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Cell cycle proteins in epithelial cell differentiation

Implications for breast cancer

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Abbreviations: CDK, cyclin dependent kinase; HLH, helix-loop-helix; Rb, retinoblastoma protein; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor

Proliferation and differentiation are tightly coupled processes, so that a final cell cycle is often linked to the initiation of cell differentiation. The flux in cell cycle proteins during this process is commonly assumed to simply control the final cell cycle exit. However it now appears that cell cycle proteins can also play a role in the decision to continue cycling or to terminally differentiate. A subset of the G_1 to S phase transition proteins, D-type cyclins, Rb family proteins and the CDK inhibitors, are particularly involved in the commitment to differentiation. Cell cycle proteins can sequester or modify activators of differentiation pathways, while simultaneously performing their cell cycle functions as illustrated by their roles in terminal differentiation in mammary epithelium. G_1 to S phase cell cycle proteins, particularly cyclin D1, are commonly altered in breast cancer and contribute to breast tumorigenesis, presumably by increasing proliferation. However the capacity for cell cycle proteins to also influence differentiation may influence tumour progression, and may alter the efficacy of differentiation-based therapeutics.

Cell Cycle Exit and Differentiation: Concurrent or Co-Dependent?

Cell cycle proteins have considerable redundancy such that many of the individual genes can be deleted and cellular proliferation continues relatively unperturbed.[1] More recently however, several non-redundant functions of cell cycle proteins have become apparent, functions that lie outside the traditionally understood cell cycle roles, including apoptosis, quiescence, motility and differentiation.[2-5] The terminal differentiation of a cell is closely linked to exit from the cell cycle, where during the final cell cycle a program of cell differentiation is initiated. However, cell cycle exit is not invariably associated with differentiation, but can also lead to a state of quiescence, senescence or cell cycle arrest. This suggests that distinct molecular events must occur during cell cycle exit associated with differentiation. Several master regulator proteins of differentiation have been identified in different tissue types, such as c/EBPβ in adipocytes[6] and Id2/PU.1 in erythropoiesis,[7] and these have well-characterised cell cycle targets that allow for differentiation-associated cell cycle exit.

The question arises as to whether changes in cell cycle proteins merely enforce cell cycle exit, or do these proteins actually take part in the differentiation process? There is now growing evidence that the flux in cell cycle proteins during the proliferation/differentiation switch may reinforce or initiate signals required for differentiation, and that each tissue relies on a different subset of cell cycle proteins for terminal differentiation. The major players of S, G_2 or M phases—cyclin E-Cdk2, cyclin A-Cdk2, cyclin A-Cdk1 and cyclin B-Cdk1 appear to have, at best, a limited role in the commitment to differentiation. Instead commitment to differentiation occurs during late G_1 phase, coinciding with the restriction point of the cell cycle where many mitogenic signals may influence proliferation.

The G_1 phase of the cell cycle is characterised by the action of D and E-type cyclins, the retinoblastoma protein (Rb) and the Cdk inhibitor proteins $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$. Initially there is an increase in cyclin D-Cdk4/6 activity leading to phosphorylation of Rb and the release of E2F transcription factors. This in turn upregulates cyclin E, leading to further Rb phosphorylation and phosphorylation of target proteins for S phase progression. The CDK inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ are downregulated and redistributed at this phase of the cell cycle, also causing increases in cyclin D-Cdk4/6 and cyclin E-Cdk2 activity. The D-type and E-type cyclins, Rb and $p21^{Cip1/Waf1}$/$p27^{Kip1}$ are independent targets of mitogenic signals, leading to altered commitment to cell cycle progression.

Alterations to G_1 cell cycle proteins, including cyclin D1 and Rb, can lead to mammary developmental defects, which raises the possibility that these proteins may affect mammary epithelial differentiation. While cell cycle proteins have established roles or associations with the differentiation of numerous other cell types including adipocytes, keratinocytes, and neural cells, the relationship of cell cycle proteins to mammary epithelial differentiation is less defined. However there is a growing body of literature that supports a differentiation function for cell cycle proteins in the mammary gland, which we outline below. This is particularly relevant to breast cancer biology where alterations

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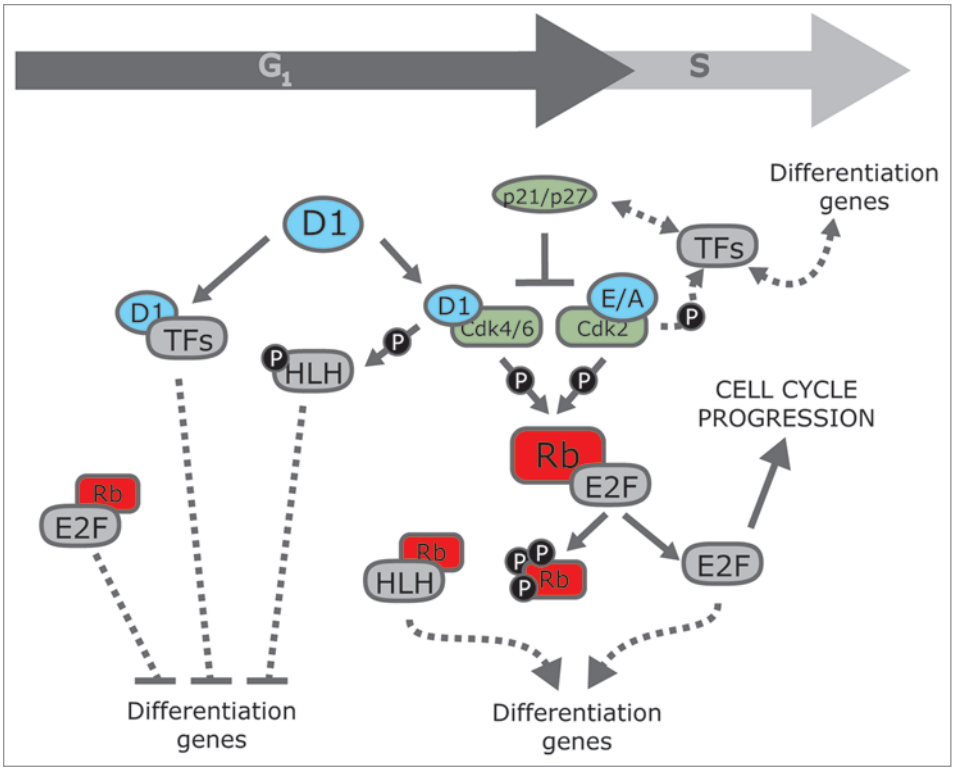

Figure 1. Terminal differentiation is intimately linked to the cell cycle machinery of G_1 phase. Both Rb and cyclin D1 can repress the expression of differentiation genes through binding to transcription factors, or via inhibitory phosphorylation by cyclin D1-Cdk4. Rb also activates differentiation specific transcription factors via direct interactions or downstream of E2F activation. The CDK inhibitors, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ may also positively or negatively affect differentiation, through direct binding to transcription factors or downstream of Cdk activity. D1, Cyclin D1; E, cyclin E; A, cyclin A; HLH, Helix-loop-helix transcription factor; TF, transcription factor; Rb, retinoblastoma protein.

in the expression of cell cycle proteins coincide with changes in tumour cell differentiation.

Cell Cycle Proteins with Roles in Differentiation

Knockout mouse models suggest specific roles for cell cycle proteins in differentiation, as certain gene deletions are associated with individual differentiation defects (reviewed in refs. 1 and 8). For example, cyclin D1 deletion leads to failed mammary gland development in mice,[8] whereas the absence of $p27^{Kip1}$ leads to female sterility as a result of failed granulosa cell luteal differentiation.[9] It can be difficult to separate whether differentiation lies downstream of changes in expression/activity of a particular cell cycle protein, or whether differentiation is induced more generally as a consequence of cell cycle arrest. However, some elegant studies have been performed where domains of proteins such as cyclin D1 and $p21^{Waf1/Cip1}$ have been specifically deleted, and cells are able to arrest, but not differentiate, or vice versa.[10-12] Studies in mouse models and in vitro cell line experiments have shown that different tissues utilise distinct subsets of cell cycle proteins during differentiation. However, there are also some frequent players that are involved or correlated with the differentiation of multiple tissue types, and these include cyclin D1, the CDKs, the Cip/Kip inhibitor proteins and the Rb pocket protein family.

Cyclin D1 and the regulation of adipocyte maturation. In many cell systems cyclin D1 is downregulated, or sequestered in the cytoplasm, with the advent of differentiation.[13-15] Reduction of cyclin D1 appears particularly crucial to adipogenesis, as cyclin $D1^{-/-}$ MEFs have enhanced adipocyte differentiation, which can be reversed by the reintroduction of cyclin D1.[16] The differentiation of adipocytes is governed by growth arrest that is carefully synchronised with the sequential expression of two main transcription factors, C/EBPβ and PPARγ.[6] Initially C/EBPβ is briefly induced, leading to the induction of PPARγ. PPARγ is subsequently activated by fatty acid and prostaglandin ligands to induce the transcription of genes involved in fatty acid metabolism and adipogenesis.[6]

There are two mechanisms by which cyclin D1 may directly inhibit the differentiation of adipocytes. First, cyclin D1 acts as an antagonist of C/EBPβ, inhibiting its transcriptional function, possibly through direct binding.[10] This function is independent of Cdk activation by cyclin D1, as a cyclin D1 mutant unable to activate Cdk4/6 also regulates C/EBPβ targets.[10] The second mechanism is through inhibition of PPARγ. This may be mediated by direct binding of the cyclin D1 helix-loop-helix (HLH) motif to PPARγ to inhibit ligand activation,[16] or by the recruitment of histone deacetylase complexes to the *PPARγ* response elements to prevent transcription.[17] It is interesting to note that *PPARγ* is also a transcriptional target of E2F complexes,[18] and that both E2Fs and Cdk4 have been identified as promoting PPARγ expression, and hence adipogenesis.[18,19] There thus appears to be a complex interplay of events where cyclin D1-Cdk4 activity is initially necessary to activate E2Fs through Rb phosphorylation, hence promoting PPARγ expression. Subsequently the further activation of PPARγ,[16] and possibly C/EBPβ, requires the downregulation of cyclin D1 to induce the full program of adipogenesis.

Interaction of cyclin D1 with differentiation-specific HLH proteins. Differentiation is frequently coordinated through the action of helix-loop-helix (HLH) proteins, which are tissue-specific transcription factors that dimerise prior to interacting with DNA. In numerous tissue types cyclin D1 modulates the activity of HLH differentiation factors in a manner that utilises both its Cdk-dependent and -independent functions (**Fig. 1**). Cyclin D1-Cdk4/6 phosphorylates and inhibits certain HLH proteins, hence maintaining cell cycle progression.[20,21] For example in B-cells, where the HLH protein E2A controls tissue-specific

function, cyclin D1-Cdk4/6 phosphorylates E2A homodimers to prevent their growth-suppressive functions such as the induction of $p21^{Waf1/Cip1}$.[20] However cyclin D1 also acts independently of Cdk4 activity; in B-cells E2A proteins with inactivated phosphorylation sites are still inhibited by cyclin D1.[20] This may occur via a physical association between HLH factors and cyclin D1, such as direct binding of the cyclin D1 HLH-like domain[16] or cyclin D1 C-terminus[22] to target HLH transcription factors, or indirect binding through the p300 coactivator.[23] Cyclin D1 also has a more general role in suppressing p300 coactivated genes, which extends to non-HLH transcription factors such as forkhead factors.[24]

Other cyclins. The role of the cyclin D family in differentiation appears cyclin-specific rather than redundant between the family members. Cyclin D1 appears to be the most potent regulator of certain transcription factors, being the most effective in the regulation of BETA2/NeuroD,[23] v-Myb[25] and the estrogen and androgen receptors.[26,27] In addition, both cyclin D1 and D2 regulate MyoD, while cyclin D3 is ineffective against this transcription factor.[21] The potential for distinct roles of the D-cyclins is also reflected in their individual expression patterns during differentiation. For example, while cyclin D1 is decreased during adipogenesis leading to increased activity of adipogenic transcription factors, cyclin D3 increases during adipogenesis, and promotes the activity of PPARγ.[28] Similarly, in myoblasts, cyclin D3 expression is induced during differentiation, concomitant with the decrease in cyclin D1,[29] and high expression has been observed in other differentiated tissues.[30] However, cyclin D3 is not always upregulated with differentiation: in myeloid cells cyclin D3 binds and inhibits Runx1, thus preventing the transcription of genes involved in myeloid differentiation.[31]

Cyclin E has a crucial role in asymmetric division and polarity in the development of early embryonic tissues.[32-34] Otherwise cyclin E and cyclin A appear to inhibit differentiation of adult cell lineages primarily through Rb hyperphosphorylation,[35] leading to alterations in the binding of Rb to differentiation specific transcription factors (**Fig. 1** and and see below).

Cyclin dependent kinases. The Cdk proteins, as part of cyclin/Cdk complexes, inhibit differentiation through the phosphorylation and downregulation of transcription factors that drive differentiation, or through their sequestration. Cdk2 phosphorylates Cdx2, a homeodomain transcription factor important in determination of intestinal cell fate, leading to its ubiquitination and degradation.[36] Cdk4 directly binds and inhibits some differentiation promoting HLH transcription factors such as MyoD,[37,38] although this action can be dependent on expression of cyclin D1 relocalising Cdk4 to the nucleus.[37] In other tissue types Cdk4 promotes differentiation; in adipocytes Cdk4 activates E2F transcription factors as well as activating PPARγ via a direct interaction.[19]

The downregulation of Cdk6 is necessary for differentiation in numerous cell types, although a Cdk6-dependent mechanism of regulation of differentiation is yet to be established.[39] Recent array data by Slomiany and others indicates that Cdk6 overexpression alters cytoskeletal pathways consistent with failure of differentiation, in addition to blocking cell cycle exit.[40] Despite these specific interactions of Cdks with transcription factors, it is likely that a major mechanism by which Cdks affect differentiation is via phosphorylation of Rb, in partnership with cyclin proteins.

CDK inhibitors (Cip/Kip family). The inhibitor proteins $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ bind to cyclin E-Cdk2 preventing kinase activity, while simultaneously stabilizing the complex by inhibiting phosphorylation of cyclin E.[41,42] The availability of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ to inhibit cyclin E-Cdk2 can be altered through sequestration by cyclin D1-Cdk4/6[43] and by cytoplasmic relocalisation.[44] While $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ effectively inhibit the cyclin E-Cdk2 complex, the kinase complex also positively regulates its own activity by phosphorylating $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ so that they are targeted for degradation.[43,45] The three members of the Cip/Kip family appear to have unique associations with differentiation, where they may play distinct roles in a number of tissues. $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ may induce separate pathways of differentiation in the same tissue,[46] and $p57^{Kip2}$ is uniquely required for cell cycle exit in some systems.[47]

The role of $p21^{Waf1/Cip1}$ in differentiation is tissue-specific, as modulation of its expression may either antagonise or induce differentiation, depending on the model system.[48] Certainly while the differentiation of multiple tissues is associated with an increase in $p21^{Waf1/Cip1}$ levels,[49,50] there are also several instances where $p21^{Waf1/Cip1}$ is downregulated during differentiation.[11,51,52] The downregulation of $p21^{Cip1/Waf1}$ in murine granulocytes predisposes cells to differentiation, and overexpression prevents differentiation.[52] This may be independent of cell cycle function as the cyclin-Cdk binding domain of $p21^{Waf1/Cip1}$ does not need to be intact in order for $p21^{Waf1/Cip1}$ to inhibit differentiation.[11,12] $p21^{Waf1/Cip1}$, independently of its cyclin-Cdk domain, induces upregulation of the *IGF-1* gene and hence MAPK signalling pathways to inhibit the differentiation of keratinocytes.[12] In those instances where high $p21^{Waf1/Cip1}$ is thought to promote differentiation, a possible mechanism of action is via the inactivation of cyclin D1-Cdk4/6 complexes, which has been suggested as occurring in the differentiation of oligodendrocytes.[53]

Expression of $p27^{Kip1}$ increases with differentiation of multiple cell lineages, both in vivo and in cell line studies.[4] Mice lacking $p27^{Kip1}$ show differentiation defects and excessive proliferation in several cell types, including the failure of differentiated luteal cells to undergo growth arrest, leading to infertility.[54] More generally, the overexpression of $p27^{Kip1}$ induces several cell lines to either express markers of differentiation or become more sensitive to differentiation, although differentiation is not necessarily induced.[4] $p27^{Kip1}$ itself thus may not actually regulate genes involved in differentiation in many tissues, but rather create an appropriate molecular environment for differentiation to occur. However, in neuronal development overlapping domains of $p27^{Kip1}$ are associated specifically with either cell cycle exit or induction of differentiation, identifying that $p27^{Kip1}$ plays an active role in the two processes.[55] The N-terminal domain of $p27^{Kip1}$ interacts with and inhibits cyclin-Cdk complexes to induce cell cycle exit. The same region also stabilises Ngn2, a differentiation factor in neurogenesis, and a $p27^{Kip1}$ mutant

incapable of binding cyclin-Cdk complexes still causes Ngn2 stabilisation.[55,56]

Rb/p130/p107. Rb, p130 and p107 form the pocket protein family which, when hypophosphorylated, block S phase progression by binding and inhibiting E2F transcription factors. Hyperphosphorylation of pocket proteins by cyclin-Cdk complexes leads to E2F release and transcription of genes, including cyclin E, necessary for cell cycle progression. There is now accumulating evidence that the activity of these pocket proteins is not confined to cell cycle control but in fact extends to DNA repair, apoptosis, differentiation and development.[57]

The best characterised of the pocket proteins is Rb. $Rb^{-/-}$ mice die during embryogenesis due to defects in differentiation of multiple tissues, believed to be the result of both direct and indirect interference with differentiation.[58] In vitro models of macrophage, neuron and myeloid differentiation have identified cell-type specific molecular pathways that utilise Rb, where hypophosphorylated Rb may interact directly with transcription factors such as C/EBP-β, NPR/B and MyoD, and enhance their induction of differentiation.[59] More recently it has become apparent that Rb can displace differentiation inhibitors from other transcription factors. For example, Iavarone and others have identified that in red blood cell maturation, Rb sequesters the HLH inhibitor Id2 to allow transcriptional activation by PU.1 of CSF-IR, a master regulator in erythroid differentiation.[7]

Counter-intuitively, another role of hypophosphorylated Rb is to interact with E2F transcription factors to form repressor complexes that can inhibit differentiation-specific gene sets,[57] presumably to prevent precocious differentiation in developing tissues. This accounts for the influence of many cell cycle genes on differentiation, since by altering the phosphorylation of Rb, proteins such as cyclins, Cdks and CDK inhibitors will influence binding to E2Fs and hence differentiation. However, the role of Rb in differentiation is by no means completely dependent on E2F binding and hence linked to cell cycle functions,[58] as Rb mutants that are incapable of binding E2F are still able to induce differentiation.[60]

The other pocket proteins, while believed to also have some differentiative roles, are not as well characterised. In some systems p107 and p130 mirror the effects of Rb in differentiation, but in others they have opposing functions. For example, p107, like Rb, binds to and enhances transcription promoted by the NeuroD HLH transcription factor, leading to differentiation of pituitary cells.[61] By contrast, in adipogenesis the presence of Rb is needed for complete adipocyte differentiation and it activates the transcription factor C/EBPα, whereas overexpression of either p107 or p130 inhibits differentiation probably through inhibition of PPARγ.[62] Further studies are required to separate the roles of Rb, p107 and p130 in differentiation, but this is confounded by their redundant roles in cell cycle progression and E2F binding.

Cell Cycle Proteins in the Terminal Differentiation of Mammary Epithelial Cells

The mammary gland is developmentally unusual in that only a rudimentary structure is present at birth. Some morphological differentiation occurs at puberty, with the development of a branched ductal tree. Under the influence of pregnancy hormones, including progesterone and prolactin, a rapid burst of proliferation and differentiation lead to increased sidebranching and the development of hollow spherical lobular alveoli at the end of each of the ducts.[63] This fully differentiated structure requires further hormonal changes after parturition before milk production will begin. At the end of lactation the gland regresses but retains the capacity for new rounds of epithelial expansion and differentiation in subsequent pregnancies. The mammary gland thus undergoes differentiation in stages, each controlled by a distinct set of hormonal cues, and retains the capacity for self-renewal during adulthood.[64]

Complete mammary glands can be reconstituted from a single mammary stem cell in mouse models, and transcription factors such as GATA-3, N-p63 and Elf5 are required for lineage determination to form the distinct cell types of the mammary gland.[64,65] Other transcription factors such as Hox, Stat, Ets, Msx, Id, C/EBP and PPAR proteins also influence lineage determination in the mammary gland as well as contributing to ductal and alveolar morphogenesis and lactation.[66,67] In addition these transcriptional regulators modulate proliferation through the regulation of cell cycle proteins that include cyclin D1, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, Rb and p16, with cyclin D1 being a common target.[66]

Cell cycle proteins are regulated during mammary gland development as cells undergo proliferative arrest and terminally differentiate. For example, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ expression increase at involution,[68,69] whereas cyclin D1 is downregulated during lactation.[70] Mouse models that express cell cycle transgenes or knockout models can present with strong mammary phenotypes.[8,69,71] This may result from changes in early mammary gland development leading to altered levels of the progenitor cell populations that develop into the terminally differentiated alveolar cells. As a consequence, it is difficult to draw conclusions about whether cell cycle proteins are then involved in terminal epithelial differentiation. Despite these limitations, there is increasing evidence for a differentiation specific role for cell cycle proteins, especially cyclin D1, in the mammary gland.

Cyclin D1. In cyclin D1 knockout mice the branching mammary ductal tree develops normally during puberty, but fails to develop lobular alveoli during pregnancy,[8] and this occurs in a cell autonomous manner.[72] This phenotype has been attributed to the fact that cyclin D1 is the major D-type cyclin of the mammary gland, and its expression is high during pregnancy.[70,73] Cyclin D1 is upregulated during mammary development via a number of transcription factors that promote the proliferation of lobular alveolar cells, including RankL and Msx1, and also via steroid hormones estrogen and progesterone.[66,67,70] Additionally, other regulators such as Caveolin-1, an anti-proliferative protein found in the differentiated mammary gland,[74] directly downregulate cyclin D1 expression in the differentiated mammary gland.[75] While cyclin D1 is directly targeted by many of these transcription factors, it is now evident that cyclin D1 can feed back into these pathways through the regulation of certain differentiation regulators such as C/EBPβ and PPARγ.

C/EBPβ is necessary for both alveolar expansion and lactation during mammary gland differentiation, through the direct targeting of genes required for milk production and modulation of other transcription factors.[76] C/EBPβ acts as an effector of cyclin D1 action in mammary epithelial cells via the constitutive repression of cyclin D1 target proteins.[10,77] The overexpression of cyclin D1 antagonises this repressor function,[10,77] and the introduction of cyclin D1 antisense oligonucleotides induces a set of lipogenic genes, probably through C/EBPβ.[78] The interaction between cyclin D1 and C/EBPβ is Cdk4-activity independent, as a kinase-dead mutant of cyclin D1 also antagonises C/EBPβ.[10] C/EBPβ is expressed as the active LAP and inactive LIP isoforms, where in differentiated cells there is a high LAP to LIP ratio, resulting in greater C/EBPβ activity. This is frequently disrupted in breast cancer by the overexpression of the LIP isoform, where a low LAP/LIP ratio is associated with high-grade cancers.[79] The phenotypes of both mammary transplants overexpressing the inhibitory C/EBPβ LIP and mammary glands of knockout C/EBPβ mice are similar to the mammary phenotype of cyclin D1 transgenic mice,[72,79,80] confirming the likelihood that there is a regulatory relationship between C/EBPβ and cyclin D1.

PPARγ and cyclin D1 also have inverse expression patterns in breast epithelial cells,[16] where PPARγ upregulation is causative for mammary epithelial cell differentiation,[81,82] and cyclin D1 is downregulated during differentiation.[83] The inverse expression patterns of cyclin D1 and PPARγ are at least partially due to the negative regulation of cyclin D1 by PPARγ, and vice versa. Activated PPARγ inhibits proliferation via downregulation of cyclin D1 in breast cancer cells, which is reversed by cyclin D1 overexpression.[84,85] Cyclin D1 prevents PPARγ ligand activation and transcription in a kinase-independent fashion in mouse embryonic fibroblasts and likely acts similarly in breast cancer cells.[16,17] Curiously the Cdk4 kinase activates PPARγ in adipocytes,[19] suggesting there may be both positive and negative effects of cyclin D1 on PPARγ, dependent on Cdk4 activation. PPARγ can also upregulate $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, further contributing to cell cycle arrest.[86] When the related protein, PPARα, is constitutively activated in the mouse, the mammary epithelium displays impaired proliferation during pregnancy and a reduction in cyclin D1 levels.[87]

Observations on the overexpression of cyclin D1 in experimental models support the conclusion that it may impair differentiation: cyclin D1 overexpression leads to sustained proliferation of mammary epithelial cells, which is associated with a delay in acinar development in in vitro models[88] and a failure to terminally differentiate in mouse models.[89] Overexpression of cyclin D1 under a MMTV promoter in mouse mammary glands leads to the development of mammary adenocarcinoma,[90] though this occurs with long latency. Furthermore, the overexpression of cyclin D1, while inducing carcinoma, does not greatly increase epithelial cell mass, implying that cyclin D1 expression maintains proliferation and inhibits differentiation, rather than solely increasing proliferation.[89] Cyclin D1 is also targeted by the developmental protein, Msx1, whose overexpression during late pregnancy leads to failed differentiation and lactation via maintenance of expression of cyclin D1.[70] Again, this phenotype is not associated with any hyperproliferation, but is instead characterised by a failure to terminally differentiate and produce milk proteins.[70] Notably, while cyclin D1 is required for complete mammary gland development, the phosphorylation of Rb via cyclin D1 action is not required, as cyclin D1-Cdk4/6 activity is dispensable to normal mammary gland development during pregnancy.[91] Consequently the Cdk-independent roles of cyclin D1, such as interactions with C/EBPβ and PPARγ, are likely to be important in mammary gland development.

Rb. While the retinoblastoma protein Rb affects differentiation in several cell types (see above), it was initially believed to be dispensable in normal mammary gland development.[92] However, through the overexpression of a non-phosphorylatable mutant (RbΔK) in mouse mammary glands, Jiang et al. demonstrated that it was also important in mammary epithelial differentiation.[71] RbΔK expression retards ductal proliferation in mammary glands and leads to precocious differentiation of the mammary gland of virgin females, characterised by the expression of the milk protein β-casein and a morphology reminiscent of lactation.[71] In addition, the overexpression of other Rb mutants that cannot bind E2F co-repressors but are still phospho-competent, leads to mammary phenotypes with normal epithelial differentiation but excessive proliferation in the form of hyperplasia.[93] Together these data suggest that the interaction of Rb with distinct sets of transcription factors, dependent on its phosphorylation status, may lead to either cell differentiation or proliferation. In other cell systems Rb can bind the transcription factor C/EBPβ[59] and also Id2,[7] which are both important in mammary epithelial differentiation, but these interactions have not yet been demonstrated in mammary epithelial cells.

Other cell cycle proteins. Cell cycle proteins other than cyclin D1 and Rb may also influence the differentiation of mammary epithelial cells. $p21^{Waf1/Cip1}$ overexpression induces morphological changes and lipid production in MCF-7 breast cancer cells[94] and $p21^{Waf1/Cip1}$ binds in a complex with the co-activator protein p300/CBP, which activates differentiation pathways in other tissues.[94] However, $p21^{Waf1/Cip1}$ knockout mice have a normal phenotype, although the mammary phenotype, if any, has not been described explicitly.[95] Deletion of $p27^{Kip1}$ in the mouse mammary gland has given rise to distinct outcomes in different laboratories, either with no overt phenotype,[96] or having impaired proliferation and lobular alveolar morphogenesis.[69] In another study it was found that mammary-derived cells with high $p27^{Kip1}$ levels fail to undergo alveologenesis and also lack hyperproliferative potential.[97] Consequently the role of $p27^{Kip1}$ in differentiation of mammary epithelial cells is still to be clarified, although it may have an antagonistic role to cyclin D1, as $p27^{Kip1}$ deletion is able to rescue the phenotype of cyclin $D1^{-/-}$ mice.[98] At this stage it does not appear that $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ have core regulatory roles in mammary differentiation as has been observed in keratinocytes and neuronal cells, respectively.

Overall it appears increasingly likely that, like other tissue types, certain cell cycle proteins play a role in differentiation in the mammary gland. In particular, cyclin D1 lies downstream of multiple differentiation regulators, but provides feedback through proteins C/EBPβ and PPARγ. Cyclin D1 is also a key regulator of

adipocyte differentiation through its interactions with C/EBPβ and PPARγ.[78] Since adipocytes form a significant component of the mammary stroma and affect differentiation of the mammary epithelium through paracrine signalling,[74] it is not surprising to identify overlapping regulatory networks between adipocytes and the mammary epithelium.[78] High cyclin D1 expression is also postulated to maintain an active stem cell pool which is consistent with a decrease in the incidence of terminal differentiation,[89] and cyclin D1 may also be required for the action of other genes involved in lineage determination such as Id1.[99]

Cyclin D1 functionality may also reside in its interaction with Rb, as the mammary phenotype of cyclin D1 knockout mice can be rescued by both cyclin E1 knockin[100] and $p27^{Kip1}$ deletion.[98] A cyclin D1-unique function is still likely however, as cyclin D1/Cdk4 kinase activity is not required for full mammary gland development,[91] and cyclin D2 is unable to substitute for cyclin D1 in the retina which is also a cyclin D1-dependent tissue.[101] It is also probable that Rb may affect differentiation independently of cyclin D1, given its propensity to interact with numerous differentiation factors in other tissues, such as Id2. However, further investigation is required to gain a full understanding of the complete role of cyclin D1, Rb and the Cdk inhibitors, in the terminal differentiation of the mammary epithelium.

Differentiation Status of Breast Tumours, and Relationship to Cell Cycle Proteins

Breast cancer is a heterogenous disease that includes tumours with distinct cellular morphology and degrees of differentiation. Upon initial detection, breast cancers are subclassified by grade, histology and molecular markers such as estrogen receptor and Her2/Neu status, which allow personalised disease management based on predicted outcome and response to therapy. Surprisingly, only some cell cycle defects are associated with tumours that present with a "more proliferative" phenotype, as measured by mitotic index or high expression of molecular markers such as Ki67.[102] Consequently, with the possible exception of cyclin A, cell cycle proteins cannot be used indiscriminately as surrogate markers for proliferation.[103] By contrast, there can be a strong association between distinct cell cycle protein profiles and degrees of differentiation of breast tumours, such that invasive and non-invasive lesions of similar differentiation status can have similar alterations in cell cycle proteins.[104,105] This suggests that the expression of cell cycle proteins in breast cancers may reflect aspects of tumour biology such as differentiation, apoptosis and hormone receptor status, rather than simply being a readout of proliferative rate.

A key example of the different phenotypes associated with cell cycle defects arises from a comparison of tumours with high expression of cyclin D1 and cyclin E1. Both cyclins are overexpressed in a large proportion of breast cancers, although frequently in distinct subsets, e.g., ER positive and ER negative.[106] Overexpression of either cyclin D1 or E1 can be associated with poor prognosis in breast cancer, but cyclin E1 has a much stronger association with proliferative markers or mitotic index.[107-109] Indeed, cyclin D1 overexpression has not been associated with markers of cancer cell cycle activity in breast cancers[110,111] or with Cdk4 activity in a panel of breast cancer cell lines,[112] supporting a hypothesis that non cell cycle roles of cyclin D1 may play a role in breast tumorigenesis. In fact the non-Cdk functions of cyclin D1 are strongly implicated in carcinogenesis, as the regions of the protein outside the cyclin box are required for transformation.[113] Additionally, overexpression of cyclin D1 that is unable to activate Cdk4 leads to a gene signature characteristic of cyclin D1-overexpressing breast cancers.[10] This is consistent with the growing body of evidence that implies that cyclin D1 potentially mediates effects in breast cancer through the transcription factor C/EBPβ,[10] and by altering differentiation potential.[89]

The association of cyclin D1 with breast tumorigenesis appears to be unique among the D-cyclins, despite the fact that the D-cyclins can generally substitute for one another in proliferation.[114] Cyclin D1 and D3 overexpression in mice also gives rise to phenotypically distinct mouse mammary tumours, where the MMTV-cyclin D1 mouse produces adenocarcinomas and cyclin D3 induces squamous cell carcinomas.[115] Cyclin D3 is also overexpressed in a proportion of breast cancers (~10%),[116] but unlike cyclin D1, cyclin D3 expression increases with grade,[117] and is associated with a subset of estrogen receptor negative, ErbB2 negative aggressive cancers.[118]

Other proteins of the G_1 to S phase transition of the cell cycle, including $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p16^{INK4a}$, are also frequently altered in breast cancer. Generally the reduced expression of the Cdk inhibitor protein, $p27^{Kip1}$, has a strong association with high grade and increases in markers of proliferation such as PCNA or Ki67 expression, or mitotic index.[102,119] Studies on the expression of $p21^{Cip1/Waf1}$ are less clear, with high $p21^{Cip1/Waf1}$ associated with both high and low grade tumours,[102,120] and high $p21^{Cip1/Waf1}$ both positively and negatively associated with markers of proliferation.[121,122] The levels of Rb are not altered in the majority of breast cancers, but recently it has been identified that Rb is expressed at low levels in the poorly differentiated, highly proliferative basal-like breast cancers.[123,124] In addition, Rb-responsive genes are commonly deregulated, suggesting that Rb inactivation may be more common than deletion or mutation.[125] $p16^{INK4a}$ is also expressed at high levels in this subgroup of cancers, even though high expression would be presumed to have an anti-proliferative effect.[123,124]

Gene expression arrays have identified "proliferation signatures" in breast cancer that are associated with poor prognosis.[126-128] While some cell cycle genes such as cyclin B and cyclin E2 are included in these signatures, they do not include many cell cycle proteins with known central roles in breast cancer cell proliferation, including cyclin D1 and $p21^{Waf1/Cip1}$.[126] Changes in these proteins are however, very common in breast tumorigenesis, suggesting that alterations in their expression do not necessarily lie downstream of increases in proliferation, or conversely, do not always contribute to tumorigenesis through increases in proliferation. Indeed, the expression of certain proteins is highly correlated with core aspects of breast cancer biology such as hormone receptor status rather than proliferation per se: cyclin D1 is a well-established estrogen receptor target and is high in estrogen receptor positive tumours,[129] whereas

cyclin E and $p27^{Kip1}$ are generally associated with ER negative tumours.[102,108,109]

Alterations to many cell cycle proteins can promote hyperplasia and tumour formation in mouse models, which argues that they are sufficient to induce tumorigenesis. $p21^{Waf1/Cip1}$, $p27^{Kip1}$, cyclin D1, $p16^{INK4a}$ and the retinoblastoma protein can contribute to changes in apoptosis, cell motility and differentiation, which are all important aspects of cancer development.[2-5] Breast cancer has been described as a disease of defective differentiation as much as a disease of excessive proliferation,[130] and given the prominent association of cell cycle proteins with breast cancer progression, a better understanding of their role in breast epithelial differentiation may shed further light on their role in breast cancer development.

Differentiation Therapeutics in Breast Cancer, and Their Effect on Cell Cycle Proteins

Several differentiation agents have been evaluated as therapeutics for breast cancer, including PPARγ agonists, melatonin, HDAC inhibitors, Vitamin D and glucocorticoids. These drugs can have multiple effects such as the induction of growth arrest, changes to morphology and increases in apoptosis, which may or may not be associated with terminal differentiation.[131] Nevertheless, since differentiation has been a successful therapeutic strategy in leukaemia, there is ongoing research into the mechanism of action and efficacy of differentiation therapeutics in breast cancer.

Many differentiation agents induce breast cancer cells to undergo growth arrest by targeting the G_1 to S phase proteins. For example, PPARγ agonists cause breast cancer cells to undergo growth arrest by targeting the cell cycle through increases in $p27^{Kip1}$,[132] and by preventing estrogen induction of cyclin D1.[81,86,133] Treatment of mice with the PPARγ agonist, 1,1-Bis(3'-indolyl)-1-(*p*-biphenyl)methane, retards expansion of xenografted mammary tumour cells, associated with increases in $p27^{Waf1}$ and differentiation marker Caveolin-1.[132] Likewise, the PPARγ agonist, GW7845, caused a delay in the appearance of chemically induced mammary tumours, and alterations in tumour morphology which has a molecular signature consistent with increased differentiation.[134] These data suggest that PPARγ agonists induce breast epithelial cells to differentiate in association with cell cycle arrest. While the in vitro and mouse model data appear promising, clinical trials in breast cancer patients with PPARγ agonists have not to date proved successful.[135-137] This reflects the generally poor performance of PPARγ agonists as therapeutics in multiple tumour types, which is partially attributed to mutations in the PPARγ signalling pathway.[135]

Vitamin D is also known to target breast cancer cells for growth arrest, apoptosis and differentiation. Vitamin D-induced differentiation is associated with a G_0/G_1 cell cycle arrest,[138] which is itself strongly linked with changes in cyclin D1, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, and c-Myc.[131,139] As described for PPARγ agonists, Vitamin D therapy has generally not been successful as a stand alone treatment of any tumour type, due to a combination of resistance and toxicity.[131,140] Vitamin D causes some, but not all, breast cancer cell lines to undergo growth arrest and differentiation, where resistance to vitamin D treatment is associated with changes in the levels of the Vitamin D receptor and metabolising enzymes.[131,141]

The most successful use of a differentiation agent for cancer therapy has been the treatment of acute promyelocytic leukaemia (APL) with the activator of the retinoic acid pathway, ATRA.[142] Retinoids, including ATRA, target malignant cells by inducing growth arrest, apoptosis and differentiation.[143] APL is uniquely susceptible to retinoid therapy, which is at least partially due to a common translocation event in APL that fuses the promyelocytic leukaemia (PML) and retinoid receptor alpha gene (RARα) to create a dominant negative RARα transcription factor. Retinoid treatment dissociates the PML-RARα fusion protein from promoter regions and also promotes its degradation.[143] Retinoids also affect growth and differentiation independently of this fusion protein, which is relevant to the therapeutic use of retinoids against other tumour types. Cell cycle arrest is induced via increased turnover of cyclin D1,[144,145] and by increasing $p21^{Cip1/Waf1}$ or $p27^{Kip1}$ expression.[146,147] Retinoids are generally not effective in solid tumours such as breast cancers, although they may have potential in reducing the occurrence of breast cancer.[143] The reduced susceptibility of breast cancers to retinoid therapy is probably due to epigenetic modifications or mutations in components of the retinoic acid signalling pathway.[148]

Overall differentiation agents have not been successful as primary therapy in breast cancer, due to a combination of changes in the expression of receptor proteins, epigenetic modifications and treatment toxicity. However, differentiation agents are still under consideration in combination therapy or as adjuvant therapy, which means it is important to understand their mechanism of action. Studies of differentiation agents such as PPARγ, vitamin D and retinoic acid have identified cyclin D1, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ as common cell cycle targets in breast cells that are involved in the growth arrest induced by these agents. As discussed in the preceding section, cyclin D1, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ frequently have altered expression in breast cancers. Consequently, another factor in the effectiveness of differentiation treatment may be the cell cycle profile of the target cells. For example, the overexpression of cyclin D1, which is commonly observed in breast cancer, is able to prevent retinoid-induced cell cycle arrest and differentiation in human mammary epithelial cells.[149]

Cancer cell lines derived from other tumour types have also shown an altered response to differentiation agents due the modulation of levels of cell cycle proteins. Moreover, the change in expression of the cell cycle proteins does not only prevent growth arrest, it also appears to alter the overall response to the differentiation agent. For example, $p21^{Waf1/Cip1}$ is upregulated by a PPARγ agonist in thyroid carcinoma cells, and siRNA-mediated knockdown of $p21^{Waf1/Cip1}$ prevents both growth arrest and apoptosis induced via the agonist.[150] Retinoic acid can also induce different cell fates in association with cell cycle arrest dependent on the overall expression profile of cell cycle proteins. Neuroblastoma cells with high $p21^{Cip1/Waf1}$ and cyclin D1 levels differentiate in response to retinoids, whereas cells with

low $p21^{Cip1/Waf1}$ and expression of $p16^{INK4a}$ and $p18^{INK4c}$ enter a senescent state.[151]

These data indicate that the molecular profile of a tumour, including expression levels of signalling components for differentiation pathways and cell cycle proteins, may be informative in relation to whether a differentiation therapeutic could be efficacious. Currently breast tumours are not subjected to extensive genomic profiling as part of clinical practice, except for major markers such as estrogen receptor, progesterone receptor and Her2. However, this is a rapidly developing area, and molecular profiling tools such as Oncotype DX, a 21 gene assay, are coming into use to stratify patients into those predicted to respond better to hormonal therapy or chemotherapy.[152] Given the poor outcome from the use of differentiation therapies to date, the use of molecular profiling may improve their successful application in the clinical setting.

Concluding Remarks

Changes in cell cycle proteins are some of the earliest events noted in pre-neoplastic lesions in the mouse mammary gland,[153] and this also appears true of human breast cancer.[104,154,155] Many of the proteins involved in G_1 to S phase control have altered expression in breast cancer, including cyclin D1, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and changes in these proteins are likely to be significant in the increased proliferation observed in hyperplasia.

A review of the literature suggests the breast epithelium also utilises cyclin D1, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and Rb during differentiation, to provide positive or negative feedback to differentiation pathways. Cyclin D1, in particular, is a central player both in the differentiation of mammary epithelium and breast cancer development. It appears to be closely involved in the regulation of mammary epithelial differentiation by C/EBPβ and PPARγ, as both a regulator and a target of these transcription factors. Cyclin D1 is also a conduit for the action of differentiation-inducing therapeutics, including PPARγ agonists. Consequently the alteration of proteins such as cyclin D1, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and Rb in breast cancer may not only alter proliferation, but may also create a more permissive environment for other events in tumorigenesis, such as failure to differentiate. As such, a differentiation stimulus may have reduced efficacy against a neoplastic cell with altered expression of cell cycle proteins. In the continuing study of differentiation pathways and therapeutics it will be relevant to observe the effect of expression of cell cycle proteins. This may allow a further understanding of the role of cell cycle proteins in differentiation, and also how they influence the progression of breast cancer.

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