PROJECT:

Time is central to embryogenesis. Cells must be born in the right order and at the right time to create the complex 3D organization of the developing organism. Yet, the molecular mechanisms controlling the timing of cell diversification are still poorly understood in animals let alone humans. This is illustrated in the context of the *'HOX* clock', one of the most important molecular timers instructing cell diversity during embryogenesis[1]. *HOX* genes are grouped in 4 genomic complexes (*HOXA/B/C/D)* and encode 39 transcription factors differentially expressed along the body axis along which they instruct cell diversity. *HOX* spatial patterns are initiated by their sequential activation along their complexes from early 3' to late 5' genes[1]. This '*HOX* clock' is activated in axial progenitors, a population of caudally located cells that feed vertebrate body extension. As axial progenitors generate progressively more caudal tissues, *HOX* sequential activation translates into spatial patterns along the body axis[1]. While the tempo of *HOX* activation is critical for proper development, the timer mechanisms and its link with spatial patterns remain unclear, in particular in humans. Beside its fundamental biological relevance, decoding these mechanisms is essential for *in vitro* engineering of human cells and tissues and a better understanding of HOX deregulation in disease. However, tracking temporal processes at the single cell level in complex tissues is extremely challenging in animals developing in utero and impossible in humans. In vitro models of human embryogenesis such as organoids and embryoids derived from human pluripotent stem cells (hPSCs) provide the first experimentally accessible, tractable models of human development amenable to genetic modification and live imaging to tackle such questions.

The lab of S. Nedelec (PhD advisor 1, P1) generated axial progenitors from human pluripotent stem cells (hPSCs) in which *HOX* sequential activation is recapitulated[2]. Using this model, P1 discovered that the temporal progression of *HOX* sequential induction is paced by the levels of two extrinsic factors, FGF2 and GDF11, a member of the TGF-β family. Inhibiting their activity stops *HOX* induction while their precocious increase precipitates their induction and results in the generation of neuronal subtypes located in the caudal end of the human embryonic spinal cord[2]. Pharmacological experiments and transcriptomic analysis showed that *HOXs* are direct targets of the MEK1-ERK1/2 MAPKinase pathway downstream FGF2 and of the SMAD2/3 pathway downstream GDF11[2]. Yet, the mechanisms by which the activity of these two signaling pathways can control the activation of distinct *HOX* genes over time remains unknown. This might lie in the adaptative capacity of these signaling pathways (i.e. return to baseline in presence of constant stimuli).

The lab of P. Hersen (PhD co-director 2, **P2)** including **B. Sorre (co-supervisor)** largely contributed to the demonstration that the temporal dynamic of MAPK and SMAD2/3 pathway adaptation is controlled by the induction of negative retro-feedbacks of the pathways, such as phosphatases or secreted inhibitors of ligands. The level and dynamic of feedbacks result in changes in the duration, amplitude or frequency of signaling that can induce distinct cellular responses such as different gene expression programs[3–5]. P1 transcriptomic data on axial progenitors showed that together with *HOXs*, typical negative feedbacks of FGFs or GDF pathways are activated upon stimulation[2] (and unpublished). Furthermore, ERK and SMAD2 activities are rapidly inhibited following stimulations and thus display adaptation. This raised the possibility that negative feedbacks induction upon stimulation tune the level or duration of ERK1/2 and SMAD2/3 activity which in turn control the activation of more and more 5' *HOX* genes. Hence, the *HOX* clock might be encoded in the temporal dynamics of these signaling

pathways. We thus propose to combine our expertise to supervise a PhD project at the interface of biophysics, bioengineering and human developmental biology to test this hypothesis and provide a breakthrough in this long-standing question with basic and translational implications.

AIM 1: how FGF and GDF pathway dynamics controls the HOX clock?

Our preliminary results indicate that FGF and GDF11 pathways are adaptative in axial progenitors (i.e. return to baseline despite ligand presence). **P2** previously showed that adaptation of the TGF-b/SMAD2 pathway depends on the temporal profile of stimulation. Short pulses of ligand, insufficient to induce retro-feedbacks, lead to a stronger cellular response than a sustained signal that triggers feedbacks and pathway adaptation [4,5]. We will thus investigate how axial progenitor response depends on FGF2 and GDF11 temporal profile of stimulations and how this impacts *HOX* activation. As the parameter space of stimulation (dose, duration and frequency) is too big to be explored empirically, the student will predict the temporal stimulations inducing the highest and lowest adaptation using a biophysical model developed by P2[5]. This model can be constrained with a limited number of parameters (adaptation timescale and dose response EC50). To define them the student will use hPSC lines carrying live reporters of ERK1/2 and SMAD2/3 pathway activity and fluorescent time-lapse microscopy to monitor the responses to step increase of FGF2 and GDF11 concentrations

Then to correlate the history of signaling activity with induction of specific *HOX* genes at the single cell level, the student will introduce in those hPSC lines fluorescent proteins under the control of regulatory elements of Hox genes[6]. Using a fully automated microfluidics cell culture platform available in P2 lab the most promising profiles predicted by the model will be tested[5]. This system allows to stimulate cells with arbitrary temporal profiles of up to 16 morphogen combinations while recording cells' response in real time. With this series of experiments, we will demonstrate the causal link between *HOX* genes induction and the temporal profile of pathway activity.

AIM 2: Retro-feedback roles on *HOX* induction, patterns and cell fate

P1 recently performed a temporal transcriptomic analysis on axial progenitors exposed to FGF and GDF11. *HOX* genes are rapidly induced together with genes encoding typical negative feedbacks of ERK1/2 and SMAD2 pathways[3]. To test the roles of these feedbacks in *HOX* regulation, the student will genetically-modify the reporter iPSCs lines described above to perform inducible gain (GOF) and loss (LOF) of function of candidates. Upon the stimulation paradigms and experimental set-up defined above he/she will determine: **1)** the dynamics of signaling and gene induction **2)** spinal neuron subtype specification which depend on HOX genes. Finally, the student will test whether changes in temporal induction change HOX spatial patterns and neuronal subtype allocation. For that, she/he will use a new innovative human embryoid model developed by **P1** which recapitulate many aspects of human embryo caudal development including the formation of HOX spatial patterns. Embryoids will be cultured in a new type of microfluidic chambers developed by **P2** compatible with 3D cultures and live imaging. He/she will determine pathway temporal dynamic, *HOX* reporter induction, HOX patterns and spatial allocation of cell types in LOF and GOF embryoids.

AIM 3: Signaling dynamics-dependent genomic recruitment of SMAD2/3 and HOX induction?

Then, we will determine how changes in signaling dynamics translate into differential induction of *HOX* genes by focusing on the transcription factor SMAD2/3. An increase in signaling duration/amplitude might induce SMAD2/3 recruitment to progressively more 5' *HOX* enhancers leading to chromatin opening and 5' *HOX* induction. To test this, cells will be stimulated with different GDF11 paradigms identified in aim 1 followed by 1) chromatin immunoprecipitation and sequencing to determine SMAD2/3 binding sites in *HOX* complexes, and 2) ATAC-sequencing to determine whether these binding events are linked to changes in chromatin accessibility. The role of the binding sites will then be tested by mutagenesis. This will provide a model for how pathway temporal dynamics is integrated at the genomic level to induce *HOX* sequential activation.

Overall this PhD project combining biophysical, bioengineering and in vitro models of human development should provide a breakthrough in the longstanding question of how *HOX* genes are sequentially activated during embryogenesis, a process essential for the organization of cell diversity in embryos. Beside its fundamental biological relevance, decoding this mechanism will improve *in vitro* engineering of human cells and organoids from hPSC considering the pleiotropic role of HOX transcription factors. The use of microfluidic devices will favor the establishment of organ- and embryo-on-chips, an important development for basic and translational applications such as toxicology tests. Finally, this joint PhD will foster a long-term collaboration between the two labs to develop synthetic gene circuits to probe and hack signaling pathways and transcriptional programs to control developmental processes.

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