TGFß induces transdifferentiation of iBREC to αSMA-expressing cells

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Abstract. Transforming growth factor ß (TGFß) both inhibits proliferation of macrovascular endothelial cells and promotes their transdifferentiation to α-smooth-muscle-actin (αSMA)-expressing mesenchymal cells *in vitro*. Recently, we have confirmed that proliferation of immortalized bovine retinal microvascular endothelial cells (iBREC) is strongly inhibited by TGFß2. We now demonstrate a complete transition of both parental iBREC and single cell-derived subclones from cobblestone morphology to a ragged appearance as a consequence of incubation for a few days with 10 ng/ml TGFß1 or TGFß2. Depending on the type of culture medium, 5-40% of these cells strongly expressed αSMA after approximately 6 days whereas expression of the endothelial cell-specific marker proteins von Willebrand factor and VE Cadherin (CD144) declined. Expression of αSMA, associated with formation of stress fibers, was first detected in single cells and then spread to adjacent cells, and declined slowly after prolonged cultivation in medium without TGFß2. However, re-constitution of vWF expression was not observed. TGFß2-induced phenotypic alterations were specific, as they were not caused by treatment of iBREC with VEGF, IGF-1 or bFGF. Induction of αSMA expression but not effects on morphology was strongly inhibited by bFGF, whereas IGF-1 enhanced TGFß2-induced αSMA expression. These findings may have an important impact on the understanding of development of microvascular complications of diabetes such as diabetic retinopathy.

Introduction

Transforming growth factor ß (TGFß) is an important regulator of various cellular processes in endothelial, epithelial and other cells including proliferation, apoptosis and transdifferentiation in embryonic lineages and into mature cells. As a consequence of binding of TGFß, specific type I and type II receptor serine/threonine kinases associate on the cell surface resulting in activation of SMAD proteins and their subsequent translocation to the nucleus followed by activation of cell type-specific transcription (1-3). Recently, an ubiquitously expressed TGFß receptor has been discovered which is involved in growth inhibition in a SMAD-independent manner (4,5). TGFß2 is also crucially involved in transdifferentiation of epithelial cells to mesenchymal cells which occurs during embryogenesis but is also thought to be an important step in the genesis of various diseases.

Of the three highly homologous isoforms TGFß1, 2 and 3, which are all expressed in different regions of the eye (6,7), TGFß2 was found to be the predominant isoform in the vitreous, neural retina and retinal pigment epithelium of the monkey eye (8). TGFß2 inhibits proliferation of retinal endothelial cells (9,10) and pericytes (11), and it also seems to be involved in fibrosis and transdifferentiation of lens epithelial cells into mesenchymal cells, as indicated by induction of αSMA (12). Similar TGFß-induced transdifferentiation into αSMA-expressing mesenchymal vascular cells was demonstrated for mature macrovascular endothelial cells (13-15). However, it has not been investigated if TGFß can affect the differentiation state of microvascular endothelial cells. In this study, we used well-characterized immortalized microvascular endothelial cells derived from bovine retina [iBREC; (10)] as a model for endothelial-mesenchymal transdifferentiation in the microvasculature. Indicative of transdifferentiation, these cells lose endothelial marker proteins and start to express αSMA during incubation with TGFß2. We also showed that this process is co-regulated by growth factors which are aberrantly expressed in diabetic retinopathy (DR), the major cause of blindness in industrial countries. Therefore, our results are an important contribution to the further revelation of pathogenic mechanisms leading to DR, in which the early loss of αSMA-expressing pericytes is frequently observed (16).

Materials and methods

Materials. Mouse monoclonal antibodies (Mabs) binding to the human cell type-specific proteins desmin (clone D33) or α-smooth muscle actin (clone 1A4), and rabbit polyclonal antibodies specific for human von Willebrand factor (vWF) were purchased from Dako Cytomation (Hamburg, Germany). Rabbit polyclonal antibodies specific for human VE Cadherin

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(CD144, VECad) were obtained from Acris (Hiddenhausen, Gemany). Recombinant human growth factors (rhVEGF$_{121}$, rhbFGF, rhIGF-1, rhPDGF-AA, rhPDGF-BB and rhTGFß1 or rhTGFß2) were from R&D Systems (Wiesbaden, Germany). Laboratory reagents were generally purchased from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany) with the few exceptions indicated when relevant. Complete endothelial microvascular growth medium (ECGM) and basal endothelial microvascular growth medium (ECBM) based on MCDB131 (17) were from Promocell (Heidelberg, Germany). ECGM was supplemented with premixed additives resulting in final concentrations of 0.4% ECGS/H, 5% fetal calf serum, 10 ng/ml epidermal growth factor, and 1 µg/ml hydrocortison. ECBM was used with 1 µg/ml hydrocortison and 1% FCS. DME media supplemented with either 4.5 g/l (DMEM-nG) or 1 g/l (DMEM-lG) glucose and 1% FCS were purchased from Invitrogen (Karlsruhe, Germany). Antibiotics were not added to media used for cultivation of iBREC prior to experiments.

Cell culture. Immortalized microvascular endothelial cells from bovine retina (iBREC) were established by transfection of primary retinal endothelial cells with plasmid pCI-neo hTERT encoding the catalytic subunit of human telomerase reverse transcriptase under control of a CMV promotor/enhancer (10). iBREC were maintained in ECGM in 25-cm^2 flasks coated with human fibronectin (10 µg/ml; BD Biosciences, Heidelberg, Germany) in a humidified atmosphere containing 5% CO_2 at 37˚C with passaging every 3 days. To ensure expression of hTERT during cultivation of iBREC, all media were supplemented with 300 µg/ml geneticin.

Subcloning of iBREC. iBREC were subcloned by limiting dilution to 0.6 cells/well and re-cultivated in a 96-well microtiter plate in ECGM with 300 µg/ml geneticin. Single-cell clones that reached confluency were characterized by analyses of expression of vWF and αSMA.

Induction of transdifferentiation with TGFß2. iBREC were cultivated at a density of ~3x10^3 cells/cm^2 overnight on two-chamber slides (BD Biosciences or Nunc, Roskilde, Denmark) coated with fibronectin. Medium was then changed to either ECBM, DMEM-nG or DMEM-lG supplemented with 10 ng/ml TGFß1 or TGFß2. Under these conditions, cells were incubated for up to 10 days with renewal of supplemented media every three days. To investigate the effects of potential co-regulators on the induction of αSMA expression, iBREC were incubated with TGFß2 as described above in the presence of growth factors (10 ng/ml bFGF, VEGF, IGF-1, or PDGF-AA/PDGF-BB), hydrocortisone (1 µg/ml) or $MgCl_2$ (9.2 mM). Alternatively, cells which had been treated with 10 ng/ml TGFß2 for 6 days were further incubated with 50 ng/ml bFGF or 10 ng/ml TGFß2. In additional experiments, cells were cultivated with 10 ng/ml TGFß2 for 2 weeks and then for 7 days in either ECBM or DMEM-lG without or with only 5 ng/ml TGFß2. Cells were fixed at different time points after addition of growth factors, and expression of αSMA, vWF and VECad was assessed by immunohistochemical staining. Adhesion of cells in long-term experiments was better

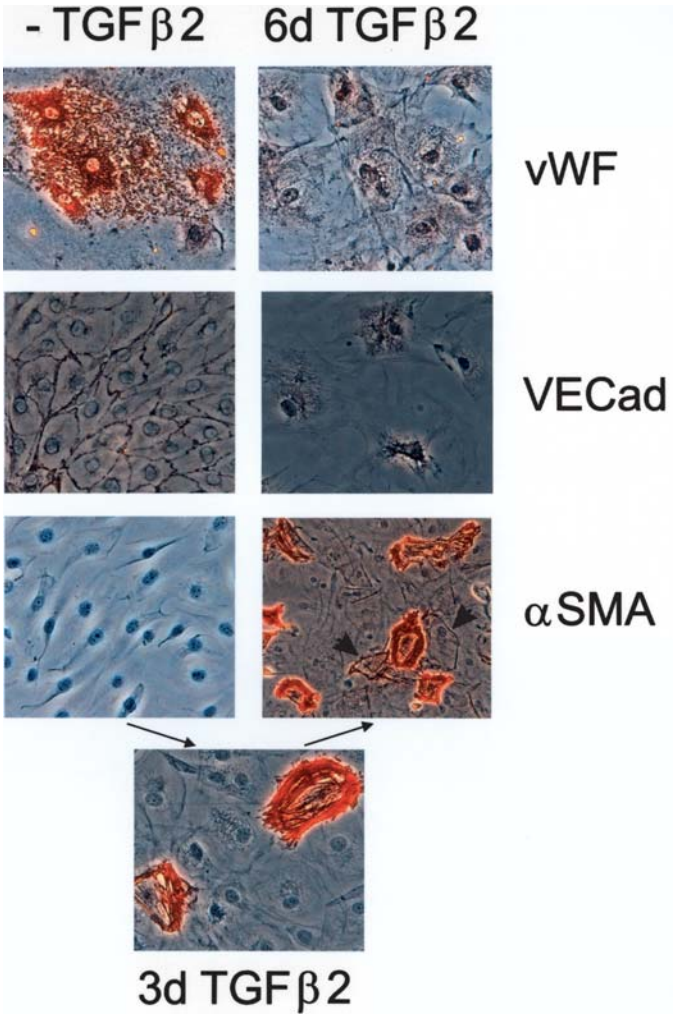

Figure 1. Expression of endothelial cell-specific markers vWF, VECad and the SMC-specific marker αSMA in iBREC before and after incubation with TGFß2. iBREC were maintained in culture medium (left column) or were additionally treated with 10 ng/ml TGFß2 for 6 days (right column). Cells were fixed and immunostained with specific antisera or a monoclonal antibody as indicated. Untreated iBREC strongly expressed endothelial marker proteins vWF and VECad and were negative for αSMA. Expression of vWF and VECad was lost during treatment of iBREC with TGFß2 whereas αSMA expression was induced. Expression of αSMA in TGFß2-induced transdifferentiated iBREC was first seen after 3 days in isolated spots and was associated with stress fibers. αSMA-expressing cells appeared in clusters after prolonged exposure to TGFß2 and its expression was induced in adjacent cells (arrows).

in the presence of 5-10 mM Mg^{2+}, but at these concentrations induction of αSMA was slightly affected.

Proliferation assay. Incorporation of [^{3}H]-thymidine into proliferating iBREC in the presence of growth factors was measured essentially as described previously (10). Results were presented as mean ± standard deviation. The Mann-Whitney U test was used to analyze experimental data and resulting p-values <0.05 were considered indicative of significant differences.

Immunocytochemistry. Immortalized BREC and transdifferentiated cells cultivated on two-chamber slides were fixed in methanol/acetone at -20˚C and incubated with prediluted

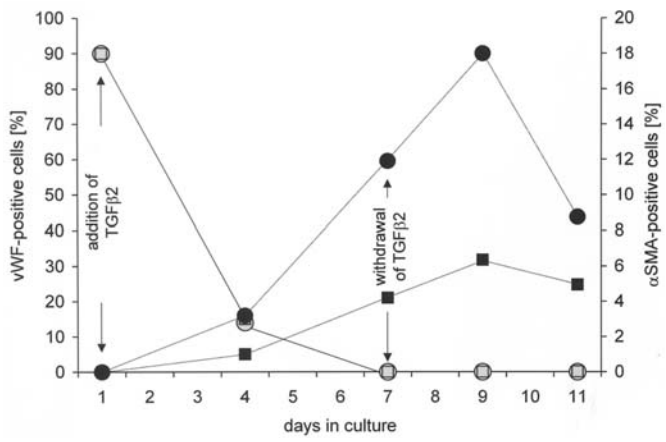

Figure 2. TGFß2-induced transdifferentiation during cultivation in different media. iBREC were cultivated for 6 days with 10 ng/ml TGFß2 in either ECBM (squares) or DMEM (4.5 g/l glucose; circles) and subsequently in TGFß2-free medium for 4 days. The numbers of vWF-positive cells (grey symbols) and αSMA-positive cells (black symbols) were counted after immunocytochemical staining. The fraction of αSMA-expressing cells was higher in DMEM than in ECBM. αSMA expression increased even after removal of TGFß2 to a maximum and then declined, whereas re-induction of vWF expression was not observed.

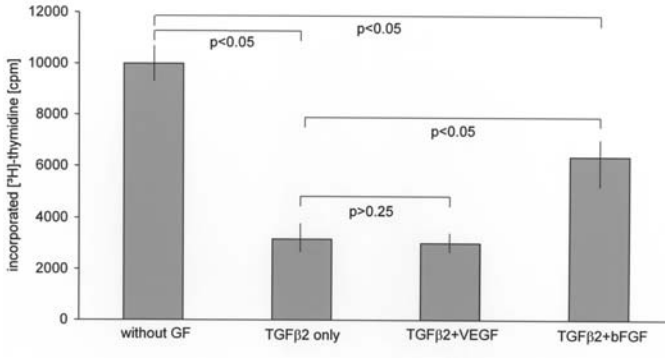

Figure 3. bFGF but not VEGF counteracts inhibition of proliferation of iBREC by TGFß2. iBREC were cultivated for 24 h in serum-deprived medium and then incubated in medium supplemented with 10 ng/ml of growth factors in different combinations for 48 h. [^{3}H]-Thymidine was added for the last 24 h and incorporated radioactivity was determined by scintillation counting. The Mann-Whitney U test was used to confirm significance of observed differences.

primary antibodies specific for vWF, cytokeratin, desmin, αSMA or VECad (10 ng/ml), respectively. Binding of these antibodies was detected with the Universal LSAB2 kit (Dako Cytomation) according to the manufacturer's protocol. Immunostained cells were counted and expressed as a fraction of the total number of cells per chamber. Each experiment was repeated at least 3 times and results of a typical experiment are shown.

Results

Transdifferentiation of iBREC induced by TGFß2. iBREC are a homogenous cell line of immortalized microvascular endothelial cells derived from bovine retina expressing endothelial markers vWF and VECad but not αSMA (10; Fig. 1). To induce transdifferentiation, iBREC were treated with 1-10 ng/ml TGFß2 for several days and changes in morphology or expression of vWF, VECad and αSMA were analyzed. Within a few days after addition of TGFß2, iBREC began to loose their cobblestone morphology and to develop thin spike-like extensions of the plasma membrane. After continued exposure for 1 week, endothelial cells were not detected any longer and all cells showed a ragged morphology (Fig. 1). Morphological changes were also accompanied with loss of cell-cell contact which also correlated with the loss of expression of the cell surface adhesion molecule VECad (Fig. 1). Immunocytochemical staining showed that expresssion of vWF in the cells also rapidly declined within 4 days after treatment with TGFß2 and that the ragged cells had lost this endothelial marker protein completely (Fig. 1), indicating that these cells resemble a cell type phenotypically distinct from endothelial cells. First ragged cells expressing αSMA were detected after 4 days and αSMA expression of ≤40% of all ragged cells was observed after approximately 1 week of exposure to TGFß2 (Fig. 1). αSMA-expressing ragged cells were termed BRSMC, an acronym for bovine retinal smooth muscle actin-expressing cells. In the process of conversion of iBREC, loss of endothelial cell-specific vWF preceded the rise of αSMA expression. Untreated cells constantly expressed vWF during prolonged cultivation and induction of αSMA, even in single cells, was never observed (Fig. 2). iBREC converted by TGFß2 did not express desmin although this pericyte marker protein had been detected in primary pericytes isolated from bovine retina (unpublished data). Transition of iBREC into ragged cells and induction of αSMA by TGFß2 was similarly seen in single cell-derived subclones of iBREC. In addition, TGFß2 and TGFß1 appeared not to differ in their effect on cell morphology and expression of αSMA (data not shown). In order to confirm specificity of the TGFß-induced conversion, iBREC were incubated either in medium (DMEM/ECBM + 1% FCS) without TGFß2 or in the presence of other growth factors (10 ng/ml VEGF, bFGF or IGF-1). In accordance with a TGFß-specific effect, induction of αSMA expression or loss of cobblestone-like morphology were never observed under any of these conditions. Expression of αSMA was first seen synchronically in several isolated spots consisting of single cells (Fig. 1), underlining that these were derived from iBREC and were not due to proliferation of pericyte-like cells present in the culture or generated by other processes during prolonged cultivation. Induction of αSMA expression was generally accompanied by formation of stress fibers [Fig. 1; (18)]. After longer exposure to TGFß2, clusters of ragged cells had formed from which induction of αSMA spread to adjacent cells (Fig. 1). In accordance with antiproliferative and proapoptotic effects of TGFß2 (19), conversion of endothelial cells into αSMA-expressing cells occurred with a massive decline of the total number of cells (>70%, Fig. 3).

Although all iBREC lost their cobblestone morphology and converted into ragged cells in a TGFß2-treated culture, only a fraction (in its size dependent on the type of culture medium) of these cells eventually expressed αSMA (Fig. 2). Ragged cells (≤12%) expressed αSMA when cultivated in DMEM whereas only 5% were αSMA-positive in ECBM (Fig. 2, day 7, $p<0.03$). This might be due to the presence of a number of additives in ECBM which is optimized for cultivation of endothelial cells, e.g. hydrocortisone and a higher

Table I. Influence of growth factors on TGFß2-induced αSMA expression in transdifferentiated iBREC.[a]

	Relative numbers of αSMA-positive cells		Effect on αSMA expression	
	TGFß2	TGFß2 + GF		
bFGF	1.00±0.19	0.03±0.01	0.03x	(p<0.008)
IGF-1	1.00±0.034	2.34±0.22	2.3x	(p<0.0001)
PDGF-AA	1.00±0.082	1.39±0.19		(p>0.09)
PDGF-BB	1.00±0.25	1.30±0.28		(p>0.5)
$VEGF_{121}$	1.00±0.036	0.72±0.06	0.7x	(p<0.005)

[a]iBREC were incubated with 10 ng/ml TGFß2 in DMEM-lG containing additional 10 ng/ml bFGF, IGF-1, VEGF, PDGF-AA, or PDGF-BB for 6 days before expression of αSMA was detected by immunostaining. The numbers of αSMA-expressing cells were determined and normalized on basis of αSMA-positive cells in cultures treated with TGFß2 only. Statistical significance of differences were calculated with the Mann-Whitney U test.

Table II. Effect of bFGF on TGFß2-induced expression of αSMA in transdifferentiated iBREC.[a]

	6 d TGFß2	+4 d TGFß2	+4 d bFGF
Relative number of αSMA-positive cells	1.00±0.26	9.24±1.53	0.13±0.048
Effect on αSMA expression	-	9x (p<0.04)	0.13x (p<0.005)
Number of cells	4980±110	2980±710	12400±1540
Effect on cell number	-	None (p>0.2)	2.5x (p<0.04)

[a]iBREC were incubated with 10 ng/ml TGFß2 for six days before 50 ng/ml bFGF or 10 ng/ml TGFß2 were added for an additional 4 days. The numbers of αSMA-expressing cells were determined and normalized on basis of αSMA-positive cells in cultures treated with TGFß2 only for 6 days. Statistical significance of differences were calculated with the Mann-Whitney U test.

Mg^{2+}-concentration (17). Indeed, addition of 1 µg/ml hydrocortisone to DMEM-IG significantly lowered the number of αSMA-positive cells (2.2±1.9% versus 11.1±4.2%, p<0.03). The number of αSMA-expressing cells was also slightly higher in DMEM-lG containing 1 mg/ml glucose compared to DMEM-nG with 4.5 mg/ml glucose. Prolonged exposure of cells to TGFß2 did not lead to a higher number of αSMA-expressing cells but rather to an increased loss of cells, even if the concentration was lowered to 5 ng/ml.

Transdifferentiation of iBREC into BRSMC is not reversible. To investigate if TGFß-induced transdifferentiation into mesenchymal αSMA-expressing cells is reversible and marker proteins of endothelial cells are re-expressed when TGFß2 is removed, we incubated iBREC in 10 ng/ml TGFß2 for 6 days and then in TGFß2-deficient culture medium (Fig. 2). After 6 days all cells showed a ragged morphology and expression of αSMA which increased to a maximum of 18% of all cells in DMEM-nG at day 2 after withdrawal of TGFß2. During further cultivation without TGFß2, expression of αSMA decreased and finally dropped below the detection limit (Fig. 2). However, restoration of vWF expression in transdifferentiated iBREC was never found, even after prolonged maintenance in ECBM or ECGM, media optimized for endothelial cells (Fig. 2).

Influence of growth factors on expression of αSMA in transdifferentiated iBREC. A number of growth factors are known to modulate the action of TGFß through linked signaling pathways. We therefore studied the influence of those growth factors which are considered to play a major role in the development of diabetic retinopathy (bFGF, IGF-1, PDGF-AA, PDGF-BB and VEGF) on TGFß2-induced transdifferentiation of iBREC and expression of αSMA (Table I). None of the added growth factors inhibited the transition from cobblestone to ragged morphology of iBREC and down-regulation of vWF. Whereas PDGF-AA and PDGF-BB did not significantly influence αSMA expression, the addition of VEGF lead to a slightly but significantly lower expression. In contrast, TGFß2-induced αSMA expression was strongly (2.3x) stimulated in the presence of IGF-1. However, bFGF strongly inhibited the expression of αSMA, nearly all cells were negative for αSMA expression. When iBREC were transdifferentiated and αSMA expression was induced by TGFß2, addition of bFGF strongly reduced αSMA expression whereas that of TGFß2 increased it (Table II). The decrease of the total number of cells during TGFß2-induced transdifferentiation was similarly observed in the presence of IGF-1, VEGF, PDGF-AA and PDGF-BB, but bFGF was found to have a protective effect. In accordance with the assumption that the inhibition of proliferation by TGFß2 is at least partly compensated by bFGF, incorporation of [^{3}H]-thymidine in iBREC as a measure of their DNA synthesis was higher in the presence of 10 ng/ml of both TGFß2 and bFGF compared with cultures supplemented only with TGFß2 (Fig. 3).

In summary, we demonstrated that TGFß2 induced transdifferentiation of iBREC into αSMA-expressing cells possessing ragged morphology and that it strongly inhibited proliferation of iBREC. The effects of TGFß2 on proliferation and expression of αSMA but not on morphological alteration of iBREC were found to be attenuated by bFGF.

Discussion

Transdifferentiation of endothelial cells (EC) of the macrovasculature to αSMA-expressing mesenchymal cells (SMC) is considered an important process in normal development and mechanisms leading to diseases. *In vitro* studies revealed that such conversion of mature macrovascular endothelial cells can be induced by TGFß1 or by removal of the TGFß1 antagonist bFGF (13-15,20). The role of TGFß in transdifferentiation of microvascular endothelial cells has not been investigated previously, mainly because of the limited number of primary cells and their life-span *in vitro*. In this study, we used the

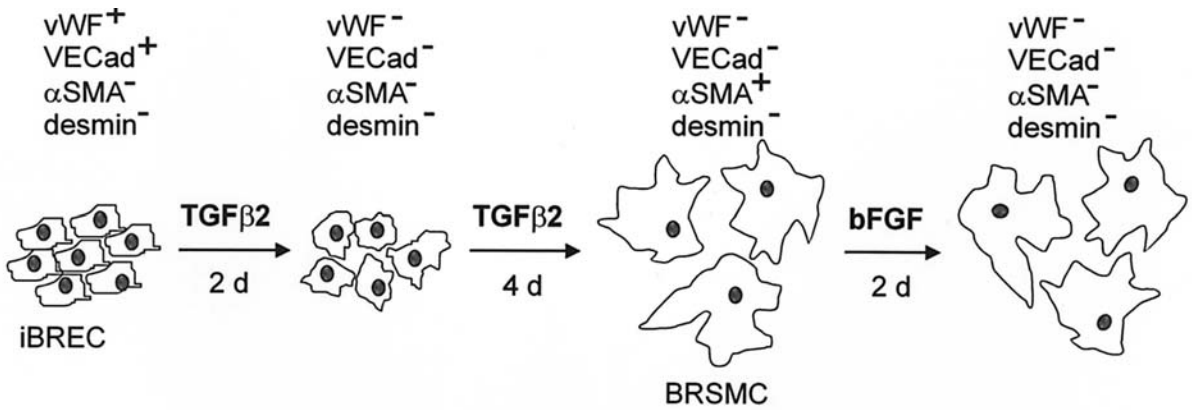

Figure 4. *In vitro*-transdifferentiation of iBREC induced by TGFß2. iBREC express EC-marker proteins vWF and VECad and their expression is lost after short incubation with TGFß2 whereas the expression of the SMC-marker protein αSMA is induced in a fraction of the transdifferentiated cells after 6 days (d). Induction of αSMA is accompanied by loss of cobblestone-morphology. In addition, bFGF reduces expression of αSMA indicating cross-talk between the two signaling pathways.

recently established cell line iBREC, consisting of immortalized microvascular endothelial cells derived from bovine retina (10), as a model for TGFß2-induced endothelial-mesenchymal transition in the microvasculature of the eye. As a clear consequence of treatment with TGFß, iBREC lost their cobblestone morphology as well as marker proteins of endothelial cells, and started to express αSMA (summarized in Fig. 4). This effect was TGFß-specific and not reversible during long-term cultivation or addition of EC-stimulating factors, which is in accordance with similar conversions macrovascular EC can undergo (14). In contrast to the behavior of aortic valve EC (14) and HUVEC (20), these differentiation processes were never observed in the absence of TGFß. Although all iBREC lost their cobblestone morphology after addition of TGFß, only a fraction of the resulting ragged cells expressed αSMA. These cells (BRSMC), however, did not express desmin which is considered a specific marker for pericytes, the major cell type of αSMA-expressing vascular cells in the microvessels of the adult retina (21). Our experiments point to an irreversible transdifferentiation induced by TGFß1/2 in iBREC. The observation that expression of αSMA was initially detected in several independent isolated cells and then spread to adjacent cells, and the fact that all results were confirmed with single cell-derived subclones of the parental iBREC, clearly exclude the possibility of proliferation of somehow persisting pericytes giving rise to a subpopulation of apparently transdifferentiated EC. Whether the ragged BRSMC derived from iBREC are able to differentiate into functional pericytes remains to be demonstrated. Further characterization of transdifferentiated iBREC will be facilitated by future development of tools for expression profiling and immunophenotyping of bovine cells.

The growth factors bFGF, VEGF, IGF-1 and TGFß1 have antagonistic functions in a broad range of cells including microvascular endothelial cells such as iBREC (10). VEGF and IGF-1 stimulated proliferation of iBREC but did not induce transdifferentiation and αSMA expression in iBREC or BRSMC. Only weak synergistic effects of VEGF and TGFß2 were observered, whereas combined treatment with IGF-1 and TGFß2 synergistically enhanced the induction of αSMA expression in BRSMC, indicating cross-talk between signaling pathways activated by these factors, which has been shown for other cell types (5). Cross-talk of bFGF and TGFß signaling has been studied in several αSMA-expressing cells. bFGF activates proliferation of retinal pericytes and inhibits their expression of αSMA. These effects are counteracted by TGFß1, most likely through induction of the transcriptional regulator myf-5 and nuclear translocation of SMAD2 and myf-5 (22). Up-regulation of differentiation markers for SMC such as αSMA, SM22α, or calponin by TGFß1 *in vitro* involves suppression of the serum response factor and this effect is ameliorated by bFGF (23). TGFß2-induced transdifferentiation of iBREC included up-regulation of αSMA and inhibition of proliferation. bFGF inhibited induction of αSMA by TGFß2 and suppressed TGFß2-induced αSMA expression in BRSMC, indicating that TGFß2- and bFGF-activated pathways are linked in iBREC and BRSMC. This invites the hypothesis that transcriptional regulation of αSMA expression in BRSMC and pericytes may be regulated by similar mechanisms. The observed modulation of the inhibitory effect of TGFß1/2 on proliferation by bFGF again confirmed that pathways triggered by other factors can interfere with TGFß1/2 signaling (4,22,23).

Pericytes control proliferation of microvascular EC *in vivo* by expressing TGFß2 and the loss of these cells seems to be an early event in the development of diabetic retinopathy, a major complication of diabetes mellitus and the main reason for blindness in the Western world (16). Changes in the gene expression pattern of pericytes induced by bFGF has been suggested to be an important mechanism of modulation of pericyte functions (22). We demonstrated here that EC originating from the retinal microvasculature can be transdifferentiated into SMC *in vitro*, indicating that these cells have the potential to differentiate to pericytes or pericyte-like cells. It should be interesting to further investigate the implication of this process *in vivo* in maturation of the microvasculature and development of diabetic retinopathy.

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