Developmental Cell, Vol. 7, 21–32, July, 2004, Copyright ©2004 by Cell Press

ERK and MMPs Sequentially Regulate Distinct Stages of Epithelial Tubule Development

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Summary

Epithelial cells undergo tubulogenesis in response to morphogens such as hepatocyte growth factor (HGF). To organize into tubules, cells must execute a complex series of morphogenetic events; however, the mechanisms that underlie the timing and sequence of these events are poorly understood. Here, we show that downstream effectors of HGF coordinately regulate successive stages of tubulogenesis. Activation of extracellular-regulated kinase (ERK) is necessary and sufficient for the initial stage, during which cells depolarize and migrate. ERK becomes dispensable for the latter stage, during which cells repolarize and differentiate. Conversely, the activity of matrix metalloproteases (MMPs) is essential for the late stage but not the initial stage. Thus, ERK and MMPs define two regulatory subprograms that act in sequence. By inducing these reciprocal signals, HGF directs the morphogenetic progression of tubule development.

Introduction

Tubules are the architectural hallmark of epithelia such as the kidney, lung, and mammary gland. During embryogenesis, tubules develop in response to morphogenetic growth factors (Hogan and Kolodziej, 2002). Such factors induce a wide range of cellular behaviors, including migration, proliferation, differentiation, and polarization. To create a tubular architecture, these behaviors must be coordinated over space and time. How growth factor signaling achieves such coordination is fundamental to understanding how individual cells organize into complex structures.

Hepatocyte growth factor (HGF)-induced tubulogenesis of Madin-Darby canine kidney (MDCK) cells provides a cell culture system for analyzing tubule development (O'Brien et al., 2002). When embedded in a collagen matrix, MDCK cells form cysts, spherical monolayers of cells enclosing a central lumen (Figure 1A). HGF causes cysts to develop multicellular tubules, a response analogous to the induction of tubules by growth factors in vivo (Montesano et al., 1991a, 1991b).

HGF-induced MDCK tubulogenesis consists of two morphologically defined stages: a partial epithelial-mesenchymal transition and a subsequent redifferentiation (Figure 1A) (O'Brien et al., 2002). Epithelial-mesenchymal transition (EMT) involves the transformation of noninvasive, cuboidal epithelial cells into invasive, spindle-shaped mesenchymal cells. Cells undergoing EMT lose the apicobasolateral polarity characteristic of epithelia and acquire the leading edge-trailing edge polarity typical of motile cells (Hay and Zuk, 1995). During embryogenesis, EMT enables cells to populate and establish tissues in distant body regions, while during malignant tumor progression, EMT allows cancer cells to metastasize to other organs (Thiery, 2002).

Early in tubule development, MDCK cells exhibit many features characteristic of EMT (Figure 1A) (Pollack et al., 1998). Cells begin morphogenesis by forming long, invasive cytoplasmic extensions. Extensions next develop into single-file chains that are from one to three cells long. Cells in chains are invasive and elongated, like cells undergoing EMT. They have also lost apicobasolateral polarity and gained leading edge-trailing edge polarity. However, cells undergoing EMT lose all intercellular adhesion, while cells in chains do not. Thus, the process of extension and chain formation represents a partial EMT (p-EMT).

The p-EMT that initiates tubulogenesis is transient. To complete development, cells in chains redifferentiate (Pollack et al., 1998). They lose their mesenchymal qualities and form multilayered cords by expanding regions of cell-cell contact and reestablishing a cuboidal shape. Nascent lumens and incomplete apical and basolateral domains appear. Eventually, cords mature into tubules through formation of a single continuous lumen and coordinated apicobasolateral polarization of individual cells.

Both p-EMT and redifferentiation necessitate that cells modulate their motility, polarity, morphology, and adhesion. How does HGF elicit this complex response? The biphasic nature of tubulogenesis raises the possibility that distinct HGF effectors control each developmental stage.

One critical HGF effector is the MAP kinase pathway Raf-MEK-ERK. This cassette transduces signals from the HGF receptor Met to the nucleus. Constitutive extracellular-regulated kinase (ERK) activation is essential for complete EMT in both MDCK cells and in vivo models of epithelial tumor metastasis (Janda et al., 2002; Lehmann et al., 2000; Montesano et al., 1999; Oft et al., 2002; Schramek et al., 1997). Furthermore, the ERK pathway is essential for MDCK tubulogenesis (Khwaja et al., 1998). However, its role during tubule formation is unknown.

Matrix metalloproteases (MMPs) represent another class of HGF effectors. In epithelia, HGF has been reported to upregulate numerous MMPs (Balkovetz et al.,

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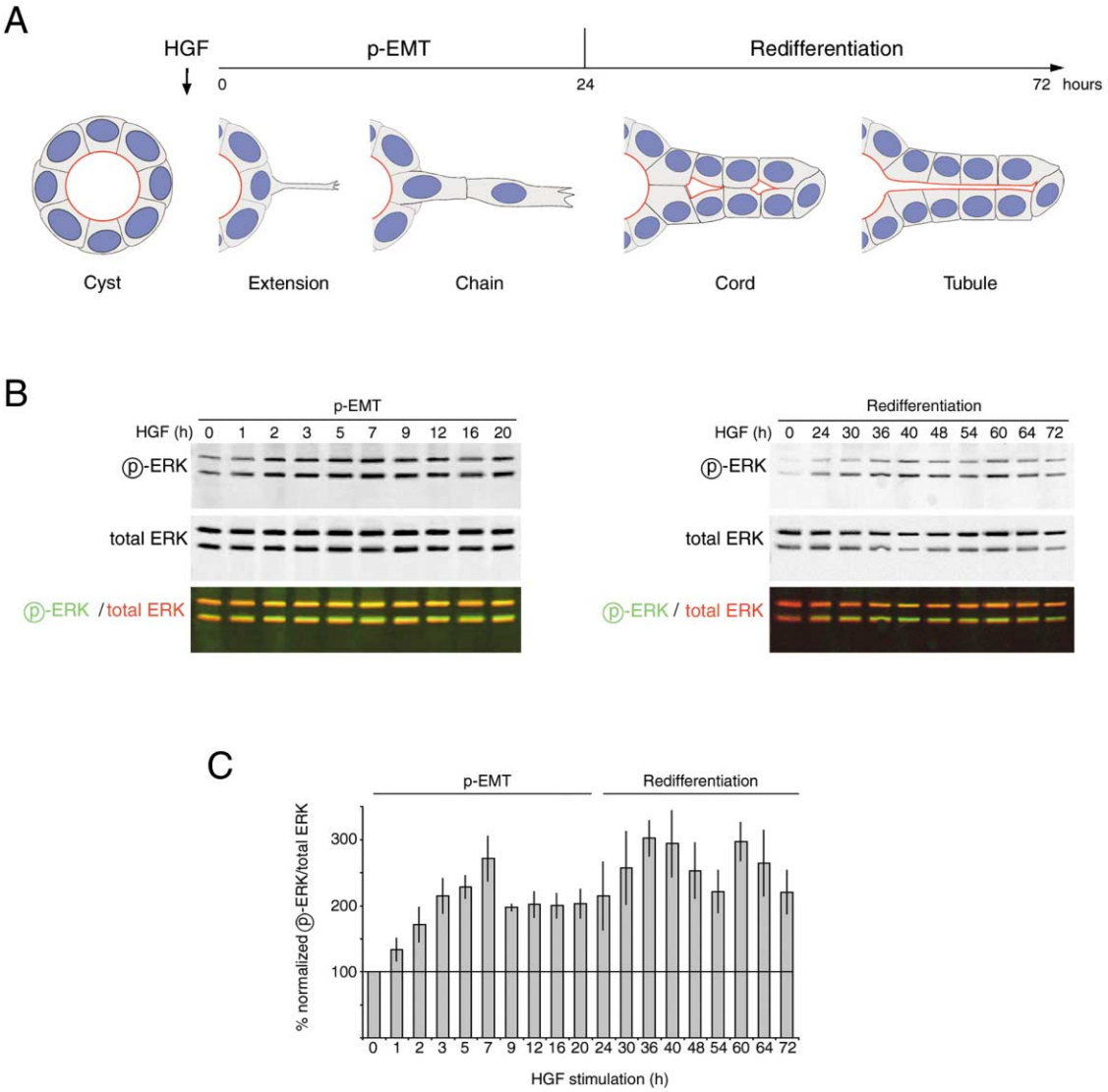


Figure 1. Kinetics of ERK Activation during HGF-Induced Tubulogenesis

(A) Stages of HGF-induced MDCK tubulogenesis. In cross-section, spherical MDCK cysts appear as a monolayer of cells surrounding a central lumen. The apical plasma membrane (red) faces the lumen. HGF causes cysts to form tubules through a series of stereotyped events. The initial p-EMT stage lasts approximately 24 hr. During p-EMT, cells form cytoplasmic extensions that develop into single-file chains. Apical surfaces are absent from these chains. The subsequent redifferentiation stage lasts approximately 48 hr. During redifferentiation, chains evolve into multilayered cords with nascent lumens and incomplete apical surfaces. Some cords develop into mature tubules, which are characterized by continuous lumens and complete apicobasolateral polarity.

(B) ERK activation during p-EMT and redifferentiation. Activated, phosphorylated ERK and total ERK were assessed in lysates of HGF-treated MDCK cysts at different times during tubule development. Phospho- and total ERK were detected simultaneously on the identical blot using two different antibodies. The antibodies were distinguished with distinct fluorophores. During p-EMT, phospho-ERK levels increase steadily in the first 7 hr (top left). From 9 to 20 hr, phospho-ERK decreases slightly but remains above baseline. Total ERK levels are relatively constant (middle left). Phospho-ERK levels stay elevated during redifferentiation (top right), although local fluctuations are apparent. Total ERK levels are still fairly constant (middle right). In overlays of phospho- and total ERK signals, phospho-ERK comigrates with the slower fraction of the total ERK population (bottom; green, phospho-ERK; red, total ERK; yellow, overlap).

(C) Quantification of ERK activation. The ratios of phospho-ERK to total ERK in tubulating cysts were quantified. The values shown are means ± SEM for three separate 0–20 hr experiments and three separate 24–72 hr experiments. Values are normalized relative to the phospho-ERK/total ERK ratio of untreated cysts. Following a 7 hr induction period, ERK activation levels fluctuate but remain significantly above baseline during both stages of tubulogenesis.

2004; Gong et al., 2003). MMPs are capable of remodeling extracellular matrix (ECM), influencing cell morphology, and altering the function of secreted growth factors. These abilities make them ideally suited to regulate morphogenesis. Indeed, MMPs are required during MDCK tubulogenesis (Hotary et al., 2000; Kadono et al., 1998). MMPs have been proposed to enable ECM invasion by developing tubules, but their exact role remains unclear.

Here, we examine the role of ERK and MMPs during HGF-induced MDCK tubulogenesis. We find that these

HGF effectors define reciprocal subprograms that regulate successive developmental stages. ERK activation is necessary and sufficient for p-EMT but dispensable for redifferentiation. Conversely, MMP activity is essential for the onset of redifferentiation but not required for ECM invasion during p-EMT. Thus, the two stages of tubulogenesis are mechanistically as well as morphologically distinct. Our results provide insight into the molecular mechanisms that control the organization of cells into tubules.

Results

HGF Activates ERK during Both p-EMT and Redifferentiation

Prior studies have shown that ERK signaling downstream of HGF is highly sustained; HGF activates ERK for 3–12 hr, in contrast to many growth factors that activate ERK for only 15–20 min (Liang and Chen, 2001; Paumelle et al., 2000; Potempa and Ridley, 1998). These studies used plastic-grown cells, which scatter in response to HGF after several hours. Because tubules develop in a three-dimensional ECM over multiple days, the kinetics of ERK activation during tubulogenesis are likely to differ.

To understand ERK function during tubulogenesis, we began by analyzing ERK activation kinetics in HGF-treated, tubulating cysts. Cysts were rapidly lysed in situ at different stages of tubule development. To precisely compare activated and total ERK, total ERK immunoprecipitates were immunoblotted simultaneously with two different antibodies. One antibody recognized phospho-Tyr204, an epitope specific to activated ERK. The other recognized a different epitope and was used to detect the total pool. These antibodies were distinguished with distinct fluorophores, and their signals were quantified with a LI-COR Odyssey Infrared Imager.

In response to HGF, levels of phospho-ERK rose steadily for 7 hr to a local maximum of 274% ± 34% above basal levels (Figures 1B and 1C). This initial peak coincides with the formation of nascent extensions that are the first manifestations of p-EMT (data not shown). After 7 hr, ERK activation declined but remained above 200% of baseline for the rest of development. During redifferentiation, phospho-ERK ranged from 215% ± 51% at 24 hr to 302% ± 27% at 36 hr, with local peaks at 36 and 60 hr (Figures 1B and 1C). Whether these oscillations are biologically significant or merely stochastic is unclear.

These data profile the activation of ERK by HGF during tubulogenesis. After a 7 hr stimulation phase, phospho-ERK levels remain 200%–300% above baseline during both p-EMT and redifferentiation. Thus, the activation kinetics of tubulating cysts are significantly slower and more sustained than the kinetics previously observed with plastic-grown cells.

p-EMT but Not Redifferentiation Requires Raf-MEK-ERK Activation

Since ERK is activated during both p-EMT and redifferentiation, we next examined whether ERK is required during each stage. Cysts treated for 24 hr with HGF developed extensions and chains characteristic of p-EMT (Figure 2B). Blocking the ERK activator MEK with the inhibitor U0126 abrogated HGF-induced p-EMT. Cysts treated for 24 hr with HGF and U0126 resembled untreated cysts (Figures 2A and 2C). Combined HGF and U0126 still produced no morphogenetic response after 72 hr, showing that MEK inhibition prevents p-EMT rather than delaying it (Figure 2G). Intermediate concentrations of U0126 partially inhibited p-EMT, and ERK activation levels correlated with the vigor of extension and chain formation (data not shown). PD98059, a distinct MEK inhibitor, produced identical effects (data not shown). These data indicate that p-EMT requires activation of ERK through MEK.

After 72 hr of HGF, cysts exhibited cords and tubules indicative of redifferentiation (Figure 2E). Strikingly, MEK inhibition did not affect this stage. Adding U0126 for the last 48 hr of development, after p-EMT had already occurred, did not visibly impact cord and tubule formation (Figure 2F).

The requirement for Raf-MEK-ERK during p-EMT but not redifferentiation implies that tubulogenesis involves a transition from ERK dependence to ERK independence. To define when this transition occurs, cysts were initially treated with HGF alone; after development had proceeded for 6, 12, or 18 hr, MEK was inhibited with U0126. MEK inhibition after 6 hr completely blocked p-EMT, indicating that extension formation requires more than 6 hr of ERK activation (Figure 2D). MEK inhibition after 12 or 18 hr did not prevent p-EMT or redifferentiation, but the resulting structures were fewer and smaller than normal (data not shown). These data suggest that ERK dependence gradually decreases during the latter half of p-EMT and is not completely lost until the onset of redifferentiation.

In summary, while Raf-MEK-ERK is activated during both p-EMT and redifferentiation, it is needed only for p-EMT. Initiation of p-EMT requires 6–12 hr of sustained activation. ERK dependence declines in the latter half of p-EMT, between 12 and 24 hr. ERK becomes dispensable after 24 hr, concomitant with the switch to redifferentiation.

Activation of Raf-MEK-ERK Is Sufficient for p-EMT

To further understand the role of ERK, we used a MDCK cell line in which ERK activation is conditionally regulated (Hansen et al., 2000). These cells stably express an inducible form of Raf-1 kinase, EGFPΔRaf-1:ER (Raf:ER). The Raf:ER construct is a fusion of the Raf-1 kinase domain, the estrogen receptor ligand binding domain (ER), and EGFP (Woods et al., 1997). Binding of the estrogen analog 4-hydroxytamoxifen (4-HT) to the ER moiety activates the Raf-1 domain, which leads to stimulation of MEK and ERK. When induced, Raf:ER accumulates in the cytoplasm and is detectable through fluorescence of the GFP moiety.

We examined whether Raf:ER induction is sufficient for p-EMT in the absence of HGF. After 24 hr of 4-HT, Raf:ER cysts fluoresced green (Figures 3D and 3J), confirming upregulation of the Raf:ER fusion protein. Furthermore, structures resembling HGF-induced extensions and chains had developed (Figures 3A, 3D, 3G, and 3J). Both HGF and Raf:ER extensions formed from

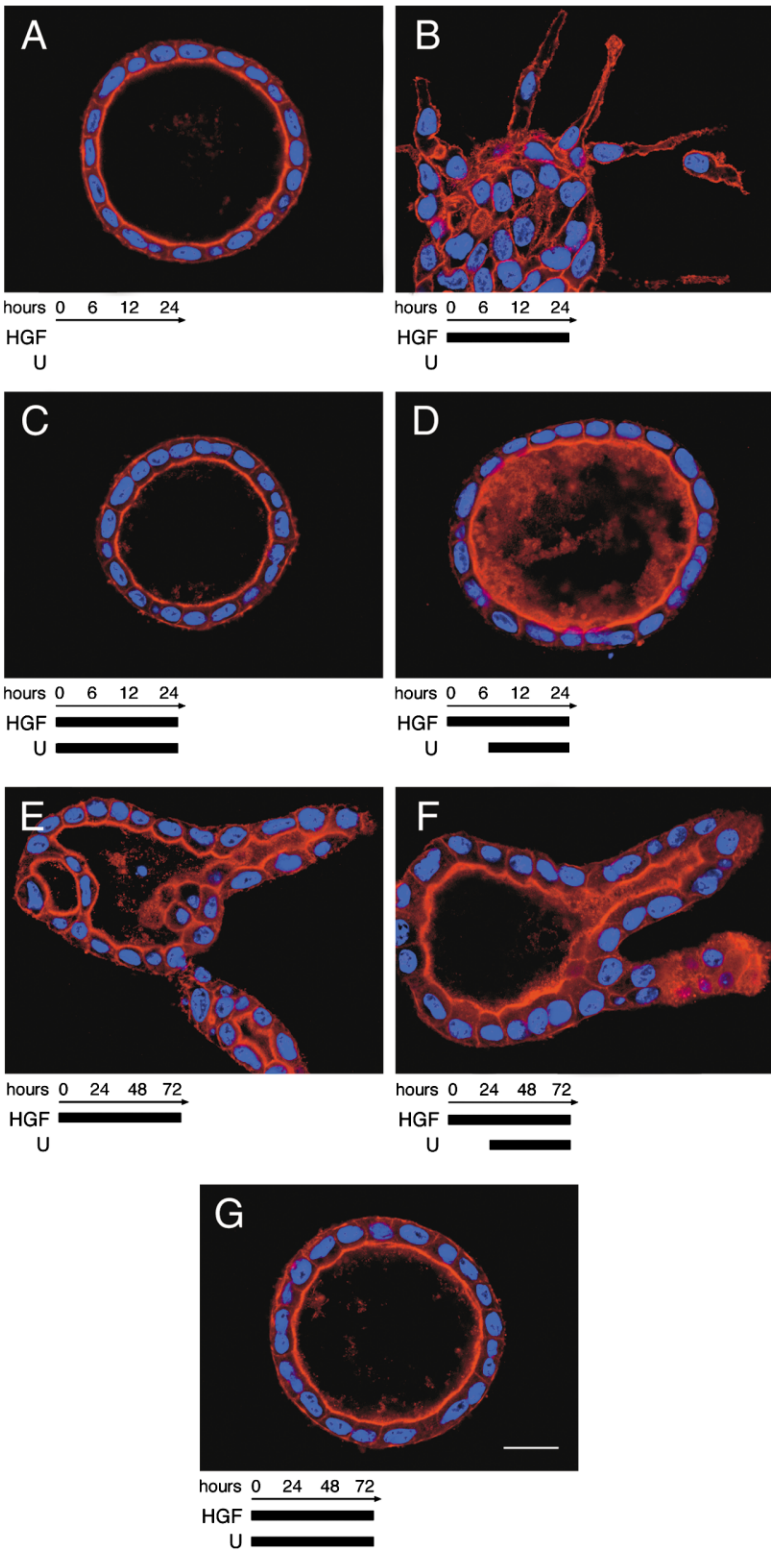


Figure 2. p-EMT but Not Redifferentiation Requires the Raf-MEK-ERK Pathway

MDCK cysts were treated with the MEK inhibitor U0126 during one or both stages of HGF-induced tubulogenesis and then stained for nuclei (blue) and actin (red) to identify individual cells. Intense actin staining at the lumenal surface represents apical microvilli, and cortical actin outlines basolateral surfaces. Single confocal optical sections are shown. (A) Cysts exhibit no tubulogenetic activity in the absence of HGF. (B) HGF treatment alone for 24 hr induces p-EMT, as evidenced by the development of extensions and chains. (C) Treatment with HGF and U0126 together for 24 hr prevents p-EMT. (D) Treatment with HGF alone for 6 initial hours followed by HGF and U0126 together for 18 subsequent hours also blocks p-EMT. (E) After 72 hr of HGF alone, the formation of cords and tubules indicates that tubulogenesis has reached the redifferentiation stage. (F) The addition of U0126 specifically during redifferentiation does not alter cord and tubule development. The middle of the cord is out of the plane of section. (G) The presence of U0126 during both p-EMT and redifferentiation completely blocks tubulogenesis. Scale bar, 20 μm.

the p58-positive basolateral domain, while a gp135-positive apical domain remained at the lumenal surface of extension-forming cells (Figures 3B, 3C, 3E, and 3F). Untransfected MDCK cysts did not fluoresce green or exhibit a detectable morphological response to 4-HT (data not shown).

Raf:ER cysts also formed chains. Like HGF chains, Raf:ER chains consisted of elongated cells (Figures 3G and 3J). Chain cells in both cases lacked apicobasolateral polarity; the apical marker gp135 was undetectable, and the basolateral marker p58 was nonpolar (Figures 3H, 3I, 3K, and 3L). HGF chains were often two to three

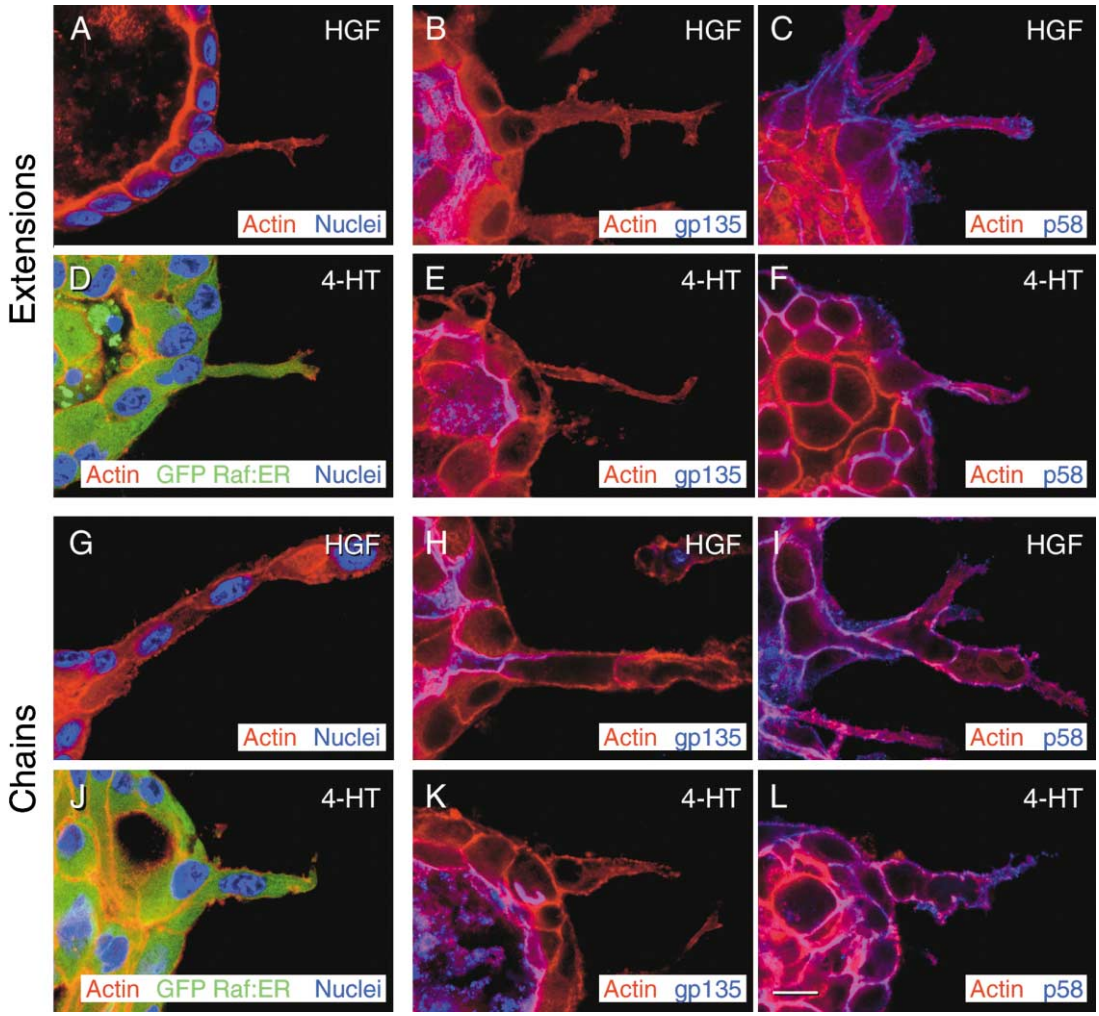


Figure 3. Exogenous Raf-1 Induction Is Sufficient for p-EMT

Cysts transfected with EGFPΔRaf-1:ER were treated for 24 hr with either HGF (A–C and G–I) or 4-HT (D–F and J–L). 4-HT activated the Raf:ER construct. The resulting structures were stained for actin (red; all panels) and either nuclei (blue; [A, D, G, and J]), the apical marker gp135 (blue; [B, E, H, and K]), or the basolateral marker p58 (blue; [C, F, I, and L]). Overlap between red and blue appears pink. (A–F) Morphology and polarity of HGF and Raf:ER extensions. (A and D) Raf:ER induction causes formation of cytoplasmic protrusions that resemble HGF-induced extensions. Green fluorescence in (D) indicates upregulation of Raf:ER. (B, C, E, and F) HGF and Raf:ER extensions exhibit similar polarity. In both cases, extension-forming cells retain an apical domain facing the cyst interior (B and E), while extensions develop from the basolateral membrane (C and F). In (E) and (F), cytoplasmic green fluorescence was observed but is not shown because it obscured the blue channel. (G–L) Morphology and polarity of HGF and Raf:ER chains. (G and J) Raf:ER induction also causes formation of structures that resemble HGF-induced chains. As in HGF chains, cells in Raf:ER chains are elongated; however, there are generally fewer cells per chain. (H, I, K, and L) HGF and Raf:ER chains exhibit similar polarity. In both cases, the apical marker gp135 is undetectable in chain cells, and the basolateral marker p58 is nonpolar. As in (E) and (F), green fluorescence was observed in (K) and (L) but is not shown. Scale bar, 10 μm.

cells long, while Raf:ER chains were usually one cell long. The shorter length of Raf:ER chains might reflect a lower rate of cell proliferation; whereas HGF is mitogenic for MDCK cells (Weidner et al., 1993), Raf:ER induction is not (Hansen et al., 2000). When treated simultaneously with 4-HT and the MEK inhibitor U0126, Raf:ER cysts fluoresced green but did not tubulate (data not shown), verifying that Raf:ER induces p-EMT through downstream MEK and ERK. These experiments used 1 μM 4-HT, which leads to ERK activation at levels approximately 3-fold higher than HGF (data not shown). Treatment with 10 nM 4-HT similarly induces p-EMT, but at levels of ERK activation that are comparable to HGF (data not shown).

These data show that Raf:ER upregulation induces p-EMT in the absence of HGF. Thus, the Raf-MEK-ERK pathway is not only activated by HGF and necessary for HGF-induced p-EMT, it is also sufficient for p-EMT. Taken together, these findings compellingly demonstrate that Raf-MEK-ERK is central to the p-EMT stage of tubulogenesis.

MMPs Control the Switch from p-EMT to Redifferentiation

We next sought to identify other regulators of tubule development. MMPs are essential for HGF-induced tubulogenesis (Hotary et al., 2000; Kadono et al., 1998) and have been proposed to enable developing tubules

to invade ECM. We thus examined whether MMPs are needed for invasion during p-EMT. Surprisingly, extensions and chains formed normally in the presence of the broad-spectrum MMP inhibitors BB-94 (Figures 4A and 4B), GM6001 (data not shown), or AG3340 (data not shown). These data are consistent with those of Montesano, Schaller, and Orci (Montesano et al., 1991b), who reported the appearance of "thin cytoplasmic processes" when HGF treatment was combined with an MMP-1 inhibitor. Several tumor cell lines are able to invade ECM without MMPs by changing from an elongated to an amoeboid shape (Sahai and Marshall, 2003; Wolf et al., 2003). In contrast, MMP inhibition does not alter the morphology of MDCK extensions and chains.

Equally unexpectedly, MMP inhibition prevented redifferentiation. Extensions and chains did not form cords and tubules but instead persisted in p-EMT after 72 hr of simultaneous treatment with HGF and either BB-94, GM6001, or AG3340 (Figures 4C and 4D and data not shown). Adding BB-94 after 24 hr of HGF alone produced a similar effect (data not shown).

Redifferentiation normally involves cell proliferation. After 72 hr of HGF, cyst cultures contain about twice as many cells as untreated cultures (data not shown). BB-94 inhibited proliferation in these cultures by 35% $\pm$ 5% ($n = 4$ samples from two independent experiments). Whether this reduced growth is a cause or a consequence of developmental arrest is currently unclear. Importantly, however, this antiproliferative effect specifically occurred in ECM; on plastic, BB-94 inhibited proliferation by only 3% $\pm$ 1% after 72 hr of HGF ($n = 4$ samples from two independent experiments). These data corroborate work by Hotary et al. (2003) and imply that MMPs specifically regulate morphogenesis in three dimensions.

Exogenous tissue inhibitors of metalloproteases (TIMPs) were used to further explore MMP function. TIMPs are cell-secreted proteins that bind and inhibit MMPs. Four mammalian TIMPs have been identified to date, although their specificity in vivo for different MMPs is largely unknown (Baker et al., 2002). We investigated the effects of recombinant TIMP-1, TIMP-2, and TIMP-3 on tubule formation. None of these TIMPs affected p-EMT when added either individually or as a combination of all three (Figures 4E and 4F and data not shown). Additionally, no single TIMP affected redifferentiation when added individually (Figure 4G and data not shown). In contrast, simultaneous addition of all three TIMPs prevented redifferentiation. After 72 hr, cysts either exhibited a few extensions or chains (Figure 4H) or completely lacked tubular structures. The specific block of redifferentiation by combined TIMPs thus resembles that of synthetic MMP inhibitors.

The requirement for MMPs during redifferentiation but not p-EMT might predict that MMP levels would peak during redifferentiation. In a recent microarray study of MDCK monolayers, MMP-13, a collagenase traditionally associated with skeletal development, emerged as a major downstream target of HGF (Balkovetz et al., 2004). HGF is also known to upregulate the gelatinase MMP-9 and the integral membrane protease MT1-MMP in kidney cells (Gong et al., 2003; Kadono et al., 1998). We therefore examined MMP-13 and -9 and MT1-MMP in tubulating cyst cultures by immunoblot. Both pro and active forms of MMP-13 increased throughout development and reached maximal levels during redifferentiation (Figure 4I). In particular, active MMP-13 grew from 220% $\pm$ 126% of baseline during p-EMT to 698% $\pm$ 188% during redifferentiation. By contrast, MMP-9 and MT1-MMP levels remained constant (data not shown). The coincidence of peak MMP-13 levels with redifferentiation is consistent with the requirement for MMPs at this stage and suggests that MMP-13 may have an important role.

Overall, these results indicate that the function of MMPs in HGF-induced tubulogenesis differs significantly from earlier models. MMPs are dispensable for ECM invasion during p-EMT. Instead, they enable the redifferentiation of developing tubules.

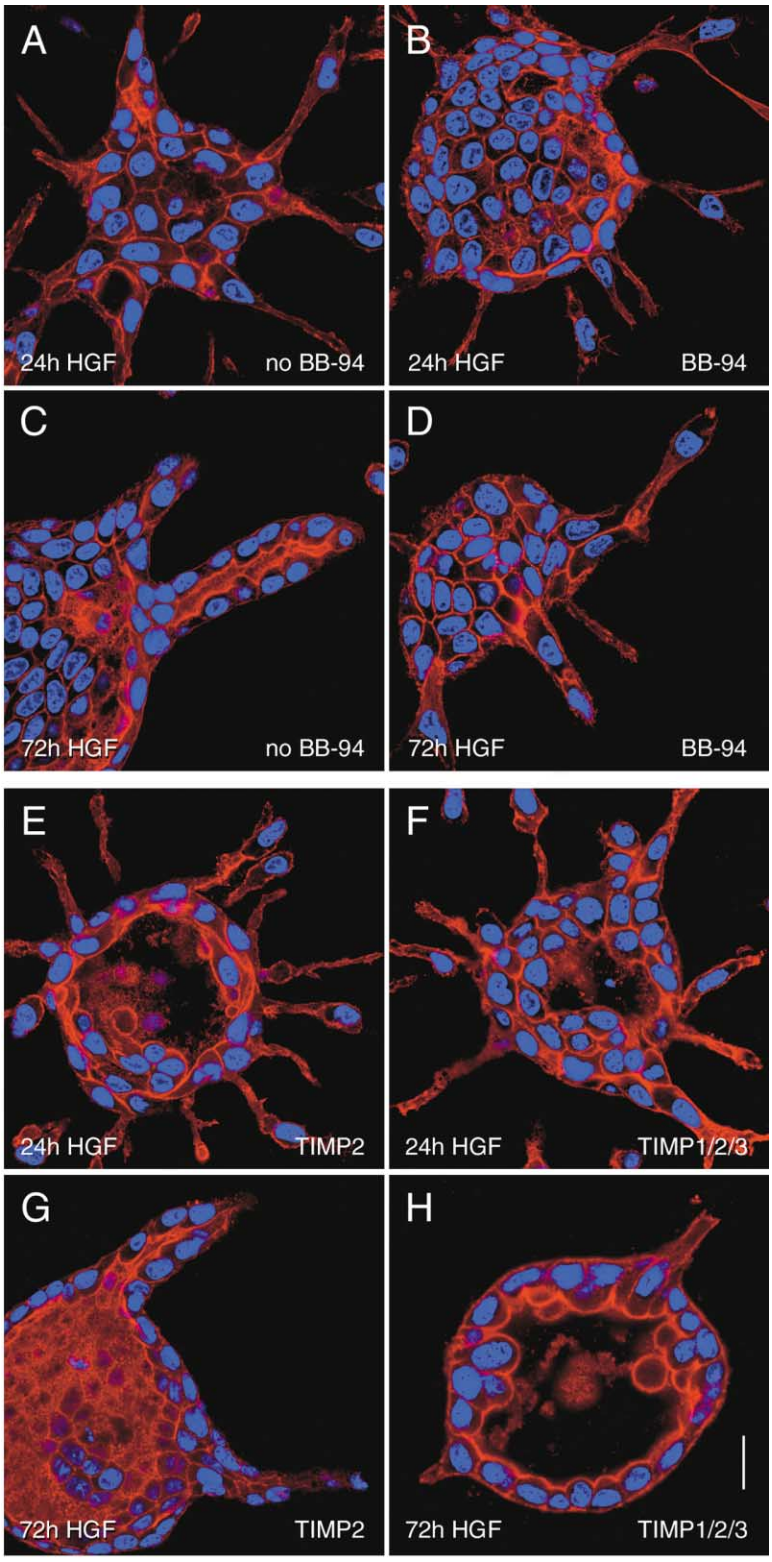
Raf:ER Downregulation Promotes Redifferentiation in the Absence of HGF

Thus far, we have shown that the ERK pathway controls p-EMT and that redifferentiation requires MMPs. To gain further insight into how redifferentiation is regulated, we examined the effects of prolonged, conditional ERK activation in Raf:ER cysts. Since Raf:ER induction is sufficient for p-EMT (Figure 3), we wanted to determine whether Raf:ER-induced p-EMT could progress to redifferentiation in the absence of HGF.

Raf:ER cysts fluoresced green after 72 hr of either 1 μM or 10 nM 4-HT, but no tubular structures were apparent (Figure 5G and data not shown). Instead, scattered single cells and clumps of two to three cells surrounded the cysts. These cells likely represented failed tubules that had lost contact with their cysts. Many dispersed cells exhibited membrane protrusions indicative of motility. They also lacked apicobasolateral polarity; the apical marker gp135 was absent or intracellular, and the basolateral marker p58 was nonpolar (Figures 5H and 5I). Thus, prolonged Raf:ER induction does not lead to redifferentiation.

We next tested whether downregulation of Raf:ER after extensions and chains had formed might allow these structures to redifferentiate. Raf:ER cysts were treated for 24 hr with 4-HT to induce p-EMT. The 4-HT was then washed out, and the cysts were analyzed 48 hr later. Washout cysts did not fluoresce green, indicating that Raf:ER had been downregulated (Figures 5J and 5M). Biochemical analysis confirmed that ERK activation returned to baseline 6–12 hr after washout (data not shown).

Washing out 4-HT allowed Raf:ER extensions and chains to redifferentiate into cords and tubules. Inhibiting MEK with U0126 after 24 hr of Raf:ER induction also led to redifferentiation, even with continual 4-HT, confirming that redifferentiation was due to MEK-ERK downregulation (data not shown). Like HGF cords, washout cords consisted of multilayered, cuboidal cells that had formed small, discontinuous lumens (Figures 5A and 5J). Some but not all cells in these cords exhibited distinct apical and basolateral domains (Figures 5B, 5C, 5K, and 5L). Like HGF tubules, washout tubules were cylindrical monolayers of cells surrounding a central lumen (Figures 5D and 5M). Each cell in these tubules was completely polarized (Figures 5E, 5F, 5N, and 5O). Washout cords and tubules developed at a lower frequency and were shorter than HGF-induced structures.



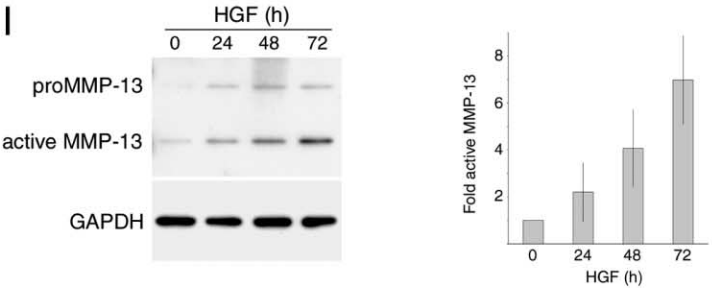


Figure 4. Redifferentiation but Not p-EMT Requires MMP Activity

MDCK cysts were treated with HGF and either the synthetic, broad-spectrum MMP inhibitor BB-94 (A–D) or TIMPs (E–H). The resulting structures were stained for actin (red) and nuclei (blue). Additionally, MMP-13 protein was examined in cyst cultures at different stages of development (I). (A–D) Inhibition of MMPs by BB-94 blocks redifferentiation. Cysts treated for 24 hr with HGF alone (A) or with HGF and BB-94 (B) exhibit extensions and chains characteristic of p-EMT. Cysts treated for 72 hr with HGF alone (C) exhibit cords and tubules characteristic of redifferentiation. Cysts treated for 72 hr with HGF and BB-94 (D) have no cords or tubules. Development is stalled in the p-EMT stage; only extensions and chains are present. (E–H) TIMP-1, -2, and -3 block redifferentiation when combined but not individually. Cysts exhibit normal p-EMT when treated for 24 hr with HGF and either TIMP-2 (E) or TIMP-1, -2, and -3 combined (F). Cysts form cords and mature tubules after 72 hr of HGF and TIMP-2 (G). In contrast, cysts exhibit no cords or tubules after 72 hr of TIMP-1, -2, and -3 combined (H). TIMP-2 was used at 200 ng/ml in (E) and (G), and TIMP-1, -2, and -3 were used at 200 ng/ml per TIMP in (F) and (H). TIMP-2 at 600 ng/ml and TIMP-1 alone or TIMP-3 alone at either 200 or 600 ng/ml also did not inhibit tubulogenesis (data not shown). Scale bar, 20 μm. (I) MMP-13 levels peak during redifferentiation. MMP-13 levels during tubulogenesis were assayed in solubilized cultures of HGF-treated cysts by immunoblot (left). The identities of pro and active forms were confirmed with a pro-specific antibody (data not shown). The fold increase of active MMP-13 is shown relative to untreated cysts (right). Values are the mean ± SEM of three independent experiments and are normalized against GAPDH. MMP-13 levels increase throughout development and reach peak levels during redifferentiation.

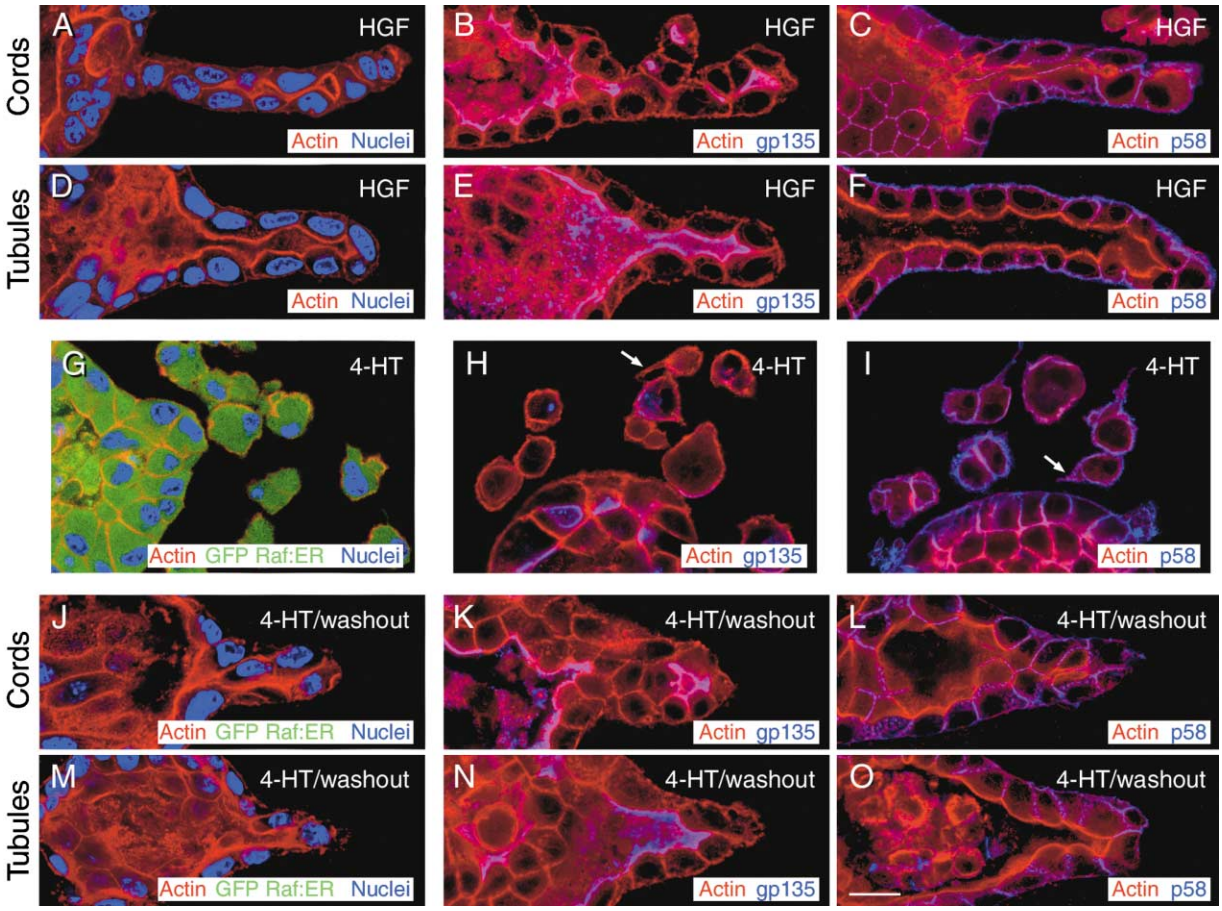


Figure 5. Downregulation of Raf:ER Promotes Redifferentiation

Raf:ER cysts were treated with HGF for 72 hr (A–F), 4-HT for 72 hr (G–I), or 4-HT for 24 hr followed by washout of 4-HT for 48 hr (J–O). The third treatment transiently activated Raf:ER for the initial 24 hr of the 72 hr experiment. The resulting structures were stained for actin (red; all panels) and nuclei (blue; [A, D, G, J, and M]), the apical marker gp135 (blue; [B, E, H, K, and N]), or the basolateral marker p58 (blue; [C, F, I, L, and O]). (A–F) Morphology and polarity of HGF-induced cords and tubules. HGF cords consist of multilayered, cuboidal cells with nascent lumens between the cell layers (A). Apical surfaces appear at the developing lumens (B) while basolateral domains have formed in some but not all cells (C). HGF tubules possess a single continuous lumen (D), and each cell exhibits distinct apical and basolateral domains (E and F). (G–I) Cell dispersion in cysts treated for 72 hr with 4-HT. Round, detached cells and clusters of two to three cells surround the cysts (G). The apical marker gp135 is largely absent or intracellular (H), and the basolateral marker p58 is nonpolar (I). Several dispersed cells exhibit filopodial structures indicative of motility (arrows in [H] and [I]). Cytoplasmic green fluorescence in (G) reflects Raf:ER activation. Green fluorescence was also observed in (H) and (I) but is not shown because it obscured the blue channel. (J–O) Morphology and polarity of cords and tubules formed after transient Raf:ER induction. Washout cords contain fewer cells than HGF cords but otherwise exhibit a similar morphology (J) and polarity (K and L). Likewise, washout tubules are shorter than HGF tubules, but their morphology (M) and polarity (N and O) are comparable. The absence of GFP fluorescence in (J) and (M) confirms downregulation of Raf:ER. Green fluorescence was also not observed in (K), (L), (N), and (O). Scale bar, 15 μm.

Since elongation requires cell proliferation (Yu et al., 2003), the difference in length may reflect a slower growth rate in 4-HT-treated cultures.

In summary, prolonged Raf:ER induction leads to cell dispersion. This finding contrasts with the activation of Raf-MEK-ERK throughout HGF-induced tubulogenesis and indicates that the redifferentiation stage has different tolerances for ERK activation depending on whether tubulogenesis is initiated by HGF or Raf:ER. By comparison, *transient* Raf:ER induction is sufficient to replicate the morphological steps of HGF-induced tubulogenesis. This result is consistent with the finding that ERK is essential for p-EMT but not redifferentiation during HGF-induced tubulogenesis. It also corroborates the idea that the primary role of ERK during tubule formation is the induction of p-EMT.

Redifferentiation Requires MMPs Even after ERK Downregulation

The switch from p-EMT to redifferentiation during HGF-induced tubulogenesis requires MMP activity (Figure 4). Since ERK remains upregulated at this switch, one possible scenario is that MMPs promote redifferentiation by antagonizing downstream effectors of ERK-induced p-EMT. If this were the case, then redifferentiation of Raf:ER extensions and chains should not require MMPs, because downregulation of ERK by 4-HT washout would preclude the need to antagonize ERK.

To test this possibility, we examined the effects of MMP inhibition on Raf:ER-induced tubulogenesis. As with HGF-induced tubulogenesis, MMPs were dispensable for p-EMT. The broad-spectrum MMP inhibitors BB-94 or AG3340 did not affect formation of extensions

and cords after 24 hr of 4-HT (Figures 6A and 6B and data not shown). Notably, MMPs were still required for redifferentiation. Extensions and chains remained in p-EMT 48 hr after 4-HT washout when BB-94 or AG3340 was present despite downregulation of Raf:ER (Figures 6C and 6D and data not shown).

Thus, MMP inhibition blocks redifferentiation whether tubulogenesis is initiated by Raf:ER or HGF. Since ERK activity is downregulated in the former case and sustained in the latter, this finding implies that MMPs do not simply antagonize ERK signaling during redifferentiation but instead fulfill a distinct role.

Discussion

Induction of epithelial tubules by growth factor signaling is a common theme in embryogenesis. Formation of these multicellular structures involves precise execution of a series of morphogenetic events. HGF-induced MDCK tubulogenesis provides a system for exploring the regulation of this complex process. Here, we have shown that distinct HGF effectors control successive stages of tubule development: p-EMT is ERK dependent and MMP independent, while redifferentiation is MMP dependent and ERK independent (Figure 6E). By inducing these complementary effectors, HGF directs the formation of a tubular architecture. We have further shown that ERK signaling is fundamental to the organization of cells into tubules. Exogenous, transient activation of Raf-MEK-ERK replicates the stages of HGF-induced tubulogenesis in the absence of HGF, and these Raf-induced stages also exhibit inverse ERK and MMP requirements. Collectively, our findings provide a mechanistic framework for understanding the morphogenetic events that create tubules.

Raf-MEK-ERK Signaling during Tubulogenesis

The Raf-MEK-ERK pathway is a crucial transducer of many growth factor signals, including HGF. We examined HGF-induced ERK activation in the context of a three-dimensional ECM. Compared to plastic, signaling kinetics in ECM differ in two key respects. First, the time required for ERK activation to reach maximal levels is much longer: 7 hr in cysts (Figure 1C) versus 15–60 min on plastic (Liang and Chen, 2001; Paumelle et al., 2000; Potempa and Ridley, 1998). Second, the time that ERK remains activated is also much longer: at least 72 hr in cysts versus 3–12 hr on plastic. EGF-induced ERK activation is also slower and more persistent in cysts than in plastic-grown cells (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/7/1/21/DC1 and data not shown). Thus, delayed, prolonged kinetics may be a general feature of growth factor signaling in ECM.

Different growth factors activate ERK for different lengths of time. The duration of the ERK signal can determine the phenotypic outcome of growth factor treatment (Rosario and Birchmeier, 2003). In cysts, the hypersustained nature of HGF-induced ERK activation is critical for tubulogenesis; indeed, p-EMT induction requires at least 6 hr of activity (Figure 2D). SH2-containing phosphatase (SHP-2) prolongs ERK activation by HGF on plastic (Maroun et al., 2000; Schaeper et al., 2000) and may function similarly in developing tubules. In contrast to HGF, EGF-induced ERK activation attenuates after 3 hr (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/7/1/21/DC1). This shorter signal may be at least partly responsible for the poor tubulogenetic response to EGF.

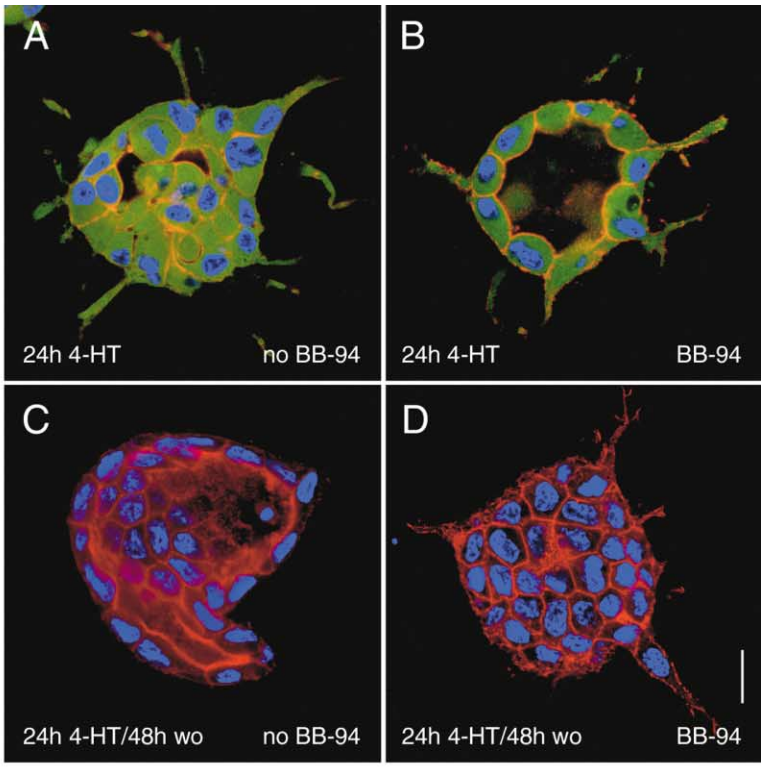
Raf-MEK-ERK and SHP-2 are far from the only transducers of HGF signaling. Adaptors such as Cbl and Gab1 and effectors such as PI-3 kinase and STAT3 also contribute (reviewed in Rosario and Birchmeier, 2003). Raf:ER cells enabled us to study the role of Raf-MEK-ERK in isolation, without other HGF-induced signals. Raf:ER induction is sufficient for p-EMT without HGF, and subsequent Raf:ER downregulation leads to redifferentiation (Figures 3 and 5). The necessity of downregulating Raf:ER to complete development contrasts with the activation of Raf-MEK-ERK during HGF-induced redifferentiation. We suggest that this difference likely reflects the absence of signals from other HGF effectors in the Raf:ER system. Crosstalk between one or more of these molecules and Raf-MEK-ERK could modulate the effects of sustained ERK activation.

The Biphasic Nature of Tubule Development

Morphological differences were the original basis for distinguishing the stages of p-EMT and redifferentiation (O'Brien et al., 2002). Here, we show that the successive actions of ERK and MMPs underlie these stage-specific phenotypes. The stepwise nature of this mechanism implies that ERK and MMPs define separate signaling modules for the two stages. The existence of these modules, coupled with the ability of ERK and MMPs to be upregulated by HGF, suggests an attractive explanation for how HGF elicits this complex developmental process—namely, by inducing distinct effectors that act sequentially.

The central role of Raf-MEK-ERK in tubulogenetic p-EMT complements its well-documented role in complete EMT. A key distinction between complete EMT and p-EMT is the retention of cell-cell contact in the latter; to create tubules, cells must be invasive enough to form chains but not invasive enough to break away from their neighbors. In the Raf:ER system, downregulation of Raf:ER is needed to prevent dispersal of cell chains, but how HGF-induced chains avoid dispersal is unclear. Interestingly, detached cells are frequently visible in the vicinity of HGF-induced tubules (see Figures 2B and 5C). Their presence indicates that misregulation of p-EMT and consequent loss of cell-cell contact may regularly occur.

The onset of redifferentiation is a critical transition during which cells cease p-EMT and reestablish an epithelial architecture. MMPs are essential for this switch, though how precise timing of the switch is determined is presently unclear. Levels of MMP-13 (and perhaps other MMPs) increase throughout tubulogenesis (Figure 4I), and one idea is that these MMPs trigger redifferentiation when their activities reach a critical threshold level. In osteoclasts, ERK is necessary for MMP-13 upregulation (Loeser et al., 2003; Yang et al., 2004). This finding raises the intriguing possibility that ERK activation during the first 24 hr of tubulogenesis may not only induce p-EMT but also lay the groundwork for redifferentiation by upregulating MMP-13.



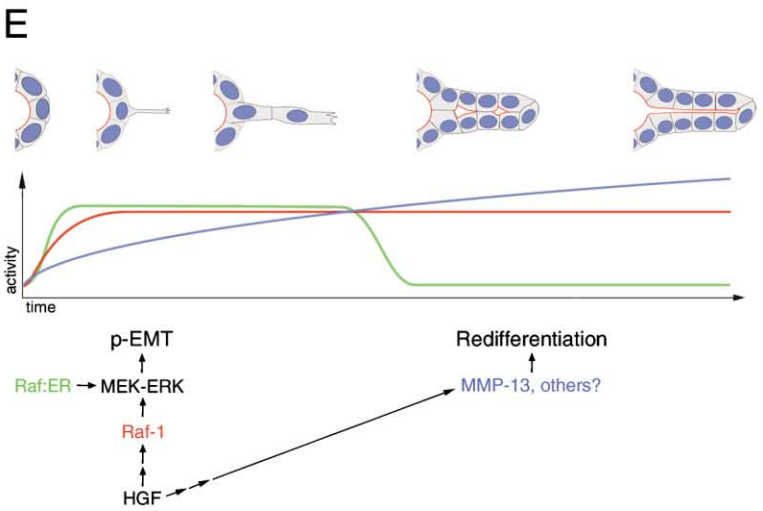


Figure 6. Redifferentiation after Raf:ER Washout Requires MMP Activity

(A–D) Effect of MMP inhibition on Raf:ER tubulogenesis. Raf:ER cysts were treated either with 4-HT alone or with 4-HT and BB-94 for 24 hr. Some samples were analyzed at this point, while other samples were subjected to 4-HT washout for 48 additional hours in the continued absence or presence of BB-94. Cysts were stained for actin (red) and nuclei (blue). (A) Cysts treated with 4-HT alone for 24 hr exhibit extensions and chains indicative of p-EMT. GFP fluorescence reflects Raf:ER induction. (B) BB-94 does not prevent Raf-induced p-EMT. (C) Treatment with 4-HT alone for 24 hr followed by washout for 48 hr leads to redifferentiation. Loss of GFP fluorescence confirms downregulation of Raf:ER. (D) BB-94 blocks washout-induced redifferentiation. Despite Raf:ER downregulation, tubulogenesis stalls in p-EMT. Only extensions and chains are present. Scale bar, 20 μm.

(E) Model. Regulatory modules during HGF and Raf:ER tubulogenesis. HGF activates Raf-MEK-ERK (red) to induce p-EMT. HGF-induced ERK activity is sustained throughout development but is dispensable for redifferentiation. HGF also upregulates MMPs (blue), which are necessary for redifferentiation but not p-EMT. Exogenous activation of MEK and ERK by Raf:ER (green) is sufficient for p-EMT; however, in the absence of HGF, ERK must be downregulated for redifferentiation.

MMPs could control redifferentiation by numerous means. Indeed, the distinct effects of individual and combined TIMPs may suggest that multiple MMPs are involved and that their roles are overlapping or redundant. MMPs have been implicated in growth control in ECM (Hotary et al., 2003) and may directly regulate the proliferation required for tubule formation. MMPs are also essential for lumen formation in mammary epithelial cysts and might have a similar role in tubulogenesis (Montesano and Soulie, 2002). A third scenario is that MMPs could remodel ECM during redifferentiation; in fact, MMP-13 is a collagenase.

HGF, ERK, and MMPs all have been implicated in epithelial organogenesis. During kidney development, the temporal and spatial expression patterns of HGF, Met, and an HGF-activating protease suggest a regulatory role, and HGF-blocking antibodies inhibit tubulogenesis of kidney explant cultures (reviewed in Rosario and Birchmeier, 2003). ERK is essential for development of the renal ureteric bud and the submandibular gland (Fisher et al., 2001; Kashimata et al., 2000). Finally, MMPs promote processes such as blood vessel formation and mammary ductile branching (Hiraoka et al., 1998; Sympson et al., 1994). Thus, tubulogenesis in vivo and MDCK tubulogenesis share key regulatory elements. This congruence may indicate that growth factor-induced ERK and MMP subprograms could underlie tubule development in other systems.

Proper timing and sequence of cellular events is crucial for successful morphogenesis of multicellular structures. Our results provide insight into the molecular mechanisms that orchestrate the creation of a tubular architecture. The sequential requirement for two classes of HGF effectors, the ERK pathway and MMPs, during the two stages of MDCK tubulogenesis illustrates how HGF can induce distinct regulatory subprograms that act at different times and effect divergent morphological outcomes. By dividing the complex process of tubule development into subprograms, these findings provide a basis for exploring the mechanism of tubulogenesis in vivo.

Experimental Procedures

Antibodies and Reagents

Primary antibodies were mouse anti-p58 (gift of Karl Matlin); mouse anti-gp135 (gift of George Ojakian); rabbit anti-ERK1/2 (Santa Cruz); mouse anti-phosphoERK1/2 (Santa Cruz); rabbit anti-MMP-13, anti-MMP-9, and anti-MT1-MMP (all from Triple Point); and mouse anti-GAPDH (Chemicon). Secondary antibodies were rabbit anti-mouse Alexa Fluor 647 (Molecular Probes); goat anti-mouse IRDye 800 (Rockland); goat anti-rabbit HRP (Jackson); and goat anti-mouse HRP (Jackson). Actin filaments were stained with Alexa Fluor 546 phalloidin (Molecular Probes). Nuclei were stained with TO-PRO3 or Hoescht (Molecular Probes). Reagents used were HGF (Genentech); 4-HT (Calbiochem); U0126 (Promega); BB-94 (British Biotech); AG3340 (Agouron); GM6001 (Chemicon); and recombinant, purified human TIMP-1, -2, and -3 (Chemicon).

Cell Culture

MDCK cells were maintained in MEM with 5% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. EGFPΔRaf-1:ER cells have been characterized extensively elsewhere (Hansen et al., 2000) and were maintained as previously described.

Growth of cysts in three-dimensional collagen I gels was performed as detailed previously (Pollack et al., 1998). To induce tubulogenesis, MDCK or Raf:ER cysts were treated with 10 ng/ml HGF for 24–72 hr. To induce Raf:ER activity, Raf:ER-transfected cysts were treated with 4-HT (1 μM except where indicated otherwise) for 24–72 hr. HGF- and 4-HT-containing media were refreshed each 24 hr period. To downregulate Raf:ER, cysts were washed extensively with media. Samples in particular experiments were also treated with U0126 (10 μM except where indicated otherwise), BB-94 (10 μM), AG3340 (20 μM), GM6001 (25 μM), or TIMPs (200 or 600 ng/ml).

Immunofluorescence and Confocal Microscopy

Cysts were processed for immunofluorescence and analyzed as previously described (O'Brien et al., 2001).

Immunoblotting

For ERK experiments, cyst-containing collagen gels were treated with phosphatase inhibitors for 45 min and then boiled in 0.5% SDS lysis buffer plus protease inhibitor cocktail to rapidly denature the collagen matrix and lyse the cysts. The solubilized gel mixture was immunoprecipitated with an antibody that detects phosphorylated and unphosphorylated forms of ERK. Immunoprecipitates were then simultaneously immunoblotted for total ERK and phospho-ERK with, respectively, a rabbit anti-ERK 1/2 antibody directly conjugated to Alexa Fluor 680 and a mouse anti-phospho-ERK 1/2 primary antibody followed by a goat anti-mouse IRDye 800 secondary. Blots were analyzed using an Odyssey Infrared Imager (LI-COR).

For MMP-9 and -13 experiments, cyst-containing gels were solubilized, electrophoresed, and immunoblotted. For MT1-MMP experiments, cysts were isolated from collagen and lysed as previously described (O'Brien et al., 2001); lysates were then electrophoresed and immunoblotted.

Acknowledgments

We are grateful to Ralph Schwall for HGF and David Shalinsky for AG3340. We thank Henry Bourne, Josh Thaler, Martin McMahon, Roberto Montesano, and Anne Pollack for critical reading of the manuscript. We also acknowledge Pascale Leroy, George Lemieux, Anirban Datta, Kathleen Liu, Paul Brakeman, and Mirjam Zegers for helpful discussions. L.E.O. is supported by an American Heart Association predoctoral fellowship and a NIH training grant. J.H.L. is supported by grants from the Kidney Foundation of Central Pennsylvania and the Polycystic Kidney Disease Foundation. This work was supported by NIH grants to K.E.M.

Received: March 16, 2004
Revised: April 22, 2004
Accepted: April 22, 2004
Published: July 12, 2004

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