Cell-nonautonomous function of the retinoblastoma tumour suppressor protein: new interpretations of old phenotypes

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Loss of the retinoblastoma protein (pRb) induces a cell-nonautonomous defect in both erythroid and neuronal differentiation. It has previously been thought that this reflects a requirement for pRb function in cells that normally support erythropoiesis and neurogenesis, rather than in the erythrocytes or neurons themselves. However, recent studies have challenged this interpretation, and it appears that erythrocytes and neurons themselves have the intrinsic requirement for pRb function. This requirement can be bypassed by signals supplied by wild-type erythroid or neuronal cells. The existence of such a signalling mechanism has implications not only in understanding pRb function but also in the interpretation of other cell-nonautonomous phenotypes.

The pRb protein

The retinoblastoma protein (pRb) is the founding member of the family of so-called pocket proteins, which also includes the proteins p107 and p130 (Lipinski and Jacks, 1999). Although pRb plays a pivotal role in controlling gene expression to regulate cellular proliferation, apoptosis and differentiation (Lipinski and Jacks, 1999; Vooijs and Berns, 1999; Nevins, 2001; Zhang and Dean, 2001), it lacks a DNA-binding domain and is tethered to promoters through its interaction with other transcription factors (Zhang and Dean, 2001). The interaction of pRb with such factors is inhibited by its phosphorylation (Sherr, 1996), a process that is mediated by cyclin-dependent kinases (CDKs), which are themselves regulated by the CDK inhibitors (Sherr and Roberts, 1999). The E2F family of transcription factors is among those bound and regulated by hypophosphorylated pRb (Nevins, 2001). The latter acts both by directly inhibiting E2F transactivating activity and by recruiting transcriptional repression

complexes to promoters containing E2F sites (Lipinski and Jacks, 1999). In fact, pRb phosphorylation results in disruption of pRb/E2F complexes and is thought to be a crucial event in the G₁ to S phase transition (Sherr, 1996; Sherr and Roberts, 1999). Hypophosphorylated pRb also binds to and regulates the function of other proteins, including the helix-loop-helix protein Id2 (Lasorella *et al.*, 2000), CAAT/enhancer binding proteins (Chen *et al.*, 1996) the HMG family member HBP1 (Shih *et al.*, 1998) and, possibly, basic-helix-loop-helix proteins such as MyoD (Gu *et al.*, 1993). pRb also appears to regulate gene transcription by recruiting chromatin remodelling factors such as histone deacetylases, the SWI/SNF complex, DNA methyltransferases and histone methyltransferases (Ferreira *et al.*, 2001; Nielsen *et al.*, 2001; Zhang and Dean, 2001).

Loss of pRb in mice

Recent work on the role of pRb in erythropoiesis has revealed a novel link between this cell cycle regulatory protein and intercellular signalling. pRb regulates the development and differentiation of various tissues, including central and peripheral nervous system, muscle, retina, lens and blood (Lipinski and Jacks, 1999; Vooijs and Berns, 1999). However, pRb does not induce a simple cell-autonomous defect in some of these tissues, i.e. the phenotype of the cell does not directly correlate with its genotype. This was demonstrated by the finding that, in pRb null chimeric animals, the mutant cells contributed significantly to both the erythroid and neuronal lineages (Maandag *et al.*, 1994; Williams *et al.*, 1994). It is important to understand how pRb functions in this cell-nonautonomous manner in these lineages.

The requirement for pRb function was demonstrated by gene disruption experiments in mice. Complete loss of pRb resulted in

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lethality during gestation at around 14 days post coitum (d.p.c.). pRb null embryos displayed excessive neuronal cell death and were anemic. The latter effect appears to have been due to the disruption of fetal liver erythropoiesis. Normally, at this stage in development, definitive (or adult) enucleated erythrocytes replace primitive (or embryonic) nucleated yolk-sac derived erythrocytes in the circulation. The first and major site of definitive erythropoiesis at this stage is the fetal liver. Definitive erythropoiesis in the fetal liver (and later in the spleen and bone marrow) occurs in erythroblastic islands (Bessis *et al.*, 1983). These contain a central macrophage surrounded by erythroid cells at different stages of maturation. The latter are arranged in a characteristic pattern, with immature cells close to the centre and mature cells near the edge (Figure 1) (Bessis *et al.*, 1983; Bernard, 1991). In pRb null embryos, the number of the definitive erythrocytes was dramatically reduced. Instead, immature (nucleated) fetal liver derived cells were present in the circulation in high numbers (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Furthermore, erythroid colonies grown from pRb null fetal livers were defective in differentiation, i.e. the colonies were more numerous and the cells were paler and failed to enucleate efficiently (Jacks *et al.*, 1992).

From these experiments, it is unclear whether pRb is intrinsically required in the erythroid cells for their normal differentiation or in a cell type that supports erythropoiesis, such as a growth factor producing stromal cell. Chimeric mice are usually generated to determine whether a gene functions autonomously or nonautonomously in a particular lineage. Gene function is considered cell autonomous when a cell displays a phenotype corresponding to its genotype, regardless of the genotype of surrounding cells (Figure 2A). In the case of the pRb null chimeric mice (Maandag *et al.*, 1994; Williams *et al.*, 1994), the function of pRb in the erythroid lineage would be considered cell autonomous if pRb null cells failed to differentiate and the surrounding wild-type erythroid cells differentiated normally. Gene function is defined as cell nonautonomous when a cell displays a phenotype that does not correspond to its genotype (Figure 2B) (Apfeld and Kenyon, 1998): in other words, where wild-type cells display the phenotype of mutant cells or mutant cells display the phenotype of wild-type cells. In the chimeric animals, pRb null cells contributed highly to the erythroid compartment (Maandag *et al.*, 1994; Williams *et al.*, 1994). Importantly, the differentiation of pRb null erythroid cells was indistinguishable from that of wild-type cells. The only phenotypic change in a few mice was a transient persistence of nucleated cells (both wild-type and pRb null) in the circulation of chimeras with a relatively higher contribution of pRb null cells (Maandag *et al.*, 1994). Since genotypically pRb null erythroid cells display a wild-type phenotype, the function of pRb in erythropoiesis is, by definition, cell nonautonomous.

The cell that requires pRb

These observations beg the question: in which cell type is the function of the pRb gene required for normal erythropoiesis? If the defect had been cell autonomous, the interpretation would have been that the pRb gene is required in the erythroid cells themselves for their normal development and/or differentiation. Conversely, when cell nonautonomy occurs, as is the case in the pRb chimeras, the gene is thought to function in cells other than

Fig. 1. A simplified model of the erythroblastic island. The position of the central macrophage and the arrangement of the erythroid precursors within the macrophage cytoplasmic extensions are shown (as indicated by the key, in order of maturation). The processes of enucleation and phagocytosis of expelled pyknotic nuclei are represented.

those that exhibit the original phenotype (Rossant and Spence, 1998). In principle, if a cell-nonautonomous phenotype is displayed by cell A, then the function of gene X is required in cell B (Figure 3). In such a model, cell B could be supplying a signal that is required for the wild-type phenotype of cell A. Cell A then responds to this signal by displaying a wild-type phenotype, regardless of whether or not gene X is present in its own genome. In the case of the erythroid pRb phenotype, it was proposed that pRb is required in a stromal cell (cell B), which supports erythroid differentiation, and that pRb is not intrinsically required by the erythroid cells themselves (cell A) (Figure 3A) (Maandag *et al.*, 1994; Williams *et al.*, 1994; Vooijs and Berns, 1999). This interpretation is a heterotypic signalling model, where cell A is of a different lineage to cell B. However, subsequent experiments cast doubt on this interpretation. When lethally irradiated wild-type animals (all hematopoietic cells essentially eliminated) were repopulated with pRb null hematopoietic cells, all hematopoietic lineages examined appeared normal, with the exception of the erythroid lineage. In this case, the erythroid cells failed to differentiate normally, showing increased proliferation and a block in differentiation, manifest as

Fig. 2. Cell autonomy and cell nonautonomy in heterocellularly mutant cell populations. (A) Gene function is considered cell autonomous when a cell displays a phenotype corresponding to its genotype, regardless of the genotype of surrounding cells. The pink cell on the left, with a wild-type genome, becoming a red cell represents normal differentiation. The cell on the right has a mutation (shown as a crossed gene) that prevents differentiation. (B) In cell nonautonomy, mutant cells display the phenotype of wild-type cells (left panel) or wild-type cells display the phenotype of mutant cells (right panel; this situation is not discussed in this paper).

Fig. 3. Two possible interpretations of cell nonautonomy. (A) In a heterotypic signalling model, gene function is not required in cell A. The function of the gene is required by cell B, which is of a different lineage. Cell B supplies a signal (red arrows) that is required for the differentiation of cell A. (B) In a homotypic signalling model, the function of the gene is required for the differentiation from cell A to cell B. Cell B supplies a signal that activates the differentiation of wild-type cell A and is required for the differentiation of mutant cell A.

a stable persistence of nucleated erythroid cells in the circulation and extensive erythropoiesis outside the bone marrow (Hu *et al.*, 1997). There are two possible outcomes of the irradiation relevant to this experiment, i.e. the stromal cells in the recipient animal survive or not. If the host stromal cells did not survive the irradiation, all donor derived hematopoietic cell lineages requiring stromal cells would be affected, regardless of their pRb requirement (i.e. the same would be observed with wild-type donor tissue). In contrast, if the host stromal cells (pRb positive) persisted after irradiation, the heterotypic model described above predicts that all hematopoietic lineages (pRb negative) would be normal. Neither of these possibilities occurred. This indicates that pRb function must be required in a critical transplantable cell that is lost in the recipient during the lethal irradiation. This cell, in turn, expresses a signal that is required for the normal differentiation of pRb null erythroid cells. This may be a non-erythroid cell, consistent with a heterotypic signalling model. However, such a critical non-erythroid cell has not been identified, making this model unsubstantiated.

In contrast to a heterotypic model, one can propose a homotypic signalling model (Figure 3B). In this case, cell B, which supplies the signal required by cell A, has differentiated further

along the same lineage. According to this model, in the pRb null chimeric mice, the surrounding wild-type erythroid cells would supply the signal that activates the differentiation of less mature pRb null erythroid cells. This is distinct from a heterotypic model, as it is based on the premise that pRb functions in the erythroid cell itself. In a homotypic model, loss of pRb in erythroid cells disrupts the expression of the signal required for the differentiation of pRb null cells. If the gene disrupted is, for example, a paracrine growth factor expressed and required by cells of a particular lineage, such a homotypic model is intuitively obvious. However, pRb is not a paracrine growth factor and it is unlikely that it would regulate only the expression of such factors by erythroid cells. Nevertheless, pRb may indirectly affect the expression of paracrine factors by regulating the differentiation process itself. Indeed, many studies suggest that pRb plays an intrinsic role in regulating the proliferation and differentiation of erythroid cells. During hematopoiesis, pRb is expressed in the erythroid lineage and down-regulated in other lineages (Condorelli *et al.*, 1995). Accumulation of hypophosphorylated pRb is characteristically observed during erythroid differentiation (Sehy *et al.*, 1992; Kiyokawa *et al.*, 1994;

Zhuo *et al.*, 1995; Hseih *et al.*, 2000). pRb-mediated G₁ arrest is thought to be important in chemically induced differentiation of virally transformed murine erythroleukemia (MEL) cells (Zhuo *et al.*, 1995) and in activin-A induced differentiation of human erythroleukemia cells (Sehy *et al.*, 1992). Inhibition of MEL cell differentiation by the overexpression of the erythroid transcription factor GATA-1 is also associated with reduced levels of hypophosphorylated pRb and the inhibition of differentiation-associated G₁ arrest (Whyatt *et al.*, 1997). Antisense experiments suppressing pRb activity have suggested that pRb regulates erythropoiesis (Condorelli *et al.*, 1995; Bergh *et al.*, 1997; Konishi *et al.*, 1999). Various studies have proposed functional cross-regulation between pRb and factors such as Fli-1, PU.1, PML and Tal-1 during erythropoiesis (Konishi *et al.*, 1999; Labbaye *et al.*, 1999; Tamir *et al.*, 1999; Vitelli *et al.*, 2000). When considered alongside the transplantation experiment (Hu *et al.*, 1997), these studies argue that erythroid cells have an intrinsic requirement for pRb activity. A homotypic signalling model reconciling this with the cell nonautonomy found in the chimeras is shown in Figure 3B. In this model, when an erythroid cell is pRb null, the requirement for pRb activity can be bypassed by signals supplied by cells that have already undergone the maturation process.

In support of the above, a similar model has already been proposed that is based on an experiment in which the transcription factor GATA-1 was overexpressed in erythroid cells (Whyatt *et al.*, 2000). GATA-1 is a zinc-finger-like transcription factor required for erythroid differentiation, and loss of this factor induced cell-autonomous apoptosis in immature erythroid cells (Pevny *et al.*, 1991; Weiss and Orkin, 1995). Interestingly, mice overexpressing GATA-1 under erythroid-specific activating sequences had an erythroid phenotype similar to that of the pRb null mice (Whyatt *et al.*, 2000). The mice were anemic due to a failure in fetal liver derived erythropoiesis and died at around 13.5 d.p.c. Erythroid cells overexpressing GATA-1 were intrinsically defective and failed to differentiate in mice overexpressing GATA-1 in all erythroid cells. However, this defect was also cell nonautonomous. It is postulated that GATA-1 overexpressing erythroid cells differentiate in response to a signal (designated REDS, for red cell differentiation signal) that is supplied by relatively mature erythroid cells (Whyatt *et al.*, 2000) in the erythroblastic island (Figure 4). In mosaic animals, immature erythroid cells overexpressing GATA-1 would be juxtaposed with wild-type 'mature' cells in the same erythroblastic island and/or the neighbouring island, making REDS signalling possible and thereby reversing the defects induced by high GATA-1 levels. In mice overexpressing GATA-1 in all cells there would be no such juxtaposition, explaining the failure of erythroid differentiation (Whyatt *et al.*, 2000).

Nonautonomous pRb function in the nervous system

Although pRb null neurons in chimeric mice autonomously display ectopic S-phase entry, the neuronal apoptosis that otherwise occurs in the absence of pRb is suppressed (Lipinski *et al.*, 2001). Thus, the effect of pRb loss is partially cell nonautonomous in neuronal tissues. Theoretically, pRb null cells could secrete an apoptosis-inducing factor that is diluted in chimeras (Lipinski *et al.*, 2001). However, it is unlikely that pRb would

Fig. 4. Homotypic signalling within the erythroblastic island. Arrows indicate the signal REDS being produced by relatively mature erythroid cells. The bar represents the switch from predominantly proliferation to differentiation during erythropoiesis.

specifically regulate the expression of such a secreted factor by neurons. In fact, loss of pRb in the neuronal cells themselves is likely to disrupt their differentiation and thereby lead to apoptosis (Lee *et al.*, 1994; Macleod *et al.*, 1996; Tsai *et al.*, 1998). It has been suggested that a signal supplied by wild-type cells might inhibit the apoptosis of pRb null neurons (Lipinski *et al.*, 2001). If this were the case and the cells supplying the signal were wild-type neurons, homotypic signalling would again be the factor that overcomes the loss of pRb in neurogenesis.

Homotypic signalling in other systems

Homotypic signalling mechanisms also appear to explain cell-nonautonomous phenomena in other systems. One precedent for the homotypic model presented here exists in the nematode *Caenorhabditis elegans*. In this organism, the insulin/IGF receptor homologue DAF-2 regulates adult development and ageing. A mild decrease in *daf-2* activity can lengthen lifespan, and a more severe decrease in activity can cause larvae to enter a state of diapause (dauer formation) rather than progressing to adulthood. Mosaic analysis demonstrated that *daf-2* functions cell nonautonomously in both processes. For example, during development, larvae null for *daf-2* become dauers. In contrast, larvae mosaic for *daf-2* can become adults and all cells, regardless of genotype, display an adult phenotype. Importantly, an adult phenotype was not associated with a requirement for *daf-2* activity in any particular cell. It is thought that secondary signals operate to ensure that all cells adopt the same developmental fate (Apfeld and Kenyon, 1998). Similarly, in mice, homotypic signalling between neural crest cells has been proposed to explain the cell-nonautonomous defect in colonization

of the lower gut by endothelin-receptor B null enteric neuroblasts (Kapur *et al.*, 1995).

Perspectives

Homotypic signalling potentially activates pathways that allow defects, such as pRb loss or GATA-1 overexpression, to be tolerated. However, what would be the function of such a signalling mechanism during erythropoiesis when such defects are not present? Mature erythroid cells regularly leave the erythroblastic island and must be replaced. Signals, such as the postulated REDS, may normally regulate the balance of proliferating and differentiating cells within the island. REDS produced by mature cells may activate the maturation and differentiation (at the expense of proliferation) of more immature cells. This would lead to the reinforcement or acceleration of maturation among all responsive cells in the island. This, in turn, would create a feedback mechanism in which the departure of mature cells would allow immature cells to proliferate and expand, whereas the accumulation of mature cells would inhibit the proliferation and activate the differentiation of immature cells. In addition to a normal role in regulating the balance between differentiation and proliferation, REDS could also provide a mechanism to activate differentiation during periods of rapid proliferation, e.g. during Epo-induced erythroid expansion following hypoxia (Krantz, 1991). As immature cells moved towards the source of REDS, i.e. relatively mature cells on the periphery of the island, differentiation would become synchronized, maintaining the distribution of mature cells on the periphery. This could explain the observation that the process of erythroid differentiation within islands, including the timing of cell division, can be highly synchronous (Allen and Testa, 1991). Such synchronization may be functionally important in the efficient release of mature cells into the circulation, as it would result in a large proportion of cells being at the same late stage of maturation (Allen and Testa, 1991).

In conclusion, it appears that the intrinsic requirement for pRb function in the differentiation of some cell types can be bypassed by signals supplied by wild-type cells, particularly in systems in which mature and immature cells are often in contact with one another. This phenomenon has implications in cancer, as loss of signal responsiveness could contribute to the tumorigenicity of cells that have lost pRb function. Furthermore, the existence of such a signalling mechanism indicates that previous interpretations of cell-nonautonomous defects may, in fact, attribute gene function to incorrect cell types.

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