Unraveling Growth Factor Signaling and Cell Cycle Progression in Individual Fibroblasts*§

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Cultured cells require the actions of growth factors to enter the cell cycle, but how individual members of a population respond to the same stimulus remains unknown. Here we have employed continuous monitoring by live cell imaging in a dual-reporter cell model to investigate the regulation of short-term growth factor signaling (protein kinase B (PKB/Akt) activity) and longer-term progression through the cell cycle (cyclin-dependent kinase 2 activity). In the total population, insulin-like growth factor-I (IGF-I)-enhanced cell cycle entry by >5-fold compared with serum-free medium (from 13.5 to 78%), but at the single cell level we observed a broad distribution in the timing of G1 exit (4–24 h, mean ~12 h) that did not vary with either the amount or duration of IGF-I treatment. Cells that failed to re-enter the cell cycle exhibited similar responses to IGF-I in terms of integrated Akt activity and migration distance compared with those that did. We made similar observations with EGF, PDGF-AA, and PDGF-BB. As potential thresholds of growth factor-mediated cell cycle progression appeared to be heterogeneous within the population, the longer-term proliferative outcomes of individual cells to growth factor stimulation could not be predicted based solely on acute Akt signaling responses, no matter how robust these might be. Thus, although we could define a relationship at the population level between growth factor-induced Akt signaling dynamics and cell cycle progression, we could not predict the fate of individual cells.

Movement through the cell cycle is controlled at multiple stages. Extensive studies in cultured cells have identified a key regulatory step termed the restriction point, which was initially defined as the time after which the presence of growth factors was no longer needed for cell cycle progression (1–3). From a more modern perspective, the restriction point represents commitment to subsequent DNA replication (S phase), and appears to coincide with a buildup of enzymatic activity of cyclin-dependent kinase 2 (Cdk2)2 (4, 5). In mechanistic terms, the restriction point is accompanied by progressive inactivation of the retinoblastoma protein, pRb, caused by an increase in Cdk activity and a decline in Cdk inhibitor abundance (4–8). Consequently, the transcription factor E2F is released from pRb inhibition (7). At or near the restriction point, this combination of effects promotes enhanced transcription of Cyclin E and Cdk2 genes by E2F, further inactivation of pRb by active Cdk2, and additional degradation of Cdk inhibitors (7, 9), such that the transition to S phase becomes rapid and irreversible (10, 11). The collection of events leading to cell cycle commitment at the restriction point functions as a bi-stable switch (7, 9–11). As a consequence, a threshold of signaling activity must be attained to promote the events that activate the switch; anything below the threshold will not result in cell cycle progression.

The earliest studies of the restriction point in mammalian cells separated the actions of different growth factors based on their ability either to make cells competent for the cell cycle, or to promote progression past the restriction point (12–14). From work with the mouse Balb/c 3T3 fibroblast cell line, PDGF was defined as a competence factor, and IGF-I and EGF were shown to be progression factors (12–14). Subsequent studies found that these definitions could not be generalized to other cultured cells (3, 7, 9, 15), and additional experiments demonstrated that depending on the cell type, short incubations with individual growth factors at two distinct time points separated by several hours were sufficient to drive S phase entry (9, 15). Other studies identified a second restriction point in non-quiescent cells that occurs in the preceding cell cycle (4).

More recently, advances in live-cell imaging have been applied to understand the steps required to cross the restriction point and enter S phase (5, 8, 11). It has become clear from these studies that the average measurements of a population tend to obscure the range of responses seen in single cells, and thus hide important differences about how within-population heterogeneity influences long-term outcomes. Results also have revealed how single time point measurements may limit the understanding of dynamic behaviors that occur at different times in different cells as they transit through the cell cycle (4, 5). Despite advances in fundamentals of cell cycle control described in these publications, the observations collectively

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1 This article contains supplemental Movies S1–S6.

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2 The abbreviations used are: Cdk2, cyclin-dependent kinase 2; IGF-I, insulin-like growth factor-I; pRb, retinoblastoma protein; HDHB, human DNA helicase B; SFM, serum-free medium.
have provided only limited insights into how growth factors influence cell cycle progression.

Here we have developed an experimental cell system that allows simultaneous tracking in real time of initial and longer-term responses to different growth factors, which we have applied to assess cell cycle progression in C3H10T1/2 fibroblasts. We find that at the population level there are clear dose- and time-thresholds for individual growth factors to promote the G1 to S phase transition, but that in single cells these thresholds are variable. As a consequence, although growth factor signaling activity correlates with cell cycle progression in the overall population, it does not in individual cells. Hence an acute signaling response cannot be used to determine if a cell will commit to S phase. Thus, our studies reveal how growth factor signaling is encoded into cell cycle progression at the population level but not in single cells.

Results

Analyzing Akt and Cdk2 Signaling in Individual Cells—Growth factors play a key role in cell proliferation, but their actions have been found to be temporally limited (1–3, 15). Before reaching the restriction point in the G1 phase of the cell cycle, growth factors are required; after it, cell cycle movement to S phase is growth factor independent. However, it is unclear if individual cells show behaviors similar to the entire population with regard to responsiveness to growth factor signaling and cell cycle progression. To address this issue, we developed an experimental system in C3H10T1/2 fetal fibroblasts that allows simultaneous tracking of growth factor actions in individual cells, as assessed by activity of the Akt protein kinase (16, 17), and cell cycle progression, as defined by Cdk2 activity. To dynamically measure Akt signaling, we monitored the real time subcellular distribution of a chimeric molecule composed of the Akt substrate, FoxO1, and the green fluorescent protein, clover (FoxO1-clover (16) (Fig. 1A). Increased Akt activity causes nuclear to cytoplasmic translocation of the reporter molecule (16). For tracking different phases of the cell cycle, we used a protein consisting of a segment of the Cdk2 substrate, human DNA helicase B (HDHB), fused to the red fluorescent protein, mKate2 (mKate2-HDHB, Ref. 4) (Fig. 1B). In response to increased Cdk2-mediated phosphorylation during the G1 to S phase transition, HDHB localizes from the nucleus to the cytoplasm (4, 11) (Fig. 1, C and D).

To test the effects of growth factors on cell cycle progression, we first analyzed cells incubated in medium with 10% FBS. In this treatment protocol, each individually tracked cell progressed through mitosis with average cell cycle duration of 19.4 ± 0.7 h. In >80% of the cells analyzed, the mKate2-HDHB reporter relocated from the nucleus to the cytoplasm within 6.5 h after mitosis (Fig. 1D, black circles mark S phase); under these conditions the average duration of G1 was 5.5 ± 0.4 h (range of 2.25 to 19.5 h, Fig. 1E).

To test the effects of a growth factor on both sensor proteins, cells first were incubated for 24 h in SFM to promote cell cycle arrest in early G1. This resulted in 82.7 ± 2.5% of cells being in the G0/G1 phase of the cell cycle, as assessed by nuclear localization of the mKate2-HDHB reporter (4, 11). SFM or IGF-1 was then added, and cells were tracked at 15-min intervals for the next 24 h. In the continuing presence of SFM, the mKate2-HDHB reporter remained predominantly nuclear for 24 h in 22/25 cells analyzed (Fig. 2A). In contrast, after addition of IGF-I, the reporter was retained in the nucleus in only 5/25 cells (Fig. 2, B and C, Supplemental Movie S1). FoxO1-clover was exported to the cytoplasm in cells incubated with IGF-I, and was maintained there in nearly all cells for the duration of the experiment (Fig. 2C, lower panels). Thus, using our dual reporter cell line, we can simultaneously track Akt activity and cell cycle reentry.

IGF-I Signaling Duration and Cell Cycle Progression Are Correlated in the Population, but Not at the Single Cell Level—To test the time dependence of growth factor actions on cell cycle progression, we incubated cells in SFM for 24 h, and then added IGF-I with or without the IGF-I receptor inhibitor, linsitinib (18), at 0, 3, 6, 9, or 12 h later. IGF-I promoted sustained Akt activity, leading to persistent nuclear to cytoplasmic relocation of FoxO1-clover (Fig. 3A). This was rapidly and completely inhibited by linsitinib (Fig. 3A), although the nuclear intensity of the reporter molecule was apparently greater the later that linsitinib was added, possibly reflecting changes in cell shape and in rates of protein synthesis from prolonged stimulation by IGF-I. Linsitinib also decreased the percentage of cells that moved from the G1 to the S phase of the cell cycle in a manner that was dependent on the time that it was added. Addition coincident with or 3 h after IGF-I prevented cell cycle progression in 80–90% of the population compared with no inhibitor, but addition at 6 h after IGF-I caused only a 43% decline. When linsitinib was added 9 or 12 h after IGF-I there was a minimal reduction in cell cycle progression compared with cells incubated solely with IGF-I (Fig. 3B). Our results thus indicate a restriction point in C3H10T1/2 cells that is dependent on the duration of growth factor signaling.

To separate population averages from single cell responses, we quantified cell cycle reentry in individual cells. This analysis revealed remarkable variability in the timing of the G1-S transition, with a range of <4 to 24 h after exposure to IGF-I (n = 104 cells, Fig. 3C). This broad distribution was not dependent on the duration of IGF-I receptor activity, since G1 to S phase transition times remained similar regardless of when linsitinib was added (Fig. 3C). Thus, the main effect of more sustained IGF-I action was to recruit more cells to transit to S phase, rather than altering the timing of cell cycle progression (Fig. 3, C and D).

In these experiments, 30.7 ± 8.7% (n = 3 experiments) of cells did not undergo the transition from G1 to S phase when incubated with IGF-I (Fig. 3B, dark blue bar). These cells could have been unresponsive to growth factor stimulation. To test this idea, we compared the integrated Akt signaling activity of cells that exited G1 with those that did not. Although Akt activity varied among individual fibroblasts, there were no consistent differences between cells that remained quiescent and those that entered the cell cycle (Fig. 3D). Note that signaling activity accumulated at a relatively steady rate over the course of 24 h in cells that entered the cell cycle, and that there was no direct relationship with timing of G1 exit (Fig. 3F). We thus found that the rate or extent of IGF-I-initiated Akt signaling in
a single cell was not strictly linked to its progression through the cell cycle.

Threshold Effects of IGF-I Signaling Amplitude on Cell Cycle Progression—At sub-maximal growth factor concentrations, signaling effects might accumulate in cells over time and eventually trigger a longer-term outcome such as cell cycle progression. Alternatively, each individual cell may have its own signaling threshold, which must be overcome to promote the G1 to S phase transition. To distinguish between these two possibilities, cells were incubated for 24 h with SFM, and then IGF-I plus different concentrations of linsitinib were added concomitantly. Under these conditions, IGF-I robustly stimulated Akt activity, as measured by an initial 80% decline in nuclear levels of the FoxO1-clover reporter within 30 min compared with little effect of SFM (Fig. 4A). IGF-I also led to a 6.5-fold increase in the fraction of cells undergoing the G1 to S transition by 24 h compared with SFM, from 13.5 ± 2.9% versus 78.0 ± 5.2% (n = 4 experiments) (Fig. 4B). The IGF-I receptor inhibitor caused a dose-dependent reduction in both sustained Akt activity and in cell cycle progression that was particularly

FIGURE 1. Application of a live-cell imaging reporter for cell cycle progression. A, top: schematic of FoxO1-clover reporter protein showing locations of three consensus Akt phosphorylation sites (arrows) and the nuclear localization sequence (NLS) and nuclear export sequence (NES) of FoxO1; FP, fluorescent protein (see Ref. 16 for details). Bottom: diagram of the expected location of FoxO1-clover in cells with low Akt activity, where FoxO1 is predominantly nuclear, or high Akt activity, where FoxO1 is highly phosphorylated (P) and is primarily cytoplasmic. B, top: schematic of mKate2-HDHB (amino acids 994–1097) reporter protein showing locations of four consensus Cdk2 phosphorylation sites (arrows at residues 1005, 1021, 1048, 1058) and NLS and NES of HDHB; FP, fluorescent protein. Bottom: diagram of the expected location of the mKate2-HDHB reporter in cells with low cell cycle activity (e.g. during early G1 phase), where HDHB is not phosphorylated (P) and is predominantly nuclear, or high activity (e.g. during S-G2 phases), where HDHB is highly phosphorylated and is primarily cytoplasmic. C, image showing location of mKate2-HDHB in cells incubated in medium with 10% FBS. D, time course results for nuclear localization of the mKate2-HDHB reporter for each of 25 cells incubated in medium with 10% FBS. Each open black circle represents the time an individual cell entered S-phase, and each closed black circle depicts the time of mitosis. Individual cell traces were aligned beginning with the time of the preceding mitosis. E, line graph showing cell cycle progression for each of 50 individual cells analyzed for up to 40 h in medium containing 10% FBS. Cells have been aligned computationally beginning with the time since the preceding mitosis.
evident at the highest concentrations of linsitinib (Fig. 4, A and B). Thus, the results from Figs. 3B and 4B show that at the population level there is a duration and dose threshold of IGF-I-mediated signaling that must be met for a cell to progress from G1 to S phase.

Although the percentage of cells in the population that exited G1 and entered S phase declined with increased IGF-I receptor blockade, the time of the G1 to S phase transition remained dynamically broad, with a range of < 5 to 24 h regardless of the presence or dose of linsitinib (Fig. 4C). The main effect of more IGF-I receptor activity was an increase in the number of cells entering S phase without variation in the time of entry (Fig. 4, C and D). This observation argues against cumulative signaling activity being critical for cell cycle progression, since at higher effective growth factor doses this level should be reached sooner, resulting in an earlier G1 exit. Rather, it appears that a signaling threshold must be exceeded that differs among individual cells in the population.

To examine the concept of a signaling threshold for cell cycle progression in a second way, we analyzed IGF-I-mediated cell migration and compared it with effects on S phase entry after coincident treatment with varying concentrations of linsitinib (Fig. 5). Incubation with IGF-I promoted cell migration that varied in distance traveled from ~50 to >300 μm over 24 h (Fig. 5A). Under these conditions 22/25 cells entered S phase from 4–21 h after initiation of growth factor treatment (Fig. 4A, black circles). Linsitinib added at the same time as IGF-I caused a dose-dependent decline in integrated Akt signaling and in cell migration in the population as a whole (Fig. 5B). In single cells, however, there was not a strict relationship between the summed migration distance and cell cycle progression to S phase over 24 h. Although mean migration distance was diminished in the presence of linsitinib in the population, it varied substantially over this interval in individual cells, even at the highest inhibitor concentration (from <50 to >300 μm at [250 μM], Fig. 5C). Thus, as seen in Figs. 4 and 5, a cell can demonstrate both growth factor-stimulated migration and Akt activity, but not exhibit cell cycle progression.

Akt Signaling Dynamics and Cell Cycle Progression for Different Growth Factors—Growth factors differ in their potency as mitogens in the same cell types despite the presence of appropriate receptors (12–14). The mechanisms responsible for this variability are uncertain, but have been linked to the activity of various feedback and feed-forward signaling loops (19, 20). In several cultured cell lines, we have found that Akt signaling dynamics differed dramatically among growth factors during a 90-min incubation period (17). IGF-I produced sustained responses, effects of EGF were transient, and PDGF actions varied depending on the cell type and growth factor concentration (17). We thus investigated potential relationships between growth factor-encoded Akt signaling activity and cell cycle progression.

We initially quantified the subcellular localization of the FoxO1-clover reporter molecule in cells incubated in SFM for 24 h, and then treated for 24 h with IGF-I, EGF, PDGF-AA, or PDGF-BB (Fig. 6A). In cells maintained in SFM, FoxO1-clover remained predominantly nuclear for the entire tracking period, with the slight reduction in nuclear levels after 12 h possibly reflecting effects of growth factors produced and secreted by the cells (Fig. 6A, supplemental Movie S2). In contrast, addition of IGF-I caused sustained cytoplasmic relocation of the reporter (Fig. 6A, supplemental Movie S3). Long-term treatment with EGF was minimally different from SFM: after an initial transient movement of FoxO1-clover to the cytoplasm, the reporter protein became fully nuclear within 2 h, and remained predominantly nuclear for the duration of the 24 h...
EGF incubation (Fig. 6A, supplemental Movie S4). PDGF-AA caused a more prolonged nuclear to cytoplasmic translocation of FoxO1-clover than EGF in the population (Fig. 6A, supplemental Movie S5), although single cell behavior was quite varied (see below). As noted with IGF-I, exposure of cells to PDGF-BB also caused sustained Akt signaling responses, with > 50% of FoxO1-clover maintaining a cytoplasmic distribution at 24 h after growth factor addition (Fig. 6A, supplemental Movie S6), although the pattern differed from that seen with IGF-I, in which ~75% of the reporter was retained in the cytoplasm at 24 h.

Tracking the effects of different growth factors on the cell cycle, we found that the transition from G1 to S phase was modest in cells incubated with SFM or EGF (19.0 ± 7.6% versus 25.0 ± 7.0% (n = 4 experiments) over the 24-h observation period), but was 68–84% after exposure to PDGF-AA, IGF-I, or PDGF-BB (Fig. 6B). The results in Figs. 6, A and B show a correlation between a minimal level and/or duration of growth factor-mediated Akt signaling activity and the overall extent of cell cycle progression in the population. By contrast, analysis of individual cells failed to show a temporal relationship between the extent of growth factor actions and the timing of the G1-S transition, as it varied from <3 to ~24 h after growth factor exposure (Fig. 6C). This variability was not altered by any growth factor, since it was similar regardless of incubation type (Fig. 6C). Rather, at the level of individual cells, growth factors...
that induced larger and longer Akt signaling recruited more cells to reenter the cell cycle (Fig. 6D, compare EGF to PDGF-AA, PDGF-BB, or IGF-I).

Variability in the Temporal Patterns of Growth Factor-mediated Signaling in Individual Cells—Our analysis of single cells by live-cell imaging revealed that long-term responses to different growth factors were variable. Although population data after incubation in SFM showed that FoxO1-clover was maintained in the nucleus over a 24-h tracking period (Fig. 6A), in single cells the extent of sustained nuclear localization varied from minimal to >50% (Fig. 7A, supplemental Movie S2). A different dynamic was seen with PDGF-BB, in which the reporter was retained primarily in the cytoplasm for the first 12 h of growth factor exposure, but then showed substantial individual variability, with full relocation to the nucleus in some cells, complete cytoplasmic retention in others, and a more complex pattern in still others (Fig. 7B, supplemental Movie S6). In contrast, after treatment with IGF-I, the reporter was predominantly cytoplasmic for the entire 24-h tracking period, with only a few cells showing some nuclear re-localization (Fig. 7C, supplemental Movie S3). The most varied signaling pattern was observed for cells incubated with PDGF-AA. Growth factor addition led to a coordinated initial response of nuclear to cytoplasmic to nuclear translocation of FoxO1-clover that lasted for ~2.5 h, and that was followed by oscillatory patterns of cytoplasmic-to-nuclear-to cytoplasmic movement of the reporter (Fig. 7, D and E). These oscillations were observed in nearly all cells, continued for up to 24 h, and tended to become out of phase with one another (Fig. 7, D and E, supplemental Movie S5). Taken together, these results demonstrate remarkable plasticity in long-term PDGF-mediated signaling dynamics.

Growth Factor-mediated Migration Fails to Predict Individual Cell Cycle Progression—Other growth factors, in addition to IGF-I, can stimulate cell migration (19, 21). Compared with cells incubated in SFM for 24 h, PDGF-BB promoted ~4.5-times further distance traveled of individual fibroblasts (Fig. 8, A and B). Although there was a correlation in single cells between 24-h migration distance and growth factor-stimulated Akt activity as measured by integrated cytoplasmic localization of FoxO1-clover (Fig. 8C), again there was not a strict relationship between the summed migration distance over time and cell cycle progression through S phase (Fig. 8D). Taken together, these data add further support to the idea that growth factor-mediated cell cycle progression occurs via a threshold effect, and that although signaling activity correlates with migration and generally with cell cycle progression, they are not inextricably linked in individual cells.

Discussion

Non-cancerous cells require growth factor signaling to stimulate movement through the cell cycle, but how individual cells respond within a population remains unknown. Here we have
established a model to investigate how different growth factors can regulate both short-term Akt signaling and longer-term progression through the cell cycle. Using continuous monitoring by live cell imaging, we have found variable dose and time thresholds that limit IGF-I-mediated actions on cell cycle progression in individual members of a population. Signaling below these thresholds does not promote movement into S phase, and more surprisingly does not correlate with other more immediate effects of IGF-I signaling, including acutely stimulated Akt activity and growth factor-mediated cell migration. We made similar observations with EGF, PDGF-AA, and PDGF-BB. As these thresholds appear to be heterogeneous within a population, the longer-term proliferative outcomes of individual cells to growth factor stimulation cannot be predicted based solely on their acute or sub-acute Akt signaling responses, no matter how robust these may be. Thus, even though we could define a relationship at the population level between growth factor-induced Akt signaling dynamics and cell cycle progression, in which more extensive responses led to a larger fraction of the entire group being recruited into the cell cycle, we could not predict the fate of individual cells.

Growth Factor Signaling Increases the Fraction of Cells Entering S phase, but Does Not Alter the Rate or Timing of Cell Cycle Progression—In response to maximal concentrations of IGF-I, ~70–80% of cells re-entered the cell cycle after a period of quiescence achieved by incubation in SFM. However, ~20–30% of IGF-I-treated cells remained in G0-G1 (Figs. 3B, 4B, 6B). Potentially this latter subset had become unresponsive to growth factor stimulation, an assessment that could not be evaluated in prior studies that solely examined population responses (2, 3, 12–14). By using live cell imaging to track signaling in individual cells, we found that both groups showed equivalent induction of Akt activity and migratory behavior (Figs. 3D, 4D, and 5C). Generally similar results were observed after exposure of cells to PDGF-AA or PDGF-BB (Figs. 6D and 8D). Thus, although the lack of acute signaling responses as seen for EGF correlated with minimal growth factor-mediated cell cycle progression at the population level, (Figs. 6D and 8D), robust signaling did not predict the ability of a growth factor to promote the G1 to S phase transition in any individual cell.

The G1 Restriction Point Shows Temporal Variability—Cells treated with specific growth factors demonstrated a broad response with regard to timing of entry into S phase, ranging from ~2 to ~24 h after growth factor addition (Fig. 6C). At the single cell level, this range of times neither correlated with the strength of Akt signaling (Fig. 6D), nor with the extent of growth factor-stimulated migration (Fig. 8D), although in the overall population more robust Akt activity corresponded to a higher fraction of cells undergoing progression to S phase (Fig. 6D). In cells treated concurrently with IGF-I and an IGF-I receptor inhibitor, there was a similarly broad range of G1 exit times, even when cells incubated in the highest doses of lin-sitinib were compared with those treated with IGF-I alone (Fig. 4C). Varying the timing of IGF-I receptor inhibition additionally revealed that unabated exposure to IGF-I for ~9 h was needed to ensure maximal progression through the cell cycle in

FIGURE 5. IGF-I-mediated signaling dynamics correlate with cell migration. A, migration distance over time in microns for 25 cells incubated with IGF-I (500 pM) for 24 h. Each black circle represents when an individual cell entered S-phase. B, dot plot showing on the ordinate the 24-h integrated Akt signaling activity (cytoplasmic localization of FoxO1-clover) and on the abscissa the 24-h migration distance of individual cells incubated in SFM (purple), IGF-I (blue), and IGF-I plus lin-sitinib (50 nM, green); 100 nM, orange), or 250 nM, red). C, dot plot of the 24-h migration distance of individual cells (n = 200 cells/treatment group) treated as in B. Cells have been separated into those that entered S phase during the tracking period (+, left side) from those remaining in G1 (−, right side), based on redistribution of the mKate2-HDHB reporter molecule. Color-coding in B and C is identical.
the population as a whole, although a significant fraction responded to briefer treatment (Fig. 3B). Taken together, these results indicate not only that the signaling responses required to promote cell cycle progression cannot be predicted at the individual cell level but also that the restriction point shows temporal variability.

The mechanisms responsible for causing variable restriction points throughout a population remain unclear. Recent work suggests that alterations in the abundance of Cdk inhibitors, p21 and p27, may play a central role in creating this heterogeneity (5, 11). In the absence of p21, all MCF10A cells progressed through the cell cycle, whereas in cells re-expressing p21, approximately half of the population resumed cycling in response to EGF and half remained quiescent (11). In HeLa cells, rapid degradation of p27 mediated by Cdk2-CyclinE appeared to be the rate-limiting step for S phase entry (5), potentially because of growth factor dependent regulation of the Skp2-containing ubiquitin-proteasome complex, which targets p27 for destruction (22). Thus, within a cell population a range of concentrations of Cdk inhibitors may act as gatekeepers for the restriction point. Cells with less inhibitor may require less or shorter-term growth factor stimulation to progress through the cell cycle, while those with more may need larger or more prolonged growth factor activity.

Complex Signaling Dynamics Revealed—Patterns of Akt activity varied dramatically among the growth factors studied, as seen by analysis of long-term monitoring of the live-cell sensor, FoxO1-clover (Figs. 6A and 7, B–D). Cells treated with EGF showed only transient Akt activity at the population level, while the effects of IGF-I and PDGF-BB were more extensive and sustained. PDGF-AA exhibited an intermediate pattern (Fig. 6A). These results generally mirrored effects identified previously in cells exposed to growth factors for shorter time periods (17), and are consistent with other reports that used population averages to identify early and late phases of PDGF-BB signaling (15). Yet, at the individual cell level, we found that PDGF-AA, and to a lesser extent PDGF-BB, stimulated a cyclical pattern of signaling activity that was hidden in population averages (compare Fig. 7, D and E, and supplemental Movie S5 with Fig. 6A). The mechanisms responsible for this complex signaling behavior are unknown, but could be influenced by patterns of receptor translocation (23) or by the actions of signaling intermediates (e.g. negative feedback by Erk on the Gab1 adaptor, inhibitory receptor phosphorylation by PKC, or de-phosphorylation by PTP/SHP1/2 (19, 20). Alternatively, PDGF-α receptor activation by PDGF-AA could result in induction of other growth factors, thus driving a long-lasting autocrine signaling cascade (24).

Implications and Limitations of Dual-reporter Live-cell Imaging—Growth factor receptors are ligand-activated tyrosine kinases that typically control multiple downstream signaling pathways (19). It is likely that interactions among these cascades define the specifics of longer-term growth factor effects, including those that control progression through the cell cycle (25). For example, it is possible that, even though Erk and Akt pathways are both stimulated by upstream growth fac-

![FIGURE 6. Reporter dynamics and cell cycle progression after exposure of cells to different growth factors. A, time course of the relative nuclear intensity of the FoxO1-clover reporter in C3H10T1/2 cells incubated in SFM for 24 h, and then exposed to SFM, EGF (4.2 nM), PDGF-AA 1.4 nM), PDGF-BB (1.4 nM), or IGF-I (500 pM) for 24 h. Population means are presented (n = 150–200 cells per group). The nuclear intensity of the reporter in each cell was normalized to its value at the start of imaging. B, percentage of cells (mean ± S.E.; n = 4 independent experiments) that exited G1 during incubation with different growth factors for 24 h. C, time of G1 exit in each of 150–200 individual cells incubated with different growth factors. Each black horizontal line represents the mean time of G1 exit after initiation of growth factor treatment. D, integrated Akt signaling activity in individual cells measured during 24 h of growth factor exposure using the nuclear intensity of the FoxO1-clover reporter protein as in A. Vertical lines separate cells that entered S phase (+, left side) from those remaining in G1 (−, right side), based on redistribution of the mKate2-HDHB reporter molecule. Color-coding in B–D is identical to A.](image)
tor receptors, Erk activity can predict cell cycle progression in an individual cell but Akt activity cannot. It thus will be essential to develop other robust live-cell imaging readouts to define the full picture of how growth factor-mediated signaling dynamics are translated into distinctive cellular behaviors, and how these behaviors influence physiological processes and disease mechanisms. It also will be important to assess in cycling cells if growth factors have actions in the latter parts of the cell cycle (4, 5). It is likely that the relationships identified here will not be the same in all cells, because of variation in the types and amounts of different growth factor receptors, and because of variability in cell cycle regulatory components and other key molecules. From a more fundamental perspective, comprehensive live-cell imaging studies with multiple readouts, coupled with other biochemical and cell biological approaches at the single cell level, should allow better understanding of how signaling pathways are controlled in time and space to trigger discrete longer-term outcomes (26, 27).

**Materials**—Reagents for cell culture were purchased from Gibco-Life Technologies (Carlsbad, CA), including Dulbecco’s modified Eagle’s medium (DMEM), phosphate-buffered saline, FluoroBrite imaging medium, penicillin-streptomycin, and trypsin/EDTA solution. Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Growth factors were obtained from Gro- Pep (Adelaide, Australia) IGF-I analogue, R3-IGF-I, Invitrogen (Carlsbad, CA) recombinant human PDGF-BB, Gibco-Life Technologies mouse EGF, and Thermo Scientific (Rockford, IL), recombinant human PDGF-AA. Growth factors were dissolved in 10 mM HCl with 1 mg/ml bovine serum albumin, stored in aliquots at −80 °C, and diluted into FluoroBrite imaging medium immediately prior to use. Linsitinib (ApexBio, Houston, TX), was solubilized in DMSO, and diluted into imaging medium just prior to use. Cells were grown on 6-well tissue culture plates (Greiner, Monroe, NC). Restriction enzymes, buffers, ligases, and polymerases were purchased from Roche Applied Sciences (Indianapolis, IN) and BD Biosciences-Clontech (Palo Alto, CA). Other chemicals and reagents were from commercial suppliers.

**Production of Recombinant Lentiviruses and Creation of Stable Cell Lines**—Construction of the FoxO1-clover lentivirus and development of stably infected C3H10T1/2 cells were described previously (16). To produce the mKate2-HDHB len-
tiviral plasmid, DNA was first synthesized encoding codons 994–1087 of human DNA helicase B (4) plus recognition sites for restriction enzymes Bgl2 and Xho1 at the 5′/H11032 and 3′/H11032 ends, respectively (Invitrogen: Gene strings). The DNA fragment was digested and ligated in-frame to an mKate2 expression plasmid to generate the mKate2-HDHB fusion protein. A DNA fragment containing mKate2-HDHB was then digested, and ligated into the pWPXL lentiviral plasmid (Addgene, Cambridge, MA 12257), replacing EGFP. Concentrated lentivirus was prepared as described previously (28, 29).

C3H10T1/2 mouse embryonic fibroblasts (ATCC CCL226) stably expressing FoxO1-clover (16) were transduced with concentrated mKate2-HDHB lentivirus in the presence of 6 μg/ml polybrene. Seven days later, cells were sorted by green (excitation 488 nm, emission 530/40 nm) and red (excitation 561 nm, emission 615/24 nm) fluorescence intensity using a Becton-Dickinson Influx cell sorter at the Oregon Health & Science University Flow Cytometry Core Facility.

Live Cell Imaging—Live cell imaging was performed with an EVOS FL Auto microscope using a stage top incubator maintained at 37 °C and 5% CO2. Images were collected at 100× magnification using a 10× Fluorite objective (numerical aperture: 0.3), and GFP (excitation peak, 472/22 nm; emission peak, 510/42 nm) and Texas Red LED light cubes (excitation peak 585/29 nm; emission peak, 624/40 nm). Images were analyzed with NIH ImageJ plug-in Fiji (NIH, Bethesda, MD) using the Polynomial Fit plug-in to remove background fluorescence, the Stack Reg plug-in (rigid registration) to register images, and the Gaussian Blur plug-in (2-pixels) to average fluorescence across pixels. To follow both FoxO1-clover and mKate2-HDHB, nuclei of individual cells were manually tracked using the mTrackJ plug-in (30). The same tracking was used to quantify cell migration. Cells that died, divided, migrated out of frame, expressed only one of the two fluorescent reporters, or did not have nuclear localized mKate2-HDHB after 24 h in serum-free medium (SFM) were excluded from analysis. Nuclear intensities were normalized to values recorded at time 0 of each treatment protocol (see below). Exit from G0/G1 to S phase of the cell cycle was defined as the time when the nuclear intensity of the mKate2-HDHB reporter was less than its cytoplasmic intensity (4, 5). Integrated localization of FoxO1-clover was determined by summing relative differences in nuclear intensity from time 0 at 15-min intervals over a 24-h monitoring period.

Imaging Protocols—Cells expressing both reporter molecules were grown in DMEM containing 10% FBS for 24 h. After two washes with DMEM, cells were then incubated in serum-free Fluorobrite imaging medium containing penicillin and streptomycin for 24 h. One of three different treatment protocols was then initiated. In the first protocol, cells were incubated with IGF-I (500 pM) in serum-free Fluorobrite imaging medium at time 0; the IGF-I receptor tyrosine kinase inhibitor, linsitinib

FIGURE 8. Growth factor-induced signaling dynamics correlate with cell migration. A, migration distance in microns for 10 cells incubated in SFM for 24 h. B, migration distance in microns for 10 cells incubated in PDGF-BB (1.4 nM) for 24 h. C, dot plot showing on the abscissa the 24-h integrated cytoplasmic FoxO1-clover localization and on the ordinate the 24-h migration distance of individual cells incubated in SFM (purple) or PDGF-BB (1.4 nM, green). D, dot plot of the 24-h migration distance of individual cells (n = 150–200 cells per treatment group) treated with SFM (red), EGF (4.2 nM, orange), PDGF-AA (1.4 nM, green), PDGF-BB (1.4 nM, blue), or IGF-I (500 pm, dark blue). Cells have been separated into those that entered S phase during the tracking period (+, left side) from those remaining in G1 (−, right side), based on redistribution of the mKate2-HDHB reporter molecule.
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(250 nM), was added 0, 3, 6, 9, or 12 h later, and cells were imaged every 15 min for 24 h. In the second protocol, cells were incubated with IGFl-1 (500 pm) in serum-free Fluorobrite imaging medium in the presence of linsitinib (0, 50, 100, or 250 nM), and imaged every 15 min for 24 h. In the third protocol, cells were incubated with different growth factors in serum-free Fluorobrite imaging medium (PDGF BB (1.4 nM), PDGF AA (1.4 nM), IGFl-1 (500 pm), or EGF (4.2 nM)), and imaged every 15 min for 24 h. For all studies multiple experiments were performed. Data in the text are presented as the mean ± S.E.

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