

Retinoblastoma protein: a central processing unit

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The retinoblastoma protein (pRb) is one of the key cell-cycle regulating proteins and its inactivation leads to neoplastic transformation and carcinogenesis. This protein regulates critical G₁-to-S phase transition through interaction with the E2F family of cell-cycle transcription factors repressing transcription of genes required for this cell-cycle check-point transition. Its activity is regulated through network sensing intracellular and extracellular signals which block or permit phosphorylation (inactivation) of the Rb protein. Mechanisms of Rb-dependent cell-cycle control have been widely studied over the past couple of decades. However, recently it was found that pRb also regulates apoptosis through the same interaction with E2F transcription factors and that Rb–E2F complexes play a role in regulating the transcription of genes involved in differentiation and development.

[Poznic M 2009 Retinoblastoma protein: a central processing unit; *J. Biosci.* **34** 305–312]

1. Introduction

The retinoblastoma protein (pRb) belongs to a ‘pocket protein’ family with a highly conserved sequence in the pocket domain. This sequence mediates interactions with cellular proteins to exhibit their biological function, interacts with viral oncoproteins (Dyson *et al.* 1989; Ludlow *et al.* 1989; Felsani *et al.* 2006) and is conserved throughout a wide variety of taxa, from plants to invertebrates and mammals (Du and Pogoriler 2006; Miskolczi *et al.* 2007). The mammalian ‘pocket protein’ family consists of three proteins: Rb, p107, p130; all of which contain a pocket domain separated into A and B pockets by a spacer region that is not conserved (figure 1).

The *Rb* gene is located on 13q14.2 (Xing *et al.* 1999) and encompasses 27 exons that span 180 kb of chromosome 13. The gene produces a 4.7 kb long transcript which encodes a 928 amino acid protein with an MW of 110 kDa. This product is a nuclear phosphoprotein that represses transcription of genes required for G₁-to-S phase transition, thereby causing G₁ cell-cycle arrest (Weinberg 1995; Giacinti and Giordano 2006). Arrest is executed throughout interaction of the Rb protein with its target E2F family of

transcription factors leading to transcriptional inactivation (Hamel *et al.* 1992; Weintraub *et al.* 1995). Additionally, it was reported that microinjection and ectopic expression of the Rb protein causes arrest in cell-cycle progression at G₁-to-S transition (Goodrich *et al.* 1991; Hinds *et al.* 1992). Cyclin-dependent kinases (Cdks) in complex with their cyclin partners inactivate the Rb protein by phosphorylation (Sherr 1996; Wong and Weber 2007), which is required for progression through the G₁ and S phases of the cell cycle. Phosphorylation by cyclin D-Cdk4/Cdk6 and cyclin E-Cdk2 complexes is necessary for complete inactivation of Rb leading to progression of the cell cycle (Lundberg and Weinberg 1998). Both cyclins and their corresponding Cdks are activated during the late G₁ phase of the cell cycle and they phosphorylate Rb on specific amino acid residues (Knudsen and Wang 1996; Garnovskaya *et al.* 2004); cells expressing inhibitor of kinase 4 (INK4) proteins cannot proliferate since these proteins inhibit activation of cyclin D-dependent kinases (Hirai *et al.* 1995). However, Ezhevsky *et al.* (1997) reported that some functions of the Rb protein, such as binding to E2F-4, require phosphorylation by cyclin D-dependent kinases, which may direct Rb to complex with some cellular proteins enabling its other functions

Keywords. Cell cycle control; E2F transcription factor; oncological transformation; retinoblastoma protein; transcription repression

Abbreviations used: Cdk, cyclin-dependent kinase; HDAC, histone deacetylase; INK4, inhibitor of kinase 4; pRb, retinoblastoma protein; TNF, tumour necrosis factor

and not necessarily inactivating protein binding of Rb. In addition, high instability of cyclin D and the need for its continuous synthesis makes cyclin D-dependent kinases effective sensors of various intra- and extracellular changes by providing an instant cellular response. Cyclin E is usually present in cells in the form of an inactive complex with p27^{Kip1} Cdk inhibitor. Its inactivation is dependent on *de novo* synthesis of cyclin E (Perez-Roger *et al.* 1997), leading to phosphorylation of p27^{Kip1} by cyclin E–Cdk2 complexes and activation of previously inactive cyclin E–Cdk2 complexes (Morisaki *et al.* 1997). Although cyclin E-dependent kinases are capable of complete inactivation of Rb protein, this may not be the case during the cell cycle since their activation is directly associated with Rb phosphorylation by cyclin D-dependent kinase. Heavily phosphorylated Rb is present in the cell during most of the division cycle while the unphosphorylated or poorly phosphorylated state is present only during the G₁ phase. Dephosphorylation arises in a step-wise fashion, leading to an accumulation of partially

dephosphorylated forms of Rb in cells following exit from mitosis (Rubin *et al.* 2001). The main Rb-phosphorylating kinase during mitosis is the cyclin B–Cdk1 complex (Lin and Wang 1992).

2. Rb-mediated transcriptional repression – molecular mechanism

Rb protein was reported to inhibit transcriptional activators via the ability of the E2F complex and recruit Rb to the promoter, thereby inhibiting the activity of other promoter elements (Hiebert *et al.* 1992; Weintraub *et al.* 1992; Siddiqui *et al.* 2007). It was also demonstrated that Rb is a transcriptional repressor of expression of genes transcribed by RNA polymerases I and III (Cavanaugh *et al.* 1995; White *et al.* 1996), pointing to Rb being a repressor of all three classes of RNA polymerases. There are at least two different mechanisms of transcriptional repression by Rb protein (figure 2).

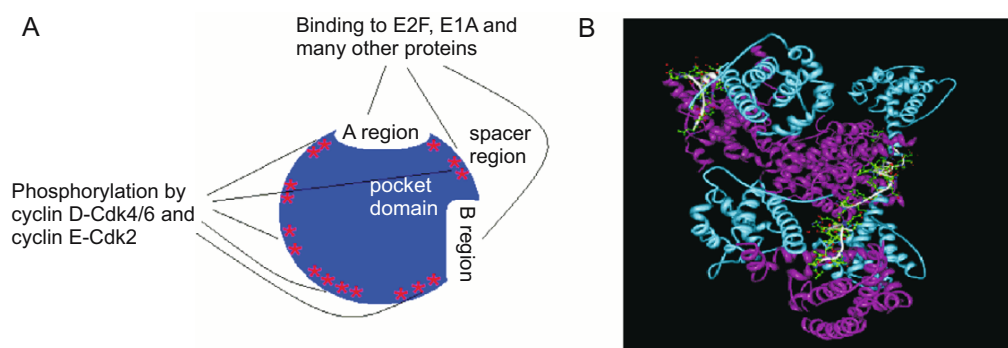


Figure 1. (A) Schematic structure of Rb protein. Pocket domain composed of A and B regions separated by a spacer region is responsible for most protein–protein interactions; * indicates phosphorylation sites. (B) 3D structure of the A pocket (purple) and B pocket (blue) in complex with E2F Rb-binding domain (atoms and ribbons); Protein Workshop software (Moreland *et al.* 2005) was used to visualise 3D structure resolved by Xiao *et al.* (2003).

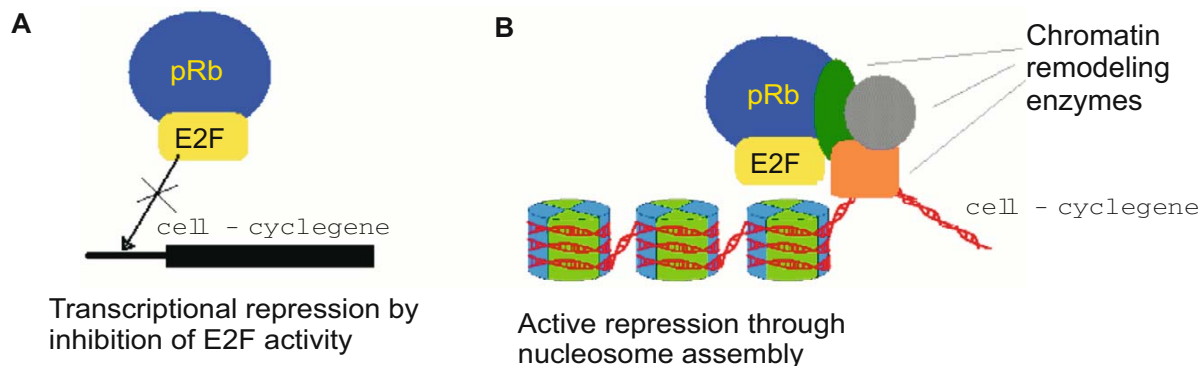


Figure 2. Mechanistic model of Rb regulation of cell cycle. Direct interaction with E2F transcription factors masks its transactivation domain and directly inhibits transcriptional activity (A); alternatively, recruitment of chromatin-remodelling enzymes by the Rb–E2F complex leads to active repression of E2F-dependent gene transcription (B).

Direct binding to the transcription domain of E2F blocks its ability to activate transcription (Helin *et al.* 1993). Also, as a complex with E2F, Rb binds to the promoter and actively represses transcription (Adnane *et al.* 1995; Weintraub *et al.* 1995) and this active involvement is crucial for Rb-mediated cell cycle arrest. Sellers *et al.* (1995) reported that chimeric protein constructed from E2F-1, whose transactivation domain is replaced with the pocket domain of the Rb protein, induces growth arrest in SAOS-2 cells. In addition, the overexpression of E2F that lacks an Rb-binding or transactivation domain causes displacement of Rb–E2F complexes from promoters (Zhang *et al.* 1999). He *et al.* (2000) reported that introduction of plasmids with multiple copies of E2F-binding sequences into cells sequester Rb–E2F complexes away from their endogenous promoters, pointing towards Rb being a potent repressor of gene transcription when it is anchored to E2F-regulated promoters. Since it was demonstrated that blocking the Rb–E2F complex from interacting with its endogenous genes is sufficient to prevent Rb-mediated growth suppression, active repression through nucleosome assembly is likely the most important mechanism of cell cycle arrest caused by the Rb protein.

How exactly does the Rb–E2F complex actively repress transcription once it is anchored to E2F-regulated promoters? One important mechanism of gene transcription regulation is through modification of chromatin structure, mainly by histone acetylation (Felsenfeld 1992; Parra and Wyrick 2007). Many transcription factors were found to be associated with histone acetyltransferases, which act as coactivators allowing access for transcription factors through alteration of chromatin structure (Wolffe and Pruss 1996; Grunstein 1997). On the other hand, histone deacetylases (HDACs) are usually associated with repressor complexes causing transcriptional inhibition (Hasig *et al.* 1997; Pazin and Kadonaga 1997; Siddiqui *et al.* 2003) by removal of acetyl groups from core histones forming a tighter association between DNA and nucleosomes. It has been shown that the Rb protein can simultaneously bind E2F and HDACs (Brehm *et al.* 1998) leading to the translocation of HDAC–Rb–E2F complexes onto promoters of cell-cycle regulating genes where they actively repress transcription by histone deacetylation of promoter sequences (Luo *et al.* 1998). Moreover, RbAp48, one of the first isolated Rb-binding proteins (Qian *et al.* 1993), was later shown to be associated with HDACs (Taunton *et al.* 1996). Also, inhibitors of HDAC interfere with pRb repression of E2F-dependent transcription. Ferreira *et al.* (1998) reported that overexpression of cyclin D-Cdk4 decreases interaction between p107 and HDAC1, suggesting that binding of pocket proteins to HDAC is disrupted when cells progress through the G₁-to-S commitment point and that this interaction is critical for the control of cell proliferation.

Phosphorylation of Rb by cyclin D-Cdk4/6 does not disrupt its inhibition of E2F transactivation activity but relieves active repression from the Rb–E2F complex associated with HDAC (Harbour *et al.* 1999). Zarkowska and Mitnacht (1997) reported that phosphorylation of Rb complexed with E1A adenoviral oncoprotein by cyclin E-dependent kinase leads to dissociation of the complex, which does not happen when it is phosphorylated by cyclin D-activated kinases, implying that phosphorylation by cyclin D-activated kinases is insufficient for complete inactivation of the Rb protein.

3. Disruption of the Rb network in cancer

Mutation of the Rb-coding gene in sporadic cancers is rare (with the exception of a few types of human tumours) and usually arises from disruption of the sequence coding the central ‘pocket’ domain of Rb protein in the Rb-coding gene (Horowitz *et al.* 1990). This domain is also a target for viral oncoproteins (figure 3) with an inactivating effect on the Rb protein (Whyte *et al.* 1988; Dyson *et al.* 1989; Ludlow *et al.* 1989; Felsani *et al.* 2006). More frequently, oncogenic transformation is a result of alterations that inactivate the Rb protein leading to constant transcription of E2F-controlled genes involved in progression of the cell cycle with consequent uncontrolled cell proliferation.

One of the most frequent mutations affecting Rb–E2F regulation in human cancers involves p16^{INK4a} and represents a hallmark of cancer (Sherr and McCormic 2002). p16^{INK4a} controls cell proliferation through maintenance of a hypophosphorylated state of pRb, and its loss of function by gene deletion, promoter methylation and mutation within the reading frame have been found in various cancers (Shapiro *et al.* 1995; Sherr 1996). In addition, deregulations of Cdk4 and Cdk6 leading to their overexpression such as gene amplification, resulting in increased tolerance to kinase inhibitors, or point mutations in the coding region of corresponding genes, with subsequent inability of INK4 proteins to complex with and inhibit the activity of kinases have also been reported (Khatib *et al.* 1993). Finally, amplification and translocation of the cyclin D1 gene resulting in increased expression has been observed (Reissmann *et al.* 1999).

Viral oncogenic transformation by adenoviral E1A, papilloma E7 or polyoma virus large T antigen targeting and inactivating Rb protein with subsequent increased E2F activity is basically substituting for increased cyclin D-dependent kinase activity (Lukas *et al.* 1994; Helt and Galloway 2003; Liu *et al.* 2006). Moreover, E1A is capable of displacing factors such as HDAC1 from a complex with Rb family proteins, thus further releasing active repression of cell-cycle related genes. Apart from the direct inhibitory interaction of Rb by viral oncoproteins, there are a few types of herpesviruses that encode cyclin D-like proteins which

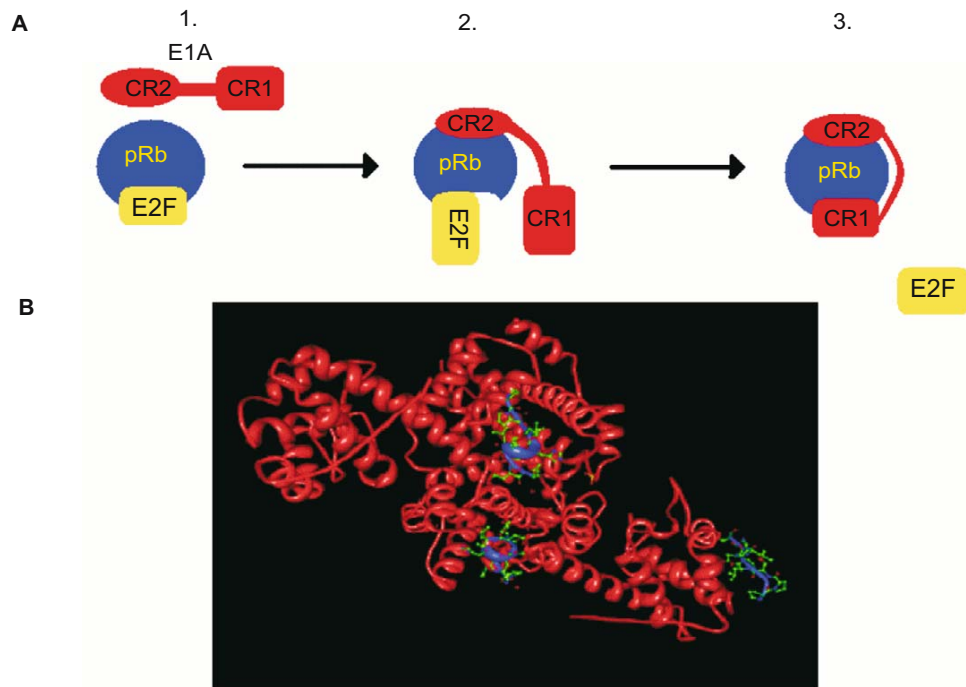


Figure 3. (A) Disruption of the Rb–E2F complex by E1A adenoviral oncoprotein. Through its CR2 domain (1) E1A binds to pRb (2) allowing the CR1 domain to interact with the E2F-binding site of Rb protein, thus disrupting the complex (3). (B) 3D structure of the pRb pocket domain (red) in complex with the CR1 domain of the Ad5-E1A protein (atoms and ribbons); Protein Workshop software (Moreland *et al* 2005) was used to visualise 3D structure resolved by Liu and Marmorstein (2007).

possess kinase activity causing phosphorylation of Rb and p27^{Kip1} proteins and leading to the release of previously inhibited cyclin E–Cdk2 complexes (Mittnacht and Bosshoff 2000).

4. Control of apoptosis by Rb protein

E2F transcription factors, apart from inducing expression of cell-cycle related genes, also regulate the expression of pro-apoptotic proteins. Hence, the ability of Rb to repress E2F-targeted genes implicates it as a repressor of apoptotic signalling even though some authors have reported that Rb regulation of apoptosis depends on the type of apoptotic stimuli (Masselli and Wang 2006). Most findings about Rb regulation of apoptosis came from knock-out mice models. Rb-null embryos undergo widespread apoptosis of cells in the central nervous system, lens and skeletal muscles (Clarke *et al.* 1992), which is diminished by p53 or E2f-1 gene mutation but not in skeletal muscles (Macleod *et al.* 1996; Tsai *et al.* 1998). Mice bearing these mutations suppress apoptosis caused by Rb deficiency and undergo prenatal development, but die in a matter of minutes after birth with severe apoptosis of the skeletal muscles (Chau and Wang 2003). The common target gene whose expression is regulated by E2F-1 and p53 is the gene coding Apaf-1, a component of apoptosome and

one of the key regulators of apoptotic signal transduction in mitochondria-dependent apoptosis that activates the caspase cascade. Since Apaf-1 knock-out rescues apoptosis in the central nervous system (Guo 2001) and caspase-3 knock-out abolishes apoptosis in the peripheral nervous system of Rb-null embryos (Simpson *et al.* 2001), it is obvious that apoptosis due to Rb dysfunction depends on activation of the apoptotic machinery. Furthermore, Rb protein was shown to possess several caspase-3 cleavage consensus sites indicating that, once the apoptotic signal is transduced, enhanced degradation of Rb contributes to more rapid progression of apoptosis (Fattman *et al.* 2001). Mutation of the C-terminal cleavage site generates a caspase-resistant form of Rb protein and the expression of this Rb-mutant was reported to repress apoptosis induced by tumour necrosis factor (TNF)- α in fibroblasts (Tan *et al.* 1997). Chau *et al.* (2002) reported that mice whose wild-type Rb was replaced with the caspase-resistant form became resistant to bacterial endotoxin-induced apoptosis which was, moreover, tissue-specific. Apoptosis induced through the widely expressed type I TNF- α receptor in mice expressing caspase-resistant pRb was blocked, but induction via TNF receptor type II (TNFR_{II}) remained active. The expression of TNFR_{II} is restricted primarily to the haematopoietic lineage of cells, suggesting that Rb-dependent control of apoptosis

is tissue-type or differentiation-type specific. Rb protein can also suppress apoptosis through direct binding to and inhibition of some other pro-apoptotic proteins such as c-ABL tyrosine kinase (Borges *et al.* 2007) and JNK kinase, which are involved in stress-induced apoptosis (Chau and Wang 2003).

Dysfunction of Rb protein leads principally to p53 accumulation and subsequently to p53-induced apoptosis. Higher activity of E2F-1 transactivates expression of p19^{ARF}, the accumulation of which blocks Mdm2, a p53 ubiquitin ligase, thus allowing for an accumulation of p53 (Zhang *et al.* 1998; Zhu *et al.* 1999). This is probably the reason why viral oncogenic transformation targeting the Rb protein usually also targets and inhibits p53.

5. Rb-dependent regulation of gene transcription in differentiation

Differentiation is a process characterised by permanent cell-cycle withdrawal and transcriptional activation of a cell-type specific gene-set. This results in a terminally differentiated cell with strictly defined morphology and function. Rb protein plays a very important role in this process due to its interactions with various differentiation-type transcription factors and chromatin modification enzymes. Furthermore, in muscle cells undergoing terminal differentiation, Rb expression is upregulated and p130/E2F-4 complexes play a key role in silencing cell-cycle genes, suggesting that high levels of pRb in differentiating cells are due to its regulation of differentiation-specific expression (Deléhouzée *et al.* 2005). Additionally, Sage *et al.* (2005) reported that a loss of Rb function in fully differentiated hair cells causes them to re-enter the cell cycle and proliferate without de-differentiation before cell-cycle entry, suggesting that continued expression of Rb is important for maintenance of cell-cycle withdrawal in this cell type. Moreover, Sankaran *et al.* (2008) reported that Rb protein serves as an intrinsic promoter of erythropoiesis and its absence leads to differentiation block in early-to-late erythroblast transition, probably because of the inability to inhibit E2F-2 leading to aberrant S-phase entry instead of an exit from the cell cycle (Dirlam *et al.* 2007). Thus, it seems that the role which Rb protein plays in differentiated cells depends on the cell type or possibly stage of differentiation.

Active repression by chromatin modification in cycling cells is based on deacetylation of histones with constant levels of its methylation (Ait-Si-Ali *et al.* 2004). However, upon permanent exit from the cell cycle, for example, in Ras-induced senescence, chromatin becomes deacetylated and histone H3 is methylated on lysine residue 9. During myogenic differentiation, this methylation is a result of the activity of the Suvar-39h enzyme which also interacts with pRB (Siddiqui *et al.* 2007). Rb protein interacts with many

transcriptional regulators; some of them are differentiation-specific transcription factors and their coordinated regulation by pRb is important to enable transcription programmes for terminal differentiation.

A key regulator of melanocyte differentiation is Mitf-1, which was found to cooperate with pRb in transcriptional activation of p21^{Cip1} and some other proteins included in terminal differentiation, suggesting that this interaction is important for cell-cycle withdrawal as well as terminal differentiation (Carreira *et al.* 2005). Through interaction with PU.1 protein, pRb is recruited to the α -globin promoter and represses its transcription, switching cell differentiation from the erythroid to the myeloid line. PU.1 activates macrophage differentiation-specific genes and requires active pRb to be able to bind to its target promoters. In Rb-deficient cells, PU.1 is not bound to target genes because of the activity of its negative regulator Id2, which can also bind to pRb (Rekhtman *et al.* 2003; Iavarone *et al.* 2004). Therefore, during myeloid differentiation, PU.1–Rb transcriptional repression is significant for driving cells through this transcriptional programme. However, in a terminal differentiation programme of macrophages, the role of pRb switches towards inhibition of Id2 so that PU.1 could activate the transcription of genes involved in the execution of terminal differentiation of macrophages. But how exactly is specificity of Rb towards transcription factors in differentiating cells determined? One possible solution for this problem could be found in the report by Nguyen *et al.* (2004), who discovered that pRb undergoes acetylation upon cellular differentiation leading to maintenance of Rb in its active hypophosphorylated form resulting in a permanent growth-arrested state (Wong and Weber 2007). Acetylation by P/CAF acetyltransferase on lysine residues 873/874 in the C-terminal region of pRb is required for expression of differentiation-specific genes and permanent withdrawal from the cell cycle. Furthermore, using the acetylation-resistant mutant, Nguyen *et al.* discovered that Rb-dependent cell-cycle arrest and repression of E2F transcription activity is not affected, indicating that this post-translation modification only determines pRb affinity to certain differentiation-specific protein partners.

5. Conclusion

The Rb protein is one of the key regulators of various stages of cell development. It regulates transmission from extracellular signals and controls the response of a cell to these signals. In cycling cells, Rb regulates progression through and exit from the cell cycle, and also regulates permanent withdrawal from the cell cycle preceding differentiation. The involvement of Rb in apoptosis perfectly completes its position in the determination of cell fate. During the cell cycle Rb is inactivated which, among

all, results in higher sensitivity to apoptotic stimuli and this makes sense since cycling cells are very susceptible to many genetic defects making them a high risk for subsistence of an organism. Furthermore, embedded caspase-3 cleavage consensus sequences amplify suicide signalling if anything goes wrong with the cell. However, differentiation requires a large amount of material, energy and time to produce cells with a defined function and specific purpose, making their development less dispensable. Furthermore, terminally differentiated cells have permanently exited from the cell cycle and are not multiplying any more, and so they do not pose any threat to the organism. Hence, it makes much more sense to render differentiated cells more tolerant to various defects and keep them to fulfil their function until the programmed lifetime expires.

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MS received 13 October 2008; accepted 20 January 2009

ePublication: 18 March 2009

Corresponding editor: MARÍA ELIANA LANIO