Molecular ties between the cell cycle and differentiation in embryonic stem cells

Victor C. Li and Marc W. Kirschner[1]

Department of Systems Biology, Harvard Medical School, Boston, MA 02115

Contributed by Marc W. Kirschner, May 21, 2014 (sent for review March 11, 2014)

Attainment of the differentiated state during the final stages of somatic cell differentiation is closely tied to cell cycle progression. Much less is known about the role of the cell cycle at very early stages of embryonic development. Here, we show that molecular pathways involving the cell cycle can be engineered to strongly affect embryonic stem cell differentiation at early stages in vitro. Strategies based on perturbing these pathways can shorten the rate and simplify the lineage path of ES differentiation. These results make it likely that pathways involving cell proliferation intersect at various points with pathways that regulate cell lineages in embryos and demonstrate that this knowledge can be used profitably to guide the path and effectiveness of cell differentiation of pluripotent cells.

proliferation control | differentiation modeling | guided differentiation | systems biology

As cells differentiate during embryonic development, they progress through a stereotypical sequence of events, starting from highly potent embryonic precursors to germ-layer intermediates, then to lineage-restricted progenitors, and finally to terminally differentiated cell types. Any of these stages may consist of further states of differentiation and may be difficult to recognize. Most of our knowledge about the differentiation process comes from studies in the latter stages of differentiation (i.e., terminal model systems), where cells are one step away from their final fate and are usually restricted to differentiate to one type of cell. Less is known about what happens during early embryonic stages, where the differentiation process is just beginning and many alternative pathways of differentiation may still be available.

In terminal somatic cell culture models, inhibition of the cell cycle is almost always a requisite for differentiation. Forced inhibition of the cell cycle very often induces terminal differentiation and vice versa (1–3). The molecular pathways that couple the cell cycle to differentiation involve molecules of the G_1/S transition including growth factors, downstream signaling pathways, Myc, the Rb/E2F pathway, and the CDK inhibitors (e.g., p21). We previously investigated the role of G_1 length on embryonic stem (ES) cell self-renewal and found that in contrast to the terminal stages, it did not accelerate the loss of pluripotency or facilitate differentiation (4). We now address the state of the cell cycle molecular network in the ES cell system and ask how the cell cycle may be recoupled to differentiation to redirect lineage pathways, ultimately for practical benefit.

As we began experimenting with cell cycle perturbation, we found it useful to summarize for ourselves a version of some of what is now known or thought about these pathways in somatic and ES cells in simple flow diagrams. Because these pathways are a convenient road map for readers, we present them first. We know that they are certain to be simplifications, and in time, they may not be all found to be accurate and certainly not complete. We are not trying to prove these as models, although largely our results correspond to what could be expected from them, but instead use them to highlight some of the cell cycle differences between embryonic, somatic cycling, and terminally differentiated cells that we exploit in our experiments.

Results

In terminally differentiated cells the cell cycle and differentiation are linked together through a molecular network rooted in the G_1/S transition. A wiring diagram summarizing such a network is shown in Fig. 1*A*, with explanations and justifications provided in Fig. S1, which was constructed from known or postulated relationships in normal somatic cycling cells and interactions between the cell cycle machinery and terminal transcription factors, such as MyoD.

During the process of differentiation, the network changes. For cultured cells at the terminal stage of differentiation, this involves an exit from the cell cycle, activation of terminal transcription factors, and a shift toward insulin signaling away from other growth factors for survival and growth. These changes to the network are shown in Fig. 1*B*. At the other end of the differentiation process are ES cells, which have a number of unique features. They can be maintained in an undifferentiated state with the combination of leukemia inhibitory factor (LIF) and high amounts of serum, or LIF and Bmp4, as shown in Fig. 1*C*. The actual differentiation process from ES cells to terminally differentiated cells spans at least three states in a defined order (ES to somatic cycling cells to terminal differentiation) but may pass through other intermediate stages of differentiation, expressing genes and behavior different from terminal cells and pluripotent stem cells; little is known concerning the cell cycle and their state of differentiation in these largely uncharacterized cell cycle states.

Guided Differentiation and Cell Cycle Manipulation of ES cells. To probe the effect of the cell cycle on differentiation we used an ES cell line that constitutively overexpressed the transcription factor MyoD driven off an EF1α promoter (5) activated by tamoxifen-induced Cre recombination. The use of this cell line facilitated our analysis by channeling differentiation away from a diverse collection of phenotypes into a more uniform population of cells expressing muscle genes, such as myosin heavy chain (MHC).

Significance

The role of the cell cycle as an inhibitor of the late stages of cellular differentiation is well known, but in early embryonic development its role is mysterious. Normally, embryonic stem (ES) cells proliferate quickly during differentiation and do not differentiate simply by cell cycle slowing. In this paper, it is shown that the role of the cell cycle in terminal stages of differentiation can be adapted for and extended into ES cells. Methods aimed at inhibiting the cell cycle drive a rapid, condensed differentiation to terminally differentiated cells, demonstrating that the cell cycle is a principle rate-limiting step of differentiation throughout early and late stages. This is beneficial as well for accelerating differentiation in ES cell applications.

Author contributions: V.C.L. and M.W.K. designed research; V.C.L. performed research; V.C.L. contributed new reagents/analytic tools; V.C.L. and M.W.K. analyzed data; and V.C.L. and M.W.K. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: marc@hms.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1408638111/-/DCSupplemental.

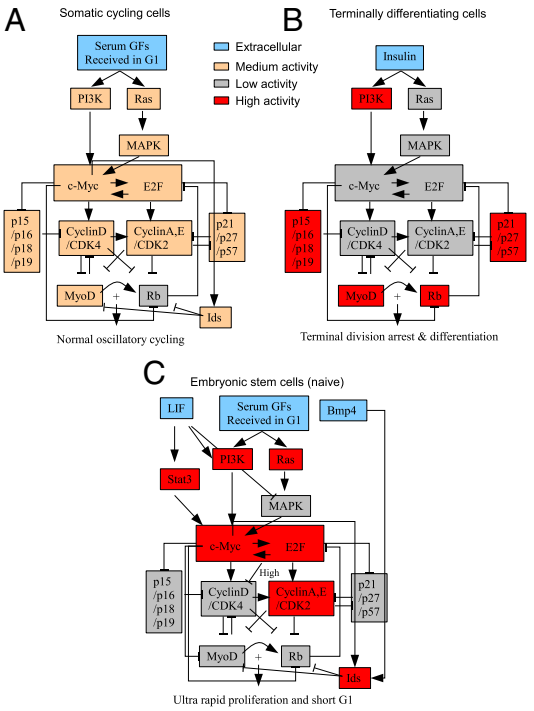

Fig. 1. Suggested summary of the proliferation/differentiation network adapted for ES cells. (*A*) Reconstructed summary of main network components and interactions in normal somatic cycling cells. Extracellular growth factors (GFs) either purified or in serum activate downstream signaling pathways (PI3K, MAPK), which then trigger a transcriptional activation (Myc, E2F) that drives the core cell cycle machinery (Cyclins and Cyclin-dependent kinases). For a full explanation and justification of the summary, see Fig. S1. The cellular behavior associated with this model is normal oscillatory cycling. (*B*) Changes to the network that occur during terminal division arrest and differentiation. Cells switch to insulin signaling for survival and growth and shut down cycling activity. Terminal transcription factors become fully active, leading to complete differentiation. (*C*) Composite adaptation of network for ES cells. ES cells are normally maintained by LIF and high serum or LIF and Bmp4. This hyperactivates PI3K, Myc, E2F, CDK2, and Id family activities. Meanwhile, MAPK, CDK4, p16 family, p21 family, and Rb family activities are highly suppressed. This leads to an ultrarapid proliferation and short G_1 phase.

Our first cell cycle manipulation was growth factor or serum withdrawal, which has a long history of use in studies of terminal differentiation. Using the cell line that continuously expressed MyoD, we removed LIF at what we nominally call zero time to initiate differentiation and reduced serum at various times thereafter from the standard 15% serum to 2% (vol/vol) with additional insulin (10 μg/mL). As shown in Fig. 2*A*, in the continuous presence of 15% serum, MHC is completely suppressed, despite constitutive MyoD overexpression. When serum is reduced 1 d after MyoD induction, MHC begins to accumulate 4 d later. By day 12, 20–30% of cells express MHC and show characteristic morphology of mature skeletal muscle fibers, including elongation, an increase in volume, and significant multinucleation (Fig. S2). Myofibrillar striations were also observed by immunostaining for sarcomeric α-actinin (Fig. S2). If serum removal is delayed relative to LIF removal, the cells still begin to express MHC with a 2- to 4-d delay after serum reduction. Thus, serum reduction strongly potentiates terminal muscle differentiation in a very short time under the conditions studied.

From our cell cycle summary in Fig. 1*A*, MyoD activation and hence muscle differentiation from ES cells should be blocked by either the action of LIF, which activates Myc, or Bmp4, which promotes inhibitor of differentiation (Id) protein family expression. However, any implication of growth factor effects through the manipulation of serum can be fraught with the inconsistencies and complexities of serum. To avoid these problems, we examined more defined conditions with two types of basal insulin-containing media, N2B27 and DMEM plus 20% knockout serum replacement (KOSR), neither of which contains growth factors. Use of both media in ES cells led to activation of MyoD and terminal myogenesis similar to the 2% low serum media, with some improvement (Fig. 2*B*; assessed on day 4). The N2B27 media produced ~38.5% MHC^+ nuclei, whereas 20% KOSR produced ~19.6%. As expected, when we added LIF or Bmp4 back to N2B27, differentiation was blocked (0%; Fig. 2*B*). These results confirm our expectation that the reduction of LIF and BMP in the setting of no other growth factors produces highly effective conditions for ES differentiation.

The lineage from ES cells to terminal differentiation first involves the loss of pluripotency factors, followed by passage through intermediate cell types, identifiable by expression of specific transcription factors. We found that the decline in Oct4 and Nanog mRNA levels induced by LIF removal was completely unaffected by serum reduction (Fig. 3). This is similar to our previously published results showing that extension of G_1 had no effect on Nanog levels (4).

By contrast, beyond the loss of pluripotency factors there is a dramatic effect of serum removal on the differentiation cascade toward muscle. From studies in embryos, there is a prescribed sequence of steps in setting up the myogenic lineage involving the specification of the mesoderm, the subspecification of the myotome, and the steps leading to overt cell differentiation (6, 7). When we examined the mRNA levels of genes within this hierarchy using the above protocol of serum reduction, we found Pax3, which is expressed in the dermomyotome, rising dramatically (to a peak of ~50 fold) and very prematurely within

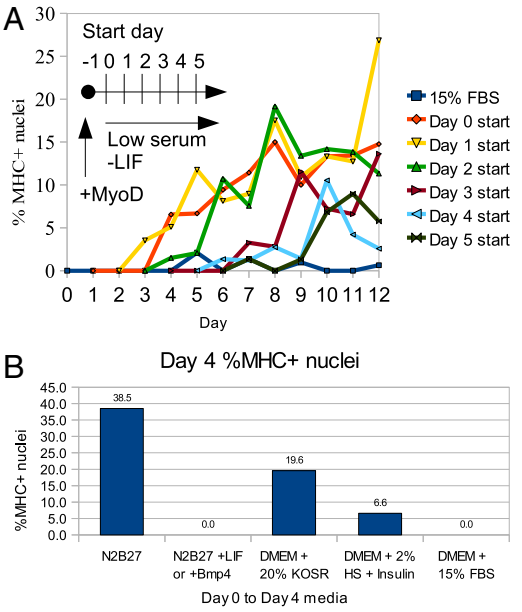

Fig. 2. Growth factor/serum reduction drives a direct terminal skeletal muscle differentiation program. (*A*) Differentiation time course of ES cells to skeletal muscle when exposed to low serum vs. high serum conditions. MyoD overexpression was initially induced with tamoxifen starting at day −1 for 24 h under ES conditions. LIF was removed at day 0. Low serum was then initiated at different starting times beginning with day 0. Cells were then fixed and immunostained for MHC expression. Differentiating cells in high serum were continuously split to prevent overgrowth. (*B*) A comparison of differentiation efficiency generated by two types of defined media (N2B27) and 20% KOSR, low serum media [2% horse serum (HS) + insulin], and high serum media (15% FBS). Cells were removed from standard ES media (LIF and serum) and incubated in the specified media starting from day 0 to day 4. Continuation of LIF (1000U/mL) or addition of Bmp4 (10 ng/mL) results in a strong block to muscle differentiation.

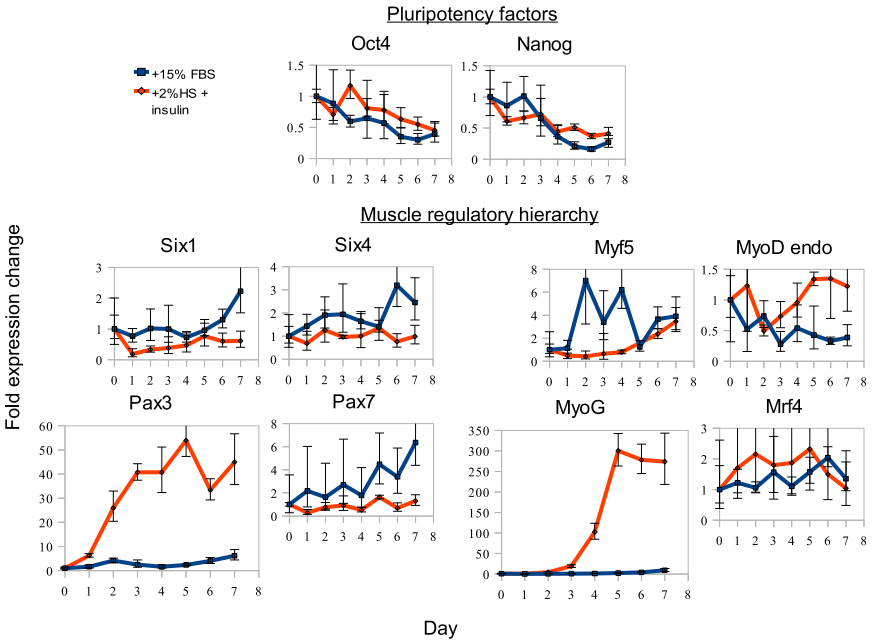

Fig. 3. Growth factor/serum withdrawal drives a condensed gene expression program of early Pax3 expression and subsequent MyoG expression but does not affect kinetics of Oct4 or Nanog loss. Gene expression time courses of differentiation over 7 d. In this experiment, low serum was initiated at day 0, 1 d after induction of MyoD expression in ES media, and maintained over the course of differentiation. The rate of decline of Oct4 and Nanog mRNA levels are not strongly affected by cell cycle inhibition. In the muscle regulatory hierarchy, only Pax3 and MyoG are strongly up-regulated. Up-regulation of additional terminal markers of skeletal muscle terminal differentiation can be found in Fig. S3. Ct values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GADPH). Error values reflect SEM ($n = 3$).

2 d of serum reduction. The premyogenic homeodomain factors Six1 (8) and Six4 (9), which are normally upstream of Pax3, are not affected or modestly suppressed, as was the case for the paired-box domain protein Pax7, which is expressed in the dermomyotome and somites during embryogenesis (10). There are small effects of serum reduction on the myogenic regulatory factor (MRF) genes, like Myf5, MRF4, and endogenous MyoD, but there is a massive (300-fold) up-regulation of myogenin (MyoG), which plays a key role in very late-stage skeletal myogenesis during the period of days 3–7 (11). Other muscle lineage markers also respond rapidly to serum withdrawal in the presence of MyoD, indicating that the entire suite of terminal muscle lineage is induced very prematurely. Seven of these—desmin, skeletal muscle actin, troponin, myosin light chain, tropomyosin, the myoblast fusion regulator Dyrk1b (12), and titin—are shown in Fig. S3. The dramatic overexpression of Pax3 and MyoG depend on the overexpressed exogenous MyoD, because without the induction of MyoD, their expression is lower. These results document the extraordinarily rapid production of some downstream muscle differentiation factors and definitive muscle proteins in the setting of growth factor or serum withdrawal.

Promoting Differentiation by Perturbing Intracellular Pathways. Based on suggestions from the pathway diagrams in Fig. 1, we focused on a few critical components of cell cycle control and measured their effects on the two markers strongly perturbed by serum withdrawal, Pax3 and MyoG. Perturbations were made both under high and low serum conditions and were extended throughout the time course of differentiation. LY294002 is a potent broad inhibitor of phosphoinositide-3-kinases (PI3Ks) and when applied continuously to ES cells over 7 d induced a significant 2.7-fold increase in Pax3 mRNA expression (Fig. 4). This increase was observed both in high serum and low serum media. However, continuing treatment with LY294002 led to cell death, and we saw no expression of myogenin (Fig. 4, *Lower Left* and *Lower Right*). A similar situation was observed with HLM006474, which broadly inhibits E2F family transcription factors in their interaction with DP proteins. Myc drives cell cycle progression and growth. Its activity can be inhibited by two compounds: JQ1, a newly-identified compound that specifically inhibits bromodomains but subsequently results in Myc down-regulation; and 10058-F4, an inhibitor that blocks the dimerization of Myc-Max complexes. Both are indirect inhibitors of Myc activity; to date, there are no direct pharmacologic inhibitors of the Myc protein. The effects of JQ1 were similar to LY294002 and HLM00647: an increase in Pax3 early expression but later suppression of MyoG expression. Of all of the drugs, the Myc-bromodomain inhibitor JQ1 had the largest effect in inducing Pax3, whereas the Myc-Max dimerization inhibitor 10058-F4 had suppressive effects on both Pax3 and MyoG.

We also examined the effects of inhibiting cyclin-dependent kinases (kinases that are more centrally involved in cell cycle control) and MAP kinase, which very often is involved in cell cycle regulation. Roscovitine is a broad CDK inhibitor that blocks a number of family members, including CDK1, CDK2, and CDK5. After continuous treatment throughout the 7 d of differentiation in our time course, we saw that roscovitine had no effect on Pax3 expression (Fig. 4). It also had little effect on the later expression of MyoG, either under high or low serum conditions. This lack of effect on Pax3 and MyoG was also observed for the more specific CDK4 inhibitor PD0332991. However, the MAPK inhibitor PD98059, which blocks MEK1/2, had no effect on Pax3 and induced MyoG only under low serum conditions (Fig. 4, *Lower*). The protein CDK inhibitor p21, which is much more specific than roscovitine, blocks CDK2 and prevents entry into S phase. In ES cells, p21 is expressed at low levels but gradually increases during the course of ES cell differentiation (13). We developed an mES cell line that constitutively overexpresses the p21 protein bicistronically with a mCherry tag (fused to the C terminus with a 2A peptide). This line exhibits an elongated G_1 and can be propagated in standard LIF^+ serum media. When induced to differentiate by the removal of LIF, we noticed that this line up-regulated MyoG under low serum media but had no effect on Pax3, a behavior similar to the MAPK inhibitor. Thus, within the set of cell cycle inhibitors we examined, we observed stage-specific and condition-specific effects on gene expression.

Induction of Unguided Differentiation. Although forced expression of MyoD nicely served to focus differentiation into the skeletal muscle cell lineage, we also wanted to examine what happens in ES cells that are not guided in their differentiation path by MyoD. When LIF is removed in ES cells without MyoD, there is differentiation into a heterogeneous mixture of cell types. Under standard culture conditions of 15% serum, which promotes expansion, ES cells deprived of LIF normally differentiate first into

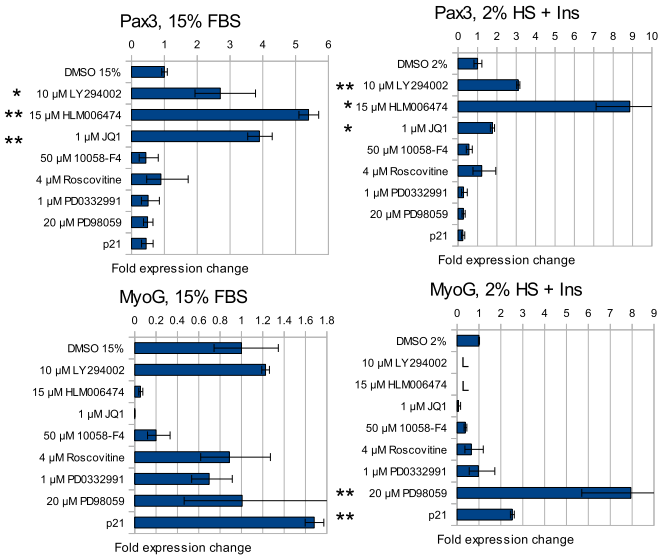

Fig. 4. Specific cell cycle inhibitors have stage and condition-specific effects on Pax3 and MyoG expression. Time courses of differentiation were run for 7 d, similarly to the previous growth factor withdrawal experiments. MyoD was induced at day −1, and LIF was removed at day 0. Cells were kept in either high serum (15% FBS) or low serum [2% horse serum (HS) plus insulin] media throughout the time course. Drug treatments were applied continuously at the designated concentrations for the entire duration of the time course, with daily media change. mRNA expression levels were measured by quantitative RT-PCR. Pax3 was measured on day 3 (because it is up-regulated early), and MyoG was measured on day 7 (because it is up-regulated late). All measurements are relative to a DMSO control for the specific condition (i.e., low serum values are still higher than in high serum). L, lethal (drug had strong anti-survival effects, so no data were collected). Error bars indicate SEM (n = 2).

general mesodermal, endodermal, and ectodermal tissues and then later into a heterogeneous mixture of terminal cell types (7, 14). We examined the effects of serum withdrawal on this system. When we compared high and low serum time courses in ES cells differentiating without exogenous MyoD over a period of 7 d, there was premature expression of genes that are normally associated with multiple cell lineages (Fig. 5). Low serum induced the expression of many lineage-specific factors. For example, an increase in the neural marker Delta (Dll1) was observed. The cardiac muscle factors Sox6, Smyd1, GATA4, and GATA6 all increased, as well as the neural/muscle transcription factor Mef2c. For adipose genes, we detected a very large increase in PPARγ expression (>400 fold). The early endoderm genes Sox17 and Nkx2.2 also were elevated in low serum compared with high serum. Similarly, increases in Runx2 (osteoblast differentiation), Mitf (melanocyte), and Sox9 (chondrocyte differentiation) were also observed. For hematopoietic factors, we noticed increases in the erythrocyte factor GATA1 (Fig. 5) and the progenitor factor GATA2 (Fig. S4). A full list of factors we profiled and their time course data are available in Fig. S4.

Although we have not tried to unscramble the temporal progression of the various lineages represented in this heterogeneous population, the early up-regulation of such a large number of somatic lineage factors suggests that growth factor/serum reduction is permissive for a wide variety of differentiated gene expression. Many of the up-regulated factors have been reported to function in terminal differentiation. Perhaps most interesting is the failure to express many of the markers of the early lineages. As we saw in the MyoD-guided system, only Pax3 and MyoG were significantly activated but not other factors in the muscle lineage hierarchy. In the unguided system, in addition to the terminal factors that were up-regulated, there were numerous intermediate lineage factors that were not (e.g., Pax6, C/EBPα, C/EBPβ, Pdx1, Cdx2, etc.) (Fig. S4 and Table S1).

Discussion

Our understanding of cell differentiation comes mainly from two different sources: studies of cell culture systems and studies of embryonic systems. Although the embryo remains the gold standard for the functional process of embryogenesis, there is today a strong incentive to understand alternative in vitro pathways that can be exploited for therapeutic purposes. Furthermore, there is no reason why we should consider embryonic lineages as mechanistically the most informative. Embryos have to accomplish feats other than differentiation, such as morphogenesis and cell proliferation, and many intermediate behaviors of cells may reflect those roles.

Much ingenuity and decades of effort has resulted in the discovery of ways to manipulate cells isolated and cultured from various tissues, so that they can differentiate into one or a very few cell types. We now recognize that these processes take cells from an already determined state and drive them to a state of clear expression of specific markers, rather than starting from a very early precursor state. Such manipulations can drive presumptive myoblasts to muscle, neuroblasts to neurons, fibroblasts to adipocytes, etc. A very different source of cells are pluripotent ES cells of the mouse and now of human. These cells start at an earlier state and can be driven to differentiate either by recreating some early embryonic state through embryoid bodies or by going through a series of steps in culture, thought to parallel the various intermediate states of differentiation found in the embryo itself. As work in stem cells and ES cells in particular exploded in the last few years, there has been a serious effort to identify and recreate in culture the series of signals that drive the pluripotent state to the differentiated state. In the embryo, these include the addition and removal of factors like Wnts, Nodals, BMPs, EGFs, etc. There is also a practical side to this endeavor: to either generate differentiated cells that can generate replacement tissues and organs or to find ways to stimulate the body's regenerative potential to repair worn or diseased cells.

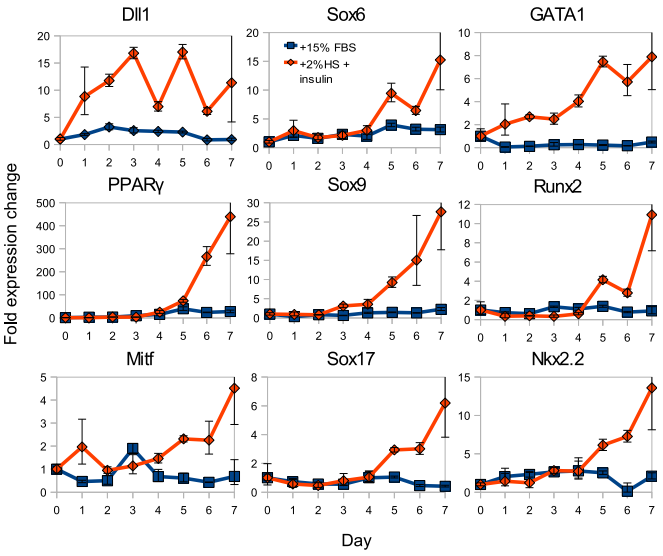

Fig. 5. In an unguided or heterogeneous differentiation setting, growth factor/serum withdrawal up-regulates the expression of numerous genes associated with differentiated cell types. Gene expression time courses of unguided (without MyoD) differentiation after release from ES cell media at day 0. Low or high serum was applied at day 0 for the full time course. Up-regulated factors include Dll1, Sox6, GATA1, PPARγ, Sox9, Runx2, Mitf, Sox17, and Nkx2.2 (continued in Fig. S4). These genes are involved in the differentiation of multiple cell types, including neurons, chondrocytes, erythrocytes, adipocytes, cardiomyocytes, osteoblasts, melanocytes, and β cells. Error values indicate SEM (n = 2).

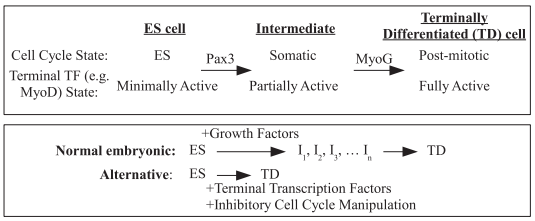

Fig. 6. Use of network-based cell cycle manipulation may provide an alternative strategy to generating terminal cell types that is more efficient than recapitulating embryogenesis. (*Upper*) Analytical scheme of how cell cycle states drive activation of a terminal transcription factor. In our MyoD overexpression system, we propose that transitions from the three states of ES to somatic cycling to a terminally differentiated state influence the subsequent activation of MyoD and hence the progression of differentiation. The first transition is correlated with Pax3 activation, and the second transition is correlated with MyoG activation. Accelerating the cell cycle transitions accelerates the differentiation process. (*Lower*) Current strategies of differentiating ES cells into cells involves growth-factor based recapitulation of embryogenesis. The alternative strategy suggested in this paper (and shown in the case of skeletal muscle) is to artificially induce differentiation by accelerating cell cycle inhibition combined with addition of a terminal transcription factor. Because ES cells are susceptible to cell cycle-related pathways, this can lead to a faster, more efficient differentiation.

There has also been a long-standing interest in understanding how the cell cycle could further the process of differentiation. Inhibition of cell proliferation in G_1 is almost always accompanied by cell differentiation. In this paper, we endeavored to see whether this effect of cell cycle inhibition can be observed early in the differentiation process and whether the paths taken are the same as seen in the absence of cell cycle inhibition.

In our experiments, cell cycle perturbations, starting with a reduction or removal of growth factors/serum, change the timing and the course of differentiation to muscle in a model that involves the continuous expression of MyoD. Notably none of the perturbations had an effect on exit from the ES cell state as reflected in the loss of Oct4 and Nanog. We were surprised that this progression to the differentiated state seemed not to seem to follow the normal sequence of gene expressions seen in ES cells in culture or by embryonic lineages in the embryo. However, we probably should not have been surprised to find more than one path to differentiation. For example, bone can be generated through somitic lineages or through the neural crest (15). Even within the somite of the chick, back muscles are generated through a somatic lineage that depends on early Myf-5 induction and abdominal and limb muscles that depend on MyoD (16).

To inform our experiments, we built heuristic descriptions of somatic cycling cells, terminally differentiating cells, and ES cells from data in the literature (Fig. 1). Terminally differentiated cells typically maintain their size or grow slowly, stimulated commonly by the insulin pathway. In this cell cycle state, the drivers of the cell cycle are inhibited and the expression of cell cycle inhibitors are stimulated, leading to the expression of terminally differentiated genes like MyoD, neurogenin, etc. The proliferative state that preceded terminal differentiation is reduced in the expression of CDK inhibitors and particularly in the activity of Rb. In this case MyoD activity is also reduced. Lastly, the pluripotent embryonic state has new extracellular players: LIF, serum growth factors, and Bmp4. In this state, there is more complete suppression of Rb and MyoD through the activation of proliferative signals in the cell cycle and the downstream suppression of antiproliferative factors such as CDK inhibitors.

Our experiments perturbing the cell cycle suggest that in our MyoD-guided system, there are minimally three states and two transitions (Fig. 6, *Upper*). The first stable state is pluripotency. When the cell cycle is suppressed, there is a rapid transition to an intermediate somatic state, which correlates with an up-regulation in Pax3. Manipulations that promote this transition also promote Pax3 up-regulation. For example, this transition could be facilitated by growth factor/serum withdrawal, LY294002 (PI3K inhibition), HLM006474 (E2F inhibition), and JQ1 (bromodomain/Myc inhibition). Subsequently, there is a second transition to the terminally differentiating state, which correlates with up-regulation of MyoG. Manipulations that promote this transition also promote MyoG. MyoG could be facilitated by PD98059 (MEK1/2 inhibitor) and p21 (CDK inhibitor) when combined with low serum but not with roscovitine (broad CDK family inhibitor) or PD0332991 (CDK4 inhibitor).

Although this scheme is likely to be a simplification for any lineage and may differ in different lineages, it nevertheless helped us make sense of a number of observations. The removal of extracellular factors in the form of LIF and Bmp4, and the replacement of serum with insulin leads to activation of MyoD and full induction of terminal myogenesis. When applied early, this leads to a very direct form of ES-to-terminal differentiation, and Pax3 and MyoG up-regulation is observed. PI3K inhibition is predicted to activate MyoD by removing the stimulus to Myc but is also expected to be inhibitory on the last steps of terminal differentiation as it becomes necessary for metabolic cell growth of the final differentiated cell. Accordingly, we see that LY294002 up-regulated Pax3 but promoted poor survival, which could have suppressed MyoG expression. This suggests that the step of MyoG up-regulation corresponds to what happens in the terminal phase in our model, which is consistent with what is known about myogenin's role from terminal models. From our heuristic description, it is also expected that Myc or E2F suppression would help activate MyoD. We observed that JQ1 and HLM006474 both induced Pax3, which is consistent with this prediction. However, we also noticed that they suppressed MyoG expression, which is unexplained. Whether this effect is caused by the same reason as the LY294002 or some off target effect of these inhibitors is unknown. Also unexplained is why the Myc-Max inhibitor 10058-F4 suppressed both Pax3 and MyoG.

The cell cycle schemes also correctly predict that some CDK inhibitors and the MAPK inhibitor would have little effect on Pax3 expression by themselves but would facilitate MyoG expression. This is attributable to the fact that in ES cells Myc and Id proteins are highly expressed and can independently repress the expression of endogenous MyoD and other myogenic regulatory factors outside of CDK activity (Fig. 1*C*). As ES cells differentiate, they transition to the somatic cell cycling state and then to the terminally differentiated model (Fig. 6, *Upper*, and Fig. 1 *A* and *B*). Throughout this transition, Myc and Id activity decline until CDK activity is the predominant factor blocking differentiation. Hence, at the early stage of Pax3 activation, CDK inhibition is not expected to have a significant effect, whereas at the late stage of MyoG activation the expectation is that it will. Moreover, CDK4 activity is suppressed in ES cells, so additional inhibition of their activity by PD0332991 should reveal no effect on Pax3. MAPK (MEK1/2) activity is also suppressed in ES cells but is up-regulated during differentiation. Thus, its inhibition should stimulate MyoG induction but not Pax3 induction. Our understanding is also consistent with the observation that the CDK inhibitors and the MAPK inhibitor promote differentiation only under low serum conditions, because high levels of growth factor/serum conditions are expected to induce higher levels of Myc and Id expression. What we do not understand in some cases is why CDK inhibitors can have different effects. We found that the p21 protein, but not roscovitine or PD0332991, is highly effective at promoting MyoG expression. This may have to do with the differing specificities of the kinase inhibitors used.

Although we intentionally focused our studies on muscle differentiation, these specific manipulations may be generalizable to other cell types with other terminal factors in place of MyoD. Indeed, many terminal cell systems couple cell cycle inhibition with differentiation (1), and many potent transcription factors interact with cell cycle components in ways similar to MyoD, including Ngn2, Pdx1, Smad3, Mitf, Runx2, PU.1, Hnf4α, and C/EBPβ (17–24). Whereas experimental validation and extension of our findings with MyoD to other lineages remains to be

explored, we saw that the expression of several genes associated with diverse differentiated cell types could be stimulated by cell cycle inhibition in a heterogeneous differentiation system where MyoD was not expressed (Fig. 5). Extension into human primed or naïve ES cells also remains to be explored. Traditional human ES cells that resemble primed mouse epiblast ES cells have been reported to have cell cycle features that are more characteristic of somatic cycling cells (25) and hence may be represented by the intermediate phase in our model.

It is important to note that our system is clearly artificial and that the manipulations probably do not reflect the situation in normal embryogenesis, where there is generally a proliferative period during which the cells divide, germ layers and somites form, and the embryo consequently expands. However, it may not be necessary nor desirable to recapitulate the embryonic steps to achieve differentiated tissues. The forced silencing of proliferative pathways and the resulting rapid differentiation in vitro may be a useful strategy to generate terminal cell types compared with trying to recapitulate embryonic differentiation pathways, which often takes weeks (Fig. 6, *Lower*) (26–31). Altogether, improvements in our understanding of the cell cycle and differentiation should allow us to better interpret the pathways of embryonic development and help us to better manipulate stem cell fates for regenerative medicine.

Materials and Methods

ES Culture and Differentiation. The tamoxifen-inducible MyoD-overexpressing ES cell line was graciously provided by Eva Thoma (University of Wuerzburg, Wuerzburg, Germany) (5). The ES cells contain MyoD (and associated puromycin resistance marker) expressed from a EF1α promoter. MyoD could be expressed once a loxP segment inserted between the promoter and transgene was excised by a Cre recombinase fused to the estrogen receptor. ES cells were cultured in LIF and standard conditions containing 15% FBS, nonessential amino acids, L-glutamine, penicillin/streptomycin, and β-mercaptoethanol. To induce MyoD, cells were treated for 24 h with 1 μM 4-hydroxytamoxifen (Sigma) in ES media. Reduced serum media consisted of DMEM and 2% horse serum (Invitrogen) plus 10 μg/mL insulin (to maintain cell survival) (Sigma) with sodium pyruvate and penicillin/streptomycin. During differentiation, cells were treated with 1 μg/mL puromycin continuously to select for cells that maintained MyoD expression. N2B27 components/supplements and KOSR were purchased from Invitrogen. LIF was used at a 1,000 U/mL and Bmp4 at 10 ng/mL.

Drugs. The PI3K inhibitor LY294002, roscovitine, the MEK1/2 inhibitor PD98059, and 10058-F4 were purchased from Sigma. The E2F inhibitor HLM006474 was purchased from Millipore. PD0332991 was purchased from SelleckChem. JQ1 was purchased from ApexBio.

Immunostaining. MHC expression was detected with use of the MF20 antibody (R&D Systems). α-Actinin was detected using EA-53 antibody (Abcam). Cells were fixed in 4% PFA, permeabilized in 0.1% Triton-X, and costained with antibody and DAPI (Sigma).

RNA Isolation and RT-PCR. RNA was isolated using RNAeasy plus kit (Qiagen). Reverse transcription was performed using iScript cDNA synthesis (Bio-Rad). Real-time quantitative PCR was done on a CFX96 PCR machine using SYBR Green Supermix (Bio-Rad). A complete list of primers used is provided in *SI Materials and Methods*.

Microarray Analysis. RNA time-course samples were hybridized to Illumina Ref8 BeadChip arrays. Data analysis was performed with GenomeStudio software and the help of the Children's Hospital Boston Intellectual and Developmental Disabilities Research Center Molecular Genetics Core.

Cloning. The mouse p21 ORF was cloned into the pmCherry-N1 plasmid (Clontech) with a self-cleaving P2A peptide and mCherry fused to its C terminus. The plasmid was transfected into the MyoD-inducible ES cell line and selected by G418. The final line was derived from the picking of a single-cell clone colony.

ACKNOWLEDGMENTS. V.C.L. thanks R. Jaenisch, A. Klein, and S. Gruver for discussions and S. Oh for microscopy assistance. This work was supported by National Institute of General Medical Sciences Grant GM26875 (to M.W.K.).

1. Muñoz-Alonso M, León J (2003) G1 phase control and cell differentiation. *G1 Phase Progression*, ed Boonstra J (Landes Bioscience, Georgetown, TX), pp 236–264.
2. Budirahardja Y, Gönczy P (2009) Coupling the cell cycle to development. *Development* 136(17):2861–2872.
3. Hindley C, Philpott A (2012) Co-ordination of cell cycle and differentiation in the developing nervous system. *Biochem J* 444(3):375–382.
4. Li VC, Ballabeni A, Kirschner MW (2012) Gap 1 phase length and mouse embryonic stem cell self-renewal. *Proc Natl Acad Sci USA* 109(31):12550–12555.
5. Thoma EC, Maurus K, Wagner TU, Schartl M (2012) Parallel differentiation of embryonic stem cells into different cell types by a single gene-based differentiation system. *Cell Reprogram* 14(2):106–111.
6. Bentzinger CF, Wang YX, Rudnicki MA (2012) Building muscle: Molecular regulation of myogenesis. *Cold Spring Harb Perspect Biol* 4(2):a008342.
7. Keller G (2005) Embryonic stem cell differentiation: Emergence of a new era in biology and medicine. *Genes Dev* 19(10):1129–1155.
8. Gianakopoulos PJ, et al. (2011) MyoD directly up-regulates premyogenic mesoderm factors during induction of skeletal myogenesis in stem cells. *J Biol Chem* 286(4): 2517–2525.
9. Niro C, et al. (2010) Six1 and Six4 gene expression is necessary to activate the fast-type muscle gene program in the mouse primary myotome. *Dev Biol* 338(2):168–182.
10. Galli LM, et al. (2008) Identification and characterization of subpopulations of Pax3 and Pax7 expressing cells in developing chick somites and limb buds. *Dev Dyn* 237(7): 1862–1874.
11. Venuti JM, Morris JH, Vivian JL, Olson EN, Klein WH (1995) Myogenin is required for late but not early aspects of myogenesis during mouse development. *J Cell Biol* 128(4):563–576.
12. Deng X, Ewton DZ, Pawlikowski B, Maimone M, Friedman E (2003) Mirk/dyrk1B is a Rho-induced kinase active in skeletal muscle differentiation. *J Biol Chem* 278(42): 41347–41354.
13. White J, et al. (2005) Developmental activation of the Rb-E2F pathway and establishment of cell cycle-regulated cyclin-dependent kinase activity during embryonic stem cell differentiation. *Mol Biol Cell* 16(4):2018–2027.
14. Odorico JS, Kaufman DS, Thomson JA (2001) Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* 19(3):193–204.
15. Hall BK (1981) The induction of neural crest-derived cartilage and bone by embryonic epithelia: An analysis of the mode of action of an epithelial-mesenchymal interaction. *J Embryol Exp Morphol* 64:305–320.
16. Ordahl CP, Le Douarin NM (1992) Two myogenic lineages within the developing somite. *Development* 114(2):339–353.
17. Ali F, et al. (2011) Cell cycle-regulated multi-site phosphorylation of Neurogenin 2 coordinates cell cycling with differentiation during neurogenesis. *Development* 138(19):4267–4277.
18. Kim YC, et al. (2011) RB regulates pancreas development by stabilizing Pdx1. *EMBO J* 30(8):1563–1576.
19. Matsuura I, et al. (2004) Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* 430(6996):226–231.
20. Carreira S, et al. (2005) Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. *Nature* 433(7027):764–769.
21. Thomas DM, et al. (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol Cell* 8(2):303–316.
22. Rekhtman N, et al. (2003) PU.1 and pRB interact and cooperate to repress GATA-1 and block erythroid differentiation. *Mol Cell Biol* 23(21):7460–7474.
23. Hanse EA, et al. (2012) Cyclin D1 inhibits hepatic lipogenesis via repression of carbohydrate response element binding protein and hepatocyte nuclear factor 4α. *Cell Cycle* 11(14):2681–2690.
24. Chen PL, Riley DJ, Chen Y, Lee WH (1996) Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes Dev* 10(21):2794–2804.
25. Singh AM, Dalton S (2009) The cell cycle and Myc intersect with mechanisms that regulate pluripotency and reprogramming. *Cell Stem Cell* 5(2):141–149.
26. Mallanna SK, Duncan SA (2013) Differentiation of hepatocytes from pluripotent stem cells. *Curr Protoc Stem Cell Biol* 26:Unit 1G.4.
27. Lian X, et al. (2013) Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nat Protoc* 8(1):162–175.
28. Oldershaw RA, et al. (2010) Directed differentiation of human embryonic stem cells toward chondrocytes. *Nat Biotechnol* 28(11):1187–1194.
29. Lu SJ, Feng Q, Park JS, Lanza R (2010) Directed differentiation of red blood cells from human embryonic stem cells. *Methods Mol Biol* 636:105–121.
30. Hosoya M, Kunisada Y, Kurisaki A, Asashima M (2012) Induction of differentiation of undifferentiated cells into pancreatic beta cells in vertebrates. *Int J Dev Biol* 56(5): 313–323.
31. Albini S, et al. (2013) Epigenetic reprogramming of human embryonic stem cells into skeletal muscle cells and generation of contractile myospheres. *Cell Reports* 3(3): 661–670.