Oncogene (2010) 29, 392–402

www.nature.com/onc

ORIGINAL ARTICLE

Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells

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Cell–cell adhesion is an elementary process in normal epithelial cellular architecture. Several studies have shown the role mediated by cadherins in this process, besides their role in the maintenance of cell polarity, differentiation and cell growth. However, during tumour progression, these molecules are frequently altered. In breast cancer, tumours that overexpress P-cadherin usually present a high histological grade, show decreased cell polarity and are associated with worse patient survival. However, little is known about how this protein dictates the very aggressive behaviour of these tumours. To achieve this goal, we set up two breast cancer cell models, where P-cadherin expression was differently modulated and analysed in terms of cell invasion, motility and migration. We show that P-cadherin overexpression, in breast cancer cells with wild-type E-cadherin, promotes cell invasion, motility and migration. Moreover, we found that the overexpression of P-cadherin induces the secretion of matrix metalloproteases, specifically MMP-1 and MMP-2, which then lead to P-cadherin ectodomain cleavage. Further, we showed that soluble P-cadherin fragment is able to induce *in vitro* invasion of breast cancer cells. Overall, our results contribute to elucidate the mechanism underlying the invasive behaviour of P-cadherin expressing breast tumours.
Oncogene (2010) **29,** 392–402; doi:10.1038/onc.2009.338; published online 9 November 2009

Keywords: P-cadherin; migration; invasion; MMPs; sP-cad

Introduction

Cell migration and invasion are critical properties that characterize malignant neoplastic cells. Recently, a number of molecular mechanisms have been identified in transformed cells that become migratory and invasive during carcinogenesis. These includes alterations in cell–cell and cell–matrix adhesion, activation of small GTPases or modulation of receptor tyrosine kinase-mediated signal transduction pathways (Cavallaro and Christofori, 2004).

Cell–cell adhesion is crucial for the maintenance of normal epithelial cellular architecture and is frequently altered in tumour progression, inducing a multistep process termed epithelial-to-mesenchymal transition (EMT) (Thiery, 2002). During EMT, tumour cells progressively downregulate their cell–cell adhesion epithelial-specific proteins, such as E-cadherin, and express *de novo* mesenchymal adhesion molecules, such as N-cadherin. This cadherin switch leads to the inhibition of cell–cell contacts and elicits active signals, which support tumour cell migration, invasion and metastatic dissemination (Frixen *et al*., 1991; Chen *et al*., 1997). However, regardless the major progress in understanding the *in vitro* molecular processes underlying EMT and tumour progression, a number of questions remain unsolved. For instance, the majority of breast cancer subtypes are diagnosed as invasive and malignant under pathological criteria, but rarely loose complete E-cadherin expression and infrequently gain *de novo* N-cadherin expression (Sarrio *et al*., 2008).

Our group has been focused in studying the role of another classical cadherin in breast cancer, namely P-cadherin. As mentioned before, in breast cancer, many of the highly aggressive tumours do not actually show a cadherin switch. In contrast, these tumours overexpress P-cadherin, maintaining the normal E-cadherin expression (Paredes *et al*., 2005, 2008). In clinical terms, these P-cadherin-overexpressing tumours present high histological grade, with decreased cell polarity, aggressive behaviour and worse patient survival (Peralta Soler *et al*., 1999; Gamallo *et al*., 2001; Paredes *et al*., 2002, 2005). On the basis of these observations, we aimed to understand how P-cadherin, in an E-cadherin wild-type background, could influence the behaviour of these tumours. Thus, we have studied earlier the expression of classical cadherins in a small collection of breast cancer cell lines (Paredes *et al*., 2007), and we associated this expression with the invasiveness potential of these cells.

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Received 13 February 2009; revised 9 September 2009; accepted 16 September 2009; published online 9 November 2009

Indeed, it is possible to see that there is a significant association between P-cadherin overexpression and the invasion capacity of breast cancer cells that maintain the expression of wild-type E-cadherin.

In this study, we show for the first time that P-cadherin overexpression, in wild-type E-cadherin breast cancer cells, is able to induce increased cell invasion, motility and migration. Additionally, we found that the presence of P-cadherin is able to provoke the secretion of pro-invasive factors, such as matrix metalloproteases (MMPs), MMP-1 and MMP-2, which then lead to P-cadherin ectodomain cleavage. Interestingly, we observed that this formed soluble P-cadherin fragment (sP-cad) is responsible for the *in vitro* invasion of wild-type E- and P-cadherin expressing cells, which clarifies the mechanism associated to cell invasion and may explain the poor prognosis of patients harbouring breast tumours expressing these two molecules (Paredes *et al*., 2008).

Results

P-cadherin overexpression in wild-type E-cadherin breast cancer cell lines is associated with an increase in cell invasion

In a recent study, we analysed the mRNA expression levels of the major classical cadherins (E-, P- and N-cadherins) among a small collection of different breast cancer cell lines, using RT–PCR (Paredes *et al*., 2007). Comparing these results with the knowledge acquired about these cell lines in earlier publications (Figure 1a), especially concerning their invasiveness potential, EMT phenotype and gene expression profile (Charafe-Jauffret *et al*., 2006; Lombaerts *et al*., 2006; Neve *et al*., 2006; Blick *et al*., 2008), we can clearly see that E-cadherin mRNA is only expressed by cell lines that maintain an epithelial phenotype, whereas N-cadherin is expressed by cells that show a mesenchymal phenotype. Besides the phenotype, the expression of these cadherins is also highly correlated with cell invasiveness capacity, where E-cadherin-positive cell lines show low invasive potential, whereas N-cadherin expressing cells are widely described as highly invasive. When breast cancer cell lines are classified in accordance with their gene expression profile, E-cadherin is also predominantly expressed by both Luminal and Basal A cell lines, whereas N-cadherin transcripts are restricted to Basal B cell lines.

Although these associations are already well known for E- and N-cadherin, similar findings were never found, concerning P-cadherin expression and these cell properties. Looking carefully to these associations (Figure 1a), P-cadherin mRNA transcripts are predominantly detected in E-cadherin-positive cells, being mostly associated with an epithelial phenotype. Curiously, this cadherin is strongly expressed by cell lines classified as Basal A, being also associated with an increased cell invasive capacity, compared with other epithelial cell lines harbouring a Luminal gene expression profile. Using Matrigel invasion assay, we confirmed the invasion potential of two Luminal cell lines, namely MCF-7/AZ and T47D, and two Basal A cell lines, MDA-MB-468 and BT-20. Figure 1b shows that cell lines with higher levels of P-cadherin expression revealed an increased ability to invade through Matrigel. More importantly, these results are in agreement with what has been described in invasive primary breast tumours, showing the clinical relevance of P-cadherin expression in the diagnosis and prognosis of patients with aggressive mammary carcinomas (Peralta Soler *et al*., 1999; Paredes *et al*., 2005).

To determine whether or not P-cadherin expression is in part responsible by the moderate/high invasive capacity of Basal A cells lines, we set up two E-cadherin-positive cancer cell models: (1) the stable induction of P-cadherin overexpression in non-invasive MCF-7/AZ cells, by retroviral infection; and (2) the transient silencing of P-cadherin in invasive BT-20 cells, using specific small interference RNA (siRNA). As can be noticed in Figure 2a, these two cell models were established and the role of P-cadherin in cell invasion was, therefore, analysed. P-cadherin-overexpressing MCF-7/AZ cells showed a significant increase in cell invasion capacity, which was repressed when cells were treated with a P-cadherin inhibitory-function antibody, pointing to a P-cadherin-dependent cell invasion mechanism (Figure 2b). Identically, BT-20 invasive potential was significantly inhibited when P-cadherin was silenced by siRNA (Figure 2c).

As a conclusion, we can state that P-cadherin-overexpressing breast cancer cells, although maintaining its epithelial phenotype, have selective advantage to invade when compared with cell lines that mostly express E-cadherin.

P-cadherin overexpression promotes cell motility and cell migration of breast cancer cells

To further explore the function of P-cadherin in breast cancer, its effect on cell motility and cell migration was also tested.

We evaluated breast cancer single cell motility by time-lapse microscopy, during 6 h of culture (see movies in Supplementary data). Figure 3 represents snapshot images from time-lapse movies, where differences were seen between the motility of MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells. Figure 3 also shows the results obtained with BT-20 cells transfected with a control siRNA or with an siRNA that specifically abolish P-cadherin expression. The trajectory of the cell's nuclei from both cell lines is showed in Figure 3a. MCF-7/AZ.Mock cells barely showed significant movement, whereas MCF-7/AZ.Pcad cells exhibited increased cell motility. Besides this significant increase in the total number of cells with motile capability, MCF-7/AZ.Pcad cells also showed a fourfold increase in cell speed (Figure 3b). P-cadherin silencing in BT-20 cells, turns these cells significantly less motile, and the cell speed was decreased for half of the one showed by BT-20 control cells (Figures 3c and d).

a

Cell line	E-CAD	P-CAD	N-CAD	Invasiveness (1,2)	EMT phenotype (3-5)	Gene expression profile (6)
SKBR3	-	-	-	Low	Epithelial	Luminal
ZR-75.1	++	+	-	Low	Epithelial	Luminal
MCF-7/AZ	++	+	-	Low	Epithelial	Luminal
T47D	++	+	-	Low	Epithelial	Luminal
BT-474	++	+	-	Low	Epithelial	Luminal
MDA-MB-468	++	++	-	Mod	Epithelial	BasalA
BT-20	++	+++	-	Mod/High	Epithelial	BasalA
BT-549	-	+	++	High	Mesenchymal	Basal B
HS578T	-	+	+++	High	Mesenchymal	Basal B
MDA-MB-231	-	-	+	High	Mesenchymal	Basal B
MDA-MB-435	-	-	+	High	Mesenchymal	Basal B
HBL-100	-	-	++	High	Mesenchymal	Basal B

1) Paredes et al, 2007; 2) Blick et al, 2008; 3) Perou et al, 2000; 4) Lombaerts et al, 2006; 5) Charafe-jauffret et al, 2006; 6) Neve et al, 2006

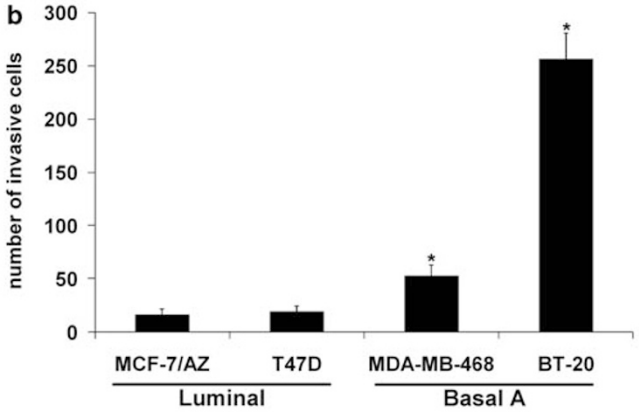

Figure 1 Association between P-cadherin expression and cell invasiveness in wild-type E-cadherin breast cancer cell lines. (**a**) Table comparing the pattern of expression of classical cadherins (namely E-, P- and N-cadherin) in different breast cancer cell lines with their invasiveness potential, EMT (epithelial-to-mesenchymal transition) phenotype and gene expression profile. (**b**) Matrigel invasion assay was used to analyse the invasion potential of two Luminal cell lines, MCF-7/AZ and T47D and two Basal A cell lines, MDA-MB-468 and BT-20. The basal cell lines showed significant increase in cell invasion, when compared with the Luminal cell lines (*$P<0.005$ compared with MCF-7/AZ non-invasive cells).

Besides analysing cell motility, we evaluated cell migration capacity, using a wound-healing migration assay. As observed in Figures 4a and b, P-cadherin-positive cells migrated significantly faster into the wound compared with control cells; importantly, this result was not due to an increase in cell proliferation rate of P-cadherin-overexpressing cells (Figure 4c). To confirm that induced migration was dependent of P-cadherin expression in these cells, the same experiment was performed in the presence of a functionally blocking antibody against P-cadherin activity (the NCC-CAD-299 clone). As shown in Figure 4b, the presence of this antibody significantly inhibited the migration capacity of P-cadherin-overexpressing cells into the wound area, whereas a non-specific IgG did not. An obvious observation of P-cadherin-induced cell migration was the evident pattern of collective cell migration, as P-cadherin-overexpressing cells do not lose their E-cadherin cell–cell contacts, and cells migrate faster and cohesive. Time-lapse microscopy movies clearly show this result (see Supplementary data).

In both assays, besides the increase in cell motility and migration, it was also possible to observe clear differences between cell's phenotype (see Supplementary data), where P-cadherin-overexpressing cells presented an increased number of membrane protrusions, structures that are usually associated to moving cells.

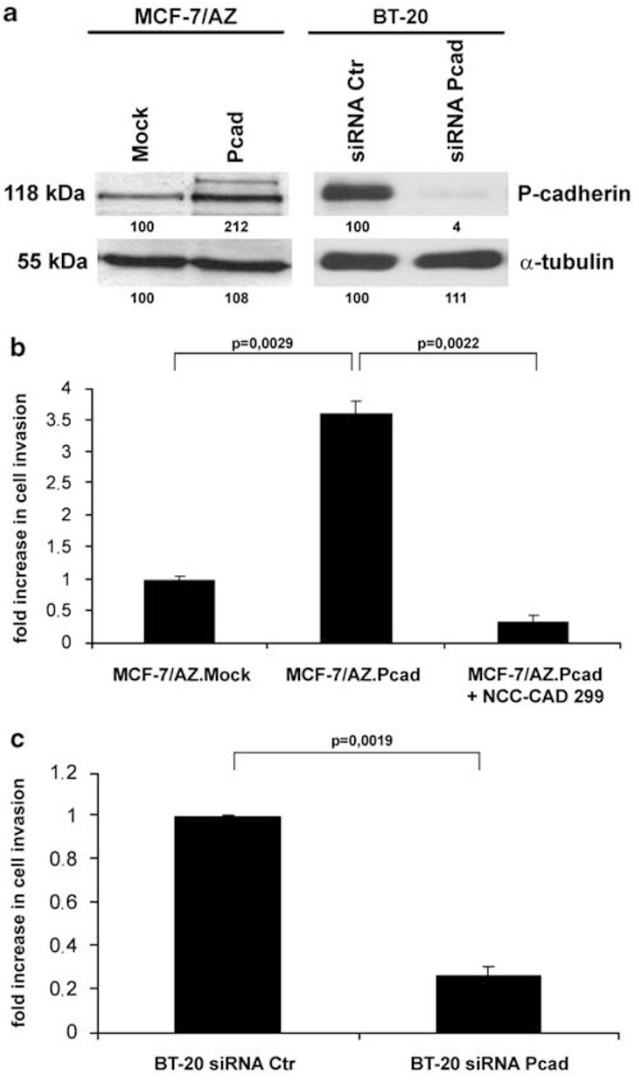

Figure 2 P-cadherin is involved in breast cancer cell invasion. (**a**) P-cadherin and α-tubulin protein expression in two different breast cancer cell models: induction of P-cadherin overexpression in MCF-7/AZ cells, by retroviral infection, and silencing of P-cadherin overexpression in BT-20 cells, using specific small interference RNA (siRNA). (**b**) Matrigel invasion assay of control MCF-7/AZ.Mock cells, and MCF-7/AZ.Pcad cells in the absence or presence of a function-blocking anti-P-cadherin antibody (NCC-CAD-299, $P=0.0029$ and 0.0022, respectively). (**c**) Matrigel invasion assay of BT-20 siRNA control cells compared with BT-20 with P-cadherin knockdown expression ($P=0.0019$).

P-cadherin overexpression induces the formation of cell membrane protrusive structures

An effective cell migration requires the integration of localized and transient signalling events, leading to changes in cellular architecture, namely in the re-organization of the actin cytoskeleton. Given that P-cadherin affects cell migration, motility and invasion, we analysed the effect of P-cadherin overexpression on the actin cytoskeleton organization, by F-actin fluorescence immunostaining. Differences between control and MCF-7/AZ.Pcad cells were evident as P-cadherin-overexpressing cells showed an increase in membrane ruffling and in actin cellular extensions, as well as in their cytoplasmic area (Figure 5). Indeed, P-cadherin-overexpressing cells, including MCF-7/AZ.Pcad and BT-20, show an upper cell localized nuclei and appear as rounded and flat cells with actin-rich sheet-like membrane protrusive structures that, according with the literature, are essentially observed during crawling cell motility and spreading (Yamazaki *et al*., 2005; Chhabra and Higgs, 2007).

Using double F-actin and p120ctn staining, MCF-7/AZ.Mock versus MCF-7/AZ.Pcad cells showed distinct aggregation ability, where MCF-7/AZ.Mock cells, in contrast to P-cadherin-overexpressing cells, showed well-formed and tight aggregates (Figure 5). Indeed, microscopic fluorescence imaging of P-cadherin expressing cells showed that P-cadherin destabilizes cell–cell adhesion, promoting cytoskeleton changes, leading to a different cell phenotype. The cells resemble the aggressive morphology observed in primary basal-like P-cadherin-positive breast carcinomas, as these cells normally acquire a large cytoplasm and several membrane protrusions, and cell–cell adhesion is not usually mediated by a compact zipper-like structure (Yamazaki *et al*., 2005; Chhabra and Higgs, 2007).

These results clearly show an association between P-cadherin expression and actin cytoskeleton reorganization, suggesting that P-cadherin has a role in the mechanism that regulates the cellular architecture changes that are needed to promote cell migration and invasion.

P-cadherin overexpression induces active MMPs that are responsible for its extracellular cleavage and shedding

One of the families of proteases, which are well known to be involved in cell invasion induction, namely in the extracellular matrix (ECM) degradation process, is the family of MMPs. On the basis of this, MMP activity levels were assessed in the conditioned medium from P-cadherin-overexpressing breast cancer cells, using β-casein and gelatin zymography. The results obtained showed that P-cadherin expression induces significantly the levels of active MMP-1 (42 kDa) and active MMP-2 (66 kDa) in the conditioned medium compared with control cells (Figure 6a). These observations were further confirmed by western blot, using antibodies specifically to analyse the expression of inactive and active forms of MMP2 and MMP1 (Figure 6b). Accordingly, also high levels of active MMP-1 and MMP-2 were found in the conditioned medium from BT-20 cells, although transient P-cadherin silencing was not enough to abrogate the expression levels of these active MMP forms (data not shown).

Given that MMPs were already described to shed the extracellular domains of membrane glycoproteins, including E-cadherin, giving rise to a soluble fragment with pro-invasive activity (Lochter *et al*., 1997; Herren *et al*., 1998; Noe *et al*., 2001), we looked forward for the presence of soluble fragments of cadherins in the conditioned medium from the studied cell lines. Surprisingly, no significant differences were observed

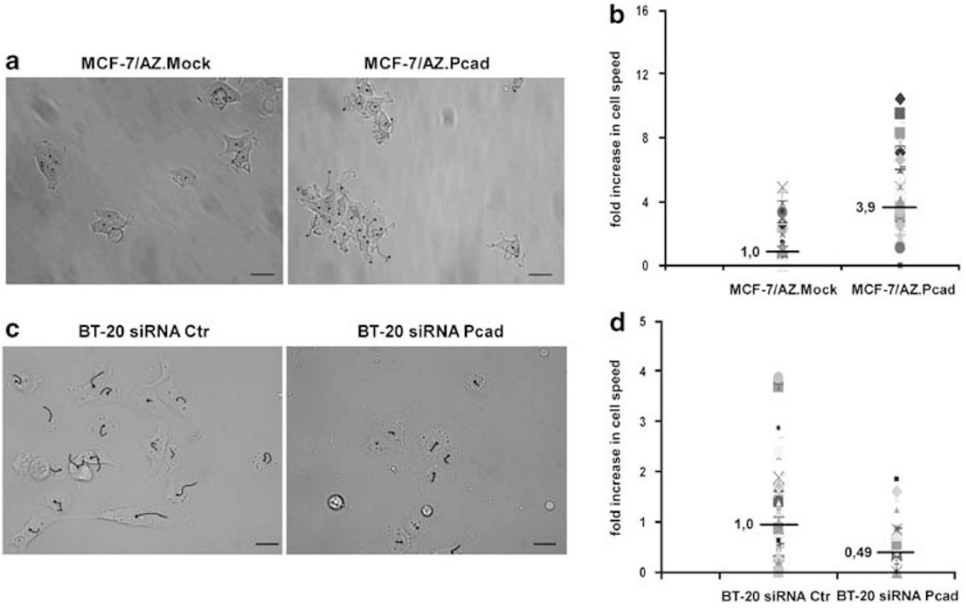

Figure 3 Promotion of cell motility by P-cadherin overexpression in breast cancer cells. Snapshot images from time-lapse movies of MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells (**a**) and BT-20 siRNActr and BT-20 siRNAPcad (**c**). Cells were monitored during 6 h, and trajectories for each cell were determined manually based on the centre of the nuclei along time. Black lines represent the trajectories performed by cells. Cell speed, considering the ratio from the distance made by cells versus the time of movement, was quantified for MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells (**b**) and for BT-20 siRNActr and BT-20 siRNAPcad (**d**), where 300 cells were analysed per cell line ($P<0.001$). In both cell models, is possible to conclude that P-cadherin expression promotes an increase in cell motility and cell speed.

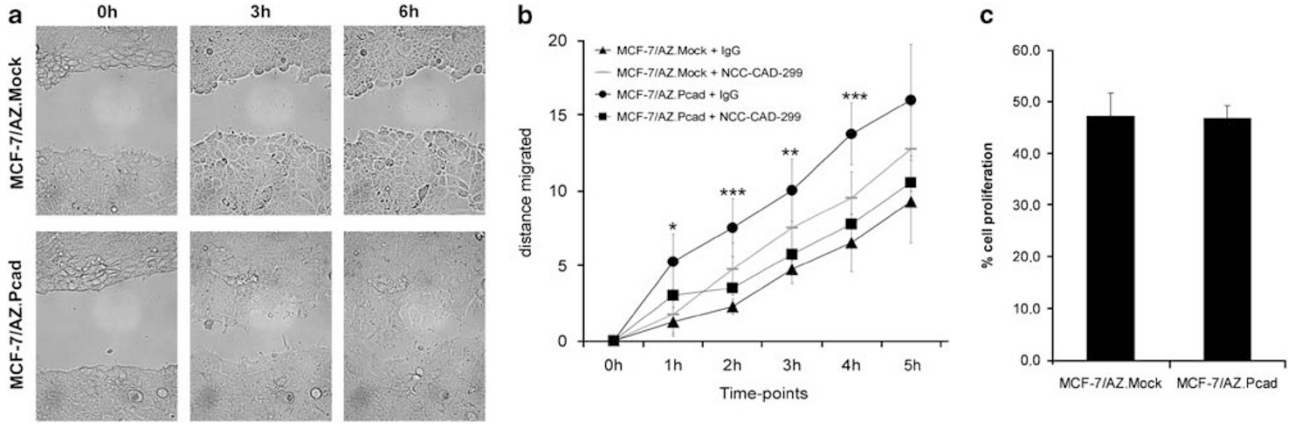

Figure 4 Induction of cell migration by P-cadherin overexpression in breast cancer cells. (**a**) Cell migration was estimated by means of wound-healing migration assay, and monitored by time-lapse microscopy. The distances migrated by breast cancer cells were measured at several time points: 0, 1, 2, 3, 4, 5 and 6 h. The experiment shown is relative to a representative experiment that was repeated three times. (**b**) Migration of MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell lines, in the presence or absence of a function-blocking anti-P-cadherin antibody (NCC-CAD-299) was evaluated by wound-healing migration assay (*$P<0.05$, corresponding to P-values from MCF-7/AZ.Pcad + IgG compared with MCF-7/AZ.Mock + IgG cells; **$P<0.05$, ***$P<0.01$, corresponding to P-values from MCF-7/AZ.Pcad + IgG compared with MCF-7/AZ.Pcad + NCC-CAD-299 antibody treatment). P-cadherin expression is directly implicated in the migratory capabilities of this cell line. (**c**) BrdU proliferation assay was performed in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells, to exclude that differences observed in cell migration were due to altered cell proliferation. No differences were observed in the percentage of cell proliferation in both cell lines.

concerning the presence of soluble E-cadherin (sE-cad); however, sP-cad was increased 8.7-fold in the conditioned medium from MCF-7/AZ.Pcad cells (Figure 6c). BT-20 cells also showed high levels of sP-cad, which were significantly decreased when these cells were transfected with P-cadherin siRNA (Figure 7a). These results showed, as expected, a direct association between full-length P-cadherin overexpression at the cell membrane and the presence of sP-cad in the conditioned media.

Finally, our goal was to assess if MMPs might have a role in P-cadherin shedding, as was described earlier for

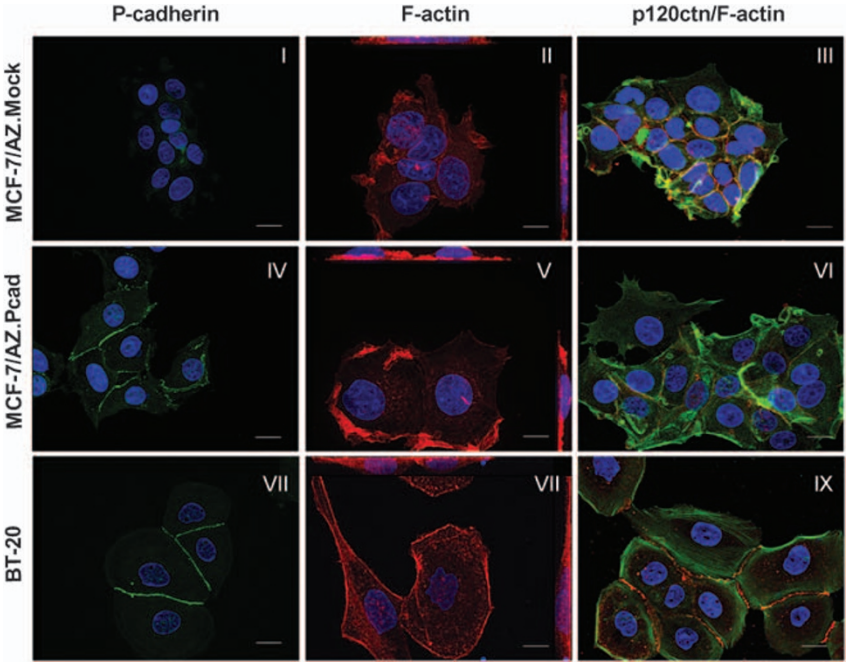

Figure 5 P-cadherin overexpression induces the formation of cell membrane protrusions and large cytoplasm. Breast cancer cells were seeded on a glass surface, fixed and stained for P-cadherin (I, IV, VII). FITC-phalloidin staining was also performed (II, V, VII), to visualize actin filaments (1000 × amplification), where it was possible to confirm alterations in the actin cytoskeleton re-organization in P-cadherin-overexpressing cells. Confocal microscopy imaging of aggregates from MCF-7/AZ.Mock (III), MCF-7/AZ.Pcad (VI) and BT-20 cells (IX), stained for F-actin (green) and p120ctn (red) (400 × amplification) was performed. Cell phenotype and structure of cell clusters were different, attributing a role for P-cadherin in the induction of loose aggregates. The white line in each picture represents 20 μm scale.

E-cadherin. Thus, MCF-7/AZ.Pcad cells were treated with a MMP inhibitor (with higher affinity to inhibit MMP-1 and MMP-2), the conditioned medium was recovered and the presence of sP-cad was evaluated by western blot. Remarkably, the treatment with the MMP inhibitor reduced the levels of sP-cad, showing that MMPs have an important role in P-cadherin cleavage and shedding (Figure 6d). To understand if P-cadherin shedding had also a role in the production of MMPs, inducing a mechanism of positive feedback, the parental cell line MCF-7/AZ was treated with human recombinant P-cadherin (hrP-cad). This peptide should mimic the effect of sP-cad, as it only harbours the extracellular part of this complete adhesion molecule. Interestingly, we found that hrP-cad induced the secretion of active MMP-1 and MMP-2, as happens in MCF-7/AZ.Pcad cells (Figure 6e). We also showed that hrP-cad is also cleaved by MMPs, originating an 80 kDa fragment, identical to sP-cad; the levels of this fragment were decreased when cells were treated with hrP-cad in combination with MMP inhibitor, confirming the importance of these MMPs to full-length P-cadherin cleavage (Figure 6f).

These results show that P-cadherin overexpression, sP-cad cleavage, as well as active MMPs secretion, are highly correlated. Briefly, P-cadherin expression leads to the secretion of active MMPs, enzymes that then cleave the extracellular domain of P-cadherin, giving rise to sP-cad. This soluble fragment is able to induce and maintain the secretion of active MMPs.

sP-cad has pro-invasive activity in breast cancer cells

As a crucial step for invasion and metastasis is the destruction of biological barriers (basement membrane and ECM) by activated proteolytic enzymes, we aimed to assess whether the medium from P-cadherin-overexpressing breast cancer cells, rich in MMPs and sP-cad, was enough to facilitate cell invasion of non-invasive cells. Thus, we performed the same invasion assay using the parental MCF-7/AZ cell line, treated with the conditioned medium obtained from MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells (Figures 7a and b). Interestingly, only the conditioned medium from P-cadherin-overexpressing cells itself was able to significantly induce invasion. This effect was not observed in the presence of the conditioned medium secreted by cells without P-cadherin overexpression (MCF-7/AZ.Mock). To confirm these results, these same cells were treated with the conditioned medium from BT-20 cells, which also contain high levels of sP-cad and MMPs, and '*de novo*' invasive cell behaviour was observed. These results show that P-cadherin-overexpressing cells secrete factors to the medium that are crucial for cell invasion induction (Figures 7a and b).

In the past, *in vitro* studies showed that sE-cad has a pro-invasive role in tumour cell lines, although its mechanism of action is still not well understood (Noe *et al*., 2001). However, the potential activity of sP-cad in cell invasion induction has never been described in *in vitro* studies. To understand whether sP-cad would have a role in breast cancer cell invasion, we depleted the

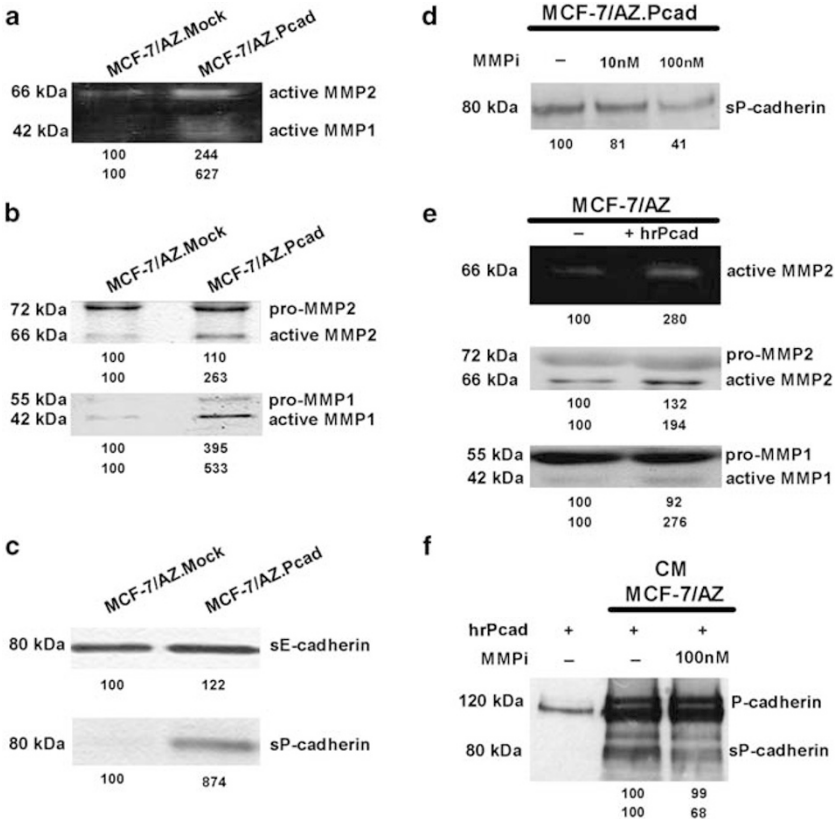

Figure 6 P-cadherin overexpression induces increased levels of MMP-1 and MMP-2 activity, which have a role in P-cadherin shedding. (**a**) β-casein zymography, which allows identification of activity of MMP-1 and MMP-2, was performed using the conditioned medium from mock and MCF-7/AZ.Pcad cells, cultured in collagen type-I. (**b**) Western blot to detect the secretion of active-MMP-1 and active-MMP-2 into the conditioned medium was evaluated and compared between mock and P-cadherin-overexpressing cells. (**c**) Detection for the presence of sE-cadherin and sP-cadherin fragments in the medium was achieved by performing western Blot for these proteins, using the conditioned medium obtained from each cell line. (**d**) Effect of MMPs in the shedding of P-cadherin was evaluated by analysing the presence of the sP-cad fragment in the conditioned medium from MCF-7/AZ.Pcad cells treated with different concentrations of the MMP inhibitor III. (**e**) Zymography detecting increased MMP-2 activity levels in parental MCF-7/AZ cells treated with a human recombinant fragment of P-cadherin (hrP-cad). Western blot for active-MMP-1 and active-MMP-2 present in the conditioned medium from control cells treated with hrP-cad. (**f**) Protein expression analysis for the conditioned medium from control cells treated with hrP-cad and MMPi, to confirm that hrP-cad can be shed to an 80 kDa form and that this process is MMP dependent.

sP-cad fragment from the conditioned medium of MCF-7/AZ.Pcad cells by repeated immunoprecipitations, using a specific P-cadherin antibody (Figures 7a and b). Moreover, we treated the cells with hrP-cad, which was added to the conditioned medium collected from MCF-7/AZ.Mock cells. Afterwards, we performed Matrigel invasion assay using the parental non-invasive MCF-7/AZ cells, treated with these mediums: or with the hrP-cad-rich medium or with the depleted (without sP-cad) conditioned medium of P-cadherin-overexpressing cells. Although hrP-cad-rich medium promoted cell invasion capacity to a non-invasive cell line, this effect was completely abolished when sP-cad was depleted from the medium (Figures 7a and b). Moreover, when MCF-7/AZ cells were treated with the conditioned medium from BT-20 cells, where P-cadherin was silenced by siRNA, its invasion was significantly reduced comparing with BT-20 control cells. Together, these results clearly show a critical role for sP-cad in breast cancer cell invasion.

Finally, to understand whether the invasion effect induced by sP-cad was due to homotypic interactions with endogenous P-cadherin on the surface of the cells, we decided to measure the invasion rates of BT-20 target cells, with P-cadherin siRNA knockdown, exposed to conditioned medium rich in sP-cad or rich in hrP-cad (Figure 7c). Interestingly, we found that in both situations, it is possible to restore the invasion capacity of these cells. These results show that the invasive effect mediated by sP-cad is not due to a homotypic interaction with endogenous P-cadherin at the cell membrane.

Discussion

Previous studies have shown that P-cadherin expression in breast carcinomas is able to identify a subgroup of lesions with a more aggressive behaviour and poor

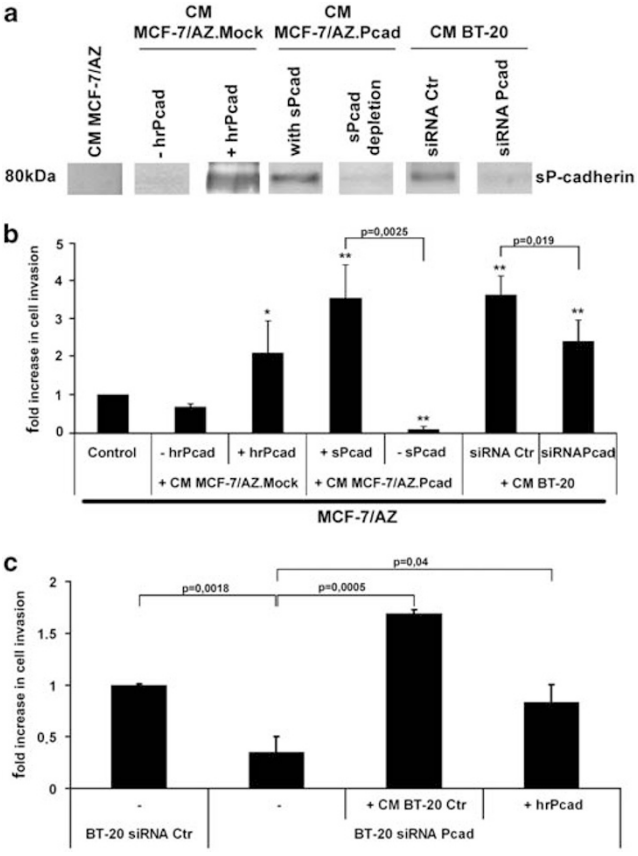

Figure 7 sP-cad has pro-invasive activity. (**a**) Western blot for several conditioned medium, showing different levels of sP-cad. Conditioned medium from MCF-7/AZ.Pcad cells (with sP-cad) was subjected to serial immunoprecipitations for the sP-cad fragment, using a specific monoclonal P-cadherin antibody, originating the conditioned medium with sP-cad depletion. (**b**) To clarify the specific role of s-Pcad in breast cancer cell invasion, matrigel invasion assay was performed in non-invasive parental MCF-7/AZ cells incubated with the conditioned medium from the different cell lines that present different levels of sP-cad (shown in **a**). Control cells were treated conditioned medium from MCF-7/AZ.Mock cells (with and without hrP-cad), conditioned medium from MCF-7/AZ.Pcad cells (with and without sP-cad depletion) and with conditioned medium from BT-20 cells (with and without P-cadherin knockdown). Student's *t*-test was performed and the statistically different values are indicated (*$P<0.05$ and **$P<0.01$ compared to control conditioned medium). (**c**) To understand whether the invasion effect induced by sP-cad was due to homotypic interactions with endogenous P-cadherin on the surface of the cells, we calculated the invasion rates of BT-20 with P-cadherin knockdown, exposed to conditioned medium from BT-20 control cells (rich in sP-cad) or rich in hrP-cad. We observed a rescue in the invasion capacity of these cells.

patient survival (Peralta Soler *et al*., 1999; Gamallo *et al*., 2001; Paredes *et al*., 2002, 2004, 2005, 2007, 2008). Similar associations between P-cadherin expression and poor prognosis are described in other cancer models, namely endometrial, cervical, gastric and pancreatic carcinomas (Stefansson *et al*., 2004; Longatto Filho *et al*., 2005; Taniuchi *et al*., 2005).

Our group has previously shown *in vitro* that, using HEK 293 T cells as a model system, P-cadherin has a pro-invasive activity, through its juxtamembrane domain (Paredes *et al*., 2004). In contrast, in highly invasive melanoma cell lines, P-cadherin overexpression is able to promote the formation of cell–cell contacts and counteract invasion (Van Marck *et al*., 2005), showing that it may function either as an invasion promoter or as an invasion suppressor depending on tissue specificity. On the basis of clinical evidences and on its contradictory biological role in distinct tumour tissues, we aimed at understanding how P-cadherin influences tumour aggressiveness. Furthermore, we aimed to clarify, besides cell invasion, its role in migration, motility and activation of MMPs, using breast cancer-derived cell lines.

Using *in vitro* cell models, we found for the first time that overexpression of exogenous P-cadherin is able to promote single cell motility, inducing an increase in the number of moving cells and speed when compared with cells with low levels of this protein. Furthermore, P-cadherin-overexpressing cells not only showed increased single cell motility, but also increased directional cell migration, as well as, invasion capacity through the Matrigel. This behaviour was shown to be directly dependent on P-cadherin, as when overexpressing cells were treated with a P-cadherin blocking antibody or transfected with a siRNA to inhibit P-cadherin transcripts, there was an inhibition of both effects (migration and invasion). Interestingly, Simpson *et al*. (2008) also identified CDH3 (the P-cadherin codifying gene) as one of the genes involved in the regulation of breast cell migration using an siRNA approach. Further, in other studies, using different cancer cell models, P-cadherin was shown to have a role in promoting cell migration. Namely, Taniuchi *et al*. (2005) showed that a pancreatic cancer cell line, transfected with wild-type P-cadherin, migrated faster than the cells without this molecule. Epithelial cell migration requires the coordination of three basic cellular processes: actin cytoskeleton reorganization, matrix adhesion and matrix re-modelling (Lauffenburger and Horwitz, 1996; Fenteany *et al*., 2000). In this present study, we also show, by time-lapse microscopy and actin phalloidin staining, that P-cadherin is able to induce phenotypic changes involving alterations in cell polarity and leading edge morphology, formation of membrane protrusions, as well as, increase of their cytoplasmic area, which usually is characteristic from cells with a motile behaviour.

Further, we aimed to determine the molecular mechanisms underlying P-cadherin overexpression and its cellular-associated effects in breast cancer cells. It has been described that the degradation of the ECM, with recruitment of proteolytic enzymes, occurs in a variety of cellular events requiring tissue reorganization, such as embryonic development, wound healing and cancer progression (Vu and Werb, 2000). Among these enzymes, MMPs are able to degrade almost all the ECM components, and have largely been involved in both tumour invasion *in vitro* and in early and late stages of tumour progression *in vivo*. When we evaluated the cell invasion capacity of non-invasive parental cells exposed to conditioned media from P-cadherin-over-

expressing cells, we conclude that indeed P-cadherin induces the secretion of factors that facilitate cell invasion of non-invasive MCF-7/AZ cells that we identified as being active forms of MMP-1 and MMP-2. More importantly, we showed that these enzymes are responsible by the shedding of a soluble extracellular fragment of P-cadherin harbouring pro-invasive activity, as its depletion from the conditioned medium of P-cadherin-overexpressing cells re-establish the non-invasive phenotype of E-cadherin breast cancer cells.

Our results are in accordance with several recent studies, concerning the role of MMP-2, MMP-1 and sP-cad in breast cancer. *In vivo*, MMP-2 is described as a key enzyme for the degradation of the ECM and facilitating tumour invasion and metastasis, being its active form present in half of all human breast carcinomas (Stetler-Stevenson *et al*., 1993; Remacle *et al*., 1998). Not only MMP-2 has pro-invasive activities, but also MMP-1 overexpression has been shown in a variety of advanced carcinomas, being associated with poor prognosis (Murray *et al*., 1998a, b; Ito *et al*., 1999; Fujimoto *et al*., 2008; Okuyama *et al*., 2008). High levels of MMP-1 expression have been detected in human breast cancer cells with elevated metastatic capacity towards the bone, providing evidence for its role in cancer cell invasion and metastasis (Kang *et al*., 2003; Okuyama *et al*., 2008). However, the molecular mechanism by which P-cadherin expression induces the secretion of these both enzymes to the medium remains unsolved.

The mechanism of ectodomain cleavage of adhesion proteins, mediated by MMPs, has been already well described (Lochter *et al*., 1997; Herren *et al*., 1998; Noe *et al*., 2001). MMP-3 and MMP-7 have a role in the shedding of the extracellular domain of E-cadherin, generating a soluble 80 kDa fragment (Noe *et al*., 2001). Interestingly, the sE-cad fragment, when released, inhibits E-cadherin functions in a paracrine way, inducing cell invasion into collagen type I and inhibiting E-cadherin-dependent cell–cell aggregation (Noe *et al*., 2001). Very recently, Mannello *et al*. (2008) showed a significant increased shedding of soluble fragments of P-cadherin in nipple aspirate fluids from women with breast cancer, when compared with healthy subjects or with women with pre-cancer conditions. This suggests its possible release through proteolytic processing in cancer cells. Until nowadays, there are only few studies showing the presence of sP-cad in biological human fluids (such as milk (Soler *et al*., 2002) and sperm (De Paul *et al*., 2005)), but no specific cellular effect has been attributed to this fragment. Our results show, for the first time, that sP-cad is produced by proteolytic enzymes (MMP-1 and MMP-2) and is responsible for the invasive capacity of breast cancer cells. Moreover, this invasive effect induced by sP-cad is independent from the endogenous expression of P-cadherin at the cell membrane. Further research is needed to elucidate which signalling pathways are activated by this pro-invasive factor.

In conclusion, the results herein described contribute to clarify the role of P-cadherin expression in breast cancer, as they unravel the molecular mechanism and the associated cellular effects mediated by this protein. In breast cancer cells, P-cadherin expression gives an advantage to cells to migrate and move, as well as the possibility to secrete pro-invasive factors, such as MMPs and sP-cad.

Materials and methods

Cell culture and transfection

Human cancer cell lines were obtained as described: MCF-7/AZ (kindly given by Prof. Marc Mareel, Ghent University, Belgium), T47D, MDA-MB-468 and BT-20 from American Type Culture Collection (Manassas, VA, USA). Cell lines were routinely maintained at 37 °C, 5% CO_2, in the following media (Invitrogen Ltd, Paisley, UK): 50% DMEM/50% HamF12 (MCF-7/AZ), DMEM (BT-20, T47D, MDA-MB-468). All the media contained 10% heat-inactivated fetal bovine serum (Greiner bio-one, Wemmel, Belgium), 100 IU/ml penicillin and 100 μg/ml streptomycin. MCF-7/AZ cell line was retrovirally stable transduced to encode P-cadherin (MCF-7/AZ.Pcad cell line), as described earlier (Paredes *et al*., 2004). MCF-7/AZ.Mock cell line, encoding only EGFP, was used as a control. BT-20 transient transfection with siRNA specific for P-cadherin (50 nM, Hs_CDH3_6, GW Validated siRNA, Qiagen, Cambridge, MA, USA) was carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer's procedures. A negative control, with no homology to any gene, was also used (Qiagen).

For the conditioned medium assays, cells were grown until confluence in collagen-type I-coated flasks (0.2 mg/ml—Sigma, Steinheim, Germany) and incubated in serum-free medium for 48 h. The conditioned medium was filtered and the proteins secreted were quantified in the recovered supernatant.

Antibodies and chemicals

Primary antibodies. P-cadherin (Western blot: clone 56, BD Biosciences, Lexington, KY, USA; immunofluorescence: Cell Signalling tecnhology, Boston, MA, USA; P-Cadherin-blocking-function antibody: clone NCC-CAD-299, Zymed Laboratories, San Francisco, CA, USA), E-cadherin (clone HECD-1, Takara Bio Inc., Shiga, Japan), p120ctn (clone 98, BD Biosciences), β-actin (I-19, Santa Cruz Biotechnologies, CA, USA), MMP-1 and MMP-2 (Ab-6 and Ab-7 respectively, Neomarkers, Fremont, CA, USA).

Chemicals. MMP inhibitor III (treatement with 10 nM or 100 nM, for 48 h, Calbiochem, EMD Chemicals, Darmstadt, Germany); Recombinant human P-cadherin/Fc chimaera (20 μg/ml; hrPcad, R&D Systems, Inc., Minneapolis, MN, USA). For detailed description see Supplementary data.

Cancer cell motility and wound-healing assay

For the motility assay, cells were monitored with an inverted time-lapse controller (Leica FW 4000, DMIRE 2, Pecon, Leica, Bensheim, Germany) and distance moved by the cells was determined, as well as cell speed (μm/h).

For the wound-healing migration assay, wounds were made across the cell monolayer and distances between the wound edges were determined. Cells were also treated with NCC-CAD-299 (100 μg/ml) or control mouse immunoglobulin G (IgG; 100 μg/ml, Upstate, Millipore, Billerica, MA, USA).

BrdU proliferation assay was performed to exclude that difference in cell migration was due to cell proliferation. For detailed description see Supplementary data.

Immunofluorescence and confocal microscopic analysis
Cells were plated on glass coverslips (Becton Dickinson Labware, Franklin Lakes, NJ, USA), fixed with 4% formaldehyde. For protocol details see Supplementary data.

Gelatin and β-casein zymography
The conditioned medium was analysed for proteinases activity using gelatin and β-casein zymography (gels loaded with 12 and 100 μg of protein, respectively) as described earlier (Oliveira *et al*., 2003). Quantification of band density was done using the Quantity One software (version 4.0, Bio-Rad, Hercules, CA, USA), unless specified.

Immunodepletion and western blot
For sP-cad immunodepletion, 300 μl of conditioned medium collected from MCF-7/AZ.Pcad cells was incubated with NCC-CAD-299 and immunoprecipitated twice. Western Blot was performed as described earlier (Paredes *et al*., 2007). The experiments selected to show are representative ones. For more detailed description on immunoprecipitation and western Blot see Supplementary data.

Matrigel invasion assay
Matrigel invasion assay was performed according to manufacter's instrutions (BD Biosciences). For more detailed description see Supplementary data.

Statistical analysis
Data are expressed as mean values of at least three independent experiments ± s.d. Student's *t*-tests were used to determine statistically significant differences ($P<0.05$).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The work presented was mainly supported by a scientific project (POCI/BIA-BCM/59252/2004) financed by the Portuguese Science and Technology Foundation (FCT). FCT also provided research grants as follows: Programa Ciência 2007 (FCT) for Joana Paredes, and PhD research grants for Ana Sofia Ribeiro (SFRH/BD/36096/2007) and André Albergaria (SFRH/BD/15316/2005).

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)