

Smooth Muscle Cells Differentiated From Reprogrammed Embryonic Lung Fibroblasts Through DKK3 Signaling Are Potent for Tissue Engineering of Vascular Grafts

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Rationale: Smooth muscle cells (SMCs) are a key component of tissue-engineered vessels. However, the sources by which they can be isolated are limited.

Objective: We hypothesized that a large number of SMCs could be obtained by direct reprogramming of fibroblasts, that is, direct differentiation of specific cell lineages before the cells reaching the pluripotent state.

Methods and Results: We designed a combined protocol of reprogramming and differentiation of human neonatal lung fibroblasts. Four reprogramming factors (OCT4, SOX2, KLF4, and cMYC) were overexpressed in fibroblasts under reprogramming conditions for 4 days with cells defined as partially-induced pluripotent stem (PiPS) cells. PiPS cells did not form tumors *in vivo* after subcutaneous transplantation in severe combined immunodeficiency mice and differentiated into SMCs when seeded on collagen IV and maintained in differentiation media. PiPS-SMCs expressed a panel of SMC markers at mRNA and protein levels. Furthermore, the gene dickkopf 3 was found to be involved in the mechanism of PiPS-SMC differentiation. It was revealed that dickkopf 3 transcriptionally regulated SM22 by potentiation of Wnt signaling and interaction with Kremen1. Finally, PiPS-SMCs repopulated decellularized vessel grafts and ultimately gave rise to functional tissue-engineered vessels when combined with previously established PiPS-endothelial cells, leading to increased survival of severe combined immunodeficiency mice after transplantation of the vessel as a vascular graft.

Conclusions: We developed a protocol to generate SMCs from PiPS cells through a dickkopf 3 signaling pathway, useful for generating tissue-engineered vessels. These findings provide a new insight into the mechanisms of SMC differentiation with vast therapeutic potential. (*Circ Res.* 2013;112:1433-1443.)

Key Words: animal models ■ smooth muscle cells ■ stem cells ■ vascular progenitors ■ vascular tissue engineering

Vascular tissue engineering has the potential to provide biological substitutes for repair or replacement of damaged or blocked vessels in patients.¹ Recent reports demonstrated that several techniques using different sources of endothelial cells (ECs) and smooth muscle cells (SMCs) were developed to produce small diameter conduits.¹⁻⁴ However, the lack of high numbers of autologous vessels for complicated surgeries remains problematic.^{4,5} Because SMCs are a major component of vessels and play an important role throughout the vascular system, undeniably they would be expected to improve the functional characteristics of bioengineered vessels.⁶ SMCs have been isolated from adult arteries and used for tissue-engineered vessels. These SMCs divide a finite number

of times before undergoing growth arrest, a state known as senescence, attributable to progressive telomere erosion that proceeds with each cell division.^{7,8} Finding a new cell source that will provide large amounts of SMCs is important for the development of vascular tissue engineering as treatment for vascular diseases. We have previously demonstrated that embryonic stem cells can differentiate into SMCs via different signal pathways.⁹⁻¹³ However, the initial signals that stimulate the differentiation of embryonic stem cells *in vitro* and *in vivo* still remain unclear.

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Nonstandard Abbreviations and Acronyms

Ctl	control
DKK3	dickkopf 3
DM	differentiation medium
ECs	endothelial cells
iPS cells	induced pluripotent stem cells
PiPS cells	partially-induced pluripotent stem cells
SCID	severe combined immunodeficiency
SMCs	smooth muscle cells

Somatic cell reprogramming is a stochastic process during which extensive epigenetic modifications and chromatin remodeling takes place to accommodate the activation of pluripotent loci and the inactivation of lineage-specific genes.¹⁴ Studies with inducible vectors have shown that during reprogramming, intermediate populations that can ultimately give rise to induced pluripotent stem (iPS) cells are generated.^{15,16} This knowledge combined with the use of transcription factors for induction of pluripotency, led to the hypothesis that accordingly specific combinations of transcription factors and culture conditions could lead to reprogramming from one somatic cell fate to another without an intermediate pluripotent state. Consequently, partially-iPS (PiPS) cells were generated.¹⁷

Dickkopf 3 (DKK3) is the most divergent member of the dickkopf family, which seems to have a decisive function in myogenic cell fate, because it was recently found to be highly expressed in different skeletal muscle subtypes.¹⁸ Further evidence showing that DKK3 positively regulates the expression of *myf5*, a transcription factor regulating muscle differentiation, adds to its role in myogenesis.¹⁹ DKK3 could also have an intracellular function because it was recently shown to directly interact with β TrCP (negative regulator of β -catenin).²⁰ However, the involvement of DKK3 in SMC differentiation from stem/progenitor cells remains unknown. In the present study, the potential of PiPS cells to differentiate into functional SMCs was thoroughly investigated. We demonstrated a method that generates PiPS-SMCs via a DKK3 pathway and provided evidence that PiPS-derived SMCs can be used for the generation of functional tissue-engineered vessels, capable of mimicking native vessels in *in vivo* animal models.

Methods**Generation of PiPS Cells**

PiPS cells were generated as previously described.¹⁷ Briefly, human embryonic lung fibroblasts were nucleofected with a polycistronic plasmid containing all 4 factors (Oct4, Sox2, Klf4, and cMyc; Addgene Plasmid 20866: pCAG2LMKOSimO).²¹ They were then maintained in reprogramming media on gelatin for 4 days.¹⁷

Cell Differentiation

PiPS cells were seeded on collagen IV (5 μ g/mL) and maintained in differentiation media (DM). The cells were cultured in DM for 4 days after which they were harvested and further analyzed.

Generation of Tissue-Engineered Vessels and Artery Graft Procedure

PiPS cells or fibroblasts were seeded in decellularized vessels and differentiated to SMCs and ECs for the generation of double-seeded,

tissue-engineered vessels. These vessels were then grafted in the carotid artery of severe combined immunodeficiency (SCID) mice for 1 to 3 weeks.

Statistical Analysis

The data were analyzed using Graph Pad Prism Software. The data expressed as the means \pm SEM were analyzed with a 2-tailed student *t* test for 2 groups. A value of *P*<0.05 was considered significant. Detailed Methods are included in the Online Data Supplement.

Results**Characterization of PiPS Cells**

During somatic cell reprogramming extensive molecular changes occur, leading to the differential expression of several genes. Microarray analysis of samples at 4, 7, 14, and 21 days during fibroblast cell reprogramming revealed that the expression levels of a great number of genes were altered from as early as day 4 (Online Figure I and Online Table II). Therefore, PiPS cells were generated and characterized as previously shown.¹⁷ Human fibroblasts were nucleofected with a linearized plasmid encoding all 4 genes and maintained in reprogramming media for 4 days. Simultaneously, fibroblasts, nucleofected with an empty vector, were maintained in reprogramming media and used as controls (ctl). Although PiPS cells did not form colonies at this stage (Figure 1B), they displayed morphological differences when compared with ctl cells (Figure 1A). PiPS cells expressed the 4 genes at the mRNA level (Figure 1C) and expressed kinase insert domain receptor (vascular endothelial growth factor receptor 2), as revealed by fluorescence-activated cell sorting analysis (Figure 1D). Finally, PiPS cells were negative for alkaline phosphatase (Figure 1E–1G) and did not form teratomas *in vivo* 2 months after subcutaneous transplantation in SCID mice, as shown by hematoxylin/eosin staining (Figure 1H–1M), and immunofluorescence for markers specific for the 3 germ layers (Online Figure II). PiPS cells were labeled with Vybrant to demonstrate successful incorporation within the tissues (Online Figure IV).

PiPS Cells Can Differentiate Into SMCs

We have recently shown that PiPS cells can differentiate into functional ECs in response to vascular endothelial growth factor under specific culture conditions.¹⁷ Hence, in this study, the potential of PiPS cells to differentiate into vascular SMCs was assessed. PiPS cells were seeded on collagen IV and maintained in DM, based on a previously established SMC differentiation protocol.⁹ After 4 days of differentiation, PiPS cells exhibited a SMC-like morphology (Figure 2B) comparable to primary SMCs (Figure 2C) as opposed to the ctl population where cells maintained their fibroblast morphology (Figure 2A). Furthermore, they expressed SMC-specific markers in a time-dependent manner at the mRNA level (Figure 2D and 2E). More specifically, on day 4, the differentiated PiPS cells expressed a full panel of SMC-specific markers at the mRNA level (Figure 2F) and smooth muscle actin, calponin, and SM22 at the protein level (Figure 2G). Additionally, immunofluorescence for calponin and SM22 demonstrated a SMC-like morphology when compared with ctl cells (Figure 2H), with an efficiency of 42.5% for calponin and 38% for

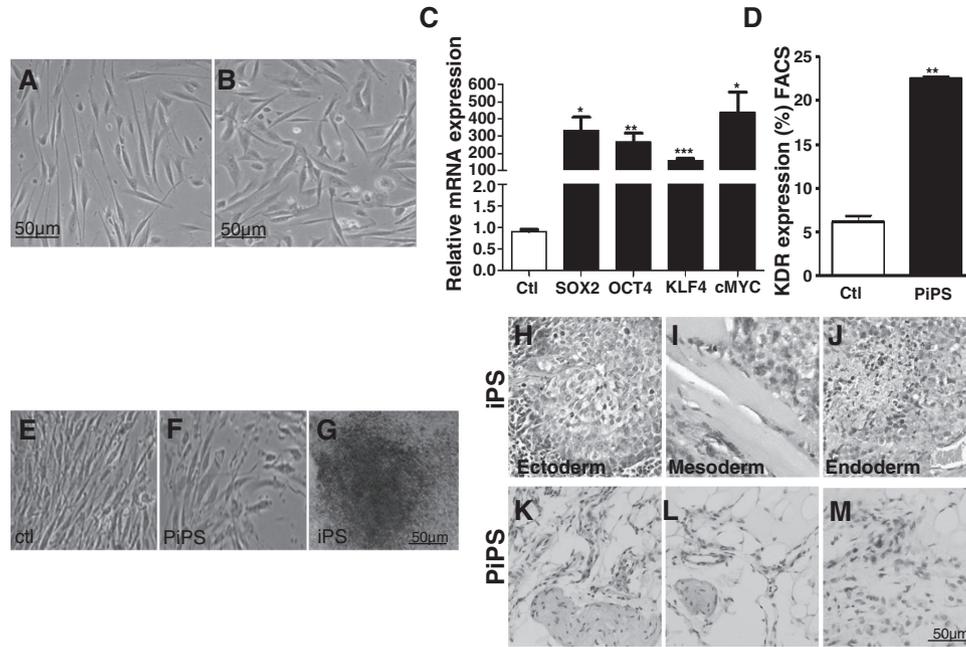


Figure 1. Characterization of partially-induced pluripotent stem (PiPS) cells. Human embryonic fibroblasts were nucleofected with pCAG2LMKOSimO (PiPS cells) or empty vectors (control [ctl] cells) and maintained in reprogramming conditions for 4 days as previously described. Morphological differences between ctl (A) and PiPS cells (B) are apparent after 4 days of reprogramming. PiPS cells express the 4 reprogramming factors SOX2, OCT4, KLF4, and cMYC at the mRNA level (C) as revealed by quantitative real-time polymerase chain reaction (mean±SEM of n=3; * P <0.05; ** P <0.01; *** P <0.001). Fluorescence-activated cell sorting (FACS) analysis revealed an increased expression of kinase insert domain receptor (KDR, vascular endothelial growth factor receptor 2) in PiPS cells when compared with the ctl cells (D; mean±SEM of n=3; ** P <0.01). PiPS cells were negative for the embryonic stem cell marker alkaline phosphatase (E–G) and did not proceed to teratoma formation after 2 months of in vivo transplantation in severe combined immunodeficiency mice as revealed by hematoxylin/eosin staining (K–M), contrary to the induced pluripotent stem (iPS) cells (H–J).

SM22 (Figure 2I). Moreover, PiPS-SMCs showed elevated levels of elastin and collagen 1A1, which are characteristic of large artery SMCs (Figure 2J). Interestingly, selection of PiPS cells with neomycin during reprogramming revealed a 100% efficiency of differentiation to SM22-positive cells, whereas the nonreprogrammed cells remained in a fibroblastic state (Online Figure V). Finally, fluorescence-activated cell sorting analysis for the proliferation marker Ki67 revealed an almost identical proliferation rate between PiPS-SMCs and human SMCs (Figure 2K), whereas stimulation of PiPS-SMCs with 60 mmol/L KCl caused them to contract as shown in Online Movie I in a way similar to human SMCs (Online Movie II). It should be noted that investigation of additional lineage markers revealed that this protocol is specific for SMC generation (Online Figure VI). Collectively, these results demonstrate that PiPS cells can differentiate to functional SMCs, which were defined as PiPS-SMCs.

DKK3 Is Involved in the Differentiation of PiPS Cells to SMCs

When elucidating the mechanism of PiPS-SMC differentiation, a member of the dickkopf family, DKK3, was identified from the microarray analysis to be correlated to the reprogramming process toward SMCs. DKK3 was upregulated in parallel with SMC markers during PiPS-SMC differentiation at both the mRNA (Figure 3A) and protein levels (Figure 3B). Furthermore, immunofluorescence confirmed the concomitant expression of DKK3 with SMC-specific markers, revealing a stronger perinuclear pattern of DKK3 in the differentiated PiPS-SMCs when compared with the ctl cells (Figure 3C and

3D). Additionally, DKK3 was transiently overexpressed in PiPS-SMC during differentiation. Upregulation of DKK3 led to further induction of SMC-specific markers at the protein level (Figure 3E and 3F). These results suggest that DKK3 is involved in PiPS-SMC differentiation. To address whether DKK3 is sufficient to drive PiPS-SMC differentiation, the aforementioned gene was suppressed via lentiviral delivery of short hairpin DKK3 during differentiation. SMC-specific markers were downregulated at the mRNA level (Figure 3G) concomitant to the downregulation of DKK3. Interestingly, further investigation of cardiovascular lineage markers after DKK3 overexpression or knockdown did not reveal any significant expression changes, suggesting that the effect of DKK3 is SMC-specific (Online Figure VIII). These results show that DKK3 is essential for PiPS-SMC differentiation.

Cytokine Role of DKK3 in PiPS-SMC Differentiation

DKK3 is a secreted glycoprotein expressed in a variety of tissues, and its role as a cytokine has previously been reported.²² However, its physiological role remains unclear. To investigate the role of DKK3 as a cytokine in PiPS-SMC differentiation, the supernatant of 4-day differentiated PiPS and ctl cells was collected. DKK3 ELISA measurements revealed that there was an increased release of DKK3 in the supernatant of PiPS-SMCs when compared with that of ctl cells (Figure 4A). To ascertain whether DKK3 has a cytokine effect in our system, a human recombinant DKK3 cytokine was used to stimulate PiPS-SMCs. PiPS cells were differentiated for 3 days and then stimulated with human recombinant DKK3 for

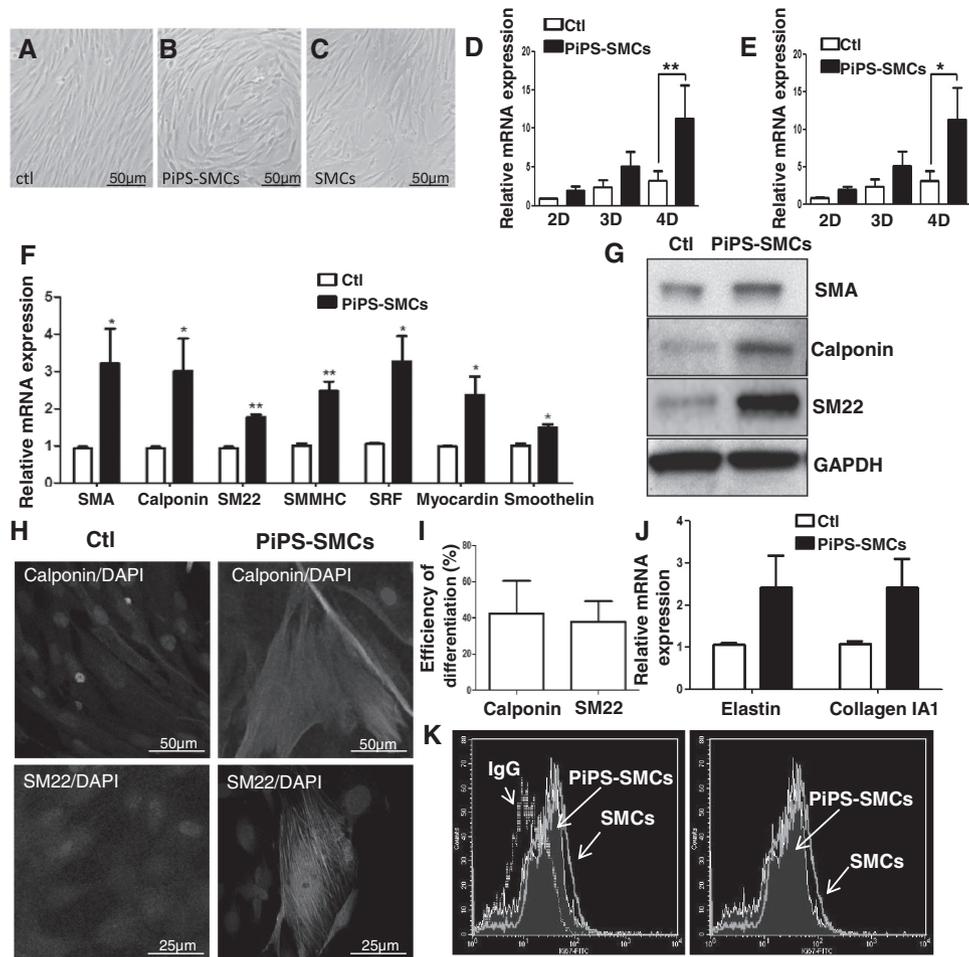


Figure 2. Partially-induced pluripotent stem (PiPS) cells can differentiate to smooth muscle cells (SMCs). PiPS cells or control (ctl) cells were seeded on collagen IV and maintained in differentiation media for 4 days. Contrary to ctl cells (A), PiPS-SMCs (B) have adopted a SMC-like morphology as comparable with native SMCs (C) and expressed SMC-specific markers, such as smooth muscle actin (SMA; D) and calponin (E), on a time-dependent manner (mean \pm SEM of n=3; * P <0.05; ** P <0.01). More specifically, on day 4, PiPS-SMCs expressed a full panel of SMC markers at the mRNA level (F) and the SMC markers, SMA, calponin, and SM22 at the protein level (G). Immunofluorescent staining showed a typical SMC staining for calponin and SM22 as revealed by confocal microscopy (H) and quantitative immunofluorescence revealed an efficiency of 42.5% for calponin and 38% for SM22 positive cells (I). Quantitative real-time polymerase chain reaction analysis for elastin and collagen I A1 revealed an upregulation in PiPS-SMCs when compared with the ctl cells (J; mean \pm SEM of n=3; * P <0.05; ** P <0.01). FACS analysis for the proliferation marker Ki67 revealed similar proliferation patterns between SMCs and PiPS-SMCs (K). SRF indicates serum response factor.

24 hours. Analysis of the samples revealed an upregulation of SMC specific marker expression at both the mRNA and protein levels between stimulated and unstimulated PiPS-SMCs, as shown by quantitative real-time polymerase chain reaction (Figure 4B) and Western blot (Figure 4C and 4D), respectively. These results suggest that DKK3 secretion plays an important role in PiPS-SMC differentiation.

DKK3 Regulates the Transcriptional Activation of SM22 Through Activation of Wnt Signaling

To clarify the molecular mechanism regulated by DKK3 during PiPS-SMCs differentiation, the effect of DKK3 in the transcriptional activation of SMC-specific markers was evaluated. Luciferase assays were performed, and the promoter activity of SM22 was detected. PiPS cells during SMC differentiation were cotransfected with a reporter gene pGL3-Luc-SM22 vector²³ and a plasmid encoding DKK3. The results revealed that there was an upregulation of the

SM22 promoter activity on DKK3 overexpression (Figure 5A), suggesting that DKK3 induced the activation of SM22 at the transcriptional level. To further confirm the effect of DKK3, the promoter activity of SM22 was also detected after knockdown by short hairpin DKK3, revealing a decrease in the transcriptional activation of SM22 (Figure 5B). These results confirmed the effect of DKK3 in the transcriptional regulation of SM22 during PiPS-SMC differentiation. It has previously been shown that β -catenin, the downstream mediator of Wnt signaling, directly regulates the transcription of SM22 by binding to its -213 to -192 upstream promoter region.²⁴ In our PiPS-SMCs differentiation model, an increased level of β -catenin expression was detected in PiPS-SMCs when compared with the ctl cells at both the mRNA (Figure 5C) and protein levels (Figure 5D and 5E). Furthermore, DKK3 as part of the dickkopf family of Wnt inhibitors has been implicated in activation or deactivation of Wnt signaling, although its role remains unclear.^{25,26} Therefore, to test whether DKK3 induced

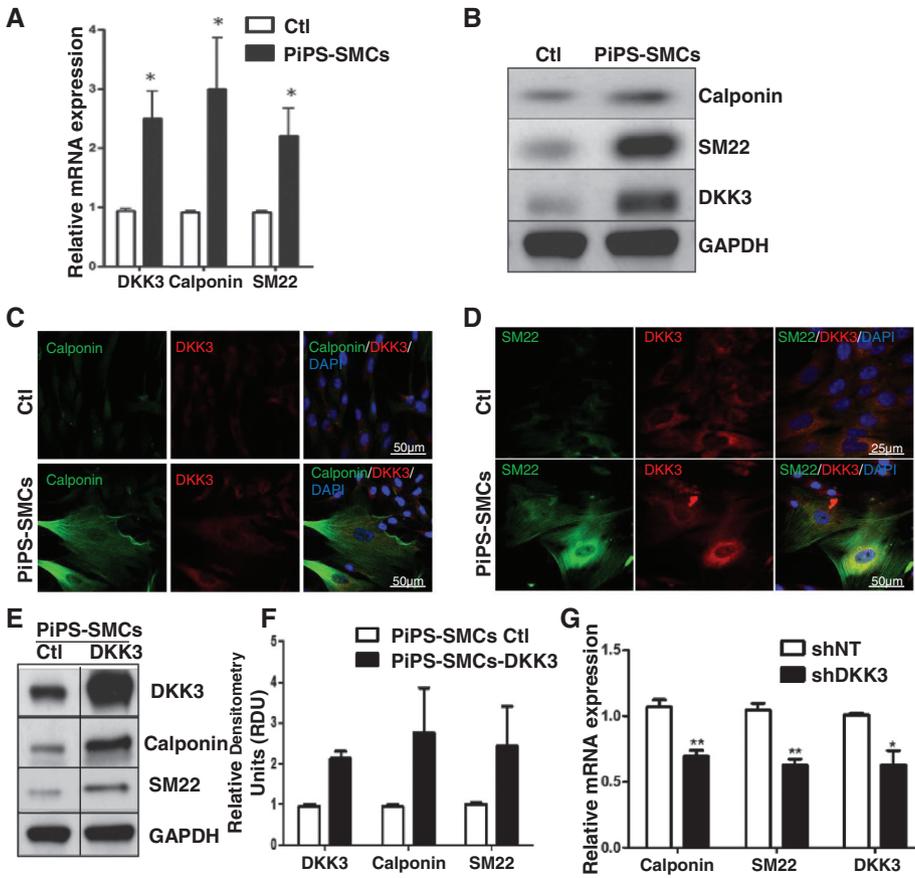


Figure 3. Dickkopf 3 (DKK3) is involved in partially-induced pluripotent stem-smooth muscle cell (PiPS-SMC) differentiation. DKK3 expression was upregulated in parallel with SMC-specific markers on day 4 of differentiation in PiPS-SMCs at both the mRNA and protein levels as revealed by quantitative real-time polymerase chain reaction (A; mean±SEM of n=3; *P<0.05) and Western blot analysis, (B) respectively. Immunofluorescent staining revealed a concomitant expression of DKK3 and the SMC markers, calponin and SM22, in PiPS-SMCs, with DKK3 appearing in a perinuclear pattern (C and D). DKK3 overexpression with a DKK3 plasmid or a pCMV5 empty vector in PiPS-SMCs at 2 days revealed a further induction of SMC markers at the protein level (E and F) when assessed on day 4. DKK3 was knocked down with shDKK3 in PiPS-SMCs at day 1 of differentiation, showing a suppression of SMC markers on day 4 at the mRNA level (G; mean±SEM of n=3; *P<0.05; **P<0.01).

PiPS cell differentiation to SMCs through its interaction with the Wnt signaling pathway, the activation status of Wnt was initially assessed. A TopFlash vector (transcription factor reporter plasmid) was used to measure the promoter activity of the Wnt signaling target genes on DKK3 overexpression

in differentiating PiPS cells. Detection of the luciferase activity revealed an upregulation in the reporter activity of TopFlash reporter genes during induction of DKK3 (Figure 5F). Investigation of the transcriptional patterns of the Wnt pathway targets β -catenin, transcription factor 1 and Axin2

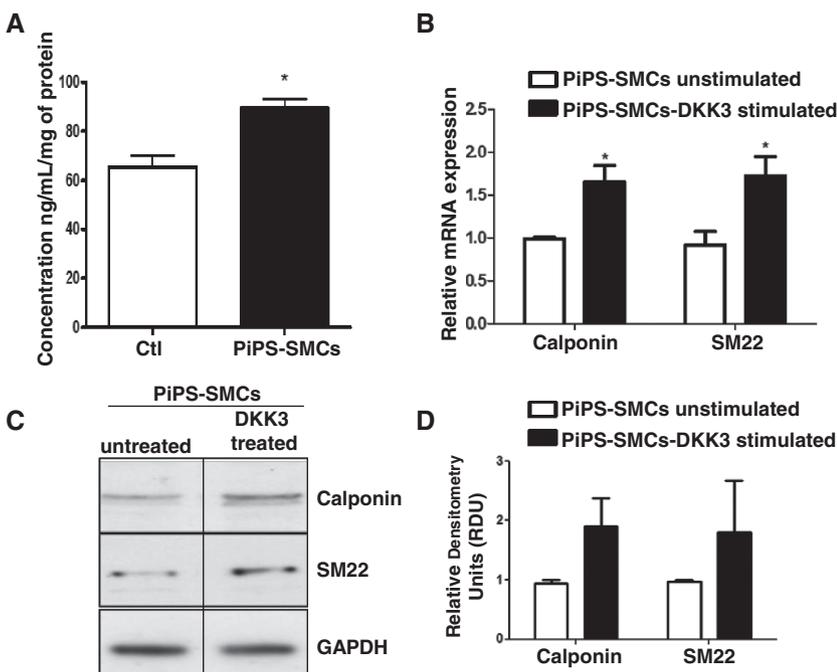


Figure 4. The cytokine role of dickkopf 3 (DKK3) in partially-induced pluripotent stem-smooth muscle cell (PiPS-SMC) differentiation. Supernatant from PiPS-SMCs and control (ctl) cells was collected at day 4 of differentiation and subjected to a DKK3 ELISA assay (A). It was revealed that there is an increased release of DKK3 in the supernatant of PiPS-SMCs when compared with the ctl cells (mean±SEM of n=3; *P<0.05). Stimulation of PiPS-SMCs with 50 ng/mL of human recombinant DKK3 cytokine in serum-free media for 24 hours revealed a further upregulation of SMC markers at the mRNA and protein levels as revealed by quantitative real-time polymerase chain reaction (B; mean±SEM of n=3; *P<0.05) and Western blot (C and D).

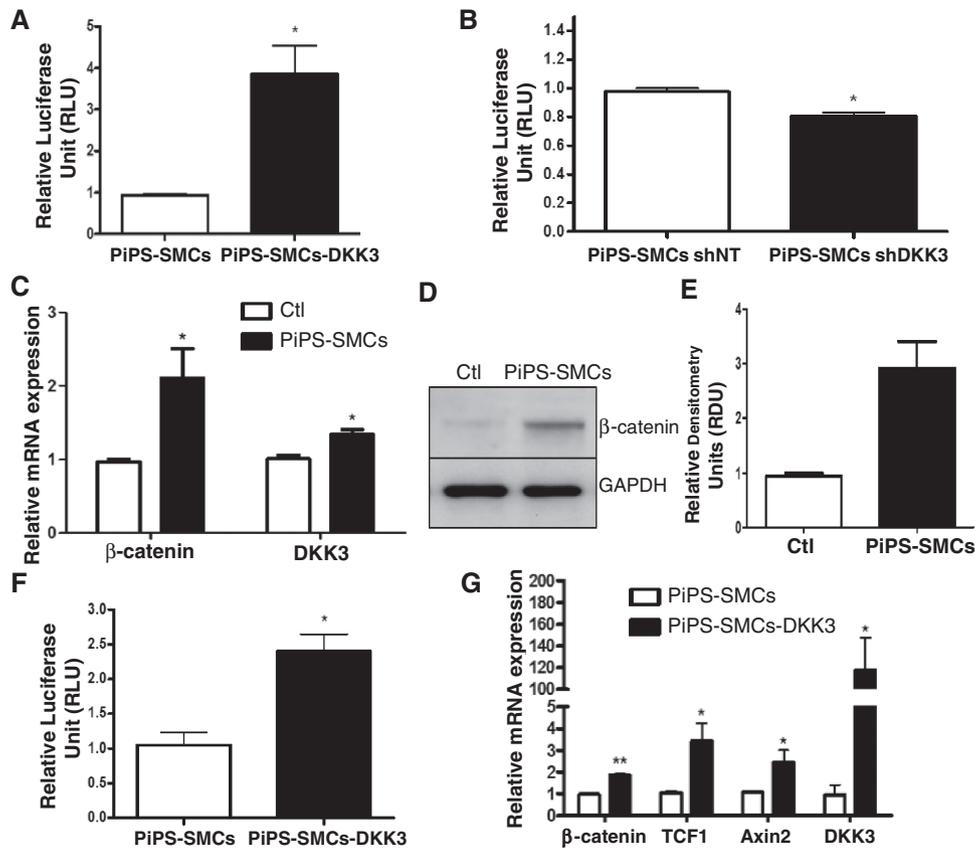


Figure 5. Dickkopf 3 (DKK3) regulates the transcriptional activation of SM22 through activation of Wnt signaling. Luciferase assays were performed in cells overexpressing DKK3 or an empty pCMV5 vector at day 4 of differentiation (A). It was revealed that there is an increased promoter activity for SM22 in the presence of DKK3 (mean±SEM of n=3; * P <0.05). Luciferase assays were also performed in cells, where DKK3 was silenced by shDKK3 at day 4 of partially-induced pluripotent stem-smooth muscle cell (PiPS-SMC) differentiation (B), where it was found that there is a decreased promoter activity for SM22. Quantitative real-time polymerase chain reaction (q-PCR) results (C; mean±SEM of n=3; * P <0.05) and Western blot analysis (D and E) show an increased expression of β -catenin in PiPS-SMCs when compared with the control (ctl) cells. Luciferase assays in cells overexpressing DKK3 or an empty pCMV5 vector at day 4 (F) revealed an increased promoter activity for TopFlash in the presence of DKK3, suggesting Wnt signaling activation (mean±SEM of n=3; * P <0.05). q-PCR revealed a further upregulation of β catenin after overexpression of DKK3 in PiPS-SMCs, as well as an increase of the Wnt signaling targets transcription factor (TCF) 1 and Axin2 at the mRNA level (G; mean±SEM of n=3; * P <0.05; ** P <0.01).

in PiPS-SMC-DKK3 cells revealed an upregulation in the expression of these targets (Figure 5G). Interestingly, further investigation into the expression of a number of Wnt elements in both PiPS-SMCs and PiPS-SMC-DKK3 cells did not reveal any significant changes in the expressions of LRP6, SFRP5, and DKK3 but revealed an upregulation of Wnts and the Frizzled receptor 1 (Online Figure IX). These findings further support the notion that upregulation of DKK3 leads to activation of Wnt signaling during PiPS-SMC differentiation.

DKK3 Regulates PiPS-SMC Differentiation Through β -catenin Nuclear Translocation

It has previously been shown that after inhibition of the β -catenin destruction complex, the cytoplasmic β -catenin, is stabilized and can enter the nucleus to interact with the transcription factor/lymphoid enhancer-binding factor family of transcription factors, promoting specific gene expression.²⁷ Therefore, we postulated whether DKK3 could induce the translocation of β -catenin into the nucleus and subsequently the transcriptional regulation of SM22 and the Wnt signaling target genes. Indeed, immunofluorescent staining showed that β -catenin was translocated into the cell nucleus on DKK3

stimulation in PiPS-SMCs (Figure 6A). To confirm that the DKK3-induced regulation of SM22 is mediated by β -catenin, the reporter activity of the SM22 promoter was assessed after silencing of β -catenin in PiPS-SMCs (Figure 6B) and in PiPS-SMC-DKK3 cells with simultaneous knockdown of β -catenin (Figure 6C). Interestingly, sh β -catenin leads to a dramatic downregulation of the SM22 promoter activity in PiPS-SMCs, whereas knockdown of β -catenin in PiPS-SMC-DKK3 cells leads to a downregulation of the SM22 promoter activity negating the effect of DKK3. Additionally, luciferase assays for the activation of the SM22 promoter after overexpression of DKK3 that were performed after mutation of the Wnt-responsive element (−213 to −192) within the promoter revealed the attenuation of the DKK3 effect on the SM22 promoter activation (Figure 6E). Collectively, these results demonstrate that the transcriptional activation of SM22 by DKK3 is mediated by β -catenin nuclear translocation.

DKK3 Activates Wnt Signaling Through Interaction With Kringle Containing Transmembrane Protein 1

Thus far, our data have demonstrated that transcriptional activation of SM22 by DKK3 was mediated by β -catenin.

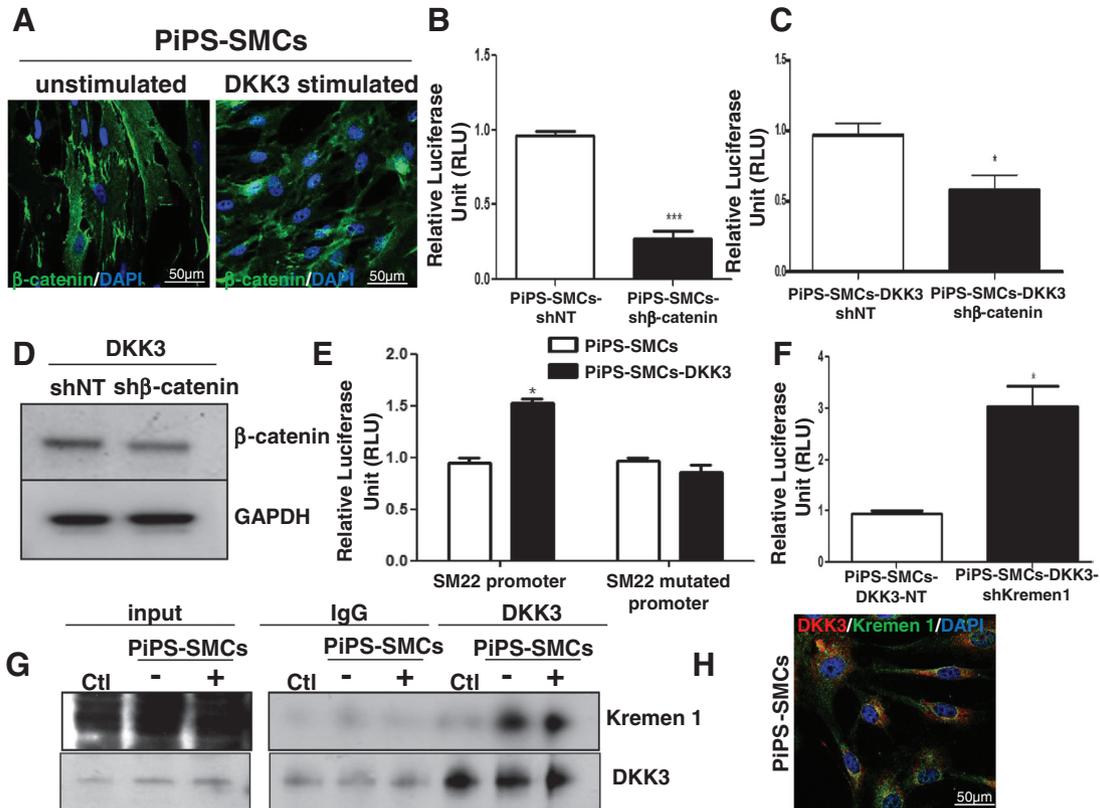


Figure 6. Activation of Wnt signaling through β -catenin translocation and binding of dickkopf 3 (DKK3) on Kringle containing transmembrane protein 1 (Kremen1). Partially-induced pluripotent stem-smooth muscle cell (PiPS-SMC) were stimulated for 6 hours with 50 ng/mL human recombinant DKK3 cytokine in serum-free and cytokine-free conditions. Immunofluorescent staining revealed the translocation of β -catenin in the nucleus of PiPS-SMCs on 6 hours of DKK3 stimulation (A). Luciferase assays performed in PiPS cells after β -catenin knockdown revealed a significant downregulation of the SM22 promoter activity (B; mean \pm SEM of n=3; *** P <0.001). Additionally, luciferase assays were performed in PiPS-SMC-DKK3 cells, whereas β -catenin was silenced by sh β -catenin (C). It was revealed that the upregulation of SM22 promoter activity in the presence of DKK3 is ablated on knockdown of β -catenin (mean \pm SEM of n=3; * P <0.05). Downregulation of β -catenin was confirmed by Western blot analysis (D). Luciferase assays for activation of the SM22 promoter after overexpression of DKK3 were also performed after mutation of the Wnt-responsive element within the promoter. The DKK3-induced activation of SM22 is attenuated after mutation of the -213 to -192 upstream promoter region (E; mean \pm SEM of n=3; * P <0.05). Luciferase assays for TopFlash activity were also performed in PiPS-SMCs overexpressing DKK3, whereas Kremen1 was knocked down. The results revealed an even further induction of Wnt signaling after the combined effect of DKK3 induction and Kremen1 knockdown (F; mean \pm SEM of n=3; * P <0.05). Control (ctl), PiPS-SMCs, and PiPS-SMCs stimulated with 50 ng/mL human recombinant DKK3 cytokine were subjected to immunoprecipitation with rabbit DKK3. Western blot analysis of the precipitated complexes (G) revealed binding of DKK3 with Kremen1 in PiPS-SMCs and stimulated PiPS-SMCs when compared with the ctl cells. Indirect immunofluorescent experiments in PiPS-SMCs revealed a colocalization of Kremen1 and DKK3 in a perinuclear pattern (H).

To elucidate the underlying mechanism of this regulation, the interaction of DKK3 with proteins of the Wnt signaling pathway was assessed. It has recently been reported that DKK3 can potentiate Wnt signaling through interaction with the kringle containing transmembrane protein 1 (Kremen1).²⁸ Thus, we questioned whether the activation of Wnt in our system is mediated through interaction of DKK3 with Kremen1. Whole cell lysates from ctl cells, PiPS-SMCs, and PiPS-SMCs stimulated with 50 ng/mL human recombinant DKK3 were subjected to immunoprecipitation using DKK3. Subsequent immunoblotting with Kremen1 showed that although no binding between DKK3 and Kremen1 was revealed in the ctl cells, the aforementioned proteins show strong binding in PiPS-SMCs and PiPS-SMC-DKK3-stimulated cells (Figure 6G). To further confirm this interaction, double immunofluorescent staining for DKK3 and Kremen1 was performed, which revealed a clear colocalization of the 2 proteins in a distinct perinuclear pattern (Figure 6H).

Finally, to confirm the function of Kremen1 in association to Wnt signaling, luciferase assays for TopFlash, in PiPS-SMC-DKK3 cells where Kremen1 was silenced by shKremen1, were performed revealing further induction of Wnt activation after concomitant Kremen1 knockdown and DKK3 induction (Figure 6F). These findings postulate that the response of PiPS-SMCs to DKK3 and the subsequent activation of Wnt signaling occurs through binding of DKK3 to Kremen1.

PiPS-SMCs Display SMC Properties Ex Vivo and In Vivo

To test the functionality of the PiPS-SMCs, an ex vivo model generated in our laboratory was used. Fibroblasts or PiPS cells selected with neomycin were seeded on a decellularized vessel scaffold in a specially constructed bioreactor in DM suspension containing 25 ng/mL platelet-derived growth factor-BB. Both vessels were harvested 5 days later. The ex vivo PiPS-SMC vessels stained positive for several SMC markers

and exhibited the characteristic morphology and localization of SMCs in the media of the vessels. Such staining was not obtained by the fibroblast-derived vessels (Online Figure XI).

In addition, the functionality of PiPS-SMCs and PiPS-ECs, as well as their potential to generate patent vessels comparable with native vessels, was investigated. A population of PiPS cells after selection with neomycin was seeded on a decellularized vessel in DM containing 25 ng/mL platelet-derived growth factor-BB, where it was initially differentiated toward SMCs for 48 hours. The circulating medium was then exchanged to endothelial growth media 2 (EGM-2 as endothelial specific medium) and a second seeding step of selected PiPS cells was performed. The vessels remained under constant shear stress for 5 days after which they were harvested. Hematoxylin/eosin staining of normal mouse vessels, decellularized vessels, as well as the ex vivo double-seeded vessels, revealed that their recellularization with PiPS-SMCs and PiPS-ECs resulted to vessels that greatly resembled a native mouse artery (Figure 7A–7C). Furthermore, double immunofluorescence revealed characteristic staining for the EC-specific markers, CD31 and CD144, and SMC markers, smooth muscle actin and SM22, as well as characteristic localization of PiPS-SMCs and PiPS-ECs, within the vessel scaffold (Figure 7D–7I). Subsequently, to assess the patency of the tissue-engineered vessels in *in vivo* conditions, normal mouse arteries, decellularized arteries, and the PiPS cell-derived vessels were grafted into mice (Online Movie III). It was revealed that the mice engrafted with the decellularized vessels presented with high mortality rates from as early as day 1, whereas the mice that received the double-seeded, tissue-engineered vascular grafts presented with a survival rate of 60%, 3 weeks after transplantation (Figure 8). Additional *in vivo* studies revealed that although transplantation of decellularized vessel grafts leads to rupture and transplantation of fibroblast-derived vessel grafts leads to occlusion of the vessel, transplantation of the double-seeded, tissue-engineered grafts leads to the generation of patent vessels mimicking healthy native vessels (Online Figure XII). Furthermore, the 3-week-old grafts were fully characterized in terms of the presence of human PiPS-SMCs, ECs, and host macrophage infiltration (Online Figures XIII and XIV). These results indicate that PiPS cells are capable of differentiating into both vascular lineages and are potent for generating functional tissue-engineered vessels, capable of substituting native vessels *in vivo*.

Discussion

Vascular SMCs play a critical role in both the physiological maintenance of the cardiovascular system and the pathophysiology of vascular diseases in adults.^{29,30} They are also a major component in engineered vascular grafts, particularly, in the generation of large vessels. In the present study, we used a shortcut method to successfully reprogram fibroblasts to functional SMCs, that is, PiPS-SMCs. PiPS-SMC differentiation was found to be regulated by a DKK3 signaling pathway. As demonstrated by our *ex vivo* experiments, PiPS-SMCs form vascular structures when seeded in a specially constructed bioreactor and differentiated into mature SMC phenotypes. Importantly, when engrafted into SCID mice, the aforementioned tissue-engineered vessels displayed a function *in vivo*.

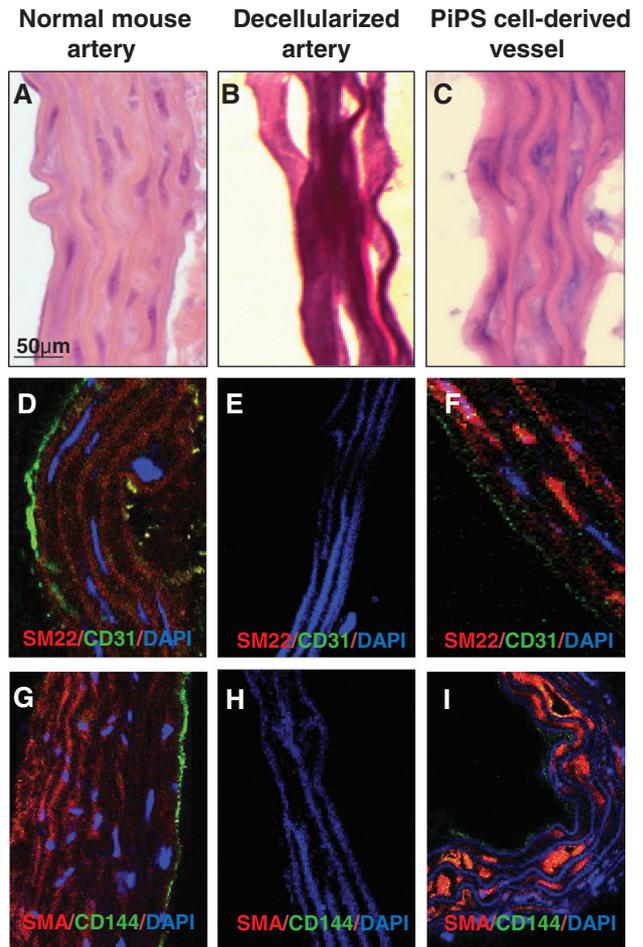


Figure 7. Generation of double-seeded, tissue-engineered vascular grafts. Partially-induced pluripotent stem-smooth muscle cell (PiPS-SMC) and PiPS-endothelial cells (Ecs) display SMC and EC properties, respectively, as well as recellularization properties in tissue-engineered vessels. Hematoxylin/eosin (HE) staining of the double-seeded vessels (C) revealed an architecture similar to that of native vessels (A) with multiple layers of SMCs and a monolayer of ECs. Successful decellularization is confirmed by the absence of cells in the HE-stained decellularized vessel (B). Double-seeded, tissue-engineered vessels (F and I) stained positive for EC and SMC markers. The concomitant expression of SMC and EC markers, as well as the characteristic morphology and localization of PiPS-SMCs and PiPS-ECs, within the media and intima was comparable to that of native vessels (D and G). SMA indicates smooth muscle actin.

Therefore, it can be concluded that the strategy used here for the first time to generate PiPS-SMCs is a fast, simple, efficient, and reproducible method for obtaining SMCs, which could constitute a valuable tool for regenerative medicine, cell replacement therapy, as well as tissue engineering.

The accumulating knowledge concerning the use of transcription factor-based reprogramming enhanced the idea of direct lineage reprogramming circumventing pluripotency. In agreement with previous studies,^{31,32} our microarray analysis results reveal that the expression patterns of genes associated with fundamental processes of the cell, such as differentiation and cell growth, are altered from as early as day 4 during reprogramming. We have recently shown that 4-day reprogrammed cells termed PiPS cells

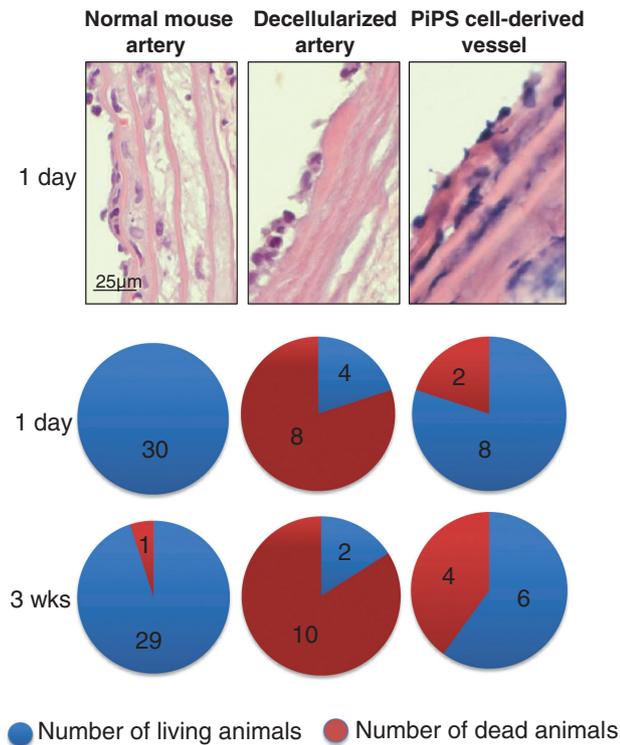


Figure 8. Partially-induced pluripotent stem (PiPS) cell-derived, tissue-engineered vascular grafts are patent for substituting native vessels in vivo. Normal mouse artery, decellularized artery, and PiPS cell-derived vessels were engrafted in the carotid artery of severe combined immunodeficiency mice. The mice engrafted with the PiPS cell-derived grafts presented with a 60% survival rate 3 weeks after surgery, whereas the survival rate of mice engrafted with decellularized arteries was significantly lower (20%; $n=10$).

can differentiate into functional ECs in response to defined media and culture conditions. PiPS cells did not form tumors after subcutaneous transplantation in SCID mice rendering them safe for clinical applications.¹⁷ Further configuration of the identity of PiPS cells did not reveal significant changes in the expression of ectodermal or endodermal markers (Online Figure III). However, PiPS cells expressed kinase insert domain receptor, the first molecule to be expressed during development with specificity to the endothelium,³³ suggesting that a vascular program has already initiated after 4 days of reprogramming.¹⁷ Thus in the present study, the potential of PiPS cells to differentiate to SMC was investigated, and PiPS-SMCs were successfully generated and characterized. Interestingly, selection of PiPS cells for the kinase insert domain receptor revealed a higher upregulation of SM22 and myocardin (data not shown), which in conjunction to the lack of upregulation of ectodermal markers in PiPS cells suggests a mesodermal origin of PiPS-SMCs within our system of differentiation. Notably, PiPS cells, generated from adult skin fibroblasts and differentiated as previously described, did not reveal significant SMC marker upregulations at day 4. This suggests that further optimization of the differentiation protocol, such as increase of the Oct-Sox-Klf-Myc transfection efficiency or longer differentiation time points, are imperative for direct differentiation of more mature cell lines to SMCs.

The generation of PiPS-SMCs has several implications. Initially, the time from reprogramming to obtaining SMC is significantly shorter when compared with the time-consuming generation of iPS cells and redifferentiation to the desired cell line. This offers the opportunity to generate patient-specific cells quickly and efficiently for personalized cell therapy. Additionally, one of the main concerns of stem cell and iPS cell therapy, which is tumorigenesis, is eliminated. Moreover, they are generated by using a nonintegrative method of over-expression of the 4 reprogramming factors and, if required, they can be selected to obtain populations of high purity, thus making them a more accessible and reliable source of cells for use in regenerative medicine.

Elucidation of the mechanism of PiPS-SMC differentiation revealed the involvement of DKK3, a gene whose expression was altered during the initial stages of reprogramming. DKK3 has mainly been implicated in cancer disease.^{34–36} Recently, DKK3 was associated with tumor angiogenesis and was considered a putative proangiogenic factor and marker for neo angiogenesis,³⁷ as well as a differentiation factor involved in remodeling of the tumor vasculature.³⁸ In the present study, it is shown that DKK3 can regulate the transcriptional patterns of SM22 via promoter activation. It is also demonstrated that DKK3 can act as a cytokine and that it can positively modulate the canonical Wnt signaling and induce β -catenin translocation and ultimately induction of Wnt target genes. Importantly, this potentiating effect of DKK3 within our system seems to be unique because DKK1 was found to negatively regulate Wnt signaling in PiPS-SMCs in accordance with previously published data³⁹ (Online Figure X). Finally, we demonstrated that DKK3 interacts with the transmembrane protein Kremen1. Our results showed that PiPS-SMC differentiation was induced through a DKK3-Wnt signaling pathway, giving DKK3 a novel function with possible developmental and therapeutic implications (Online Figure XVIII).

Short-term reprogramming could reinforce the expression of important cell fate-determining genes, which regulate several differentiation pathways and make cells amenable to responding to specific stimulus.⁴⁰ Interestingly, application of lineage-specific differentiation in PiPS cells showed that their differentiation potential may not be limited to vascular cell lineages. Preliminary studies show that PiPS cells can respond to defined culture conditions for neuronal, adipocyte, chondrocyte, and osteocyte differentiation and can express markers specific for these lineages on differentiation (Online Figure XIX). It would, therefore, be interesting to investigate the multipotent potential of PiPS cells.

After the generation of the first in vitro blood vessel more than 30 years ago, this particular research area has seen considerable progress in terms of scaffold availability, construction of the vessels, and application of stem cells.¹ Studies have shown that decellularized vessels treated with heparin alone can successfully be applied in animal models; however, it is widely accepted that this approach would not be applicable in humans and that multilayers of SMCs, as well as an endothelial layer in the vessel wall, are essential for the prevention of graft rupture and atherosclerosis.² In the present study, we developed a method to seed decellularized vessel scaffolds with PiPS cells on both the adventitial and

luminal sides. Using this method, an artery-like vessel was created containing several layers of SMCs between the elastic lamina and a monolayer of ECs investing the inner surface of the vessel. Importantly, the PiPS tissue-engineered vessels are mostly patent, whereas the majority of decellularized grafts presented with occlusion or ruptured within 24 hours of grafting into SCID mice. Failure of some tissue-engineered vessels over time could be the result of inflammatory responses caused by macrophages. Nevertheless, the major advantage of these tissue-engineered vessels is primarily the source of the cells used. PiPS cells are capable of differentiating into both ECs and SMCs making the technique of generating the grafts in ex vivo conditions less complicated and significantly faster. Additionally, transplantation of PiPS or fibroblasts in vivo does not lead to tumorigenesis. Interestingly, ex vivo contractility assays of the tissue-engineered vessels revealed that although the cells can successfully contract within the decellularized scaffold, tension bath contraction assays may not be suitable for assessing the functionality of the generated vessels (Online Figures XV and XVI). Thus, the present study provides a method of generating tissue-engineered vessels ex vivo, as well as a suitable mouse model, for assessing the function of the generated vessel grafts.

Summarizing, we have established a method to generate PiPS cells using human fibroblasts, which can effectively differentiate into SMCs via a DKK3 signaling pathway. PiPS cells can incorporate in decellularized vessel scaffolds by differentiating into both ECs and SMCs, leading to the generation of patent tissue-engineered vessels capable of mimicking native vessels when engrafted into an animal model. Although direct reprogramming between lineages is currently in its infancy, usage of the current knowledge and the continuous effort to expand our basic understanding of somatic cell reprogramming can revolutionize the field of regenerative medicine and make patient-specific therapy a reality in the near future.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- The sources of obtaining smooth muscle cells (SMCs) for the generation of tissue-engineered vessels are scarce.
- SMCs could be directly reprogrammed from one lineage to another.

What New Information Does This Article Contribute?

- Fibroblasts subjected to short-term reprogramming can successfully differentiate into SMCs termed partially-induced pluripotent stem (PiPS)-SMCs.
- PiPS-SMCs can contribute to the generation of functional tissue-engineered vessels, which can mimic native mouse vessels when engrafted into an animal model.

SMCs constitute a large part of the vessel wall, which makes them an important component of tissue-engineered vascular

grafts. However, the sources by which they are isolated are limited, and de novo generation of SMCs from pluripotent cells can be challenging because of inefficient and time-consuming protocols. Herein, we report that we have successfully differentiated semireprogrammed fibroblasts termed PiPS cells into functional SMCs. In addition, we have identified Dickkopf 3 as novel SMC differentiation regulator and have elucidated the signaling pathway by which Dickkopf 3 regulates PiPS-SMCs differentiation. Furthermore, using PiPS-SMCs, we have generated tissue-engineered vascular grafts that when engrafted into a mouse model will function like native vessels. These findings add to the current knowledge of direct reprogramming between lineages providing an ample source of functional SMCs for use in tissue engineering. Moreover, we have identified a simple and fast method for generating tissue-engineered vascular grafts as well as a suitable mouse model for testing the functionality of these vessels in vivo.