c-Jun N-terminal kinase activity supports multiple phases of 3D-mammary epithelial acinus formation

SARA MCNALLY*, EMMETT MCARDLE*, EMER GILLIGAN*, SILVIA NAPOLETANO, MALGORZATA GAJIEWSKA, ORLA BERGIN, SARAH MCCARTHY, JACQUELINE WHYTE, ALESSANDRO BIANCHI, JULIANNE STACK and FINIAN MARTIN*

UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Ireland

ABSTRACT Primary murine mammary epithelial cells cultured on a laminin-rich-extracellular matrix (ECM) require c-Jun N-terminal kinase (JNK) activity for acinus formation. Inhibition of JNK (using SP600125) or small interfering RNA-mediated knockdown of JNK1 blocked acinus formation, impaired cell polarisation and lumen clearance and allowed sustained extracellular signal-regulated kinase (ERK) phosphorylation, cell proliferation, adhesion-independent cell survival and expression of epithelial-mesenchymal transition markers. ERK inhibition abolished the effects of JNK blockade. Interestingly, inhibition of JNK from the time of cell seeding blocked cell polarisation and lumen clearance; later inhibition (>6 h) only affected lumen clearance. ERK inhibition effectively protected cell polarisation but less so, lumen clearance. SP600125-treatment similarly affected acinus formation by the ‘normal’ human mammary epithelial MCF10A cell line. Expression of dominant-negative JNK1 in MCF10A cells also undermined acinus formation, generating large ‘multi-acinar spheres’ whose formation is probably driven by excessive luminal cell proliferation and cell survival. As JNK activity must be suppressed from the time of cell seeding to block cell polarisation, we studied the behaviour of MCF10A cells immediately after seeding in laminin rich matrix: we detected engagement of cells with the matrix, early polarisation, movement of cells into clusters and ‘epithelial-cell- like’ behaviour of clustered cells. Inhibition of JNK activity or expression of dominant-negative JNK1 allowed cell engagement to the matrix, but blocked cell polarisation and all subsequent ‘behaviours’. While integrin activation occurred, tyrosine-phosphorylation of paxillin, Fak and Src was significantly damped by JNK inhibition. These results emphasise the multi-phase dependency of the organisation of mammary cells in 3D on JNK activity and suggest a ‘permissive’ support of ECM-integrin ‘outside-in’ signalling and a ‘damping’ of growth-factor ERK signalling as its two key cell physiological effects.

KEY WORDS: mammary epithelial cell, acinus, JNK, ERK, MAP kinase, EMT

Introduction

Culturing mammary epithelial cells on a reconstituted laminin-rich extracellular matrix (ECM) (Engelbreth-Holm-Swarm (EHS) tumour derived matrix) generates 3D-cell assemblies that mature to form acini: spherical mono-layers of cells that enclose a central lumen. Acinus formation in this context requires firstly, cell polarisation and exit from the cell cycle of the outer mono-layer of cells in intimate contact with the ECM, and secondly, lumen clearance which occurs, at least in part, by an apoptotic mechanism (reviewed in Blatchford et al., 1999; Debnath and Brugge, 2005). The spatial organisation of cells within acini is maintained through ECM-integrin and cell-cell interactions (Reginato and Muthuswamy, 2006; Saelman et al., 1995). The essential cell-cell interactions occur via the

Abbreviations used in this paper: ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; shRNA, small hairpin RNA; siRNA, small interfering RNA.

*Address correspondence to: Finian Martin. UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland. Tel: +353-1-716 6734. Fax: +353-1-283 7211. e-mail: finian.martin@ucd.ie  # Note: These authors contributed equally to this paper.

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epithelial juncional complexes: tight junctions, adherens junctions and desmosomes (O’Brien et al., 2002; Underwood et al., 2006). Acinus formation also requires the cultures to be supplemented with a growth factor (EGF and/or bovine foetal serum), insulin or IGF-1 and hydrocortisone (Murtagh et al., 2004). The growth factor will stimulate that number of rounds of cell replication necessary to allow acinus formation initiate where, for instance, single MCF10A cells are used as starting material (e.g. [Debnath et al., 2002]) or

epithelial juncional complexes: tight junctions, adherens junctions and desmosomes (O’Brien et al., 2002; Underwood et al., 2006). Acinus formation also requires the cultures to be supplemented with a growth factor (EGF and/or bovine foetal serum), insulin or IGF-1 and hydrocortisone (Murtagh et al., 2004). The growth factor will stimulate that number of rounds of cell replication necessary to allow acinus formation initiate where, for instance, single MCF10A cells are used as starting material (e.g. [Debnath et al., 2002]) or

a limited number of rounds of replication where dispersed primary mammary mammary epithelial cells rapidly associate to initiate acinus formation. Insulin or IGF-1 support cell survival (Green and Streuli, 2004; Marshman and Streuli, 2002). Murtagh et al., (Murtagh et al., 2004) showed that the glucocorticoid, hydrocortisone, causes mitogen-activated protein (MAP) kinase, JNK, activation through a novel Bcra1-dependent signalling cascade and that JNK activity was essential for acinus formation. It was concluded that JNK probably acts, at least in part, though c-Jun to sustain expression of adhesion molecule gene expression (Murtagh et al., 2004).

Acinus formation has proved to be an excellent model system in which to study normal mammary epithelial cell behaviour and with which to elucidate functional differences between normal and transformed or oncogene challenged mammary epithelial cells. For instance, Weaver et al., (Weaver et al., 1997) established that the tumorigenic T-42 mammary epithelial cells fail to organise as acini in culture because of a hyper-activated α1-integrin-EGFR-ERK MAP kinase axis and would revert to behave like their related ‘normal’ S-1 cells (which form acini in culture) if α1-integrin or EGFR function was inhibited. Importantly, this behaviour was only observed in 3D cultures; no reciprocal down-regulation occurred when neutralizing antibodies were added to T4-2 cells cultured as mono-layers (Wang et al., 2002; Wang et al., 1998; Weaver et al., 1997).

The complex nature of genetic alterations in transformed cells makes the identification of individual events responsible for functional alterations observed difficult. Thus, 3D culture of normal mammary epithelial cells has been used to assess the biological effects of the activation of particular oncogenes. For example, over-expressing activated ErbB2 receptor in MCF10A mammary epithelial cells, Muthuswamy et al., (Muthuswamy et al., 2001) could document a failure of lumen clearance and the failure of associated luminal cell apoptosis and so, aberrant acinus maturation. That this required a combined triggering of sustained proliferation and an anti-apoptotic/pro-survival effect was clear from the failure of over-expression of either cyclin D1 or Bcl2 alone to achieve the same effect. Again the effects of active ErbB2 over-expression were associated with sustained activation of the core intracellular signalling cascades: the MEK-ERK and PI-3-kinase signalling pathways
Markers of EMT expressed in 3-D structures exposed to SP600125

- [see Supplemental Fig. S2]
- Suppressed occludin expression and localisation
- Induced occupation of E-boxes in occludin promoter by Snail1; and loss of AP-1 occupation;
- Elevated fibronectin and vimentin expression
- Depressed E-cadherin and increased N-cadherin expression
- Increased MMP-2 and -9 expression; and Snail1 occupation of MMP promoters; and
- In monolayer culture, increased nucleus-to-nucleus distance, transition to mesenchymal phenotype, and induction of focal adhesions at wound borders.

Fig. 2. During acinus formation JNK inhibition impairs localisation of, and expression of, cell-cell junction protein but induces expression of markers of EMT. (A) Treatment of acini forming from primary murine mammary epithelial cells with SP600125 caused failure of localisation of ZO-1 (green) to tight junctions (top panels), reduced ZO-1 expression (Western analysis, middle panels) and reduction in c-Jun and c-Fos occupation of AP-1 sites in the proximal ZO-1 gene promoter but increased occupation of E-boxes by snail1 (ChIP analysis). Bar 10 μm. (B) List of markers of EMT whose expression was induced in cells of acini forming in the presence of SP600125, further details are given in Fig S2. (C) Western analysis shows induction of EMT marker, vimentin, in both acini forming from cells treated with SP600125 (top panels) and acini expressing JNK specific shRNA (bottom panels). A greater than 50% reduction in JNK1 expression in cells infected with the JNK1 shRNA is also shown.
(CSF-1R) had been induced. This resulted in a severe disruption of MCF10A acinus architecture where excessive proliferation and aberrant survival was accompanied by progressive disruption of cell–cell adhesion in these structures and culminated in the release of individual cells into the surrounding matrix (Wrobel et al., 2004). The adhesive defect is dependent upon the ability of CSF-1R to chronically activate Src family kinases and these CSF receptor-mediated effects could be blocked using a Src inhibitor. Thus, the disruptive effects of cell transformation or inappropriate receptor tyrosine kinase activation or oncogene expression on acinus formation is transmitted through key associated intracellular signalling pathways. The ERK MAP and PI-3 kinase pathways have, in particular, been implicated as downstream transducers in this context and their inhibition can protect from the deleterious effects on acinus formation (Debnath et al., 2003b; Janda et al., 2002; Pinkas and Leder, 2002; Regnato and Muthuswamy, 2006; Wang et al., 2002).

Here we have further investigated the requirement for JNK activity during acinus formation by primary mammmary epithelial cells. Inhibition of JNK (using SP600125) or JNK1 ‘knockdown’ (using small interfering RNA (siRNA)) resulted in the formation of cell assemblies that did not polarise nor exit the cell cycle, (paralleled by ERK-dependent BimEL phosphorylation), that expressed markers of epithelial–mesenchymal transition (EMT) and had sustained, high levels of phospho-ERK. These effects could be blocked (or reversed) by generating the spheres in the presence of a MEK-ERK signalling inhibitor. Interestingly, during normal acinus formation, ERK-specific phosphatase (MKP-2 and -3) expression was induced and this may also support the restriction of ERK pathway signalling required for normal acinus formation. JNK inhibition with SP600125 also blocked acinus formation by MCF10A cells; spheroids of non-polarised cells formed and lumen clearance failed. Interestingly, over-expression of dominant-negative JNK1 in MCF10A cells led to the formation of large ‘multi-acinar spheres’ whose formation is probably driven by excessive luminal cell proliferation and cell survival rather than compromised cell polarisation. This was a milder effect than that induced by the chemical JNK inhibitor. Lastly, by studying the behaviour of MCF10A cells immediately after seeding (30min – 6h) in laminin-rich ECM under conditions that support acinus formation: we could pin-point very early influences of JNK on organisation of mammary epithelial cells in 3D. Inhibition of JNK activity or expression of dominant negative JNK1 allowed cell engagement to matrix (and β1-integrin activation), but blocked cell polarisation and so, all subsequent ‘behaviours’ (ie. Motility, clustering etc.). And, while integrin activation occurred, phosphorylation on tyrosine of paxillin, Fak and Src (reflective to recruitment to integrin and initiation of integrin ‘outside-in’ signalling) was significantly damped by JNK inhibition. Our results emphasise the multi-phase dependency of the organisation of mammary epithelial cells in 3D on JNK activity and suggest a ‘permissive’ support of ECM-integrin ‘outside-in’

**Fig. 3.** JNK inhibition suppresses focal adhesion-associated protein phosphorylation but induces sustained phosphorylation of EGF receptor and ERK in forming acinus. (A) SP600125 treatment causes loss of polarised baso-lateral localisation of active-β1-integrin (green) and apical localisation of F-actin (red) in forming primary mammary epithelial cell acini. (B) Western analysis shows loss of phosphorylation/activation of focal adhesion associated proteins, FAK (pY397), paxillin (pY118) and Src (pY416) in acini forming in the presence of SP600125. (C) Western analysis shows increased phosphorylation/auto activation of EGFR (p-Y1173) and phosphorylation of ERK in SP600125 treated acini. In addition, SP600125 treatment caused a ‘band-shift’ in BimEL and phosphorylation of Bim on ERK targeted ser-65. Expression of the JNK1 specific shRNA in cells forming acini also induced increased ERK phosphorylation.
Results

**Inhibition of JNK activity impaired cell polarisation and lumen clearance during acinus formation**

We have previously shown that JNK is phosphorylated / activated during acinus formation by primary mammary epithelial cells supported by a laminin-rich ECM (see also Supplemental Fig. S1). In addition, we showed that omission of glucocorticoids, an essential component of the culture medium, both impaired acinus formation and stopped JNK activation (Murtagh et al., 2004). Here we show that SP600125, the small molecular weight JNK inhibitor, blocks lumen clearance as judged by EtBr staining for dead cells and loss of the apoptosis marker, cleaved caspase 3 (Fig. 1A) and so impairs acinus formation by primary mammary epithelial cells. In addition, SP600125 treatment stopped the cell polarisation and proliferation restriction (and suppression of cyclinD1 expression) that accompany acinus formation (Fig. 1A,B). That JNK activity was essential for acinus formation was validated by knocking down JNK1 using a specific small hairpin RNA (shRNA) delivered from a recombinant lentivirus: JNK knockdown again blocked both lumen clearance and cell polarisation (the latter judged by apical localisation of F-actin) (Fig. 1C).

These experiments reiterated the requirement for JNK signalling in driving cell polarisation during acinus formation (see also (Whyte et al., 2010)). A likely target of this (glucocorticoid) – JNK signalling axis is maintenance of expression of adhesion proteins (Murtagh et al., 2004), which play a role in establishing and maintaining the polarised state of organised epithelial cell aggregates. This was further investigated.

**Disruption of acinus formation by JNK inhibition triggers expression of markers of EMT**

During acinus formation, JNK inhibition with SP600125 both inhibited cell polarisation (above) and caused mis-localisation and reduced cell levels of tight junction protein, ZO-1 (Fig 2A). This was paralleled by a reduction in ZO-1 mRNA levels (Murtagh et al., 2004), reduced occupation of an AP-1 binding element in the ZO-1 gene proximal promoter by cJun and cFos and, in contrast, increased occupancy of E-boxes by snail in the same promoter. JNK action and acinus formation

**Fig. 4. Acini forming in the presence of an ERK signalling pathway inhibitor PD98059 are resistant to the effects of JNK inhibition.** (A) The MEK-ERK pathway inhibitor, PD98059, added at the time of cell seeding, protected from inhibition of lumen clearance (as judged by vital dye staining with EtBr) and luminal cell apoptosis (detected by cleaved caspase3 staining) in primary mammary epithelial cell acini forming in the presence of SP600125. (B) PD98059 also permitted cell polarisation in acini forming in the presence of SP600125 as judged by apical distribution of Factin and baso-lateral distribution of E-cadherin (both red), nuclei counterstained with DAPI (blue). (C) PD98059 treatment also suppressed the persistent cell proliferation (detected by BrDu labelling (green)) induced by SP600125. (D) Western analysis shows that addition of PD98059 at time of cell seeding to acini forming in the presence of SP600125 protects from sustained phosphorylation/activation of ERK, the increase in apparent size of BimEL and increased ERK dependent phosphorylation of Bim at ser65, and suppression of phosphorylation/activation of Src (pY416). (E) Addition of PD98059 to acini forming in the presence of SP600125 suppressed the induction of EMT marker, vimentin, as judged by confocal immune fluorescence analysis (vimentin (green), left-hand panels) and by Western analysis (right-hand panels).
Inhibiting ERK activation protects from the effects of JNK inhibition on acinus formation

Acinus formation by primary mammary epithelial cells saw the expression of activated β1-integrin at the baso-lateral surface of those cells in immediate contact with the ECM (Fig. 3A). In addition, western analysis showed the parallel phosphorylation/activation of intracellular proteins normally recruited to such activated integrins (FAK, paxillin and Src). Inhibition of JNK with SP600125 disturbed the localization of the β1-integrin and suppressed the phosphorylation of the integrin associated proteins (Fig. 3A). In contrast, inhibition or knockdown of JNK during acinus formation led to persistent increases in the phosphorylation/auto-activation of the integrin associated proteins (Fig. 3A). In addition, inhibition of JNK during acinus formation leads to a shift in balance between integrin-JNK signalling and EGFR-ERK signalling, with the latter now dominant. We therefore investigated whether inhibition of ERK activation was sufficient to protect from or reverse the effect of inhibition of JNK signalling during acinus formation. We show that inhibition of ERK activation with the kinase inhibitor, PD98059, was sufficient to block the impairment of lumen clearance caused by JNK inhibition, as judged by vital dye staining (calceinAM and EtBr) and luminal cell apoptosis, detected by cleaved caspase3 staining (Fig. 4A). Fig. S3 shows that PD98059 not only reverses the effect of SP600125 but also gives rise to small acini. In addition, inhibition of ERK activation permitted normal cell polarisation (F-actin and E-cadherin localisation, Fig. 4B) and normal suppression of cell proliferation (BrdU incorporation, Fig. 4C) and suppression of ERK-dependent Bim phosphorylation and activation of Src (Fig. 4D) and, finally, suppression of vimentin expression (Fig. 4E). Thus, inhibition of ERK activation triggered by JNK inhibition was sufficient to protect from the effects of JNK inhibition during acinus formation. The EGFR inhibitor (AG1478) was also effective in protecting acinus formation by the primary mammary epithelial cells from the effects of JNK inhibition (data not shown).

Likewise, inhibition of ERK activation was sufficient to reverse the effects of JNK inhibition after the latter was initiated 17 h before addition of the MEK inhibitor, PD98059. Again inhibition of ERK activation was sufficient to reverse all aspects of acinus formation impaired by JNK inhibition (ie cell polarisation, lumen clearance and luminal cell apoptosis, inhibition of Bim activity, suppression of expression of markers of EMT and reactivation of Src) (Fig. S4 a-f).

It is clear, therefore, that acinus formation will only execute successfully if EGFR-ERK signalling is restricted. This is primarily achieved by restricting EGFR activity but dephosphorylation/inactivation of phospho-ERK by ERK-directed phosphatases (MKPs) could also contribute. The two principal ERK-directed MKPs are MKP-2 and MKP-3 (Camps et al., 2000; Farooq and Zhou, 2004; Karlsson et al., 2004; Keyse, 2000; Zhang et al., 2002). Levels of MKP-2 and -3 were high in whole cell extracts of normal acini,
and low in the presence of the JNK inhibitor (Fig. 5A). Levels of the nuclear localised MKP-2 rose in nuclear extracts during normal acinus formation but not on JNK inhibition (Fig. 5B). MKP-3 levels measured in whole cell extracts showed a similar profile (Fig. 5B). Failure of acinus formation and sustained high p-ERK levels in cultures treated with SP600125 were associated with constitutively low levels of MKP-2,-3. Thus, MKP-2 and -3 may contribute to the efficient constraining of active ERK levels during acinus formation.

There is an interesting contrast between the expression of MKP-2 and -3 and the phospho-JNK-directed phosphatase MKP-1 during acinus formation: MKP-1 is expressed at a high level during normal acinus formation, when the levels of its active target are high (in contrast to the ERK-directed MKPs) (Fig. 5 C,D). Its expression is lost when JNK is inhibited. In a number of biological models (Llense and Martin-Blanco, 2008) active JNK induces MKP1 expression as part of an auto-regulatory negative feedback loop.

**The requirement of JNK for cell polarisation, and lumen clearance during acinus formation are temporally separable events**

Cell polarisation precedes lumen clearance during acinus formation. It was of interest to examine the dependency of both events on JNK. We observed that addition of the JNK inhibitor, SP600125, to the primary mammary epithelial cells at time of plating inhibited both events. However, if the JNK inhibitor was added to the forming acini 6h or more after cell seeding, cell polarisation was unaffected, while lumen clearance was still inhibited (Fig. 6A, b). Thus, the requirement of JNK for cell polarisation and lumen clearance during acinus formation are temporally separable. In addition, we investigated the ability of ERK inhibition to separately protect these two events from JNK inhibition. Interestingly, we found that cell polarisation was fully protected by ERK inhibition while lumen clearance was significantly less so (Fig. 6B). At this time the reason for this difference in sensitivity to ERK inhibition under these conditions is not understood.

**Inhibition of JNK also impairs acinus formation by MCF10A cells**

In order to establish JNK signalling as a general requirement for mammary epithelial cell acinus formation, we investigated the effects of JNK inhibition on acinus formation by the human mammary epithelial cell line, MCF10A. We observed MCF acini on day 8 of culture. At this time, 2 distinct sub-populations of cells are apparent: polarised cells in contact with the matrix, and, disorganised luminal cells, programmed to die (see Fig. S5a). In MCF10A 3D cultures, inhibition of JNK with SP600125 impairs acinus formation and generates spheroids of surviving / proliferating, unpolarised cells with elevated levels of activated ERK (Fig. 7 A-C) reminiscent of treating the MCF10A cultures with the standard inducer of EMT, TGF-β (Fig. S5 b-d); or of treating cultures of primary mammary epithelial cells with the same inhibitor (compare Figs. 7 A,B and 1 A,B). It should be noted that to observe this effect the SP600125 must be added at the lower concentration of 20 μM, and ≥ 3d after seeding, earlier addition results in no spheroid development.

**Expression of a dominant negative (activation deficient) JNK1 in MCF10A cells subverts acinus formation**

Using the MCF10A cells we could investigate the effect of overexpression of a dominant-negative, activation-deficient JNK mutant protein (Fig. 8A) on acinus formation. Overexpression was achieved by infecting the cells with a recombinant lentivirus carrying the mutant JNK gene. Empty-vector infected cells and cells overexpressing wild-type JNK, were used for control purposes. The viruses also delivered GFP and green fluorescence monitored infection efficiency. It was clear from forming acini at day 4 that adequate infection had been achieved and spheroid formation was proceeding (Fig. 8 A,B). However, by day 8 phase contrast microscopy showed that the JNK DN infected spheres were now large and multi acinar (Fig. 8A). Immunofluorescence confocal microscopy analysis showed that the JNK-DN expressing acini were indeed large and multi acinar, while normal acini were generated by the...
empty vector and JNK wt infected cells (Fig. 8B). In the multi-acinar spheres cell polarisation (as judged by E-cadherin and GM130 distribution) seemed normal, as did rates of luminal cell apoptosis (Fig. 8C). However, luminal cell proliferation rates were high (at d8) and luminal cell survival may have been enhanced (Fig. 8D).

By western blot analysis, the activation of dominant negative JNK was reflected by the expected reduction in JNK-dependent cjun activation/phosphorylation and corresponding reduction in levels of activated Src and Paxillin (Fig. 8E). Thus, acinus formation by MCF10A cells does indeed require JNK. However, the potency of effect of using the chemical inhibitor and dominant negative protein may differ with the latter not affecting cell polarisation, but more influencing the regulation of cell proliferation.

JNK activity is required to support immediate responses of MCF10A mammary epithelial cells to engaging ECM in 3D cultures

In the period immediately after plating MCF10A cells in EHS-ECM, under conditions that support acinus formation, we can detect a series of ‘key events’: 1. Engagement of the cells with the matrix (reflected by stable ‘suspension’ of cells within matrix and significant ‘active β1-integrin’ staining, from as early as 30min after cell plating); 2. Cell polarisation; 3. Movement of cells towards nascent clusters (reflected by mesenchymal shape and extension of processes to clusters); and 4. Formation of clusters of cells and expression of epithelial markers (ie. Adhesion molecules at cell-cell interfaces).

Inhibition of JNK with SP600125 had no immediate effect on cell engagement to matrix, as reflected by numbers of cells stably suspended in matrix (DAPI staining, 30min – 2h (Fig. 9A)) and extent of active β1-integrin activation (Fig. 9B). However, inhibition of JNK activity clearly blocked ‘early’ cell polarisation, and downstream cell behaviours (motility, and formation of clusters). Thus, at 4h JNK inhibited cultures are a collection of single un-polarised cells ‘frozen’ / suspended within the matrix. In contrast, normal cultures are an active amalgam of polarised cells moving into clusters (and adopting a more epithelial phenotype once settled within a cluster (Fig. 9C). At a molecular level, JNK inhibition seemed to impair the efficiency of phosphorylation / activation of a range of key integrin-associated proteins (paxillin, FAK and Src) (Fig. 9 D-F). Expression of the dominant negative JNK1 in MCF10A cells had a similar effect on cell behaviour in the period early after seeding the cells in the laminin-rich ECM: JNK-DN expressing cells were predominantly single and unpolarised (eg. Fig. 9G, bottom panel). In contrast, empty vector and JNK wt infected cells were predominantly in clusters. Again, phosphorylation/activation of Src, FAK and paxillin was under-powered in the presence of the dominant negative (Fig. 9G, bottom panel). Note that the phosphorylation of the integrin-associated proteins was most intense in the cells over-expressing JNK wt. We conclude that JNK is required to support molecular events downstream of integrin activation in the immediate aftermath of matrix engagement at the initiation of MCF10A cell participation in acinus formation.

Overall, our results emphasise the multi-phase dependency of the organisation of mammary epithelial cells in 3D on JNK activity and suggests a ‘permissive’ support of ECM-integrin ‘outside-in’ signalling and a ‘damping’ of growth factor ERK signalling as its two key cell physiological targets.

Discussion

We have observed a requirement for JNK activity in supporting the organisation of mammary epithelial cells in three dimensions, most particularly in the formation of acini. In these studies we have
Fig. 8. Effect of expression of an activation-deficient dominant negative JNK1 mutant protein in MCF10A cells triggers formation of multi-acinar spheres. (A) Line diagram indicates mutations in activation-deficient JNK dominant negative mutant protein. Phase contrast and fluorescence microscopy: shown are fields of MCF10A acini, infected with recombinant lentiviruses, carrying empty vector (pWPI), wild type JNK1 (pWPI-JNK1-WT) and the dominant-negative (pWPI-JNK1-DN) at day 4 and day 8 after cell seeding. Note the comprehensive GFP staining (green), reflecting successful infection. Also clear is the generation of multi-acinar spheres in the pWPI-JNK1-DN infected cells. (B) Confocal immunofluorescence microscopy analysis shows the formation of multi-acinar spheres by MCF10A cells expressing the JNK1-DN protein (dapi, blue; GFP, green). (C) Expression of the JNK1-DN causes formation of multi-acinar spheres at day 8, but with little disturbance of polarised GM130 and E-cadherin localisation. In the case of the GM130 analysis, fields of, and individual acini are shown. Expression of empty vector and JNK1-WT had no effect on acinus development. (D) Expression of the JNK1-DN did not affect levels of luminal apoptosis (cleaved caspase3 staining (red)) but did increase levels of luminal cell proliferation (Ki67 staining (red)) at day 8 of acinus formation. (E) Western analysis: expression of JNK1-DN in MCF10A acini at day 8 reduced both activated/phosphorylated cJun and total cJun levels, and also p-paxillin (Py118) and p-Src (pY416) levels. It also reduced total Bim levels but did not affect p-ERK expression at day 8. All analyses are compared to cells infected with empty vector and JNKWT.
Fig. 9. JNK activity is required to support immediate responses of MCF10A mammary epithelial cells to engaging ECM in 3D cultures. Confocal fluorescence microscopy analysis: (A) Time course (30 min – 6 h) of engagement of MCF10A cells with EHS-ECM. Effect of JNK inhibition with SP600125. DAPI staining. (B) Detection of active β1-integrin staining on engagement of MCF10A cells with EHS-ECM in the presence and absence of SP600125. (C) Inhibition of cell polarisation and cell clustering on engagement of MCF10A cells with EHS-ECM by SP600125 treatment. (D-F) Impairment of phosphorylation/activation of integrin-associated proteins, Src (pY416), FAK (pY397) and paxillin (pY118), on engagement of MCF10A cells with EHS-ECM by SP600125 treatment. (G-I) Inhibition of cell polarisation and cell clustering, and impairment of phosphorylation/activation of integrin-associated proteins, Src (pY416), FAK (pY397) and paxillin (pY118), on engagement of MCF10A cells with EHS ECM by expression of dominant negative JNK1.
used three model systems, primary mid-pregnant mouse mammary epithelial cells, cultured on a laminin rich ECM MCF10A human mammary epithelial cells, cultured within the same matrix, and, lastly, we have observed the behaviour of MCF10A cells at early time points (30 min -6h) under identical culture conditions. Each model has contributed to our understanding of the JNK requirement. In order to modulate JNK activity in these culture cells, we have used an established specific small molecular weight JNK inhibitor (SP600125), we have knocked down JNK1 using lentiviral delivery of a JNK-specific shRNA, and, finally, we have overexpressed an activation-deficient dominant negative JNK mutant. These different and independent approaches have added validity to our experimental observations.

The dispersed primary mouse mammary cells formed mature acini when cultured on the laminin rich ECM in the presence of insulin, EGF and hydrocortisone. Addition of the small molecular weight JNK inhibitor impaired cell polarisation, withdrawal from the cell cycle and lumen clearance, the necessary steps in acinus formation. The JNK inhibited cells formed loosely aggregated spheroids and expressed a range of markers of EMT. Expression of the JNK1 specific shRNA had a similar effect. This validated the requirement for JNK for acinus formation in this model system. Of particular note was that cell proliferation and EGFR-ERK signalling persisted once JNK was inhibited. This led us to show that inhibition of ERK activation was sufficient to protect acinus formation from impairment by JNK inhibition. EGFR inhibition was also sufficient to provide this protection. These data suggest that a primary requirement for acinus formation is the down regulation of EGFR-ERK signalling and associated restriction of cell proliferation. This requirement has also been described for the formation of MCF10A cell acini (Weaver et al., 1997). However, this is the first time that the requirement for JNK activity to support this proliferation restriction, has been reported.

We also provide evidence that the requirement for JNK activity for acinus formation can be separated into two temporally independent events. We show that, while addition of SP600125 to primary cultures at time of seeding inhibits both cell polarisation and lumen clearance, addition of the JNK inhibitor later (after 6h or more) inhibits only lumen clearance. Thus, there may be two cellular targets for JNK in this context. The first, which supports establishment of cell polarisation, probably lies upstream of PKCζ activation on a signalling path downstream of activated β1 integrin (Whyte et al., 2010). The later target, which supports lumen clearance, is likely to centre on rac1 activation (Keely et al., 1997; Muthuswamy et al., 2001).

Using MCF10A cells, it was possible for us to show in an independent system of acinus formation, that JNK activity was again necessary to support this organisation of mammary epithelial cells in 3D. However, the MCF10A cells proved to be more sensitive to the chemical inhibitor. In particular, it needed to be added at least 3 days after culture was initiated. Of note is the fact that we have routinely observed these acini at day 8 of culture, when two separate populations of cells are robustly apparent in the acini. Firstly, polarised cells in intimate contact with the ECM, and secondly, a separate disordered population of luminal cells (see Fig. S5a). The JNK inhibitor impaired acinus formation by the MCF10A cells in a similar fashion to its effect on the primary acinar cultures. It led to failure of cell polarisation, failure of lumen clearance and the persistent activation of ERK (Fig. 7 A-C). It led to the generation of spheroids of loosely associated disordered cells. With the MCF10A cells, it was also possible to inhibit normal JNK function by the overexpression of an activation-deficient JNK mutant protein. Again, down regulation of cellular JNK drive by this method, affected acinus formation. Control expression of the empty delivery vector, or wild type JNK1, had no qualitative effect on acinus formation. Expression of the dominant negative generated multi–acinar spheres, a phenotype that has been widely reported. For instance, overexpression of active ErbB2 (Muthuswamy et al., 2001) or overexpression of a kinase-dead PKCζ (Whyte et al., 2010) leads to such an outcome. In these multi-acinar spheres, cell polarisation seems unaffected and the impairment seems to arise due to excessive proliferation of the luminal cells and/or their persistent survival. It would seem therefore, that expression of the dominant negative has had a milder effect on acinus formation than had the small molecular weight inhibitor, SP600125.

The sensitivity of the MCF10A cells to JNK inhibition at early times of culture led us to investigate a possible requirement for JNK activity in the initial interactions between cells and ECM in these 3D cultures. We observed cell behaviour in a window from 30 min to 6h after cell seeding. We could define a series of early behaviours: the cells engaged the matrix, they polarised, they moved into clusters and, finally, within the clusters they exhibited epithelial like behaviours. Inhibition of JNK using SP600125, or by overexpressing the dominant negative protein, arrested the development of this pattern of behaviours at an early stage. It did not have an effect on engagement of the cells with the matrix, but it robustly inhibited cell polarisation and, therefore, its downstream consequences, motility, cluster formation and epithelisation. At a molecular level, it did not seem to affect β1 integrin activation, but seemed to dampen phosphorylation/activation of integrin associated intracellular signalling proteins i.e. FAK, paxillin and Src. These studies point to a requirement for JNK in supporting the efficient generation of signal from integrin that is necessary to drive the organisation of mammary epithelial cells in three dimensions.

In summary, we have comprehensively analysed the requirement for the MAP kinase, JNK in supporting the organisation of mammary epithelial cells in three-dimensional cell cultures, particularly, its effect on acinus formation. In addition, we report for the first time on the use of very short-term 3D MCF10A cultures, and note its significant suitability for mechanistic studies.

Materials and Methods

Cell culture

Mouse mammary epithelial cells were harvested from mid- to late-pregnant CD-1 mice and cultured as described previously (Furlong et al., 1996; Murtagh et al., 2004). Cells were seeded on tissue culture dishes coated with concentrated, growth factor depleted, EHS ECM (Matrigel®; BD Biosciences; 1.74 mg protein/6 cm2 plate) at a density of 2.4 X 104 cells/ml and cultured with 5 ng/ml epidermal growth factor (Promega). 5 μg/ml insulin (Sigma-Aldrich), 1 μg/ml hydrocortisone (Sigma-Aldrich), and 50 μg/ml gentamycin (Sigma-Aldrich) in F12 medium (Life Technologies, Inc.). These cells were harvested after 1-4 d in culture. Acini were recovered by scraping and were pelleted by centrifugation. The cell pellets were snap frozen and stored at -80°C before RNA or protein extract preparation. 50 μM SP600125 (Calbiochem), 10 μM PD98059 (Calbiochem) and 10 μM AG1478 (Calbiochem) were added to cells either at time of plating or after 17 h. For 2-D mono-layer studies cells were seeded onto 2 chambered plastic slides coated with diluted EHS matrix (1:5; Matrigel: Ham’s F-12) using identical culture conditions. Cells were fixed and stained at 48 h.
MCF10A cells were cultured exactly as described previously (Whyte et al., 2010) and as outlined in (Debnath et al., 2003a). Epidermal growth factor (EGF, 20ng/ml (Sigma)) was added after each cell splitting procedure (unless stated otherwise). 20μM SP600125 was added on day 3 to MCF10A cells, and cells were harvested/fixed on days 4 and day 6, or day 10 for transduction experiments. For short term 3D culture of MCF10A cells, the cells were counted and seeded (1x10^5 cells/ml) on EHS-ECM pre-coated glass chamber slides in medium supplemented with 2% Matrigel® and 20ng/ml EGF. Inhibitors were added to cells (SP600125 (20 μM) (Calbiochem)) at the time of plating, unless otherwise stated. Cells were harvested and fixed at 30 min, 2h, and 4h after seeding.

**Cell extract preparation and Western blot analysis**

Whole cell extracts were prepared by re-suspending pelleted cells in 50-100 μl of extraction buffer (Furlong et al., 1996; Murtagh et al., 2004). The samples were kept on ice for 30 min before centrifugation at 14,000 rpm at 4°C for 20 min. The supernatant constituted the whole cell extract.

Western blot analysis was performed as described previously (Furlong et al., 1996; Murtagh et al., 2004). For a list of antibodies used, see Supplemental Fig. S7a(i).

**RT-PCR analysis**

Cultured cells or cell assemblies were gently scrapped from the plates, briefly centrifuged, washed by resuspension in PBS, centrifuged, and resuspended in TRizol® Reagent (Invitrogen). Reverse transcription and limited cycle PCR were performed as described previously (Murtagh et al., 2004; O’Brien et al., 2002). Primers were designed using Primer3 software (Rozen and Skaltsky, 2000). Primer sequences are given in Fig S7e.

**Microscopy**

Acini were fixed using 2% (MCF10A) or 4% PFA (primary cells) for 20 minutes at room temperature and stained as previously outlined (Whyte et al., 2010). Cells were incubated with primary antibodies (or phallolidin/ rhodamine (Molecular Probes)) overnight at 4°C in a humidity chamber and then incubated with FITC, Texas-red conjugated, or Alexa-Fluor 555 goat anti-mouse/rabbit (Molecular Probes) secondary antibodies in darkness for 1 h. For a list of primary staining antibodies used, see Supplemental Fig. S7a(ii). Slides were mounted in Prolong Gold anti-fade reagent with Dapi (Molecular Probes). For analysis with vital dyes, acini were incubated with 10 μg/ml EtBr and 10 μM Calcein AM (Molecular Probes) for 10 min at 37°C. For BrdU incorporation, BrdU was added to forming acini for 24 h periods (0-24 h or 24-48 h). After incubation, cells were fixed and incubated with methanol at -20°C for 30 min. Cells were washed with ice cold PBS and treated with 2N HCl for 30 min with rotation. Excess HCl was removed and remaining HCl was neutralised by incubation of the cells with 0.1M sodium borate (Borax, Sigma) for 5 min with rotation. Cells were washed with PBS for 2 min and permeabilised with 0.5% Triton X-1000 for 10 min. FITC conjugated monocolonal mouse anti-BrdU-BrdU stock solution (BD Pharmingen) was added to the sample for 30 min before visualisation. Images were captured using a Bio-Rad MRC 1024 or a Carl Zeiss Laser Scanning LSM 510 Meta Confocal microscope.

**Chromatin immuneprecipitation (ChIP) analysis**

ChIP analysis was performed using a ChIP assay kit purchased from Upstate Biotechnology as we have previously described (Murtagh et al., 2004). Acini were cross-linked on the cell culture plates by adding 0.1M sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl immunocomplex buffer (0.25% LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 1mM Tris, pH 8.1) and TE, pH 8.0, with brief centrifugation before each wash to recover the beads. The immune complexes were eluted from the beads by the addition of 500 μl 1% SDS in 0.1 M NaHCO3 to the bead pellet, vortexing and rotating at room temperature for 30 min. To reverse the cross-linking process, 200 mM NaCl was added to the eluates which were then incubated at 65°C for 4 h, 10 mM EDTA, 40 mM Tris-HCl, pH 6.5, and 2 μl of 10 mg/ml protease K was then added and incubated at 45°C for 1 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Promoter sequences were detected in immunoprecipitated and input DNA by PCR using specific primers. A parallel ChIP analysis for each promoter was performed using an anti-acetylated histone H3 antibody as a positive control (data not shown). Details of promoter structure, PCR strategy and PCR primer sequences are given in Fig. S7(b-e).

**small hairpin RNA transduction using recombinant lentiviruses**

The following siRNA sequences targeted against mouse JNK1-1 mRNA (cDNA sequence (NM016700): JNK1sh1: 5'-GGGACCTAGTTGCTGATGAC-3'; JNK1sh2: 5'-AGCGAGGACCACATGGAAGT-3'; JNK1shscr: 5'-GTTTACACAAAGGTGTGAGG-3') were designed using the MIT Whitehead website (http://jura.wi.mit.edu/bioc/siRNAext/). siRNA sequences were used to make shRNA cassettes which were subsequently cloned directly into the shRNA expressing lentivector, pLVTHM, using Cla-1 / Mlu-1 restriction sites downstream of the H1 promoter. Presence of the shRNA cassette was verified by DNA sequencing. Scrambled versions of the siRNA sequences were also cloned into pLVTHM. Lentiviral particles were produced by transfecting HEK293t cells with a mix of the transfer vector (pLVTHM-JNK-shRNA), packaging vector (pCMV-SP600125, 150 μg/ml EGF. Inhibitors were added to cells (SP600125 (20 μM) (Calbiochem)) at the time of plating, unless otherwise stated. Cells were harvested and fixed at 30 min, 2h, and 4h after seeding.

**MCF10A lentivirus transduction**

Genes (cDNAs) to be expressed were purchased from Addgene (catalogue numbers 13798, 13846 and 12254) and cloned from pCDNA3, into pWPi. All MCF10A virus transduction protocol was carried out as previously described in (Whyte et al., 2010). See Supplemental Fig. S6a for schematic of JNK virus constructs used in these studies. Quicktitre Lentivirus Quantitation kit (Cell Biolabs) was used to quantitate virus particles per ml (VP/ml). Addition of 2.5 x 10^6 VP or ~250 μl of virus solution per well was sufficient for ~100% infection without toxicity.
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