Apollon gene silencing induces apoptosis in breast cancer cells through p53 stabilisation and caspase-3 activation

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We analysed the effects of small interfering RNA (siRNA)-mediated silencing of Apollon, a member of the inhibitors of apoptosis protein family, on the proliferative potential and ability of human breast cancer cell lines to undergo apoptosis. In wild-type p53 ZR75.1 cells, Apollon knockdown resulted in a marked, time-dependent decline of cell growth and an increased rate of apoptosis, which was associated with p53 stabilisation and activation of the mitochondrial-dependent apoptotic pathway. Pre-incubation of cells with a p53-specific siRNA resulted in a partial rescue of cell growth inhibition, as well as in a marked reduction of the apoptotic response, indicating p53 as a major player in cell growth impairment consequent on Apollon silencing. Apollon knockdown induced consistently less pronounced anti-proliferative and pro-apoptotic effects in mutant p53 MDA-MB-231 cells than in ZR75.1 cells. Furthermore, the activation of caspase-3 seemed to be essential for the induction of apoptosis after Apollon knockdown, as the Apollon-specific siRNA had no effect on the viability of caspase-3-deficient, wild-type p53 MCF-7 cells or the ZR75.1 cells after RNA interference-mediated caspase-3 silencing. Our results indicate that p53 stabilisation and caspase-3 activation concur to determine the apoptotic response mediated by Apollon knockdown in breast cancer cells, and suggest Apollon to be a potential new therapeutic target for this malignancy.

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Apoptosis is a tightly regulated process, which plays a central role in the development and homeostasis of multicellular organisms. Its deregulation is involved in a wide spectrum of diseases including cancer, in which an overexpression of anti-apoptotic proteins endows cells with a selective survival advantage that promotes malignancy (Green and Evan, 2002). The members of the inhibitors of apoptosis protein (IAP) family have been proved to be crucial negative regulators of apoptosis (Srinivasula and Ashwell, 2005), and some of these proteins were found to be expressed highly in human tumours (Hong et al, 2003; Yang et al, 2003; Srinivasula and Ashwell, 2008).

Apollon, also known as Bruce or BIRC6, is the largest member of the IAP family, containing one baculoviral IAP repeat domain at its N-terminal region and a C-terminal E2 motif, which can form thioester bonds with ubiquitin (Chen et al, 1999). It has also been suggested to function as an E3 ligase, and is itself regulated by ubiquitin-dependent degradation mediated by E2 UbCH5 and E3 Nrdp1 (Qiu and Goldberg, 2005). Although the physiological role of Apollon in counteracting apoptosis is largely unknown, the available information indicates that Apollon exerts its cytoprotective activity by promoting ubiquitination and degradation of the pro-apoptotic protein Smac/DIABLO and by inhibiting caspase activity (Hao et al, 2004; Qiu and Goldberg, 2005). It has been reported that Apollon is upregulated in some brain tumour cell lines that are resistant to certain DNA-damaging agents (Chen et al, 1999), and also that its overexpression is associated with poor prognosis in childhood acute myeloid leukaemia (Sung et al, 2007). These findings, together with preliminary evidence concerning the possibility of sensitising tumour cells to apoptosis induced by some anticancer drugs through antisense oligonucleotide- or small interfering RNA (siRNA)-mediated downregulation of Apollon (Chen et al, 1999; Qiu et al, 2004; Chu et al, 2008), has led researchers to consider the gene as a possible new therapeutic target.

With the aim to elucidate the molecular bases responsible for the cytoprotective activity of Apollon in breast cancer, we investigated the effects of its knockdown, accomplished through RNA interference (RNAi), on the proliferative potential and its ability to undergo apoptosis of established cell lines differing in the TP53 gene status. The results of this study indicate that wild-type p53 stabilisation and caspase-3 activation concur in determining the apoptotic response, consequent on Apollon knockdown in breast cancer cells.

MATERIALS AND METHODS

Cell lines

We used three human breast carcinoma cell lines: ZR75.1 and the caspase-3-deficient MCF-7 cell lines expressing wild-type p53, and the MDA-MB-231 cell line expressing a mutant p53...
Table 1  Sequences of control siRNA and siRNAs targeting Apollon mRNA

| Name  | Sequence                                      |
|-------|-----------------------------------------------|
| Ctr   | CAUUCUGUAUAGAUAUGAAdTdT                      |
| Apo1  | AGAAAAUGCCUUAGAUUUAAdTdT                    |
| Apo2  | GCAGUACAUUGAGUAAUUAAdTdT                    |
| Apo3  | AGACUGUCUGAGAUAUUAAdTdT                    |
| Apo4  | UGAAUCCGUGCAUAUAAAdTdT                     |

siRNA = small interfering RNA

Transfection procedures

The day before transfection, breast cancer cells were seeded at a density of 2 x 10^5 per 25-cm² flask. A given amount of each Apollon and control siRNA was mixed with Lipofectamine2000™ (Invitrogen, San Giuliano Milanese, Italy) for 20 min at room temperature. The mixtures were then applied to the cells in a humidified incubator at 37°C with a supply of 5% CO₂:95% air atmosphere.

Quantification of cytochrome c release

The cytochrome c release was measured using the Cytochrome c ELISA kit (Medical & Biological Laboratories). After colour development had stopped, the absorbance at 450 nm was measured on the microplate reader. Percent release of cytochrome c was calculated as the amount of cytosolic cytochrome c divided by the total amount of cytosolic and mitochondrial cytochrome c.

Total RNA isolation and RT–PCR

Total RNA isolated using Qiagen RNeasy Mini Kit (Qiagen, Milan, Italy) was reverse-transcribed in the presence of random hexamers using the GeneAmp RNA Core Kit (Applied Biosystems, Foster City, CA, USA). To assess the p53 mRNA expression, the resultant cDNA was amplified through optimised PCR cycling conditions in the presence of specific primer pair (Maxwell et al, 1999): the sense primer was 5'-TCTTGTACGTTGGGAGACGCC-3' and the anti-sense primer was 5'-AGCTGGTGTGTGAGGTCCTCCCT-3' (Eurofins MWG Operon). A fragment corresponding to β-actin was used as the standard of the amplification reaction. The PCR products were verified by gel electrophoresis, and the images were acquired by a ScanJET IIcx/T scanner (Hewlett Packard, Milano, Italy).
Statistical analysis

Student’s t-test was used to analyse the differences between control and siRNA-transfected cells in terms of protein expression, cell growth, rate of apoptosis, in vitro catalytic activity of caspase-9, caspase-3 and caspase-8, and release of cytochrome c. P-values < 0.05 (two-sided) were considered statistically significant.

RESULTS

siRNA-mediated Apollon knockdown affects cell growth in breast cancer cells

To gain insight into the role of Apollon in breast cancer cell survival, we used an RNAi-based strategy to downregulate its expression in three human breast cancer cell lines, characterised by a different TP53 gene status: ZR75.1 cells bearing wild-type p53 and MDA-MB-231 cells carrying mutant p53. We first tested the effectiveness of four 21-mer siRNAs targeting different portions within the Apollon mRNA (Table 1), to silence the Apollon gene expression in the ZR75.1 cell line. Western blotting experiments carried out in cells collected at different time points (24–72 h), after a 4-h transfection with 10 nM of each Apollon-specific siRNA, showed a variable degree of protein expression inhibition as a function of the different oligomer used (Figure 1A and B). Specifically, the abundance of Apollon protein was reduced significantly starting from 24 h after transfection with all siRNAs as compared with that in mock control (Figure 1A and B). The extent of the inhibition increased over time and reached its maximum at 72 h after transfection with all siRNAs.
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Apollon knockdown induces apoptosis in breast cancer cells

To investigate whether cell growth inhibition consequent on Apollon knockdown was ascribable to the induction of apoptosis, cells were stained with propidium iodide 72 h after transfection, and the presence of cells with an apoptotic nuclear morphology was assessed by fluorescence microscopy. In all cell lines, apoptosis was observed in a modest fraction of cells transfected with the control siRNA (from 2.3 to 4.8% of the overall cell population) and in mock control cells (from 1.7 to 3.8%). Conversely, after the exposure of ZR75.1 cells to Apo2, the percentage of apoptotic cells was about six-fold higher than that of mock control (21.7 vs 3.8%) (Figure 3A). A significant, although considerably less pronounced, increase in the percentage of apoptotic cells was also observed in the MDA-MB-231 cells transfected with Apo2 compared with that in mock control (9.3 vs 2.9%) (Figure 3A). Consistent with a negligible effect on cell growth, in MCF-7 cells, Apollon knockdown did not induce any increase in the apoptotic cell rate (Figure 3A).

Caspase catalytic activity was further assessed in all cell lines 72 h after transfection. The results revealed that in ZR75.1 cells, Apollon knockdown induced a 20-, 25- and 33-fold increase in caspase-9, caspase-3 and caspase-8 catalytic activity, respectively, compared with that in mock control (Figure 3B–D). A 10- and 17-fold increase in caspase-3 and caspase-8 catalytic activity, respectively, was also observed in MDA-MB-231 cells after exposure to Apo2 (Figure 3C and D), although no appreciable effect on caspase-9 activity was recorded in this cell line (Figure 3B). In MCF-7 cells, exposure to Apo2 induced a 17-fold increase in caspase-9 catalytic activity compared with that in mock control. In agreement with the lack of functional caspase-3 in this cellular model (Janicke et al, 1998; Yang et al, 1998), no enzyme activity was detectable under any of the treatment conditions (Figure 3C). Moreover, no appreciable activation of caspase-8 was observed in this cell line (Figure 3D).

The activation of caspase-9 in ZR75.1 and MCF-7 cells suggested that the intrinsic (mitochondrial) pathway of apoptosis is activated by Apollon knockdown only in breast cancer cells bearing wild-type p53. To confirm this, we assessed by western blot the total amount of p53 after Apollon silencing in the different cell lines (Figure 4A). The wild-type p53 levels in ZR75.1 and MCF-7 cells increased markedly after transfection with Apo2 (Figure 4A), whereas the inherently high levels of mutant p53 protein in MDA-MB-231 cells did not vary with the treatment (Figure 4A). The upregulation of p53 seemed to reflect protein stabilisation rather than enhanced gene transcription, as no increase in p53 mRNA levels was detected by RT–PCR in Apollon downregulated cells (Figure 4B).

To explore in more detail the molecular mechanisms of the induction of apoptosis by Apollon knockdown, we examined the expression of proteins involved in the mitochondrial pathway. Transfection with Apo2 induced the accumulation of Bax and Bad proteins and a remarkable release of the AIF from mitochondria into the cytosol in cells with wild-type p53, but not in cells expressing mutant p53 (Figure 4C). In addition, although to a variable extent, an increase in the total Smac/DIABLO was observed in all cell lines (Figure 4C). The cytochrome c release from mitochondria was also determined by ELISA after subcellular fractionation (Figure 4D). The results indicated that Apollon knockdown caused an enhancement of the cytochrome c cytosolic fraction in the two cell lines expressing wild-type p53. Specifically, the release of cytochrome c was 12- and 9-fold higher in ZR75.1 and MCF-7 cells treated with Apo2, respectively, than in cells transfected with the control siRNA (Figure 4D). By contrast, in MDA-MB-231 cells bearing mutant p53, Apollon knockdown did not cause any cytochrome c release (Figure 4D). Such findings were corroborated by western blot data obtained on isolated

![Figure 2](https://example.com/f2.png) **Figure 2** Effects of Apollon downregulation on in vitro growth of breast cancer cells. Survival curves of ZR75.1, MDA-MB-231 and MCF-7 cells exposed to mock control (●) or transfected with ctr (■) and Apo2 (▲) siRNAs. Points represent the mean values ± s.d. of at least three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs mock control.

(Figure 1A and B). Transfection with the Apollon-specific siRNA (Apo2), which was able to induce the greatest inhibition of Apollon expression in the ZR75.1 cell line, also resulted in a significant and time-dependent decline of the protein in the MDA-MB-231 and MCF-7 cell lines (Figure 1C and D). Conversely, Apo2 did not modify the expression of other anti-apoptotic proteins belonging to the IAP family, including cIAP1, cIAP2, XIAP and survivin (Figure 1E).

The effects of Apollon downregulation on the in vitro proliferative potential of breast cancer cells were further evaluated using Apo2, which was able to inhibit the protein expression by ~90% at 72 h after transfection in all cell lines (Figure 1B and D). In ZR75.1 cells, inhibition of Apollon resulted in a significant and time-dependent decrease in viable cell number as compared with that in mock control (Figure 2, upper panel). Such a growth inhibition was appreciable starting 48 h after transfection and increasing progressively over time. Although to a lesser extent, cell growth was affected by Apollon knockdown also in the MDA-MB-231 cells, and a significant reduction in cell number compared with that in mock control was appreciable at 72 and 96 h after transfection (Figure 2, middle panel). Conversely, Apollon downregulation failed to affect the growth of MCF-7 cells at any time point considered (Figure 2, lower panel).
mitochondrial and cytosolic fractions, which indicated a marked decrease in the amount of cytochrome c remaining in mitochondria in ZR75.1 and MCF-7 cells, but not in MDA-MB-231 cells (Figure 4E).

Wild-type p53 and caspase-3 seem to be necessary for the execution of apoptosis induced by Apollon knockdown

To determine to what extent wild-type p53 influences the induction of apoptosis consequent on Apollon downregulation, we knocked down p53 in ZR75.1 cells by RNAi. Pre-incubation of cells with p53-specific siRNA resulted in a partial rescue of cell growth inhibition (Figure 5) and a marked reduction of apoptosis (Figure 3). These observations, which are consistent with the modest level of apoptosis observed in Apo2-transfected MDA-MB-231 cells, bearing mutant p53 (Figure 3), suggest that the wild-type p53 protein is a major player in the induction of programmed cell death by Apollon knockdown.

It is noteworthy that apoptosis induction resulting from Apollon silencing was only reduced partially, but not abrogated completely after p53 knockdown, suggesting that Apollon downregulation can also interfere with caspase-3 activity in a p53-independent manner. To address this hypothesis, we tested the effect of the simultaneous knockdown of caspase-3 and Apollon in ZR75.1 cells. We found that the siRNA-mediated inhibition of caspase-3 catalytic activity resulted in the complete rescue of cell growth inhibition induced by Apollon silencing (Figure 5), as well as in the total abrogation of apoptosis although in the presence of activated caspase-9 (Figure 3). These findings are reminiscent of what we observed in MCF-7 cells transfected with Apo2 (Figures 2 and 3), in which induction of apoptosis was prevented by the absence of caspase-3 also in the presence of caspase-9 activation.

**DISCUSSION**

In this study, we evaluated the effects of RNAi-mediated Apollon gene silencing in human breast cancer cell lines. Transfection with a siRNA able to consistently induce an almost complete abrogation of Apollon protein expression in all cell lines, variably influenced cell growth as a function of TP53 gene status of the tumour model and the availability of a functional caspase-3. Specifically, in the wild-type-p53 ZR75.1 cells, Apollon silencing caused a marked impairment of cell growth, which was paralleled by upregulation of p53, activation of the mitochondrial apoptotic pathway including Bax upregulation, release of cytochrome c and AIF from mitochondria, and consequent enhancement of caspase-9 and caspase-3 activity. The activation of caspase-8 was also observed. Likewise, p53 upregulation and activation of the mitochondrial pathway was also observed in the wild-type-p53 MCF-7 cell line. However, as these cells lack caspase-3 (Janicke et al, 1998; Yang et al, 1998), no cell growth impairment and no evidence of caspase-8 activation or apoptosis induction was found in these cells.

Although to a lesser extent, compared with ZR75.1 cells, growth retardation, as well as apoptosis induction as a consequence of enhanced caspase-3 and caspase-8 activity were also observed in...
mutant-p53 MDA-MB-231 cells. The latter finding, together with the evidence that concomitant downregulation of p53 by RNAi only partially rescued cell growth inhibition and reduced, but not abrogated completely, apoptosis induced by Apollon knockdown in ZR75.1 cells, led us to hypothesise that Apollon downregulation can also directly or indirectly activate caspase-3 by a p53-independent signal (Figure 6). Experimental support for this hypothesis comes from experiments of simultaneous silencing of Apollon and p53 in ZR75.1 cells, which showed that the abrogation of the enzyme’s catalytic activity resulted in a complete rescue of cell growth inhibition and total abrogation of apoptosis induced by Apollon silencing.

Our findings corroborate and extend earlier observations by Ren et al (2005) indicating that inactivation of Bruce, the murine homologue of Apollon, through deletion of the C-terminal UBC domain, induced apoptosis in mouse embryonic fibroblasts through upregulation and nuclear localisation of wild-type p53, activation of the mitochondrial pathway of apoptosis and the consequent activation of caspase-9 and caspase-3. The same authors showed that the RNAi-mediated Apollon silencing in the p53++/+ human lung cancer cell line H460, also induced a strong apoptotic response as a consequence of p53-dependent activation of the mitochondrial pathway. Moreover, simultaneous silencing of Apollon and p53 in these cells induced only a partial rescue of cell viability and a limited decrease in caspase-3 activation (Ren et al, 2005). Both our studies and Ren’s studies (Ren et al, 2005) point to a central role of p53 as a downstream effector of Apollon/Brace. Although it is not known how Apollon knockdown causes p53 stabilisation, no interference with p53 gene transcription was reported in either study. Considering that Apollon is itself an E2/E3 ubiquitin ligase, it could act similarly to Mdm2, Pirh2 or COP1, directly catalysing p53 ubiquitination and proteasome degradation (Leng et al, 2003; Dornan et al, 2004).

The possibility that Apollon knockdown enhances caspase-3 catalytic activity in a direct way is supported by the observation of Bartke et al (2004), who reported that Apollon interacts strongly with caspase-3 and inhibits its activation in a cell-free extract of 293T adenovirus-transformed human embryo kidney cells. However, such findings were not confirmed by Qiu and Goldberg (2005) in the same experimental model. An alternative possibility is that increased caspase-3 activity could be the result of caspase-8 activation after Apollon knockdown. In this context, it has been reported recently that apoptosis induction after transduction of HT-1080 human fibrosarcoma cells with an oncolytic adenovirus, expressing a short hairpin RNA against Apollon, was mediated by the proteolytic activation of caspase-8 and caspase-3 (Chu et al, 2008).

In our study, we observed caspase-8 activation after Apollon knockdown only in ZR75.1 and MDA-MB-231, but not in the caspase-3-deficient MCF-7 cell line and in ZR75.1 cells after siRNA-mediated caspase-3 silencing. Such evidence would suggest a possible alternative interpretation of the results, in that the active form of caspase-3 binds pro-caspase-8 and activates it (Sartorious et al, 2001; Wajant, 2003), which in turn can activate caspase-3 (Figure 6). Somewhat surprisingly, the results we obtained in the mutant-p53 MDA-MB-231 cells did not show any caspase-9
activation after Apollon knockdown. In fact, earlier observations obtained by Qiu and Goldberg (2005) in cell-free systems indicated that immunopurified Apollon binds in vitro to pro-caspase-9 and caspase-9 (although in a different study, only a weak binding of Apollon to caspase-9 was detected (Bartke et al., 2004)), and inhibits the enzyme’s catalytic activity (Qiu and Goldberg, 2005). In addition, in 293T cells ectopically expressing Apollon, it was shown that the protein ubiquitinated and facilitated the degradation of caspase-9 (Hao et al., 2004). However, as all this evidence has been derived from cell-free systems or living cells forced to express Apollon, it is difficult to compare it with the findings we obtained in cells that were not manipulated to increase their Apollon abundance. It is worthy to note that no published data dealing with the interference of Apollon/Bruc with caspases on cells expressing mutant p53 are currently available.

Overall, our results indicate that the Apollon gene silencing causes growth impairment in breast cancer cells carrying a wild-type or mutant p53 gene, mainly as a consequence of apoptosis induction, and identifies p53 stabilisation and caspase-3 activation as major events that concur to determine the apoptotic response. These data also suggest the opportunity to conduct a survey of the tumour types that express Apollon, and to further proceed with the validation of Apollon as a potential new therapeutic target for tumour types that express Apollon, it is difficult to compare it with the findings we obtained in cells that were not manipulated to increase their Apollon abundance. It is worthy to note that no published data dealing with the interference of Apollon/Bruc with caspases on cells expressing mutant p53 are currently available.

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