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Studying Lineage Decision-Making In Vitro: Emerging Concepts and Novel Tools

Stefan Semrau¹ and Alexander van Oudenaarden^{2,3}

¹Leiden University, 2333 CC Leiden, The Netherlands; email: semrau@physics.leidenuniv.nl ²Hubrecht Institute, 3584 CT Utrecht, The Netherlands; email: a.vanoudenaarden@hubrecht.edu

³University Medical Center Utrecht, Cancer Genomics Netherlands, 3584 CG Utrecht, The Netherlands

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Abstract

Correct and timely lineage decisions are critical for normal embryonic development and homeostasis of adult tissues. Therefore, the search for fundamental principles that underlie lineage decision-making lies at the heart of developmental biology. Here, we review attempts to understand lineage decision-making as the interplay of single-cell heterogeneity and gene regulation. Fluctuations at the single-cell level are an important driving force behind cell-state transitions and the creation of cell-type diversity. Gene regulatory networks amplify such fluctuations and define stable cell types. They also mediate the influence of signaling inputs on the lineage decision. In this review, we focus on insights gleaned from in vitro differentiation of embryonic stem cells. We discuss emerging concepts, with an emphasis on transcriptional regulation, dynamical aspects of differentiation, and functional single-cell heterogeneity. We also highlight some novel tools to study lineage decision-making in vitro.

Contents

INTRODUCTION

Embryonic development and homeostasis of adult tissues rely on the ability of pluripotent stem cells to develop into the right cell type at the right time. This formidable task involves integrating many different signals and the robust, fine-tuned regulation of gene expression. Owing to limited accessibility, stem cells are difficult to study in vivo, at least in mammalian systems. Therefore, embryonic stem cells (ESCs) have been used extensively as a model system to study lineage decision-making. Despite the stability of the pluripotent state in vitro, differentiation is remarkably variable at the single-cell level. On the population level, however, differentiation is robust to fluctuations of the environment and leads to reproducible distributions of cell types. Embryonic development relies on these properties of differentiation to create cell-type diversity that is patterned in a reliable way. For in vitro differentiation, however, variability is a nuisance and limits the applicability of ESCs. Therefore, it is crucial to understand the process by which an individual cell adopts a particular fate: single-cell decision-making (Balázsi et al. 2011).

In the context of differentiation, we define decision-making as the interplay of two fundamental biological phenomena: single-cell heterogeneity and gene regulation (Enver et al. 2009). A population of genetically identical ESCs shows significant phenotypic variability, in particular with respect to lineage bias. However, in the absence of reinforcing mechanisms, fluctuations are only transient, and cells remain in a dynamic, yet stable, self-renewing equilibrium. Differentiation occurs when the pluripotent state becomes unstable in reaction to differentiation cues, such as activation of a particular signaling pathway. Consequently, an initial, stochastic lineage bias is amplified and the cell's expression profile changes until a new, stable expression state, i.e., a new cell type, is reached. Cell types are emergent properties of gene regulatory networks (GRNs). GRNs are composed of transcription factors, epigenetic marks, and other components of gene regulation, all of which modulate one another's expression and determine properties of a cell type.

Although a growing consensus about the central role of single-cell heterogeneity and GRNs in differentiation exists, many challenging questions concerning the underpinning molecular mechanisms still need to be answered. What are the sources of heterogeneity in ESCs, and what is their functional importance for decision-making? Which are the most important gene expression regulators involved in lineage decisions, and how are they wired together to constitute GRNs? How and when is the pluripotent state destabilized and substituted with a new stable state?



Figure 1

The different layers of gene regulation interact at enhancers and promoters. Master transcription factors (TFs), which are core regulators of a particular cell type, and signaling TFs, which are downstream of signaling pathways, cobind enhancers. DNA looping brings enhancers into physical proximity to the transcription initiation site. TFs also bind to promoters, which are regulatory elements that are typically proximal to the gene. Epigenetic marks, such as DNA methylation and histone modifications, modulate the activity of regulatory elements. The deposition of these marks is guided by TFs and long noncoding RNAs (lncRNAs). MicroRNAs (miRNAs) regulate gene expression post-transcriptionally by targeting messenger RNAs (mRNAs) for destabilization. Abbreviations: RISC, RNA-induced silencing complex; RNA pol, RNA polymerase.

In this review, we explore emerging concepts related to lineage decision-making, focusing largely on in vitro differentiation of ESCs. We give a brief overview of the different layers of gene regulation and how they interact to form GRNs, which control self-renewal and differentiation. We present studies on dynamical aspects of differentiation and discuss recent findings on functional single-cell heterogeneity. Finally, we highlight novel tools to study decision-making.

EMERGING CONCEPTS

The Layers of Gene Regulation

An overview of the various layers of gene regulation discussed below is shown in Figure 1.

Transcription factors. Transcription factors regulate gene expression by binding to regulatory genomic sequences and recruiting the transcription apparatus (Lee & Young 2013). Oct4, Nanog,

and Sox2 are master transcription factors of the pluripotent state in ESCs (Whyte et al. 2013, Young 2011). These transcription factors positively regulate other genes necessary for maintenance of the pluripotent state but repress lineage-specific factors to prevent differentiation. Importantly, it has been suggested that these factors also guide lineage decision-making: Subsequent to downregulation of Nanog, Oct4 and Sox2 are differentially regulated during a lineage decision between mesendoderm and neural ectoderm in mouse embryonic stem cells (mESCs) (Thomson et al. 2011). Oct4 and Sox2 are anticorrelated before the expression of specific lineage markers, which suggests causal involvement of the two transcription factors. A combination of chromatin immunoprecipitation (ChIP) measurements of Oct4 and Sox2 binding and perturbation of expression levels further suggested that Oct4 represses neural ectoderm, whereas Sox2 represses mesendoderm. These results have led to a model in which competition between differential regulation by external signals and mutual activation of the two factors explains the lineage decision. This and other studies suggest that, in mESCs, pluripotency factors double as lineage specifiers: Overexpression of individual factors leads to differentiation into particular lineages, and knockdown can impair differentiation. These observations have led to the idea that pluripotency is a metastable state that depends on the precise balance of pluripotency factors (Loh & Lim 2011). In this model, pluripotency factors must crossinhibit one another's lineage-specifying activities, which renders the pluripotent state intrinsically unstable. This model is plausible, as pluripotency is a very transient state in vivo; it is therefore unlikely that mechanisms to preserve its long-term stability would have evolved.

In marked contrast to mESCs, human embryonic stem cells (hESCs) generally do not differentiate upon overexpression of pluripotency factors, and only Oct4 and Nanog, but not Sox 2, are necessary for self-renewal (Wang et al. 2012). The difference between the roles of orthologous transcription factors in mESCs and hESCs is indicative of a more fundamental distinction between these cell types: They occupy different pluripotent states, which are termed naïve and primed, respectively (Nichols & Smith 2009) (see the sidebar Naïve Versus Primed Pluripotency). Whereas the transcription factors involved in naïve pluripotency have been studied extensively, much less is known about potential regulators of the totipotent subpopulations newly identified in mESCs (Macfarlan et al. 2012, Morgani & Brickman 2014, Morgani et al.

NAÎVE VERSUS PRIMED PLURIPOTENCY

Mouse embryonic stem cells (mESCs) are generally considered naïve because they readily differentiate into lineages from all germ layers and the germline and contribute to blastocyst chimeras. Because mESCs can be propagated when paracrine fibroblast growth factor (FGF) signaling is inhibited and in the absence of other added extrinsic instructive signals, it has been postulated that they possess an intrinsic self-renewal program: the ground state of pluripotency (Wray et al. 2010, Ying et al. 2008). It has been suggested that this program originates in the closest in vivo counterpart of mESCs, the preimplantation epiblast (Boroviak et al. 2014). Mouse epiblast stem cells (mEpiSCs) are derived from the postimplantation embryo. They do not contribute to blastocyst chimeras and they have variable differentiation bias (Brons et al. 2007, Nichols & Smith 2009, Tesar et al. 2007). They are therefore considered primed. Human embryonic stem cells (hESCs) cultured under conventional conditions, on feeders in serum and FGF2, are considered to be more similar to mouse mEpiSCs than to mESCs in terms of defining features such as expression profile and response to signaling factors (Vallier et al. 2009). Excitingly, several groups have recently reported culture conditions that support the propagation of naïve hESCs (Chan et al. 2013, Gafni et al. 2014, Ware et al. 2014).

2013). The transcription factors Rex1 (Guallar et al. 2012), Tbx3 (Dan et al. 2013), and Hex (Morgani et al. 2013) have been implicated. Notably, these transcription factors are also involved in extraembryonic endoderm specification.

Chromatin modifications and DNA methylation. The physicochemical state of chromatin forms another layer of gene regulation. Covalent histone modifications (Chen & Dent 2013) and DNA methylation (Schübeler 2015, Smith & Meissner 2013) are the best-studied chromatin features. Post-translational modifications of histones, such as acetylation or methylation, are reversible marks that indicate the activity status of regulatory regions. For example, histone H3 lysine 4 trimethylation (H3K4me3) indicates active chromatin, whereas histone H3 lysine 27 trimethylation (H3K27me3) is considered a repressive mark.

In general, ESCs have an open and dynamic chromatin state, with less tightly packed heterochromatin than do differentiated cells and large active chromatin domains characterized by histone acetylation, H3K4me3, and hypomethylation of DNA (Chen & Dent 2013). Bivalent domains, originally discovered in ESCs, are marked by both H3K4me3 and H3K27me3 and have been posited to silence lineage-specific genes while keeping them poised for activation (Bernstein et al. 2006, Mikkelsen et al. 2007). However, the importance and even existence of bivalent domains have been called into question by several recent observations (Chen & Dent 2013). Potentially, bivalent domains might simply be artifacts of the ChIP assay, which measures population averages. When hESCs are sorted based on the surface markers c-KIT and A2B5, several supposedly bivalent lineage-specific genes show only one histone mark in a sorted subpopulation (Hong et al. 2011).

Nevertheless, during differentiation, chromatin is remodeled on a global scale, which leads to extended heterochromatin. Obstruction of this remodeling process by knockout of chromatin modifiers results in specific differentiation phenotypes. For example, disruption of the polycomb group (PcG) repressive complexes in ESCs, which includes polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2), prevents the proper expression of lineage-specific genes (Surface et al. 2010). However, PcG proteins also help maintain the pluripotent state by repressing lineage-specific genes (Boyer et al. 2006).

Whereas histone modifications are thought to be highly dynamic, DNA methylation is traditionally considered relatively stable. Therefore, it supposedly functions as long-term epigenetic memory. In agreement with this notion, DNA methylation is functionally involved in stable epigenetic silencing (Schübeler 2015). However, several recent studies suggest that DNA methylation is more dynamic than previously thought (Shipony et al. 2014, Singer et al. 2014). Shipony et al. (2014) compared the distribution of methylation patterns in clones of hESCs and somatic cells with the respective polyclonal populations. In contrast to somatic cells, hESC clones show no persistence of rare methylation patterns, which argues against static maintenance of epigenetic memory in these cells. Rather, stable methylation patterns seem to be achieved by rapid, balanced methylation and demethylation. It will be interesting to see if fluctuating methylation patterns are correlated with heterogeneity in gene expression. DNA methylation levels in mESCs are also sensitive to culture conditions. When cultured in serum and leukemia inhibitory factor (LIF), mESCs resemble somatic cells in their globally high level of methylation (Ficz et al. 2013). Culture in media containing GSK and ERK inhibitors leads to a hypomethylated state, which approximates the methylation state of the inner cell mass (Habibi et al. 2013, Leitch et al. 2013). mESCs transition between the two distinct methylomes within a few cell cycles.

Noncoding RNAs. Over the past decade, it has become clear that noncoding, or regulatory, RNAs make a substantial contribution to the gene regulatory machinery (Morris & Mattick 2014).

MicroRNAs (miRNAs) are short RNAs (~22 nt) that decrease the stability of target messenger RNAs (mRNAs) and mediate gene repression (Guo et al. 2010). miRNAs target mRNAs through base pairing of the seed sequence (nucleotides 2–8 of the miRNA) with complementary sequences within the target mRNA, typically in the open reading frame and 3' untranslated region. Post-transcriptional regulation by miRNA is believed to reduce noise and increase robustness (Ebert & Sharp 2012). A recent quantitative study produced a more nuanced picture of the impact of miRNA regulation (Schmiedel et al. 2015). Schmiedel et al. (2015) showed that for genes with low expression, miRNA regulation does reduce gene expression and consequently intrinsic noise. For highly expressed genes, however, miRNA regulation acts as an additional extrinsic noise source, as miRNA expression is, itself, fluctuating. This effect disappears when an mRNA is targeted by several independently fluctuating miRNAs.

During differentiation of mESCs, miRNAs are necessary for silencing the self-renewal program and proper expression of lineage-specific genes (Wang et al. 2007). A detailed study has shown that distinct miRNA families can have opposing effects on self-renewal (Melton et al. 2010). When introduced in miRNA-depleted mESCs, *let-7* miRNAs enable silencing of self-renewal. A different miRNA family, ESC cell cycle regulating (ESCC) miRNAs, can block this effect. miRNAs also regulate epigenetic modifications, albeit indirectly: Kumar et al. (2014) observed that the expression of DNA methyltransferases was reduced in mESCs with impaired expression of mature miRNAs. In fact, the mESC-specific *mir-290* cluster of miRNAs controls DNA methyltransferase expression, as was shown earlier by Sinkkonen et al. (2008). These observations link miRNA regulatory activity directly to DNA methylation.

Another important class of noncoding RNAs is formed by long noncoding RNAs (lncRNAs), which are distinct from miRNA in size (>200 nt) and mechanism of action (Quinodoz & Guttman 2014). lncRNAs function as scaffolds for chromatin-modifying protein complexes, similar to telomerase RNA. In this way, they can fulfill a gene regulatory role in development and differentiation (Fatica & Bozzoni 2014). A large-scale knockdown experiment in mESCs showed that lncRNAs are important for pluripotency maintenance and repress early lineage-specific expression programs (Guttman et al. 2011). During differentiation, most lncRNAs expressed in mESCs are downregulated, corresponding with their function in pluripotency. By contrast, some lncRNAs are upregulated, suggesting a role in lineage specification (Klattenhoff et al. 2013). A 590-nt lncRNA dubbed Braveheart was indeed found to be necessary for the differentiation of mESCs to the cardiovascular lineage (Klattenhoff et al. 2013). Braveheart likely exerts its function through interaction with PRC2, which deposits the repressive H3K27me3 mark. lncRNAs also play an important role in hematopoiesis. A systematic search by Alvarez-Dominguez et al. (2014) identified hundreds of lncRNAs, many of them previously unannotated, which are specifically expressed in the erythroid lineage. In this study, all candidate lncRNAs predicted to be regulators of development were confirmed to be necessary for proper erythroblast maturation. These results, paired with the high tissue specificity of lncRNA expression, implicate lncRNAs as important regulators in many developmental systems.

Signaling pathways. Several signaling pathways have been implicated in the maintenance of pluripotency (Jaenisch & Young 2008, Pera & Tam 2010, Ye et al. 2014). In mESCs, autocrine FGF signaling destabilizes the pluripotent state (Kunath et al. 2007). The ERK pathway, which is activated by FGF, is specifically involved in primitive endoderm differentiation but is dispensable for the induction of neural lineages (Hamilton & Brickman 2014). Conversely, inhibition of the ERK pathway leads to an epigenetic ground state, which is characterized by global hypomethylation (Ficz et al. 2013).

The ground-state model argues that pluripotency is stable without exogenous cytokines (Ying et al. 2008), whereas the alternative model, discussed above, claims that persistent signaling is necessary to preserve a delicate balance of pluripotency factors, which also act as lineage specifiers (Loh & Lim 2011). In support of the importance of signaling, it was found that Wnt signaling is necessary to prevent differentiation of mESCs into epiblast stem cells (ten Berge et al. 2011). Typical ground-state culture conditions, in fact, contain a GSK-3 inhibitor, which activates Wnt signaling.

As exemplified by the ERK pathway, signaling is of paramount importance for differentiation. In fact, most directed differentiation protocols developed to date rely on the timed activation of signaling pathways to achieve a particular lineage outcome (Cohen & Melton 2011). Protocols typically follow the course of signaling events observed in vivo (Murry & Keller 2008). In line with the phenotypic similarity between mouse epiblast stem cells (mEpiSCs) and hESCs, similar pathways can be used to elicit similar differentiation outcomes in these two cell types (Vallier et al. 2009). For example, exposure to FGF results in neuroectoderm, whereas exposure to BMP4 induces extraembryonic lineages. Notwithstanding the remarkable success of using signaling to direct cell fate, a quantitative understanding of signaling pathways in differentiation is still largely missing. This is partially because the growth factors, cytokines, and small molecules used in differentiation typically activate multiple pathways, which can have substantial crosstalk. For example, retinoic acid (RA), which is used in many in vitro differentiation protocols and has important functions in development in vivo (Rhinn & Dolle 2012), modulates the expression of hundreds of genes (Mendoza-Parra et al. 2011). The many binding sites of its cognate receptors greatly overlap with the binding sites of core pluripotency markers (Mahony et al. 2011). In particular, RA directly represses Oct4 (Pikarsky et al. 1994), which may explain the rapid induction of ESC differentiation upon RA exposure. Further, RA modulates FGF (Stavridis et al. 2010) and Nodal (Engberg et al. 2010) signaling during in vitro differentiation and, given the promiscuous binding of its receptors, likely influences many other signaling pathways. Tellingly, RA induces neural fates (Kim et al. 2009) as well as primitive endoderm (Capo-Chichi et al. 2005) in embryoid body assays.

Given their ease of use, small molecules like RA, growth factors, and cytokines will continue to play an important role in directed differentiation. However, we need a better quantitative understanding of the signaling pathways they affect to develop more precise and efficient differentiation protocols.

Regulatory genomic sequences: promoters and enhancers. The various layers of gene regulation discussed so far converge and interact on genomic regulatory sequences, the most important of which are promoters and enhancers.

Transcription factors bind both to promoters, gene-proximal DNA elements in which transcription is initiated, and to enhancers, elements that can be hundreds of kilobases away from their target genes (Young 2011). Most gene regulation relevant to development is believed to occur at enhancers. These distal elements, which are typically a few hundred base pairs in length, are bound by multiple transcription factors and thus form platforms for combinatorial gene regulation.

Master transcription factors determine cell-type-specific effects of signaling pathways by cobinding with signaling transcription factors at enhancers (Mullen et al. 2011). In mESCs, the enhancers of many pluripotency genes are co-occupied and coactivated by the core pluripotency factors, Sox2, Oct4, and Nanog, as well as transcription factors that relay LIF, BMP, and Wnt signaling (Young 2011). Interestingly, some enhancers are arranged in large clusters called super-enhancers (Hnisz et al. 2013, Whyte et al. 2013). Super-enhancers drive high expression of key regulators of cell identity. Consequently, they control many regulators of pluripotency in mESCs.

Notwithstanding the dominant role of transcription factors in gene regulation, the epigenetic landscape at enhancers has much more than just a stabilizing function. A condensed chromatin structure can impede transcription factor binding, and epigenetic marks are believed to be prepatterned before the lineage decision and lineage-specific expression occur (Chen & Dent 2013). Some of this prepatterning is executed by pioneer factors. These factors can bind to condensed chromatin and render it competent for the binding of other factors (Magnani et al. 2011, Zaret & Carroll 2011). In particular, pioneer factors contribute to enhancer bookmarking, i.e. the deposition of epigenetic marks that determine the activity of an enhancer. Whereas pioneer factors are typically unable to activate transcription on their own, some transcription factors, such as Sox2, Oct4, or the GATA factors, also have pioneer activity.

The few examples discussed here can only hint at the many ways in which the different layers of gene regulation interact at genomic regulatory sequences. Untangling the crosstalk between different epigenetic marks and identifying the molecular drivers of chromatin remodeling and gene regulation remain formidable tasks for the future (Calo & Wysocka 2013). Another important layer of gene regulation is the topological organization of the genome in three-dimensional (3D) space. For example, enhancers are believed to exert long-range functions via direct physical interaction with their targets, which results in looping of the intervening DNA. A detailed discussion of this important field is beyond the scope of this review. We refer the interested reader to excellent recent reviews by Cavalli & Misteli (2013) and Gorkin et al. (2014).

Gene Regulatory Networks and Dynamics of Differentiation

As indicated in the previous section, much work has focused on individual molecular factors involved in gene regulation. However, a great challenge still lies ahead: the integration of molecular factors into comprehensive GRN models. Such models will allow us to make quantitative predictions, in particular about differentiation dynamics (see the sidebar Gene Regulatory Networks in Lineage Decision-Making and **Figure 2**).

Gene regulatory networks. A small network of transcription factors, the core pluripotency network, is believed to control the pluripotent state in ESCs (Jaenisch & Young 2008, MacArthur et al. 2009). A study in hESCs by Boyer et al. (2005) measured genome-wide binding patterns of the core pluripotency factors Nanog, Sox2, and Oct4 using ChIP. In hESCs, the three core factors targeted one another as well as many other transcription factors with roles in both self-renewal and differentiation. These results suggested a highly interconnected regulatory network, but systematic knockout studies have shown that the three core factors are more independent than initially thought (Wang et al. 2012). Whereas Nanog and Oct4 are coregulated, and downregulation of one factor affects the other, Sox2 is not affected by downregulation of the other core factors and can be manipulated independently. Also, most of the direct targets identified earlier (Boyer et al. 2005) are unaffected in core pluripotency factor knockouts, and most of the observed expression changes are related to indirect targets. An equivalent core pluripotency network has been discovered in mESCs (Kim et al. 2008, Loh et al. 2006). In contrast to hESCs, there seems to be significant overlap in the targets of individual core pluripotency factors (Ivanova et al. 2006). The differences in the core pluripotency networks in mESCs and hESCs show that ChIP assays, which only quantify transcription factor binding, must be complemented with functional assays to discover meaningful GRNs. In a recent study in mESCs by Dunn et al. (2014), effective interactions between pairs of transcription factors were derived from correlations between their expression (Dunn et al. 2014). A vast number of possible networks can be constructed from combinations of these effective interactions. Dunn et al. used a range of perturbations as constraints to identify a

GENE REGULATORY NETWORKS IN LINEAGE DECISION-MAKING

Gene regulatory networks (GRNs) are conceptually important because they formalize Waddington's [2014 (1957)] idea of an epigenetic landscape. Different cell types can be understood as attractor states of complex GRNs (Huang et al. 2005) (see **Figure 2***a*). In this framework, differentiation is the noise-driven choice between alternative attractors. Attractors can be visualized as local minima in a quasipotential landscape defined by the GRN. Finite potential barriers between attractors model the irreversible nature of physiological transitions between cell states (e.g., from the pluripotent state to a particular lineage) (Wang et al. 2010). Given that pluripotency can be represented by a single attractor, embryonic stem cells (ESCs) dynamically explore a region close to this attractor, known as the basin of attraction, with dynamics driven by the various sources of expression heterogeneity discussed below. It has therefore been suggested that the pluripotent state should not be considered a property of a single cell, but rather of the population, and that concepts and methods from statistical mechanics are applicable (MacArthur & Lemischka 2013).

Networks have been studied extensively as abstract objects independent of a particular interpretation of their nodes and edges. Results of these studies are valuable for understanding GRNs. The existing work is concentrated in three areas (Huang & Kauffman 2012): (*a*) network inference; (*b*) analysis of general network properties, in particular topology; and (*c*) network dynamics. The vast body of work on network topology contains many results that are particularly valuable for gene regulation. Notable examples are the scale-free topology of certain growing random networks (Barabási & Albert 1999, Barabási & Oltvai 2004), which are often found in biological contexts, and the discovery of network motifs (Alon 2007, Milo 2002), which are local structures that appear more frequently in real biological networks than in randomized networks. These motifs embody particular functions such as noise filters or pulse generators.

Finally, the modeling of network dynamics is of great importance to lineage decision-making (Huang & Kauffman 2012). As a result of the absence of genome-wide data on the strength of interactions between genes, dynamics have been studied only in small, isolated subnetworks essential for biological functions or effective networks capturing the qualitative behavior of a system. The study of such small networks can be surprisingly insightful. For example, Huang et al. (2007) analyzed the differentiation of a multipotent hematopoietic progenitor cell line, which can give rise to erythroid or myeloid cells. This binary cell-lineage decision is governed by the transcription factors GATA-1 and PU.1. A simple set of ordinary differential equations (ODEs) describing mutual inhibition and autoactivation of these two factors (see **Figure** *2b*) was able to capture essential features of the differentiation process and even quantitative properties of genome-wide expression measurements. **Figure** *2c,d* shows trajectories in gene expression space resulting from these ODEs for two different sets of parameters. The chosen parameters lead to either three or two stable states, modeling the transition from a progenitor state to one of two alternative cell lineages.

set of minimal models. Surprisingly, a network with as few as 3 inputs, 12 components, and 16 interactions (see **Figure 3**) could satisfy the constraints and have significant predictive power.

Another approach to reduce the complexity of the gene regulatory circuitry may involve the recently discovered super-enhancers (Whyte et al. 2013). Taking into account only the activity of a few hundred super-enhancers instead of many thousands of conventional enhancers would significantly reduce the complexity of models for the gene regulatory circuitry. Importantly, the transcription factors forming the core pluripotency network regulate not only each other but also other species of regulatory molecules. For example, Oct4, Sox2, and Nanog regulate large numbers of lncRNAs (Guttman et al. 2011) and miRNAs (Marson et al. 2008), which make these regulatory RNAs an integral part of the GRN in ESCs. miRNAs are involved in recurrent network motifs (Tsang et al. 2010) in various cell types, and ESCs are no exception (Marson et al. 2008).



Figure 2

Gene regulatory networks (GRNs) govern lineage decisions. (a) Waddington's [2014 (1957)] epigenetic landscape can be represented by a potential that changes shape over time during development or differentiation. The shape of this potential is defined by the GRN governing the lineage decision. In this picture, a cell is a point moving in the potential. The cell's movement is partially deterministic, driven by the potential force, and partially stochastic, driven by gene expression noise. In the pluripotent state, the potential has one global minimum, or attractor, and a stem cell (black dot) is close to that minimum in expression space. During differentiation, the initial attractor becomes unstable, and new local minima, or attractors, appear (blue and orange dots). These attractors represent alternative cell lineages. As a result of expression fluctuations, a cell can initially be close to a particular attractor and therefore biased toward the corresponding cell lineage. Which attractor the cell ends up in depends on the relative height of the minima, as well as gene expression noise along the way. It is important to note that some GRNs result in circular paths through expression space and therefore cannot be represented by a potential. (b) This scheme depicts a minimalistic GRN, which can give rise to multiple stable states. It is composed of the mutual inhibition (red *inhibition lines*) and autoactivation (green arrows) of two gene expression profiles, x_A (blue) and x_B (orange). The dynamics of this GRN are governed by a set of simple ordinary differential equations (ODEs): $\frac{dx_A}{dt} = p_A \frac{x_A^n}{S^n + x_A^n} + q_A \frac{S^n}{S^n + x_B^n} - k_A x_A \text{ and } \frac{dx_B}{dt} = p_B \frac{x_B^n}{S^n + x_B^n} + q_B \frac{S^n}{S^n + x_A^n} - k_B x_B.$ The first term in each ODE models autoactivation, which sets in beyond a threshold expression Sⁿ. The second term models mutual inhibition, again with a threshold S^n . The third term models degradation. Parameters p and q set the strengths of mutual inhibition and autoactivation. (c,d) Numerical solutions of the ODEs described in panel b are shown. Trajectories start from a grid of points in gene expression space, which is spanned by gene expression profiles x_A and x_B . For both panels c and d, $q_A = q_B = k_A = k_B = 1$, S = 0.5, and n = 4. In panel c, $p_A = p_B = 1.2$, and trajectories end up in one of three attractors, known as tristability. The progenitor state P has intermediate levels of both x_A and x_B . The two other cell fates, A and B, have high expression of x_A and x_B , respectively. In panel d, $p_A = p_B = 0.6$, and there are only two attractors; the progenitor attractor has disappeared. A differentiation process can be modeled as a down-sweep of parameters p_A and p_B . A cell initially in the progenitor state moves to either attractor A or B, depending on expression noise once the progenitor state becomes unstable.

It is important to realize that intracellular GRNs do not function in isolation but receive important inputs from signaling pathways. If taken to the extreme, external signals are even able to overrule a weak intracellular GRN, as has been demonstrated for hematopoietic progenitors (Fang et al. 2013). Even if the intracellular GRN is wired to result in mutually exclusive expression of antagonistic transcription factors, strong cytokine signaling leads to their high and



Figure 3

A minimal network for controlling pluripotency identified by Dunn et al. (2014). Transcription factors are shown as white nodes, signaling pathways as a blue node, and inputs as gray nodes. The inputs consist of the GSK-3 inhibitor CHIR99021 (CH), the MEK inhibitor PD0325901 (PD), and the cytokine leukemia inhibitory factor (LIF), which together establish ground-state conditions. The output of the network is the transcriptional profile of naïve pluripotency. Possible functional interactions were determined from the Pearson correlations between gene expression of any two factors. Gene expression was measured over a range of perturbations, such as omission of one of the inhibitors. The shown activation (*green*) and inhibition (*red*) interactions are therefore functional but not necessarily direct. The depicted network was the simplest network satisfying all experimental constraints.

ubiquitous expression. Similarly, the state of the pluripotency network in ESCs is modulated by several signaling pathways (Chen et al. 2008). For example, LIF, a cytokine that is crucial in conventional mESC culture, activates multiple signaling pathways targeting distinct members of the pluripotency network (Niwa et al. 2009). Paracrine signaling between cells effectively extends GRNs beyond single cells. Therefore, ligand-receptor interactions between different cell types must be taken into consideration for intercellular networks. Qiao et al. (2014) analyzed such interactions between various progenitor and mature cell types of the hematopoietic system. They found that cell types cluster by expression of ligands and effects of those ligands on the lineage decision of hematopoietic stem cells (HSCs). The cell-cell communication network developed for their in vitro culture system suggests that lineage decision-making in HSCs can be regulated by accessibility to ligands. Ligand concentrations are modulated in vivo by cell-type frequency or spatial segregation. For ESCs, a recent model even proposed cell-cell interactions as the driving force behind differentiation (Furusawa & Kaneko 2012, Suzuki et al. 2011). This model reconciles self-renewal and multilineage potential through oscillatory gene expression.

Differentiation dynamics. How the structure of relevant GRNs changes dynamically during exit from pluripotency and lineage decisions is still an open question and an active area of research. In

this respect, looking at snapshots of differentiation in pluripotent cells or fully differentiated cells in isolation is of limited use. Several recent studies have tracked dynamic changes in transcriptional profile, protein levels, epigenetic state, and transcription factor binding during the process of differentiation (Gifford et al. 2013, Lu et al. 2009, Tsankov et al. 2015, Xie et al. 2013). These measurements have led to the discovery of general principles in the interplay of epigenetic marks, transcription factor binding, and cell-lineage decisions (Chen & Dent 2013). For example, a study by Tsankov et al. (2015) reaffirmed the notion that transcription factors play a central role in differentiation: Targets of lineage-specific factors lose DNA methylation upon differentiation, and key regulators of particular lineages operate at super-enhancers. Ziller et al. (2015) found that transcription factors can prime the epigenetic landscape at early stages of differentiation for the subsequent activation of genes in more mature cell types.

A particularly interesting early time point during differentiation is the exit from pluripotency (Kalkan & Smith 2014). How the pluripotency network is disassembled in a controlled and robust way is not well understood. Studies focusing on the pluripotency factor Oct4 have identified a multistep process for its silencing during differentiation. Upon exposure to RA, Oct4 is repressed directly through the binding of RA receptors and other repressive factors (Pikarsky et al. 1994). Then the histone lysine methyltransferase G9a mediates methylation of histone H3 lysine 9 (H3K9), which is necessary for the subsequent DNA methylation of the Oct4 promoter, resulting in stable silencing (Feldman et al. 2006). In addition to direct repression by specific factors and epigenetic silencing, other mechanisms, including the modulation of mRNA stability and the subcellular localization of transcription factors, have been implied in the exit from pluripotency (Kalkan & Smith 2014). Genome-wide knockdown screens have identified hundreds of molecules that affect the exit from pluripotency (Betschinger et al. 2013, Leeb et al. 2014, Yang et al. 2012). For example, these screens all identified the repressive factor Tcf3, a terminal component of the Wnt pathway, as a crucial factor, and one study produced several hits in the FGF/ERK signaling pathway (Leeb et al. 2014). In line with these findings, several studies report that the sensitivity of mESCs to the activation of canonical signaling pathways is modulated in a dynamical way around the exit of pluripotency (Jackson et al. 2010, Thomson et al. 2011, Trott & Martinez Arias 2013, Turner et al. 2014). Interestingly, mESCs seem to gain competence to react to certain lineagespecifying signals only gradually, and competence is contingent on the downregulation of naïve pluripotency factors. It is tempting to speculate that this initial refractory period is necessary to remodel the epigenetic landscape and render lineage-specific enhancers competent for activation (Buecker et al. 2014, Kalkan & Smith 2014, Yang et al. 2014).

Single-Cell Heterogeneity

Most of the studies discussed so far consider ESCs to be a homogeneous population of cells, disregarding any single-cell heterogeneity. However, such heterogeneity manifests itself in all aspects of differentiation, including the response to small molecule inducers, lineage bias, and dynamics of the exit from pluripotency.

Recent surveys of single-cell gene expression in ESCs have consistently found substantial variability (Abranches et al. 2014, Grün et al. 2014, Kumar et al. 2014, Martinez Arias & Brickman 2011, Singer et al. 2014). Although such variability has previously been connected to priming toward particular fates (Canham et al. 2010, Chang et al. 2008, MacArthur & Lemischka 2013), doubt has been cast on the function or even existence of expression fluctuations (Faddah et al. 2013, Kalkan & Smith 2014, Pina et al. 2012, Smith 2013). Here we discuss common nongenetic sources of expression heterogeneity (see the sidebar Common Nongenetic Sources of Expression Heterogeneity and Figure 4) and review evidence of its functional importance.

COMMON NONGENETIC SOURCES OF EXPRESSION HETEROGENEITY

Gene expression is inherently stochastic (Elowitz 2002, Golding et al. 2005, Raj & van Oudenaarden 2008, Raj et al. 2006). Even in constant external conditions, genes randomly switch between inactive and active states, which leads to bursts of transcription. This intrinsic noise is a property of individual genes, and the parameters of the stochastic process, such as burst frequency and size, determine the level of gene activation. By definition, intrinsic noise is uncorrelated across genes or different alleles of the same gene. Extrinsic noise, by contrast, causes gene expression variability that is correlated across multiple genes (Elowitz 2002). An example of a source of extrinsic noise is a variably expressed transcription factor with multiple target genes.

Although the term noise typically suggests an underlying stochastic process, it is important to realize that deterministic origins can make substantial contributions to single-cell heterogeneity (Snijder & Pelkmans 2011). Population context, for example, influences important phenotypic characteristics (Snijder et al. 2009). Therefore, we treat stochastic and deterministic heterogeneity on equal footing, and we expect that some of the processes considered stochastic today will turn out to have deterministic underpinnings.

A fundamental process common to all actively dividing cells is the cell-division cycle, which contributes multiple sources of variability. Many genes are regulated in a cell cycle–dependent manner (Whitfield et al. 2002). Importantly, not only average gene expression levels but also transcriptional bursting frequency, and therefore gene expression noise, can depend on the cell cycle (Zopf et al. 2013): Transcriptional activity at low expression levels of a synthetic promoter was found to be restricted to the G2 phase, an effect likely related to temporary changes in chromatin state during DNA replication. Even cell division itself, despite being a highly regulated process, introduces variability. Uneven partitioning of mRNA and proteins between daughter cells randomizes molecular abundance (Golding et al. 2005, Huh & Paulsson 2011), an effect that is relatively more important for lowly expressed genes.

Heterogeneity in epigenetic marks is likely another source of variability. Singer et al. (2014) recently showed a correlation between promoter methylation and dynamic gene expression heterogeneity in single cells.

Finally, dynamic, random monoallelic expression seems to be abundant in preimplantation embryos (Deng et al. 2014), making it another likely contributor to expression heterogeneity. However, care must be taken to discriminate dynamic transcriptional bursting, which occurs randomly at both alleles, from bona fide, mitotically stable monoallelic expression (Eckersley-Maslin & Spector 2014).

Given the close relationship between gene expression and the cell cycle, it is not surprising that the differentiation propensity of stem cells depends on cell cycle phase. The G1 phase is a general window of opportunity for differentiation in embryonic carcinoma cells (Mummery et al. 1987), and recent studies have found the same in ESCs (Coronado et al. 2013, Singh et al. 2013). Importantly, the cell cycle regulates more than just the general sensitivity of ESCs to fate-specifying signals. Pauklin & Vallier (2013) have shown that the propensity of hESCs to develop into particular lineages depends on cell cycle phase. Their study and related work in mESCs (Li & Kirschner 2014) have shown that the cell cycle can be manipulated to direct ESCs to particular fates.

Cell cycle–regulated genes are not the only genes that show periodic expression fluctuations, and a recently proposed framework for understanding pluripotency has periodicity at its core (Furusawa & Kaneko 2012). Suzuki et al. (2011) have proposed that oscillatory gene expression is an intrinsic property of stem cells that reconciles a robust self-renewing state with the ability to create cell-type diversity. Experimental validation of this notion was provided by a study of neural progenitor cells (Imayoshi et al. 2013): The key transcription factors Hes1, Ascl1, and Olig2 oscillate on a timescale of hours in the multipotent state. Cells with different levels of these three factors show different propensities to develop into astrocytes, neurons, or oligodendrocytes, respectively. During differentiation, expression oscillations are supplanted by sustained expression



Figure 4

Common nongenetic sources of expression heterogeneity comprise intrinsic factors (e.g., transcriptional bursting, random monoallelic expression, and possibly epigenetic heterogeneity) and extrinsic factors (e.g., cell cycle, cell size or metabolic state, and spatial population context). Abbreviation: RNA pol, RNA polymerase.

of one of the key transcription factors, depending on the particular lineage. Remarkably, Hes1 also oscillates in mESCs (Kobayashi et al. 2009). Depending on Hes1 expression level, mESCs are biased toward the neural or mesodermal lineages. With this notable exception, most of the reported cell cycle–independent heterogeneity in ESCs has been deemed stochastic. However, one must keep in mind that to date there have been no attempts to systematically identify oscillatory expression in ESCs. Because oscillations have the potential to confer robustness at the population level (Furusawa & Kaneko 2012, Paszek et al. 2010), we might expect to discover more genes with oscillatory behavior in the future.

A classic example of functional, stochastic expression heterogeneity was discovered in a hematopoietic progenitor cell line. Chang et al. (2008) showed that the surface antigen Sca-1 is heterogeneously expressed and that significant genome-wide transcriptional differences exist between cells with high and low Sca-1 levels. Cells sorted on the extremes of Sca-1 expression could reconstitute the complete distribution and, importantly, have different propensities to develop into the erythroid or myeloid lineages. This study suggested that self-renewing stem or progenitor cells stochastically and reversibly explore lineage options, which are executed if the proper differentiation cues are present. However, a follow-up study determined that culture-reconstitution ability and lineage bias are, in fact, not present in one and the same cell. Pina et al. (2012) showed that cells with low levels of Sca-1 could be further divided into a CD34 positive, multipotent subpopulation that is able to reconstitute the parental distribution and a CD34 negative, erythroid-biased subpopulation without reconstitution ability.

Similar functional stochastic heterogeneity has been reported in mESCs using fluorescent reporters for the pluripotency markers Rex1 (Toyooka et al. 2008, Wray et al. 2010), Stella

(Hayashi et al. 2008), and Nanog (Chambers et al. 2007, Filipczyk et al. 2013, Kalmar et al. 2009, Singh et al. 2007). The extensive amount of work on Nanog heterogeneity was recently reviewed in detail by Torres-Padilla & Chambers (2014). Intriguingly, it has been proposed that dynamically regulated, monoallelic expression contributes substantially to Nanog's heterogeneity (Miyanari & Torres-Padilla 2012). Recent studies have found equal amounts of Nanog protein (Faddah et al. 2013, Filipczyk et al. 2013) and mature mRNA (Hansen & van Oudenaarden 2013) from both alleles in most cells. However, a small fraction of cells show monoallelic expression to an extent that cannot be accounted for by assuming bursty transcription (Hansen & van Oudenaarden 2013). Notwithstanding potential caveats related to fluorescent Nanog reporters (Faddah et al. 2013), the general consensus is that Nanog heterogeneity is functional: Cells expressing lower amounts of Nanog in self-renewing conditions are prone to differentiation, albeit not primed for a particular lineage, whereas higher Nanog expression goes along with increased clonogenic potential (Abranches et al. 2014, Chambers et al. 2007, Faddah et al. 2013). Whereas no difference in potency between cells with high and low Nanog levels has been reported, high expression of the endoderm marker Hex in mESCs indicates a subset of totipotent cells (Morgani et al. 2013). When injected into preimplantation embryos, mESCs contribute readily to all lineages of the embryo proper but only rarely to extraembryonic lineages (Beddington & Robertson 1989). Cells with high Hex levels, however, are primed for trophoblast and extraembryonic lineages but are still able to contribute to embryonic lineages. Another distinct subset of mESCs with increased potency was discovered by Macfarlan et al. (2012). Interestingly, those cells were identified by the activity of an endogenous retrovirus, muERV-L, whose in vivo expression is restricted to the two-cell (2C) stage.

Similar to mESCs, hESCs exhibit a considerable amount of gene expression heterogeneity and, correspondingly, varying amounts of self-renewal capacity and lineage priming (Hough et al. 2014). Notably, expression of a primate-specific endogenous retrovirus, HERVH, identified a subset of hESCs resembling the naïve state of mESCs (Wang et al. 2014). As exemplified by the studies discussed above, no culture condition developed to date is able to capture a particular developmental state in a perfectly homogeneous way. Despite the prevailing notion that mESCs are similar to cells in the preimplantation epiblast (Boroviak et al. 2014), subpopulations that exhibit totipotent fate potential extending to extraembryonic lineages exist, even when cultured in ground-state conditions (Macfarlan et al. 2012, Morgani & Brickman 2014, Morgani et al. 2013). Compared to culture in serum and LIF, ground-state conditions lead to reduced expression variability for many genes (Grün et al. 2014, Kumar et al. 2014, Singer et al. 2014, Wray et al. 2010). A recent study by Singer et al. (2014) suggests that the global DNA hypomethylation observed in ground-state conditions (Ficz et al. 2013, Habibi et al. 2013, Leitch et al. 2013) is responsible for this increase in homogeneity. When grown in serum and LIF, mESCs exist in two distinct states. These two states differ by the methylation states of many promoters, including promoters of genes encoding regulators of pluripotency. Infrequent switching between the two states, in combination with transcriptional bursting on shorter timescales, explains much of the observed expression variability. Methylation levels are correlated across states for a large subset of genes; this phenomenon is reflected by highly correlated expression fluctuations. Culture in ground-state conditions, however, reduces occupancy of the high-methylation state, which results in a switch from bimodal to unimodal expression of many measured genes (Singer et al. 2014). Similarly, a study by Singh et al. (2013) has linked fluctuating levels of 5-hydroxymethylcytosine (5hmC) to heterogeneous expression of lineage-specific factors in hESCs. Interestingly, both methylation and expression fluctuations are cell cycle dependent and peak in late G1. It will be interesting to see whether the expression and functional heterogeneity observed in hESCs cultured under conventional conditions (Hough et al. 2014) are reduced in naïve hESCs, similar to mESCs in the ground state. A phenotype resembling ground-state conditions has been found in mESCs deficient for mature miRNAs (Kumar et al. 2014). In contrast to ground-state conditions, though, expression heterogeneity is increased in these cells. This effect is consistent with the notion that miRNAs buffer expression variability (Ebert & Sharp 2012).

It is important to note that single-cell heterogeneity in gene expression and that in gene regulation are by no means independent or separable processes. Whereas GRNs shape the correlations between fluctuations of multiple genes (Pedraza 2005), stochastic noise itself can lead to bimodal expression patterns in the absence of deterministic bistability (To & Maheshri 2010). From a practical point of view, the analysis of expression variability is a powerful way to unravel mechanisms of gene regulation (Munsky et al. 2012). Stewart-Ornstein et al. (2012) have exploited correlations between expression fluctuations to identify noise regulons (Junker & van Oudenaarden 2012), groups of genes covarying under steady-state conditions. Genes belonging to a noise regulon were also found to be functionally related. Strategies to infer gene regulation from (co)fluctuation of expression have not been applied extensively to ESCs, despite their great potential to unravel the rewiring of GRNs in the differentiation process.

NOVEL TOOLS

A typical differentiation experiment with ESCs consists of these basic components: (*a*) culture of ESCs, (*b*) induction of differentiation, (*c*) measurement of differentiation dynamics, and (*d*) data analysis. Here we highlight a few recently developed tools related to these basic components.

Culture of Embryonic Stem Cells

Culture conditions can have a profound impact on expression heterogeneity and therefore lineage decision-making. Several recent studies have focused on the difference between mESCs grown in serum and LIF versus ground-state conditions (Grün et al. 2014, Kumar et al. 2014, Singer et al. 2014). Gene expression is consistently more homogeneous in ground-state conditions. However, even in a population of mESCs cultured in ground-state conditions, functionally different sub-populations can be found (Macfarlan et al. 2012, Morgani et al. 2013). Therefore, it remains to be tested whether mESCs in ground-state conditions respond to differentiation-inducing signals in a homogeneous manner. Recently developed culture conditions for naïve hESCs (Chan et al. 2013, Gafni et al. 2013, Theunissen et al. 2014, Ware et al. 2014). However, the conditions for the culture of naïve hESCs developed to date vary significantly between laboratories. Consequently, so do expression profiles and functional abilities of naïve hESCs.

Although there have been no large-scale measurements of gene expression on the single-cell level, reduced variability is expected in naïve hESCs compared with their primed counterparts. Indeed, a single-molecule fluorescence in situ hybridization (FISH) measurement of Nanog expression suggests such a trend (Theunissen et al. 2014). Correspondingly, differentiation of naïve hESCs should occur in a less biased and more homogeneous manner. The potentially improved homogeneity and the possibility to create human-mouse chimeras with naïve hESCs will be beneficial for future research and applications.

Induction of Differentiation

Induction of differentiation can be as simple as removing certain factors from ESC growth medium, such as LIF, when cells are cultured in serum/LIF, or LIF and GSK/MEK inhibitors, when cells

are cultured in ground-state conditions. Lineage decision-making can be directed to particular lineages by coculturing with other cell types, adding cytokines or other signaling molecules, or inhibiting particular signaling pathways (Cohen & Melton 2011, Williams et al. 2012). Whereas most existing protocols are strongly informed by in vivo development (Murry & Keller 2008), recent efforts have attempted to optimize protocols in a systematic way. Promising forays in that direction include (*a*) high-throughput screening of the cell line–specific response to signaling molecules in hESCs while controlling colony size (Nazareth et al. 2013) and (*b*) the systematic establishment of a signaling logic (Loh et al. 2014). In particular, Loh et al. (2014) have shown that sensitivity to signaling molecules is highly dynamic. Importantly, lineage decisions can be directed most efficiently at bifurcation points through simultaneous induction of one particular lineage while inhibiting alternative fates (Loh et al. 2014).

The use of signaling molecules to direct differentiation is advantageous because they are easily applied and induce endogenous signaling cascades. However, the level of fine control over gene expression is limited, as a particular pathway typically has many downstream targets. A recently developed mRNA transfection technique promises significant improvement in that respect. Warren et al. (2010) have used synthetic mRNA to evade the innate immune response and ensure mRNA stability. By transfecting mRNA coding for the transcription factor MyoD into mouse mesenchymal stem cells, they have achieved efficient differentiation into myogenic cells. The flexibility of this technique with respect to timing and level of exogenous gene expression will likely make it an important tool for directed differentiation, especially in combination with genetic screens to identify genes with differentiation phenotypes. The feasibility of such screens was recently demonstrated in mESCs using the CRISPR-Cas system (Koike-Yusa et al. 2013).

When culturing ESCs on a flat substrate, the material properties of that substrate can have a significant influence on differentiation. The extracellular matrix (ECM) components used to mediate cell attachment (Flaim et al. 2008) and the stiffness of the substrate (Engler et al. 2006) influence the lineage decision. A study by Warmflash et al. (2014) used micropatterning to control the size of hESC colonies before differentiation. In these experiments, colony size had a strong influence on the diversity of emerging lineages and, intriguingly, gastrulation-like events were observed. Similar micropatterning was used in a screen of the impact of developmental factors on hESC differentiation (Nazareth et al. 2013). These studies indicate that precise control over the microenvironment of ESCs makes it possible to address questions about spatial patterning, local response to signaling activity, and the influence of ECM components in vitro. Methods such as soft lithography, microfluidics (Ankam et al. 2013), and bioprinting (Tasoglu & Demirci 2013) will be invaluable to achieve the necessary level of control and to perform experiments in a high-throughput mode.

Even in the absence of an artificial, patterned environment, ESCs show remarkable selforganizing capabilities when cultured in 3D (see the sidebar Differentiation in Three Dimensions and **Figure 5**).

Measuring Differentiation Dynamics

A lineage decision is by definition a single-cell process, and as discussed above, a population of ESCs is heterogeneous and responds to differentiation cues in a heterogeneous manner. Therefore, it is imperative to measure the dynamics of differentiation at the single-cell level (Etzrodt et al. 2014, Hoppe et al. 2014).

The best-studied and most highly informative feature of a cell state is a cell's transcriptional profile. Several methods for genome-wide RNA sequencing (RNA-seq) of individual cells have been developed (for detailed reviews, see Junker & van Oudenaarden 2014, Saliba et al. 2014, Shapiro

DIFFERENTIATION IN THREE DIMENSIONS

The initial step of many differentiation protocols is the creation of free floating, differentiating aggregates, or embryoid bodies (EBs), that contain cells from all germ layers. EBs are valuable objects of study in their own right, as their development mimics early embryonic development to some extent. For example, two days after aggregation, EBs acquire an epithelial layer of cells that resembles the primitive endoderm, both functionally and in the expression of particular marker genes (Hamazaki et al. 2004). Despite being spherically symmetric at this stage, EBs show signs of symmetry-breaking: Spontaneously occurring, localized Wnt signaling activity leads to a polarized gene expression profile, with domains resembling mesendoderm and neuroectoderm, respectively (ten Berge et al. 2008). When started from a defined number of cells and under timed activation of Wnt signaling, EBs assume an ovoid shape and produce elongating protrusions (van den Brink et al. 2014). These EBs are suggested to mimic gastrulation and are therefore termed gastruloids.

Remarkably, the ability of embryonic stem cells (ESCs) to mimic in vivo development goes beyond the earliest stages of development. In several studies, ESCs have been coaxed to form structures, called organoids, that resemble adult tissues. Examples include organoids mimicking the neural tube (Meinhardt et al. 2014) or the optic cup (Eiraku et al. 2011); cerebral organoids or mini-brains (Lancaster et al. 2013) (see **Figure 5**); and intestinal organoids or mini-guts (Forster et al. 2014, Sato et al. 2009, Spence et al. 2011). Compared with their in vivo counterparts, these complex organoid structures can be produced and manipulated relatively easily and with a higher throughput. These advantages will make them important tools to study lineage decision-making in a spatial context and, in particular, the role of cell-cell contacts and patterned signaling activity, such as morphogen gradients.



Figure 5

Cerebral organoid or mini-brain, showcasing the remarkable self-organization ability of embryonic stem cells. The fluorescence micrograph shows an organoid made from human embryonic stem cells that have been differentiated for two months and stained for DNA (*blue*) as well as the neuronal markers doublecortin (*red*) and nestin (*green*). This image is courtesy of Yun Li, Jaenisch Lab, Whitehead Institute, Cambridge, MA.

et al. 2013, Wu et al. 2013). Two notable recently developed methods, Drop-seq (Macosko et al. 2015) and inDrops (Klein et al. 2015), leverage the advantages of microfluidics to achieve highthroughput single-cell RNA-seq. Both methods encapsulate cells in nanoliter droplets and thereby allow for the creation of RNA-seq libraries from thousands of individual cells. Importantly, in-Drops has been applied to undirected differentiation of mESCs (Klein et al. 2015). It revealed a highly asynchronous onset of differentiation as well as distinct subpopulations of differentiated cells (Klein et al. 2015). Single-cell RNA-seq techniques hold great potential for the study of lineage decision-making, as they facilitate an unbiased classification of the single-cell response to differentiation cues and, eventually, different cell types. Such capabilities have been demonstrated with cells from the spleen (Jaitin et al. 2014), lung epithelium (Treutlein et al. 2014), and cerebral cortex (Pollen et al. 2014). As these studies demonstrate, single-cell RNA-seq methods enable the cellular decomposition of tissues into cell types. However, they destroy all spatial relationships between cells, as they require dissociated cells as input. Techniques such as laser-capture microdissection could potentially overcome this limitation, but they are probably unfeasible for large, complex tissues such as the brain. Therefore, methods that can assess the transcriptional profile of individual cells while preserving their spatial context are highly useful. For detailed information about spatially resolved transcriptomics, we refer the reader to a recent review by Crosetto et al. (2015). For most existing methods, the trade-off for spatial information is a limit in the number of different genes that can be interrogated simultaneously. Notable exceptions are fluorescence in situ sequencing (FISSEQ; Lee et al. 2014) and multiplexed error-robust fluorescence in situ hybridization (MERFISH; Chen et al. 2015). Both techniques are able to measure the expression level and localization of thousands of different RNAs in situ.

As discussed above, important layers of gene regulation operate at the transcriptional level, but translation is regulated as well. A study by Sampath et al. (2008) of ribosomal loading during ESC differentiation showed that the translational efficiency of most genes is modulated. Several genes are even under exclusive translational control. Therefore, it can be critical to measure protein levels to get a faithful representation of the cell state. Methods to measure protein expression in individual live cells are largely based on fluorescent reporters. Signals from such reporters can be read out using imaging, to preserve the spatial context, or via flow cytometry in high-throughput studies. Because different fluorophores must be resolved spectrally, the number of genes that can be measured simultaneously using such approaches is limited, and gene-targeting strategies can potentially influence results (Faddah et al. 2013). Mass cytometry, by contrast, can only be performed on dissociated and typically fixed cells, and it is a destructive method (Bendall et al. 2011).

Methods to measure the epigenetic state of individual cells are still rare (Bheda & Schneider 2014), but they are beginning to emerge. Most methods to measure epigenetic marks or transcription factor binding patterns require thousands of cells or more as input. One possibility to meet this requirement is to sort cells based on expression of lineage markers and to perform downstream analyses on purified populations. It was recently demonstrated that cells can be sorted based on RNA abundance using RNA FISH (Klemm et al. 2014). This sorting method should allow convenient sorting and epigenetic profiling of arbitrary, specific cell types in complex tissues.

Data Analysis

New computational methods are being developed to fully exploit the wealth of information created with the novel methods discussed above, in particular single-cell RNA-seq. One key task is the quantification of expression heterogeneity in a population. Genome-wide assessment of single-cell heterogeneity is possible if proper models for technical noise are applied (Grün et al. 2014). Also, heterogeneity related to cell cycle–regulated gene expression must be taken into consideration when nonsynchronized cell populations are analyzed (Buettner et al. 2015).

Another recurring challenge is the classification of different cell types in a mixture and their ordering along a developmental trajectory. The SPADE algorithm was developed to classify cells using high-throughput cytometry measurements (Qiu et al. 2011). Cells are represented as points in an n-dimensional space, where n is the number of measured gene expression values. After using local, density-dependent downsampling to achieve equal representation of rare and abundant cell types, cells are clustered by k-means clustering. The obtained clusters are connected by a minimum spanning tree, and all cells from the original, full data set are mapped to the closest cluster, such that mean expression levels can be calculated for each cluster. In an application of this algorithm to a mixture of cells from the mouse bone marrow, the branches of the SPADE tree were shown to relate to different cell types.

Another graph-based algorithm is WANDERLUST (Bendall et al. 2014). This algorithm orders cells along a developmental timeline based on their expression profiles, starting from a user-defined initial cell. To that end, WANDERLUST first constructs a nearest-neighbor graph connecting individual cells in the *n*-dimensional expression space. Next, the shortest path between the initial cell and a target cell is calculated as a first estimate for the target's cell position on the developmental trajectory. Noise accumulated along this trajectory, along with short circuits due to cells close in expression space but distant in developmental time, are potential pitfalls of this approach. WANDERLUST uses intermediate waypoint cells to minimize the influence of noise, and averaging over ensembles of graphs mitigates the influence of rare short circuits. The algorithm was successfully validated by unraveling the B cell lymphopoiesis process in a bone marrow sample analyzed with mass cytometry.

Unfortunately, WANDERLUST can only be applied to nonbranching processes. This limitation is absent in a tool developed by Trapnell et al. (2014) called MONOCLE. Similar to the previous algorithm, MONOCLE is graph-based, but it can integrate measurements from different time points. After dimensionality reduction by independent component analysis, MONOCLE constructs the minimum spanning tree between cells and the main path through this tree, which can potentially be branched. A pseudotemporal ordering of cells is then defined as the length of the path traveled on this tree. Importantly, this algorithm addresses the common problem that differentiation is, in general, not synchronous across a population of cells. Cells sampled at the same point in time might be at very distant points along a differentiation trajectory. MONOCLE's pseudotemporal ordering allows the discovery of dynamical features that remain obscured when conventional methods are used.

Compared with MONOCLE and WANDERLUST, the SCUBA algorithm, developed by Marco et al. (2014), is substantially simpler but requires sampling at different time points. First, the SCUBA algorithm identifies bifurcation events during a differentiation or developmental process by clustering single-cell expression profiles at each time point. Then, it constructs a binary lineage tree under the assumption that gene expression changes smoothly between time points. At each bifurcation, SCUBA projects gene expression on the direction of bifurcation, such that a simple 1D potential function with two local minima can be used to capture gene expression variability. The parameters of this potential contain valuable information about robustness of gene expression and the epigenetic landscape. When applied to a data set describing early mouse embryonic development, SCUBA recovered the two well-known bifurcations that lead to the three preimplantation lineages: epiblast, primitive endoderm, and trophectoderm.

Most existing classification algorithms work robustly only for relatively abundant cell types. A new algorithm for rare cell-type identification (RaceID) developed by Grün et al. (2015) tackles this problem. RaceID identifies outliers using a background model for technical and biological

noise. When applied to single-cell RNA-seq measurements of mouse intestinal organoids, the algorithm was able to discover novel, rare cell subtypes.

After the classification of cells in a population, the identified clusters have to be interpreted biologically. For tissue samples, at least some prior knowledge about the cell-type distribution is typically available. In vitro differentiation protocols, however, can produce a priori unknown cell types. CellNet is a computational approach that addresses this problem (Cahan et al. 2014, Morris et al. 2014). In this approach, engineered cells are systematically compared with a library of known cell types. CellNet is based on the reconstruction of cell-type- or tissue-specific GRNs. Similarity between a query data set and a particular reference data set depends on the level to which certain GRNs are established. In this way, CellNet not only provides a precise metric for similarity but also identifies aberrantly expressed regulatory nodes, which can then be targeted to improve the engineered cell type.

Another innovative way to apply GRNs in the context of differentiation was developed by Bodaker et al. (2014). They showed that the most active pathways in differentiation can be predicted purely from their position in the GRN. For all methods relying on GRNs, it is important that GRN topology be known with high confidence. Methods to infer network topology typically rely on bulk expression data and specific perturbations. An algorithm developed by Chen et al. (2014) is a first attempt to improve network inference by using single-cell transcriptional measurements and exploiting known lineage relationships.

FUTURE DIRECTIONS

We believe that GRNs will continue to play an important role in the study of lineage decisionmaking. By putting Waddington's [2014 (1957)] metaphor of an epigenetic landscape on firm mathematical ground, GRNs enable methods from statistical mechanics (MacArthur & Lemischka 2013) and many-body physics (Zhang & Wolynes 2014) to be applied to biological problems.

We expect that more examples of heterogeneity considered stochastic today will turn out to have deterministic underpinnings, such as population context or cellular history. The study of such deterministic heterogeneity should lead to the identification of physiologically relevant regulatory mechanisms (Snijder & Pelkmans 2011).

Signaling molecules and small molecule inducers of differentiation, such as RA, will continue to be used frequently in in vitro differentiation and the study of developmental processes. Unraveling the mechanisms of action of these molecules will be a touchstone for systems and quantitative biology in the future. To fully understand the signaling process, we will have to combine integrative genomics approaches (Mendoza-Parra et al. 2011), quantitative measurements of signaling pathway crosstalk (Chen et al. 2012), and single-cell measurements of the heterogeneous transcriptional response.

Concerning novel tools, we believe that single-cell epigenomics is the new frontier (Bheda & Schneider 2014). Single-cell measurements of histone modifications could reveal epigenetic priming prior to a transcriptional response (Chen & Dent 2013) and resolve important questions around bivalent domains. The measurement of RNA transcribed from enhancers (eRNA; Kim et al. 2010) may be a possibility to assess enhancer activity in single cells.

Proteomics is another area that will make important contributions. In several differentiation experiments, the expression of many genes changed at the protein but not at the mRNA level (Lu et al. 2009, Sampath et al. 2008). Proteomics could help unravel the regulatory mechanisms at play.

We expect that lineage decision-making will remain a highly dynamic field for years to come, and we look forward to the exciting new concepts and clever new techniques it will produce.

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