Understanding the role of signaling in pattern formation in mouse embryonic organoids

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Abstract

Small aggregates of 300-500 mouse embryonic stem cells are able to self-organize into polarized structures that exhibit collective behavior that mirrors those observed in early mouse embryos. This includes symmetry breaking, axial organization, germ layer specification and axis elongation on a time-scale similar to that of the mouse embryo. These embryonic organoids are called Gastruloids and are a reproducible model system to understand the processes underlying early mouse development. These Gastruloids were formed from mouse embryonic stem cells containing reporters for T-Brachyury, and FGF Signaling. We were able to quantitatively assess the contribution of these signaling pathways to the establishment of a pattern in early gastrulogenesis through single time-point and live-cell fluorescence microscopy.

We found that during the first 24h-48h of culture, interactions between the Wnt/β-Catenin signaling pathways promote the initial symmetry-breaking event (elongation), manifested through polarized T-Brachyury expression. Our experiments also show that FGF/MEK signaling pathway does not play an important role in pattern formation or in Gastruloid elongation although we suspect that another FGF signaling pathway might be crucial for these events to happen. However, more experimental work should be done to better understand which FGF pathway is really involved in early gastrulogenesis. We can conclude that chemical signaling plays an important role in pattern formation but the mechanical interactions between cells is also relevant for this process to take place.

From these experimental observations, we tried to model T-Brachyury pattern formation in Gastruloids using the reaction-diffusion model from Alan Turing, which is the best known theoretical model used to explain self-regulated pattern formation in the developing animal embryo. We performed the stability analysis for a two component and a three component system were we imposed that only two molecules were able to diffuse. Also, we included time delay in the typical Gierer-Meinhardt models to see whether pattern could still be able to form. As a result, we showed that any two component reaction diffusion system with only one diffuser cannot exhibit Turing instabilities and the addition of a third diffuser in our system failed to become a Turing system because of our network structure. With all the stability analysis performed, we created a tool used to reject three component systems with two diffusers from being Turing patterns just by looking at the network structure. Finally, we were able to observe how a small time delay in protein production was able to avoid pattern formation in the most used reaction-diffusion systems.

The experimental and computational results suggest that pattern formation is a process that requires mechanical transduction and time delay due to protein formation, so as a future work we shall focus on a different modeling approach.

**Keywords:** Embryonic Stem Cells, Reaction Diffusion, Gastruloids, Pattern.
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Introduction

1 Mouse Development

The aim of this report is to understand how signaling patterning emerges by studying the role of two main signaling pathways in the early development. To address this question, 3D culture of embryoid bodies and mathematical modeling will be combined. This section will introduce the processes underlying mouse development, to provide useful background to better understand the content of the report.

1.1 Morphogenetic Processes

Embryonic development is a dynamic process that varies within different organisms and it requires several steps in order to form an embryo. As this report will be utilizing mouse cells, we will focus on the development of mouse embryo.

The first main process in the formation of a mouse embryo is the fertilization in which the gametes (ovum and sperm) fuse together in order to form a diploid cell called a zygote. Following fertilization, the development of a multicellular organism continues with the next step which is Cleavage. During this process, a series of mitotic divisions occur in which the enormous volume of the egg cytoplasm is divided into numerous nucleated cells. In Figure 1 we can observe the cleavage of a fertilized mouse egg from the two cell stage up to the formation of the Blastocyst [1]. Fertilization and the four cell stage occur in the first embryonic day (E1.0). After the eight-cell stage, these cells form the Morula in which you can no longer distinguish single cells. The internal cells of the Morula will give rise to the Inner Cell Mass in the Blastocyst as the upper bulk of cells (Figure 1). The compaction of cells, the formation of the Morula and the progression into the Blastocyst happens during E2.0 and E3.0. Once the Blastocyst is formed, it is composed by the Inner Cell Mass, the Blastocyst Cavity (Blastocoele) and the Trophoblast, the latter, will not give rise to embryonic tissue but to extra-embryonic membrane and portion of the placenta. From the Inner Cell Mass, embryonic stem cells are obtained in order to work with them in vitro as we will discuss later on. The Inner Cell Mass will then further segregate into the epiblast, which will give rise to the embryo proper and some components of the fetal extra-embryonic membranes, and the primitive endoderm [2].

During E4.0 Implantation occurs, which is the process by which the blastocyst adheres to the maternal uterus wall. After implantation, the mouse embryo starts to develop into a cylindrical structure which is different from most mammalian embryos, which tend to be more spherical. During this time, E5.0, the mouse embryo changes dramatically in size and shape; the embryonic tissue volume increases by about 40-fold, the number of cells in the inner cell mass grows and the epiblast cells become epithelial and form a cavity - this is what changes the shape from a ball to a cup shape. At this stage, the mouse embryo is called the egg cylinder and it has a well delineated extra-embryonic and embryonic regions that further define a polarized proximal-distal
axis [3]. At E6.0-E6.5, the primitive streak forms in a localized region of the epiblast located adjacent to the extra-embryonic ectoderm where it marks the posterior site of the embryo. The primitive streak will establish bilateral symmetry and initiate germ layer formation [4]. The embryo has now acquired the shape of a cup made up of two cell layers: the inner epiblast and the outer visceral endoderm.

![Figure 1: Cleavage in mouse embryo. Image obtained with permission from [1].](image)

The next step is a very important and dynamic process in which the morphology between mammalian embryos differs a lot at this stage. This process is called **Gastrulation** and mouse embryos (as well as many mammals) only need a few hundred of cells to start it [5]. The site of Gastrulation is determined at the primitive streak (E6.5) and it is a movement of epiblast cells from internal positions to wrap around the inside of the embryo. These morphogenetic movements coupled with cell proliferation and differentiation, allocate cells from the epiblast to three definitive germ layers: the ectoderm, the mesoderm and the endoderm [2] [3]. At the cellular level, gastrulation involves a sequence of highly organized epithelial-to-mesenchymal transition movements \(^1\) that propagate through the tissue in a manner that resembles a traveling wave [7]. These movements involve many signaling pathways which will be discussed in section 1.2. Gastrulation is a process that lasts until E7.5 and that has great impact on embryonic development since it is the first time that a single-layered epithelium, the epiblast, is transformed progressively into three layers and also, simultaneously the embryo acquires the axial organization characteristic of the future fetus [8].

All of the mentioned processes can be exemplified in Figure 2 in which we can see a sketch of the Blastocyst and the corresponding mouse embryonic images from E6.0 until E9.0. Later processes of mouse embryonic development involve the formation of the neural plate (which involutes into the body to create the neural tube: the precursor of the central neural system) and also the formation of the heart, the amnion and eventually, limb development. However for this study we are only interested in early mouse development so we will not enter in more detail in these later processes.

\(^1\)Process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells (which are multipotent) [6].
Figure 2: TE: Trophoderm. Mouse embryonic development sketch after the Blastocyst formation until embryonic day 9. Mouse embryonic images were obtained from emouseatlas. This web page uses the EMAP resource [9] which has embryological mouse models to provide a digital atlas of mouse development as a spatiotemporal framework for embryological studies.

1.2 Spatially localized signaling

Early development is a dynamic process in which a lot of cell movement occurs but also, a lot of signals are involved in order to form a new organism.

As shown in Figure 2, the primitive streak forms in a localized region of the epiblast located adjacent to the extra-embryonic ectoderm where it marks the posterior side of the embryo [4]. At the onset of gastrulation, expression of the T-Brachyury transcription factor [10], is restricted to the primitive streak. This gene has been reported to be required for the proper development of the primitive streak, the allantois\(^2\) and the axial and the posterior mesoderm [10]. The expression of T-Brachyury can be detected in ectoderm adjacent to the streak and in nascent mesoderm underlying the streak in mouse embryos throughout gastrulation [12]. During E6.5 T-Brachyury is restricted to the proximal posterior region of the embryo [4], and at this position, the primitive streak cells will progress towards the distal end of the epiblast [13]. However the expression of T-Brachyury in the mesoderm disappears as the cells move away from the streak

\(^2\)Hollow sac-like structure filled with clear fluid that will go on to form the umbilical cord [11].
and assume their lateral, paraxial, or extra-embryonic position. Only the head process and notochord continue to express high levels of T-Brachyury [15]. After this time, T-Brachyury expression becomes restricted to a region in the tail that will undergo caudal extension to generate the caudal spinal cord and somatic mesoderm (Figure 3).

![Figure 3: On the left: Mouse embryo with T-Brachyury expression from Valerie Kouskoff lab (CRUK). On the right: T-Brachyury expression in a Gastruloid Created with mouse embryonic stem cells Brachyury::GFP reporter line. In this picture we can observe the resemblance between the Gastruloid model and the Embryo when polarization of T-Brachyury signaling occurs as a sign of Anterior-Posterior symmetry breaking.]

As we mentioned in section 1, Gastrulation involves a sequence of organized movements called epithelial-to-mesenchymal transition. The first cells undergoing this process will ingress towards the anterior contralateral side of the embryo. This movement happens simultaneously as the distal and anterior spread of the primitive streak. By the end of the Gastrulation, two-thirds of the epiblast has been wrapped by the cells that have already gone through gastrulation [16]. These cell movements happen in synchronization with chemical signaling and pattern formation like T-Brachyury polarization [17]. The polarized expression to the proximal posterior region of the epiblast is a useful reference for the onset of gastrulation. For this event to happen, we need two main signaling pathways: Wnt/β-catenin and Nodal [18].

Signaling by the Wnt family is one of the fundamental mechanisms that direct cell proliferation, cell polarity and cell fate determination during embryonic development and tissue homeostasis [19]. A critical and most studied Wnt pathway is the canonical Wnt signaling, which functions by regulating the amount of the transcriptional co-activator β-catenin that controls key developmental gene expression programs [20], like for example T-Brachyury expression (Figure 8). On the other hand, Nodal signaling allows precise temporal and spatial control of mesoderm formation and is employed in several other critical patterning events in early development, notably in the determination of left-right (L-R) asymmetry [21].

Wnt and Nodal signaling pathways are well known pathways in early mouse development, however, there is a third signaling pathway, the fibroblast growth factor (FGF) signaling which is also crucial for embryonic development in mice but yet not so well studied. In addition to their growth-promoting activities towards a broad spectrum of cell types, FGF family is suspected of playing various roles in the control of cellular

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3The patterning signal secreted by notochord cells is sonic hedgehog, which is involved in tissue differentiation and development [14].
differentiation [22]. FGF is expressed in the Inner Cell Mass and it is required for cell survival; its suppression results in a fifth cell cycle block in preimplantation mouse embryos [23].

2 Early development in a dish

2.1 Aggregates of mouse embryonic stem cells: Gastruloids

Stem cells can be defined as cells that have the ability to self renew, they can be differentiated in cell types of an organism and they can propagate this ability indefinitely in culture, but can be exhausted in vivo. These type of cells can be characterized in two different ways depending on their source: adult stem cells, found in organs already matured where their job is mainly to regenerate tissue (for repairing, etc.) and embryonic stem cells, derived from the inner cell mass of the mammalian blastocysts [24], which give rise to all tissues in the embryo proper [25].

Embryonic stem cells are cultured to study the development of many organisms since this initial process depends mainly on the signaling environment, the state of the cells (like for example stress) and also, the stochastic processes like stem cell differentiation [26]. In this report we will study mouse embryonic stem cells, which are a group of identical cells that share a common ancestry that derive from preimplantation mouse embryos. At this stage of prenatal development, the conceptus is a blastocyst (Figure 1), which means that its cells from the Inner Cell Mass can contribute to all tissues of the embryo and integrate into the normal morphogenetic processes, i.e, they are pluripotent. As mentioned before, these cells can differentiate in vitro in all the possible cell types from a given organism, however, they cannot organize themselves into structures that resemble embryos. When they are experimentally manipulated and aggregated as embryoid bodies, they tend to form disorganized masses with different cell types and very little spatial coherence [27].

In 2014, Alfonso Martinez Arias and colleagues from the Department of Genetics of Cambridge University, created small aggregates of mouse embryonic stem cells (of around 200-500 cells) that could self-organize into polarized embryoid body systems, that exhibited collective behaviors that could be observed in the early mouse embryos, including symmetry breaking, axial organization, germ layer specification and cell behavior [27]. This system is called the Gastruloids system. This experimental model was inspired by the work of Y. Marikawa [28], who demonstrated that aggregated P19 cells can exhibit expression patterns characteristic of the mesoderm formation (which happens at the early developmental stages) and also, they showed elongation morphogenesis (with distinct anterior-posterior body axis as in the embryo).

4Embryonic carcinoma cell lines derived from an embryo-derived teratocarcinoma in mice [29].
Gastruloids are a useful developmental biology tool to investigate and understand the principles underlying behind the early mammalian development and to see the potential of stem cells. Specifically, they are useful to study symmetry breaking, morphological changes, spatiotemporal coordination of gene expression patterns and the development of three axis (anterior-posterior, dorsal-ventral, bilateral asymmetry) that can be observed from gene expression localization.

Gastruloids are not the only system that has gained researchers’ interest during the last few years: there is a recent increase in the study of organoids since they are easy to manipulate, they have a low cost and also because they are a high-throughput source of cells that mimic in vivo events. Another main reason of why organoids are a useful tool for research is due to their increasing reproducibility [30].

![Figure 4: Once the Gastruloids are formed, images are acquired with the microscope every 24 hours to observe their evolution. At 24 hours cells have already started to aggregate. At 48 hours you can observe a small circular and compact shape and within time, the elongation occurs and Gastruloids become bigger and longer at each time point. Images observed are Gastruloids formed with T-Brachyury::GFP mouse embryonic stem cells reporter line with a Wnt agonist pulse (CHI99201) at 48h-72h.](image)

Gastruloids have been reported to grow in suspension culture and show symmetry breaking and stable polarization of signaling [27] as seen in Figure 3. This pattern resembles that in the embryo at E6.0 with some differences. In the embryo, the initial localization of the primitive streak can be identified as a focus of T-Brachyury expression in the proximal posterior region of the embryo [17] and its specification follows a

5Miniaturized and simplified version of an organ produced in vitro in three dimensions that shows realistic micro-anatomy [30].
sequence of events associated with the localization of ligands for Nodal and Wnt signaling to the same region [18]. In the Gastruloids, we can observe similar behaviors when they are exposed to Wnt/β-catenin signaling: T-Brachyury polarizes to the posterior side during day 3, creating a pattern, which corresponds approximately with E5.5-E6.5 as it is the time that cell extrusion and intrusion is observed. Elongation will start during day 4 which thus corresponds to E7.5 in the embryo. The sequence and timing of the events that are observed in Gastruloids are reproducible and can be related with similar events in the embryo.

The T-Brachyury expression pattern has been reported to be dependent on Nodal signaling, while reductions in Wnt/β-catenin signaling affect the robustness of the process. These results in Turner et al. [31] suggest that the pulse of Wnt signaling stabilizes T-Brachyury expression initiated between 48 and 72 hours (after Gastruloids aggregation) by Nodal signaling and is consistent with molecular data suggesting that the effectors of both signaling systems cooperate in the expression of T-Brachyury. Surprisingly although in suspension culture, cells express Nodal and Wnt signaling, T-Brachyury can be expressed in the absence of external Nodal or Wnt signaling agonists although the system becomes highly heterogeneous. This could happen because 3D culturing creates specific conditions that affect the sensitivity of the cells or the quality of the signals. For this reason, a CHI99201 pulse (which is an agonist of Wnt signaling) is given to the Gastruloids between 48 and 72 hours after their creation. From Turner et al. [31] we can also see that FGF signaling is required for this pattern process however, in contrast with Wnt and Nodal signaling, FGF is required for some specific cell fate decision (Neruoectodermal or Posterior mesodermal) which means that its role is context dependent.

3 Turing patterns in reaction-diffusion systems

In this section we want to give some insight on what a reaction-diffusion system is and how a Turing pattern (Figure 5) may emerge from a mathematical point of view. This type of system has been used to model several biological systems and in this report we will try to see if we can use this approach to explain the interactions between Nodal, Wnt and T-Brachyury. Since FGF has been shown to be more involved in thresholding for different cell fates [32], we will not be including it in our model because our aim is to model how can this patterning event (Figure 3) emerge in Gastruloids. We will use a reaction-diffusion system to try to explain the pattern we observe however, these type of equations assume instantaneous production of the molecules. Interactions occur with a certain time delay since biological processes like transcription, translation, etc. are happening inside the cells. For this reason, we hypothesize that adding a time delay to this process will not allow the pattern to emerge in silico.
Alan Turing (1912-1954) was a famous British mathematician who gained fame after his death during the late course of the twentieth century. He made great contribution to theoretical computer science providing the first formal concept of computer algorithm. Later on during the Second World War he designed a machine, the Turing Machine, which cracked the German military codes providing the Allies great advantage in the fight against the Nazis.

His contribution to mathematical biology is less well known but has gained interest during the last decades to explain several biological patterns. He published only one paper (in 1952) called *The Chemical basis of morphogens* which triggered a new field in mathematical biology concerning pattern formation [33].

3.1 Modeling reaction-diffusion systems

A typical reaction-diffusion system consists of two interacting chemicals which react in a stable, homogeneous manner although when diffusion is added to the system, it acts as a destabilizing influence (after a lapse of certain time), giving rise to spatial oscillations: Turing patterns. The theory behind these patterns is based on short range activation, long range inhibition, and a distinction between activator and inhibitor concentrations on one hand, and the densities of their sources on the other [34].
Turing, in [35], considered a system with two or three morphogens which were supposed to be initially in a stable homogeneous condition but disturbed slightly from this state by some influences unspecified such as Brownian movement, the effects of neighboring structures or slight irregularities of the shape. Another assumption he made was that slow changes were taking place in the reaction rates of the two or three morphogens under consideration. These changes are supposed to bring the system out of the stable state. In order for the problem to be "mathematically tractable", he assumed that the system never deviated very far from the original homogeneous condition. This assumption he called the "linearity assumption".

Many systems have been described to exhibit Turing instabilities, for example the Brusselator model, which explains the competition of two chemical species in a chemical reaction (which is the simplest reaction-diffusion system capable of generating complex spatial patterns) [36]; Kondo and Asai studied the horizontal stripes in the tropical fish, Pomacanthus imperator [37]. Other well-studied examples include the regular disposition of feather buds in chick [38], and of hair follicles in mice [39]. However, the most used reaction-diffusion systems are those described by Gierer and Meinhardt in 1972 [34]. They described the dynamics of the activator-inhibitor system and of the depletion model, which have been widely used to explain pattern formation in biology.

Solving Reaction-Diffusion systems

For a better understanding of the following steps concerning the analysis of Turing systems, we will first describe analytically the general idea of a reaction-diffusion system with one partial differential equation.

Fick’s law (of conservation of mass) states that the flux of a field \( c \) goes in the opposite direction of the gradient of that field. Let us assume a flux \( \vec{J} \) and gradient \( \vec{\nabla} \) of the field \( c \) that depends on both space and time \( (c(\vec{x}, t)) \):

\[
\vec{J} = -D \vec{\nabla} c \tag{1}
\]

Where \( D \) is the diffusion coefficient. The classical approach to the diffusion equation\(^6\) is through the conservation of law of Fick’s law, and if we assume that in addition to diffusion a reaction \( (f(c)) \) is taking place, we have the following expression:

\[
\frac{\partial c}{\partial t} = f(c) - \vec{\nabla} \cdot \vec{J} \tag{2}
\]

Where \( \vec{\nabla} \cdot \vec{J} \) is the divergence of the flux, which measures the number of particles that go inside and outside a volume. By replacing equation 1 in equation 2, we obtain:

\[ \frac{\partial c}{\partial t} = D \nabla^2 c \]

\(^6\) \( \frac{\partial c}{\partial t} = D \nabla^2 c \)
\[
\frac{\partial c}{\partial t} = f(c) - \nabla \cdot \vec{J} = f(c) - \nabla(-D \nabla c) = f(c) + D \left( \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2} \right) c = f(c) + D \nabla^2 c \quad (3)
\]

This is the general expression of a Reaction-Diffusion model, where \(\nabla^2\) is the Laplace operator. These systems, as mentioned before, need to be stable in the homogeneous state in the absence of diffusion in order to reach diffusion driven instabilities. With small perturbations in the temporal frame, the system should remain stable.

We first define the equilibrium solution (in the absence of diffusion) as:

When \(f(c) = 0 \rightarrow c^*\) is the fixed point or equilibrium solution \quad (4)

The equilibrium solution (or stable fixed point) is linearly stable if its linearization attracts small perturbations:

\[ \delta_c = c - c^* \quad \text{Where } \delta_c \text{ is the perturbation. We can rewrite this as } c = c^* + \delta_c \quad (5) \]

This perturbation only includes the temporal dimension. If we want to include diffusion into our solution, we need to add space to the system, which means that the perturbation will have both temporal and spatial dimensions:

\[
\frac{\partial c}{\partial t} = f(c) + D \nabla^2 c \rightarrow c = c^* + \delta_c(\vec{x}, t) \\
\delta_c(\vec{x}, t) = \delta_{c_0} e^\lambda t e^{i \kappa \vec{x}} \rightarrow e^{i \kappa \vec{x}} = \cos(\kappa \vec{x}) + i \sin(\kappa \vec{x})
\]

Where \(\delta_{c_0}\) is the initial perturbation, \(\lambda\) is the eigenvalue\(^7\) of the Jacobian and \(\kappa\) is the spatial frequency. Since we anticipated an amplitude growth in addition to phase propagation, we seek for a solution with the perturbation of the type of equation 6 (this is the Ansatz we will use).

Rewriting the partial differential equation for \(c\) (equation 6) and including the perturbation with space (equation 6), we will obtain the following partial differential equation:

\[
\frac{\partial}{\partial t} \left( c^* + \delta_c(\vec{x}, t) \right) = f \left( c^* + \delta_c(\vec{x}, t) \right) + D \nabla^2 \left( c^* + \delta_c(\vec{x}, t) \right) \rightarrow \text{Where } \frac{\partial c^*}{\partial t} = 0 \quad (7)
\]

\[
\frac{\partial \delta_c}{\partial t} = f'(c^*) \delta_c + D \frac{\partial^2}{\partial x^2} \delta_c \rightarrow \frac{\partial \delta_c}{\partial t} = f'(c^*) \delta_c + D \nabla^2 \delta_c \quad (8)
\]

\(^7\lambda = \alpha + i w \rightarrow w = 2\pi/T\), where \(w\) is the temporal frequency.
Finally, if we combine the result from equation 8 with the initial definition of the perturbation in equation 6, we will obtain the eigenvalue expression for the system with diffusion:

$$\frac{\partial \delta_c}{\partial t} = \frac{\partial}{\partial t} \left( \delta \right) + \lambda e^{\kappa x} \rightarrow \lambda = f'(c^*) - D\kappa^2$$

From equation 9 we can observe the expression for the Jacobian (in this case of $c$) which includes the diffusion coefficient $D$. The wavenumber, $\kappa$, needs to be positive and finite in order to obtain spatial oscillations and thus a Turing pattern. Also, in this equation, we can observe that by zeroing the diffusion coefficient, we will obtain the Jacobian for the homogeneous state (i.e. in the absence of diffusion).

This expression will be used in sections 7.1 and 7.2 to calculate the stability criteria to test whether a certain system can exhibit Turing Patterns.

### 3.2 Reaction-Diffusion and time delay

An important issue that remains difficult to address with current experimental techniques is how local molecular interactions are coordinated in space and time. However, there is evidence that successful development depends not only on the generation of appropriate spatial patterns, but also on the timing of each pattern-forming event [40].

The addition of time delay into reaction-diffusion systems has not been extensively explored [40, 41] even though the incorporation of time delays in gene expression due to transcription and translation play an important role in the dynamics of the cell.

Reaction-diffusion systems take as parameter values those that can produce spatial oscillations although they cannot be related with reality because patterns would not emerge (in the majority of the cases). Which is why in this section we argue how time delay may emerge in real life with a specific case (Nodal and Lefty) and how hypothetically, this could avoid pattern formation. In section 7.3 we demonstrate computationally, the nature and cause of adding an arbitrary time delay into a reaction-diffusion system. We take as an example the production rates and diffusion coefficients taken from the literature for Nodal and Lefty system since they have been previously modeled as a reaction-diffusion system [42]. This way we can theoretically discuss why we believe a time delay may avoid pattern formation. Later on, in section 9.2 we will show the in silico model of some examples of Turing systems with time delay in the production of the molecules. We will discuss the effects in pattern formation due to this delay.

**Nodal-Lefty as a Reaction Diffusion system**

Alan Turing put forward the reaction-diffusion model, in which two interacting and diffusing species of molecules can generate complex patterns [35]. Gierer and Meinhardt, independently postulated that pattern formation in reaction-diffusion models requires
a short-range activator that enhances both its own production and a long-range inhibitor [34]. Another important characteristic was that the inhibitor must diffuse faster than the activator. All of these features can be observed in several systems in the nature, however in this section we will focus in the interactions between Nodal and Lefty as a Reaction-Diffusion System [42].

Nodal signaling is essential for mesoderm formation in vertebrates and for the polarization of T-Brachyury in early development (section 1.2). A negative feedback controls the production of Nodal targets which makes this system to act very precisely. This negative feedback is created by the Lefty family proteins, which inhibits Nodal signaling by competing for the same receptors. The Nodal/Lefty system fulfills two of the tenets of activator/inhibitor reaction-diffusion models we mentioned before (Figure 7): (i) Nodal ligands are short-to mid-range activators that enhance their own expression, and (ii) Lefty proteins are long-range inhibitors that are activated by Nodals [42].

![Figure 7: Reaction-Diffusion network of Nodal (the activator) and Lefty (the inhibitor). The activator up-regulates its own production and is less diffusible than the inhibitor [42].](image)

Instantaneous production of the reacting molecules is assumed in Reaction-Diffusion systems. However, in reality, there is a time delay in protein formation due to transcription, mRNA splicing, translation, etc. We can calculate an approximation of the amount of time Nodal and Lefty proteins need in order to be formed. We are concerned about the mouse embryo so we will use the mouse data for this time delay calculation:

- Nodal mouse gene: 7367 base pairs (bp)
- Lefty mouse gene: 3379 base pairs (bp)
- Nodal Mouse protein: 354 amino-acids (aa)
- Lefty Mouse protein: 368 amino-acids (aa)

As for the time that the different biological processes take, we can obtain an approximation from the literature:

- Transcription ≈ 3.5 Kilo-base/minutes [43],
- mRNA splicing ≈ 5-10 minutes [44],
- Translation in mouse embryonic stem cells $\approx 5.6$ amino acids/s [45].

From all this, we can calculate conservative estimates of the production of both Nodal and Lefty:

(a) Nodal production $\approx 8$ minutes  
(b) Lefty production $\approx 7$ minutes

This calculus is taken into account without knowing the time for several other processes occurring during protein production: the activation of signaling pathway after receptor binding, the diffusion of Smad (transcription factor) or its transportation to the nucleus and binding to the right promoter, the transport of newly produced Nodal outside the cell.

On the other hand, it has been experimentally obtained the Diffusion coefficients for both Nodal and Lefty proteins [42]: $D_{\text{nodal}} = 2\mu m^2/s$ and $D_{\text{lefty}} = 15\mu m^2/s$. If we assume a space of $100\mu m^2$ where our molecules can freely diffuse, then can calculate the amount of time the molecules need to diffuse through that space:

$$D = \frac{L^2}{t} \rightarrow t = \frac{L^2}{D}$$

(10)

$$t_{\text{nodal}} = \frac{100\mu m^2}{2\mu m^2/2} = 1.3 \text{ hours}$$

(11)

$$t_{\text{lefty}} = \frac{100\mu m^2}{15\mu m^2/2} = 11.1 \text{ minutes}$$

This means that if there is a single source then over the 1 hour duration, that the activator takes to diffuse, any gradients of inhibitor will be completely homogenized. But to achieve patterning, there has to be huge fluctuations in the initial conditions otherwise the super fast diffusion of lefty will homogenize everything.

With all of these, we can now conclude that in order to have a truly biological model, we need to include a time delay into the simulation. We will try this hypothesis in the two general systems described by Gierer-Meinhardt [34]: the activator-inhibitor system (which Nodal and Lefty have been hypothesized to follow) and the depletion model (which is another well known Gierer-Meinhardt system).

4 Background and question

Intracellular transcriptional regulators and extracellular signaling pathways together regulate the T-Brachyury pattern formation we observe in Gastruloids, but how the molecular activities are integrated to establish the correct timing and spatial localization is still a manner of study. How Wnt/$\beta$-catenin is necessary for symmetry breaking and T-Brachyury polarization is already reported but it still remains unknown the role that FGF plays within these processes.
Schröeter et al. [32] reported that FGF signaling can establish thresholds for the response of a cell population to an input. Wnt and FGF are well known to collaborate in many cell fate decisions, but with the results shown in [32], we observe that their main role is not to determine cell fate but to influence the probability by which the cells adopt those fates and the dynamics of the process across the population.

The first question we ask is, what is the role of FGF in mouse embryonic aggregates and which of the two main pathways, FGF/RAS-MAPK signaling or FGF/PI3K-AKT signaling, is being involved. The RAS-MAPK pathway is involved in cell growth, division, and differentiation, whereas the PI3K-AKT pathway is mainly involved in cell survival [46]. To get some insight on this question, we will work in 3D culture (with Gastruloids). Firstly, we will observe how FGF is expressed throughout the Gastruloid using an FGF-reporter cell line. Additionally, we will treat these Gastruloids with FGF inhibitors to observe how they behave (regarding elongation, signaling expression, etc.). We will use an inhibitor of the FGF receptor and an inhibitor of the MEK/ERK pathway (Figure 8). Secondly, we will use the T-Brachyury reporter line to form Gastruloids and see if the pattern is still expressing if we use the same inhibitors mentioned before. With these results, we will be able to observe the FGF expression in the Gastruloid and see how the T-Brachyury pattern behaves when FGF inhibitors are added to the 3D-culture.

The second question we would like to answer is whether reaction-diffusion is a good approximation to model the T-Brachyury pattern observed in early gastrulogenesis shown in Figure 3. Wnt is the key activator of the pattern formation, so we will first start modeling a two component reaction diffusion where Wnt is the diffusing molecule and T-Brachyury the immobile one. As we will observe, a third component had to be incorporated to the model because of reasons we will explain in section 7.1. Nodal also plays an important role in the initiation of the pattern, so we used it as the third molecule. Finally, we will test the effect of adding a time delay into the most used reaction diffusion models. We will test this in the Gierer Meinhardt reaction diffusion systems to see whether protein production timing can affect Turing pattern formation.

**Methods**

5 Experimental procedures

5.1 Mouse embryonic cell lines

- **T-Brachyury expression reporter (T/Bra::GFP):** Cells harbor a GFP targeted to the T-Brachyury locus [47]. This cell line reports on T-Brachyury gene and we will use this cell line to observe pattern formation and also, to track Wnt/β-catenin signaling.

- **Spry4 expression reporter (Spry4::H2B-Venus):** Cell line created by a previous lab member, Dr. Christian Schröter. Cells harbor a H2B Venus targeted to
Figure 8: Pathways of interest: The Wnt/β-Catenin Canonical pathway involves the activation of β-Catenin by the recognition of the Wnt ligand through the receptor. Then, β-Catenin is translocated into the nucleus where it activates Wnt-targets like T-Brachyury. The FGF main signaling pathways are MEK/ERK, which is involved cell differentiation whereas the PI3K/AKT pathway is being associated with cell survival.
the Spry4 locus. This cell line reports on Spry4 (Sprouty RTK Signaling Antagonist 4) and since this gene is a target of the protein FGF, then we will use this cell line to track FGF signaling.

5.2 Routine 2D cell culture

Mouse embryonic stem cells are cultured in 25cm$^2$ flasks which have to be previously gelatinized with 5 milliliters (ml) of 0.1% Gelatin. Once this process is completed, cells are platted with ES media, which is basically G-MEM (Sigma) media supplemented with Leukemia inhibitory factor (LIF), to promote pluripotency and with fetal bovine serum (Gibco) which contains embryonic growth promoting factors among other ingredients.

These cells need to have their media replaced every day and also, every two days, they have to be passaged, ie. moved from one flask to another previously gelatinized. The purpose of this is to let the cells self-renew so that they can remain pluripotent and we can work with cells that are not yet differentiated.

5.3 Generation of Gastruloids

The experimental procedure used to create mouse embryonic stem cells aggregates can be obtained in detail in [48]. However in this section we will give a short summary on the experimental methods and materials used to create mouse embryonic stem cell-derived Gastruloids.

The first step was to dissociate the cells using Trypsin-EDTA. Then neutralization was performed using ES media and after a series of PBS washes, the resulting cell suspension was counted. T-Brachyury Cell line needs 300 cells for aggregation and the Spry4 Cell line needs 400 cells. After the calculation of how many cells were needed for that specific experiment, the cells were resuspended in N2B27 (Takara Clontech). In order to create the Gastruloids, we need to change the cells from the G-MEM Serum+LIF media, which keeps the cells pluripotent, to this basal media N2B27 so that cells can differentiate. Later, with a multi-channel pipette, 40µl of the cell suspension was added to each well of a non-tissue-culture treated, 'U'-bottomed 96-well plate (Figure 9) and incubated in a humidified incubator at 37 °C and 5% CO$_2$.

Addition of Chemicals Day 2 (24h-48h)

When a chemical (say an inhibitor, etc.) needed to be added during day two after their creation, 20µl of N2B27 was removed from each plate with a multichannel pipette and the fresh N2B27 media was added with twice the concentration of the chemical of interest.
Addition of Chemicals Day 3, 4 or 5 (48h-120h)

During these time points if a chemical needed to be added, 150µl of N2B27 was removed from each plate with a multichannel pipette and the fresh N2B27 media was added with the exact concentration of the chemical of interest. Even if no chemical was needed to be added during these time points, 150µl fresh N2B27 media was exchanged by 150µl of old media.

5.4 Chemicals used

<table>
<thead>
<tr>
<th>Small Molecule</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHI99201</td>
<td>10µM</td>
<td>3µM</td>
</tr>
<tr>
<td>PD0325901</td>
<td>10µM</td>
<td>1µM</td>
</tr>
<tr>
<td>PD173074</td>
<td>10µM</td>
<td>150nM</td>
</tr>
</tbody>
</table>

Table 1: Chemicals used during the project with the stock concentration and the working concentration used in the experiments.

CHI99201 is a inhibitor of glycogen synthase kinase 3β (GSK-3β), which acts as an agonist of the Wnt/β canonical signaling. PD0325901 is a selective and non ATP-competitive Mitogen-activated protein kinase (MEK) inhibitor, which is one of the signaling pathways of FGF. Finally, PD173074 is a FGF receptor 1 inhibitor which will repress both the MEK pathway and the PI3K pathway.

Figure 9: Gastruloids were formed in a 96-well plate with U-bottom so that they can properly aggregate. In this study two treatments were used: PD03 (MEK inhibitor) and PD17 (FGF receptor 1 inhibitor). CHI99201, also called Chiron, is the Wnt agonist and this pulse should be included between 48 and 72 hours so that Gastruloids can properly form. The control group only has Chiron while the two treatment groups will have Chiron plus the inhibitors which will be included during different time points to check the response of the cells at different stages.
5.5 Imaging and Microscopy

Wide-field, single-time point images of Gastruloids were acquired every 24 hour after aggregation with a Nikon Eclipse Ti-E microscope at 37°C in a humidified 5% CO₂ incubator with a 20x objective. Illumination was provided by a LED white-light system in combination with filter cube GFP-1828A (Semrock, NY, USA) used for both GFP and H2B-Venus.

6 Image Analysis

In this section we explain how the segmentation of the images was performed and how the fluorescence and shape analysis was carried out. Briefly, we explain how the two channel images (Fluorescence and Bright-field) were acquired from the Nikon microscope and how the analysis, segmentation, geometric transformation, normalization and graphics were done.

6.1 Image Acquisition

The images obtained from the Microscope were exported in a Tif file which was composed by two images: one which was taken with the Brightfield and the other one that had the Fluorescence. Each Tif image had the following characteristics (which we needed to take into account in order to process them):

- **File size**: 16883473
- **Format**: Tif
- **Width**: 2048
- **Height**: 2048
- **Bit depth**: 16
- **Bits per sample**: 16
- **Minimum sample value**: 0
- **Maximum sample value**: 65535

This information was obtained using the MATLAB command *imageinfo()*. The images were uploaded to MATLAB workspace using the *imread()* function and the *sprintf()* function using a for loop and saved as cell structures with the name IMAGES\{i, j\}, where i are the number of images and j represent the fluorescent images or the bright-field.

6.2 Pre-Processing and Morphological Analysis

The segmentation steps used to obtain the edges of the images can be observed in Figure 10. There are several steps that needed to be done since the images varied a lot depending on the time point at which they were acquired (48, 72, 96 or 120 hours) and also, depending on the cell line that we used, etc. In the following lines we explain briefly each step performed in Figure 10:
Figure 10: Segmentation steps performed to obtain the edges of the image.
**Image 10.1:** This was the original Image, the Gastruloid, in the Bright-field mode. The edge detection was performed from this image.

**Image 10.2:** The first step was to smooth the images with a 2-D Gaussian smoothing kernel with standard deviation equal to 2.

\[ I = \text{imgaussfilt} \left(\text{IMAGES} \{i,1\}, \sigma\right); \]

Once the images were smoothed, the next step was to reduce the amount of objects around the Gastruloid that we wanted to segment and also we used some basic morphological operators to obtain better results.

**Image 10.3:** First, we performed an erosion of the smoothed image. The structuring element (SE) size was manually introduced in the program since the Gastruloids had very different sizes and this was the best way to obtain the most accurate segmentation. Usually images acquired at the same time point had similar characteristics so the same structuring element was used, however this varied within different time points of images.

**Image 10.4:** In this image we applied \( \text{IM} = \text{imreconstruct} \left(\text{MARKER}, \text{MASK}\right) \), which performed morphological reconstruction of the image MARKER under the image MASK. Morphological reconstruction can be thought as repeated dilations of an image, called the marker image, until the contour of the marker image fitted under a second image, called the mask image. In morphological reconstruction, the peaks in the marker image "spread out," or dilate.

**Image 10.5:** After the reconstruction of the eroded image, a dilation was performed in order to thicken objects and to fill some holes.

**Image 10.6:** This image was a dilated image which had been reconstructed with the same command as used in Image 10.4 but this time, the Marker and Mask were taken from the complement of the dilated image and from the reconstructed eroded image, respectively.

**Image 10.7:** Since we used complement in the last image, now the colors were switched, and we wanted them as they were, the idea was to have the Gastruloid filled with ones and the rest (the background) filled with zeros, for simplicity. So, the \text{imcomplement} command was used to change the colors (the black became white and the same the other way around).

**Image 10.8:** The next step, once we had a clear Gastruloid shape in the image, was to obtain a binary image by using the command \text{imbinarize}(I), which binarized the image \( I \) with a global threshold computed using Otsu’s method, which chose the threshold to minimize the intraclass variance of the thresholded black and white pixels.

**Image 10.9, 10.10:** Once the image was binarized, we performed erosion two more times to shrink the objects and remove structures of the shape and size given by the
structuring element, which in this case was a diamond of size 2. This was chosen by trial and error and it was the one that best fitted this function.

**Image 10.11:** Finally, we wanted to obtain a well segmented image in which we could observe only the edges of the Gastruloid which we filled in the next step of the simulation. For this, we used a command called `bwperim(BW)` which took a binarized image and returned you the perimeter pixels.

These were all the steps used to pre-process the images and to segment them so that we could observe the edges of all the figures. However, we could see that some of the images still had some regions around the Gastruloid that should had be taken away. This is why, using the command `[x,y]=getpts(I)` one could manually select the shape that we wanted to stay with and with the help of a function called `regFill`, the selected pixel point would be used to fill all the segmented area delimited by an edge and in the end, it remained only with the Gastruloid shape filled with ones and the rest filled with zeros.

In the segmentation function, the last step is to manually draw a rectangle in the bright-field image (outside the Gastruloid region) with the command `getrect` to get the pixel positions of the fluorescent image and make an average for each picture so that we can have an estimate of what the foreground is. This was used in the fluorescence analysis explained in section 6.3. (The background was chosen manually because in the fluorescence images there could be some plastic-like structures or some other impurities that could have gotten inside the well and they reflect a lot of brightness. This is why we manually choose regions were there was nothing that could interfere in the fluorescence analysis).

### 6.3 Geometric Transformation

Once we had the images segmented and in the binary form, we could proceed to obtain some shape properties. This was done using the MATLAB function `regioprops()`, that was used to obtain the centroid, the major and minor axis length and the orientation, which was the angle between the x-axis and the major axis of the ellipse (in this case, the Gastruloid).

The next step was to rotate all the images and re-adjust the orientation so that the major axis was now 90° with respect to the x-axis. This way all the images were vertically aligned for simplicity of later steps. Subsequently, we obtained the positions for the pixels that represented the Gastruloid which were the ones that had a pixel value of one. Then, we gathered all the points from the major axis line by using the orientation and the major axis of the image and we applied simple equations of trigonometry. Finally, we calculated the mean intensity of all the pixels from the x-axis for all points in the major axis, this way, we obtained a vector with the length of the Gastruloid and with all the mean intensities of each x-segment (Figure 11). From each of these intensity means, we removed the mean background obtained before during the segmentation of the images. The output of this section 6.3 was a vector that contained...
the intensity values of all the Gastruloid along its major axis without the background so that we could proceed to analyze this data in the next section 6.4.

Figure 11: Geometry transformation sketch. Left: Gastruloids are first rotated to be vertically aligned. The points of the major axis were obtained by trigonometry equations using the points a, b (green dots in the figure), the major axis length and the orientation. Right: Once we had the coordinates of the major axis line, we computed the mean intensities of all the pixels in the x-axis for that specific point of the major axis line (y-axis), obtaining a vector with the mean intensity value across all the major length of the Gastruloid.

6.4 Fluorescence and Morphology Analysis

The experiments performed with the Gastruloids involved different time points: pictures were taken in the microscope at 48 hours, 72 hours, 96 hours and 120 hours after their creation in the 96-well plate, and in each time point, a different treatment was included, so we needed a system that was capable of comparing all the Gastruloids from one experiment. The two main features we wanted to analyze were the Fluorescence expressed by the reporter cell lines and also the elongation of the Gastruloids.

Normalization

The first step in order to know if the fluorescence has been polarized in a region of the Gastruloids was to normalize the length. To do so, we normalized each Gastruloid by its maximum length. This way, we obtained that all the Gastruloids had a length from 0 to 1.

As for the fluorescence, we did not normalize by their own maximum since this would have avoided comparison between them but, we rather normalized them by the maximum intensity value of all the Gastruloids from the experiment, this way we were able to compare different conditions during different time points. However, an issue was that not all the Gastruloids had the same amount of pixel values since they all had different lengths. To solve this problem, instead of using interpolation as this would
had changed the output of the problem (by creating or deleting points), we used a step and included the intensity values from each vector at every step number, this way we only used the values we had and we did not create any new values using common interpolation methods. In the end we took 200 points from all the Gastruloids at one specific time point. We performed the mean of all the points encountered between every step and in the end, these 200 pixel intensities were the mean intensity values between each step value.

**Fluorescence and Flattening measures**

The average fluorescence traces of Gastruloids ± the standard deviation are shown in the Fluorescence graphics in section 8. These data were plotted within the Gastruloids normalized length to observe the different intensities along the major axis and seek for polarizations.

On the other hand, the ellipticity measure that we chose is a simple measure called *Flattening* ($f$) which measures the compression of a circle across the diameter to see whether an ellipse is formed or not.

$$f = \frac{a - b}{a}$$

(12)

Where $a$ is the major axis and $b$ is the minor axis, so it is a simple measure of how a spherical shape is getting flattened as an ellipse.

**Statistical test**

The statistical test used was the non-parametric Wilcoxon signed-rank test since we cannot assume that our data follows a normal distribution. Once the test was performed, since we were comparing more than two samples, we performed a False Discovery Rate, specifically the Benjamini Hochberg procedure in order to obtain more accurate statistical significances.

**7 Pattern formation with reaction diffusion systems**

This section is meant to explain the steps performed in order to obtain the conditions for which a two or three component system could lead to a Turing Pattern. The first two subsections are focused on how the stability criteria is achieved and we show some examples of systems that can never form a Turing Pattern. The last subsection is more focused on how time delay can disrupt spatial oscillations in the classical Reaction-Diffusion systems. Most of the work is described on this section however there are some aspects which are described in more detail in the Supplementary Material.
7.1 Two-component systems

One of the questions we wanted to answer was whether Wnt and T-Brachyury could be modeled as a Turing system. Specifically, we wanted to obtain a Turing pattern of the type of a Dissipative soliton, which is a localized solitary structure [49]. Since Wnt ligand diffuses in the media but T-Brachyury is a transcription factor, it is immobile, we started by creating a two component reaction diffusion system with only one diffuser. Firstly, we modeled the general form to test if this type of systems could lead to Turing instabilities to consequently build the equations for the Solitons.

In the classical Turing system or also called the diffusion driven instability (DDI), we need to perform a stability analysis of the model and test whether our system is stable in the homogeneous steady state and unstable when diffusion is added. We can consider the following linear system with first order differential equations which includes two molecules capable of diffusing:

\[
\begin{align*}
\frac{\partial x}{\partial t} &= D_x \nabla^2 x + F(x, y) \\
\frac{\partial y}{\partial t} &= D_y \nabla^2 y + G(x, y)
\end{align*}
\]

These equations follow the general expression obtained in section 3.1. Some examples of the equations with the explicit reaction terms can be observed in section 7.3. The next step in the typical stability analysis of a reaction-diffusion system would be to calculate the steady state in the absence of the diffusion term. In this specific case, since we are working with general expressions, we will define the homogeneous steady state as:

\[
\begin{align*}
F(x, y) &= 0 \\
G(x, y) &= 0
\end{align*}
\]

Once we have the partial differential equations and the fixed points, we can proceed to create the Jacobian matrix evaluated in the homogeneous steady state:

\[
J = \begin{pmatrix}
\frac{\partial F}{\partial x} & \frac{\partial F}{\partial y} \\
\frac{\partial G}{\partial x} & \frac{\partial G}{\partial y}
\end{pmatrix} \rightarrow \begin{pmatrix}
F_x & F_y \\
G_x & G_y
\end{pmatrix} \rightarrow \begin{cases}
\tau = F_x + G_y < 0 \\
\Delta = F_x G_y - F_y G_x > 0
\end{cases}
\]

We want the system to be stable in the absence of diffusion and for this reason we want to have a negative trace and a positive determinant. If the system can exhibit instabilities by adding the diffusion terms, then we can say that it has a Turing bifurcation or a DDI. A simple way to predict if your system will exhibit this type of behavior, is by following one of the sign structures of the Jacobian evaluated at the homogeneous steady state:
These sign structures will provide a negative trace and a positive determinant which, as mentioned before, will make our system stable (in the absence of diffusion). The terms cross Kinetics and Pure Kinetics were first described by Dillon et al. [50] and they correspond to the activator-inhibitor and depletion model respectively, from Gierer-Meinhardt [34]. From these sign structures we can observe specific behaviors of the system like when $F_x > 0$ at the homogeneous steady, then $x$ will be self-activating since it up-regulates its own production during the initiation of an instability whereas if $F_x < 0$ then $x$ will denote a self-inhibitor since it down-regulates its own production. The same will happen in the case of $y$ [51].

The homogeneous steady state (when there is no diffusion), will always be stable as long as the conditions in equations 15 are fulfilled or when we have a Jacobian sign structure matrix like the ones we observe in equation 16. Having a stable homogeneous steady state ensures that if we obtain a spatial pattern, it will be due to the diffusion term. The patterns appear when the frequency (concerning time) is zero and the wavelength (concerning space) is non zero. Without diffusion, the homogeneous fixed point is stable, so we now need to analyze the case in which we introduce the diffusion terms. To do so we will be taking space into account in our equations.

The matrix we now want to analyze is the one obtained in the previous section 3.1:

$$
\det(J - D\kappa^2 \text{Id}) = 0
$$

Where $\kappa$ is the is the wave number (the spatial frequency). It is also called the spatial Fourier mode, since we are performing a Fourier decomposition: we take an arbitrary perturbation and we decompose it in Fourier modes. We need to obtain the value of $\kappa$ that allows the system to have an unstable state. This instability is represented by a positive trace ($\tau'$) and a negative determinant ($\Delta'$).

$$
J' = \begin{pmatrix}
F_x - \kappa^2 D_x & F_y \\
G_x & G_y - \kappa^2 D_y
\end{pmatrix}
$$

$$
\tau' = \underbrace{F_x + G_y - \kappa^2(D_x + D_y)}_{\tau<0} < 0 \quad \text{This is always} \quad < 0
$$

$$
\Delta' = \kappa^4 D_x D_y - \kappa^2(F_{x2}D_y + G_y D_x) + \underbrace{F_x G_y - F_y G_x}_{\Delta>0} < 0
$$

Now we assume that $\kappa^2 \equiv x$ for simplicity to obtain a second order equation where
the solutions are the wavenumber values that allow us to have spatial oscillations:

\[ x^2 D_x D_y - x(F_x D_y + G_y D_x) + \Delta = 0 \]

\[ x = \frac{F_x D_y + G_y D_x \pm \sqrt{(F_x D_y + G_y D_x)^2 - 4D_x D_y \Delta}}{2D_x D_y} \]  

(21)

Where the inside of the square root needs to be positive in order to have a real value for the wavenumber \( \kappa \). However the minimum value for \( \kappa \) can be obtained when the square root is equal to zero, which gives us the critical value of the wavenumber with which we can obtain a Turing Pattern instability:

\[ \kappa_c = \sqrt{\frac{F_x D_y + G_y D_x}{2D_x D_y}} \]  

(22)

With the conditions obtained in equation 15 and taking into account that the inside of the square root from equation 22 has to be positive, we can obtain all the criteria we need to search for a system with spatial oscillations. This is valid for the case in which both molecules are diffusing. But in this report we are trying to test the hypothesis in which one of the molecules involved, T-Brachyury, does not diffuse. By making any of the two diffusion constants equal to zero, we can observe in equation 22, that the wavenumber will be equal to infinity. However the wavenumber needs to be real, positive and finite to have a Turing system, which rejects our hypothesis of Wnt and T-Brachyury acting as a reaction-diffusion Turing-like system.

### 7.2 Three-component systems

We have proved that a two component reaction-diffusion system with only one diffuser will not yield to a Turing pattern (section 7.1). We will try our second approach which was to add Nodal into the system. In this case we will have a three component system with two diffusers (Wnt and Nodal) and one immobile molecule (T-Brachyury) (Figure 12).

Let us first assume a general form of a three component reaction diffusion system with only two diffusers where the molecules are represented as \( x, y \), the diffusers and \( z \) the immobile molecule.

\[ \frac{\partial x}{\partial t} = D_x \nabla^2 x + F(x, y, z) \]  

(23)

\[ \frac{\partial y}{\partial t} = D_y \nabla^2 y + G(x, y, z) \]  

(24)

\[ \frac{\partial z}{\partial t} = H(x, y, z) \]  

(25)

As before, the next step would be to calculate the fixed points in the absence of
diffusion however, since we are dealing with the general form we will assume we are evaluating our stability analysis in the homogeneous steady state. To perform the stability analysis we first need to create the Jacobian and because we are working with a third order system, we will use the Routh-Hurwitz stability criteria which is a necessary and sufficient condition to represent an asymptotically stable system \[ [52] \]. We will first start analyzing the system without diffusion \( \det(Id - J) = 0 \):

\[
\begin{pmatrix}
\lambda - F_x & -F_y & F_z \\
-G_x & \lambda - G_y & -G_z \\
-H_x & -H_y & \lambda - H_z
\end{pmatrix}
\] (26)

From solving the determinant equal to zero of equation 26 we obtain a third order characteristic polynomial:

\[
\lambda^3 + \lambda^2(-\tau) + \lambda(R) - \Delta
\] (27)

Where:

\[
\tau = F_x + G_y + H_z \\
\Delta = F_x(G_yH_z - G_zH_y) - F_y(G_xH_z - G_zH_x) + F_z(G_xH_y - G_yH_x) \\
R = G_yH_z - G_zH_y + G_yF_x - F_yG_x + H_zF_x - F_zH_x
\] (28)

The Routh-Hurwitz stability criteria states that the condition to obtain a stable system in a three component model is the following:

\[
\lambda^3 + \lambda^2a_1 + \lambda a_2 + a_3 \rightarrow \begin{cases} a_1 > 0 \\ a_3 > 0 \\ a_1a_2 > a_3 \end{cases}
\] (29)

So in this case to have a stable homogeneous steady state we need to full-fill this criteria:

\[
\begin{align*}
& a_1 > 0 \rightarrow -\tau > 0 \\
& a_3 > 0 \rightarrow -\Delta > 0 \\
& a_1a_2 > a_3 \rightarrow -\tau R + \Delta > 0
\end{align*}
\] (30)

Which means that to have a stable system \( \tau < 0 \), \( R > \frac{\Delta}{\tau} \) and \( \Delta < 0 \).

In equation 30 we have the conditions that need to be fullfilled in order to obtain a linear stable system in the homogeneous steady state. If any of those conditions are not met, the homogeneous steady state will no longer be stable and thus, no Turing pattern will form.

Once we have the conditions to obtain a stable homogeneous steady state, we can proceed to analyze the system with the diffusion terms. What we want to test is whether the diffusion terms are the ones driving the instability. To do so, we will calculate \( \det(\lambda Id - (J - \kappa^2 D)) = 0 \)
Where the third order characteristic polynomial is the following:

\[
\lambda^3 + \lambda^2 \left[ \kappa^2(D_y + D_x) - \tau \right] + \lambda \left[ \kappa^4 D_y D_x - \kappa^2 \left( D_y(H_z + F_x) + D_x(H_z + G_y) \right) + R \right] + \Delta - \kappa^4 H_z D_y D_x + \kappa^2 \left( D_y(H_z F_x - F_z H_x) + D_x(G_y H_z - H_y G_z) \right)
\]

Following the Routh-Hurwitz stability criteria from equation 29, the conditions for a stable system would be the following:

1. \( \kappa^2(D_y + D_x) - \tau > 0 \)
2. \(-\Delta - \kappa^4 H_z D_y D_x + \kappa^2 \left( D_y(H_z F_x - F_z H_x) + D_x(G_y H_z - H_y G_z) \right) > 0 \)
3. \(\left( \kappa^2(D_y + D_x) - \tau \right) \left( \kappa^4 D_y D_x - \kappa^2 \left( D_y(H_z + F_x) + D_x(H_z + G_y) \right) + R \right) > \left( -\Delta - \kappa^4 H_z D_y D_x + \kappa^2 \left( D_y(H_z F_x - F_z H_x) + D_x(G_y H_z - H_y G_z) \right) \right)\)

From equation 33 we can observe that condition 1 is always met because \( \tau < 0 \), which was a condition taken from the criteria obtained to make the homogeneous steady state stable. Condition 2 is the one that can destabilize the system since it affects also condition 3. For this reason, we will further analyze this expression.

The second condition of equation 33 can be reduced into an equation dependent on the wavenumber \( \kappa \):

\[
P(\kappa^2) = -\Delta - b_1\kappa^4 + b_2\kappa^2
\]

As we did in the previous section, we will look for the critical value of the wavenumber \( \kappa \) for which a Turing pattern can emerge. As before, an important condition to have a Turing system is to obtain a real, positive and finite value for the wavenumber. For simplicity, we will assume \( \kappa^2 = x \):

\[
-b_1 x^2 + b_2 x - \Delta = 0 \rightarrow x = \frac{-b_2 \pm \sqrt{(b_2)^2 - 4\Delta b_1}}{-2b_1}
\]
Where the critical value for the wavenumber is:

$$\kappa_c = \sqrt{\frac{b_2}{2b_1}} \rightarrow \begin{cases} b_1 = H_z D_x D_y \\ b_2 = D_y (H_z F_x - F_z H_x) + D_x (G_y H_z - G_z H_y) \end{cases} \quad (36)$$

In order to have a positive and real value of the wavenumber $\kappa$ we need that $b_1$ and $b_2$ have the same sign.

As we can observe in equation 36, the only variable capable of changing the sign of $b_1$ is $H_z$ since both $D_x$ and $D_y$ are positive. So from this we can obtain a condition: if $H_z > 0$ then $b_2 > 0$ and if $H_z < 0$ then $b_2 < 0$ in order for the Turing instability to occur.

So in conclusion, in order to have a Turing Pattern we need to full-fill the criteria for a stable homogeneous steady state, and also, the criteria for an unstable state with diffusion. All of these can be summarized in:

$$\tau < 0$$
$$\Delta < 0$$
$$R > \frac{\Delta}{\tau} > 0$$
$$\kappa_c = \sqrt{\frac{b_2}{2b_1}} > 0 \quad (37)$$

These conditions need to be fullfilled in order to have a Turing pattern. We obtained the general form of a three component system with two diffusers and one immobile substrate. Now that we know the conditions to obtain a Turing system, we can test our model. As observed in Figure 12, the network underlying our system has the sign structure:

$$F_x < 0 \quad , \quad F_y > 0 \quad , \quad F_z = 0$$
$$G_x > 0 \quad , \quad G_y > 0 \quad , \quad G_z = 0$$
$$H_x > 0 \quad , \quad H_y > 0 \quad , \quad H_z < 0 \quad (38)$$

The negative signs for $F_x$ and $H_z$ are due to the degradation terms. If we test these interactions with the criteria obtained, we will observe that the system can never be a Turing Pattern since $\Delta > 0$.

From this result, we can see how just by analyzing the sign structure of the network, we were able to test if the system could lead to a Turing pattern. From equation 36, we have the four conditions needed to full-fill the stability criteria of Turing systems. However, if any of those conditions is not met, then we will not have a Turing system. From this, and from the fact that the Jacobian sign structure plays an important role in the stability analysis, we decided to build a tool capable of analyzing all possible three component systems with two diffusers to observe the cases in which we cannot have a Turing Pattern regardless of the values of the parameters. This analysis was performed using the conditions (equation 36) and looking for all the cases in which at
least one of the conditions is broken. Since we were only taking into account the sign structure, this method can be extended to any type of equations or parameter values used to model the system. The steps followed to create this tool are explained in the Supplementary Material.

7.3 Two-component system with time delay

As observed in section 3.2 with the example of Nodal and Lefty, the delay due to protein production must be taken into account in order to have a realistic model. However, the typical reaction-diffusion systems assume instantaneous production of the molecules, which is why in this section our aim is to observe in silico how time delay in the production term can affect the onset of the patterning event. We will analytically analyze the Activator-Inhibitor model and the Depletion model since they are the most used to explain pattern formation in biology.

Time delay in activator inhibitor reaction diffusion system

In the activator inhibitor system we have that the activator promotes its own production and promotes the production of inhibitor whereas the inhibitor inhibits the activator production and decays with time [53]. The general form of this type of system can be seen in the following equations:

\[ \frac{\partial U}{\partial t} = k_1 - k_2 U + k_3 \frac{U^2}{V} + D_u \nabla^2 U \]
\[ \frac{\partial V}{\partial t} = k_4 U^2 - k_5 V + D_v \nabla^2 V \]  \hspace{1cm} (39)

Where we have a constant production of activator \((k_1)\), activator and inhibitor decay, for example via an independent ligand-induced receptor endocytosis. Also, two
molecules of activator can reversibly bind a receptor to induce, ultimately, the production of a molecule of inhibitor. However, this production is delayed by a time, $\tau$. Similarly, two molecules of activator can reversibly bind a receptor to induce, ultimately, the production of an additional molecule of activator yet again, this production is delayed by a time, $\tau$ [40]. For simplicity, in this report we will be assuming that the time delay of both gene expression events is the same and constant.

So the equations with the time delays are the following:

\[
\begin{align*}
\frac{\partial}{\partial t} U(x,t) &= k_1 U(x,t) - k_2 U(x,t) + k_3 \frac{U^2(x,t - \tau)}{V(x,t - \tau)} + D_u \nabla^2 U(x,t) \\
\frac{\partial}{\partial t} V(x,t) &= k_4 U^2(x,t - \tau) - k_5 V(x,t) + D_v \nabla^2 V(x,t)
\end{align*}
\]

And for simplicity we will be working with the dimensionless form (to reduce the number of parameters):

\[
\begin{align*}
\frac{\partial}{\partial t} u(x,t) &= \gamma \left( u(x,t) - b u(x,t) + \frac{u^2(x,t - \tau)}{v(x,t - \tau)} \right) + d_u \nabla^2 u(x,t) \\
\frac{\partial}{\partial t} v(x,t) &= \gamma \left( u^2(x,t - \tau) - v(x,t) \right) + d_v \nabla^2 v(x,t)
\end{align*}
\]

Equation 41 will be modeled using Python to test for different values of the parameters how does the time delay affect in the pattern formation.

**Time delay in depletion model reaction diffusion system**

The other Reaction-Diffusion system described by Gierer and Meinhardt [34] is the Depletion model. This type of system describes the dynamics of two morphogens, U and V, that are synthesized from two substrates, A and B. Two molecules of U react with one molecule of V to create an additional molecule of U. This autocatalytic reaction creates a positive feedback loop [54]. As in the previous model, the production of both U and V is delayed by a constant time $\tau$.

The equations describing this type of behavior are the followings:

\[
\begin{align*}
\frac{\partial U}{\partial t} &= k_1 a - k_{-1} U + k_3 U^2 V + d_u \nabla^2 U \\
\frac{\partial V}{\partial t} &= k_2 b - k_3 U^2 V + d_v \nabla^2 V
\end{align*}
\]

Where $k_1, k_{-1}, k_2, k_3$ are positive definite and dictate the production rate, the decay rates and rate of gene product interactions of the morphogen.

The equations describing the time-delayed model are.
\[
\frac{\partial U}{\partial t} = k_1 a - k_{-1} U(x, t) + k_3 U^2(x, t - \tau) V(x, t - \tau) + d_u \frac{\partial^2 U}{\partial x^2}
\]
\[
\frac{\partial V}{\partial t} = k_2 b - k_3 U^2(x, t - \tau) V(x, t - \tau) + d_v \frac{\partial^2 V}{\partial x^2}
\]

Where the dimensionless form is:
\[
\frac{\partial}{\partial t} u(x, t) = \gamma \left( a - u + u^2(x, t - \tau) v(x, t - \tau) \right) + d_u \nabla^2 u
\]
\[
\frac{\partial}{\partial t} v(x, t) = \gamma \left( b - u^2(x, t - \tau) v(x, t - \tau) \right) + d_v \nabla^2 v
\]

Where \( \gamma \) is a reaction-rate coefficient; \( d_u \) and \( d_v \) are diffusion coefficients, \( a, b \) are two sources (where both \( u \) and \( v \) are produced) and \( u, v \) are the concentration of the molecules involved.

Equation 44 will be used to simulate the system with time delay in Python and check for different parameter values if the overall effect is the same.

**Results**

8 **Signaling affects early gastrulogenesis**

The rationale for these experiments was to observe how Wnt and FGF affect in the patterning of early gastrulogenesis using the 3D-culture to see this effect in both time and space. The cell lines we decided to use in this case were T-Brachyury::GFP and Spry4::H2B-Venus (see section 3.1) since we wanted to track the expression of T-Brachyury to observe if the polarized pattern still formed under the conditions we tested and also, the Spry4 reporter line, which is a target of FGF, to analyze its expression under different conditions to test the role of FGF signaling pathway in gastrulogenesis. The first thing to test is the difference on growing Gastruloids with and without the Chiron pulse at 48h-72h in both of our cell lines (time point which had been shown to be the most efficient [27]). This way we could observe how Wnt acts on the morphology of the Gastruloids and on the expression of our two reporters. As reported in [16], we expected Wnt signaling to be essential for the amplification and stability of the initial patterning event.

The next step is to test three different conditions that will be applied at 24h-48h, 48h-72h, 72h-96h and 96h-120h to both cell lines to see their effect at different time points (Figures 13):

- **Control condition**: Only a Chiron pulse will be applied during 48h-72h (Figure 13 A).
- **PD03 condition**: The MEK inhibitor, PD03, will be added to observe how this
pathway affects on Gastruloids morphogenesis and in pattern formation (Figure 13 B-E).

- **PD17 condition**: The FGF receptor 1 inhibitor will be added to see how gastrulogenesis and pattern formation occurs when both the MEK signaling pathway and the PI3K signaling pathway are inhibited (Figure 13 B-E).

As for the PD03 condition we expected similar results as the one observed in [31] in which PD03 was added before the Chiron pulse and no significant differences were observed with respect the control. In this study we were willing to observe if there was any difference if PD03 was added at later time points and compare these results with the PD17 condition. Regarding the addition of the PD17 inhibitor we did not have any previous background since it was the first time this condition was tried in a 3D culture but we could expect to see significant differences with respect the control since inhibiting the FGF signaling cascade also inhibits the PI3K pathway which is known to be involved in cell survivals [55].
8.1 Wnt/β-catenin pulse promotes patterning formation and elongation in Gastruloids

We first analyze the fluorescence plots (figures on the right of Figure 14). In the Spry4::H2B-Venus cell line, in the Chiron+ condition, we can observe a higher fluorescence during the last two time points (at 96 and 120 hours) which makes sense since SPRY4 gene has been identified as an evolutionarily conserved target of the Wnt/β-catenin signaling pathway in progenitor cells [56]. In the T-Brachyury::GFP cell line, in the fluorescence graphic in the Chiron+ condition, we observe a higher and polarized fluorescence as well as a smaller standard deviation meaning that most of the aggregates behave similarly. This is consistent with what we explained before in which Wnt (which in this case comes from endogenous cell production) up-regulates T-Brachyury production and polarization.

As for the flattening plot (figures on the left of Figure 14) we can observe that in both cell lines there is a tendency in the Chiron+ condition to elongate the higher the time point is. In the Chiron− condition, there is more variability on how cells behave meaning that we can have elongated cells with high fluorescence but also more circular cells.

As reported in [27], exposure to Chiron on the third day has elicited a simple and consistent response in fluorescence and elongation in both cell-lines. This is why this experimental condition was used as the basis to analyze the effect of the later treatments we apply. These results indicate that Gastruloids are capable of elongation in the manner that has been described for P19 EC cells [28] and that this behavior is associated with specific culture conditions.
Figure 14: A) T-Brachyury::GFP Cell line and B) Spry4::H2B-Venus Cell line, both under two conditions: in one case we applied Chiron (CHI99201) with the concentration specified in section 5.4 (Chiron⁺) and in the other case we did not apply any pulse (Chiron⁻). Both cell lines were cultured in the same media (N2B27) under the same conditions specified in section 5.2. Left images: Measure of the Flattening. Right images: Measure of the fluorescence.
8.2 T-Brachyury pattern is affected by FGF signaling

We used the T-Brachyury::GFP cell line to form aggregates and we tested at different time points the two inhibitors of the FGF signaling pathway in order to see whether FGF signaling has an effect in T-Brachyury polarization.

Adding PD03 (MEK inhibitor) at 24h-48h did not make any difference in terms of fluorescence. However, the shape of the Gastruloids remained as that of the control during all time points except at 48h-72h (after the Chiron pulse) in which the treated Gastruloids became more elongated (Figure 15 A). The PD17 (FGF receptor 1 inhibitor) at 24h-48h did make a major effect on the shape and a smaller effect on fluorescence. T-Brachyury expression was reduced at 72 and 96 hours, although we can still observe some polarization at the 96 hour point and the same amount of fluorescence at 120 hours. Gastruloids with PD17 elongated rapidly until 72 hours where they stopped elongating and displayed the same elongation as the rest of the Gastruloids. This showed that the Gastruloids had the ability to go back to "normality" in both shape and expression (Figure 15 A).

On the other hand, we also added PD03 and PD17 during 48h-72h at the same time as the Chiron pulse. The MEK inhibitor, PD03, again did not make any difference in fluorescence with respect the control but this time, the shape also remained very similar with the control (Figure 15 B). This means that by adding PD03 with Chiron, the inhibition is balanced and we do not see many differences between the treatment and the control. On the other hand, the PD17 pulse at this time point (48h-72h) abolishes the T-Brachyury fluorescence normally seen at 72h time point. The shape of the Gastruloids treated with PD17 at 48h-72h is similar to the control (during 48h-72h) however 24 hours after the PD17 and Chiron pulse we observe that Gastruloids stop flattening which makes sense since the T-Brachyury expression has also stopped (Figure 15, bottom graphics).

Adding PD03 during 72h-96h (24 hours after the Chiron pulse) resulted in barely no difference in fluorescence, however the elongation was delayed: instead of starting to elongate during the 96 hours, it started later around 120 hours. Dramatic changes were observed when we added PD17 during 72h-96h (24 hours after the Chiron pulse) in both fluorescence and shape. The T-Brachyury expression was completely abolished and the elongation never occurred, meaning that Gastruloids remained very spherical and with no signaling polarization (Figure 16, top graphics).

If we add the PD03 at 96h-120h instead, then we only observed differences in the elongation at 120 hours which is bigger than the control. As for the PD17 at 96h-120h we also only see differences in terms of elongation but in this case, Gastruloids did not elongate more (Figure 16, bottom graphics).

From all of these results we can briefly summarize that adding PD03 before the Chiron pulse does not allow the cells to elongate as much compared with the control.
However, adding PD03 with or after the Chiron pulse will make cells properly elongate. Also we can observe how PD03 does not result in any change in T-Brachyury expression at any time point. The addition of PD17 before the Chiron pulse makes the Gastruloids elongate earlier and more flattened compared to the control; fluorescence is slightly reduced in this case. Yet, adding PD17 with Chiron will allow Gastruloids to elongate until 96 hours and adding the inhibitor afterwards will stop them from elongating. The fluorescence disappears by adding PD17 with or after the Chiron pulse.

We can conclude that the inhibition of FGF signaling by PD03 had little effect on the expression of T-Brachyury within the Gastruloids. Also, the gradual increase in the elongation of the T-Brachyury Gastruloids was not significantly altered over time when compared to the control. Gastruloids treated with PD17 were severely affected in their ability to up-regulate the reporter to the same extent as either the control. This situation was not so dramatic when we added the inhibitor before the Chiron pulse. This could be due to the fact that Chiron was able to compensate the effect of PD17 since it is an agonist of the Wnt/β which up-regulates T-Brachyury production.
Figure 15: T-Brachyury::GFP Cell line under three conditions: in the first case we apply PD03 (the MEK pathway inhibitor) and Chiron (CHI99201), in the second case we applied PD17 (FGF receptor 1 inhibitor) and Chiron (CHI99201) and the control case only has Chiron. In A) we applied these conditions between 24h-72h and in B) we applied them between 48h-72h. The Chiron pulse was always applied between 48h-72h. All the concentrations used are those specified in section 5.4. Both cell lines were cultured in the same media (N2B27) under the same conditions specified in section 5.2. Left images: Measure of the Flattening. Right images: Measure of the fluorescence.
Figure 16: T-Brachyury::GFP Cell line under three conditions: in the first case we apply PD03 (the MEK pathway inhibitor) and Chiron (CHI99201), in the second case we applied PD17 (FGF receptor 1 inhibitor) and Chiron (CHI99201) and the control case only has Chiron. In A) we applied these conditions between 72h-96h and in B) we applied them between 48h-72h. The Chiron pulse was always applied between 96h-120h. All the concentrations used are those specified in section 5.4. Both cell lines were cultured in the same media (N2B27) under the same conditions specified in section 5.2. Left images: Measure of the Flattening. Right images: Measure of the fluorescence.
8.3 Spry4 expression is not affected by MEK pathway inhibition

In addition to the T-Brachyury::GFP cell line, we utilized an FGF reporter (Spry4::H2B-Venus) mouse embryonic stem cell line to assess FGF/MAPK signaling and assessed its expression following treatment with either PD03 and Chiron, PD17 and Chiron, or only Chiron for the control. The control treatment (Chiron at 48h-72h) resulted in a gradual increase in the FGF reporter over time with a slight bias towards the posterior region at 48h-72h (Figure S5), with a burst of expression throughout the whole Gastruloids at 96h-120h (which is consistent with what we observe in mouse embryos [57]).

Adding PD03 at 24h-48h (before the Chiron pulse) or at 48h-72h (with the Chiron pulse) did not affect the elongation of the Gastruloids or the expression of Spry4. However adding PD17 at 24h-48h (before the Chiron pulse) had an effect during the last two time points in fluorescence, which was a reduction of Spry4 levels at 96h-120h (Figure 17 A). Regarding the elongation, we can see how PD17-treated cells elongated rapidly after the Chiron pulse but this effect stopped during 96h-120h as we can observe how the control group at this time point is more elongated. Different situation occurs if we add PD17 with the Chiron pulse (48h-72h) in which we observe no Spry4 expression and also we do not see any elongation at 96-120h (Figure 17 B).

Similarly the 24h-48h and 48h-72h time points, adding PD03 after the Chiron pulse did not affect the Gastruloids neither in fluorescence nor in elongation, they remained the same as the control. On the contrary, adding PD17 after the Chiron pulse at 72h-96h we observe a reduction in fluorescence of Spry4 and also, we can see how Gastruloids started to elongate after the Chiron pulse but when PD17 was added this effect was lost and thus they became rounded like in the first time points (Figure 18 A). Similar effects can be observed regarding elongation when we add PD17 at 96h-120h. However, in the fluorescence levels such a big reduction was not seen after the treatment with PD17 like in the last cases because there must be accumulated expression of Spry4 from the last time points (Figure 18 B).

From these results we can conclude that inhibiting the MEK pathway with PD03 does not have any effect on the Spry4 expression meaning that the transcription of this molecule must come from another FGF signaling pathway. Also, adding PD03 did not have any effect on elongation in this cell line. As for the FGF receptor 1 inhibitor (PD17), we did observe major changes in the expression of Spry4 and also in the elongation, which was disrupted when this inhibitor was added to the media.
Figure 17: Spry4::H2B-Venus Cell line under three conditions: in the first case we apply PD03 (the MEK pathway inhibitor) and Chiron (CHI99201), in the second case we applied PD17 (FGF receptor 1 inhibitor) and Chiron (CHI99201) and the control case only has Chiron. In A) we applied these conditions between 24h-72h and in B) we applied them between 48h-72h. The Chiron pulse was always applied between 48h-72h. All the concentrations used are those specified in section 5.4. Both cell lines were cultured in the same media (N2B27) under the same conditions specified in section 5.2. Left images: Measure of the Flattening. Right images: Measure of the fluorescence.
Figure 18: Spry4::H2B-Venus Cell line under three conditions: in the first case we apply PD03 (the MEK pathway inhibitor) and Chiron (CHI99201), in the second case we applied PD17 (FGF receptor 1 inhibitor) and Chiron (CHI99201) and the control case only has Chiron. In A) we applied these conditions between 72h-96h and in B) we applied them between 48h-72h. The Chiron pulse was always applied between 96h-120h. All the concentrations used are those specified in section 5.4. Both cell lines were cultured in the same media (N2B27) under the same conditions specified in section 5.2. Left images: Measure of the Flattening. Right images: Measure of the fluorescence.
9 Constraining Turing patterning in embryonic development

In this section we will show the results obtained from \textit{in silico} simulations for the Reaction-diffusion models. We will start with the three-component networks obtained using the criteria explained in section 7.2 to reject systems that could lead to Turing pattern. Next, we will provide the simulation result for the time-delayed reaction-diffusion systems.

9.1 Coupling-architecture determinants of Turing patterning

In section 7.2, we analyzed the conditions for which a three-component system with only two diffusers may never lead to a Turing pattern. We present some results that are examples of general networks we can obtain that no matter the parameter value, no Turing Pattern can emerge.

From all possible combinations of sign structure 3x3 matrices ($-,+,0$), we can have \( \approx 2 \times 10^5 \) combinations. From all of these matrices, we can discard all the ones that have \( H_z = 0 \), which would make the wavenumber equal to zero. This will leave us with \( \approx 12 \times 10^3 \). Also, we can discard all of the possible combinations that lead to a positive trace, that is, around \( 7 \times 10^2 \) more matrices. From those \( \approx 11 \times 10^3 \) possible matrices left, we test our conditions. As a result, we obtain some hundreds of matrices which can be fused together due to similarity (Figure 19).

As an \textit{a priori} conclusion, if we have it that the immobile molecule does not self-interact \( (H_z = 0) \), then no Turing Pattern will form. Also, if the three molecules are self-activators, then no pattern will emerge either (positive trace, \( \tau \)). If we observe Figure 19, which are some of the networks obtained with the conditions for no pattern formation, we can see how there is an intrinsic pattern within the networks: many of them can be fused together by only taking the fixed connections into account, and creating general solutions. However, there are many matrices to analyze (\( \approx 6,000 \)) and we were able to group them into several hundreds, however more work and a better method to check for these network patterns should be created. a
Figure 19: Some examples of the Networks that cannot lead to Turing pattern just by analyzing the sign structure of the Jacobian. This graphs were created with Matlab. Gray lines stand for any type of connection (inhibition, activation or no connection), red lines stand for inhibition, blue lines stand for activation, red and blue dotted lines represent the inhibition and activation, respectively although they can also be neglected. In these graphs, Z is the immobile molecule whereas Y, X are the diffusers.
9.2 Time delay disrupts the pattern in activator inhibitor and depletion model

Figure 20: Activator inhibitor system simulation using equation 37 and the parameter values: $b = 1, a = 0.2, d = 20, \gamma = 10000, T = 5, \tau = 2dt$, size = 50. Simulations were performed with Python.

Figure 21: Activator inhibitor system simulation using equation 37 and the parameter values: $b = 1, a = 0.2, d = 20, \gamma = 10000, T = 5, \tau = 2dt$, size = 50. Simulations were performed with Python.

These graphics were obtained using the difference element method and taking into account the Von-Neumann stability criteria, which states:

$$D \frac{dt}{dx^2} \leq \frac{1}{2}$$ (45)

In our case we used a $dt = 0.01 \cdot dx^2$ and a $dx = 1/(2 \cdot size)$.

Adding time delay into the systems described by Gierer-Meinhardt [34] did not allow pattern formation in any case (Figure S9-S12). Spatial oscillations are not robust enough to be formed when a very small time delay is added into the simulation. All
the parameters for which different Turing pattern may emerge (spots, stripes) can be observed in Figures S10-S13. Also, in Figures S14-S17 we can observe the temporal evolution of the reaction-diffusion systems with or without time delay.

**Discussion**

We have shown that Gastruloids, embryonic organoids derived from small aggregates of mouse embryonic stem cells, undergo symmetry-breaking and gene expression polarization in a manner that mirrors events in embryos as it has already been previously reported in [27,31].

Analysis of signaling reporters in the Gastruloids reveals clear Wnt/β-Catenin signaling dependency from 48h-72h for the onset and localization of T-Brachyury expression. This signaling is required in this early period for the correct timing of expression. Furthermore, as in the embryo [58], this pattern is also dependent on Nodal even though our results do not show this dependency. The Chiron pulse will up-regulate the Wnt/β-Catenin signaling activity which will also activate the Nodal signaling pathway [59]. Which is why, without the Chiron pulse, we observe more heterogeneity within the Gastruloids elongation and both T-Brachyury expression and localization.

Although there was slight variation between replicates (in terms of expression levels and the internal standard deviations), these observations suggest that whereas FGF MAP/ERK signaling has minimal effect at the onset of the initial patterning event, another FGF signaling pathway is essential to allow not only the expression of T-Brachyury::GFP but the elongation of the Gastruloids. Taken together, we can conclude that FGF MAP/ERK signaling has a limited effect on either initial patterning of the Gastruloid, the up-regulation of T-Brachyury::GFP fluorescence or progression of the Gastruloids. However another FGF signaling pathway present at these early time-points is essential for constraining the domain of expression at the posterior region and the maintenance of increased expression during all time points.

The MAP/ERK signaling pathway has been reported to be associated with the specification of mesendodermal fates [60], which could be a reason why we do not observe variations in the 3D culture because this FGF pathway is acting as a thresholding for cell fate specification. On the other hand, we hypothesize that FGF/PI3K-Akt signaling pathway could have an important role in gastrulogenesis and in Spry4 expression. Also, with these results we were ble to observe the FGF expression in the Gastruloids. We can conclude that the observed FGF pattern (Figures S5-S8) mimics that of the embryo (expression restricted to the tail) and specifically, the Spry4 gene.

From [61] we found that the activation of the PI3K signaling pathway plays an important role in glucose metabolism of the mouse embryo and in embryonic survival; the PI3K/Akt signaling pathway is also crucial during the pre-implantation stage since the signaling proteins are expressed at all stages of preimplantation development [55].

The inhibition of the PI3K/Akt pathway leads to embryonic stem cell and blastocyst apoptosis [62]. However, the experiments performed to prove this statement
were performed under serum-free conditions, which alone can induce apoptosis. Under physiological conditions, other growth factors, produced by the embryo itself, may compensate for changes in PI3K activity. Further studies are necessary to elucidate the physiological consequences of PI3K/Akt inhibition and the mechanisms involved.

From all of these results we can conclude that pattern formation in early gastrulogenesis is dependent on Wnt signaling in order to observe localized expression of T-Brachyury. We cannot conclude that FGF/PI3K is the signaling pathway which is involved in pattern formation however, we can discard the role of FGF/MEK signaling pathway. Further experiments should be performed to better understand which is the role of PI3K signaling pathway or to test whether there are other pathways interfering in these results. We see observe a strong correlation between pattern formation and Gastruloid elongation which means that the mechanics underlying this morphogenetic process is also playing a crucial role.

With this conclusion, we tried to create a reaction diffusion system that could explain the pattern observed in Gastruloids (Figure 3). The main idea was to obtain a Turing pattern of the type of a Dissipative Soliton, which is a localized structure that exists for an extended period of time and contrary to other Turing patterns, is a self-reinforcing solitary wave packet (it is non-repetitive) that maintains its shape [49]. For this purpose, we started with a simple general reaction-diffusion model which only included Wnt, as the diffusing ligand and T-Brachyury, a transcription factor, as the immobile molecule. As a result, we observed that pattern cannot form in any two component system in which one of the elements is immobile. For this reason, we decided to include another important molecule into the system, Nodal. This ligand can also diffuse in the system so we ended up with a three component system in which only two molecules were able to diffuse.

We started to analyze this three component system in the most general way in order to obtain the criteria for which the system leads to Turing instabilities. Having a general way of approaching the problem would indicate any possible changes that could be implemented to the network for a Turing pattern in case of failure. However, for our system, this type of behavior would not be possible unless we completely changed the networks behind our model. From this result, we produced a useful tool that could help discard cases by taking into account the sign of the derivatives of the equations (i.e. the Jacobian) to reject networks that will never lead to a Turing pattern.

All the conditions are stated in the Supplementary Material which, when applied with Matlab, can show all the possible matrices combinations (positive, negative or zero sign) for which Turing Pattern will never occur. As a result, we found patterns within the networks that we were able to fuse together and obtain some general network forms. However, many matrices were obtained so more work should be done in order to obtain all possible general solutions that cannot emerge in a Turing system.

Finally, since our reaction diffusion system was not possible, we then started to question whether this type of system is a good approach for this situations. We then encountered the possibility that a reaction diffusion may not lead to a Turing pattern if time delay was introduced into the equations. Time delay can be produced by gene
transcription, protein translation, splicing, etc. and this period of time should be taken into account in order to obtain more biological situations. The test was performed with the two main Gierer-Meinhardt [34] systems, the activator-inhibitor and the depletion model since they are the most used in biology to explain pattern formation. Adding the same amount of delay as two time steps was already enough to not allow pattern formation.

From all of these results we can conclude that Reaction-Diffusion is not the best approach to address our question since we have observed from in vitro experiments that mechanical transduction might play an important role and from the in silico simulations that time delay should be taken into account. As a future work, a more mechanistic model that exhibits robustness to time delays should be created.
Bibliography


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Supplementary Material

Gastruloids images with each treatment

Figure S1: Gastruloids from T-Brachyury::GFP cell line treated with PD03, PD17 (at 24h) and the Control (only Chiron). Images were analyzed with FIJI [63].
Figure S2: Gastruloids from T-Brachyury::GFP cell line treated with PD03, PD17 (at 48h) and the Control (only Chiron). Images were analyzed with FIJI [63].
Figure S3: Gastruloids from T-Brachyury::GFP cell line treated with PD03, PD17 (at 72h) and the Control (only Chiron). Images were analyzed with FIJI [63].
Figure S4: Gastruloids from T-Brachyury::GFP cell line treated with PD03, PD17 (at 96h) and the Control (only Chiron). Images were analyzed with FIJI [63].
Figure S5: Gastruloids from Spry4::H2B-Venus cell line treated with PD03, PD17 (at 24h) and the Control (only Chiron). Images were analyzed with FIJI [63].
Figure S6: Gastruloids from Spry4::H2B-Venus cell line treated with PD03, PD17 (at 48h) and the Control (only Chiron). Images were analyzed with FIJI [63].
Figure S7: Gastruloids from Spry4::H2B-Venus cell line treated with PD03, PD17 (at 72h) and the Control (only Chiron). Images were analyzed with FIJI [63].
Figure S8: Gastruloids from Spry4::H2B-Venus cell line treated with PD03, PD17 (at 96h) and the Control (only Chiron). Images were analyzed with FIJI [63].
Critera for Turing Pattern in a three component system with two diffusers

In section 7.2 we saw how a two component system with only one diffuser is not capable of forming a Turing instability so we had to add a third molecule, which diffuses, to test if we can now obtain Turing-like patterns. A three component system requires methods like the Routh-Hurwitz in order to analyze the stability since \textit{a priori} there is no reason to think that your system will not exhibit a Turing instability. For this reason, we here present the conditions that were obtained by analyzing the three component system in order to see in which cases we will not obtain Turing instabilities just by looking at the network of our system. Depending on the sign structure of the Jacobian we will be able to reject some cases that will not yield to a Turing Pattern no matter the value as long as the sign remains the same.

From equation 36 we obtained the critical value of the wavenumber $\kappa$ with which we will obtain instabilities. This variable depended on what we called $b_1$ and $b_2$ and we saw that both had to have the same sign (and be different from zero) in order for the wavenumber to have a positive value.

If we remember, criteria to have Turing Pattern was:

$$
\frac{R}{\tau} \frac{\Delta}{\kappa} > 0
$$

All of these conditions needed to be met in order to have a Turing system. From equation 46 we can now formulate the new criteria for which we will not get Turing patterns:

$$
\tau > 0 \quad \text{OR,}
\Delta > 0 \quad \text{OR,}
R < 0 \quad \text{OR,}
H_z > 0 \quad \text{AND} \quad b_2 < 0 \quad \text{OR,}
H_z < 0 \quad \text{AND} \quad b_2 > 0 \quad \text{OR,}
$$

If any of these conditions is fullfilled, we can reject the system and ensure that no Turing Pattern will emerge from it.

Before analyzing all the possible matrices, we can discard all the ones with $H_z = 0$ since the wavenumber will be zeroed under this condition. We can also reject all posible combinations with positive trace. Finally, we can start to test our conditions.

The conditions can be divided into two: the ones for the Homogeneous steady state
and the ones for the unstable state with diffusion.

**Conditions for unstable steady state**

We want $\tau > 0$, $\Delta > 0$, $R < 0$. However, we need to analyze the system by parts since we are only taking into account the signs of the network.

$$
\begin{align*}
\tau &= F_{t_1} + G_{t_2} + H_{t_3} \\
\Delta &= F_{d_1} G_y H_z - F_x G_z H_y - F_y G_x H_z + F_y G_z H_x + F_z G_x H_y - F_x G_y H_x \\
R &= G_y H_z - G_z H_y + G_y F_x - F_y G_x + H_z F_x - F_x H_z
\end{align*}
$$

(48)

**Conditions:**

1. $((t_1 \geq 0 \text{ AND } t_2 \geq 0 \text{ AND } t_3 \geq 0) \text{ AND } (t_1 + t_2 + t_3 \neq 0))$ OR

2. $((d_1 \geq 0 \text{ AND } d_2 \geq 0 \text{ AND } d_3 \geq 0 \text{ AND } d_4 \geq 0 \text{ AND } d_5 \geq 0 \text{ AND } d_6 \geq 0) \text{ AND } (d_1 + d_2 + d_3 + d_4 + d_5 + d_6 \neq 0))$ OR

3. $((r_1 \leq 0 \text{ AND } r_2 \leq 0 \text{ AND } r_3 \leq 0 \text{ AND } r_4 \leq 0 \text{ AND } r_5 \leq 0 \text{ AND } r_6 \leq 0) \text{ AND } (r_1 + r_2 + r_3 + r_4 + r_5 + r_6 \neq 0))$

**Conditions for stable non-homogeneous state**

The condition to have a stable state in the diffusion system is to not have a positive, real and finite wavenumber. For this, we need that $b_1$ and $b_2$ have opposite signs.

$$
\begin{align*}
b_1 &= H_z D_x D_y \\
b_1 &= D_y (H_z F_x - F_z H_x) + D_x (G_y H_z - G_z H_y)
\end{align*}
$$

(49)

This sign depends on $H_z$ as long as the diffusion constants are positive (which is the only case in which there is biological sense). From this fact we can obtain two set of conditions: when $H_z > 0$ and when $H_z < 0$. Let us start with the first one: $H_z > 0$.

If $H_z > 0 \rightarrow b_1 > 0$ then $b_2$ must be negative.

In order for $b_2$ to be negative no matter the values of the diffusion or without any further assumption, we can state the following conditions:
From equation 49 we can see that we can have a negative $b_2$ if:

$$
G_y H_z - G_z H_y < 0 \text{ AND } H_z F_x - F_z H_x < 0 \text{, OR }
$$
$$
G_y H_z - G_z H_y = 0 \text{ AND } H_z F_x - F_z H_x < 0 \text{, OR }
$$
$$
G_y H_z - G_z H_y < 0 \text{ AND } H_z F_x - F_z H_x = 0
$$

From these conditions we can play with the combinations of signs of $G_y, G_z, H_y, F_x, F_z$ to obtain conditions for when this can be met and thus, a Turing Pattern could not be formed.

**Condition 1. $G_y < 0$**
1.1 $G_z > 0, H_y > 0$ Any possible combination of $G_z; H_y; G_y = 0$
1.2 $G_z < 0, H_y < 0$ Any possible combination of $G_z; H_y; G_y = 0$
1.3 $G_z < 0, H_y > 0$ AND Assuming that $G_y H_z > G_z H_y$
1.4 $G_z > 0, H_y < 0$ AND Assuming that $G_y H_z > G_z H_y$

**Condition 2. $G_y > 0$**
2.1 $G_z > 0, H_y > 0$ AND Assuming that $G_z H_y > G_y H_z$
2.2 $G_z < 0, H_y < 0$ AND Assuming that $G_z H_y > G_y H_z$

**Condition 3. $F_z < 0$**
3.1 $F_z > 0, H_x > 0$ AND Any possible combination of $F_z; H_x; F_z = 0$
3.2 $F_z < 0, H_x < 0$ AND Any possible combination of $F_z; H_x; F_z = 0$
3.3 $F_z > 0, H_x < 0$ AND Assuming that $H_z F_x > F_z H_x$
3.4 $F_z < 0, H_x > 0$ AND Assuming that $H_z F_x > F_z H_x$

**Condition 4. $F_y < 0$**
4.1 $F_z > 0, H_x > 0$ AND Assuming that $H_x F_z > F_z H_z$
4.2 $F_z < 0, H_x < 0$ AND Assuming that $H_x F_z > F_z H_z$

In order to make $b_2 < 0$, we just need to combine Conditions 1 and 2 with Conditions 3 and 4.

Now we go to the case in which $H_z < 0$.

If $H_z < 0 \rightarrow b_1 < 0$ then $b_2$ must be positive.

From equation 47 we can see that we can have a positive $b_2$ if:

$$
G_y H_z - G_z H_y > 0 \text{ AND } H_z F_x - F_z H_x > 0 \text{, OR }
$$
$$
G_y H_z - G_z H_y = 0 \text{ AND } H_z F_x - F_z H_x > 0 \text{, OR }
$$
$$
G_y H_z - G_z H_y > 0 \text{ AND } H_z F_x - F_z H_x = 0
$$

Similarly to the above case, the conditions to make $b_2 > 0$ are:

**Condition 1. $G_y < 0$**
1.1 \( G_z > 0, H_y > 0 \) AND Assuming that \( G_y H_z > G_z H_y \)
1.2 \( G_z < 0, H_y < 0 \) AND Assuming that \( G_y H_z > G_z H_y \)
1.3 \( G_z < 0, H_y > 0 \) Any possible combination of \( G_z; H_y; G_y = 0 \)
1.4 \( G_z > 0, H_y < 0 \) Any possible combination of \( G_z; H_y; G_y = 0 \)

**Condition 2.** \( G_y > 0 \)
2.1 \( G_z < 0, H_y > 0 \) AND Assuming that \( G_z H_y > G_y H_z \)
2.2 \( G_z > 0, H_y < 0 \) AND Assuming that \( G_z H_y > G_y H_z \)

**Condition 3.** \( F_z < 0 \)
3.1 \( F_z > 0, H_x > 0 \) AND Assuming that \( H_z F_x > F_z H_x \)
3.2 \( F_z < 0, H_x < 0 \) AND Assuming that \( H_z F_x > F_z H_x \)
3.3 \( F_z > 0, H_x < 0 \) AND Any possible combination of \( F_z; H_x; F_x = 0 \)
3.4 \( F_z < 0, H_x > 0 \) AND Any possible combination of \( F_z; H_x; F_x = 0 \)

**Condition 4.** \( F_y < 0 \)
4.1 \( F_z < 0, H_x > 0 \) AND Assuming that \( H_x F_z > F_x H_z \)
4.2 \( F_z > 0, H_x < 0 \) AND Assuming that \( H_x F_z > F_x H_z \)

Same as before, in order to make \( b_2 < 0 \), we just need to combine Conditions 1 and 2 with Conditions 3 and 4.

So, we can obtain all the possible networks for which a Turing Pattern will never occur. In this report we show some of the results, however further analysis on this networks should be performed since they seem to pattern into fixed network architectures that will be interesting to analyze.

**Time Delay in Reaction Diffusion System: Exploring the parameter space**

Using the equations from section 7.3 we can explore all the parameters for which we can have a pattern and see the effect of the time delay.
Figure S9: Phase plane of the parameters used to vary between the different possible Turing patterns. For both the depletion model and the activator inhibitor system, the parameters $a$ and $b$ were varied. For a fixed value of the diffusion constant and $\gamma$, we can obtain the possible combinations for which we can have spatial oscillations.
Figure S10: Depletion Model for several parameter values of $a$ and $b$ for which we would obtain strips or dots as a pattern. The simulation was performed with Python using a simulation time of 10, and a space of 50. The values used for $a = [0.025, 0.1, 0.225, 0.275]$ and for $b = [0.25, 1.25, 1.5, 2.25]$. 
Figure S11: Depletion Model with timed delay for several parameter values of $a$ and $b$ for which we would obtain strips or dots as a pattern. The simulation was performed with Python using a simulation time of 10, and a space of 50. The time delayed used was the minimum possible which was equal to a time step. The values used for $a = [0.025, 0.1, 0.225, 0.275]$ and for $b = [0.25, 1.25, 1.5, 2.25]$. 
Figure S12: Depletion Model for several parameter values of $a$ and $b$ for which we would obtain strips or dots as a pattern. The simulation was performed with Python using a simulation time of 10, and a space of 50. The time delayed used was the minimum possible which was equal to a time step. The values used for $a = [0.025, 0.1, 0.225, 0.275]$ and for $b = [0.25, 1.25, 1.5, 2.25]$. 
Figure S13: Depletion Model with timed delay for several parameter values of $a$ and $b$ for which we would obtain strips or dots as a pattern. The simulation was performed with Python using a simulation time of 10, and a space of 50. The time delayed used was the minimum possible which was equal to a time step. The values used for $a = [0.025, 0.1, 0.225, 0.275]$ and for $b = [0.25, 1.25, 1.5, 2.25]$. 
We can also observe how does the activator-inhibitor system and the depletion model behave in the temporal frame and in the final step space (Figures S14-S17).

Figure S14: Simulation using the activator-inhibitor system of Gierer-Meinhardt (section 7.3). Graphics on the left: Space evolution over time. Same parameters as on Figure 20. Graphics on the right: Space 1 and space 2 correspond to $u[i-1]$ and $v[i+1]$ respectively, at the final simulation time 50 where $i = 5$. A. Activator simulations B. Inhibitor simulations.
Figure S15: Simulation using the time-delayed activator-inhibitor system of Gierer-Meinhardt (section 7.3). Graphics on the left: Space evolution over time. Same parameters as on Figure 20. Graphics on the right: Space 1 and space 2 correspond to $u_{i-1}$ and $v_{i+1}$ respectively, at the final simulation time 50 where $i = 5$. A. Activator simulations B. Inhibitor simulations
Figure S16: Simulation using the depletion system of Gierer-Meinhardt (section 7.3). Graphics on the left: Space evolution over time. Same parameters as on Figure 20. Graphics on the right: Space 1 and space 2 correspond to $u[i; -i - 1]$ and $v[i + 1:]$ respectively, at the final simulation time 50 where $i = 5$. A. Activator simulations B. Inhibitor simulations
Figure S17: Simulation using the time-delayed depletion system of Gierer-Meinhardt (section 7.3). Graphics on the left: Space evolution over time. Same parameters as on Figure 20. Graphics on the right: Space 1 and space 2 correspond to $u[i-1]$ and $v[i+1]$ respectively, at the final simulation time 50 where $i = 5$. A. Activator simulations B. Inhibitor simulations