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Paracrine mechanisms in early differentiation of human pluripotent stem cells: Insights from a mathematical model

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ABSTRACT

With their capability to self-renew and differentiate into derivatives of all three germ layers, human pluripotent stem cells (hPSCs) offer a unique model to study aspects of human development in vitro. Directed differentiation towards mesendodermal lineages is a complex process, involving transition through a primitive streak (PS)-like stage. We have recently shown PS-like patterning from hPSCs into definitive endoderm, cardiac as well as presomitic mesoderm by only modulating the bulk cell density and the concentration of the GSK3 inhibitor CHIR99021, a potent activator of the WNT pathway. The patterning process is modulated by a complex paracrine network, whose identity and mechanistic consequences are poorly understood.

To study the underlying dynamics, we here applied mathematical modeling based on ordinary differential equations. We compared time-course data of early hPSC differentiation to increasingly complex model structures with incremental numbers of paracrine factors. Model simulations suggest at least three paracrine factors being required to recapitulate the experimentally observed differentiation kinetics. Feedback mechanisms from both undifferentiated and differentiated cells turned out to be crucial. Evidence from double knock-down experiments and secreted protein enrichment allowed us to hypothesize on the identity of two of the three predicted factors. From a practical perspective, the mathematical model predicts optimal settings for directing lineage-specific differentiation. This opens new avenues for rational stem cell bioprocessing in more advanced culture systems, e.g. in perfusion-fed bioreactors enabling cell therapies.

1. Introduction

Human pluripotent stem cells (hPSCs) hold great promises for a multitude of purposes, including regenerative medicine, drug development and toxicity testing. The enormous potential of hPSCs for modern medicine is based on their capability to give rise to essentially any somatic cell type of the human body, referred to as pluripotency. To date, broad applicability of hPSC is hampered by limited robustness and understanding of the speciation processes towards a desired cell type (Denning et al., 2016; Siller et al., 2016). This is essentially due to the limited understanding of the complex regulatory networks directing the differentiation processes.

We have recently shown that even minor perturbations, i.e. simple variation in the medium volume, drastically impact on lineage-decisions in the early differentiation phase in vitro and thereby direct subsequent differentiation outcome (Kempf et al., 2016). This early differentiation phase reflects key aspects of human development during gastrulation in vivo (Arnold and Robertson, 2009). Similar to the development in vivo, upon WNT pathway stimulation, hPSCs undergo epithelial-to-mesenchymal transition and progress towards a primitive streak (PS)-like state (Skelton et al., 2016). This transition is marked by expression of the mix paired-like homeobox transcription factor 1

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(MIXL1), which in vivo is upregulated following the invagination of cells during gastrulation (Pearce and Evans, 1999). In the course of further development, PS cells give rise to the prospective definitive endoderm and several mesodermal linages, including cardiac as well as presomitic mesoderm. These lineages form the developmental foundation of several tissues including lung, gut, heart and skeletal muscle.

By monitoring PS-like priming in the hPSC model in vitro, we have recently investigated the regulatory role of secreted proteins. Mesendodermal patterning was triggered only by the administration of CHIR99021 (Kempf et al., 2016) (a potent chemical GSK-3 inhibitor agonizing the WNT pathway (Ring et al., 2003), abbreviated in the following as CHIR) combined with variations of the bulk cell density. The study revealed that a complex interplay of stimulatory and inhibitory factors secreted by hPSC during the first 24 h of differentiation directs subsequent cell fates (Kempf et al., 2016).

To better understand the underlying mechanisms, we here complement our experimental findings with a mathematical modeling approach. This allows us to feed experimental data into mathematical simulations and, vice versa, to test the calculated predictions in our cellular model. Systems of ordinary differential equations (ODEs) have been applied to describe the first 48 h of differentiation dynamics. By adding an increasing number of regulatory feedback loops we have developed a model that is sufficiently complex to accurately describe the kinetics of the differentiation process within a broad range of experimental conditions. The model allowed us to assign specific roles to three distinct factors involved in the process. It highlights 1) the indispensability of one inhibitory factor antagonizing progression of PS priming to be released readily by undifferentiated hPSCs and 2) another inhibitory as well as one activating factor of PS progression to be upregulated and secreted at early stages of differentiation.

Moreover, we show that two secreted inhibitors of Nodal signaling, LEFTY1 and CER1, fulfill the inhibitory roles predicted by the mathematical model and also regulate expression of a MIXL1-GFP reporter used to monitor PS priming towards the expected patterns.

Finally, we are using the established model to make predictions on optimal settings for lineage-specific differentiation of perfusion-fed cells (in contrast to the typical batch-feeding used in conventional cell culture), which is relevant for advanced and automated hPSC differentiation processes.

2. Materials and methods

2.1. Experimental set-up

Differentiation was conducted on a 96-well platform applying combinations of 8 different CHIR concentrations (0–17.5 μM) and 6 medium volumes (50–300 μl) for up to 48 h using a previously established embryonic stem cell (HES-3, ESIBIe003-A) reporter line containing an eGFP in the MIXL1 locus (Davis et al., 2008) (Fig. 1A, B). For two independent replicates MIXL1 expression was determined via flowcytometry at six time points (12, 18, 24, 36 and 48 h). Experimental data of 0 μM and 2.5 μM CHIR were later excluded from the mathematical modeling as these concentrations were not sufficient for mesendodermal induction and thus represent a rather undefined population.

For validation of the mathematical model, we used a previously collected data set for mesendodermal differentiation under four different combinations of CHIR concentration and medium volume on a 12-well platform. This data set consists of six time points measured with five independent replicates. Each {CHIR/Volume} combination leads to a distinct differentiation outcome, specifically: {7.5 μM; 1 ml} to definitive endoderm, {7.5 μM; 3 ml} as well as {15 μM; 1 ml} to cardiac mesoderm and {15 μM; 3 ml} to presomitic mesoderm.

For a detailed description of experimental conditions cf. supplementary information.

2.2. Derivation of model assumptions

To develop rational assumptions for a paracrine network involved in the process, four previously reported qualitative experimental observations were considered (Kempf et al., 2016):

a) Medium refreshment experiment (Fig. 1C): The potential role of secreted factors was addressed by studying the impact of a medium change on the differentiation outcome. Refreshing the medium 6 h post induction in definitive endoderm condition (DE; 7.5 μM CHIR/50 μl, blue colour code) shifts the differentiation towards cardiac mesoderm (CM) and subsequently cardiomyogenesis (green). This accelerated PS progression upon medium refreshment consequently suggests the presence of at least one inhibitory factor in the unaltered conditions, which accumulates at an early stage and delays anteroposterior PS progression. Based on this evidence for an early release of such an inhibitory factor, we assume the factor originating from hPSCs. We refer to this factor as *X*.
b) Conditioned medium experiment (Fig. 1D): Vice versa, we studied the effect of adding enriched medium. Conditioned supernatant of the DE conditions (blue) was harvested 6 h post induction and added to the medium in presomitic mesoderm condition (PSM; 15 μM CHIR/250 μl, red). This caused a delayed differentiation progression and resulted in a shift from presomitic to cardiac mesoderm and ultimately cardiomyogenesis. Repeating the equivalent procedure by harvesting the DE supernatants after 24 h did not change the phenotype of the cells, i.e. the cells remained in the PSM condition (red). Assuming a persisting activity of the inhibitory factor X, this observation suggests the presence of at least one activatory factor, that is secreted at a later stage during PS-progression and is likely originating from MIXL1$^+$ cells. This factor is referred to as *Y*.
c) Double knock-down experiment (Fig. 1E): When two TGFβ proteins, LEFTY1 and CER1, were knocked-down by about 80%, a doubling of the fraction of MIXL1$^+$ cells was observed after 24 h (from 30% to 60%). From this observation, we hypothesized the presence of a potential second inhibitor referred to as *Z* in the mathematical model.
d) Mass spectrometry (MS) analysis of the secretome after 6 h and 24 h in DE condition: This analysis revealed that LEFTY1 is present after 6 h, while CER1 was only detected after 24 h. From these observations, we hypothesize the biological equivalent of two of the three model factors, associating X to LEFTY1 (originating from hPSCs) and Z to CER1 (originating from MIXL1$^+$ cells due to its late occurrence). No evident activatory candidates (such as growth factors from the TGFβ and WNT superfamily) as positive modulator *Y* were identified in the MS data.

In addition, the model assumptions comprise the following simplifications:

- Cell proliferation, self-renewal and apoptosis are negligible within the observed time interval (0-48 h). The total cell number is thus assumed to be constant and was experimentally determined ($\approx 8 \times 10^4$ cells/well).
- CHIR concentration and medium volume are stable over time: previous MS analysis did not show any decay of CHIR in experimental settings over 48 h (Kempf et al., 2016).
- CHIR (indirectly) inhibits the production of *X* and Z as gene expression analysis showed a ~10-fold reduction in LEFTY1 and CER1 levels at 15 μM compared to 7.5 μM CHIR independent of the medium volume (Funa et al., 2015).
- Degradation processes of *X*, *Y* and *Z* are included in the model. The degradation parameters are estimated in the model and must be interpreted as not exclusively comprising the spontaneous decay process, but a complex behavior that includes the effect of the in-vitro cultivation.