HMGA2 and p14^{Arf}: Major Roles in Cellular Senescence of Fibroids and Therapeutic Implications

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Abstract. *Aim: To address the influence of genes involved in stem cell self-renewal and senescence on the growth of leiomyoma cells in vitro and to explore possible therapeutic implications of a targeted disruption of the p53–murine double minute 2 (MDM2) interaction. Materials and Methods: Gene expression studies (qRT-PCR) of fibroid tissue and cells; β-galactosidase stain and qRT-PCR after antagonizing MDM2. Results: In fibroid cells, expression of HMGA2 decreased with passaging while that of p14^{Arf} increased. Expression of these markers significantly positively, and negatively, respectively, influenced proliferation. Administration of nutlin-3, an MDM2 antagonist, induced cellular senescence and increased the expression of BAX. This, along with a significant correlation between p14^{Arf} and BAX expression in native fibroids, suggests that p14^{Arf} triggers senescence as well as apoptosis. Conclusion: p14^{Arf} and HMGA2 seem to play a pivotal role in controlling the growth of fibroid cells. Antagonizing MDM2 induces senescence, as well as apoptosis, and may offer a chance to treat fibroids.*

At first glance, the strong variation of the size of many benign tumors such as uterine leiomyomas (UL), suggests that we see them simply at different stages of their development. Nevertheless, there is ample evidence that this is only part of the story. Individual tumors of one patient having an identical hormonal background often strongly vary as to their growth potential (1) as well as to the percentage of senescent cells (2). Thus, individual factors often seem to overrule the growth control mediated by the hormonal milieu. This suggests that an endogenous, albeit varying, growth control distinguishes these tumors from malignant neoplasms that do not have this type of growth control or find ways to overcome it. However, the molecular mechanisms limiting the growth potential of these benign tumors *in vivo* (3), as well as *in vitro*, are largely unknown, although in both cases senescence has been hypothesized to be implicated in the cessation of growth. Herein, we investigated if senescence of cells derived from UL is associated with changes of the expression levels of genes mechanistically implicated in senescence or aging of normal cells as well. Recently, an interesting relationship between high mobility AT-hook 2 (HMGA2), a protein implicated in the growth of UL, and self-renewal of stem cells with the senescence-associated cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus has been proposed by Nishino *et al*. (4). In their paper, they hypothesized that HMGA2 exerts its tumorigenic effects by repressing both genes from the *CDKN2A* locus, namely *p16^{Ink4a}* and *p19(p14)^{Arf}* (4). In a previous study, we were able to show that in UL, the expression of *p14^{Arf}* correlates with that of the senescence-associated *CDKN1A* and murine double minute 2 (*MDM2*), the latter representing part of a positive p14^{Arf}-p53 feedback loop (5). Mechanistically, protection of the cells by the intact p14^{Arf} – p53 network may explain the low tendency of UL to undergo malignant transformation (6), as well as the limited growth potential displayed by many fibroids (3) as proposed by Mooi and Peeper (7) for benign naevi. Accordingly, as a rule UL express much higher levels of the senescence-associated *p14^{Arf}* mRNA (5). Generally, senescence and its underlying mechanisms may not only represent a key mechanism explaining the growth control of UL but, translated to therapy, may also represent an Achilles' heel for

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therapeutic interventions. Herein, we have attempted to contribute to a better understanding of the mechanisms of senescence of UL and to possible therapeutic interventions based on these mechanisms.

Materials and Methods

Tissue samples. Samples of UL from 28 patients and the matching myometrium were taken during surgery, immediately frozen in liquid nitrogen, and stored at –80˚C for RNA isolation and qRT-PCR analyses. For cell cultures, tumor samples were transferred to Hank's solution.

Cell culture. Tissue samples stored in sterile Hank's solution were minced into small pieces followed by treatment with 0.26% (200 U/ml) collagenase (Serva, Heidelberg, Germany). After 1-2 h, the dissociated cells were transferred into sterile 25 cm^2 cell culture flasks containing 5 ml medium 199, supplemented with 20% fetal calf serum (FCS; Invitrogen, Karlsruhe, Germany) and 2% penicillin-streptomycin (Biochrom, Berlin, Germany). The cultures were incubated in 5% CO_2 air at 37˚C and media were changed every 2-3 days. Cultures were passaged when reaching 80% confluence using 1×TrypLE Express (Gibco, Karlsruhe, Germany) in a PBS-EDTA buffer. Subcultivation was performed until passages 14 (cells of the UL with t(2;12)), and 13 (UL with normal karyotype). Total RNA of every passage was extracted 72 h after medium change.

Treatment by nutlin-3. For treatment by nutlin-3, cells were plated in Leighton tubes at a density of 200,000 on 10 mm × 50 mm coverslips 24 h before incubation with nutlin-3 (Biomol, Hamburg, Germany). Cells were treated with 30 μM and 50 μM nutlin-3 for 24 h and 72 h. As controls, cells were cultured in medium 199 supplemented with 20% FCS without nutlin-3 for 24 h and 72 h.

In situ β-galactosidase staining and mitotic rate. After incubation with nutlin-3, β-galactosidase staining of senescent cells was performed using a commercially available *in situ* β-galactosidase staining kit (Agilent, Waldbronn, Germany) according to the manufacturer's instructions. After incubation with staining solution at 37˚C for 24 h, cells were washed twice with 1×PBS and analyzed using an axioscope (Zeiss, Göttingen, Germany). The numbers of β-galactosidase-positive cells and of mitotic cells were counted using photomicrographs of ten randomly selected fields at ×10 magnification.

RNA interference. Cells were transfected with four *MDM2*-specific siRNAs (SIH900207ABCD (SABiosciences, Frederick, USA)) by SureFECT transfection reagent (SABiosciences) by reverse transfection following the manufacturer's instruction. Cells were harvested, suspended in medium 199 supplemented with 20% FCS and divided into six groups which were treated with the four different *MDM2* siRNAs, a nonspecific siRNA (SABiosciences, Frederick, USA) as negative control, and SureFECT vehicle only. For each transfection, a 2,200 μl cell suspension containing 200,000 cells was transfected with 2 μM siRNA using 6 μl transfection reagent in 6-well plates. Cells were kept under normal culture conditions and were harvested 48 h after transfection for qRT-PCR analysis.

RNA isolation. RNA isolation was performed using the RNeasy mini kit (Qiagen, Hilden, Germany) and DNase I digestion was performed following the manufacturer's instruction.

cDNA synthesis. About 250 ng of total RNA were reverse transcribed with 200 U/μl of M-MLV reverse transcriptase (Invitrogen), RNase Out, 150 ng random hexamers and 10 mM dNTPs according to the manufacturer's instructions. RNA was denatured at 65˚C for 5 min and subsequently kept on ice for 1 min. After adding the enzyme to the RNA primer mixes, samples were incubated for 10 min at 25˚C to allow annealing of the random hexamers. Reverse transcription was performed at 37˚C for 50 min followed by inactivation of the reverse transcriptase at 70˚C for 15 min.

qRT-PCR. Relative quantification of transcription levels was carried out by real-time PCR analyses using an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). Commercially available gene expression assays (Applied Biosystems) were used for quantification of mRNA of human *HMGA2* (Hs00171569_m1), $p14^{Arf}$ (Hs00924091), *BAX* (Hs00180269_m1), *MDM2* (Hs01066930_m1), *CDKN1A* (Hs99999142), *Ki-67* (Hs00606991_m1), and *GLB1* (Hs01035162). *HPRT* served as endogenous control for the quantification of the expression of all genes examined (5). All qRT-PCR experiments were carried out in triplicate. No-RT controls were used in all experiments to rule out contamination.

Cytogenetic and molecular-cytogenetic studies. Chromosome analyses and fluorescence *in situ* hybridization (FISH) were performed following routine techniques as described previously (8). For each tumor, at least twelve G-banded metaphases were karyotyped.

Statistical analyses. The statistical significance of differences was assessed by Student's *t*-test. In all comparisons, $p<0.05$ was considered statistically significant and $p<0.01$ was considered highly significant.

Results

In vitro senescence is accompanied by a decrease of the expression of HMGA2 and an increase of $p14^{Arf}$. The *in vitro* proliferative capacity of cells from fibroids is known to be limited. Because *in vivo* hematopoietic, as well as normal stem cells, show a decline of *HMGA2* expression with age (4), we reasoned that a decrease of the *HMGA2* level may also accompany *in vitro* senescence of these benign cells. Accordingly, the expression of *HMGA2* mRNA was determined at various passages of a cell culture established from an uterine leiomyoma with an apparently normal karyotype. *HMGA2* mRNA gradually declined with *in vitro* passaging (data not shown) with a highly significant decrease between the 6th and the 13th passage (Figure 1 A). Because in somatic stem cells, a causal link between aging and the decline of HMGA2 has been found to result from the ability of HMGA to indirectly repress the *CDKN2A* locus, we examined if the decrease of *HMGA2* expression was accompanied by a

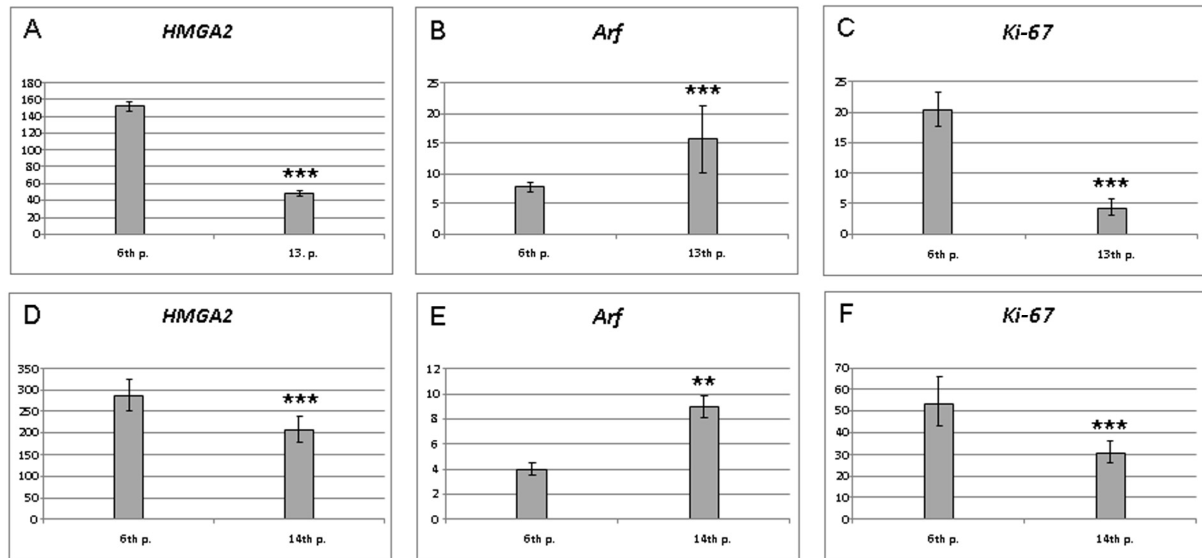

Figure 1. *In vitro senescence accompanied by a gradual decrease of HMGA2 and a simultaneous increase of p14^{Arf}. Decline of HMGA2 mRNA in an uterine leiomyoma (UL) with an apparently normal karyotype with long term passaging (A) and increase of p14^{Arf} mRNA (B) and decrease of Ki-67 mRNA (C). Decline of HMGA2 mRNA in a UL with t(2;12) with long term passaging (D) and increase of p14^{Arf} mRNA (E) and decrease of Ki-67 mRNA (F). Native myometrial tissue served as calibrator (expression=1). **p<0.01; ***p<0.001. All analyses have been performed in triplicate.*

simultaneous increase of the expression of either of the genes from that locus. Whereas a generally low but constant expression of *p16^{Ink4a}* throughout all passages was noted, *p14^{Arf}* mRNA continuously increased with passaging, again with a highly significant increase when comparing the 6th and the 13th passage (Figure 1 B). If HMGA2 positively influences proliferation of leiomyoma cells, one would expect an inverse correlation between the expression of a proliferation marker and the passage number as well. Thus, we tested whether or not such a correlation exists. Indeed, the expression of *Ki-67* significantly ($p<0.001$) decreased with increasing passage number (Figure 1 C). To exclude that this reduction of *HMGA2* expression is due to an overgrowth of normal stromal cells present in the samples, karyotype analyses were carried out at different *in vitro* passages of a leiomyoma with a 12q14~15 rearrangement (see case 0646-1, Table I, for FISH results see also (9)), the most frequent type of non-random chromosomal translocation seen in leiomyomas. At passages 7, 11, 15, and 22, each of 25 metaphases were karyotyped. In none of the passages were metaphases with a normal karyotype found, thus excluding a selection towards normal cells. However, as to the expression of *HMGA2* and *p14^{Arf}*, the same results as those observed with the karyotypically normal tumor were noted: With passaging, *HMGA2* mRNA decreased whereas *p14^{Arf}* mRNA increased (Figure 1 D, E). Akin to the tumor with an apparently normal karyotype, the expression of *Ki-67* significantly ($p<0.001$) fell with an increasing passage number (Figure 1 F).

Antagonizing MDM2 induces senescence as well as apoptosis in fibroid cells in vitro. The findings suggest that *in vitro* senescence of leiomyoma cells is controlled by the *HMGA2-p14^{Arf}* axis. Thus, a similar mechanism seems to account for the *in vivo* aging of stem cell populations as well as the *in vitro* senescence of cells from uterine fibroids. It is therefore tempting to assume that *in vivo* senescence contributes to the growth control of UL as well. There are some recent data supporting this idea; for example, we have demonstrated (5) that UL express significantly higher levels of *p14^{Arf}* than do myometrial tissue and that in leiomyomas, delicate balances along the HMGA2–p14^{Arf}–MDM2–p53–p21 axis seem to exist. Because of the possible similarities between *in vitro* and *in vivo* senescence of these tumors, we were interested to see if this balance can be disturbed *in vitro* by MDM2 inhibitors. A class of well-known small-molecule inhibitors of MDM2 that revealed promising anticancer activity in preclinical tests are nutlins (10), small molecules inhibiting the interaction of MDM2 with p53 (11). Accordingly, we used nutlin-3 to inhibit MDM2 in cell cultures from a UL with normal karyotype. To determine if this treatment can induce senescence of the cultures, the percentage of β-galactosidase-positive tumor cells with and without treatment with nutlin-3 (30 and 50 μM) for two different times (24 h, 72 h) was determined. The expression of β-galactosidase as revealed by blue-staining of the substrate is a well-established cellular marker of senescence. Compared to each of the controls, the number of positive

Table I. *Nutlin-3 influences the expression of senescence and of apoptosis markers, as well as of proliferation, in cell cultures of seven leiomyomas. All expressions are relative to the control (=100%) after treatment with nutlin-3 for 72 h. Significances as calculated by comparison with the control are given by asterisks following the values (*p<0.05; **p<0.01; ***p<0.001). n.s.=not significant.*

Case no.	Age (years)	Tumor-size (cm)	Karyotype	*CDKN1A*		*BAX*		*GLB1*		*Ki-67*	
				30 µM	50 µM	30 µM	50 µM	30 µM	50 µM	30 µM	50 µM
0503-1	40	4.0	46,XX,inv(5)(q15q31~33),t(12;14)(q15;q24)	1693.0***	2356.7***	289.1*	326.8*	215.6*	320.2**	1.8***	2.0***
0628-2	57	1.5	46,XX,?ins(12;14)(q15;q31q24)[5]/46,XX	2106.6***	2432.4***	331.1**	411.5*	189.1$^{n.s.}$	244.5$^{n.s.}$	1.1***	1.5***
0632-1	47	4.0	46,XX,t(12;14)(q15;q24)[12]/46,XX, del(4)(q31or q32),der(10),t(10;14) (q24;q32),t(12;14)(q15;q24)	8982.9***	8142.1***	467.1***	593.1***	552.6*	548.1**	0.5***	0.8***
0646-1	47	9.5	46,XX,t(2;12)(p21;p13) FISH revealed a hidden *HMGA2* rearrangement	391.7***	611.5***	117.9*	124.9***	132.8$^{n.s.}$	202.4$^{n.s.}$	6.2***	8.7***
0668-3 5.59***	57	2.5	46,XX	1515.1***	1841.1***	265.49***	322.22***	133.98**	125.1***	7.69***	
0686-2 10.4***	57	1.0	46,XX	745.1***	1189.2***	136.4*	162.4*	109.7$^{n.s.}$	155.1$^{n.s.}$	3.2***	
0691-1	55	0.8	46,XX	2122.8***	3172.6***	205.0***	268.2***	117.3$^{n.s.}$	130.7	4.7***	6.6***
Average				2508.17	2820.8	258.87	315.59	207.30	246.59	3.6	5.08

cells clearly increased after treatment (Figure 2) indicating that the cells were sensitive to inhibition of MDM2. Accordingly, mitoses were nearly absent from all of the cultures treated by nutlin-3 (Figure 2 and 3) and a drastically reduced number of cells compared to the controls was noted that could not be explained by the absence of proliferation alone but also indicates a loss of cells.

We then examined if this increase of β-galactosidase-positive cells is accompanied by an increase of the expression of *CDKN1A*, the transcription of which is directly activated by p53, making its expression a well-established marker of cellular senescence, as well as by an increase of the expression of *GLB1*. After 24 and 72 h, nutlin-3 treatment led to an increase of the expression of *CDKN1A* and of *GLB1* (Figure 3 A). The inhibition of MDM2 leads to a stabilization of p53, known to influence senescence as well as apoptosis, depending on the cellular context. To examine if the decreasing number of leiomyoma cells after treatment by nutlin-3 may result from a possible loss of cells, we then examined if the treatment by nutlin-3 *in vitro* also results in an increased expression of *BAX*, suggesting the induction of apoptosis (12). Normal fibroblasts are known to be resistant to nutlin-induced apoptosis (13). Interestingly, nutlin-3 significantly increased the expression of *BAX* after 24 and 72 h (Figure 3 A and B). The expression of the proliferation marker *Ki-67* which was tested along with the other markers significantly decreased with both concentrations of nutlin-3 and after 24 and 72 h (Figure 3 A and B).

To reproduce the results, cells from six further UL were treated with nutlin-3 (Table I). In summary, the results confirmed those obtained for the first case. On average nutlin-3 treatment for 72 h with both concentrations resulted in a more than 20-fold increase of the expression of *CDKN1A*, a more than 2-fold increase of the expression of *GLB1*, and *BAX*. In addition, a 27.8-fold (30 µM nutlin-3) and a 19.69-fold (50 µM nutlin-3) decrease of the proliferative activity as determined by the expression of *Ki-67* were noted. As a negative control, the experiments were then repeated with immortalized cell lines derived from fibroids. For these experiments, we used two cell lines transformed by the early region of SV40 (14). The transforming proteins of SV40 are known to bind and inhibit p53. Compared to the primary cells, both cell lines were practically resistant to nutlin-3, with only slight increases of the expression of *CDKN1A* and *GLB1* as well as of *BAX* and virtually no changes of that of *Ki-67* (Figure 4 A and B).

In vitro treatment of UL cells by siRNAs specifically targeting MDM2 induces changes of gene expression comparable to those induced by nutlin-3. To exclude that the effects observed after treatment with nutlin-3 are non-specific, cells from a UL (686-2, see Table I) were incubated with siRNAs specifically targeting *MDM2*. Compared to the control with non-specific siRNA either of four specific siRNAs (A, B, C, D) significantly increased the expression of *CDKN1A* and decreased that of *Ki-67*. The expression of *BAX* significantly increased with only two of the siRNAs used (Figure 5 A-D).

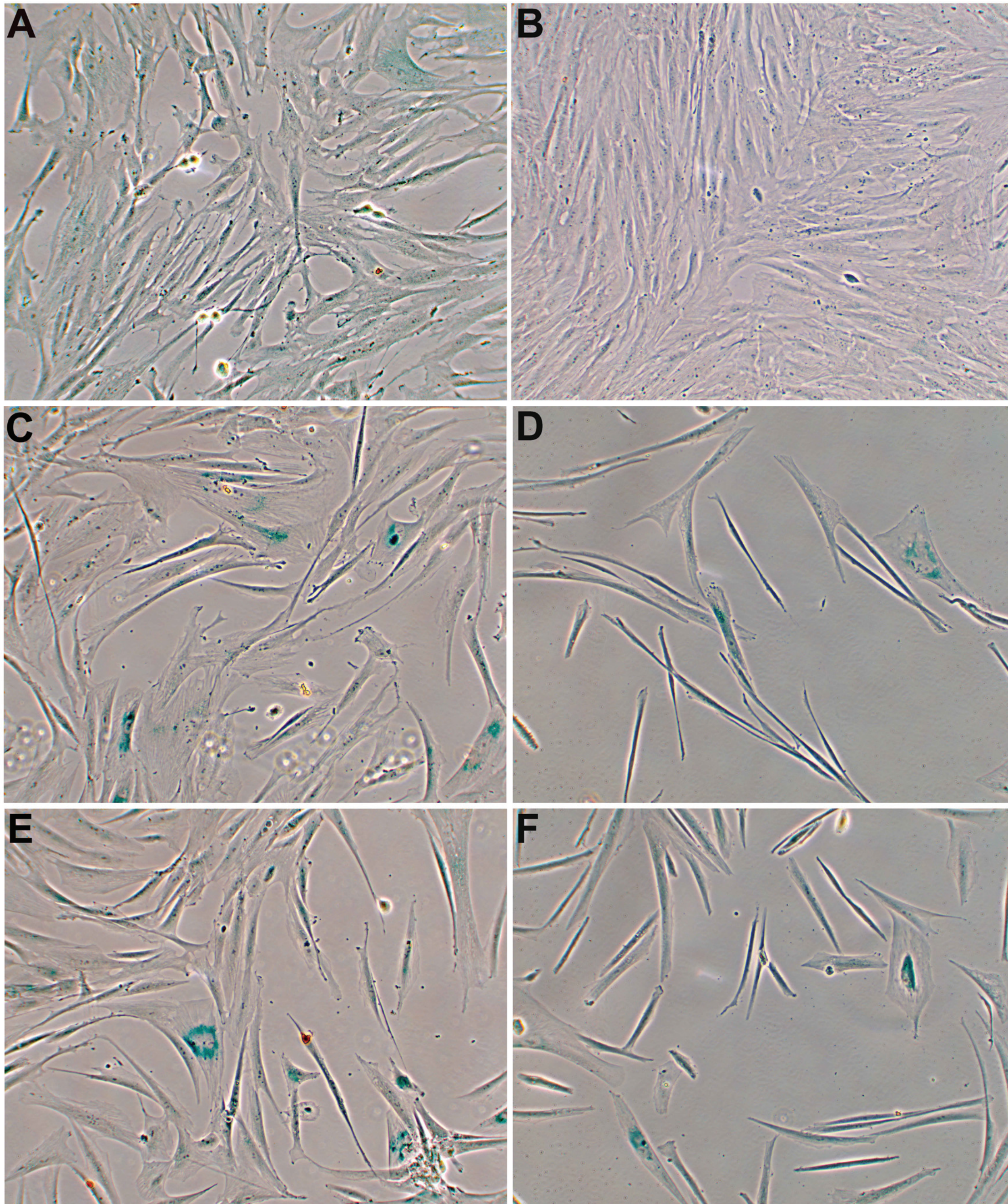

Figure 2. *In situ β-galactosidase staining increases after treatment of fibroid cells with the MDM2 antagonist nutlin-3. A: Control, UL cells grown for 24 h; B: Control, UL cells grown for 72 h; C: UL cells grown for 24 h with 30 μM nutlin-3; D: UL cells grown for 72 h with 30 μM nutlin-3; E: UL cells grown for 24 h with 50 μM nutlin-3; F: UL cells grown for 72 h with 50 μM nutlin-3.*

p14^{Arf} increases the expression of markers of senescence as well as of apoptosis in uterine fibroids. Because data showed that inhibition of MDM2 triggers the senescence pathway as well as apoptosis, we were interested if the expression of *p14^{Arf}/MDM2* may also correlate with the expression of an apoptosis marker, namely *BAX*, *in vivo*. Samples from a total of 29 UL of the two most frequent karyotype groups were analyzed for possible correlations between the expression of *p14^{Arf}* and *BAX* and *CDKN1A* and *BAX*. Interestingly, a highly significant correlation ($p<0.001$) was found in both analyses (Figure 6 A and B), suggesting that *in vivo*, *p14^{Arf}* does not only induce

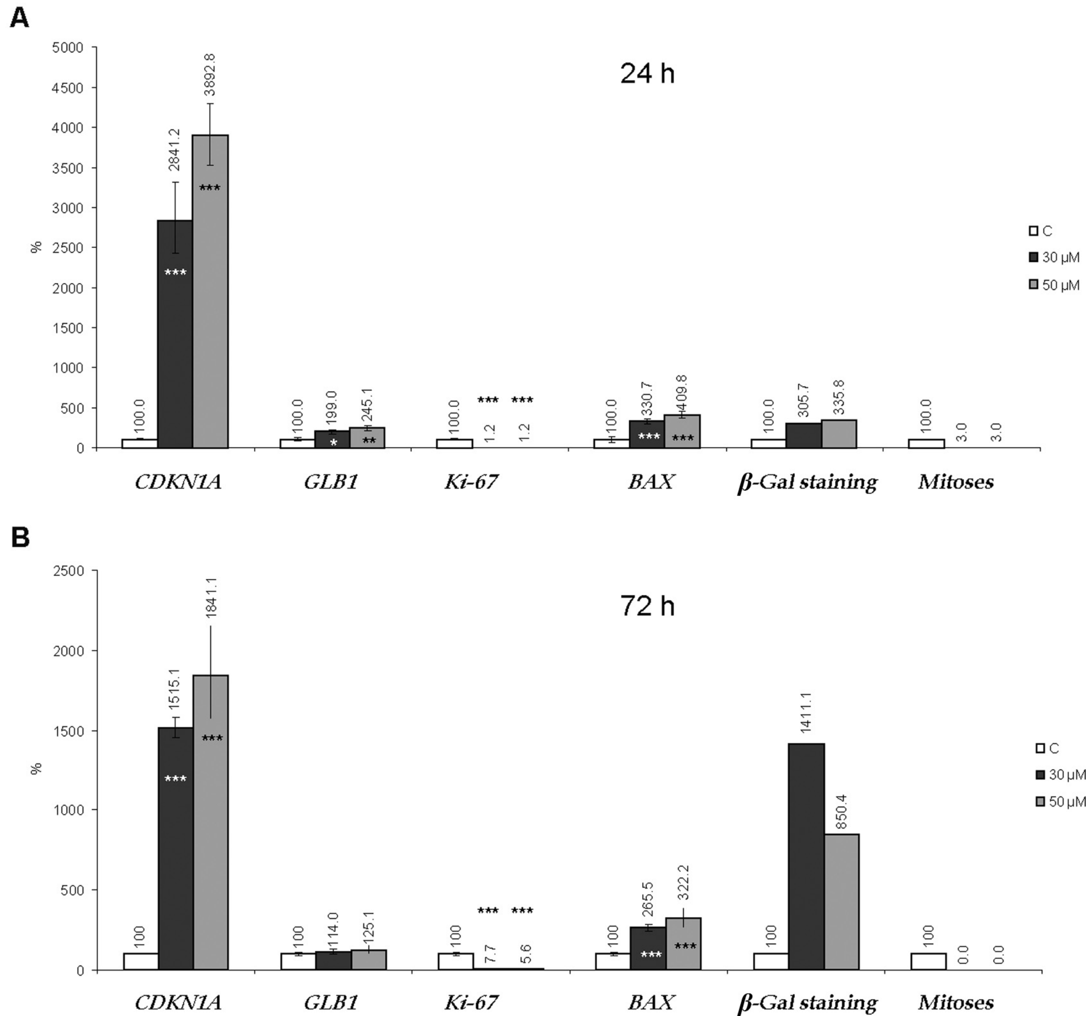

Figure 3. *In vitro treatment of UL cells by the MDM2 antagonist nutlin-3 for 24 h (A) and 72 h (B), respectively, influences important parameters associated with senescence and apoptosis. For all analyses, the control was set at 100%. Expression of CDKN1A, GLB1, Ki-67, and BAX was determined by qRT-PCR. The percentage of β-galactosidase-positive cells (total number of cells checked: control, 24 h=654; 30 µM nutlin-3, 24 h=471; 50 µM nutlin-3, 24 h=546; control, 72 h=1446; 30 µM nutlin-3, 72 h=222; 50 µM nutlin-3, 72 h=510) and mitoses were analyzed microscopically. Statistically significant differences for the qRT-PCR data are given by *p<0.05; **p<0.01; ***p<0.001.*

senescence but, simultaneously, part of the tumor cell population becomes committed to apoptosis with both these phenomena clearly depending on the level of $p14^{Arf}$.

Discussion

Akin to normal cells, senescence limits the *in vitro* proliferation of cells from benign tumors as well. Well-known examples of benign tumors with limited *in vitro* growth potential are UL clonally originating from myometrial tissue (15). Different genetic subtypes of UL exist (16) but apparently common to all subtypes is a limited *in vitro* growth potential of UL cells (14). Replicative senescence driven by telomere shortening may contribute to this limited growth potential because shortened telomeres of fibroids compared to matching myometrium have been noted (17, 18), but this does not completely explain growth restrictions seen in UL. Recently, the expression of the high mobility group protein HMGA2, causally known to be implicated in the growth of benign tumors of mesenchymal origin, has been identified as a repressor of senescence-inducing genes in neural and hematopoietic stem cells (4). Thus, we were interested to see if its expression changes during *in vitro* propagation of cells from UL. Interestingly, the results revealed a strong inverse correlation between the expression of *HMGA2* mRNA and the passage number. In contrast, the expression of $p14^{Arf}$ increased with passaging. These findings interestingly parallel those obtained for aging of somatic stem

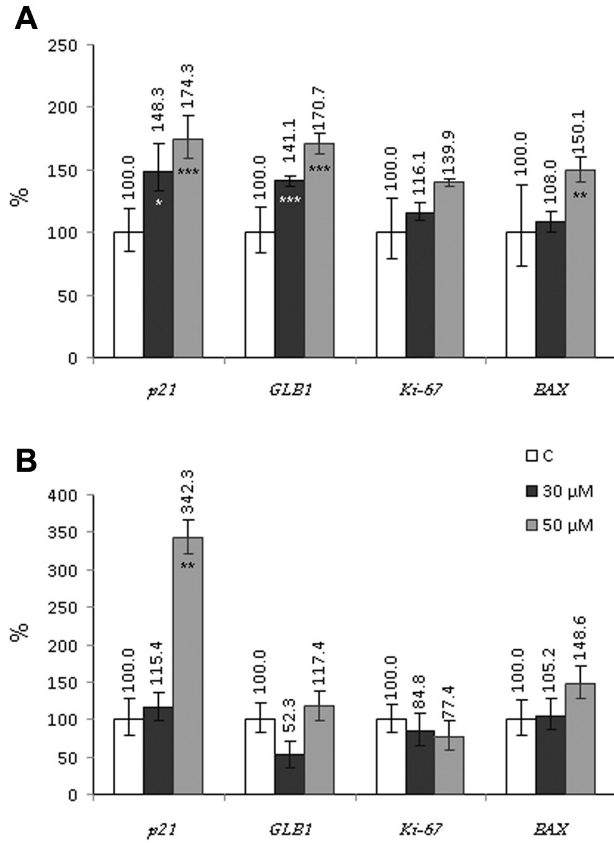

Figure 4. *Two SV40 large T-immortalized leiomyoma cell lines (A, B) used as control revealed a drastically reduced nutlin-3 sensitivity compared to primary cells. For all analyses, the control was set at 100%. Expression of CDKN1A, GLB1, Ki-67, and BAX was determined by qRT-PCR. Statistically significant increases (CDKN1A, GLB1, BAX) or decreases (Ki-67) are given *p<0.05; **p<0.01; ***p<0.001.*

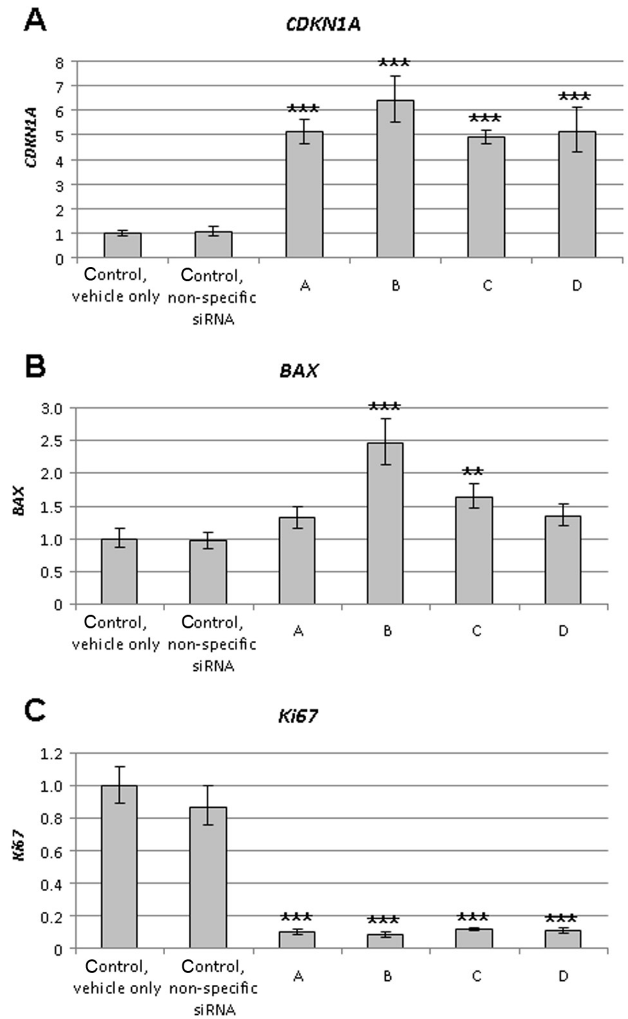

Figure 5. *In vitro treatment of UL cells by siRNAs specifically targeting MDM2 induces changes of gene expression comparable to those induced by nutlin-3. Expression of CDKN1A, Ki-67, and BAX was determined by qRT-PCR. Cells treated with the vehicle only and with non-specific siRNA were used as two negative controls. For all analyses, the expression of vehicle-only control was set at 1. Statistically significant differences compared to the control with non-specific siRNA are given by **p<0.01; ***p<0.001.*

cells, with one exception: Whereas in the stem cell populations the repression of both *p16*^{Ink4a} and *p14*^{Arf} by HMGA2 seems to be relevant for the maintenance of their self-renewal capacity, *in vitro* senescence of UL cells apparently only correlates with elevated expression of *p14*^{Arf}. Interestingly, senescence as, for example, revealed by the expression of β-galactosidase, is also a frequent finding in UL *in vivo*. In a study on 82 fibroids Laser *et al*. (2) were able to show that 11% of the tumors belonged to a highly senescent group (≥50% positive cells). No apparent relationship was noted between the expression of *p16*^{Ink4a} and the different senescence groups, suggesting that the decreasing repression of that gene by HMGA2 does not explain the accumulation of senescent cells. Our findings strongly suggest that in UL, expression of p14^{Arf} warrants consideration as a major mechanism of the endogenous growth restriction of UL. p14^{Arf} is a known regulator of p53 by its interaction with MDM2. In turn, p53 is activated by p14^{Arf} and can activate both a pro-senescence as well as pro-apoptotic response. Thus, compared to adjacent myometrium, UL can be considered a cell population of advanced age, prone to undergoing senescence. Moreover, the positive feedback between *p53* and *MDM2* revealed by gene expression studies showing a strongly positive correlation between the expression of *MDM2* and *p14*^{Arf} (5) may also have therapeutic implications. As shown herein, senescence as well as apoptosis of UL cells *in vitro* can be induced by

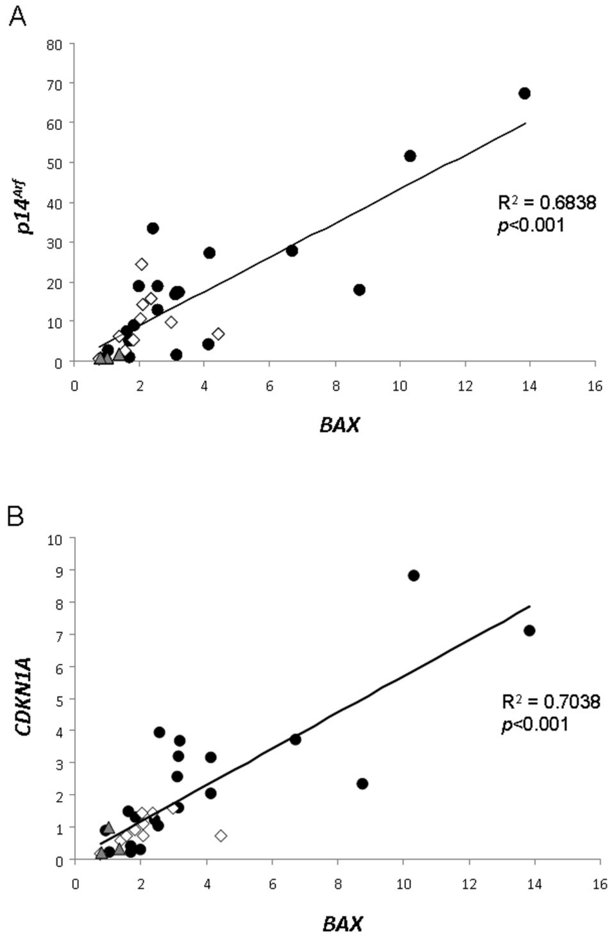

Figure 6. *A highly significant correlation (p<0.001) was found between the expression of BAX and p14^{Arf} and CDKN1A. A: In myometrium (closed triangles, n=3), UL with 12q14~15 aberrations (closed circles, n=18), and UL with a normal karyotype (open rhombi, n=10), the expression of BAX shows a highly significant correlation with that of p14^{Arf}. B: For the same samples, a highly significant association is noted between BAX and CDKN1A expression. Myometrial tissue served as calibrator (expression=1). All analyses were performed in triplicate.*

antagonizing MDM2, a negative regulator of p53 that modulates its transcriptional activity and stability (13, 19-21). In cancer, the use of MDM2 antagonists as effective therapeutics is limited by the requirement not only for wild-type p53 but also for functional signaling in the p53 pathway (22). The results of a previous (5) as well as of the present paper may suggest usefulness of MDM2 antagonists in the therapy of a UL that not only are known to show rarely if ever mutational inactivation of *TP53* (23-24) but apparently show a growth behaviour depending on p14^{Arf}–MDM2–p53 autoregulation. As to the effects induced by nutlin-3, it seems particularly noteworthy that the cells are not resistant to p53-induced apoptosis as are fibroblasts of both human and murine origin (13). The *BAX–p14^{Arf}* correlation as demonstrated in samples from native fibroids in the present study shows that *in vivo*, leiomyoma cells are not resistant to p53-dependent apoptosis. Of note, BAX protein has been shown to be detectable in leiomyoma samples but not in myometrial samples (25). In summary, the mechanisms limiting the *in vitro* proliferation of UL cells, at least in some aspects, seem to reflect an endogenous growth control of UL *in vivo*. Self-renewal induced by HMGA2 seems to be a positive regulator of UL growth, while the elements of an intact p14^{Arf}-driven network assure the high genomic stability of UL, as well as their growth control. *In vitro* antagonizing of MDM2 induces cellular senescence and apoptosis, thus offering promising new approaches towards a therapy of UL.

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