Abstract. Background: Angiogenesis is important in health and several disease states. CD105 is a proliferation-associated and hypoxia-inducible transmembrane protein abundantly expressed in angiogenic endothelial cells. CD105 is a receptor for transforming growth factors (TGF)-β1 and -β3. The exact mechanisms for CD105 regulation of vascular development have not been fully elucidated. Materials and Methods: In this study, an antisense approach to create a murine and a human stably transfected endothelial cell line expressing a reduction in CD105 protein was used. Results: We showed that inhibition of CD105 in cultured murine and human endothelial cells enhanced the ability of TGF-β1 to suppress growth and migration, and influenced TGF-β1 promoter activity. TGF-β1 not only reduced the length of the capillary-like structures, but also caused mortality in CD105-deficient murine antisense cells compared to control cultures. To determine whether CD105 affected TGF-β1-induced gene expression, a luciferase assay in transiently transfected cells with p3TP-Lux promoter constructs was performed. Both murine and human antisense transfectants showed a significant increase in p3TP-Lux promoter activity. Further studies on the functional importance of CD105 was undertaken in irradiated normoxic and hypoxic cells. The levels of pro- and anti-apoptotic markers were also evaluated. There was an increase in pro-apoptotic marker (p53), but a reduction in anti-apoptotic marker (Bcl-2) in CD105-deficient cells. Conclusion: These results provide direct evidence that CD105 antagonises the inhibitory effects of TGF-β1 on human and murine vascular endothelial cells and that normal cellular levels of CD105 are required for the formation of new blood vessels.
functional expression of receptors (TbRI and TbRII) and/or signalling mediators for TGF-β would allow cancer cells to become resistant to this potent growth inhibitor (11). The exact mechanism by which the tumour cells escape from TGF-β inhibition is uncertain, but understanding the function of those TGF-β signalling pathways that remain operative in tumour cells is of paramount importance, since most tumours with defects in TGF-β signalling still have functional components of these pathways that may play critical roles in determining the malignant phenotype. Likewise, loss of function of TGF-β1 has been noted in cells with up-regulated CD105, causing the cells to be resistant to the inhibitory effects of TGF-β1.

The existing functional studies on CD105, except the knock-out mice in ones, are based on human CD105. The murine CD105 shares 72% homology with its human counterpart and binds TGF-β1 (12), but its functions in TGF-β signal transduction and in murine endothelial cells are not known. The human studies reported have examined the effects of TGF-β1 signalling by transiently transfecting antisense oligonucleotides to human CD105. The results from transient analysis have indicated that the inhibition of CD105 expression in cultured endothelial cells enhanced the ability of TGF-β1 to suppress their growth and migration (3). To date, there are no stable human endothelial cell lines, expressing a reduction in CD105 expression, available to study this interaction further. Therefore, we have generated stable murine and human endothelial cell lines that express the antisense segment of the murine or human CD105 gene. These cell lines are unique models for the functional study of murine and human CD105 in endothelial cells, which would lead to a better understanding of the CD105 gene in TGF-β signal transduction, angiogenesis and other vascular diseases.

Materials and Methods

Endothelial cells. The murine endothelial cell line MS1 was purchased from ATCC. It strongly expresses CD105 and the VEGF receptor as well as von Willebrand factor. The human endothelial cell line, EA Hy926, was purchased from Cambrex BioScience, which expresses CD105 and von Willebrand factor. Both MS1 and EA Hy926 cells were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM:Sigma Aldrich) containing 10% fetal calf serum (Invitrogen) and 100 ìg/ml penicillin/streptomycin.

Transfection of murine MS1 cells. MS1 cells were transfected with pcDNA4, sense and antisense pcDNA4 containing mouse CD105 cDNA fragment using the LipofectAMINE reagent (Invitrogen). Eighteen hours before transfection, MS1 cells were plated into a six-well plate at a density of 2 x 10⁴ cells per well in complete DMEM and incubated at 37°C under 5% CO₂/air. For preparation of the liposome/DMEM mixture, 2 ìl LipofectAMINE reagent was incubated for 15 minutes in the presence of 1 µg plasmid DNA and 90 µl serum-free DMEM. The medium on the cells was replaced with 900 µl serum-free DMEM. After the 15-minute incubation, the LipofectAMINE/DMEM mixture was added to the MS1 cells and incubated at 37°C under 5% CO₂/air overnight. The following day, 2 ml complete DMEM containing 10% FCS was added to the transfected MS1 cells and incubated at 37°C under 5% CO₂/air for 48 hours. Fresh medium was added to the MS1 cells in the presence of 400 µg/ml zeocin (Invitrogen). The selected clones were then screened using FACS analysis to assess the levels of murine CD105 on the cell surface of the sense, antisense and vector-only transfected cells.

Transfection of human EA Hy926 cells. EA Hy926 cells were transfected with pcDNA3.1 and recombinant sense and antisense pcDNA3.1 using calcium phosphate transfection system (Invitrogen). Approximately 24 hours prior to transfection, EA Hy926 cells were plated into a 6-well plate at a density of 2 x 10⁴ cells per well in complete DMEM and incubated at 37°C under 5% CO₂/air. For preparation of the calcium phosphate/DMEM mixture, the following reactions were set up. In tube 1, 1x HBS solution was incubated with...
Fractionated proteins were electrophoretically transferred onto a PVDF membrane in 0.1 M Tris buffer pH 7.3, 0.5 mM PMSF, 1 mM pepstatin, 0.1 mM leupeptin and 1 mM EDTA). The cell lysate was solubilised in 1x10^7 cells/ml in extraction buffer (0.2% v/v Nonidet P-40 in 0.1 M Tris buffer pH 7.3, 0.5 mM PMSF, 1 mM peptatin, 0.1 mM leupeptin and 1 mM EDTA). The cell lysate was microfuged at 10,000 xg (Micro Centaur) for 10 minutes at 4°C and the supernatant was collected for immunoblotting.

**Immunoblotting of murine and human CD105 protein.** Murine and human cell extracts from 1 x 10^7 cells were subjected to immunoblotting. The total protein was measured using the Bio-Rad protein quantification kit (Bio-Rad Laboratories) and 30 mg was added drop-wise to tube 1 and incubated at room temperature for 20 minutes. Two-hundred μl of the suspension was added drop-wise to 2 ml complete media in each well. The plate was incubated at 37°C under 5% CO₂/air for 3 hours followed by two washes with pre-warmed PBS, and fixed with cold methanol for 1 hour. The cells were washed with cold PBS. After incubation with FITC-labelled rabbit anti-mouse, for human cells, or FITC-labelled rabbit anti-rat, for mouse cells (both were 1:40; DAKO) for 16 hours at room temperature in blocking buffer and incubated again at 37°C under 5% CO₂/air for a further 24 hours. Following the final incubation, the media was discarded and replaced with fresh complete media and incubated again at 37°C under 5% CO₂/air for one week. After one week in culture, independent clones were isolated in cloning cylinders and further cultured for over a month in the presence of 400 μg/ml G418-Sulfate. The selected clones were then screened using FACS analysis to assess the levels of human CD105 on the cell surface of the sense, antisense and vector-only transfected cells.

**Flow cytometry analysis.** Cell surface expression in murine and human CD105 protein was quantified by flow cytometry (Becton Dickinson Co.) using anti-CD105 monoclonal antibody E9 (13) for the human cells and anti-mouse CD105, M7/18 for the murine cells. The cells were incubated on ice for 1 hour and washed twice with cold PBS. After incubation with FITC-labelled rabbit anti-mouse, for human cells, or FITC-labelled rabbit anti-rat, for mouse cells (both were 1:40; DAKO) for 30 minutes on ice, the cells were washed and resuspended in 0.3ml 2% buffered formalin and analysed on a Becton Dickinson FACScan flow cytometer.

**Extraction of CD105 protein.** Cell extracts were obtained by solubilising 1x10^7 cells/ml in extraction buffer (0.2% v/v Nonidet P-40 in 0.1 M Tris buffer pH 7.3, 0.5 mM PMSF, 1 mM peptatin, 0.1 mM leupeptin and 1 mM EDTA). The cell lysate was microfuged at 10,000 xg (Micro Centaur) for 10 minutes at 4°C and the supernatant was collected for immunoblotting.

**Immunoblotting of murine and human CD105 protein.** Murine and human cell extracts from 1 x 10^7 cells were subjected to immunoblotting. The total protein was measured using the Bio-Rad protein quantification kit (Bio-Rad Laboratories) and 30 mg was resolved on a 12% gel for 45-60 minutes at 150 V. The fractionated proteins were electrophoretically transferred onto a nitrocellulose Hybond-P membrane (Amersham Biosciences) using the Trans-Blot system (Bio-Rad Laboratories) for 1 hour at 100 V. The membrane was subsequently blocked for 2 hours at room temperature in blocking buffer and incubated with either M7/18 or E9 mAb (in 1:1000 in blocking buffer) for 16 hours at 4°C. The membrane was thoroughly washed using PBS-Tween 20 before incubation with rabbit anti-rat or anti-mouse HRP-conjugated antibody (DAKO) diluted in 1:1000 in blocking buffer, for 2 hours at room temperature. The CD105 protein was visualised using the ECL system (Amersham Biosciences). To test for equal protein loading of the samples, the membrane was stripped and probed using rabbit anti-actin antibody (Sigma) and detected by a goat anti-rabbit HRP conjugate.

**Northern blotting analysis of CD105 mRNA.** The total RNA from the four human and murine cell lines was extracted using Trizol (Sigma), in accordance with the manufacturer’s instructions. Twenty μg of total RNA was denatured and fractionated in 1% (w/v) agarose/2.8% (v/v) formaldehyde gel and capillary blotted onto nitrocellulose. The murine CD105 cDNA probe was generated by PCR from the original sense plasmid and the 1.1Kb fragment was labelled with 32P by random prime labelling (the same method was used to generate the human CD105 probe). The blot was hybridized with the 32P labelled probe complimentary to CD105 mRNA at 65°C overnight and the extent of probe hybridization was revealed by a phosphorimagery (Molecular Dynamics). The membrane was re-probed with GAPDH as a loading control.

**Cellular DNA synthesis determination by [3H]-thymidine incorporation.** To evaluate the effects of TGF-β1 on the murine and human transfecants, [3H]-thymidine incorporation assays were carried out. Briefly 1x10^6 cells were seeded onto a 24-well plate in 0.5 ml medium containing 1% FCS and incubated for 24 hours, 10 ng/ml TGF-β1 (10 ng/ml) was added to the wells over 72 hours. The cells were labelled with 1 μCi/well of [3H]-thymidine (Amersham Pharmacia) for 3 hours followed by two washes with pre-warmed PBS, and fixed in situ with cold methanol for 1 hour. The cells were washed with ice-cold 5% (v/v) tri-chloroacetic acid (TCA) (Sigma), with five subsequent changes over 24 hours to remove any thymidine that had not been incorporated into the DNA. The cells were washed in PBS and digested by the addition of 0.4 ml/well of 0.1 M NaOH for 1 hour. Finally, the cell lysate was transferred to 4 ml of scintilliation mixture (Fisons Chemicals) and the amount of incorporated [3H]-

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**Table I. Murine and human PCR primer and probe sequences.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Murine CD105 forward primer</td>
<td>5’CTGGGACAGGGAGTGTG3’</td>
</tr>
<tr>
<td>Murine CD105 reverse primer</td>
<td>5’GCTTACGGCTTTGACCC3’</td>
</tr>
<tr>
<td>Human CD105 forward primer</td>
<td>5’CGTGGACAGCATGGACC3’</td>
</tr>
<tr>
<td>Human CD105 reverse primer</td>
<td>5’GATGCGAGGAACACTGCTG3’</td>
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<tr>
<td>Bcl-2 forward primer</td>
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<td>Bcl-2 reverse primer</td>
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<td>5’AGAAAGAACCACCTGGAGGAATAT3’</td>
</tr>
<tr>
<td>p53 reverse primer</td>
<td>5’CCCTGTAGTCACAAGGCCCTC3’</td>
</tr>
<tr>
<td>Bcl-2 probe</td>
<td>5’TCCACCTTCAGATCGGTGGCG3’</td>
</tr>
</tbody>
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**Warrington et al: The Role of CD105 in TGF-β1 Signalling in Endothelial Cells**
2.5x10^4 cells/well and transfected with 2 μg p3TP and 1 μg cells were seeded, in duplicate, in a six-well plate at a density of determine cellular responsiveness to TGF-β1. Human and murine plasminogen activator inhibitor-1 promoter region, was used to Luciferase reporter assay. The p3TP TGF-β1 inducible luciferase reporter construct (kindly provided by Professor Dimitris Kardassis, Greece), containing the luciferase gene under the control of the reporter construct (kindly provided by Professor Dimitris Kardassis, Greece), containing the luciferase gene under the control of the

**Figure 1. Flow cytometry analysis of CD105:** Both murine AS7 (A) and human AS35 (B) antisense cell lines displayed a lower percentage of membrane bound CD105 (reduced by 65% and 87%, respectively) when compared with the controls. The negative controls are background levels of parental cells which were not incubated with the primary antibody. Throughout, AS, SE and mock stand for antisense, sense and mock transfectants. Unless otherwise, stated cell experiments were performed three times.

Luciferase reporter assay. The p3TP TGF-β1 inducible luciferase reporter construct (kindly provided by Professor Dimitris Kardassis, Greece), containing the luciferase gene under the control of the plasminogen activator inhibitor-1 promoter region, was used to determine cellular responsiveness to TGF-β1. Human and murine cells were seeded, in duplicate, in a six-well plate at a density of 2.5x10^4 cells/well and transfected with 2 μg p3TP and 1 μg β-galactosidase. The cells were washed, lysed and assayed for luciferase activity using the luciferase assay system (Promega) in a MicroLumat LB96P (EG & G Berthold). The light emission by the TGF-β1-treated cells was expressed as a percentage of the emission by the untreated cells, following normalisation by subtracting the light emission from the pSV-β-galactosidase control vector assay. To assay for the control vector, 50 μl sample was loaded into a 96-well plate and 50 μl 2x assay buffer (Promega) was added, mixed and incubated at 37°C for 30 minutes. The 2x assay buffer contains the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) and, during the 30-minute incubation, the β-galactosidase hydrolyses the colourless substrate to o-nitrophenol (yellow). This reaction was terminated by addition of 150 μl 1M sodium carbonate (Promega) and the absorbance was read at 420 nm on a Titerpak Multiskan MC plate reader (Lab systems).

**Capillary formation in a 3-D collagen matrix.** The 3-D collagen matrix model is widely used to study endothelial cell behaviour because it mimics the in vivo environment (15). To make up 30 ml of 0.06% (w/v) collagen, ice-cold 7.5% (w/v) sodium bicarbonate (Sigma), 10 x DMEM (Invitrogen), complete medium containing 10% (v/v) FCS and 5 mg/ml collagen solution were mixed on ice in a universal tube at a ratio of 1:2:12:6:2.4. The 3-D sandwiched collagen gels were formed by aliquoting 500 ml/well of the 0.06% (w/v) collagen solution onto a 24-well plate (Fulka) and allowed to polymerise for 1 hour at 37°C. One ml of the cell suspension (3x10⁵ cells/ml) in medium containing 10% (v/v) FCS was then seeded on top of the matrix and the cells allowed to attach to the collagen for a further 30 minutes. The medium was gently aspirated and the cell monolayer overlaid with 500 ml 0.06% (w/v) collagen solution. The collagen was allowed to polymerise for 1 hour at 37°C and 1 ml of complete medium supplemented with 10% (w/v) FCS containing 10 ng/ml TGF-β1 were added to each well and incubated overnight at 37°C. The medium and TGF-β1 were replenished daily. Photographs were taken after 72 hours and the images were captured using black and white photography on a diavert inverted microscope (Leitz) at a magnification of x100.

**Irradiation of human AS35 and mock endothelial cell lines.** AS35 and mock cell lines were plated at a density of 5 x 10⁵ and cultured for 16 hours at 37°C, 5% CO₂/air in a 6-well plate. The following day, the cells were washed twice using PBS, the media replaced, and the cells subjected to a single dose of radiation (10 Gy, at a dose rate of 2.35 Gy/min; Faxitron N4380 X-ray source). Following irradiation, the cells were either incubated at 37°C in normoxia (5% CO₂/air) or hypoxia conditions (5% CO₂, 1% O₂, 94% N₂; BOC) for 6 hours, prior to RNA and protein extraction. The control samples were left at room temperature during irradiation of the samples.

**RNA extraction and cDNA synthesis.** Total RNA was extracted from the normoxia- and hypoxia-treated samples using Tri reagent, according to the manufacturer’s protocol (Sigma Aldrich).
Figure 2. Immunoblot analysis of CD105 protein expression in the murine (upper panel A and B) and human (lower panel A and B) endothelial cell clones: β-actin was employed for loading control. Densitometry, showing the ratios of murine or human CD105 protein expression are illustrated graphically in top and bottom right-hand panels. Throughout mean ± s.e. of three independent experiments. ** denotes the significant value p<0.001. The results show a significant reduction (p<0.001) in the amount of both murine and human CD105 proteins in the AS7 antisense cell line, when compared with the controls.

Figure 3. CD105 RNA expression in the murine (upper panels A and B) and human (lower panels A and B) endothelial cell clones: CD105 mRNA expression using the Northern blot (left hand panel) and densitometer readings (right hand panel) reveal a significant reduction (p<0.001) in the amount of mRNA in the murine and human cell line AS35, when compared to controls (parental cells SE3/SE7 or mock). The nylon membrane was stripped and re-probed for GAPDH as a loading control.
Following RNA extraction, cDNA was synthesised by reverse transcription. The reverse transcription-PCR (RT-PCR) was carried out in a 25 µl total volume using 1x reaction buffer (Bioline), 2.5 µM dNTPs (Bioline), 1U RNase inhibitor, 2 µM random p(dN)6 primer (Roche) and 2 µg total RNA. The tubes were incubated on a thermocycler (Hybaid) at 60°C for 5 minutes then placed on ice and 2.5U MMLV reverse transcriptase (Bioline) was added. The reaction mixture was incubated at 42°C for 1 hour and the reaction was stopped by incubation at 80°C for 10 minutes.

TaqMan real-time quantitative PCR analysis. A quantitative method for analysis of p53 and Bcl-2 RNA level by real-time PCR was carried out according to the manufacturer’s specifications (Applied Biosystems). Briefly, oligonucleotide primers and detection probes (Applied Biosystems) were designed (Table I) and GAPDH was used as an internal control to estimate pro- and anti-apoptotic protein expression. Amplification was carried out with the TaqMan Universal PCR Master-Mix (Applied Biosystems). Template (200 ng) was mixed with 1 x TaqMan Universal PCR Master-Mix (Applied Biosystems), 250 nM TaqMan probe (Applied Biosystems) and 900 nM of each primer (Applied Biosystems). All reactions were performed in the Model 7700 Sequence Detector (Applied Biosystems). Reaction conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of the amplification step (95°C for 15 seconds and 60°C for 1 minute). For comparative analysis of gene expression, data were obtained by using the ΔΔCt method derived from a mathematical elaboration, which is documented in the Applied Biosystems User Manual. The 2(ΔΔCt) value was plotted on a log scale to represent the increase or decrease in relative gene expression in each sample.

Statistical analysis. All assays, unless specified otherwise, were performed at least 3 times and the raw data were analysed using the one-way ANOVA (analysis of variance) and Tukey HSD post-hoc statistical tests.

Results

Stable cell lines expressing sense, antisense CD105 mRNA and the empty vector. Initially, fourteen murine clones and seven human clones of each transfectant (sense, antisense and mock) were selected and screened for the expression of CD105 by FACS. After three rounds of screening over a period of five weeks, three murine and three human clones were selected, wherein the murine antisense clone AS7 and human antisense clone AS35 expressed the lowest low level of CD105. The murine (SE3) and human (SE7) sense clones and the two mock clones expressed CD105 to the same extent as their parental cell lines (MS1 and EA Hy926 respectively).

Figure 1A illustrates that murine CD105 expression in the antisense cells (AS7) was reduced by ~65% in comparison to the sense (SE3), mock and parental MS1 cells. To further confirm that total murine CD105 protein was reduced in the AS7 but not in the SE3 and mock cells, immunoblotting was carried out using extracts from these cells. Murine CD105 appeared as one major band with a molecular weight of 180 kDa under non-reducing conditions (Figure 2A). Quantifications of the blots on a densitometer indicated that CD105 expressed in AS7 cells was approximately 11% (p<0.001) of the other three cell types, demonstrating that total CD105 was significantly reduced in the AS7 cells. Likewise, human CD105 expression was significantly down-regulated (~87%) in the antisense cell line (AS35) in comparison with the sense (SE7), mock and parental EA Hy926 cells (Figure 1B). Immunoblotting and densitometry confirmed that total human CD105 protein was significantly reduced in the AS35 (99%; p<0.001) when compared with SE7, parental and mock cells (Figure 2B).

CD105 mRNA reduced in the antisense cell line. Northern blot analysis was carried out to compare the total CD105 RNA levels in the murine and human cell lines. A
Figure 5. Effect of TGF-β1 on murine endothelial cell migration using the oil drop technique: cell migration was quantified using the oil drop technique and the images were captured using a diavert inverted microscope (Leica) at a magnification of x100. The net migration of the murine endothelial cells in mm² (shown in the top chart) was calculated using: total surface area at 24 hours (+/- TGF-β1) – area occupied at 0 hours. (T0) illustrates net growth of the four cell lines. Addition of TGF-β1 significantly reduced (p<0.001) the migration rate in the antisense cell line (AS7).
significant decrease in the amount of CD105 mRNA in both the murine antisense AS7 cells (65%; \( p < 0.001 \), Figure 3A) and human antisense AS35 cells (98%; \( p < 0.001 \), Figure 3B) were evident in comparison with the parental, sense and mock-transfected cells (Figures 3A and 3B, respectively).

Inhibitory effects of TGF-\( \beta \) on cell proliferation were enhanced by a reduction in the level of CD105. The effect of TGF-\( \beta \) on cell proliferation was quantified using a \( [3H] \)-thymidine incorporation assay (Figure 4A). Addition of 10 ng/ml of TGF-\( \beta \) resulted in a significant decrease in proliferation (70%; \( p < 0.001 \)), demonstrating that TGF-\( \beta \) exhibited augmented inhibitory effects on cell growth in CD105-deficient murine AS7 cells over either parental MS1, mock or SE3 cell lines. The untreated human cell lines showed a higher rate of proliferation than their TGF-\( \beta \)-treated counterparts, while untreated human sense cells (SE7) revealed the highest rate of proliferation (Figure 4B). The CD105-deficient human AS35 cells, on the other hand, exhibited a significant decrease in proliferation rate (\( p < 0.001 \); Figure 4B).

TGF-\( \beta \) inhibited murine and human cell migration. Figures 5A and 6A depict the net area covered by the murine and human endothelial cells, respectively, using the following calculation: total surface area at 24 hours (\( \pm +/- \) TGF-\( \beta \)) – area occupied at 0 hours (T0) = net area (mm\(^2\)).

Parental murine MS1 and human EA Hy926 cells, at T0, formed distinct circular structures similar to other cell types at time zero (Figures 5A and 6A). In untreated murine (MS1, CD105-deficient AS7, SE3 and mock cells) (Figure 5B) and untreated human cells (EA Hy926, CD105-deficient AS35, SE7 and mock cells) (Figure 6B) migration had started to occur. In the absence of TGF-\( \beta \), the increase in the area of the cells was negligible in murine and human cell lines (Figures 7 and 8, respectively). Following 10 ng/ml TGF-\( \beta \) treatment, both murine (MS1, SE3 and mock) and human (EA Hy926, SE7 and mock) cells appeared confluent and showed clear signs of migration (Figures 5C and 6C). In contrast, the CD105-deficient murine AS7 and human AS35 cell lines treated with 10ng/ml TGF-\( \beta \) exhibited significantly lower (\( p < 0.001 \) in each case) migration rates than the other cell lines (Figures 5F and 6F, respectively). Quiescent cells, which were not migrating (as observed in Figures 5F and 6F), appear ‘rounded’ at the edges, mirroring the AS7 cells at T0.

The effects of CD105 on TGF-\( \beta \)-induced gene expression using the reporter construct p3TP-Lux. To determine whether CD105 affected TGF-\( \beta \)-induced gene expression, a luciferase assay was performed. The p3TP-Lux promoter construct was transiently transfected into the murine and human cell lines. This construct contains multiple copies of TGF-\( \beta \) response elements (16). Following transfection, cells were treated with 10 ng/ml TGF-\( \beta \) for 48 hours and p3TP-Lux-mediated luminescence was determined using a luminometer. Each determination was corrected for transfection efficiency by assaying for the luminescence from the cotransfected control construct, pSV-\( \beta \)-galactosidase. The addition of 10 ng/ml TGF-\( \beta \) induced a slight increase in the p3TP-Lux promoter activity in murine control MS1 (40%), SE3 (13%) and mock (40%) in contrast to a 65% increase in the CD105-deficient AS7 cells (\( p < 0.001 \), Figure 6A). Similar to murine results, the human CD105-deficient antisense cell line AS35 displayed a significant increase in p3TP-Lux promoter activity (60%; \( p < 0.001 \), Figure 6B) compared to controls.

Murine endothelial cell tube formation in the 3-dimensional collagen matrix. The murine cell lines started to elongate and connect head-to-tail in collagen gel within 30 hours of
Figure 7. Effect of human endothelial cell migration using the oil drop technique. As in Figure 5, the addition of TGF-β1 significantly reduced (p<0.001) the migration rate in the antisense cell line (AS35).
culture and capillary-like networks formed after 50 hours in culture. In the absence of TGF-β1, the length of the capillary-like structures in parental MS1 (60.67±8.13 μm), SE3 (64.06±7.21 μm) and mock cells (64.29±10.04 μm) was similar (Figure 8A). The CD105-deficient AS7 cells, on the other hand, exhibited shorter tubules (51.5±3.04 μm) when compared to the other three cell types, although this result was not statistically significant (Figure 8B). An increase in the length of the capillary tubules in the presence of 10 ng/ml TGF-β1 was observed in MS1 (87.88±12.95 μm), SE3 (96.19±11.32 μm) and mock cells (97.33±16.22 μm). In contrast, the same concentration of TGF-β1 significantly shortened the length of the tubes in CD105-deficient AS7 cells (39.05±3.61 μm, p=0.0085) and also stimulated breakdown of the networks (Figure 8A).

**Analysis of pro-apoptotic marker p53 in AS35 and mock cell lines.** In AS35 cells, the basal level of p53 gene expression was 3.5-fold higher than in mock controls cells (Figure 9). In contrast, following irradiation with 10 Gy, mock control cells displayed a 2-fold increase in p53 gene expression, whereas AS35 cells displayed a 1.3-fold decrease (Figure 9). Culturing
the mock control cells under hypoxia induced a 4-fold decrease in p53 gene expression; conversely, treatment with 10 Gy induced a 1.5-fold increase in gene expression (Figure 2A). Following hypoxia-treatment of AS35 cells, a 3-fold increase in p53 gene expression was observed, with a 5-fold increase following irradiation (Figure 9). Protein analysis of p53 also provided the same observations in all samples except for normoxia-cultured AS35 and irradiated AS35 samples. Pro-apoptotic p53 protein expression was up-regulated in every sample following irradiation (Figure 2B). This up-regulation was more pronounced in normoxia-cultured mock cells (when compared to normoxia-cultured AS35 cells) and hypoxia-cultured AS35 cells (when compared to normoxia-cultured AS35 and hypoxia-cultured mock cells).

A decrease in anti-apoptotic Bcl-2 gene and protein expression in AS35 cells following hypoxia and radiation treatment. Basal Bcl-2 gene expression in the AS35 cell line was 2.4-fold higher than in mock control cells (Figure 10A). Following 10 Gy irradiation, 4.7-fold and 18-fold increases in Bcl-2 gene expression were seen in AS35 and mock control cells, respectively (Figure 10A). Culturing both AS35 and mock cells under hypoxic conditions induced vast differences in Bcl-2 gene expression in the two cell lines. A 95-fold and 97-fold increase in Bcl-2 gene expression was seen in non-irradiated and irradiated mock cells, respectively, when compared with the untreated control (Figure 10A). In comparison, a 6-fold and 2-fold decrease in Bcl-2 gene expression was noted in non-irradiated and irradiated AS35 cells, respectively, when compared with the cells cultured in normoxic conditions (Figure 10A). Bcl-2 protein expression was analysed in the same samples and did not markedly differ in normoxia-cultured AS35 and mock cells, irrespective of irradiation treatment (Figure 10B). In contrast, hypoxia induced a decrease in Bcl-2 protein expression in mock and AS35 cells,
which was further enhanced following irradiation in mock cells (Figure 10B). The overall levels of Bcl-2 protein expression were lower in AS35 samples cultured under hypoxic conditions (Figure 10B). The discrepancies between mRNA and protein levels may be because samples were taken only 6 hours after irradiation, which may not be sufficient time for protein levels to increase or it may be that mRNA is not translated to protein.

Discussion

How CD105 modulates TGF-β signalling remains unclear, but our study supports accumulating data indicating that it exerts a suppressive action on some of the TGF-β1 functions. The aim of this study was to create two permanently transfected CD105 antisense expression cell lines, one murine and one human, to analyse the role of CD105 in TGF-β1 signalling.

Northern blot analysis of the two antisense cell lines AS7 (murine) and AS35 (human) revealed a significant decrease (p<0.001 in both cases) in the total CD105 mRNA levels (65% and 98%, respectively) when compared with the respective mock cell lines. The surface and total levels of CD105 protein, evaluated by flow cytometry and immunoblotting, were significantly decreased in the murine and human antisense (p<0.001 in both cases) when compared with the control cells (by 89% and 99%, respectively). This suggests that the antisense mRNA is specifically binding to and substantially preventing the translation of the target mRNA. The morphology of the murine and human cells in pre-confluent and confluent states was examined (data not shown). In a pre-confluent state, both murine and human cell lines showed similar characteristics. In contrast, once the cells reached confluence, the presence of multiple holes in the monolayers and disorganised cells were observed in both murine and human antisense cells, but in none of the control cell lines. These properties, which indicate fragility of the monolayer, are reminiscent of the characteristics of the vascular endothelium of CD105 null embryos. The CD105 null mice exhibit multiple vascular and cardiac defects leading to death at an early embryonic stage (4, 17, 18). From embryonic day 9, the primitive vascular plexus of the yolk sac fails to remodel into mature vessels, causing vascular channel dilation, rupture and haemorrhage. Mice expressing a single CD105 allele develop external signs of disease similar to human HHT1 (4). These severe vascular impairments observed in CD105 knockout and heterozygous mice suggest a critical role for CD105 in vascular development.

The proliferation of the murine and human cell lines was partially inhibited in response to 10 ng/ml TGF-β1 treatment, which is consistent with the published data (3). Li and colleagues used an antisense oligonucleotide to transiently inhibit CD105 protein expression in HUVEC cells and observed a 24% decrease in proliferation when compared to the untreated controls (3). Data from this study supports the published results, but demonstrate a higher inhibition of proliferation in the CD105-deficient murine and human cell lines (Figures 4A and 4B, respectively). These data suggest that CD105 protects the cell from the inhibitory actions of TGF-β1.

Endothelial cell migration is necessary for the formation of new blood vessels from pre-existing ones. Cell motility in response to TGF-β1 was measured using a wound healing system. Treatment with 10 ng/ml TGF-β1 induced inhibition of migration in both murine and human antisense cell lines (Figures 5, murine and 6, human). These data are consistent with a previous report showing that TGF-β1 caused a 32% decrease in migration in CD105-deficient HUVEC cells (3) and suggest that CD105 prevents the inhibitory effects of TGF-β1. Down-regulation of CD105 protein was also shown to interfere with VEGF signal transduction (19). Thus, the lack of migration seen in CD105-deficient antisense cells may involve VEGF, which promotes cell motility and stimulates wound healing (20).

CD105 appears to prevent TGF-β1 promoter activity, as demonstrated by increased p3TP-Lux in response to exogenous TGF-β1 in CD105-deficient human and murine cell lines compared to control cells.

It has been observed that endothelial cells form extensive networks of capillary-like structures, with lumen following 24-hour culture in a three-dimensional collagen gel system (15). TGF-β1 was shown to inhibit tube formation by bovine aortic endothelial cells and to induce apoptosis in vitro (21, 22). However, the presence of TGF-β1 in established vessels in vivo may initiate resolution by inducing endothelial cell quiescence and vessel maturation, therefore stabilising the vessel (3). In our studies, TGF-β1 was added to the MS1 cultures immediately after seeding the cells when networks had not formed. Whereas untreated MS1, AS7, SE3 and mock cells displayed an equal ability to form capillary-like structures, the addition of 10 ng/ml TGF-β1 caused an increase in the length of the capillary tubules in the control cell lines, suggesting stabilisation of the networks. However, TGF-β1 significantly shortened tube length in the CD105-deficient AS7 cells and stimulated breakdown of the networks, suggesting that it cannot act on endothelial cells with CD105 levels reduced below a certain threshold. The human endothelial cells were not used in the capillary formation assay, as they can only form capillary-like structures on Matrigel or in collagen saturated with phorbol myristate acetate (PMA) (23). This was not appropriate for the present study as Matrigel contains a high concentration of exogenous TGF-β1 (although not disclosed by the company).

Furthermore, culturing cells in PMA and collagen stimulates capillary formation without growth factors or cytokine stimulation, therefore the results could be misleading.

Culturing cells in low oxygen stimulates angiogenesis and apoptosis and it is the balance between these two processes
that determines cell fate. VEGF, p53 and other angiogenic factors are induced by oxygen deprivation to facilitate angiogenesis and, considering the pro-angiogenic function of CD105, an association between hypoxia and radiation-induced endothelial cell apoptosis and CD105 gene expression was investigated. Under normoxic conditions, irradiated mock control cells overexpressed p53 and Bcl-2, both mRNA and protein. Under normoxia, CD105-deficient AS35 cells also overexpressed Bcl-2 and p53, but this was further enhanced following irradiation. Ionising radiation up-regulates both Bcl-2 and p53, the latter by phosphorylation of serine-18 on p53 protein (24, 25, 26). Although ionising radiation induces apoptosis, the corresponding overexpression of anti-angiogenic Bcl-2 may lead to prevention of apoptosis. In such a case, the subsequent up-regulation in p53 is sufficient to activate protein kinase c-mediated p53 gene transcription and to induce G1-phase arrest and promote cellular repair mechanisms in mock cells (27, 28).

Under hypoxic conditions, as present in areas of solid tumours, a dramatic difference in progression of apoptosis was observed in CD105-deficient AS35 and CD105-expressing mock cells. CD105-expressing mock cells exhibited down-regulation of p53 and overexpression of Bcl-2 genes and proteins. Furthermore, Bcl-2 gene expression was substantially elevated in both non-irradiated and irradiated samples. Cell survival rather than cell death is more probably the net outcome due to the co-operation between Bcl-2 and p53-mediated mechanisms. It has been postulated that overexpression of Bcl-2 will regulate the intrinsic apoptotic pathway and prevent cell death by apoptosis. Moreover, a recent report has demonstrated that hypoxia induces up-regulation of CD105 via stimulation of a multiprotein complex (Sp1- Smad 3-HIF-1) in the CD105 promoter (29). The transcription factor p53 lies at the centre of a protein network that controls cell cycle progression and commitment to apoptosis. A novel pathway for hypoxia-induced p53 activation has been reported (30). This pathway involves MDM2 repression of p53 activity through the recruitment of the transcriptional co-repressor, human c-terminal binding protein 2 (hCtBP2). This interaction, and consequent repression of p53-dependent transcription, is relieved under hypoxic conditions. Hypoxia induces nicotinamide adenine dinucleotide (NADH)-stimulated conformational change to CtbP proteins, which results in a loss of their MDM2-binding ability. Moreover, it has been reported that HIF-1 alpha directly binds MDM2 both in vitro and in vivo and blocks MDM2-mediated nuclear export of p53 (31) and protects p53 against MDM2-induced degradation. This induces activation of p53-mediated transcription in cells. Overexpression of Bcl-2 confers cellular survival due to Bcl-2 exceeding and augmenting Bax-mediated apoptosis (32). The induction of the Bcl-2 promoter cyclic AMP response element, by hypoxia in neuronal cells, reduces the level of hypoxia-induced cell apoptosis (32). This induction of Bcl-2 could be due to up-regulation of CD105 by hypoxia (33), which influences progression of angiogenic responses in favour of apoptotic responses.

Hypoxia-cultured CD105-deficient AS35 cells revealed contrasting results when compared with corresponding mock cells. Overexpression of p53 in a treatment-dependent manner was coupled with down-regulation in the Bcl-2 gene and protein expression. Under hypoxic conditions, HIF-1α-mediated repression of hCtBP2 and dissociation of p53 from MDM2 could induce up-regulation of p53 (34). This mechanism also induces p53-mSin3A interaction, which prevents MDM2 transcription and the negative feedback loop to down-regulate p53 (35). Down-regulation of Bcl-2 ensues by p53-mediated up-regulation of Bax. Although hypoxia stimulates cyclic AMP response elements in the Bcl-2 promoter, the substantial increase in p53 augments Bcl-2 function, possibly by increasing the Bax:Bcl-2 ratio and inducing cytochrome c release. The overexpression of p53 and down-regulation of Bcl-2 could commit AS35 cells to apoptosis in hypoxia environments. Therefore, it could be suggested that, under hypoxic conditions, CD105 protects the cell against apoptosis.

The results presented here are consistent with published data. Both human and mouse models revealed identical roles, which are indicative of in vitro responses of CD105 repression of TGF-β1-mediated effects. Much remains to be understood about the functional role of CD105 and on the mechanism(s) regulating its interaction with TGF-β1. The availability of two permanently transfected CD105 cell lines should facilitate such studies.

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References
