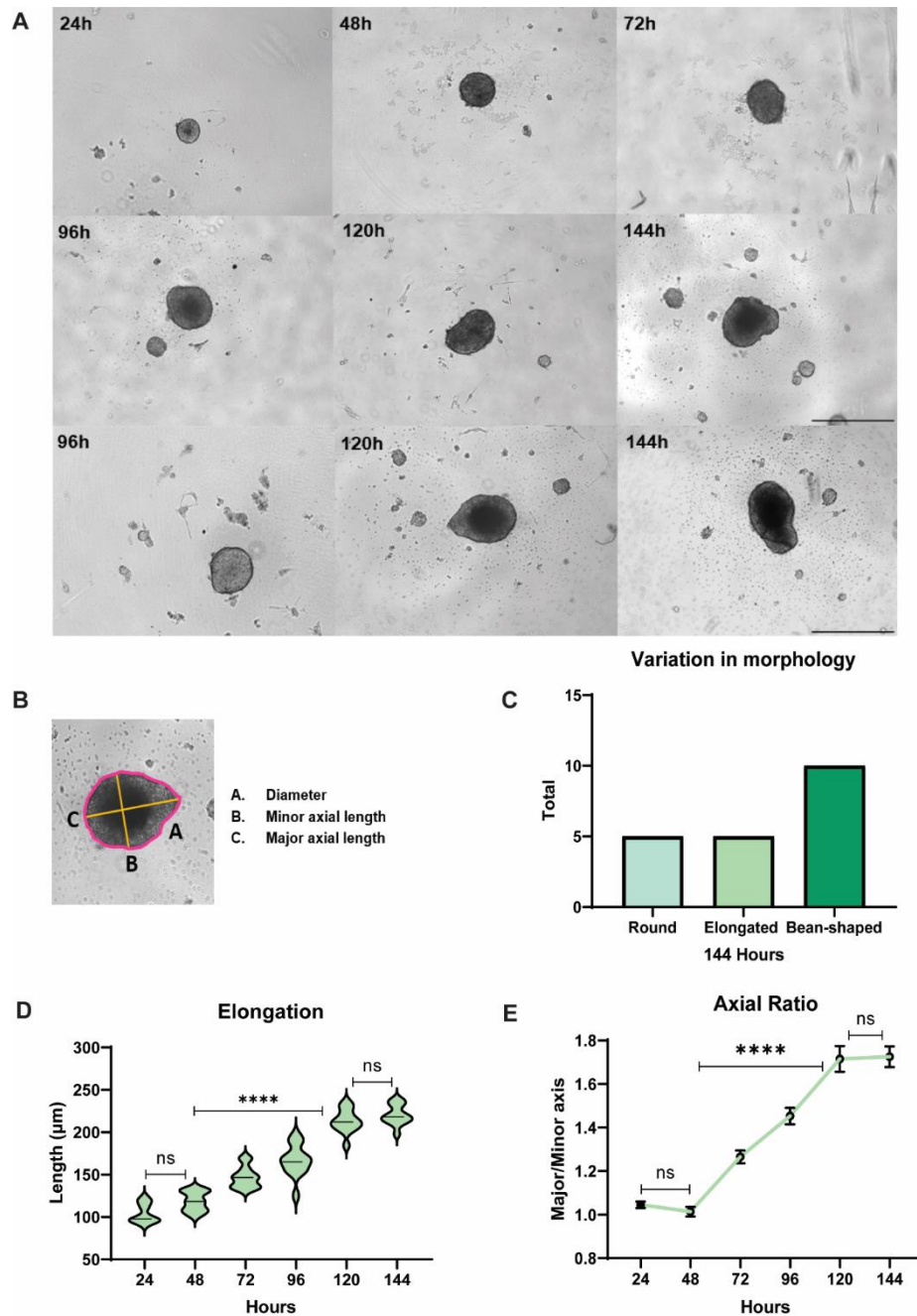


Figure 17: Morphology of Condition D - BMP4 SC gastruloids. 150 mESCs were aggregated in 96-well plates in N2B27 medium for 40 hrs whereafter the aggregates were incubated with BMP4 for 8 hrs. The BMP4 treated aggregates were merged with 250 mESC naïve aggregates and left to grow until 144 hrs. B: Schematic illustration of the measurements taken for quantification. C: Aggregates were categorized according to their variation in morphology at 144 hrs. D: Aggregate elongation was calculated by measuring the major axis length of each aggregate every 24 hrs until 144 hrs. E: Axial ratios was determined using the following formula: major axis length/minor axis length. One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing time points. Statistical significance is indicated on top of each measurement where ****: $p < 0.0001$, ***: $p < 0.001$,

******: $P < 0.05$, *ns*: non-significant, while error bars represent the SEM. $N = 20$ EBs, 4 independent experiments. Scale bar: 600 μm . ¶

4.2 Construction of PEG-Ac-SH hydrogels for differentiation protocols

The gastruloids formed in Conditions B – D were all grown in suspension (Figure 13 B-D) which do not fully recapitulate the maternal ECM environment in which embryos develop *in vivo*. In order to investigate whether the ECM environment may have an effect on *in vitro* differentiation of stem cells, a hydrogel scaffold was constructed using 4-arm PEG Acrylate and 4-arm PEG-SH. Hydrogels are hydrophilic and have a water content greater than 30% which mimics the ECM. This high-water content provides an excellent transport regime enabling the diffusion of nutrients and waste. PEG hydrogels are synthetic polymers that has been used extensively as a scaffold material in tissue engineering due to their low cost, biocompatibility, reproducibility, and tendency for modification. As a result, PEG hydrogels can be tailored to meet the design requirements. This research aim wanted to address whether mechanical stiffness alone has an effect on tissue morphogenesis by culturing gastruloids in a PEG hydrogel [234].

The mechanical properties of PEG hydrogels can be fine-tuned by changing the crosslink density. To investigate the mechanical stiffness of the gels, hydrogel scaffolds were prepared by mixing 4-arm PEG-Ac peptide conjugates with 4-arm PEG-SH in an Iso-PBS buffer. The mechanical properties of the hydrogels were varied by changing the dilution of the PEG-Ac-PEG-SH macromers expressed as a percentage volume/volume. All crosslinked polymeric hydrogels swell in water and retain water without dissolving [265]. One of the most important qualities of hydrogels is its swelling rate which depend on network density. To measure the swelling rate, the swelling capacity versus time of a hydrogel sample is often obtained by the following formula: $(W_s - W_d)/W_d \times 100$ of hydrogels measured as consecutive time intervals. The weight of the dry gels (W_d) was measured after incubation at 37°C for 1 hour. Gels were subsequently placed in Iso-PBS and incubated for 37°C for 144 hrs. Weight measurements were taken every 24 hrs. Results depicted in Figure 18 indicates the swelling rates of the range of dilutions tested between 1.5 % - 7.5 %. As shown, dilutions 2.5% - 7.5% had slower water absorption rates and was very stiff when gently touching the gels with a scalpel. The 1.5% dilution took up the most water and was not stiff when gently touching with a scalpel. However, due to the water uptake, the 1.5% dilution was too soft at 144 hrs making it technically difficult to handle.

As a result, the 1.5% dilution was introduced to BMP4 SC culture at 72 hrs. Gastruloids were grown as described in Figure 13 Condition E and cultured within the hydrogel by adding the 1.5% PEG-Ac-PEG-SH to each well containing a gastruloid prior to polymerization. Interestingly, even though the

aggregates showed a break in symmetry at 72 hrs, placement into the hydrogels exhibited no morphological changes (Figure 19 A) and instead all remained spherical in shape (n = 20) (Figure 19 A and C). however, aggregate length changed from 50 μm – 140 μm , indicating that the aggregates did indeed grow in size (Figure 19 D). There was no significant difference in the axial ratios of the aggregates where aggregates at 24 hrs had a ratio of 1.06 compared to aggregates at 144 hrs which had a decreased axial ratio of 1.04.

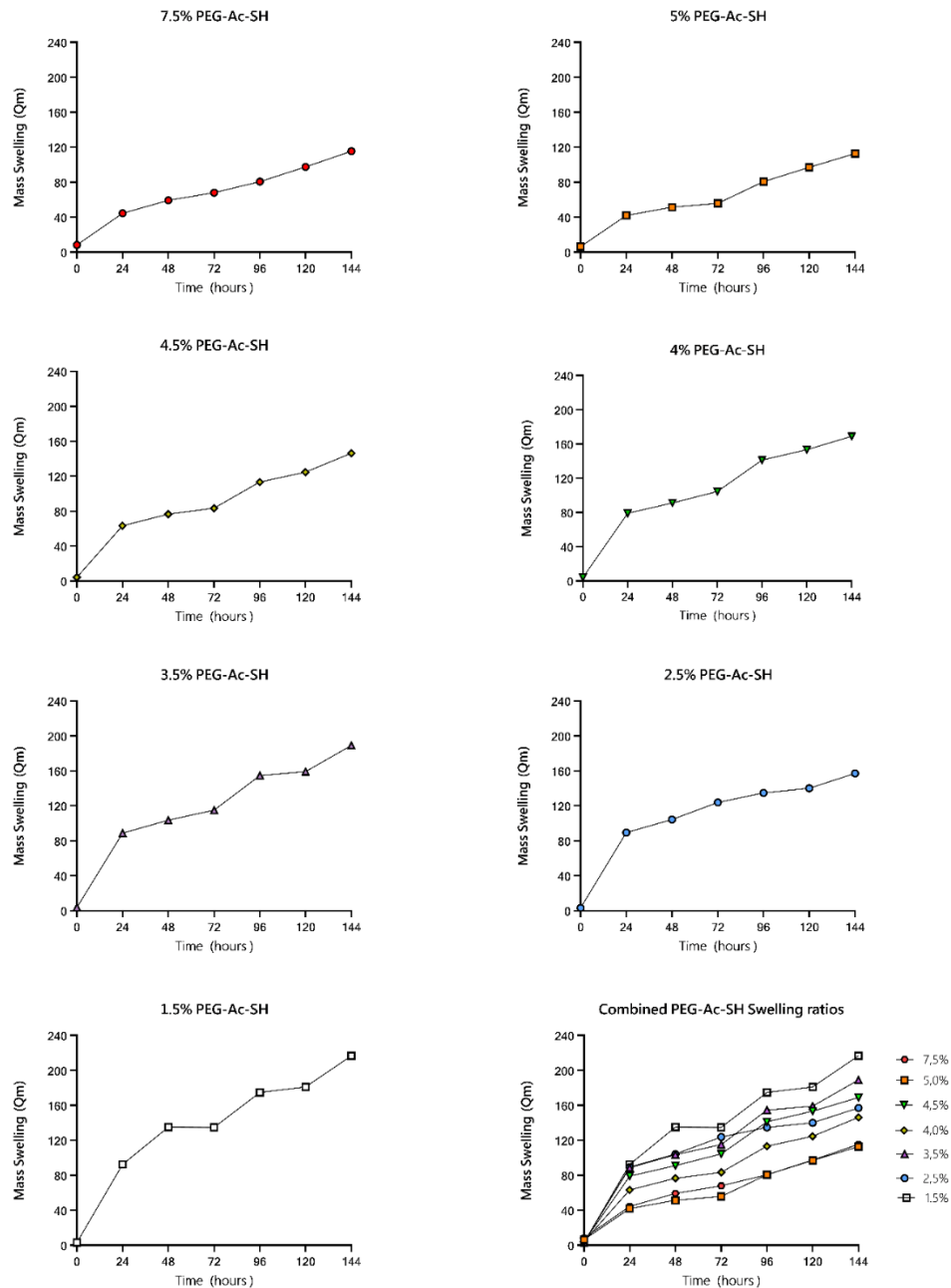


Figure 18: Swelling ratios of different composition PEG-Ac-SH hydrogels. 100 μl PEG-Ac-SH hydrogels were constructed in isotonic PBS, allowed to set, and measured (W_d). Gels were placed in isotonic PBS for 6 days and weighed daily (W_s). The swelling ratio was calculated using the formula $(W_s - W_d)/W_d$

x 100. Results shown are representative of the average of two hydrogels per percentage composition PEG-Ac-SH.

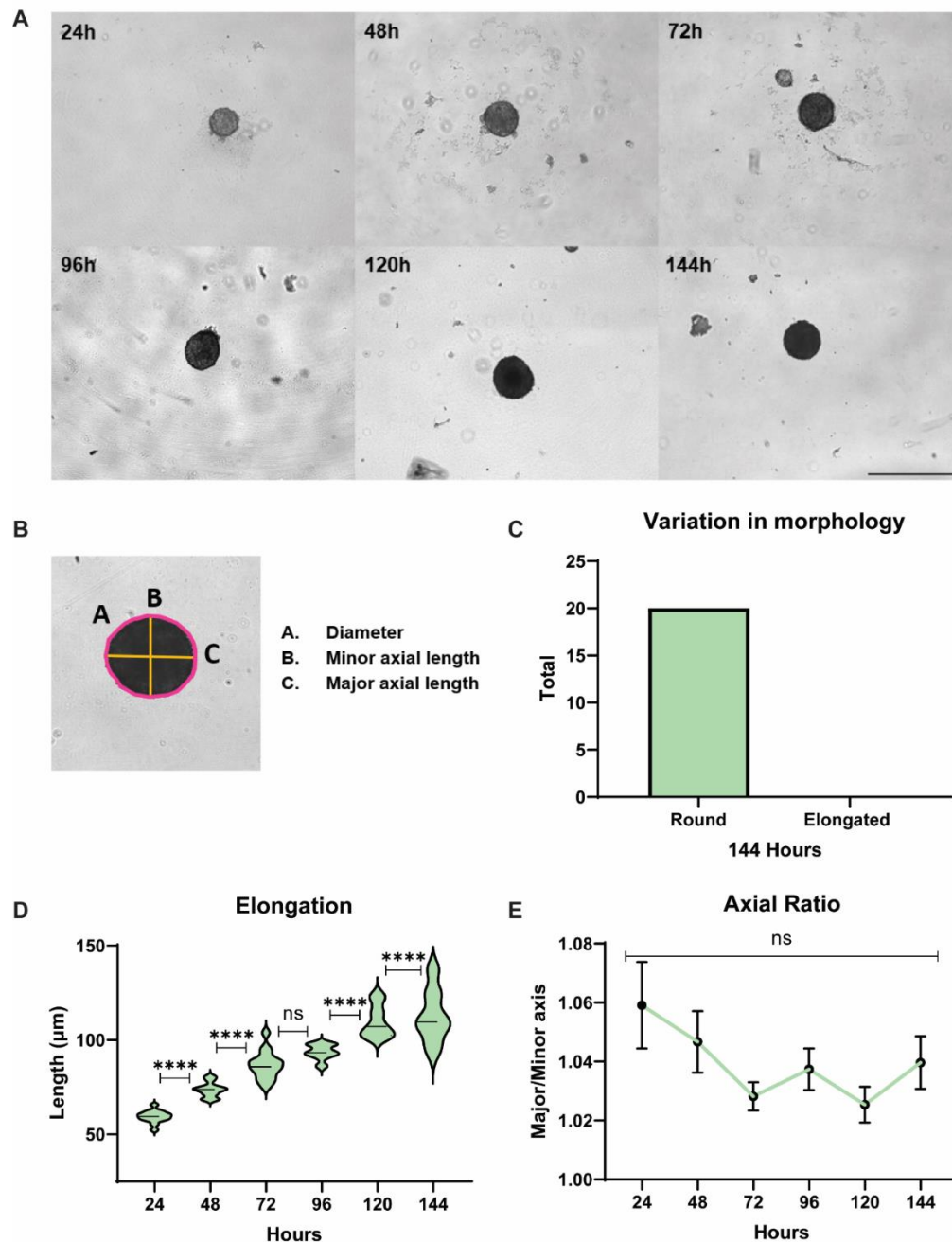


Figure 19: Morphology of Condition E - BMP4 SC in 1.5% PEG-Ac-SH hydrogels. 150 mESCs were aggregated in 96-well plates in N2B27 medium for 40 hrs whereafter the aggregates were incubated with BMP4 for 8 hrs. The BMP4 treated aggregates were merged with 250 mESC naïve aggregates and left to grow until 144 hrs. B: Schematic illustration of the measurements taken for quantification. C: Aggregates were categorized according to their variation in morphology at 144 hrs. D: Aggregate elongation was calculated by measuring the major axis length of each aggregate every 24 hrs until 144 hrs. E: Axial ratios was determined using the following formula: major axis length/minor axis length.

One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing time points. Statistical significance is indicated on top of each measurement where *****: $p < 0.0001$, ***: $p < 0.001$, **: $P < 0.05$, ns: non-significant, while error bars represent the SEM. $N = 20$ EBs, 4 independent experiments. Scale bar: $600\ \mu\text{m}$.

It is clear that Chiron gastruloids and BMP4 SC gastruloids grew largest in size, around $250\ \mu\text{m}$, compared to BMP4 + CHIR gastruloids which had a maximum length of around $220\ \mu\text{m}$ (Figure 15 – 17). In addition, all gastruloids cultured in suspension had variations in morphology compared to the BMP4 SC gastruloids cultured in 1.5% PEG-Ac-SH which had no change in morphology despite being a low dilution (Figure 19). The greatest variation in morphology was observed within the BMP4 SC gastruloids. In summary, suspension gastruloids followed a similar growth trajectory *in vitro*, indicative that the gastruloids underwent self-organization and differentiation in culture.

4.3 Differentiation is accompanied by a disruption on the pluripotency gene network

While all the gastruloid variations follow the same growth trajectories *in vitro*, except for those cultured in the hydrogel scaffolds, it is important to examine what is occurring at gene expression level within these gastruloids. By examining the expression of genes involved during *in vivo* gastrulation of the embryo within the gastruloid variations, inferences can be made whether these gastruloids do in fact undergo a gastrulation-like process *in vitro* and whether these gene expression changes resemble the *in vivo* embryo.

As discussed in Chapter 1, by adding differentiation cues to culture medium, such as Chiron and BMP4, causes a disruption in the core PGRN thereby triggering the collapse of all pluripotency pathways. In order to investigate whether the mESCs exited their pluripotent state, qPCR was used to determine the expression levels of OCT4 and NANOG within the gastruloid variations at 120 hrs and 144 hrs.

Technical challenges arose when attempting to remove BMP4 SC gastruloids from the 1.5% PEG-Ac-SH hydrogels. RNA was extracted from multiple biological replicates, however, the concentration and purity of the majority of the samples collected at 144 hrs was too low for subsequent analysis (Appendix, Figure 38 A-D). As a result, only two biological replicates at 120 hrs could be extracted that was pure enough and therefore qPCR from two biological replicates was performed only on 120 hr BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH gels.

A significant decrease in OCT4 and NANOG is observed in the EBs and all gastruloid variations at both 120 hrs and 144 hrs (Figure 20 A-D). NANOG expression was significantly lower compared to OCT4 expression. This reduction in both OCT4 and NANOG compared to mESCs does indicate that the cells

did in fact exit their pluripotent state and underwent differentiation. However, in all conditions, there is still OCT4 and NANOG present at both 120 hrs and 144 hrs (Figure 20 A-D).

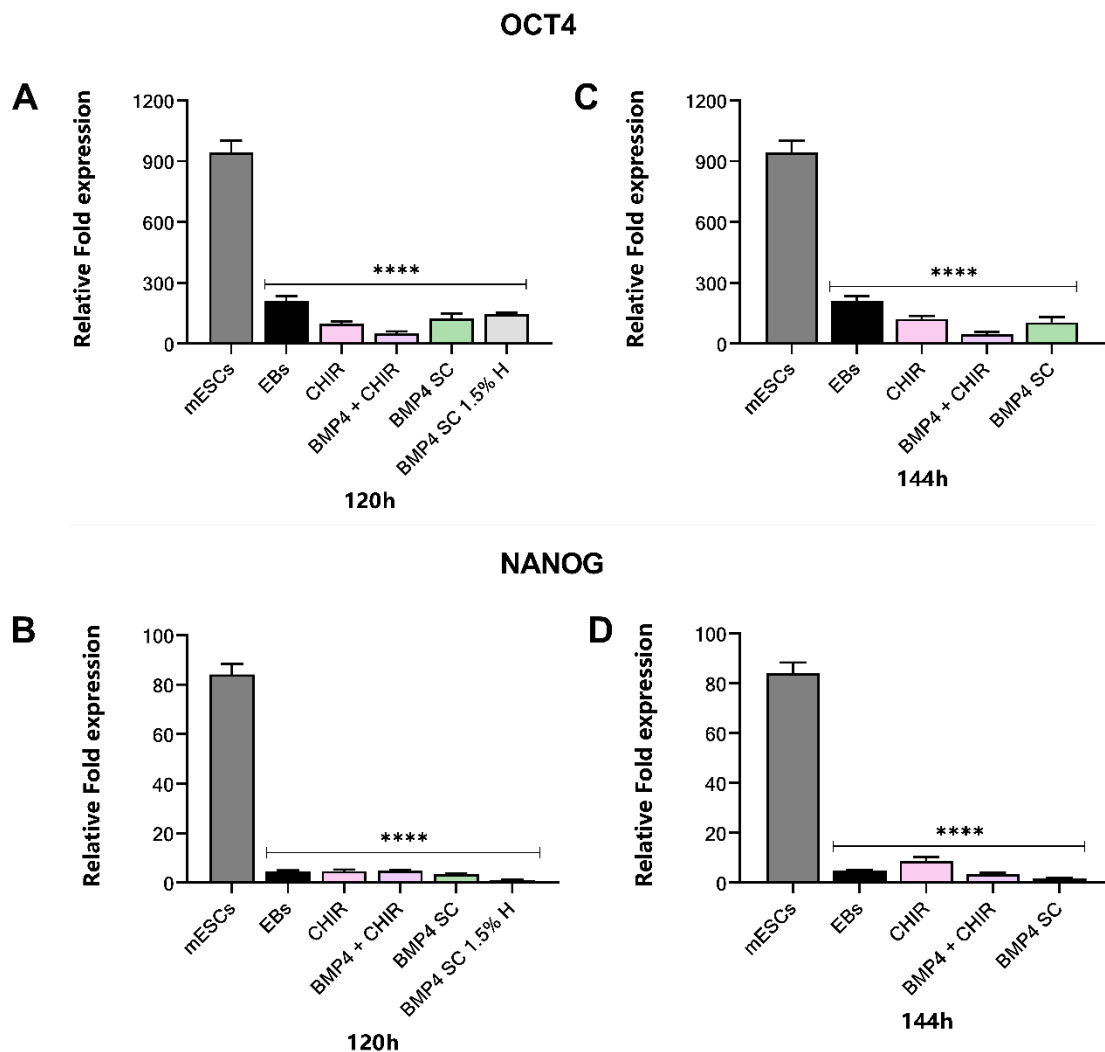


Figure 20: Relative OCT4 and NANOG gene expression in EBs and gastruloids. *qPCR was performed on reverse transcribed RNA collected at 120 hrs and 144 hrs using primers specific to OCT4 and NANOG. mRNA was normalized to GAPDH. Undifferentiated mESCs were used as a control. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing EBs and all gastruloid variations to the mESC control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$ while error bars represent the standard error of the mean (SEM). This Figure showed pooled data from two biological experiment repeats performed in triplicate.*

4.4 Discussion

While we can gain insights from stem cells grown in adherent culture, 3D models that mimic development *in vitro* allows for greater insights into cell dynamics, gene expression, and tissue interactions in a manner more similar to the *in vivo* embryo.

EBs are used as controls throughout this study as they are thought to mimic some aspects of gastrulation in a spatially disorganized manner [266]. While the EBs were mostly circular in shape and varied in size, elongation was observed in 40% of the aggregates by 144 hrs. As EBs were formed in the absence of external differentiation cues or N2B27, a neural differentiation medium, this observed spontaneous symmetry breaking event could have arisen from pre-existing heterogeneities in the starting culture of cells [210] or simply point to the innate ability of the cells to self-organize. It was also observed that the EBs formed were very small in size as they tended to form small clusters instead of large EBs. While valuable inferences can be drawn from EB research, by relying solely on the ability of mESCs, when placed closely to one another, to exchange external signals and regulate developmental programs offer restricted opportunities to experimentally control development. In contrast, the gastruloid model offers an advancement in EB research that relies solely on the activation of WNT signalling through a pulse of WNT agonist, Chiron. Interestingly, by experimentally controlling differentiation by adding Chiron, aggregates spontaneously break symmetry and undergo self-organization. As a result, EBs and gastruloids have numerous differences ranging from protocol variations, morphology, and gene expression levels.

The starting cell population is varied between EBs and gastruloids. In EBs, the starting cell number is not defined as mESC colonies spontaneously aggregate after 24 hrs forming EBs. In contrast, gastruloid formation is sensitive to the starting cell number used. While the original protocol uses 300 mESCs per well of a 96-well plate [210], cell populations of 200 – 700 mESCs have been used to form gastruloids depending on the cell line used. This study reported greater gastruloid formation using a starting cell population of 400 mESCs per well as the greater variation in morphology. In addition, aggregates formed using 200 – 350 mESCs did not undergo reproducible morphological changes while aggregates formed from 450 - 500 mESCs adhered to the plates around 72 hrs. This lack in morphology observed in low starting cell numbers have been suggested to be as a result of aggregates not generating enough diversity to respond to the Chiron stimulus whereas larger aggregates fail to self-organize [212]. The starting cell number used for gastruloid formation may largely be dependent on the cell line used which has been reported to influence the molecular properties of the cells and therefore their capacity to differentiate [267]. Unfortunately, the history of the mESCs used in this study is not known to us which may have impacted gastruloid formation. However, the use of 450 mESCs per well may have

been the optimal number required to respond to the Chiron and BMP4 stimuli in the gastruloid variations.

Because mESCs are maintained in a pluripotency state which resembles the pre-implantation Epi, the 48-hr aggregation period allows the cells to reach a state similar to the post-implantation Epi thereby rendering them competent to respond to signals from the secondary medium at 48 hrs [210, 268]. The secondary medium used for Chiron gastruloid and Chiron and BMP4 gastruloid formation contains Chiron, a WNT agonist that triggers to WNT3 signalling within the gastruloids causing the aggregates to break symmetry and undergo elongation in a manner similar to the PS in the mouse [210, 211]. Incubation with Chiron in the Chiron gastruloids resulted in symmetry breaking at 72 hrs observed by a narrowing in a region resulting in an ovoid appearance. At 96 hrs, notable elongation was observed from one axis with continued elongation observed until 144 hrs. This morphology change signifies the importance of Chiron in initiating symmetry breaking and elongation [210, 211]. However, to prevent the aggregates from drying out during medium changes, a small volume of medium must be left in the bottom of the well. The consequence is that not all secondary medium is removed at 72 hrs resulting in residual concentrations of Chiron remaining in the wells. While this has not been addressed, it is thought that daily medium changes minimize the residual effects, but this has not yet been confirmed. Another significant limitation of the gastruloid protocol is the timeline during which they can be cultured as their size renders them competent under gravity to sink to the bottom where they adhere to the bottom of the wells even on non-coated suspension plates [212]. As a result, gastruloids are typically cultured until 120 hrs, however, this study was able to continue culture until 144 hrs, the timepoint at which maximum elongation was observed.

As discussed in Chapter 1, BMP4 drives both NODAL and WNT3 signalling to induce the expression of the PS marker, Bra, which is a hallmark of gastrulation. The absolute requirement for BMP4 has been shown previously when embryos mutant for BMP4 fail to undergo gastrulation, forming a defective PS and failing to generate mesendoderm tissues [88]. Although gastruloids do not require BMP4 to elongate and pattern correctly, the fact that they lack extraembryonic tissues and require NODAL and WNT3 signalling interactions, make them an ideal model to examine the effects of BMP4 on a gastrulating system. Interestingly, BMP4 can maintain mESC self-renewal alongside LIF by preventing spontaneous differentiation [157]. However, the introduction of BMP4 in differentiation medium promotes differentiation [269, 270]. Previous work has also shown that Bra induction in gastruloids by Chiron and Activin A, an activator of Nodal signalling, occurs between 24 and 96 hours with a peak between 48 hrs and 72 hrs [268]. Based on this finding, BMP4 would be required earlier than Activin A and Chiron to activate Bra expression within the gastruloids, hence the introduction of 1 ng/ml BMP4 at 24 hrs of culture prior to the Chiron pulse to see whether BMP4 would have an effect on WNT

signalling levels within the gastruloids, which will be analysed in Chapter 5. As shown in the results, the size and shape of gastruloids treated with BMP4 and Chiron are different than those of Chiron gastruloids. This is apparent in the 144 hr timepoint of Figure 16 A, that although Chiron gastruloids become elongated by this timepoint, BMP4 + Chiron gastruloids became circular. Because BMP4 was the only variable between the two gastruloid models, it is most likely the presence of BMP4 that is interfering with gastruloid elongation. The addition of Chiron did not seem to mitigate this effect thereby indicating the effect of BMP4 on the shape of the gastruloid may be independent of WNT3 signalling or perhaps that BMP4 along with Chiron may be causing the circularity of the gastruloids. This observation was confirmed in a study that found that BMP4 interferes with the expression of both NODAL and WNT3, which are both required for correct elongation and patterning [269]. This study established that both WNT3 and NODAL signalling is required to increase gastruloid length and may suggest that it is the function of BMP4 alone that result in the circularity of the BMP4 + Chiron gastruloids [269]. Interestingly, the BMP4 + Chiron gastruloids still increased in size and area in a manner similar to the Chiron gastruloids, however, they are slightly smaller. One possible explanation for this points to the importance of WNT signalling in gastruloid growth. In the previous study, WNT inhibition in gastruloids led to ineffective NODAL signalling to induce the expression of its own inhibitor, Lefty1, thereby resulting in an increase in NODAL, more than what is required for elongation [269]. As a result, the gastruloids grow, but lose elongation thereby signifying the importance of the balance between WNT and NODAL signalling for elongation to proceed normally [269]. It could be that the BMP4 pulse induce WNT3 and NODAL expression at an earlier timepoint and that the addition of Chiron result in increased WNT3 levels and insufficient NODAL levels thereby ensuing its increase and lose of elongation in the BMP4 + Chiron gastruloids.

We next asked whether a consistent gastrulation-like event could occur in the absence of Chiron when mESCs are induced to differentiate in the presence of BMP4. In an attempt to create a concentration gradient of WNT3 and NODAL signalling, BMP4 was introduced by creating an asymmetric morphogen SC into the resulting structure [236, 237]. In this manner, the intensity of the BMP4 concentration and duration can be controlled. This strategy initiated developmental programmes that through downstream response factors, lead to successive developmental steps similar to those occurring during normal mouse development [236]. The concept is that the BMP4 treated aggregate will act as the ExE component to induce WNT3 and NODAL signalling within the naïve aggregate and thereby triggering a gastrulation-like event within the aggregates, similar to *in vivo* pre-gastrulation patterning discussed in Chapter 1.

Based on the morphological changes observed in the BMP4 SC gastruloids, the previous suggestion made that BMP4 may act alone in promoting circularity in the gastruloids was confirmed. The BMP4

SC gastruloids had the most variation in morphology with the majority bending into a bean shape with the rest either elongating or remaining circular in shape. By only evaluating changes based on morphology, it is unclear what could potentially be causing these variations, however, one potential avenue is gene expression differences that occur due to signalling molecules being used. This will be discussed in Chapter 5.

It is also worth noting that the gastruloids formed in this study did not elongate to the extent reported in research [205, 210-212]. Gastruloid growth may have also been influenced by the mESC cell line used as all the gastruloids in this study grew slower and did not undergo elongation to the extent reported from previous studies suggesting that the mESCs might have taken longer to differentiate or respond to the Chiron and/or BMP4 stimuli. In addition, the occurrence of small satellite structures persisted throughout culture despite washing twice with PBS and ensuring an accurate cell count. This observation may also indicate that the cell line used may not have been optimal for gastruloid formation as these cells did not come together as one large aggregate. Gastruloids with satellite structures were carefully monitored to ensure that these small satellite structures did not fuse with the growing gastruloid aggregate. In addition, the formation of satellite structures also points to the fact that not all the gastruloids formed from 450 mESCs per well and that in some cases gastruloids formed effectively from around 400 mESCs.

One restriction of suspension culture is the lack of an ECM microenvironment. Matrigel is traditionally used in gastruloid culture but has a limitation in that it is derived from a transformed cell line and is, in principle, not amenable to clinical application [226]. PEG hydrogels are able to circumvent this limitation as it is chemically defined thereby signifying their importance in translation of experimental research to clinical research in the future. PEG is a synthetic base polymer that is hydrophilic and resistant to non-protein adsorption [265, 271]. As a result, cells cannot adhere to it making them an ideal candidate to evaluate 3D aggregate growth. In addition, by modifying PEG-chain ends with acrylate, polymerization of the hydrogel can be achieved. 4-arm PEG-Ac was used as it provides a faster gelation time compared to 8-arm PEG-Ac. Sulfhydryls, also referred to as thiols, are present in most proteins and often linked by disulfide bonds within polypeptide bonds, and as a result, crosslinking in PEG gels therefore does not significantly modify the underlying protein structure. Crosslinking 4-arm PEG-Ac with 4-arm PEG-SH at a low dilution of 1.5% allowed for the dynamic softening of the hydrogels over time. Interestingly, no change in morphology was observed, indicating that although the aggregates broke symmetry prior to the addition of placement in the hydrogel, the gel prevented elongation as the aggregates remained spherical. This may be as a result of various factors. In addition, this study was limited in that no rheological assessment could be performed on the PEG-Ac-SH hydrogels due to instrument problems. By determining the stiffness of each gel over

time, insights into how mechanical properties affect gastruloid growth could be analysed and gels could have been tuned to resemble a stiffness of the maternal ECM in which embryos are naturally grown.

Cells remodel their surroundings to create space for cell proliferation by degrading the ECM via their deposition of enzymes during tissue development. A study by Magyar *et al* [272] encountered that mESCs encapsulated in alginate microspheres were unable to degrade the matrix which may have resulted in the inhibition of EB formation when using 1.6% alginate gels as opposed to 1.1% alginate gels. Crosslinked PEG hydrogels do not readily degrade under physiological conditions unless degradable sequences are added to the polymer. The 1.5% PEG-Ac-PEG-SH gel used in this research was not modified with a degradable sequence and relied solely on the uptake of water to soften the gel through spontaneous hydrolysis and the mESCs degrading the gel. Perhaps the 1.5% dilution was still too stiff to allow degradation by the mESCs. However, work by Baillie-Johnson using PEG-VS/PEG-Ac dilutions suggested that hydrogels with lower concentrations of PEG-Ac soften too rapidly to support the maintenance of elongation while low concentrations of PEG-VS may remain too stiff [227]. In the same study, the 2.5% PEG-Ac/PEG-VS gel enabled cell migration from the anterior of the aggregate thereby demonstrating that this cell behaviour is dependent of mechanical properties in the microenvironment rather than interactions with the ECM proteins in Matrigel [227]. It was also evident that gastruloids are sensitive to the time at which they are imbedded within the gels and that this behaviour is dependent on the stiffness and degradability of the matrix to allow for continued growth of the tissues. This study highlights that matrix stiffness is a crucial biochemical property affecting gastruloid elongation. While complex morphological alterations were not observed when aggregates were embedded in hydrogels in this study, this study highlights the importance of fine-tuning such scaffolds and the need for further experiments to determine how these cells respond to their environment so that growth can be controlled in the future with such scaffolds. One important aspect to take into consideration is emphasised by Loganathan *et al* [273] which explains that the ECM changes its physical characteristics over time and undergoes its own flows during morphogenesis which may be technically difficult to do as large-scale flows are difficult to engineer.

While morphologically the embedded aggregates did not undergo elongation as observed in the suspension gastruloids, they did in fact undergo proliferation and illustrated a minimal increase in length over time (Figure 19 D). This observation indicates that the 1.5% PEG-Ac-PEG-SH dilution is soft enough to permit expansion of the growing tissue but may be too stiff to allow for continued growth of the elongation, as observed in suspension and Matrigel cultures. However, further experiments are required to determine how the mechanical microenvironment affects the structure of the forming

tissues. In addition, the inability to extract RNA from the hydrogel cultures needs to be investigated and optimised so that gene expression within these gastruloids can be examined.

Finally, having looked at the morphological changes that occurred within all the conditions, we next wanted to evaluate whether the cells did indeed exit their pluripotent state and underwent gastrulation. While varied, all conditions exhibited a significant reduction in OCT4 and NANOG gene expression compared to undifferentiated mESCs cultured in 2iLIF medium. It can therefore be concluded that the removal of 2iLIF was sufficient to induce differentiation, as observed in the case of EBs. There was no significant difference between MEF medium and N2B27 medium when looking at the changes of both OCT4 and NANOG. It is worth noting that although OCT4 and NANOG was significantly downregulated, they are still present in within the gastruloids at 120 hrs and 144 hrs at low levels. This observation is in accordance with fact that OCT4, NANOG, and SOX2 is no longer expressed throughout the entire Epi during gastrulation, and instead becomes expressed only by cells fated to differentiate into certain germ layers [274]. OCT4 has been implicated in mesoderm differentiation [275, 276] while NANOG and SOX2 has been shown to play a role in endodermal [274] and ectodermal differentiation [68], respectively. Given this lineage-restricted expression by the core pluripotency PGRN, it may suggest that OCT4, NANOG, and SOX2 function as lineage specification factors to direct mESC differentiation to a specific lineage while inhibiting commitment to mutually exclusive lineages.

Conclusively, gastruloids cultured in suspension exhibit greater morphological changes compared to the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH. Variation in morphology was observed in gastruloids exposed to Chiron, BMP4 and Chiron, and BMP4 as a SC thereby indicating the importance of the type, concentration, and exposure of signalling molecules in driving mESCs growth, morphology, proliferation, and differentiation. The significant reduction in OCT4 and NANOG in the EBs and all gastruloid conditions indicate that the aggregates did in fact exit their pluripotency state and became primed for differentiation to specific lineages.

CHAPTER 5

Gastruloids express markers of the mesoderm and endoderm

Gastrulation is a spatially regulated and temporal event which require the precise interaction of several gene regulatory networks regulated by signalling molecules to take place correctly. The BMP4 morphogen is critical as gastrulation cannot commence without its expression from the ExE and subsequent activation of WNT3 and maintenance of Nodal in the Epi [88]. The importance of these signalling molecules in a concentration dependent manner has been shown extensively to ensure the correct specification of all three germ layers as misregulation during this process can result in congenital malformation and even embryonic death [1]. In addition, the BMP4 concentration gradient ensures that a portion of the Epi is devoid of Nodal, BMP4 and WNT3 expression, which is required for anterior neural induction in the mouse embryo [88] As gastruloids have no extraembryonic tissues, they provide an excellent platform to test the effects of BMP4 without the confounding effects of signalling from extraembryonic tissues. Conventional gastruloids, which are treated with Chiron as a WNT agonist, provide an excellent comparison when the effect of BMP4 on gastruloid formation is examined at gene expression level. Thus, this chapter examines gene expression changes at both mRNA and protein level within the gastruloid variation in order to determine whether a gastrulation-like event took place.

5.1 The signalling gene network governing gastrulation is activated in gastruloids

The gene regulatory network that governs gastrulation results from the interplay between BMP4, WNT3, and NODAL. In order to determine whether this signalling network is present within the gastruloids, the mRNA levels of BMP4, NODAL, WNT3 and its target β -catenin were measured by qPCR at 120 hrs and 144 hrs.

BMP4 was expressed within the gastruloids (Figure 21 A and B). However, Chiron gastruloids had no BMP4 present at 120 hrs but exhibited at 35-fold increase compared to the EB control at 144 hrs. It is important to note the difference in BMP4 expression in the BMP4 + Chiron gastruloids and the BMP4 SC gastruloids. The introduction of BMP4 as a 24-hr pulse followed by a Chiron pulse (BMP4 + Chiron gastruloids) exhibited a 40-fold increase in BMP4 from 120 hrs to 144 hrs. In contrast, the introduction of BMP4 as an asymmetric signalling centre, resulted in a 40-fold decrease in BMP4 from 120 hrs to 144 hrs. BMP4 expression was also increased 80-fold compared to the EB control within the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH at 120 hrs.

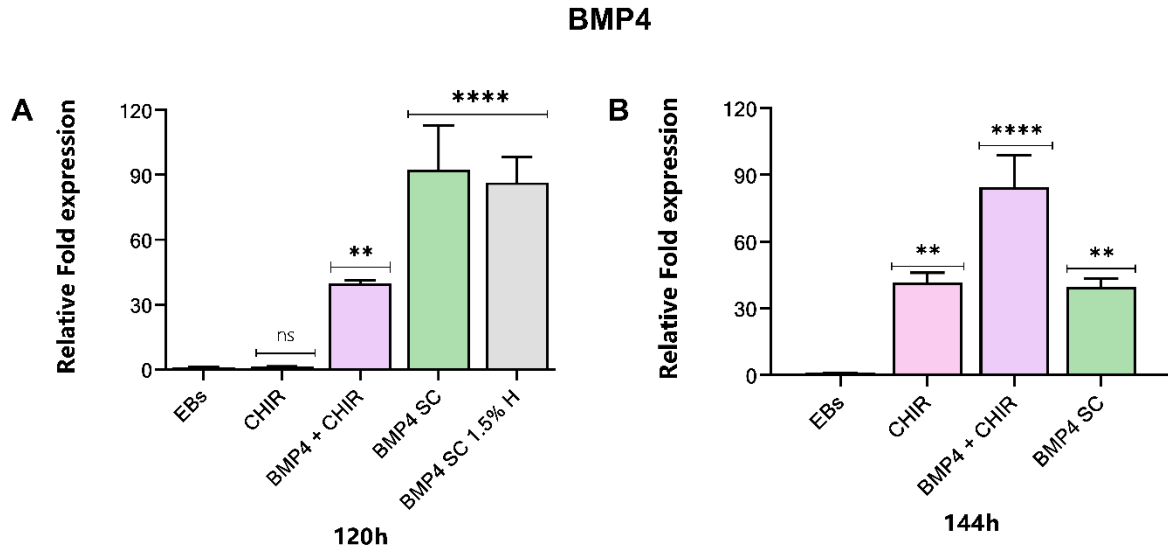


Figure 21: Evaluation of BMP4 expression in EBs and gastruloids. *qPCR* was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to BMP4. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.

WNT3 expression was highest in Chiron gastruloids, exhibiting a 75-fold increase at 120 hrs compared to the EB control (Figure 22 A) and this expression correlated to higher expression of its only downstream target β -catenin (Figure 22 C and D). Interestingly, BMP4 induced WNT3 expression to a much lower extent than Chiron (Figure 22 A and B). This can be seen by the 25-fold increase in BMP4 at 120 hrs within the BMP4 SC gastruloids and 5-fold increase in the BMP4 SC gastruloids embedded in the hydrogels compared to both Chiron gastruloids and BMP4 + Chiron gastruloids. Peak WNT3 signalling is observed at 120 hrs within all gastruloids after which its expression becomes significantly reduced. It is important to note that although higher WNT3 expression was observed in the BMP4 + Chiron gastruloids compared to the BMP4 SC gastruloids, β -catenin expression was significantly reduced within the BMP4 + Chiron gastruloids (Figure 22 C and D). β -catenin signalling was highest at 144 hrs in all gastruloids, which is 24 hrs after peak WNT3 activity observed at 120 hrs (Figure 22 D). Overall, WNT3 and β -catenin expression was induced by Chiron and BMP4.

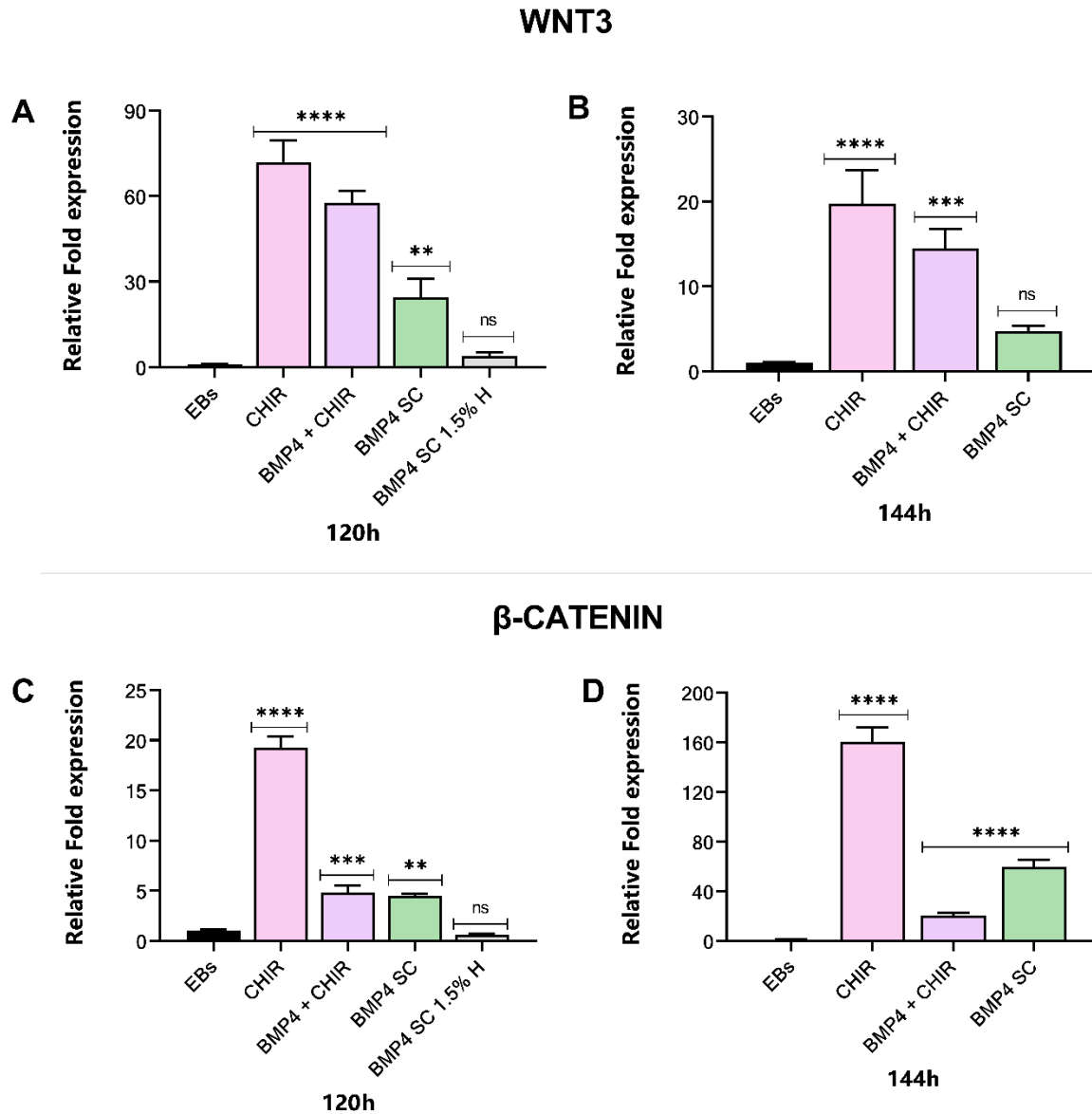


Figure 22: Expression of WNT4 and B-catenin in EBs and gastruloids. *qPCR* was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to WNT3 and its target, β -catenin. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.

Chiron treated gastruloids exhibited a 7-fold increase in NODAL at 120 hrs which decreased to 5-fold by 144 hrs compared to the EB control (Figure 23 A and B). It is important to note that both the BMP4 + Chiron gastruloids and the BMP4 SC gastruloids illustrated an increase in NODAL expression from 120 hrs to 144 hrs in contrast to the Chiron gastruloids. The BMP4 SC gastruloids exhibited higher

NODAL signalling observed by a 11.5-fold increase by 120 hrs and 17-fold increase by 144 hrs. Highest NODAL signalling was observed within the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH. In conclusion, BMP4 resulted in higher NODAL signalling compared to gastruloids treated with Chiron.

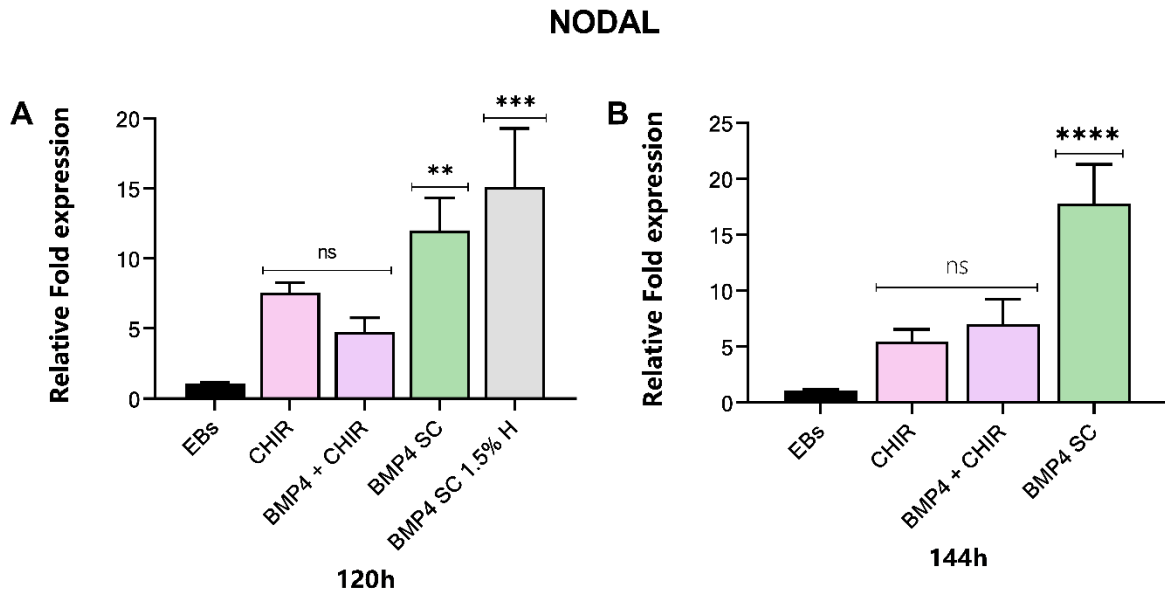


Figure 23: Expression of Nodal in EBs and gastruloids. qPCR was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to NODAL. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.

5.2 Presence of EMT-related genes in gastruloids

Having confirmed the presence of WNT3 and NODAL activation by Chiron and BMP4, we investigated whether both WNT3 and NODAL signalling induced downstream signalling events as seen in *in vivo* gastrulation. The first hallmark of gastrulation is the expression of a cohort of genes including SNAIL1, EOMES, MIXL1, and Bra by a group of cells in the posterior of the Epi at the site of the PS. As mentioned in Chapter 1, these cells that pass through the PS will undergo an EMT giving rise to the mesoderm and definitive endoderm. ECAD is a adhesion molecule that becomes downregulated during gastrulation thereby allowing the Epi cells to detach from the basement membrane and pass through the PS [102]. ECAD downregulation during gastrulation is accompanied by the upregulation of SNAIL1 [103, 104] which binds directly to ECAD thereby inhibiting its activity.

This was only true for Chiron gastruloids as they showed a decrease in ECAD at 144 hrs and increased SNAIL-1 expression (Figure 24 A - D). In contrast, BMP4 + Chiron gastruloids increased 5-fold in ECAD expression at 144 hrs while exhibiting no SNAIL-1 expression at 120 hrs but exhibited a 1-fold increase at 144 hrs compared to the EB control. The same was observed in the BMP4 SC gastruloids which had relative similar ECAD expression at both time points and no SNAIL-1 expression except at 144 hrs which had a 1-fold-increase in SNAIL-1 expression. Interestingly, BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH showed an upregulation in SNAIL-1 expression compared to ECAD, which was expressed in reduced levels compared to SNAIL-1.

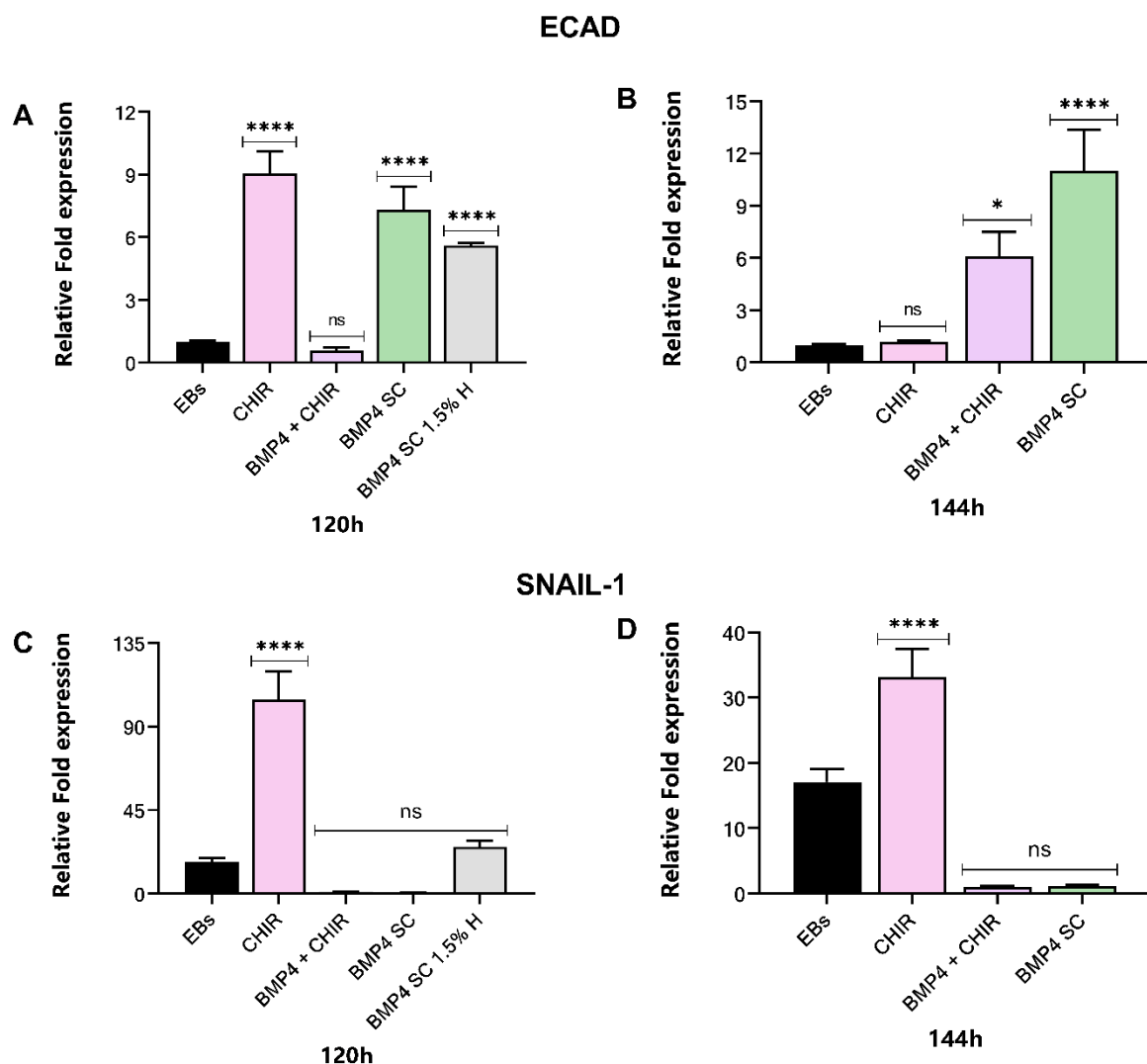


Figure 24: Expression of ECAD in EBs and gastruloids. *qPCR was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to ECAD and SNAIL-1. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.05$.*

p<0.0332, *ns*: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.

Immunofluorescence staining of ECAD revealed its presence within the gastruloids at both 120 hrs and 144 hrs (Figure 25 A-C). Contradictory to qPCR analysis of ECAD, its expression was more enhanced at 144 hrs than 120 hrs within the Chiron gastruloids. At 120 hrs in the Chiron gastruloids, ECAD seems to be restricted at the borders of the gastruloids whereafter its expression is throughout the gastruloids at 144 hrs (Figure 25 A). In contrast, ECAD expression is restricted to the borders at both 120 hrs and 144 hrs within the BMP4 and Chiron gastruloids (Figure 25 B). Interestingly, ECAD is expressed throughout the aggregate in the BMP4 SC gastruloids, however, the expression of ECAD seems to occur in clusters throughout these gastruloids (Figure 25 C). It is worth noting, due to technical difficulties, that no immunocytochemistry could be performed on the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH and was therefore not included in any immunofluorescence analysis.

Another gene evaluated was EOMES which has been shown to play a critical role during the EMT, specifically with regards to migration of mesodermal cells, and is thought to be a direct target of NODAL [106]. In addition, EOMES is required for the specification of the definitive endoderm and mesoderm [106]. Chiron gastruloids exhibited a 35-fold increase in EOMES expression at 144 hrs whereas EOMES expression became absent within BMP + Chiron gastruloids at 144 hrs (Figure 26 A and B). Both the Chiron gastruloids and BMP4 + Chiron gastruloids had relatively low levels of NODAL expression. Interestingly, NODAL expression was greatest within BMP4 SC gastruloids which correlated to greater EOMES expression levels at both 120 hrs and 144 hrs. It is worth noting that while NODAL was significantly upregulated in BMP4 SC gastruloids at 120 hrs, EOMES was expressed in low levels showing a 4-fold increase (Figure 26 A and B).

FGF signalling in the posterior Epi is also required for left-right axis formation and successful induction of the mesoderm [277]. While FGF signalling is not essential for mesoderm formation, it is involved in coordinating movements during gastrulation. In particular, analysis of FGF8 have revealed its role in the above-mentioned events as FGF8 mutant embryos illustrate EMT defects during PS formation along with mesodermal patterning defects [105, 278]. FGF8 is expressed in the PS and VE during mouse gastrulation. At E7.5, Nodal gradients triggers Left-right axis establishment with FGF8 serving as a marker of the posterior Epi. FGF8 signalling was significantly upregulated within Chiron gastruloids at both time points (Figure 27 A and B). While FGF8 expression is significantly reduced within all other gastruloids, except the Chiron gastruloids, at 120 hrs, FGF8 expression increased 7.5-fold within the BMP4 SC gastruloids at 144 hrs compared to the EB control while the BMP4 + Chiron gastruloids had no FGF8 expression (Figure 27 B).

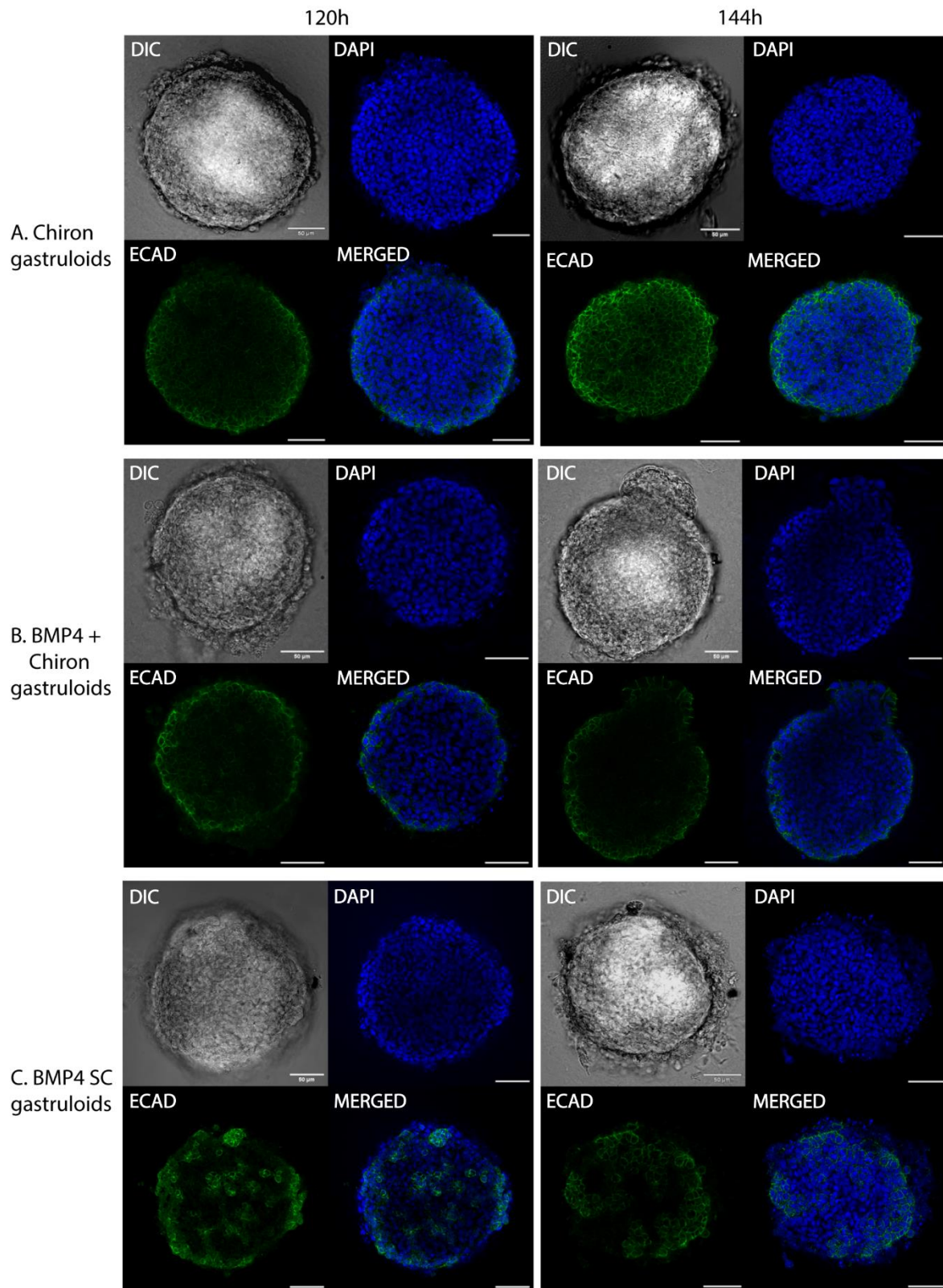


Figure 25: ECAD expression and localization in gastruloid variations by immunocytochemistry. Gastruloids were processed for immunofluorescence using monoclonal anti-ECAD (1:500) and a secondary antibody Donkey-anti-mouse conjugated to a Alexa488 fluorophore (1:500, green channel) and visualized by confocal microscopy. All gastruloids were co-stained with Hoechst and viewed with

the DAPI filter to determine the location of the nuclei (Blue channel). The Representative images for each gastruloid variation at 120 hrs and 144 hrs are shown (scale bars, 50 μ m).

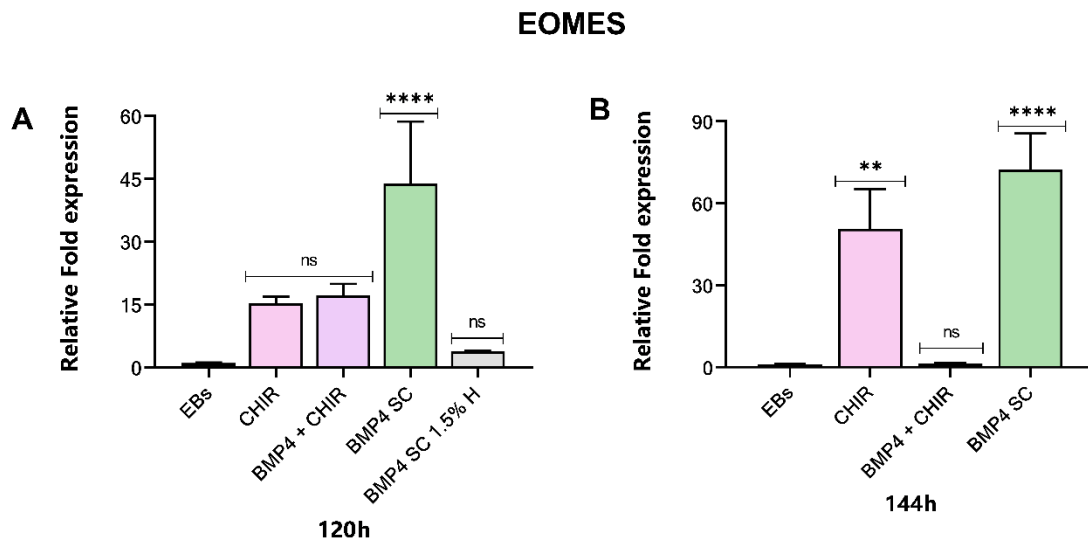


Figure 26: Expression of EOMES in EBs and gastruloids. *qPCR* was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to EOMES. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.

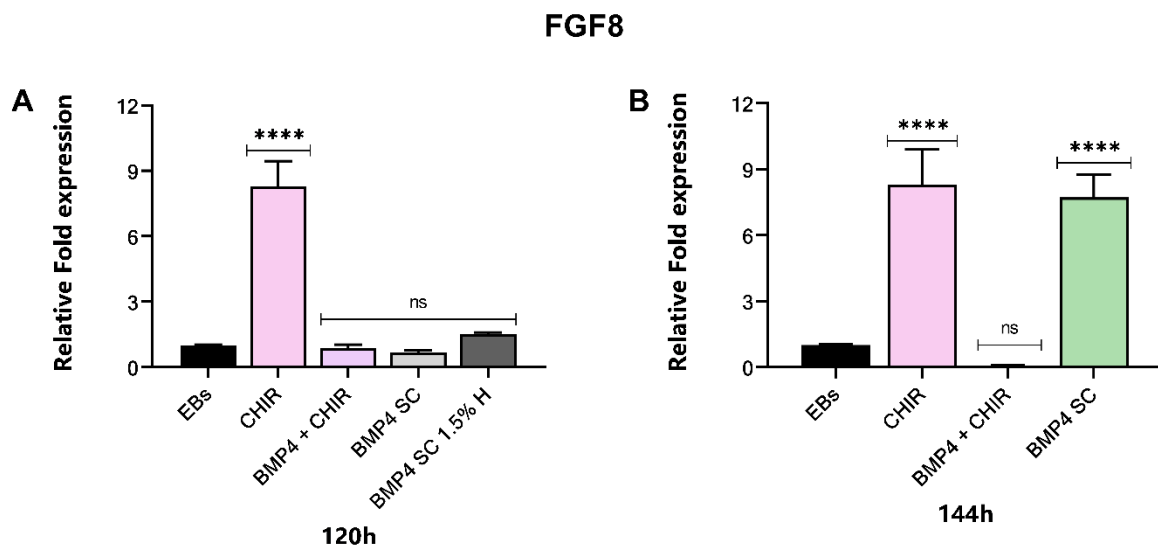


Figure 27: Expression of FGF8 in EBs and gastruloids. *qPCR* was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to FGF8 and CDX2. mRNA levels were

*normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.*

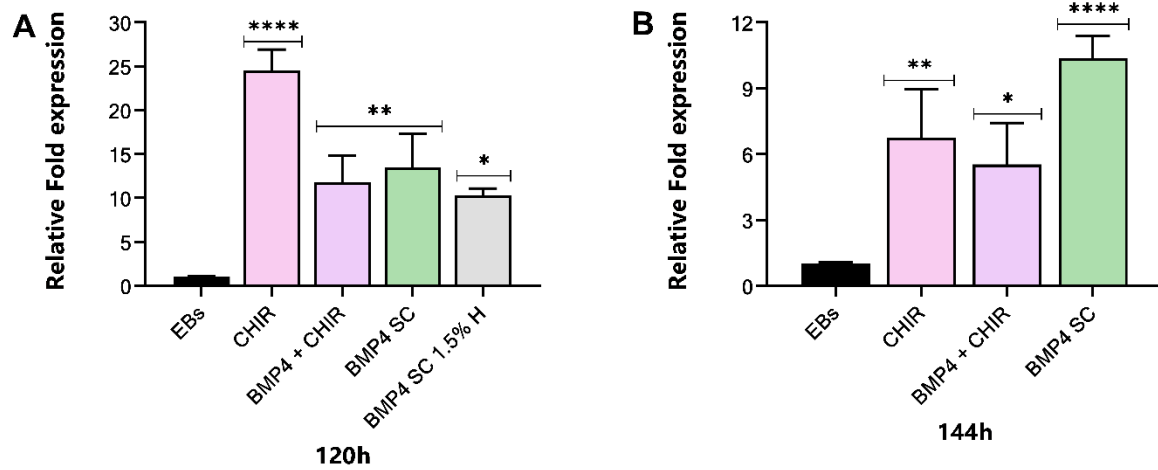
Expressed in the PS of the gastrulating embryo, MIXL-1 is induced by BMP4, and marks Epi cells destined to form the definitive endoderm and mesoderm [279]. Higher MIXL1 expression was observed within the Chiron gastruloids at 120 hr (Figure 28 A) as seen by a 24-fold increase, which decreased to 6.5-fold at 144 hrs compared to the EB control (Figure 28 B). Interestingly, BMP4 expression was highest within the BMP4 + Chiron gastruloids but showed relatively low MIXL-1 levels which decreased from 10-fold to 5-fold at 144 hrs. MIXL-1 expression also decreased within the BMP4 SC gastruloids from 12.5-fold to 10.5-fold. Collectively, both Chiron and BMP4 was able to induce the expression of MIXL-1 within all the gastruloid conditions (Figure 28 A and B).

Bra is also expressed at the site of the PS and marks cells destined to become the mesoderm [77, 280]. Bra is one of the direct targets of β -catenin, which acts downstream of WNT3, however, NODAL signalling has also been shown to drive Bra expression [281-283]. Bra expression was highest within the Chiron gastruloids as shown by a 4.5-fold increase in Bra at 120 hrs which decreased to around 2-fold at 144 hrs (Figure 28 C and D). Interestingly, BMP4 + Chiron gastruloids had significant low levels of Bra present at 120 hrs which increased to just below 2-fold at 144 hrs. In contrast, Bra expression within the BMP4 SC gastruloids peaked at 120 hrs as shown by a 3-fold increase at 120 hrs which decreased to below 1.5-fold at 144 hrs (Figure 28 C and D). The BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH had no Bra expression at 120 hrs. conclusively, both Chiron and BMP4 was able to induce the expression of Bra (Figure 28 C and D).

In addition, immunocytochemistry analysis was used to examine the spatial expression patterns of brachyury within the gastruloids (Figure 29 A-C). In the Chiron gastruloids, Bra was highly expressed at 120 hrs compared to 144 hrs (Figure 29 A). This expression of Bra seemed to localise towards one end of the gastruloid. At 144 hrs, the remaining Bra expressing cells was also clustered to one end of the aggregate. Bra expression was very weak within the BMP4 + Chiron gastruloids (Figure 29 B), however, its expression was also localised to one end of the gastruloid. It is worth noting that its expression at 144 hrs might be membranous. Bra expression was more prominent within the BMP4 SC gastruloids (Figure 29 C). While its expression also localises to one end of the aggregate, there appears to be a few clusters of Bra expression cells within the gastruloids as opposed to one defined cluster (Figure 29 C). At 120 hrs, three Bra clusters form of which two are on the same end of the

aggregate. However, at 144 hrs, all clusters can be observed within one end of the aggregate. In conclusion, all gastruloids expressed Bra and although no large clear Bra expressing pole was observed within any of the gastruloids, it is evident that Bra did localise to one end of the aggregate.

MIXL-1



BRACHYURY

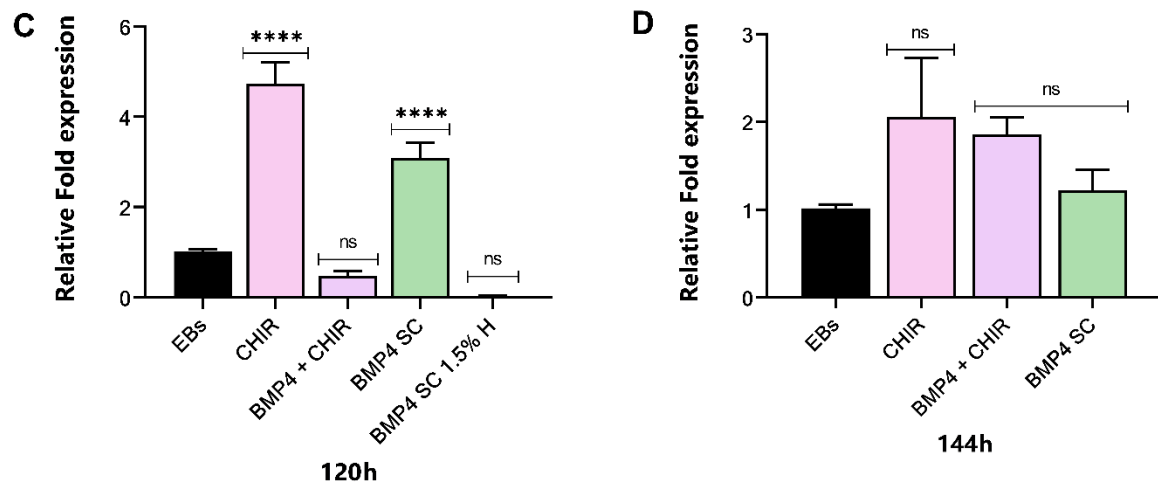


Figure 28: Expression of MIXL1 and Brachyury in EBs and gastruloids. *qPCR* was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to MIXL-1 and Bra. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.

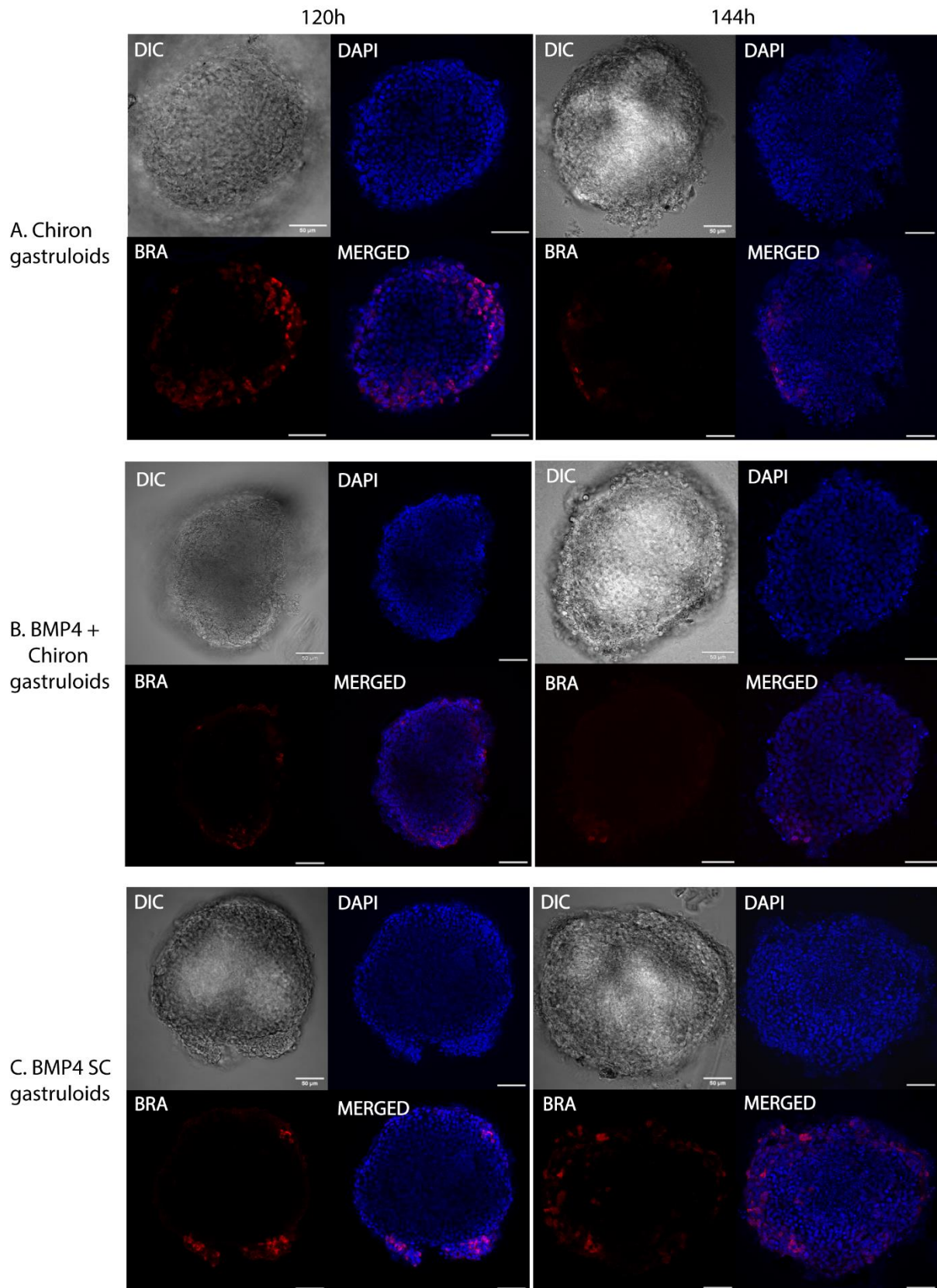


Figure 29: Brachyury expression and localization in gastruloid variations by immunocytochemistry. *Gastruloids were processed for immunofluorescence using polyclonal anti-brachyury (1:200) and a secondary antibody Donkey-anti-goat conjugated to a Cy3 fluorophore (1:500, red channel and*

visualized by confocal microscopy. All gastruloids were co-stained with Hoechst and viewed with the DAPI filter to determine the location of the nuclei (Blue channel). The Representative images for each gastruloid variation at 120 hrs and 144 hrs are shown (scale bars, 50 μ m).

5.3 Presence of Endoderm-specific genes in gastruloids

The second germ layer to emerge from the posterior Epi is the definitive endoderm. It has been reported that GATA6 is essential for visceral endoderm formation during gastrulation [214]. GATA6 expression increased from 120 hrs to 144 hrs in the Chiron gastruloids (Figure 30 A and B) whereas its expression remained the same within BMP4 + Chiron treated gastruloids which had the lowest expression levels of GATA6. The BMP4 SC gastruloids also had no significant difference in GATA6 expression levels between the two time periods, however, highest GATA6 expression was observed within the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH as seen by a 7-fold increase (Figure 30 A). During gastrulation, the cells fated to become the definitive endoderm start to express SOX17 [116] whose expression becomes restricted to the definitive endoderm, mid- and hindgut endoderm, and the endothelial cells forming vasculature [118]. Chiron gastruloids had reduced SOX17 expression at both 120 hrs and 144 hrs compared to the gastruloids that had been treated with BMP4 (Figure 30 C and D). While SOX17 was initially reduced in BMP4 + Chiron gastruloids at 120 hrs, its expression increased up to 15-fold at 144 hrs compared to the EB control. Interestingly, in contrast to the high GATA6 expression observed within the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH, SOX17 was absent at 120 hrs.

Immunocytochemistry analysis indicated that SOX17 was weakly expressed within the Chiron gastruloids, similar to what was observed by qPCR analysis, and the few cells that did express SOX17 did so all over the aggregate (Figure 31 A-C). SOX17 was also weakly expressed at 120 hrs in the BMP4 + Chiron gastruloids, however, its expression increased by 144 hrs with its expression all over the gastruloid. Interestingly, SOX17 was expressed in clusters in different portions of the BMP4 SC gastruloid at 120 hrs (Figure 31 C). However, while it was still expressed in clusters at 144 hrs, SOX17 expression was very weak.

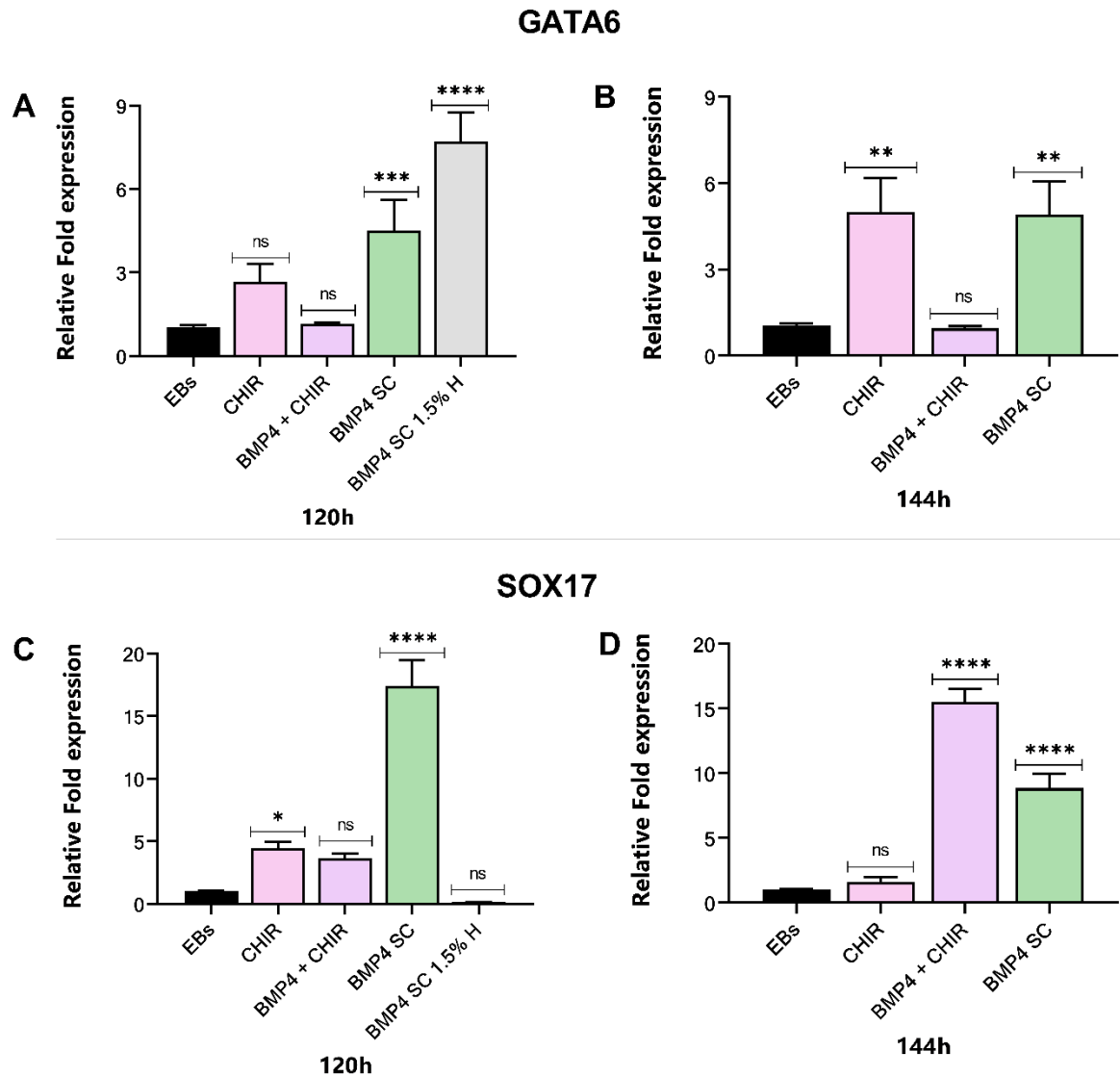


Figure 30: Expression of GATA6 and SOX17 in EBs and gastruloids. *qPCR* was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to GATA6 and SOX17. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.

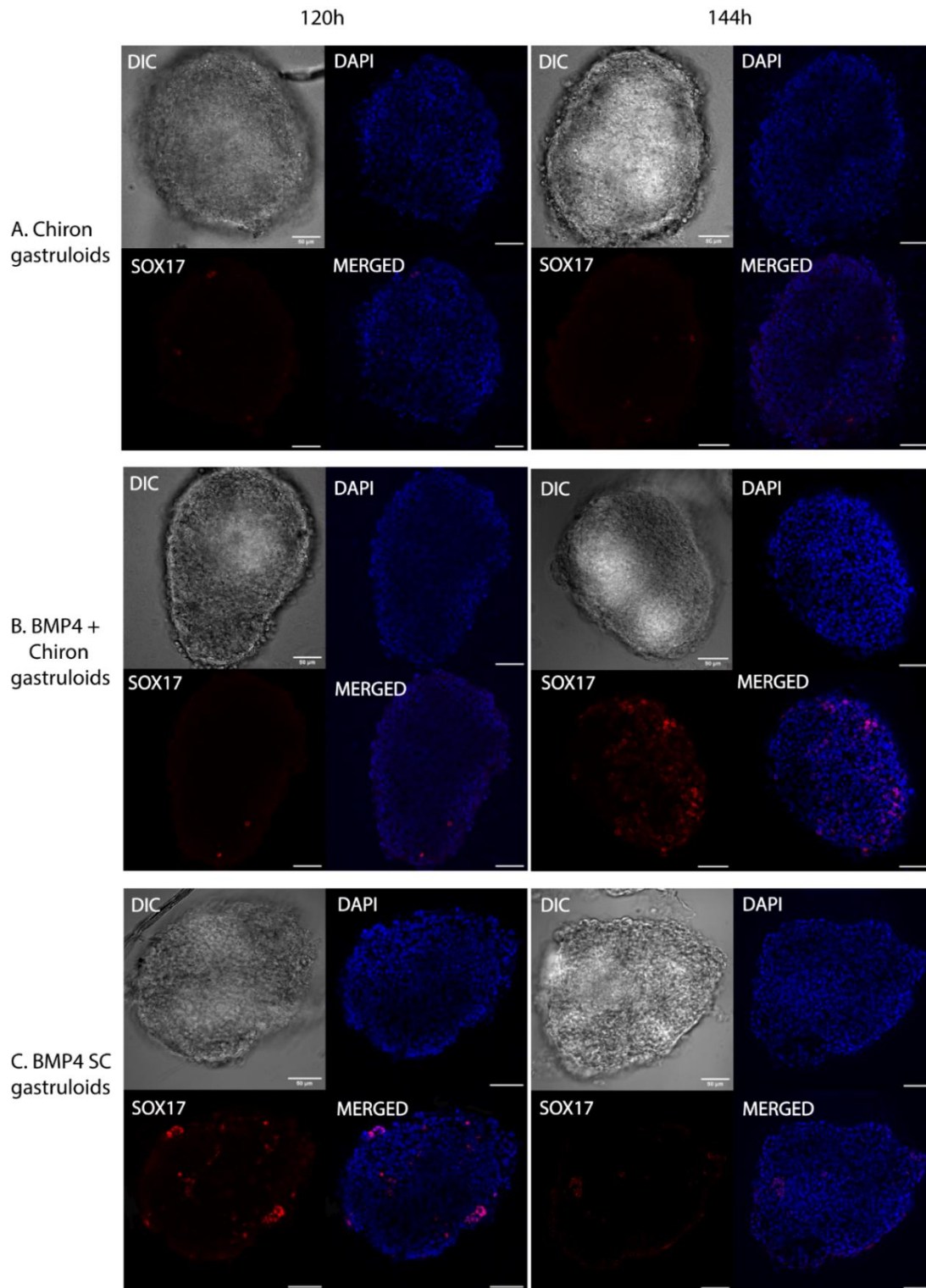


Figure 31: SOX17 expression and localization in gastruloid variations by immunocytochemistry. Gastruloids were processed for immunofluorescence using polyclonal anti-SOX17 (1:100) and a secondary antibody Donkey-anti-goat conjugated to a Cy3 fluorophore (1:500, red channel and visualized by confocal microscopy. All gastruloids were co-stained with Hoechst and viewed with the DAPI filter to determine the location of the nuclei (Blue channel). The Representative images for each gastruloid variation at 120 hrs and 144 hrs are shown (scale bars, 50 μ m).

5.4 Discussion

Once loss of pluripotency in mESCs occurs, BMP4 directs lineage commitment by favouring mesoderm and endoderm formation above NE formation [284]. Gastruloids provided an opportunity to study the effects of BMP4 at gene expression level as gastruloids have no extraembryonic lineage present. It was therefore interesting to note the expression of BMP4 in Chiron gastruloids at 144 hrs (Figure 21 B). In Chapter 3, FBS has been shown to be a possible factor in promoting differentiation of pluripotent mESCs as it contains various unknown factors. However, research has found that the suppression of myogenesis and stimulation of osteoblastic differentiation *in vitro* was as a result of a BMP-like factor present in FBS [285]. This factor was purified and found to correspond to the amino acid sequence of BMP4 [285]. Therefore, because FBS was present in small volumes during gastruloid culture, the presence of BMP4 in FBS may have triggered the mESC aggregates to produce their own BMP4 by 144 hrs. In addition, WNT signalling is known to induce BMP4 [286]. This may also be a possible explanation for the expression of BMP4 in Chiron gastruloids where WNT activation by Chiron may have triggered the expression of BMP4 in the aggregates.

In addition, it may provide a probable explanation for the increase in BMP4 at 144 hrs within the BMP4 + Chiron gastruloids as these gastruloids along with Chiron gastruloids had higher WNT3 expression. The reduction in BMP4 at 144 hrs observed within the BMP4 SC gastruloids compared to the upregulation in BMP4 at 144 hrs in the BMP4 + Chiron gastruloids may have been as a result of the duration of exposure to BMP4. Within the BMP4 pulse gastruloids, all the cells were exposed to BMP4 and for a duration of 24 hrs whereas in the BMP4 SC gastruloids, not all the cells were exposed to BMP4 as only the smaller aggregates were exposed to BMP4 and for a duration of 8 hrs. Therefore, the increased number of cells exposed to BMP4 in the BMP4 and Chiron gastruloids may have increased the effect of BMP4 in the gastruloids. In addition, BMP4 SC gastruloids also expressed lower levels of WNT3 which may have been insufficient to maintain and induce BMP4 expression.

WNT3 was activated to greater levels in gastruloids that had been treated with Chiron as opposed to BMP4 alone. This presence of increased WNT in Chiron gastruloids also yielded higher levels of its only target, β -catenin. Because there is only one β -catenin in the mouse, canonical WNT signalling activity can be completely abolished if β -catenin is absent [287]. As emphasised throughout this study, Chiron is a WNT activator, and functions by forming a complex with GSK3- β . As a result, GSK3- β can no longer form a destruction complex by binding to β -catenin and degrading it, thereby allowing β -catenin to translocate to the nucleus where it further triggers the activation of genes required during gastrulation. Much less is known regarding WNT3 activation via BMP4. While it has been well

established that BMP4 is a transcriptional target of WNT3/ β -catenin signalling [288], much less is known regarding the mechanism by which BMP4 induces WNT3 activation in the embryo.

Nodal is present in the Epi prior to gastrulation and is maintained by both BMP4 and WNT signalling through β -catenin [77]. Mentioned in Chapter 4, higher Nodal expression levels due to low WNT3 signalling may inhibit the role Nodal plays in elongating the PS. Therefore, the Nodal expression levels observed in Figure 23 may explain the increase in circularity observed within the BMP4 + Chiron and BMP4 SC gastruloids. This may suggest that Nodal acts in a dose dependent manner during gastrulation and that these levels need to be finely controlled. However, contradictory to this statement, this study reported that higher WNT3 levels corresponded to lower expression of Nodal whereas low levels of WNT3 corresponded to an increase in Nodal expression. We see in the Chiron gastruloids, WNT3 and β -catenin expression was highest, however, these gastruloids were able to maintain Nodal expression as its expression decreased from 120 hrs to 144 hrs. This may suggest that Nodal expression is to be maintained through multiple pathways. This was seen in the BMP4 SC gastruloids which had highest Nodal expression. As both WNT3 and BMP4 were present within these gastruloids, Nodal expression was maintained and induced due to the increase in expression from 120 hrs to 144 hrs. However, while no WNT3 was expressed in the BMP4 SC gastruloids embedded in hydrogels, these gastruloids had the highest levels of Nodal expression which may indicate that while WNT3 cannot maintain Nodal signalling alone, the BMP4 pathway is sufficient to maintain Nodal expression. Several studies have also suggested that Nodal expression levels within the PS is likely to regulate cell fate [129] as moderate levels of Nodal contribute to mesoderm and endoderm whereas low Nodal levels result in the loss of endoderm [289]. These results highlight the importance of the interplay between BMP4, WNT3 and Nodal in initiating the start of gastrulation and the maintenance of these molecules is required for the elongation of the PS in the embryo.

Nodal and WNT3/ β -catenin signalling is required to activate the EMT program during gastrulation. As discussed in Chapter 1, the EMT program is activated through the downregulation of ECAD and upregulation of SNAIL-1 which allows cells to break away from the basement membrane and migrate through the PS. This was only observed within the Chiron gastruloids. ECAD was found to increase within the BMP4 + Chiron gastruloids and BMP4 SC gastruloids which had little to no SNAIL-1 expression.

The EMT occurs only at the site of the PS and qPCR is the method of analysis for expression across all cells as opposed to individually and is therefore impossible to identify spatial patterning and localised gene expression. Therefore, immunocytochemistry was carried out to examine the location of ECAD throughout the gastruloids. Interestingly, the expression of ECAD at the borders at 120 hrs may

indicate that the cells in the centre underwent an EMT-like process, however ECAD was expressed throughout the aggregate at 144 hrs. This observation was contradictory to the qPCR analysis of ECAD in the Chiron gastruloids which showed an upregulation of ECAD at 120 hrs and its downregulation at 144 hrs. Like many genes involved during embryogenesis, ECAD has also been shown to define the endoderm region during gastrulation [290]. Researchers found that a group of ECAD/SOX17 expressing cells localise at the tip of the aggregate near the Bra expressing region [290]. This may explain the increased presence of ECAD at 144 hrs, however as a result of technical difficulties, the gastruloids in this study could not be co-stained and therefore cannot be definitively concluded that the expression of ECAD is due to its role in the EMT or during endoderm formation. Interestingly ECAD mRNA expression was upregulated in the BMP4 SC gastruloids at both 120 hrs and 144 hrs while immunocytochemistry revealed pockets of ECAD expressing cells throughout the gastruloids. This observation is surprising as it has been suggested that endodermal progenitors, which do not emerge through an EMT-like mechanism, emerge as small islands [291]. It may have been that the BMP4 SC gastruloids is at a later stage of development than the Chiron and BMP4 + Chiron gastruloids, and that this observation may point to the role of ECAD during endoderm specification. Another explanation could be that these gastruloids did not undergo an EMT-like process similar to what is observed *in vivo*.

EOMES serves as a marker for both the extraembryonic ectoderm during early embryogenesis and the cells leaving the PS during gastrulation. As gastruloids do not have extraembryonic lineages it can be assumed that the observed expression of EOMES is related to its role during gastrulation [77]. EOMES has also been found to regulate ECAD expression where the loss of EOMES results in the partial downregulation of ECAD and failure to execute the EMT [106]. The results presented in this study did not illustrate this, as loss of EOMES at 144 hrs in the BMP4 + Chiron gastruloids resulted in an upregulation of ECAD by 144 hrs. In addition, BMP4 SC gastruloids had significantly upregulated EOMES expression as well as higher ECAD expression compared to the other gastruloids. EOMES is the main transcriptional target of Nodal through which Nodal favours specification of the definitive endoderm and anterior mesoderm progenitors [86, 106, 274]. Research has shown that definitive endoderm specification relies on high Nodal signalling levels and the attachment of EOMES to the definitive endoderm genes [101]. This was confirmed within this study, where higher levels of NODAL and EOMES in BMP4 SC gastruloids corresponded to the significant upregulation of definitive endoderm genes SOX17 and GATA6.

In addition, low or absent Nodal levels trigger EOMES to activate key determinants for the anterior mesoderm [110] which may explain why Chiron gastruloids expressed the posterior mesoderm marker, Bra, in higher levels compared to GATA6 and SOX17 definitive endoderm markers being

expressed in very low levels. However, excessive EMT within the embryo has been found to lead to increased mesoderm formation during gastrulation [292] which may also explain the upregulation in Bra within the Chiron gastruloids. In addition, this upregulation may simply point to the increased WNT3/ β -catenin signalling in the Chiron gastruloids, as Bra is a direct transcriptional target of β -catenin. Because β -catenin was expressed in reduced levels within the BMP4 + Chiron gastruloids and BMP4 SC gastruloids, this may also explain the reduced levels of Bra in conjunction with the upregulation of Nodal and EOMES.

Additionally, it is worth noting the Bra is expressed in significantly reduced levels compared to the definitive endoderm markers. This observation could be due to several factors. Firstly, as the mesoderm is formed through the process of an EMT and the endoderm not, perhaps the gastruloids did not undergo an EMT-like process, or did so inefficiently, which led to the decrease in mesoderm markers and upregulation of endoderm markers. This explanation is most likely as the mRNA and protein expression levels of ECAD and mRNA expression levels of SNAIL-1 did not recapitulate an EMT-like behaviour as observed *in vivo*. Secondly, it is known that the endoderm is formed second to the mesoderm in a response to delayed Nodal signalling. It could be that Nodal signalling interfered with cell fate specification by favouring endoderm formation due to its upregulated expression in the gastruloids. Thirdly, the gastruloids could have been in a later developmental stage as initially thought which means that mesoderm specification could have occurred prior to 120 hrs in some of the gastruloids. The latter may be confirmed when looking at the expression of MIXL1, a gene expressed in the PS that marks cells destined to become mesoderm [114].

MIXL1 is regulated by both Nodal and BMP4 and serves as a marker and regulator of the posterior mesendodermal cells [111, 279]. Research has found that the concentration and timing of MIXL1 is critical to definitive endoderm specification as the loss of MIXL1 result in the loss of definitive endoderm genes and MIXL1 upregulation increasing the expression of endodermal genes [293]. It could be that the high expression levels of MIXL1 at 120 hrs in the gastruloids favoured definitive endoderm formation opposed to mesoderm, as seen by the upregulation in definitive endoderm markers and reduced levels of Bra, a mesoderm marker.

While Bra, the mesoderm marker, was expressed in significantly reduced levels compared to all other genes involved during gastrulation, its expression does indicate that some sort of EMT-like behaviour must have taken place within the gastruloids. Additionally, the observation that all gastruloids illustrated Bra expression towards one end of the aggregate indicates that a process of cell migration and organisation took place within the gastruloids. This observation is consistent with established research in the field [205, 210-212, 268]. Based on existing literature, it can be assumed that the

localised expression of Bra observed occurs in the posterior pole of the aggregate. Additionally, Bra expression was highest within the Chiron gastruloids which also had the lowest levels of SOX17, thereby indicating that the balance of genes specifying the mesoderm and definitive endoderm formation needs to be finely controlled as the misregulation of these factors can have a significant impact on metabolic diseases and organ malformation [294].

GATA6 is a critical regulator of endoderm formation as its depletion results in developmental arrest and reduction of endodermal markers [295]. GATA6 binds to EOMES to activate genes involved during definitive endoderm formation [295]. Lower EOMES levels, as observed within the BMP4 + Chiron gastruloids, correlated to lower GATA6 levels within these gastruloids whereas higher GATA6 levels, as seen in the Chiron gastruloids and BMP4 SC gastruloids, correlated to higher GATA6 expression levels. However, GATA6 expression levels were significantly lower than that of SOX17. Immunocytochemistry revealed dispersed SOX17 expression throughout the gastruloids in contrast to Bra, which had localised expression, thereby indicating that there was not specific endoderm-like domain within the gastruloids present. In addition, the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH had no Bra or SOX17 expression but had GATA6 and MIXL1 expression. This unusual expression pattern may be as a result of the matrix inhibiting some of the signals or gene differentiation within the gastruloid. However, further research is needed to examine the extent to which this matrix can influence differentiation and cell fate specification.

FGF8 signalling is required for cell migration away from the PS during gastrulation and therefore marks cells of the posterior embryo [105]. The significant upregulation of FGF8 in the Chiron gastruloids may suggest that the entire gastruloid is posteriorized due to the uniform expression of Chiron during culture as WNT has also been implicated in strong posteriorizing capacities [296]. In contrast, while BMP4 and Chiron was also uniformly induced during BMP4 + Chiron gastruloid formation and these gastruloids showed little to no FGF8 expression. Reports has illustrated antagonism between BMP4 and FGF8 [297], therefore the addition of BMP4, prior to Chiron, may have inhibited FGF8 signalling. Additionally, FGF signalling has been shown to be required for axial elongation in the mouse embryo [277]. Therefore, its upregulation in the Chiron gastruloids may explain why these gastruloids exhibited the most elongated structures, whereas the BMP4 + Chiron gastruloids remained more circular in shape, due to BMP4 inhibiting the function of FGF8. Furthermore, the introduction of BMP4 as a restricted signalling centre may have provided a model in which FGF8 expression was not completely lost which may explain why some of the BMP4 SC gastruloids did in fact elongate due to the presence of FGF8. Finally, FGF8 has also been shown to promote the continued expression of Bra [298], which may explain the increased Bra levels in the Chiron gastruloids. This may also point to a function of FGF8, which is to maintain mesoderm formation in the posterior Epi of the embryo,

however, its expression is not essential for mesoderm formation and it has no effect on EOMES expression [277].

The research presented in this chapter shows that BMP4 and Chiron induce a GRN that drives gastrulation-like events leading to the expression of markers that specify the mesoderm and definitive endoderm. It is evident that the choice of molecule induced during culture, either Chiron, BMP4, or both, result in variation of genes involved during gastrulation. In summary, the gastruloids may have undergone some sort of EMT-like behaviour as seen by the expression of mesoderm marker, Bra. Interestingly, Bra expression was localised to the posterior tip of each gastruloid indicating that the cells may have undergone migration and organization along the gastruloid. However, Bra was expressed in significantly reduced levels compared to definitive endoderm markers thereby suggesting that definitive endoderm formation was favoured within the gastruloids. Finally, high FGF8 levels may have contributed to posteriorizing and elongation in the Chiron gastruloids whereas its activity was inhibited by BMP4 in BMP4 + Chiron gastruloids thereby explaining the increased circularity. Finally, the BMP4 SC gastruloids could have provided a model in which FGF8 was expressed in a manner that allowed elongation of the gastruloids.

Chapter 6

Neural induction within gastruloids

Progenitors of the ectoderm germ layer do not pass through the PS by undergoing an EMT, and instead migrate to the anterior of the embryo where they give rise to the ectoderm germ layer. As a result, formation of the ectoderm occurs in the absence of both WNT3 and Nodal signalling. While Chiron gastruloids are excellent models to study the events that occur in the posterior embryo, they are limited in that they do not generate anterior neural structures. However, there is a body of work that has consistently illustrated that BMP4 is a critical regulator of both gastrulation and anterior neural induction. While BMP4 is required for the start of gastrulation, its inhibition is required for neural induction in the mouse embryo [299]. In the embryo, BMP4 is inhibited by the Chordin, which signals the nearby Epi region that will become specified as NE. As BMP4 is critically lacking within Chiron gastruloids, the modification of the gastruloid protocol to include BMP4 will provide an unparalleled opportunity to try and gain an understanding of neural induction which occurs in the anterior half of the embryo during gastrulation. In addition, by introducing BMP4 as a restricted signalling centre may shed light on its activity in a concentration dependent manner. The presence of markers for the forebrain, midbrain, and hindbrain within the gastruloids was evaluated by qPCR.

6.1 Expression of NE marker in gastruloids

The transcription factor SOX2 has various critical roles during embryonic development. During early development, SOX2 plays a crucial role in the maintenance of pluripotency and self-renewal by forming a complex with OCT4 [57]. During gastrulation, SOX2 becomes restricted to the anterior ectoderm that gives rise to the NE and SE. SOX2 expression directs differentiation into neural progenitors, specifically the NE [300], which consists of all tissues that will comprise the CNS. Its expression is also required for neural plate formation and therefore functions as a switch in neuronal development.

Interestingly, a 20-fold and 50-fold increase in SOX2 at 120 hrs was observed in the BMP4 SC gastruloids cultured in suspension and 1.5% PEG-Ac-SH, respectively (Figure 32 A). In contrast, the Chiron gastruloids and BMP4 + Chiron gastruloids showed a significantly smaller increase in SOX2 as seen by a 2-fold and 3-fold increase in SOX2 at 120 hrs (Figure 32 A). While SOX2 expression decreased significantly at 144 hrs in all gastruloid conditions (Figure 32B), SOX2 expression was still highly expressed within BMP4 SC gastruloids, observed by a 7.5-fold increase compared to Chiron gastruloids and BMP4 + Chiron gastruloids at 144 hrs (Figure 32 B).

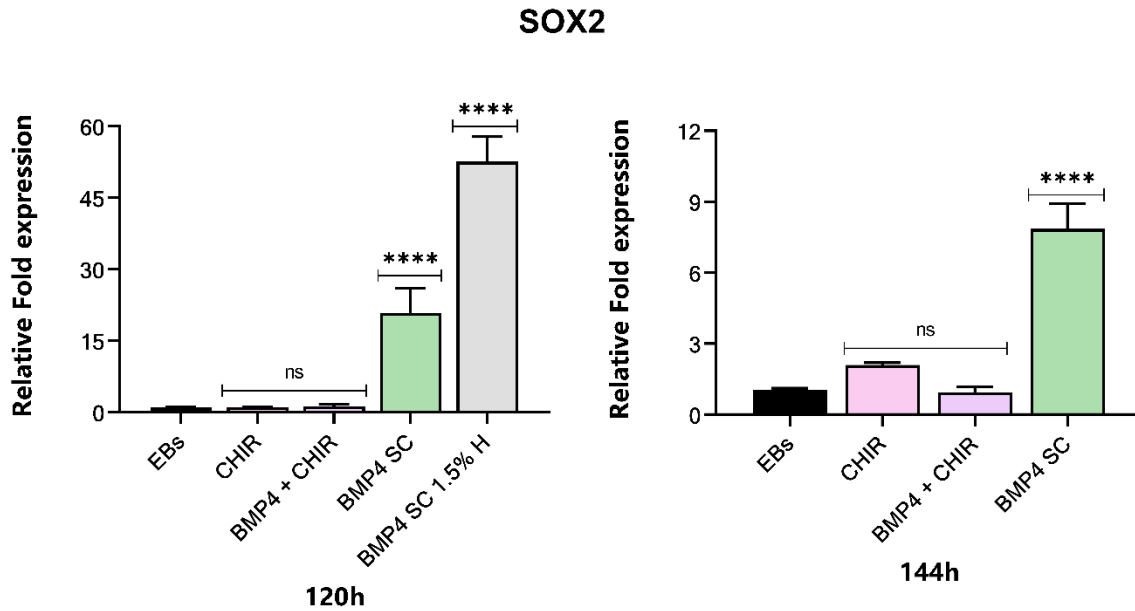


Figure 32: Expression of SOX2 in EBs and gastruloids. *qPCR was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to SOX2. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.*

6.2 Forebrain marker POU3F1 is present in BMP4 SC gastruloids

POU3F1 expression is restricted by NANOG to the anterior Epi during late gastrulation at E7.5 [301]. At the anterior Epi, POU3F1 promotes the formation of forebrain identities and has a role in the development of the neural tube. The Chiron gastruloids had a 1-fold increase in POU3F1 compared to the EB control at 120 hrs which became absent by 144 hrs (Figure 33 A and B). A similar trend was observed within the BMP4 and Chiron gastruloids which showed a 4-fold increase in POU3F1 at 120 hrs compared to the EB control, which decreased to 1-fold at 144 hrs. Interestingly, the BMP4 SC gastruloids had a 22-fold increase in POU3F1 expression at 120 hrs compared to the EB control which increased to 85-fold at 144 hrs. The BMP4 SC gastruloids in 1.5% PEG-Ac-SH also had higher POU3F1 expression, seen by the 8-fold increase at 120 hrs, compared to the Chiron gastruloids and BMP4 + Chiron gastruloids (Figure 33 A).

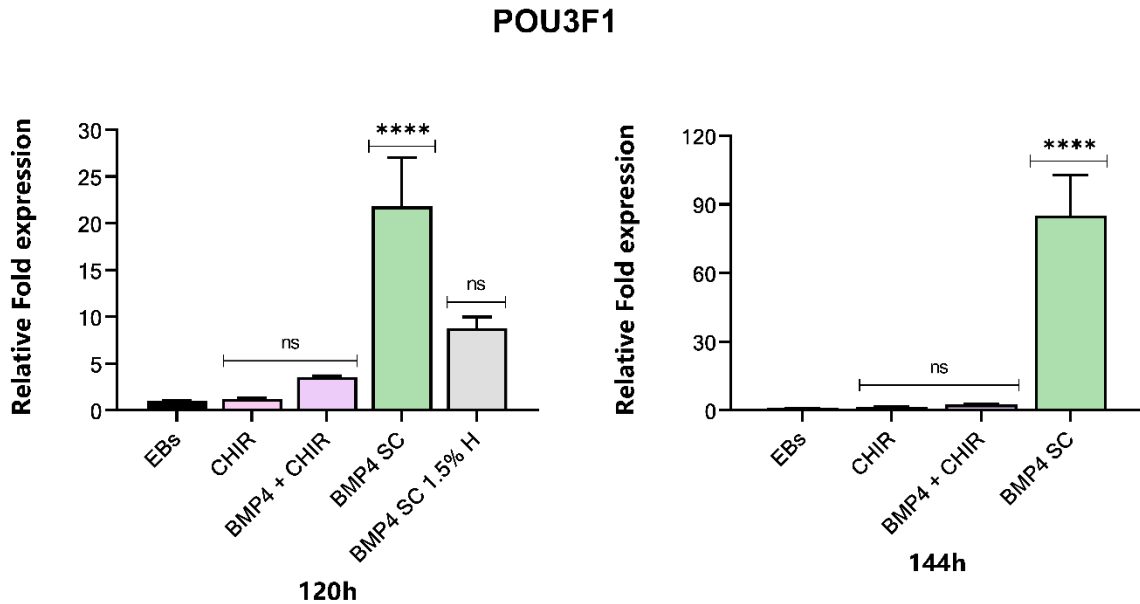


Figure 33: Expression of POU3F1 in EBs and gastruloids. *qPCR was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to POU3F1. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.*

6.3 Midbrain specific markers in gastruloids

The next two genes analysed were genes that would, if expressed together, indicate possible midbrain identity: OTX2 and EN1 [121, 122, 269]. Following the late gastrulation stage, head folds emerge from the anterior NE that will originate the cranial and brain regions together with the anterior mesoderm and endoderm [302]. OTX2 increased from 1.5-fold at 120 hrs to 4-fold at 144 hrs compared to the EB control in the Chiron gastruloids (Figure 34 A and B). In contrast, OTX2 expression decreased from 2-fold at 120 hrs to 1-fold at 144 hrs compared to the EB control in the BMP4 SC gastruloids. Finally, a significant increase in OTX2 expression was observed in the BMP4 SC gastruloids which showed a 3.4-fold increase at 120 hrs followed by a 24-fold increase at 144 hrs compared to the EB control (Figure 34 A and B).

EN1 expression was significantly reduced within the Chiron gastruloids as indicated by a 1-fold increase compared to the EB control (Figure 35 A and B). The BMP4 + Chiron gastruloids showed a slight increase in EN1 expression from 1-fold at 120 hrs to 2-fold at 144 hrs compared to the EB control. The BMP4 SC gastruloids had a significant upregulation in EN1 as seen by the 28-fold increase at 120

hrs which decreased to 11-fold at 144 hrs. The BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH showed a 2-fold increase in EN1 expression at 120 hrs compared to the EB control (Figure 35 A and B).

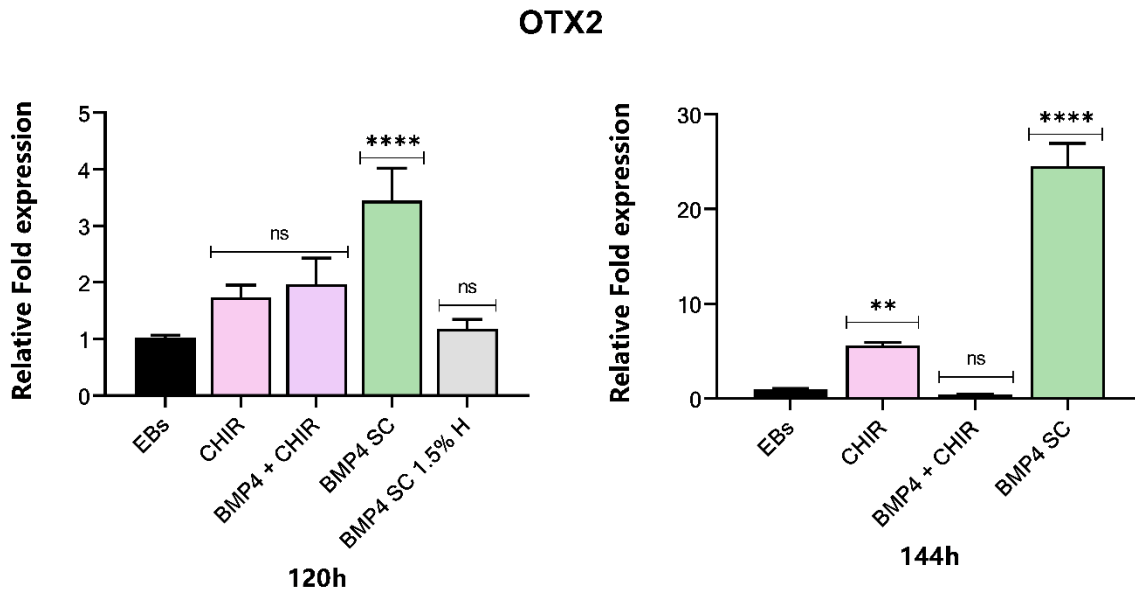


Figure 34: Expression of OTX2 in EBs and gastruloids. *qPCR was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to OTX2. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.*

Overall, EN1 and OTX2 expression was significantly upregulated within the BMP4 SC gastruloids, whereas its expression was reduced or absent within the Chiron gastruloids and the BMP4 + Chiron gastruloids.

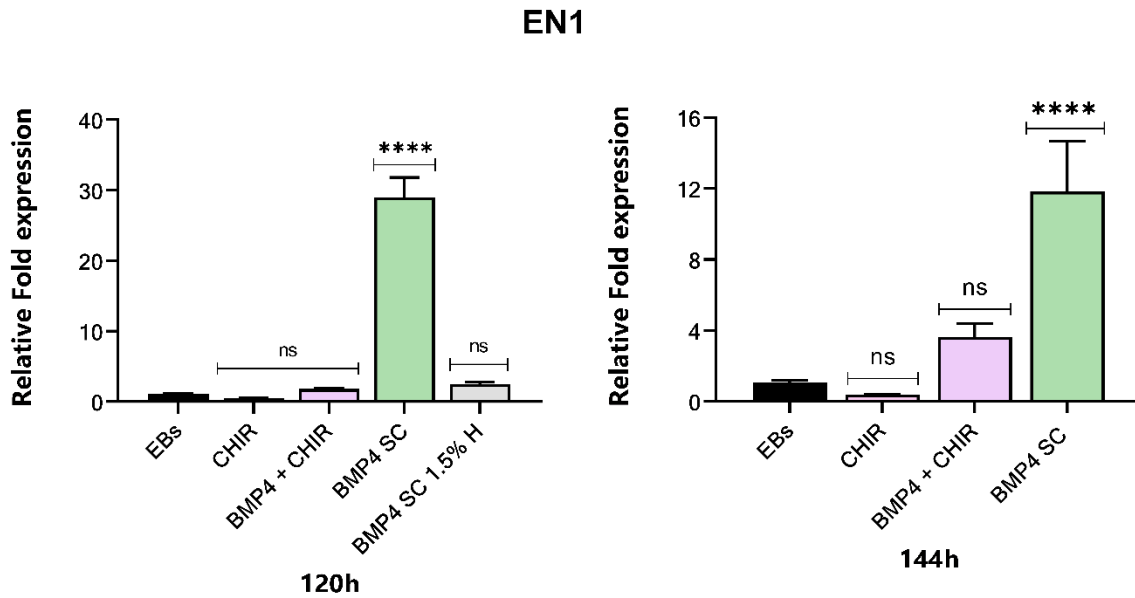


Figure 35: Expression of EN1 in EBs and gastruloids. *qPCR* was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to EN1. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.

6.4 Hindbrain markers are present within gastruloids

The last set of genes analysed corresponding to neural development is markers for hindbrain identity: IRX6 and SCUBE1. Within the Chiron gastruloids, there was a 1-fold increase in IRX6 expression at 120 hrs whereafter its expression became absent at 144 hrs compared to the EB control (Figure 36 A and B). IRX6 expression remained unchanged at both timepoints in the BMP4 + Chiron gastruloids, as seen by the 1.5-fold increase compared to the EB control. Interestingly, BMP4 SC gastruloids had significantly upregulated IRX6 levels as seen by an 8-fold increase at 120 hrs which further increased to 11-fold at 144 hrs compared to the EB control. However, the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH did not have the same IRX6 expression and instead only exhibited a 0.5-fold decrease in IRX6 compared to the EB control (Figure 36 A and B).

Interestingly, SCUBE1 expression was reduced within all the gastruloids at 120 hrs when compared to the EB control (Figure 36 C and D). SCUBE 1 expression increased 1-fold within the Chiron gastruloids at 144 hrs while its expression remained unchanged within the BMP4 + Chiron gastruloids. The BMP4 SC gastruloids increased to 3.5-fold at 144 hrs compared to the EB control (Figure 36 C and D).

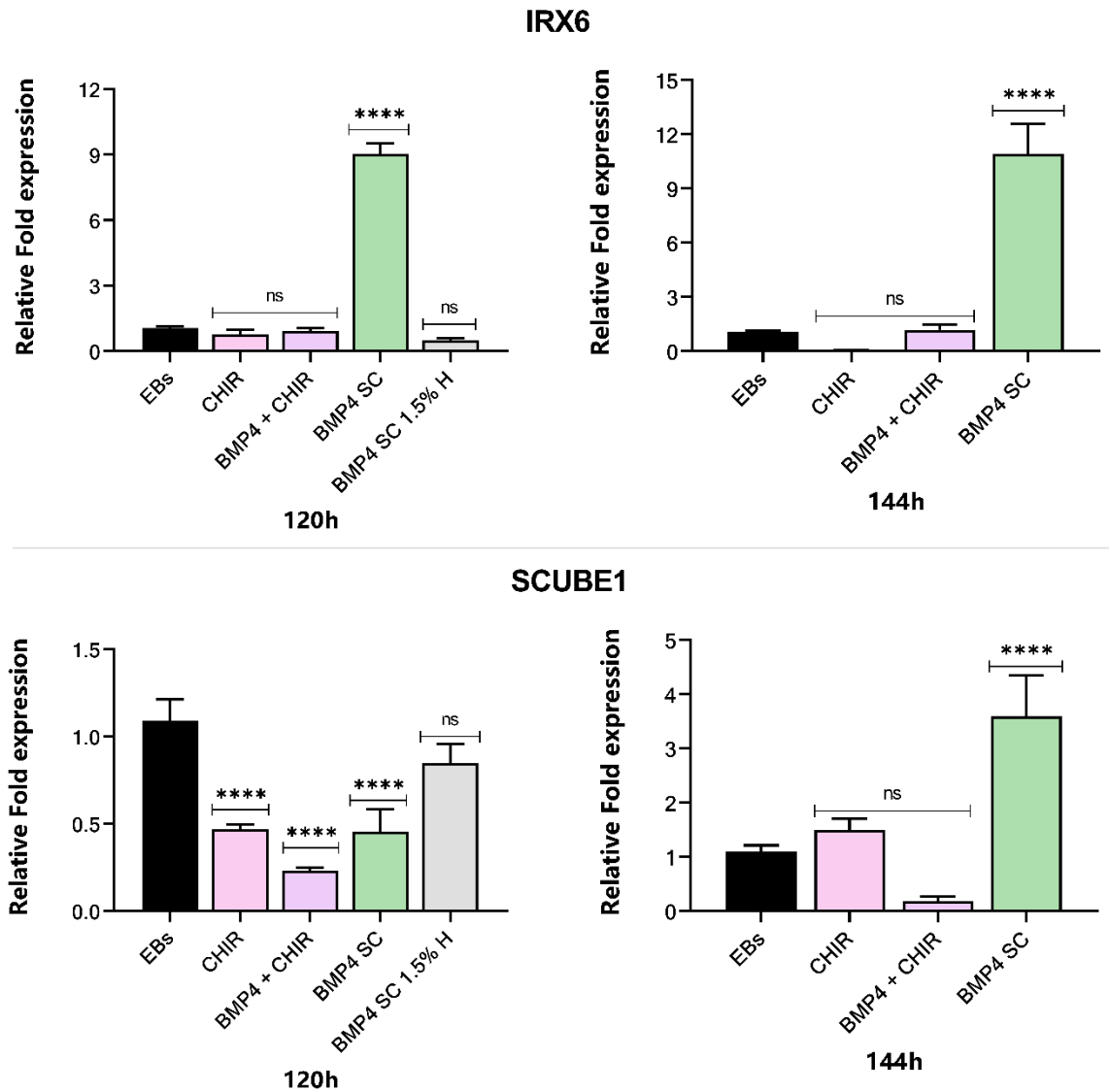


Figure 36: Expression of IRX6 and SCUBE1 in EBs and gastruloids. *qPCR was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to SCUBE1 and IRX6. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.*

Overall, the expression of IRX6 and SCUBE1 was significantly upregulated within the BMP4 SC gastruloids whereas its expression was reduced or absent within the Chiron gastruloids, BMP4 + Chiron gastruloids, and the BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH.

6.5 Overexpression of posteriorizing marker may inhibit anterior neural induction

CDX2 is a marker of posterior embryonic regions and of elongation [211]. Chiron gastruloids showed significantly upregulated levels of CDX2 as seen by a 60-fold increase at 120 hrs and 144 hrs compared to the EB control (Figure 37 A and B). In contrast, the BMP4 + Chiron gastruloids showed a decrease from 15-fold at 120 hrs to 7.5-fold at 144 hrs compared to the EB control while the BMP4 SC gastruloids increased in CDX2 expression as seen by the 2-fold increase to 13-fold at 144 hrs compared to the EB control (Figure 37 A and B).

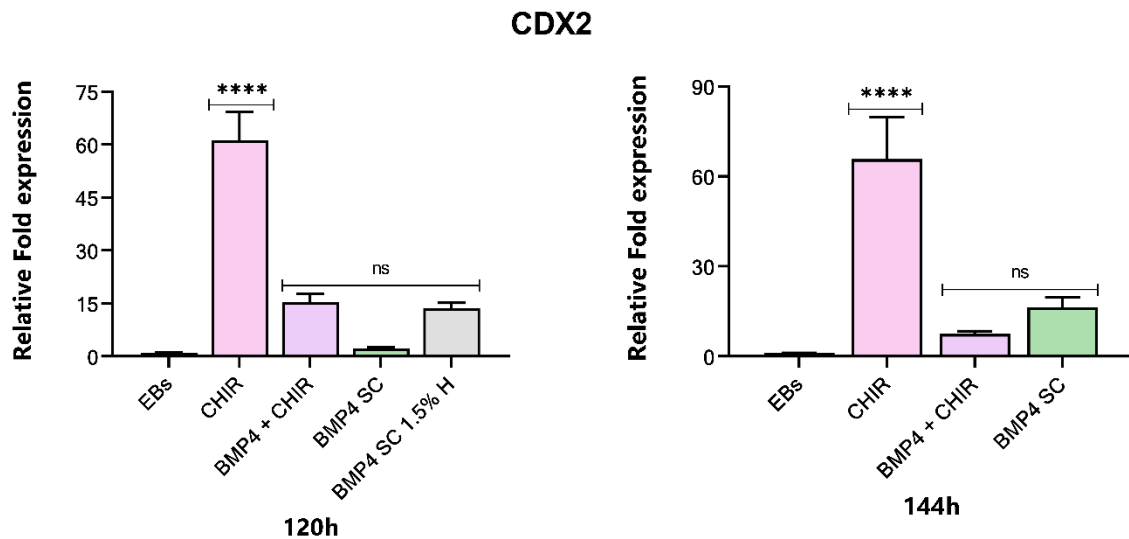


Figure 37: Expression of CDX2 in EBs and gastruloids. *qPCR was performed on reverse transcribed RNA extracted at 120 hrs and 144 hrs using primers specific to FGF8 and CDX2. mRNA levels were normalized to GAPDH. GraphPad Prism software was used to perform a One-way ANOVA with Dunnett's Multiple Comparison's post-test when comparing gastruloids to the EB control. Statistical significance is indicated on top of each group where ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$, ns: 0.1234 while error bars represent the SEM. This Figure represents pooled data from two biological experiments performed in triplicate.*

In conclusion, the BMP4 SC gastruloids showed a significant upregulation of markers for the midbrain, forebrain, and hindbrain while these markers were significantly reduced or absent in the Chiron and BMP4 + Chiron gastruloids. In contrast, the Chiron gastruloids showed a significant upregulation of the posteriorizing marker, CDX2, whose expression was significantly reduced in the BMP4 + Chiron gastruloids and the BMP4 SC gastruloids.

6.6 Discussion

The results depicted in this chapter when analysing SOX2 expression and anterior neural markers of the forebrain, midbrain, and hindbrain could be explained by the exposure of the signalling molecules Chiron, BMP4, or both. As discussed in Chapter 5, FGF8 also serves as a marker of the posterior embryo and its expression was significantly upregulated in the Chiron gastruloids. In addition, when evaluating CDX2, another marker of the posterior embryo, its expression was also significantly upregulated within the Chiron gastruloids. As the role of WNT3 in posterization has been shown previously [296], the introduction of Chiron into the culture medium uniformly may have resulted in the overexpression of genes FGF8 and CDX2, and as a result, the gastruloids may have become posteriorized. This observation is in accordance with studies indicating that Chiron gastruloids resembles a model of the post-occipital embryo [210-212]. In addition, this may explain why the Chiron gastruloids expressed SOX2 and all other neural markers at very low or absent levels as these are signals present in the anterior half of the embryo, which are seemingly lacking in Chiron gastruloids.

Furthermore, the BMP4 + Chiron gastruloids also had little to no SOX2 expression or expression of anterior neural markers. However, they expressed CDX2 and FGF8 in significantly low levels indicating that the reduced expression of anterior neural markers may not have been as a result of the entire aggregate becoming posteriorized. Instead, the absence of anterior neural markers, whose formation occurs where BMP4, WNT3, and NODAL expression is lacking, may be explained by the uniform addition of BMP4 into culture which may have inhibited the formation of anterior neural markers as BMP4 expression was highest within the BMP4 + Chiron gastruloids (Figure 21 A and B). The uniform addition of Chiron after the BMP4 pulse may also have further directed the cells towards posterior fates through the upregulation of WNT3 and Nodal.

The introduction of BMP4 as a restricted signalling centre therefore provides an unparalleled opportunity in which WNT3 and Nodal can be activated while simultaneously allowing a portion of the aggregate to differentiate into anterior neural structures where BMP4, WNT3 and NODAL is absent. This method of culture may explain why anterior neural markers for the forebrain, midbrain, hindbrain were significantly upregulated within the BMP4 SC gastruloids. Unfortunately, culture of BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH did not yield the same results as many of the genes required for anterior neural induction was significantly reduced. This is most likely due to the PEG-Ac-SH hydrogel scaffold in which the gastruloids were grown, where the stiffness may have interfered with differentiation. In addition, PEG is a base hydrogel, and no ECM signalling components of the *in vivo* ECM environment was introduced to the scaffold which may point to a possible role of ECM molecules in driving differentiation towards various cell types. This has been observed in gastruloids cultured in

Matrigel, that could lead to the generation of somite-like and heart-like structures [220, 223]. As Matrigel has some components present in the *in vivo* ECM, it may point to the importance of the ECM in driving gastrulation and neurulation.

POU3F1 expression is found to be negatively correlated with that of its direct target, NANOG, during early differentiation [301]. During gastrulation, NANOG promotes posterior differentiation and restricts anterior fates. At E6.5, POU3F1 is restricted to the anterior Epi by NANOG where it induces the formation of forebrain identities. Studies found that the upregulation of NANOG in embryos yielded a significant downregulation of POU3F1 [303, 304]. This study reported increased NANOG expression at 144 hrs within the Chiron gastruloids which correlated to reduced and absent levels of POU3F1 at 144 hrs.

It is also worth noting that EN1 is a gene indicating that the midbrain is being generated, whereas upregulation of OTX2 would not be a definitive indication of neural induction since it is also expressed in the Epi and placodal ectoderm [305]. During gastrulation, OTX2 becomes restricted to the anterior of the embryo as the PS continues to elongate and functions in the development of the head [306]. The loss of OTX2 function in mice has indicated its specific role for establishment of the midbrain-hindbrain boundary [305, 307]. In order to investigate whether the OTX2 present within the gastruloids were in fact due to the role of OTX2 in mediating anterior neural induction, future work can focus on examining downstream targets of OTX2 involved in midbrain formation. In addition, a study has suggested that OTX2 may interfere with WNT3/ β -catenin pathway signalling to ensure anterior neural development [308]. Given this finding, it could be that the levels of WNT3 and β -catenin were too high for OTX2 to interfere with its signalling which may provide a possible explanation for why the Chiron gastruloids and BMP4 + Chiron gastruloids lacked anterior neural markers. However, the most likely explanation points to the uniform introduction of the signalling molecules in these gastruloids which may have inhibited anterior gene markers within the gastruloids.

While the work presented in this chapter has generated preliminary data, there are several limitations to this study we have identified. Firstly, the expression of only a set number of genes was tested via qPCR. As discussed earlier, qPCR cannot examine individual cell expression as it evaluates expression across all cells making it impossible to identify spatial patterning. Secondly, no immunocytochemistry analysis was carried out on any of the anterior neural markers. As this is a preliminary study, more data is needed, specifically with regards to spatial patterning which can be examined through immunocytochemistry or *in-situ* hybridization to locate the position of the genes throughout the gastruloids. Performing single cell RNA-sequencing on all genes would also be beneficial in order to compare the gene expression profiles to *in vivo* mouse gastrulating embryos. Finally, as most of the

genes during embryogenesis have multiple roles, performing live cell imaging on the gastruloids may also be helpful in gaining an understanding of cell migration and specification within the gastruloids.

In summary, BMP4 SC gastruloids are able to show increased neural markers, such as SOX2, and markers indicating possible brain identity such as OTX2, POU3F1, IRX6, SCUBE1, and EN1. The possible overexpression of posterior marker, CDX2, may explain why Chiron gastruloids express reduced or absent abovementioned markers while the addition of BMP4 uniformly may explain the reduced or absent expression of the abovementioned markers.

CHAPTER 7

Conclusions

The work presented in this thesis was an effort to evaluate the action of BMP4 signalling during gastrulation and potentially neural induction by utilising the gastruloid *in vitro* system that could model these events. The results presented here illustrate the potential of 3D stem cell culture models as a powerful system to advance our understanding of mammalian development with specific emphasis on understanding cell fate decisions and signalling mechanisms independent of the embryo.

We hypothesised that in 3D culture, the GRN described in Section 1 could be elicited by applying BMP4, which would demonstrate the ability of BMP4 to modify the underlying GRN taking place in gastruloids. The introduction of 1ng/ml BMP4 was successful in inducing both WNT3 and NODAL expression, the GRN, with the gastruloids and could therefore be considered a substitute to the Chiron pulse. The activation of the GRN drove gastrulation-like events in both BMP4 + Chiron gastruloids and BMP4 SC gastruloids. However, BMP4 has been shown to negatively impact elongation in the BMP4 + Chiron gastruloids whereas its introduction as a signalling centre may have saved the BMP4 SC gastruloids from remaining circular in shape. In addition, while elongation was less prominent in the gastruloids cultured with BMP4, the aggregates were still able to grow in size and taken together with their polarised Bra expression, indicates that elongation is not merely a product of proliferation, but also of cell rearrangement thereby indicating that BMP4 could induce the cells in the gastruloid to likely undergo morphogenetic movements. BMP4 was also able to drive an EMT-like behaviour within the gastruloids as seen by the presence of Bra, although not in a manner similar to what has been reported *in vivo*.

Most importantly, the introduction of BMP4 as a signalling centre has provided an unparalleled opportunity to examine anterior neural induction. The BMP4 gradient within the BMP4 SC gastruloids may have resulted in the expression of WNT3 and NODAL in a manner that left a portion of the aggregated devoid of WNT3, NODAL, and BMP4 expression thereby allowing the significant upregulation of anterior brain markers within these gastruloids. This finding confirms the importance of signalling gradients and interactions between signalling molecules in inducing cell fate specification during gastrulation which signifies the importance of understanding the precise mechanisms between these signalling molecules.

Chiron gastruloids, which served as a representation of the original gastruloid protocol, was found to elongate best, however, may be posteriorized due to significant high expression of posterior markers

FGF8 and CDX2. As a result, while mesoderm and definitive endoderm markers were present within these gastruloids, they failed to express markers corresponding to possible brain identities further highlighting the limitation of this model.

An interesting observation from this study was that modification of the gastruloid and embryoid protocol led to a different type of gastruloid, termed BMP4 SC gastruloids, which can be used not only to examine gastrulation-like events, but to also probe into neural induction, a mechanism that could not be examined using the original gastruloid protocol. Neural induction occurs in the anterior half of the embryo and gives rise to anterior neural tissue. As this model had no BMP4 inhibitors present, it relied solely on the BMP4 concentration gradient, and the evidence showed that this model was able to induce the expression of anterior neural markers. While a lot of work remains on the characterisation of the BMP4 SC gastruloids, the data so far seem like a promising system alternative to study the GRN that take place during gastrulation and lead to neural induction while illustrating the potential importance of BMP4 signalling gradients in regulating differentiation into the three germ layers during gastrulation, either through its activation or absence.

A great limitation of the models in this study is that any developmental conclusion drawn from them must eventually be validated in the embryo. The gastruloid field is still fairly novel and has several limitations that needs to be addressed before they can be considered an accurate model of the *in vivo* mouse embryo. Firstly, variability in gastruloids needs to be reduced in order to establish their ability to be used for disease modelling and drug testing [230]. This can be accomplished by forming gastruloids in completely defined medium to reduce differences in morphology. Furthermore, future research should aim to incorporate the *in vivo* ECM environment into culture as mechanical forces provided by the uterus have been found to be critical for cell fate specification [233]. There is a distinct need for studies forming constructed hydrogels that mimic the exact mechanical properties of the maternal ECM in order to evaluate its role in driving morphogenesis during gastrulation. The use of hydrogels may also address technical limitations by prolonging gastruloid culture beyond what is currently possible.

A thought-provoking experiment that could be performed in the future that is specific to this study is to examine the growth of BMP4 SC gastruloids in a hydrogel matrix that recapitulate the *in vivo* ECM architecture and one in which maternal signalling cues from the *in vivo* ECM are present. This would help elucidate the role of maternal signalling during gastrulation as this study only examined stiffness and not ECM signalling. Finally, future work should aim to achieve validation of the gastruloid model while demonstrating continued reproducibility during gastruloid formation [230]. However, the methodologies described above are paving the way towards establishing *in vitro* simplified embryo-

like structures and help us gain an understanding into mechanisms that have been previously not well understood. The body of data on gastruloids holds great promise for their use for potential embryotoxicity, drug screening assays, and disease modelling [230].

CHAPTER 8

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CHAPTER 9

APPENDIX

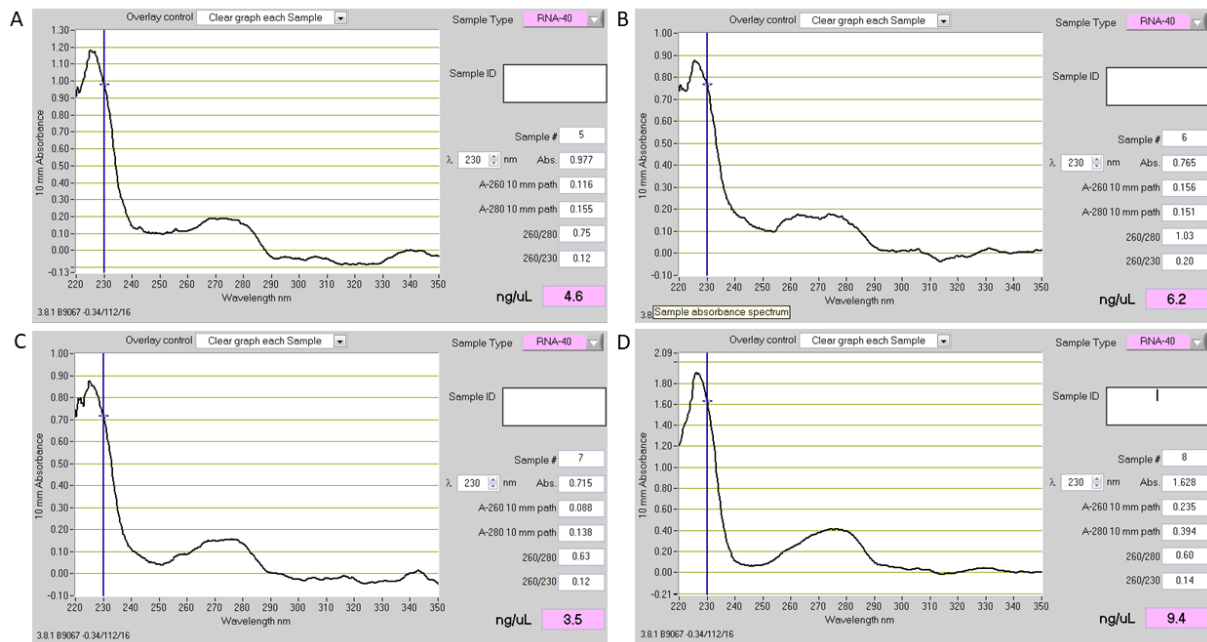


Figure 38: Concentration and purity of BMP4 SC gastruloids at 144 hrs via Nanodrop. RNA was extracted as described in section 2.5.1. Concentration and purity of eluted RNA was determined by spectrophotometry using a Nanodrop ND-100 spectrophotometer as described in sections 2.5.2. A-D represent four independent biological samples of BMP4 SC gastruloids embedded in 1.5% PEG-Ac-SH taken for RNA extraction at 144 hrs. RNA purity of all four samples was measured using the absorbance maxima of nucleic acids (260 nm) to the absorbance at 280 nm ($A_{260/280}$) ratio yielding readings significantly below 1.8. In addition, the concentration of each sample was too low for quantification during DNA synthesis. As a result all BMP4 SC gastruloid samples embedded in 1.5% PEG-Ac-SH at 144 hrs were excluded from subsequent analysis.

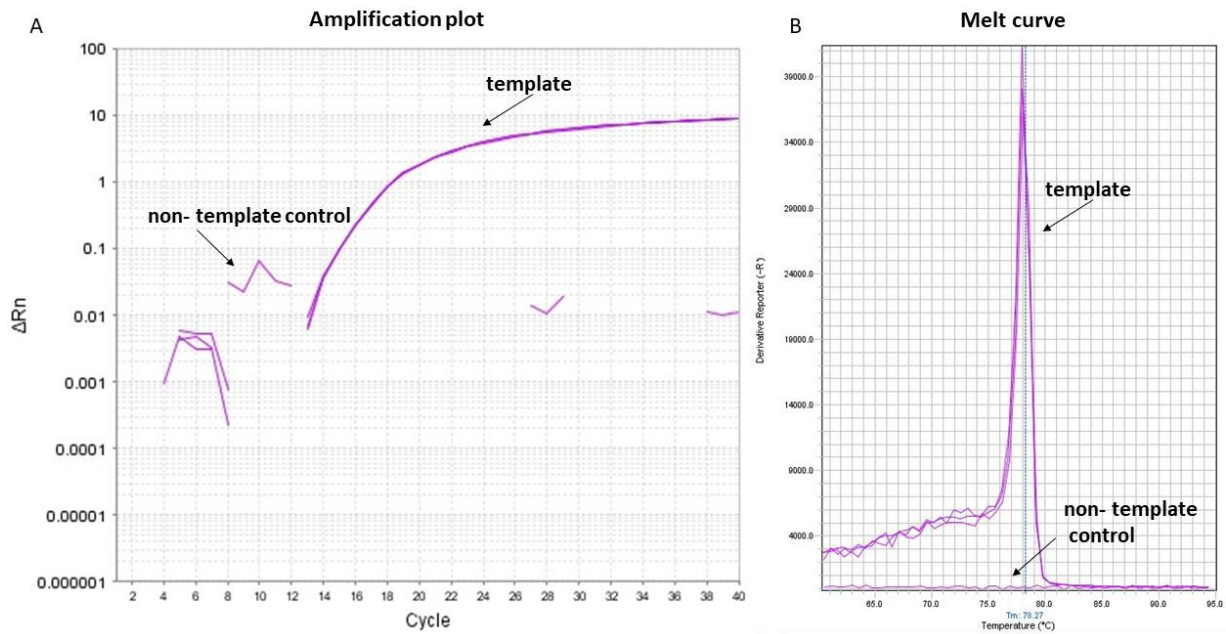


Figure 39: Amplification plot and melt curve for NANOG primer set. A: evaluation of the amplification plot was carried out to examine variation of $\log(\Delta Rn)$ with PCR cycle number. Rn is the reporter signal normalised to the fluorescent signal where ΔRn is $Rn - \text{baseline}$. The primer set is sigmoidal in shape with no variation in amplification between the replicates or PCR cycle numbers. There was no amplification of the non-template control. B: melting curve analysis was performed to confirm the amplification of a single product, i.e. the specificity. The single distinct peak observed represents a pure, single amplicon.

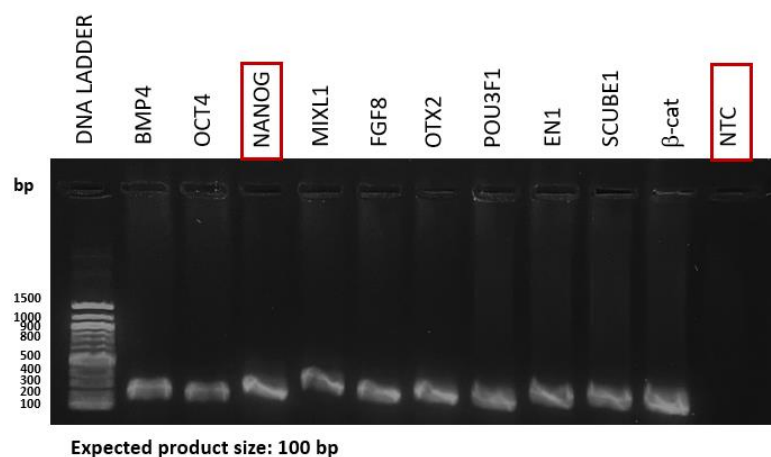


Figure 40: Agarose gel electrophoresis PCR product gel for NANOG primer set. To confirm the correct amplicon size of the amplified PCR product, agarose gel electrophoresis was performed. PCR products were separated by 1% (w/v) agarose gel electrophoresis. 100 bp DNA ladder in the first lane, NANOG primer set in the fourth lane, and the NTC with no amplicon in the last lane.