 DOI: 10.1002/masy.201100043

Enhanced Collagen Type IV Based Differentiation of Embryonic Stem Cells Towards Flk-1 Expressing Vascular Progenitors by the Wnt/β-Catenin Synergist QS11

Mark W. J. Poels,[*1] Lieke A. Golbach,[1] André A. Poot,[1] Jan Feijen,[2] Dirk. W. Grijpma,[1] Wiebe Kruijer[3]*

Summary: Mouse embryonic stem (mES) cells when plated onto collagen type IV in the presence of serum-containing medium differentiate into a mixed cell population that contains low levels of Flk-1 expressing vascular progenitor cells. When isolated and re-plated onto collagen type IV, Flk-1+ cells further differentiate into PECAM1+ endothelial and SMA+ mural cells. However, the low abundance and transient nature by which Flk-1+ cells are generated during embryonic stem (ES) cell differentiation presents limitations in case large numbers of Flk-1+ cells or its derivates are to be obtained. To optimize Flk-1 progenitor induction from undifferentiated mES cells, the effects of QS11, a small organic molecule that synergistically activates canonical Wnt signalling, were investigated. In the presence of QS11 the percentage of Flk-1+ cells in differentiating cultures of mES cells increases 1.5 fold. Furthermore, QS11 enhances cell proliferation in differentiating mES cells that results in a 2 fold increase in cell numbers when Flk-1 induction is maximal. The combined effects of QS11 on differentiation and proliferation increase the efficiency by which Flk-1 progenitors can be generated by approximately 300%, thereby providing a novel tool for vascular progenitor cell production for use in fundamental research and applications such as tissue engineering.

Keywords: collagen type IV; endothelial cell; Flk-1; mouse ES cells; mural cell; QS11; tissue engineering; vascular progenitor; Wnt/β-catenin pathway

Introduction

For the treatment of vascular disease, functional blood vessel prostheses with an inner diameter less than 5 mm are not available. Although synthetic vascular grafts made from Dacron or Teflon perform reasonably well in large-diameter applications, these grafts fail in small-diameter arterial reconstructions due to thrombus formation and intima hyperplasia.[1] Therefore, autologous vascular graft transplantation remains the method of choice for the latter type of reconstruction. Unfortunately, suitable arterial vascular grafts are often not available or are of low quality. In these situations, transplantation of a functional tissue-engineered graft represents an attractive alternative. In general, the strategy for generating vascular grafts by tissue engineering consists of seeding of suitable

[1] Biomaterials Science and Technology (BST), MIRA – Institute for Biomedical Technology and Technical Medicine, Faculty of Science and Technology, University of Twente; P.O. Box 217, 7500 AE Enschede, The Netherlands
E-mail: M.W.J.Poels@utwente.nl
[2] Polymer Chemistry and Biomaterials (PBM), MIRA – Institute for Biomedical Technology and Technical Medicine, Faculty of Science and Technology, University of Twente; P.O. Box 217, 7500 AE Enschede, The Netherlands
[3] Faculty of Science and Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

cells in a porous biodegradable tubular scaffold followed by culture of the construct in a bioreactor under physiological pulsatile flow and shear stress conditions that mimic blood pressure and blood flow.[2] Although production of autologous grafts using patient-derived cells is preferred, this choice is often hampered by limited availability or accessibility of appropriate cell type(s). The use of induced pluripotent stem (iPS) cells can overcome these limitations as these cells are patient-derived and can be mass propagated for subsequent differentiation into the desired cell types.[3] The use of iPS cells for vascular tissue engineering is feasible as both vascular endothelial and mural cells have been derived from both human and mouse ES cells and iPS cells.[4–5]

A well established method for generating vascular endothelial and mural cells from mES cells involves differentiation in monolayer on a collagen type IV substrate in serum-containing media.[6] Under these conditions, differentiating mES cells generate Flk-1+ vascular progenitor cells which upon isolation and re-plating on collagen type IV results in differentiation of a mixed population of vascular endothelial and mural cells. Moreover, when re-plated on collagen type IV in the presence of PDGF-BB or $VEGF_{165}$, Flk-1+ cells can be directed to differentiate into mural and endothelial cells, respectively, further underscoring the bi-potential differentiating capacity of Flk-1 progenitor cells.

Although Flk-1 progenitor cells have intrinsic potency for vascular tissue engineering, their prevalence within the heterogeneous differentiated cell population is low, limiting their use in tissue engineering approaches that require large cell numbers for the seeding of scaffolds. We therefore sought for methods that improve the yield of Flk-1 vascular progenitor cells from differentiating cultures of ES cells.

Here we report that QS11, a synergist of canonical Wnt signalling, increases the number of Flk-1 vascular progenitor cells in differentiating cultures of mES cells, thereby improving the first cell abundance limiting step in vascular tissue engineering.

Materials and Methods

Embryonic Stem cell Culture

G-Olig2 mES cells (American Type Culture Collection, ATTC) were cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) in Knock-Out DMEM (Gibco) with 15% Knock-Out Serum Replacement (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Gibco), 1000 U/ml Leukemia Inhibitory Factor (LIF) (Millipore), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Medium was replaced daily and mES cells were passaged every 2 days by trypsinization (0.25% trypsin, Gibco). CF-1 MEFs (Globalstem) were used as feeder cells to maintain pluripotent growth of mES cells. MEFs were expanded in DMEM (Gibco) containing L-glutamine, sodium pyruvate, 4.5 g/L glucose, 10% fetal bovine serum (FBS, Cambrex), 100 U/ml penicillin and 100 μg/ml streptomycin until passage 5, dissociated and mitotically inactivated by 25 Gray gamma irradiation. Subsequently, the cells were aliquoted and stored in liquid nitrogen until use. Cells were cultured at 37 °C in humidified air with 5% CO2.

Flow Cytometry and Immunofluorescence

For flow cytometry, cells were dissociated with TrypleLE Express (Gibco), washed and suspended in FACS medium consisting of MEM without phenol red (Gibco) with 10% Knock-Out Serum Replacement, 10 mg/ml bovine serum albumin (BSA, Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin. After dissociation, cells were stained against SSEA1 or Flk-1 by adding Alexa 488 conjugated anti-SSEA1 mAB (clone-MC-480, eBioscience) or APC conjugated anti-Flk-1 mAB (clone-AVAS12α1, BD Bioscience) to the cell suspension. To detect and compensate for any non-specific antibody binding, isotype

control experiments were done in parallel using Alexa 488 conjugated IgM (Santa Cruz) and APC conjugated IgG2a (eBioscience). Antibodies were incubated for 40 min on ice in the dark. After incubation, the cells were washed twice, re-suspended in cold FACS medium and kept on ice prior to fluorescence-activated cell sorting (FACS). Stained cells were analyzed and sorted with the FACSAria II cell sorter (BD Biosciences).

For Immunofluorescence staining of 2D mES cell cultures, cells were washed once with PBS and incubated for 10 min with FACS medium. Next, unfixed cells were incubated with Alexa 488 conjugated anti-SSEA1 mAB in FACS medium for 30 min in the dark at room temperature. After incubation, cells were washed twice, covered with PBS to prevent dehydration and subsequently analyzed under mercury light (excitation filter 470/40 nm, emission filter 520/35 nm).

In Vitro Differentiation

To induce differentiation towards Flk-1 expressing vascular progenitors, SSEA1-sorted mES cells were seeded (75.000 cells/well) on collagen type IV pre-coated 6-wells plates (BD BioCoat^{TM}) and maintained in differentiation medium consisting of α-MEM (Gibco) with 2.2 g/L sodium pyruvate (Sigma-Aldrich), 10% fetal bovine serum, 0.05 mM β-Mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. When indicated, differentiation medium was supplemented with 2.5 μM QS11 (Sigma-Aldrich) dissolved in DMSO or equal amounts of DMSO as control. Cells were differentiated for 3 to 6 days before Flk-1 expression was analyzed by flow cytometry. Total cell numbers were quantified using the Scepter automated cell counter system (PHCC00000, Millipore). To induce endothelial and mural cell differentiation, day 5 sorted Flk-1 expressing cells were re-cultured on new collagen type IV pre-coated 6-wells plates with standard differentiation medium for an additional 6 days. Medium was changed carefully every day and differentiation was performed at 37 °C in humidified air with 5% CO2.

Immunohistochemistry

Immunohistochemistry was performed on sorted Flk-1 expressing cells that were re-cultured for an additional 6 days on collagen type IV. Primary antibodies used were anti-PECAM1 mAb (clone-390, eBioscience) and anti-SMA mAb (clone-1A4, Acris). Secondary antibodies used were alkaline phosphatase-conjugated rabbit anti-rat IgG (Abcam) and horseradish peroxidase-conjugated goat anti-mouse IgG (Abcam). Cells were fixed in 10% neutral buffered formalin solution (Sigma-Aldrich) for 10 min at room temperature. After fixation, cells were rinsed with PBS and antigen retrieval was done by adding warm (70 °C) citrate buffer followed by incubation for 30 min in a pre-heated oven at 70 °C. After incubation, cells were slowly cooled to room temperature. When room temperature was achieved, cells were washed twice with PBS, and treated with 0.3% H_2O_2 in MeOH-PBS (1:1) for 30 min at room temperature. After two washes with PBS, the cells were treated with blocking buffer (2% BSA in PBS) for 30 min at room temperature. Subsequently, the cells were incubated with primary antibodies in PBS with 1% BSA and 0.5% Triton-X100 (Sigma-Aldrich) overnight at 4 °C. After washing three times for 5 min with TBS-Tween (0.05% Tween-20, Sigma-Aldrich) supplemented with 2 mM levamisole (DAKO), cells were incubated with secondary antibodies diluted in TBS-Tween for 75 min in the dark at room temperature. After incubation, cells were washed twice with TBS-Tween and visualized with diaminobenzidine (DAB, DAKO) and 5-bromo-4-chloro-3-indoxyl phosphate/Nitro Blue Tetrazolium Chloride (BCIP/NBT, DAKO) substrate following the manufacturer's instructions. After the staining procedure, cells were covered with PBS to prevent dehydration and analyzed by inverted light microscopy.

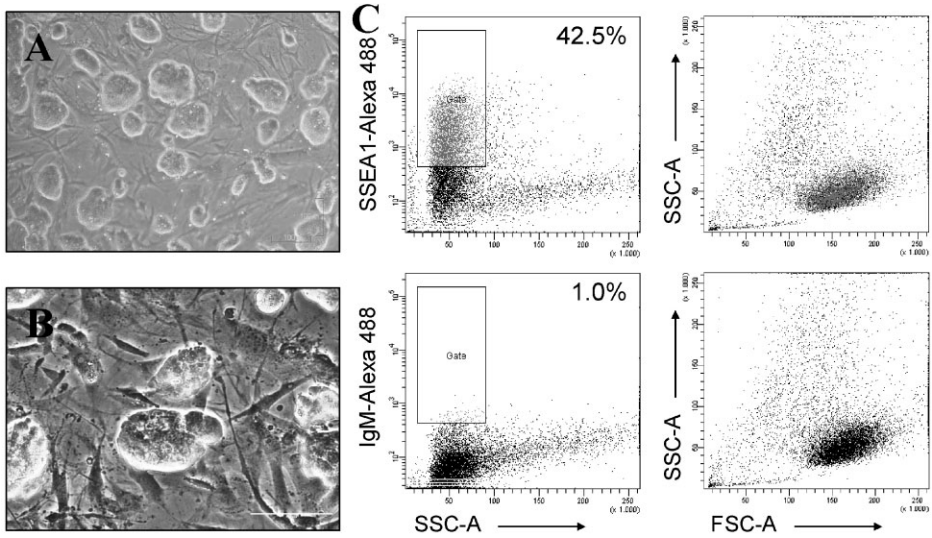

Figure 1.
Separation of mES cells from MEF feeder cells using SSEA1-based cell sorting. A, mES cells were maintained and expanded on gamma-irradiated MEF feeder cells. B, Immunofluorescent SSEA1 staining of mES cells. C, FACS dot plots of a typical cell sample stained with anti-SSEA1 antibody (top), or with a non-specific isotype control antibody (bottom). Left dot plots display the fluorescence intensity as function of the side scatter area (SSC-A). Right dot plots display the SSC-A as function of the forward scatter area (FSC-A). Outlined gated cells (top-right) resemble SSEA1 expressing mES cells, which were sorted for further differentiation.

Results

Isolation of Undifferentiated mES Cells from a Mixed Single Cell Population Containing Feeder Cells

mES cells were cultured on gamma-irradiated MEFs in the presence of LIF (Figure 1A). Under these conditions mES cells grew as tightly packed colonies that express SSEA1, a cell surface marker characteristic for undifferentiated ES cells (Figure 1B). Only mES cells expressed the SSEA1 surface marker, whereas MEF feeders were negative for this marker. To ensure a homogeneous starting cell population prior to differentiation into Flk-1+ progenitor cells, mES cells were separated from contaminating feeder cells by FACS. As shown in Figure 1C, approximately 42.5% of the cells could be sorted into a cell fraction expressing SSEA1. Depending on cell density, the percentage of the SSEA1+ mES cell fraction varied between experiments. The outlined gated cells (Figure 1C, top-left) represent the SSEA1 expressing mES cells, which were sorted and used for further differentiation studies. These results show that prior to differentiation, undifferentiated mES cells expressing SSEA1 can be separated efficiently from contaminating cells by cell sorting.

Generation of Flk-1+ Progenitor Cells from mES Cells

Flk-1 is one of the receptors for vascular endothelial growth factor (VEGF) and represents the earliest differentiation marker for endothelial cells.[7–8] To investigate Flk-1 induction during mES cell differentiation, the SSEA1+ sorted fraction, isolated as outlined in Figure 1C, was plated onto collagen IV pre-coated dishes in the absence of LIF. The differentiating mES cells changed morphology and reorganized into flat compact cell clusters. Optimal Flk-1 induction was observed at day 5 following plating on collagen type IV as determined

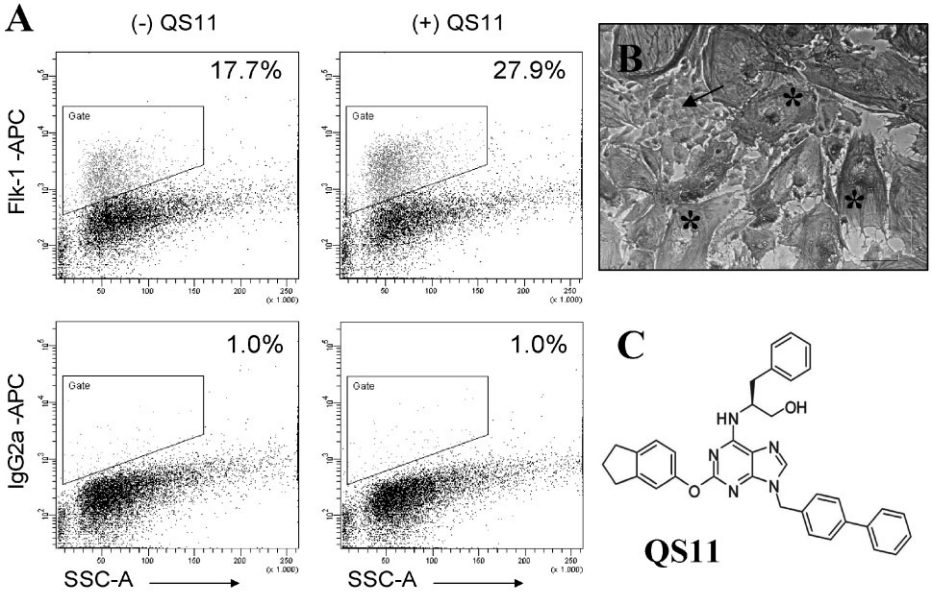

Figure 2.
Enhanced Flk-1+ vascular progenitor generation during mES cell differentiation by QS11. A, Flk-1 expression at day 5 in differentiating mES cells in the absence (left) or presence (right) of QS11 as determined by FACS analysis. FACS dot plot of a cell sample stained with an antibody specific to Flk-1 (top), or with a non-specific isotype control antibody (bottom). B, Immunohistochemistry of Flk-1 sorted cells re-cultured for an additional 6 days on collagen type IV. The sorted Flk-1 expressing cells gave rise to both PECAM1 (purple) and SMA (brown) expressing cells resembling endothelial (arrow) and mural (asterisks) cells. C, Chemical structure of the small molecule QS11.

by FACS analysis. As shown in Figure 2A, approximately 17% of the mES derived differentiated cells express the Flk-1 marker.

Previous studies have shown that mES cell-derived Flk-1+ cells have potential to differentiate into SMA or PECAM1 expressing cells, resembling mural and endothelial cells, respectively.[6] To ensure that induced Flk-1+ cells indeed resemble vascular progenitors, the Flk-1+ cells were sorted, re-plated and further differentiated on new collagen type IV plates in standard differentiation medium for another 6 days. Subsequently, expression of the mural marker (SMA) and the endothelial marker (PECAM1) was analyzed by immunohistochemistry (Figure 2B). Most of the cells expressed SMA and displayed a flat morphology. Some PECAM1 expressing cells consisting of packed sheets between the SMA+ cells were found. Endothelial cells were however present to a much lesser extent when compared to mural cells in line with previous observations.[6]

These results show that mES differentiation on collagen type IV generates Flk-1+ cells that can be isolated by FACS and after re-plating induced to differentiate into mural and endothelial cells.

The Small Molecule QS11, a Synergist of the Wnt/β-Catenin Signalling Pathway, Promotes Flk-1+ Vascular Progenitor Induction

Activation of canonical Wnt signalling has been shown to be required for generating Flk-1+ progenitor cells from undifferentiated ES cells.[9,10] QS11 modulates Wnt signalling by binding and inhibiting the GTPase activating protein of ARF-1 (ARF-GAP1).[11] Inhibition of ARF-GAP1 leads to increased levels of active ARF-1 which is associated with intracel-

lular protein transport, including β-catenin translocation. QS11 therefore only affects an already activated canonical Wnt signalling pathway. In line with the requirement for canonical Wnt signalling, we tested the effect of QS11 on Flk-1+ progenitor production during differentiation of mES cells. Sorted SSEA-1+ mES cells were plated onto collagen type IV pre-coated plates in differentiation medium supplemented with QS11 (Figure 2C). Next, Flk-1 induction was determined with FACS analysis after 5 days differentiation. Remarkably, when QS11 was added, the Flk-1 induction rate increased from 17% to approximately 27% (Figure 2A). Furthermore, no differences were observed between Flk-1 progenitors generated with or without QS11 treatment when differentiated into SMA+ mural cells and PECAM1+ vascular endothelial cells. This indicates that QS11 has no detrimental effect on further differentiation of generated Flk-1 progenitors towards their mural and endothelial cell derivates.

Time Dependent effects of QS11 on Flk-1 Expression and Cell Proliferation

To investigate whether the differentiation rate and hence optimal time of Flk-1 induction was changed by QS11, Flk-1 progenitor induction was determined at three, four, five and six days after ES cell plating (Figure 3A). Increased Flk-1 progenitor generation by QS11 was observed from day 4 till 6 when compared to control DMSO or untreated cells. Maximal Flk-1 induction was observed for all conditions at day 5 following induction of mES cell differentiation. At day 6, the amount of Flk-1+ progenitors rapidly declined, both in QS11 treated as well as DMSO control or untreated cells.

Since activated canonical Wnt signalling in general enhances cell proliferation, total cell amounts were quantified from day 3 till

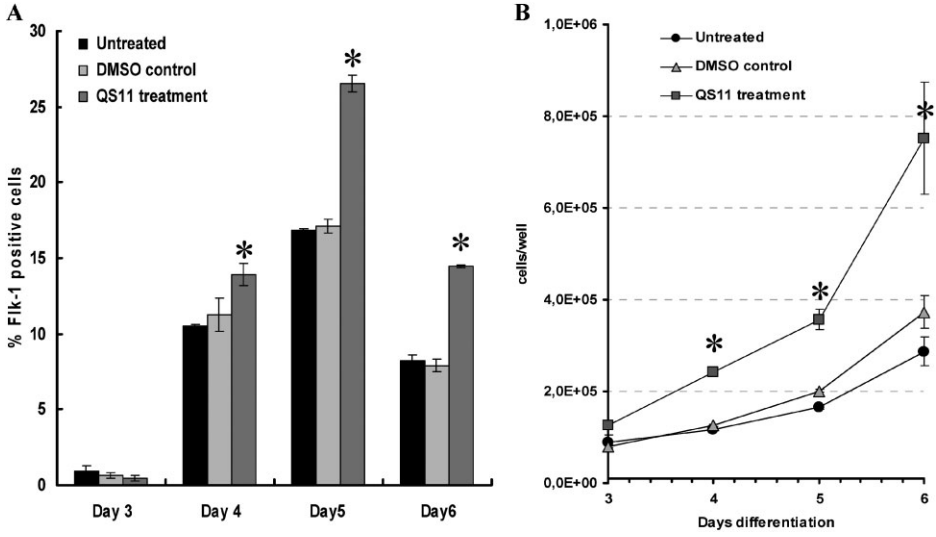

Figure 3.

Time dependent effects of QS11 on Flk-1 induction and cell proliferation. SSEA1-sorted mES cells were differentiated on collagen type IV in standard differentiation medium with or without QS11 or DMSO supplementation. A, Flk-1 expression was measured by flow cytometry after 3 to 6 days differentiation. (Relative Flk-1 expression was adjusted for non-specific antibody affinity by subtracting isotype values from specific Flk-1 antibody values.) B, Determination of cell proliferation during differentiation of mES cells on collagen type IV. Values in A and B represent the mean ± SD ($n = 2$) of a representative experiment out of 3 independent experiments. *Significant difference compared to untreated cultures ($p < 0.05$, two-tailed unpaired Student t-test).

day 6 after initiation of differentiation. As shown in Figure 3B, an increase in cell proliferation was observed in case of QS11 treatment as compared to the control treatments. At day 5, when the Flk-1 induction was optimal, QS11 treatment increased the total cell number 2 fold. Taken together, the amount of Flk-1+ progenitors that can be generated in a single experiment by QS11 treatment can be increased by a factor 3 due to a 1.5 fold increase in Flk-1 progenitors and a 2 fold increase in the total cell number. For further differentiation into mural and endothelial cells, the Flk-1+ progenitors should be harvested and replated on day 5 of QS11 treatment, since at later time points the percentage of Flk-1+ progenitors rapidly declines.

Discussion

Wnt proteins have been shown to activate self-renewal in mES cells as well as to be required for the formation of the primitive streak, the embryonic region containing Flk-1+ progenitors from which vascular endothelial and mural cells are derived.[12,13] The generation of Flk-1+ progenitors during mouse development can be mimicked *in vitro* by differentiation of ES cells using a well-established procedure that involves plating on type IV collagen in the presence of serum.[6] Since an efficient *in vitro* procedure of Flk-1+ progenitor generation is required for various experimental and/or tissue engineering applications, the potential enhancing effects of the small molecule QS11, a canonical Wnt synergist, was investigated. Our results demonstrate that the population of Flk-1 expressing cells was increased in the presence of QS11. The effect of QS11 treatment is first detectable four days after initiation of differentiation of mES on the collagen type IV and thereafter continues for an additional two days. At day 5, Flk-1 expression is maximal and at this time point approximately 27% of the cells are Flk-1 +, representing a 1.5 fold increase compared to untreated cells. In addition, QS11 also promotes cell proliferation that results in a 2 fold increase after 5 days of QS11 treatment. Therefore, the combined effects of QS11 on differentiation and cell proliferation results in a 3 fold overall increase in yield of Flk-1+ vascular progenitor cells.

Previously, Wnt/β-catenin based reporter studies have shown that QS11 synergizes with Wnt3a in enhancing the activation of the canonical Wnt/β-catenin signalling pathway.[11] QS11 only increases canonical Wnt signalling in cells in which the Wnt/β-catenin signalling pathway is already active. QS11 binds to and inactivates ARF-GAP, an activator of GTP hydrolyses of GTP-bound ARF-1. This leads to increased levels of active GTP-bound ARF-1, which in turn is linked to dissociation of membrane bound β-catenin, increased nuclear translocation of β-catenin and subsequent activation of its target genes.[11] In the absence of canonical Wnt signalling, Gsk3 will induce degradation of free cytoplasmic β-catenin and hence QS11 will have no effect. The QS11 promoting effect on Flk-1+ progenitor generation should therefore be correlated with increased nuclear location of β-catenin. Whether increased nuclear translocation of β-catenin is indeed observed during Flk-1 progenitor generation in mES cells awaits further investigation. In conclusion, the use of QS11 as described here represents an attractive tool for enhanced generation of Flk-1+ progenitor cells. Since production of Flk-1+ progenitor cells during differentiation of human ES or iPS cells is similarly controlled by Wnt/β-catenin signalling,[14] QS11 is a potentially useful tool to increase the efficiency of the production of human mural and endothelial cells for fundamental research and applications including tissue engineering.

Acknowledgements: We thank Christian Breukers and Joost Swennenhuis from the Medical Cell BioPhysics (MCBP) group of the University of Twente for assistance with the flow cytometry procedures and analyses. We also thank the department of Radiation Therapy of the MST hospital Enschede for assistance with the irradiation of the MEF feeder cells. This work was

financially supported by the Dutch Program for Tissue Engineering (DPTE, TGT.6732).

[1] P. Zilla, D. Bezuidenhout, P. Human, *Biomaterials.* **2007**, *28*, 5009.
[2] D. Pankajakshan, D. K. Agrawal, *Can J Physiol Pharmacol.* **2010**, *88*, 855.
[3] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, *Cell.* **2007**, *131*, 861.
[4] D. Taura, M. Sone, K. Homma, N. Oyamada, K. Takahashi, N. Tamura, S. Yamanaka, K. Nakao, *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 1100.
[5] H. Suzuki, R. Shibata, T. Kito, M. Ishii, P. Li, T. Yoshikai, N. Nishio, S. Ito, Y. Numaguchi, J. K. Yamashita, T. Murohara, K. Isobe, *BMC Cell Biol.* **2010**, *11*, 72.
[6] J. Yamashita, H. Itoh, M. Hirashima, M. Ogawa, S. Nishikawa, T. Yurugi, M. Naito, K. Nakao, S. Nishikawa, *Nature.* **2000**, *408*, 92.
[7] T. P. Yamaguchi, D. J. Dumont, R. A. Conlon, M. L. Breitman, J. Rossant, *Development.* **1993**, *118*, 489.
[8] A. Eichmann, C. Corbel, V. Nataf, P. Vaigot, C. Breant, N. M. Le Douarin, *Proc Natl. Acad. Sci. USA.* **1997**, *94*, 5141.
[9] H. Wang, P. C. Charles, Y. Wu, R. Ren, X. Pi, M. Moser, M. Barshishat-Kupper, J. S. Rubin, C. Perou, V. Bautch, C. Patterson, *Circ. Res.* **2006**, *98*, 1331.
[10] R. C. Lindsley, J. G. Gill, M. Kyba, T. L. Murphy, K. M. Murphy, *Development.* **2006**, *133*, 3787.
[11] Q. Zhang, M. B. Major, S. Takanashi, N. D. Camp, N. Nishiya, E. C. Peters, M. H. Ginsberg, X. Jian, P. A. Randazzo, P. G. Schultz, R. T. Moon, S. Ding, *Proc. Natl. Acad. Sci. USA.* **2007**, *104*, 7444.
[12] K. Ogawa, R. Nishinakamura, Y. Iwamatsu, D. Shimosato, H. Niwa, *Biochem Biophys. Res. Commun.* **2006**, *343*, 159.
[13] O. A. Mohamed, H. J. Clarke, D. Dufort, *Dev. Dyn.* **2004**, *231*, 416.
[14] P. S. Woll, J. K. Morris, M. S. Painschab, R. K. Marcus, A. D. Kohn, T. L. Biechele, R. T. Moon, D. S. Kaufman, *Blood.* **2007**, *111*, 122.

