Cancer-associated fibroblasts (CAFs), the most abundant and probably the most active cellular component of breast cancer-associated stroma, promote carcinogenesis through paracrine effects; however, the molecular basis remains elusive. We have shown here that p16\textsuperscript{INK4A} expression is reduced in 83% CAFs as compared with their normal adjacent counterparts cancer-free tissues isolated from the same patients. This decrease is mainly due to AUF1-dependent higher turnover of the CDKN2A mRNA in CAFs. Importantly, p16\textsuperscript{INK4A} downregulation using specific siRNA activated breast fibroblasts and increased the expression/secretion levels of stromal cell-derived factor 1 (SDF-1) and matrix metalloproteinase (MMP)-2. Consequently, media conditioned with these cells stimulated the proliferation of epithelial cells. Furthermore, the migration/invasion of breast cancer cells was also enhanced in an SDF-1-dependent manner. This effect was mediated through inducing an epithelial–mesenchymal transition state. By contrast, increase in p16\textsuperscript{INK4A} level through ectopic expression or AUF1 downregulation, reduced the secreted levels of SDF-1 and MMP-2 and suppressed the pro-carcinogenic effects of CAFs. In addition, p16\textsuperscript{INK4A}-defective fibroblasts accelerated breast tumor xenograft formation and growth rate in mice. Importantly, tumors formed in the presence of p16\textsuperscript{INK4A} defective fibroblasts exhibited higher levels of active Akt, Cox-2, MMP-2 and MMP-9, showing their greater aggressiveness as compared with xenografts formed in the presence of p16\textsuperscript{INK4A}-proficient fibroblasts. These results provide the first indication that p16\textsuperscript{INK4A} downregulation in breast stromal fibroblasts is an important step toward their activation.

The presented data clearly show that in addition to its well-known cell autonomous tumor suppressor functions, p16 has also a major cell nonautonomous tumor inhibitory role.

**RESULTS**

p16 is downregulated in breast cancer-associated stromal fibroblasts

The p16 expression was first investigated in 12 human breast CAFs and their counterpart fibroblasts isolated from histologically normal cancer-free tissues (TCFs). CAF/TCF pairs were always used simultaneously at similar passages. Whole-cell extracts were prepared and specific anti-p16 and anti-GAPDH (used as internal control) antibodies were utilized for immunoblotting analysis. Figures 1a and b show that the level of p16 is lower in 10 out of 12 CAFs (83%) as compared with their corresponding TCFs. However, p16 level was similar in 2 CAF/TCF pairs (114 and 148) (Figures 1a and b). In addition, a great interindividual variation in p16 expression was observed between the various CAFs and also TCFs (Figures 1a and b). The p16 expression was first investigated in 12 human breast CAFs and their counterpart fibroblasts isolated from histologically normal cancer-free tissues (TCFs). CAF/TCF pairs were always used simultaneously at similar passages. Whole-cell extracts were prepared and specific anti-p16 and anti-GAPDH (used as internal control) antibodies were utilized for immunoblotting analysis. Figures 1a and b show that the level of p16 is lower in 10 out of 12 CAFs (83%) as compared with their corresponding TCFs. However, p16 level was similar in 2 CAF/TCF pairs (114 and 148) (Figures 1a and b). In addition, a great interindividual variation in p16 expression was observed between the various CAFs and also TCFs (Figures 1a and b).

The level of the CDKN2A mRNA was also assessed in the same cells by reverse transcription (RT)-PCR. Therefore, total RNA was prepared and specific primers for CDKN2A as well as β-actin (used as internal control) were utilized for amplification. Figures 1c and d show a clear decrease in the CDKN2A mRNA level in 10 out of 12 (83%) CAFs, as compared with their adjacent TCFs, while CDKN2A mRNA was similar in the two CAF/TCF pairs 114 and 148. These results were confirmed for some CAF/TCF pairs with quantitative
RT–PCR (data not shown). This shows that the expression of the CDKN2A mRNA reflects that of the corresponding protein in the majority of the TCF/CAF pairs (Figures 1b and d), indicating that the decrease in p16 protein level is due, at least in part, to a decrease in the level of its corresponding transcript.

To provide evidence that p16 downregulation in CAFs is also taking place in breast tissues, frozen sections including both tumor and their adjacent normal tissues were double immunostained by fluorescent anti-p16 and anti-vimentin (fibroblast cells marker) antibodies. Figure 1e shows lower positive staining for p16 in fibroblasts present in tumors than in their counterparts present in normal parts. This indicates that p16 is also downregulated in CAFs in vivo.

Figure 1. p16 Expression level is downregulated in CAFs. (a) Whole-cell lysates were prepared from the indicated cells and 50 μg of proteins were used for immunoblot analysis using the indicated antibodies. (b, d) Histograms, the values were determined by densitometry and normalized against the internal controls glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. (c) Total RNA was extracted and the amount of CDKN2A mRNA was assessed by RT–PCR. The amplified fragments were electrophoresed on ethidium bromide stained agarose gel. Error bars represent means ± s.d. (e) Immunofluorescence analysis using the indicated frozen sections N, T and antibodies. N, normal; T, tumor.

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The CDKN2A mRNA turnover is faster in CAFs versus TCFs. Next, we sought to study the CDKN2A mRNA half-life in the TCF/CAF-180 pair. To this end, cells were treated with the transcription inhibitor, actinomycin D (5 μg/ml) for different periods of time. Subsequently, total RNA was purified and quantified by RT–PCR using specific primers for CDKN2A and β-actin. Figure 2a shows that the CDKN2A mRNA is more stable in TCF cells as compared with their corresponding CAFs. Indeed, while the CDKN2A mRNA half-life is >6 h in TCF-180, it is only 45 min in CAF-180 (Figure 2a). Similar results were obtained for other CAF/TCF pairs (data not shown), which strongly suggests that the lower level of the CDKN2A mRNA in CAFs is due to its faster turnover in these cells as compared with their adjacent counterparts.

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The level of the AUF1 protein is higher in CAFs than in TCFs. To explore the cause of the CDKN2A mRNA instability in CAFs, we assessed the level of the RNA decay-promoting protein AUF1, which is known to regulate the RNA stability of the CDKN2A mRNA, by immunoblotting. To this end, cell extracts from the 12 CAF/TCF pairs, and specific anti-AUF1 antibodies were used. Figure 2c shows that the AUF1 protein (46 kDa band) is clearly upregulated in 8 out of 12 CAFs (66%) as compared with their corresponding TCFs. Importantly, the CDKN2A mRNA was downregulated in all CAFs were AUF1 was found highly expressed (Figures 1d and 2c).

To further elucidate the link between AUF1 and p16 downregulation in CAFs, we tested the effect of AUF1 knockdown using specific siRNA on the expression level of the CDKN2A mRNA in CAF-64. Therefore, these cells were transfected with plasmids harboring either AUF1-siRNA or control siRNA for 24 h, and then cells were harvested. Total RNA was purified and the level of the CDKN2A mRNA was assessed by RT–PCR. Figure 2d shows that the AUF1 level decreased significantly in the AUF1-siRNA-expressing cells as compared with the control cells. Concomitantly, the CDKN2A mRNA level increased in the AUF1-siRNA-expressing cells (Figure 2d), indicating that AUF1 is indeed a negative regulator of CDKN2A in breast stromal fibroblasts, and it’s increase reduced p16 expression in these cells.

p16 Downregulation upregulates the α-SMA, SDF-1 and MMP-2 proteins

In an effort to study the various effects of p16 downregulation, TCF-64 cells were transfected with a vector expressing either CDKN2A siRNA or a nonspecific control siRNA as previously described. Subsequently, specific antibodies were used for immunoblotting analysis. Figure 3a shows that p16 protein level declined 7.7-fold in the CDKN2A-siRNA-treated cells (T64-si) as compared with their corresponding control cells (T64C). However, the level of p14 was not affected, while the expression of p21 decreased, as expected, like in CAF-64 cells (Figure 3a).

Interestingly, like in the corresponding CAF-64 cells, this decrease in the p16 protein level was accompanied by strong increase in the levels of α-SMA (10-fold) and SDF-1 (threefold; Figure 3a), two important markers of activated stromal fibroblasts. Likewise, p16 downregulation increased the expression of MMP-2 (twofold). This suggests a possible role of p16 in repressing breast stromal fibroblasts.

The effect of the p16 knockdown was also assessed on the MMP-2 and SDF-1 mRNA levels by real-time RT–PCR. Figure 3b shows that the mRNA levels of the two genes increased by about 50% in p16-defective cells as compared with their corresponding controls, with P-values of 5 × 10−5 and 5 × 10−4, respectively. Similar results were obtained when p16 was downregulated with a specific shRNA targeting different sequence of the gene and used transiently (data not shown). To further show this, CAF-64 cells were transfected with adenovirus-based plasmids-expressing CDKN2A (C64-ORF (open reading frame)) or not (C64C). Total RNA was purified from these cells 3 days post infection and real-time RT–PCR using specific primers was performed. Figure 3c upper panel shows the increase in the expression of CDKN2A in cells expressing the gene. The lower panel shows that the introduction of the CDKN2A ORF in CAF-64 cells significantly reduced the expression of the MMP-2 and SDF-1 mRNAs. Therefore, the mRNA levels of these genes are modulated in a p16-dependent manner.

p16 Suppresses the secretion of SDF-1 and MMP-2 from breast stromal fibroblasts

CAF-64 and TCF-64 as well as T64-si and T64C cells were cultured in complete media for 24 h, and CM from each culture were collected, and the levels of SDF-1 and MMP-2 were assessed by enzyme-linked immunosorbent assay (ELISA)-based assay. The effect of the p16 suppression was also assessed on the secretion of SDF-1 and MMP-2 from breast stromal fibroblasts. CAF-64 and TCF-64 as well as T64-si and T64C cells were cultured in complete media for 24 h, and CM from each culture were collected, and the levels of SDF-1 and MMP-2 were assessed by ELISA. Figure 3e shows that the increase in the level of the p16 coding gene reduced the secreted levels of SDF-1 and MMP-2. This indicates that p16 restrains the secretion of these cancer-promoting proteins in breast stromal fibroblasts.
p16-Deficient fibroblast secretions stimulate breast cancer cell invasion/migration in an SDF1-dependent manner

Next, we sought to study the effect of p16 downregulation in fibroblast cells on the migration/invasion of breast cancer cells. To this end, we used serum-free-CM (SF-CM) collected from T64-si and T64C after 24 h of culture. In addition, CpM and SFM were used as controls. Subsequently, 10^5 MDA-MB-231 cells in 200 μl SF-CM were seeded in the upper compartment of the migration and invasion plates for 24 h. The migration and invasion of MDA-MB-231 cells were twofold higher in the presence of SFM than in the presence of SFM (Figure 4a). Interestingly, the migration and invasion of cancer cells were more significant when these cells were incubated with media from p16-defective cells. Indeed, the migration and invasion of MDA-MB-231 cells were, respectively, two and threefold higher in the presence of SFM from T64si cells than from the control cells (P-values = 0.0039 and 1.92 × 10^-5, respectively; Figure 4a). Likewise, the invasion and migration of MDA-MB-231 cells were twofold higher in the presence of SFM from T64C cells than from the control (data not shown). Similarly, SF-CM from p16–/– mouse embryonic fibroblasts (MEFs) stimulated the migration/invasion of MDA-MB-231 cells more than SF-CM from p16+/+ MEFs (Figure 4b). Interestingly, increasing the expression of the CDKN2A gene through ectopic expression in the CAF-64 cells (C64-ORF) significantly reduced the ability of these cells (as compared with their control counterparts) in enhancing the invasion/migration of breast cancer cells (Figure 4c).

In an attempt to investigate the possible role of SDF1 in this stimulation in both human and mouse cells, SF-CM from T64-si and p16–/– MEFs were either challenged either with immuno-globulin G1 antibody or with anti-SDF-1 inhibitory antibody, and the effect on the migration/invasion of the MDA-MB-231 cells was studied as described above. Figure 4d confirms the stimulatory effect of CDKN2A downregulation and also shows that the inhibition of SDF1 in SF-CM from both human and mouse fibroblasts reduced the migration/invasion potential of breast cancer cells to levels similar to those obtained with SF-CM from the respective cells.

p16-Deficient fibroblast secretions stimulate an epithelial–mesenchymal transition-like state in breast cancer cells

Next, the level of the active forms of extracellular signal-regulated kinase and Akt was assessed by immunoblotting in MDA-MB-231 cells treated with SF-CM from TCF-64, CAF-64 as well as T64-si and T64C. Figure 5a shows that while the levels of the inactive forms of these proteins did not change, the levels of the phosphorylated extracellular signal-regulated kinase and Akt increased in MDA-MB-231 cells treated with SF-CM from TCF-64 and T64-si, as compared with their respective controls. These factors are known to induce the motile and invasive capacities of cancer cells via promotion of epithelial–mesenchymal transition state.16 To further confirm this transition, we assessed the levels of N-cadherin, CD90 and vimentin as important markers of mesenchymal cells.17 Figure 5a shows that the level of the N-cadherin protein increased in MDA-MB-231 cells treated with SF-CM from CAF-64 and T64-si, as well as T64-si and T64C. Together, these results indicate that p16-defective breast stromal fibroblasts trigger epithelial–mesenchymal transition state in a paracrine manner.

p16 Downregulation in stromal fibroblasts stimulates epithelial cell proliferation through paracrine signaling

CAF-64, TCF-64, T64-si and T64C were cultured in SFM for 24 h, counted and SF-CM was collected and then added separately to MCF-10 cells in 96-well plates. The real-time cell electronic sensing system (ACEA Biosciences Inc., San Diego, CA, USA) was used to monitor the effect of each SF-CM as well as SFM and complete media (used as controls) to the proliferation. Although MCF-10 cells proliferated rapidly in the presence of SFM, their multiplication...
was very slow in the presence of SFM (Figure 5c). Furthermore, MCF-10A proliferated faster under the effect of CAF-64 SF-CM than in the presence of SF-CM from the corresponding cells (TCF-64). Interestingly, SF-CM from p16-defective T64-si cells enhanced more than twofold the proliferation rate of MCF-10 cells as compared with their proliferation in the presence of SF-CM from control cells (Figure 5c). This indicates that p16 in breast stromal fibroblasts inhibits epithelial cell proliferation through paracrine secreted factors.
AUF1 downregulation suppresses the effects of p16-deficient stromal fibroblasts

After showing that AUF1 downregulation upregulates p16 in CAF-64 cells (Figure 2d), we sought to investigate the effect of this downregulation on the level of secreted SDF-1 and MMP-2. To this end, the levels of these proteins were assessed by ELISA in CM from CAF-64 expressing AUF1-siRNA or control-siRNA. Figure 6a shows that AUF1-defective cells wherein p16 level increased showed a strong decrease in the secreted levels of SDF-1 and MMP-2.

Next, we sought to study the effect of AUF1 downregulation in CAF-64 cells on the migration/invasion of breast cancer cells using SF-CM collected from CAF-64 expressing AUF1-siRNA or control-siRNA. The invasion and the migration of MDA-MB-231 cells were assessed as described above, and were found to be lower in the presence of SF-CM from C64-si cells than from C64C cells (P-values = 0.002246 and 0.000608, respectively; Figure 6b). This indicates that AUF1 downregulation reduces the pro-carcinogenic effects of p16-defective stromal fibroblasts, may be through p16-upregulation.

p16-Deficient fibroblasts stimulate breast cancer xenograft growth in mice

To investigate the effect of p16 deficiency in fibroblasts on tumor growth and angiogenesis in vivo, 25 nude mice were randomized into five groups and breast cancer xenografts were created subcutaneously by coimplantation of MDA-MB-231 cells (2 x 10^6) with p16−/− or p16+/+ MEFs (10^6) into the right leg of each mouse. As controls 5 x 10^6 and 2 x 10^6 MDA-MB-231 cells and 10^6 p16−/− cells were injected separately. MEF cells were used in these experiments because of their fast growth as compared with the transfected breast stromal fibroblasts (T64si) and the corresponding T64C. Although all mice coimplanted with MDA-MB-231 and p16−/− MEF cells developed tumors, only two out of five mice coimplanted with MDA-MB-231 and p16−/− MEFs had tumors. Interestingly, tumors containing p16-deficient fibroblasts (T-MEFp16−/−) appeared only 1 week post injection and grew faster than those containing p16-proficient fibroblasts (T-MEFp16+/+), which appeared after 3 weeks (Figure 7a). No tumors were obtained by injecting p16−/− or MDA-MB-231 (2 x 10^5) cells alone, while four out of five mice developed tumors following the injection of MDA-MB-231 (5 x 10^5) cells alone and appeared only after 4 weeks. However, the histology of the formed tumors was similar in both cases (data not shown). These results show that p16-defective fibroblasts promoted breast cancer tumor formation and enhanced their growth rate in nude mice as compared with their isogenic normal cells.

To further elucidate the role of p16 in the effect of these fibroblasts on cancer cells in vivo, whole-cell extracts were prepared from the two tumor xenografts T-MEFp16+/+ and 2 T-MEFp16−−, and the levels of various cancer- and metastasis-promoting genes were assessed by immunoblotting. Figure 7b shows that while the level of Akt is similar in all the four tumors, the level of phosphorylated Akt was higher in the two T-MEFp16−− than in the T-MEFp16+/+ tumors. Then, we assessed the levels of important downstream targets of the Akt/NF-kB pathway, namely Cox-2 and cyclin D1. In fact the levels of these two important oncogenes were markedly higher in the two T-MEFp16−− than in the T-MEFp16+/+ tumors (Figure 7b). We also assessed the level of MMP-9 and MMP-22 and have found that these two proteins are also highly expressed in the two tumors with p16DEF+/+ and T-MEFp16+/+ (data not shown). Furthermore, we assessed the level of p16, cyclin D1 and MMP-9 in MEFp16−− and MEFP16+/+ and have shown that p16 knockdown did not affect the level of MMP-9 and rather decreased the level of cyclin D1 in pure MEF cells (data not shown), confirming that the observed effects were rather on breast cancer epithelial cells.

DISCUSSION

In this study, we have shown that the tumor suppressor p16 protein has an important role in controlling mammary stromal...
fibroblast autocrine and paracrine signaling. We started this study by showing that p16 is downregulated in CAFs as compared with their corresponding TCFs, isolated from the same patients. This p16 decrease suggests a role of cancer cells in suppressing p16 expression in fibroblast cells present in their vicinity, either directly or indirectly through sustained proliferative stress. In fact, several lines of evidence indicate that neoplastic cells have the ability to affect their microenvironment and modulate gene expression in the surrounding fibroblasts through secreted factors. In addition, p16 expression was also low in some TCF cells, which could be owing to interindividual variation in the expression of p16.

The reduction in p16 level did not result from methylation of the p16 promoter (data not shown), but was due to a decrease in the stability of the CDKN2A mRNA in CAFs, which resulted from the increase in the expression of the CDKN2A mRNA destabilizing protein AUF1. AUF1 knockdown in CAF cells increased the expression of the CDKN2A mRNA and also suppressed the pro-carcinogenic effects of these cells. Intriguingly, AUF1 level was found higher in TCF-114 than in its counterpart CAF-114, with no much effect on p16 level. This could suggest alteration in other p16/AUF1-regulatory pathway(s) in these cells. Interestingly, AUF1 controls also the expression of many other important breast cancer-related proteins such as Cox-2, cyclin D1, C-Myc, Bcl-2, ErbB and many others. This indicates that the increase in the level of AUF1 in breast stromal fibroblasts could constitute a major change in CAFs that could explain, in great part, the significant modulation in gene expression that has been previously reported in such cells.

In addition, we have shown that p16 negatively regulates the expression of both SDF-1 and MMP-2 in breast stromal fibroblasts. SDF-1, a promoter of tumor growth and angiogenesis, is also negatively controlled by p53. This indicates that this important cancer-promoting factor is under the control of two master tumor suppressor proteins p16 and p53. Indeed, inhibition of SDF-1 in CM severely reduced the pro-invasive/migratory effects of p16-defective human and MEF cells (Figure 4). Regarding MMP-2, several lines of evidence suggest that p16 negatively regulates this pro-invasion protein in various cell types. Indeed, p16 expression inhibited the expression of MMP-2 in lung cancer cells and in glioma. The present findings corroborate these results and provide clear evidence that MMP-2 expression is modulated in a p16-dependent manner in breast stromal fibroblasts. These results raise an important question on how p16 regulates the expression/secretion of these factors. In fact, several lines of evidence indicate that p16 is not a mere CDKI. p16 regulates the expression/secretion of these factors. In fact, several lines of evidence indicate that p16 is not a mere CDKI. p16 interacts with several proteins involved directly or indirectly through sustained proliferative stress. In fact, several lines of evidence indicate that neoplastic cells have the ability to affect their microenvironment and modulate gene expression in the surrounding fibroblasts through secreted factors. In addition, p16 expression was also low in some TCF cells, which could be owing to interindividual variation in the expression of p16.

In addition, p16-deficient fibroblasts enhanced the proliferation of epithelial cells in vitro and the formation and growth of breast tumor xenografts in mice, which is another major feature of activated fibroblasts. The tumors formed in the presence of p16-deficient cells expressed high levels of Cox-2 and MMP-9, known to promote tumor cell invasion and has been recently shown to be an important mediator of CAF-induced tumor growth and progression. These tumors showed also high levels of MMP-2, cyclin D1 and active Akt, which explains their faster onset and growth.

In addition to p16, other important tumor suppressor genes such as p53, p21, PTEN and CAV-1 are also implicated in repressing the pro-carcinogenic effects of breast stromal fibroblasts both in vitro and in vivo.

In summary, the present findings have shown that in addition to its well-known cell-autonomous tumor suppressor function within incipient pro-carcinogenic epithelial cells, stromal fibroblast p16, like other tumor suppressor genes, exerts also cell nonautonomous effects on breast tumorigenesis. Therefore, stromal fibroblast p16 might constitute a valid therapeutic target to stop tumor progression and/or recurrence.

**MATERIALS AND METHODS**

**Cells, cell culture and chemicals**

Breast fibroblast cells were obtained, characterized and cultured as previously described. MEF cell lines (a generous gift from Dr R A DePinho) were cultured as described previously and used at low passages (6–8). In the present experiments, CAFs and their corresponding TCFs were always cultured simultaneously, in the same conditions and at similar passages (4–8). CAFs grow slightly faster than their corresponding TCFs and slight variation in cellular growth was also observed between various CAFs and various TCFs, and both can reach passage 12 with no sign of senescence.

MDA-MB-231 and MCF-10 cell lines were obtained from ATCC (Manassas, VA, USA) and were cultured following the instructions of the company. All supplements were obtained from Sigma (St Louis, MO, USA) except for antibiotics and antymycotics solutions, which were obtained from Gibco (Grand Island, NY, USA).

**Immunohistochemical staining**

Frozen tissues plated on coverslips were used as detailed in the Supplementary Materials and methods.

**Cellular lysate preparation and immunoblotting**

This has been performed as described previously. See Supplementary Materials and methods for details.

**RNA purification, RT–PCR and real-time RT–PCR**

Total RNA was purified using the TRI reagent (Sigma) according to the manufacturer’s instructions. RT–PCR and real-time RT–PCR were performed as described in the Supplementary Materials and methods.

**Analysis of mRNA stability**

Cells were challenged with 5 μg/ml actinomycin D for various periods of time (0–6 h) and then total RNA was purified and assessed using RT–PCR.
siRNA transfection
The permanent transfection using CDKN2A-siRNA and control-siRNA was performed as described previously.14 pSILENCER-AUF1siRNA and control-siRNA plasmids15 were used to carry out transient transfection using the human dermal fibroblast nucleofector kit (Amaza BioSystems, Gaithersburg, MD, USA) following the protocol recommended by the manufacturer. Low passage cells (2–4) were used for transfection.

Cell proliferation analysis
A volume of 100 µl of complete medium containing 2–4 × 10³ cells was loaded in each well of the 96-well microtiter E-plates with integrated microelectronic sensor arrays at the bottom of each well, and cell proliferation was assessed by the real-time cell electronic sensing system as described previously16 and detailed in the Supplementary Materials and methods.

Chemotaxis and invasion assay
The 24-well BD BioCoat Matrigel Invasion Chambers were used as per the manufacturer guideline (BD Bioscience, Chicago, IL, USA). See Supplementary Materials and methods for a detailed description.

Conditioned media
Cells were cultured in media ± serum for 24 h, and then the media were collected and centrifuged. The resulting supernatant was either used immediately or frozen at −80 °C until needed.

Tumor xenographs
Animal experiments were approved by the KFSH&RC institutional Animal Care and Use Committee (ACUC) and were conducted according to relevant national and international guidelines. See Supplementary Materials and methods for a detailed description.

Statistical analysis
Statistical analysis was performed by the Student’s t-test and P-values of 0.05 and less were considered as statistically significant.

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