Transglutaminase 2 Promotes PDGF-Mediated Activation of PDGFR/Akt1 and β -Catenin Signaling in Vascular Smooth Muscle Cells and Supports Neointima Formation

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Key Words

Vascular smooth muscle cells · Neointima · Transglutaminase 2 · Platelet-derived growth factor · Akt1 · β -Catenin

Abstract

Background: Phenotypic switch of vascular smooth muscle cells (VSMCs) accompanies neointima formation and associates with vascular diseases. Platelet-derived growth factor (PDGF)-induced activation of PDGFR/Akt1 and β -catenin signaling pathways in VSMCs has been implicated in vessel occlusion. Transglutaminase 2 (TG2) regulates these pathways and its levels are increased in the neointima. **Objective:** The aim of this study was to evaluate the role of TG2 in PDGF/ β -catenin signaling cross-talk and assess its contribution to neointima. **Methods:** Aortic VSMCs from wild-type and TG2 knockout mice were tested in vitro for levels of VSMC markers, proliferation, migration and PDGF-induced activation of PDGFR/Akt1 and β -catenin pathways. Neointima in these mice was studied ex vivo in coronary vessels using a heart slice model and in vivo using a carotid artery ligation model. **Results:** Genetic deletion of TG2 attenuated the PDGF-induced phenotypic switch of aortic VSMCs, reduced their proliferation and migration rates, and inhibited PDGF-induced activation of PDGFR/Akt1 and β -catenin pathways in both ex vivo and in vivo neointima models. Importantly, genetic dele-

tion of TG2 also markedly attenuated vessel occlusion. **Conclusions:** TG2 promotes neointima formation by mediating the PDGF-induced activation of the PDGFR/Akt1 and β -catenin pathways in VSMCs. This study identifies TG2 as a potential therapeutic target for blocking neointima in blood vessels.

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Introduction

The success of surgical procedures impacting the vasculature, such as angioplasty, vascular bypass surgery and solid organ transplantation, is adversely affected and often compromised by the eventual thickening of the vessel wall due to neointima formation [1–8]. The hyperplastic response in the vessel wall and the formation of neointima involve a phenotypic transition of contractile vascular smooth muscle cells (VSMCs) into secretory cells. This phenotypic switch is induced by growth factors and inflammatory cytokines and results in VSMC dedifferentiation, proliferation, migration, and increased extracellular matrix (ECM) protein synthesis and deposition [9–11]. Impaired control of the VSMC differentiation state is also associated with other vascular pathologies, including atherosclerosis and arterial calcification [12, 13]. Notably, recent findings implicated a multifunctional protein transglutaminase 2 (TG2) in these diseases [14–21], as well as

in small artery remodeling [22–24], arterial hypertension [25–30] and age-dependent aortic stiffening [31–33].

TG2 is a ubiquitously expressed member of the transglutaminase family of protein cross-linking enzymes [34–36]. This protein is localized to the cytoplasm, nucleus, mitochondria and extracellular surface of various cell types [36]. In addition to its enzymatic activity, TG2 has important nonenzymatic functions, including the regulation of several signaling pathways [reviewed in 34–36]. TG2 is abundant in vascular cells, including VSMCs, endothelial cells, leukocytes, monocytes/macrophages and fibroblasts [35–38]. Previously we observed significant accumulation of TG2 in the ECM of arterial neointima in response to vessel wall injury in vivo [38]. In addition, our studies in cultured VSMCs revealed a novel role for extracellular TG2 in the activation of two principal signaling pathways implicated in neointima formation in blood vessels [39–46], namely the platelet-derived growth factor (PDGF)/PDGFR [37, 38] and β -catenin pathways [47–50]. We detected an increase in the overall and extracellular levels of TG2 in VSMCs exposed to PDGF and, using an shRNA approach [38], showed a requirement for TG2 in the dedifferentiation, migration and proliferation of VSMCs controlled by PDGF [39–42]. Furthermore, our data supported a role for direct interaction of TG2 with PDGFR on the VSMC surface in sensitizing these cells to the action of PDGF [37, 38]. In addition, it has been shown that PDGF modulates VSMC proliferation via the activation of β -catenin signaling [44]. Importantly, we characterized TG2 as a novel agonist of β -catenin activation acting via the key receptors of the canonical β -catenin pathway, LRP5/6 (low-density lipoprotein receptor-related proteins 5 and 6) [19, 50]. Here, we examined whether TG2 mediates PDGF/ β -catenin cross-talk in VSMCs and determined the impact of TG2-mediated activation of these signaling pathways on neointima formation in blood vessels ex vivo and in vivo using genetic deletion of this protein in mice.

Materials and Methods

Mice

All experimental procedures were approved by the Animal Care and Use Committee of the University of Maryland Medical School, Baltimore, Md., USA, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals established by the US National Institutes of Health (NIH) publication N85–23 (revised in 1996). TG2^{–/–} mice (a kind gift from Dr. Graham, Victor Chang Cardiac Research Institute, Darlinghurst, N.S.W., Australia [51]) were back-crossed with C57BL/6 mice and the obtained heterozygotes were then bred to establish related colonies of wild-type (TG2^{+/+}) and TG2^{–/–} mice used for these studies.

Models of Neointima Formation

Neointima formation was induced by PDGF in organotypic heart slice cultures ex vivo [48, 52]. Briefly, 1-mm-thick transverse sections of hearts from 3-day-old wild-type TG2^{+/+} or TG2^{–/–} mice, sliced with a rodent heart matrix (Harvard Apparatus), were cultured on a Millicell-CM 0.4- μ m membrane (Millipore) covered with a thin layer of heart medium (Dulbecco's modified Eagle's F12 medium supplemented with 20% knockout serum replacement, 1% nonessential amino acids, 2 mM L-glutamine, 0.1% β -mercaptoethanol and 0.1% penicillin/streptomycin) in the presence or absence of 0.5 nM recombinant mouse PDGF-BB (ProSpec, East Brunswick, N.J., USA). Heart slices were maintained for 5 days at 37°C in a humidified atmosphere containing 5% CO₂. Cardiac vessel occlusion was evaluated as the difference between the area occupied by smooth muscle (SM) α -actin-positive cells and the total luminal area. To study the impact of TG2 on neointima in vivo, a carotid artery ligation (CAL) model of restenosis (as described originally by Kumar and Lindner [53]) was employed in wild-type (TG2^{+/+}) and sibling TG2^{–/–} mice. In this model, extensive arterial remodeling and neointima formation is induced by complete ligation of the left carotid artery for 4 weeks.

Histology, Immunohistochemistry and Morphometric Analysis of Carotid Artery

For histological and immunochemical staining, 5- μ m paraffin sections of entire necks of TG2^{+/+} and TG2^{–/–} mice were deparaffinized on glass coverslips. Hematoxylin and eosin (HE), Verhoeff-van Gieson (EVG; elastic tissue fibers) and Masson trichrome (collagen) histological stains were applied to define the vascular tissue structure and determine the extent of lumen occlusion in the ligated carotid arteries. The VSMC content in ligated arteries was detected by SM α -actin staining, and macrophage infiltration was detected by staining for Mac-2 antigen. To quantify neointimal and medial areas, 6 carotid artery sections from each mouse (identical section numbers 10, 20, 30, 40 and 50), each 5 μ m in thickness, 50 μ m apart, and within 250 and 500 μ m from the carotid bifurcation, were taken for analysis. These sections were analyzed digitally using VelocityTM image software (PerkinElmer, Waltham, Mass., USA) to quantify the neointimal areas between the internal elastic lamina and the lumen, and the medial area demarcated by the external and internal elastic lamina with negligible error and variability. These values represented individual rather than repeated measures. Given that the sections cover an identical segment of the vessel and are separated by an identical and defined distance, the mean of these 6 measurements represents an approximation of the neointimal and medial volume within that segment for a given mouse. The resulting values for neointima, media areas and intima/media ratios were expressed as means $\pm$ SEM for 8 mice representing each genotype.

Cell Culture, Proliferation and Migration Assays

Primary VSMCs were isolated from aortae of 8-week-old male wild-type (TG2^{+/+}) C57BL/6 and TG2^{–/–} mice combining aortae from 2–3 animals for each isolation [54] and cultured on fibronectin-coated plastic dishes in DMEM with 20% FBS and antibiotics/antimycotics. Aortic VSMCs were used at passage 4 for immunostaining, immunoblotting with antibodies to VSMC markers, and analysis of PDGF-BB-induced PDGFR, Akt1 and β -catenin activation. To analyze the PDGF-BB-induced signaling, cells were deprived of serum (0.1% serum) for 48 h prior to stimulation with 0.5 nM PDGF-BB for the indicated periods of time. Proliferation

rates of TG2^{+/+} and TG2^{-/-} mouse aortic VSMCs in DMEM containing 0.5 nM PDGF-BB were measured over a 72-hour period using Cell Titer 96[®] Aqueous MTS-based reagent (G3580; Promega, Madison, Wisc., USA) for 4 h of culture prior to each time point. The quantity of formazan product proportional to the number of metabolically active live cells was measured at 490 nm and converted to cell numbers. Chemotactic migration of TG2^{+/+} and TG2^{-/-} mouse aortic VSMCs (5×10^4 cells per well) in DMEM with 0.1% BSA towards 0.1, 1 and 10 nM PDGF-BB in the bottom chambers was measured using gelatin-coated Transwell inserts containing 8- μ m pores (Corning Inc., Corning, N.Y., USA). After 5 h of incubation, cells on both sides of the membrane were fixed and stained with Diff-Quick staining kit (Baxter Healthcare, Deerfield, Ill., USA). Cells on the upper side were removed with a cotton swab. The transmigrated cells on the lower side of the membrane were counted in 10 randomly chosen nonoverlapping fields ($\times 100$).

Other Methods

The following antibodies were used: TG2 (polyclonal Ab-4, Neomarkers, Fremont, Calif., USA), vinculin/metavinculin (7F9), aciculin (14F8), SM α -actin, β -catenin, SM myosin and β -tubulin (all from Santa Cruz Biotechnology, Santa Cruz, Calif., USA), PDGFR β , pTyr751-PDGFR β , Akt1, pThr308-Akt1 and pSer473-Akt1 (all from Cell Signaling, Danvers, Mass., USA). The mean cell area and the number of vinculin-containing focal adhesions per cell were quantified for 80 randomly selected nonadjacent cells in the populations of TG2^{+/+} and TG2^{-/-} VSMCs using Velocity[™] image analysis software (PerkinElmer). For quantitative immunoblotting, densitometry with NIH Image 1.63f software was employed. For immunostaining analysis, DAPI (4',6-diamidino-2-phenylindole, Life Technologies, Carlsbad, Calif., USA) was used to counterstain nuclei. mRNA was purified with RNAeasy kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocol. Real time PCR amplification was performed with EVA green chemistry (Bio-Rad, Berkeley, Calif., USA) using an CFX96 thermo cycler. Primers for Lef1, Tcf4, CycD1, p21, numb, axin2 and housekeeping gene RPL19 were as described [47]. Reactions were performed in triplicate for each primer pair to amplify mRNAs encoding mouse β -catenin downstream signaling targets.

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. Student's t test was used for comparison between two groups. For more than two groups, significance was determined using one-way analysis of variance (ANOVA) with comparison between groups by Tukey-Kramer post hoc analysis. A p value <0.05 was considered statistically significant.

Results

Characterization of the *In vitro* Phenotype of TG2^{-/-} Aortic VSMCs

Primary TG2^{+/+} and TG2^{-/-} mouse aortic VSMCs were cultured on fibronectin-coated plates for four passages and then analyzed for general morphology, cytoskeletal organization and differentiation state (fig. 1). Double labeling for TG2 and nuclei confirmed the lack of

Fig. 1. TG2 regulates cytoskeletal organization and spreading of VSMCs *in vitro*. Analysis of primary TG2^{+/+} and TG2^{-/-} mouse aortic VSMCs cultured on fibronectin-coated plastic wells for four passages. Immunofluorescence costaining was performed for TG2 (green; colors refer to the online version only) and nuclei (blue), or labeling for focal adhesion component vinculin and markers of VSMC phenotype, aciculin and SM α -actin. Note the increased spreading, more numerous focal adhesions and more developed actin stress fibers in TG2^{-/-} compared with TG2^{+/+} VSMCs.

this protein in the aortic VSMCs from mice with genetic deletion of TG2. In the TG2^{-/-} VSMCs, we detected a ~2.5-fold increase in cell spreading by immunostaining for vinculin ($1.28 \pm 0.07 \times 10^3$ vs. $0.52 \pm 0.03 \times 10^3 \mu\text{m}^2$ for TG2^{-/-} vs. TG2^{+/+} cells) and a ~70% increase in the number of focal adhesions (38 ± 6 vs. 22 ± 4 for TG2^{-/-} vs. TG2^{+/+}) compared to wild-type cells. Unlike the majority of the TG2^{+/+} cells, most of the TG2^{-/-} cells also displayed prominent parallel arrays of stress fibers containing the cytoskeletal markers of VSMC phenotype

Fig. 2. TG2 regulates the VSMC phenotype in vitro. **a** Increased levels of cytoskeletal markers of the VSMC phenotype, such as metavinculin, SM α-actin, SM myosin and aciculin, revealed in the TG2^{-/-} cells by immunoblotting. **b** Proliferation rates of TG2^{+/+} and TG2^{-/-} mouse aortic VSMCs were measured over 72 h in the medium containing 0.5 nM exogenous PDGF-BB. Note that the TG2^{-/-} cells proliferated significantly slower than the TG2^{+/+}

cells (* $p < 0.05$ at 24 h; ** $p < 0.01$ at 48 and 72 h). **c** Chemotactic migration of TG2^{+/+} and TG2^{-/-} mouse aortic VSMCs toward PDGF-BB was measured in Transwells. Note the reduced migration of the TG2^{-/-} cells (* $p < 0.05$ with 10 nM PDGF-BB; ** $p < 0.01$ with 0.1 and 1 nM PDGF-BB). The means $\pm$ SEM for two independent experiments performed in triplicate with VSMCs derived from 6 animals of each genotype are shown (**b**, **c**).

such as aciculin [55] and SM α-actin. Increased levels of the SM markers metavinculin, SM α-actin, SM myosin and aciculin in the aortic VSMCs lacking TG2 were also confirmed by Western blot analysis (fig. 2a). Enhanced expression of the genes encoding SM-specific cytoskeletal proteins in vitro was accompanied by a ~40% decrease in the cell proliferation rate of TG2^{-/-} aortic VSMCs (fig. 2b). The TG2^{-/-} cells also displayed decreased chemotactic migration towards PDGF-BB, with the strongest inhibitory effect observed at low concentrations of this growth factor (fig. 2c). These findings show that genetic deletion of TG2 attenuates the transition of VSMCs from a contractile to synthetic phenotype and reduces their PDGF-BB-induced proliferation and migration.

Loss of TG2 Reduces PDGF-Induced Activation of the PDGFRβ/Akt1 Signaling Pathway in Aortic VSMCs

Akt1 kinase acts as a major downstream mediator of the PDGF-induced effects of PDGFR on proliferation and differentiation in VSMCs [56]. Since our previous data suggested a direct role for TG2 in the regulation of PDGFR and Akt1 activity [37, 38], we analyzed whether TG2 is required for activation of the PDGFR/Akt1 signaling pathway in mouse aortic VSMCs (fig. 3). While we observed a similar time course of PDGFRβ activation by PDGF in both cell populations, the magnitude of PDGFRβ activation evidenced by receptor autophosphorylation at the Tyr751 residue was decreased by 2.7 ± 0.4 -fold in the

TG2^{-/-} cells (fig. 3a, b). Similarly, Akt1 activation, evidenced by its phosphorylation at Thr308 and Ser473, was reduced by 3.2 ± 0.5 -fold in the TG2^{-/-} aortic VSMCs. Yet, the overall protein levels of PDGFRβ and its signaling targets were similar in the TG2^{+/+} and TG2^{-/-} aortic VSMCs (fig. 3 and data not shown). Thus, we concluded that TG2 significantly enhances activation of the PDGFR/Akt1 signaling axis in the aortic VSMCs in response to PDGF.

Deletion of TG2 Reduces PDGF-Mediated Induction of β-Catenin Signaling in Aortic VSMCs

To examine the role for TG2 in PDGF/β-catenin signaling cross-talk, we analyzed two common hallmarks of β-catenin pathway activation, nuclear translocation of β-catenin protein and transcription of downstream β-catenin target genes in serum-starved mouse aortic VSMCs exposed to PDGF-BB (fig. 4). In agreement with previous studies, we did not detect nuclear β-catenin in wild-type TG2^{+/+} VSMCs in the absence of PDGF [44, 48, 50]. PDGF treatment resulted in nuclear accumulation of β-catenin protein in the majority of TG2^{+/+}, but only in a few TG2^{-/-} cells (fig. 4). Accordingly, the transcription of β-catenin downstream target genes *Lef1*, *Tcf4*, *cycD1*, *p21*, *numb* and *axin2* was significantly induced (up to 12-fold) by PDGF-BB in the TG2^{+/+} VSMCs (fig. 5a). Consistent with the lack of β-catenin nuclear accumulation in the PDGF-treated TG2^{-/-} VSMCs, this growth factor induced either relatively little (e.g. *Lef1*,

Fig. 3. Genetic deletion of TG2 reduces PDGF-induced activation of PDGFR β and Akt1 in VSMCs in vitro. **a** TG2 $^{+/+}$ and TG2 $^{-/-}$ mouse aortic VSMCs cultured on fibronectin-coated plastic wells were starved for 48 h in 0.1% FBS and then treated with 0.5 nM PDGF-BB for up to 45 min (as indicated). Activation levels of PDGFR β and Akt1 were determined by immunoblotting with antibodies to pTyr751-PDGFR β , PDGFR β , pThr308-Akt1, pSer473-Akt1, Akt1 and TG2. All samples were normalized to tubulin levels. **b** Phosphosite signals for pTyr751-PDGFR β , pThr308-Akt1 and pSer473-Akt1 were quantified, averaged and expressed for the TG2 $^{-/-}$ cells as fold activation over those in the TG2 $^{+/+}$ cells. The means $\pm$ SEM for two independent experiments with VSMCs derived from 3 animals of each genotype are shown (* $p < 0.05$).

Fig. 4. TG2 is required for nuclear translocation of β -catenin in VSMCs in response to PDGF. Primary TG2 $^{+/+}$ and TG2 $^{-/-}$ mouse aortic VSMCs were cultured in the presence or absence of 0.5 nM PDGF-BB for 24 h and then double-stained for β -catenin and DAPI to visualize nuclei. Overlays are shown in the lower panels. Note a prominent nuclear localization of β -catenin in the PDGF-treated TG2 $^{+/+}$ cells (arrowheads) that is lacking in the TG2 $^{-/-}$ cells.

Fig. 5. TG2 is required for PDGF-dependent activation of β -catenin target genes in VSMCs. TG2^{+/+} and TG2^{-/-} mouse aortic VSMCs were cultured in the presence or absence of 0.5 nM PDGF-BB for 24 h. Where indicated, 100 μ M TG cross-linking inhibitor cystamine was used. Quantitative real-time PCR analysis of β -catenin target genes in untreated and PDGF-treated TG2^{+/+} (a) and TG2^{-/-} (b) VSMCs. Expression levels of *Lef1*, *Tcf4*, *cycD1*, *p21*, *numb* and *axin2* genes were normalized to the expression of ribosomal protein L19. Changes in normalized levels of expression in response to PDGF-BB were expressed as the fold change over those without this growth factor. n = 6 animals of each genotype analyzed in 2 independent experiments; PCR runs were conducted in triplicate. * p < 0.05; ** p < 0.01. Note a robust inhibition of PDGF-induced expression of the β -catenin downstream target genes in the TG2^{-/-} cells.

cycD1) or no (e.g. *Tcf4*, *p21*, *numb*, *axin2*) upregulation of β -catenin target genes in these cells (fig. 5b). Similarly, the transglutaminase inhibitor cystamine partly reversed the PDGF-induced transcription of several β -catenin targets in wild-type cells (fig. 5a), further supporting a role for TG2 in PDGF/ β -catenin signaling cross-talk. Together, these data demonstrate that PDGF requires TG2 for efficient activation of the β -catenin signaling pathway in VSMCs.

Deletion of TG2 Blocks PDGF-Induced Cardiac Vessel Occlusion and β -Catenin Activation in VSMCs in Ex vivo Cultured Mouse Heart Slices

PDGF/ β -catenin cross-talk has been implicated in increased cell proliferation in VSMCs [44] which, in turn,

contributes to neointima formation [2, 4, 5, 7–10]. Based on the established requirement for TG2 in the PDGF-dependent β -catenin activation in VSMCs, we inquired whether the loss of TG2 would prevent the PDGF-induced neointima formation. To model neointima formation ex vivo, we employed organotypic cultures of neonatal heart slices. In the absence of PDGF, vessel morphology remained intact in control TG2^{+/+} heart slices (fig. 6) [52]. A 5-day-long treatment with PDGF-BB induced an extensive lumen occlusion by SM α -actin-positive cells (fig. 6a arrow, b). Of note, this effect was specific for PDGF-BB, as bFGF did not induce such hyperplastic response (data not shown). In contrast to the TG2^{+/+} heart slices showing PDGF-induced cardiac vessel narrowing and lumen occlusion by SM α -actin-expressing cells,

Fig. 6. TG2 is required for PDGF-dependent cardiac vessel occlusion in the ex vivo neointima formation of organotypic culture of mouse heart slices. **a** Detection of vascular occlusion in heart slices by visualization of vascular SM α -actin-positive cells. One-millimeter heart slices from the wild-type TG2+/+ and TG2-/- mice were cultured ex vivo for 120 h in growth medium either with or without 0.5 nM exogenous PDGF-BB. Immunofluorescence labeling of cardiac sections for SM α -actin combined with DAPI stain-

ing to identify cell nuclei is shown; overlays are shown in the lower panels. Note that cardiac vessel occlusion due to migration and proliferation of SM α -actin-expressing cells occurs in the TG2+/+ but not in the TG2-/- heart tissue and only in the presence of PDGF-BB. **b** Quantification of the PDGF-BB effects on cardiac vessel occlusion in the TG2+/+ and TG2-/- heart tissue. Data are expressed as percent of the occlusion areas in untreated control heart slices (n = 15 vessels from 3 animals; * p < 0.05).

these responses were essentially blunted in the TG2-/- cardiac tissue (fig. 6a, b).

Similar to the results of our in vitro studies with mouse aortic VSMCs (fig. 4), nuclear β -catenin was also detected in the SM α -actin-positive cells comprising the neointima of the TG2+/+ cardiac blood vessels ex vivo (fig. 7 inserts), but was absent in the quiescent SM α -actin-expressing cells in the media layer of either untreated TG2+/+ or PDGF-treated TG2-/- cardiac blood vessels. Therefore, TG2 is essential for both PDGF-dependent cardiac vessel occlusion ex vivo and for PDGF-induced β -catenin activation in proliferating neointimal VSMCs.

Deletion of TG2 Suppresses the PDGF-Induced Neointima Formation and Inhibits Activation of the PDGFR/Akt1 Signaling Pathway In vivo in the Ligated Carotid Artery

To establish in vivo the essential role of TG2 as a mediator of PDGF-dependent neointima formation, we employed the well-established mouse model of CAL [53]

in which neointima formation is dependent on activities of PDGF [8, 10, 40–43] (fig. 7). In TG2+/+ mice, extensive neointima containing mostly proliferating SM α -actin-positive cells and a few infiltrating macrophages formed 4 weeks after CAL (fig. 8a). In contrast, in the TG2-/- mice, the process of lumen occlusion with SM α -actin-positive cells was significantly attenuated and macrophage infiltration was reduced (fig. 8a). Gross ECM structure in the carotid vessel wall, as detected by elastin (EVG) and collagen (trichrome) histological staining, was similar between the TG2-/- and TG2+/+ littermates, and morphometric analysis of the ligated carotids in the TG2+/+ and TG2-/- mice revealed no changes in the mean media area. However, a ~4.5-fold decrease in the neointima area and the intima/media ratio were observed in the TG2-/- mice compared to wild-type littermates (fig. 8b). Biochemical analysis showed decreased PDGFR and Akt1 phosphorylation (activation) in the injured blood vessels of the TG2-/- mice compared with those of the wild-type TG2+/+ littermates (fig. 8c). Therefore, we conclude that TG2 is re-

Fig. 7. TG2 is required for PDGF-dependent activation of β -catenin in the ex vivo model of neointima formation. One-millimeter heart slices from TG2^{+/+} and TG2^{-/-} mice were cultured ex vivo for 120 h in growth medium either with or without 0.5 nM exogenous PDGF-BB. Double immunofluorescence staining of cardiac sections for β -catenin and SM α -actin combined with DAPI staining to identify cell nuclei is shown. Merged images are

shown in the right panels. Note that in the TG2^{+/+} heart slices, exposure to exogenous PDGF-BB stimulates both β -catenin nuclear targeting (activation) in VSMCs and occlusion of the cardiac vessel's lumen with proliferating VSMCs (dashed inserts are shown below at higher magnification). Importantly, both these outcomes require TG2, as they appear strongly repressed in the TG2^{-/-} heart slices.

quired in vivo for efficient PDGF-mediated activation of the PDGFR/Akt1 signaling axis and neointima formation in injured arteries.

Discussion

The protein cross-linking enzyme TG2 has been implicated in various vascular diseases [17–36]; however, the molecular basis for the involvement of TG2 in these processes remains unclear. TG2 is a multifunctional member of the transglutaminase family, which in addition to its enzymatic role has important nonenzymatic activities, including the regulation of several signaling pathways [14–16]. Using ex vivo and in vivo approaches, in this study we demonstrate a novel role for TG2 as a critical mediator of PDGF-induced neointima formation. Ex vivo, PDGF failed to stimulate occlusion of TG2^{-/-} cardiac vessels with SM α -actin-positive cells although this growth factor effectively promoted vessel occlusion in wild-type TG2^{+/+} cardiac vessels. In vivo, neointima development in ligated carotid arteries, which depends on PDGF and its downstream signaling [8, 10, 40–43], was dramatically attenuated by genetic ablation of TG2.

While several TG2-dependent mechanisms may be involved in the stimulatory impact of this protein on neointima development, our findings presented here indicate that TG2 impacts the PDGF/ β -catenin signaling cross-talk that regulates VSMC proliferation [44]. The PDGFR/Akt1 and β -catenin signaling pathways are largely dormant (inactive) in quiescent, contractile VSMCs, but become activated by PDGF and inflammatory cytokines and are critically important for pathophysiological response to vessel wall injury and intimal hyperplasia [39–46].

Several Wnt-independent mechanisms contributing to PDGF/ β -catenin cross-talk have been proposed. These include proteolytic shedding of N-cadherin that results in increased cytoplasmic levels of β -catenin [44], displacement of the destruction complex from β -catenin favoring its translocation in the nucleus [57], and interactions between LRP6 (a key receptor of the canonical β -catenin pathway) and PDGF receptor- β at the cell membrane [58]. Our data indicate that TG2 may affect the PDGF/ β -catenin cross-talk via direct interactions with the key receptors of both signaling pathways. Previously, we showed that extracellular TG2 is capable of noncovalently binding to PDGFR, thus raising its affinity for PDGF and increasing receptor avidity (clustering) [37, 38]. In turn, el-

Fig. 8. Neointima formation in vivo in the carotid artery and concomitant activation of the PDGFR β /Akt1 signaling pathway are both impaired in TG2 $^{-/-}$ mice. Complete ligation of left carotid artery in 12-week-old male wild-type (TG2 $^{+/+}$) and sibling TG2 $^{-/-}$ C57BL6 mice was applied for 28 days as described [53]. **a** Histological and immunocytochemical characterization of neointima formation in ligated carotids of the TG2 $^{+/+}$ and TG2 $^{-/-}$ mice. Five-micrometer transverse serial sections of the ligated carotids were stained with HE to visualize both the general tissue structure and extent of lumen occlusion. Immunocytochemical staining for SM α -actin and Mac-2 was used to identify VSMCs

and macrophages, respectively, in the vessel wall. Elastin and collagen fibers were detected by EVG and Masson trichrome histochemical stains, respectively. **b** Morphometric analysis of neointima formation in carotid arteries of the TG2 $^{+/+}$ and TG2 $^{-/-}$ mice. ** $p < 0.01$; $n = 8$. **c** Analysis of the in vivo state of the PDGFR β /Akt1 signaling axis in the ligated carotids of the TG2 $^{+/+}$ and TG2 $^{-/-}$ mice. SDS extracts of ligated carotids were used for SDS-PAGE and immunoblotting to determine the levels of PDGFR β , pTyr751-PDGFR β , pThr308-Akt1, pSer473-Akt1, Akt1 and TG2 in the samples normalized to tubulin levels.

evated TG2-induced activation of PDGFR increases the activity of multiple downstream signaling targets, in particular Akt1 [37, 38]. In parallel, extracellular TG2 directly binds to and enzymatically cross-links the extracellular domains of LRP5/6, thereby triggering activation and nuclear translocation of β -catenin, which results in transcription of its downstream target genes [19, 50]. Interestingly, in vitro the accumulation of nuclear β -catenin was described as a common outcome of Wnt and growth factor signaling in VSMC proliferation [59]. However, the

activation of β -catenin in the occluded cardiac vessels of rejected mouse heart allografts was not accompanied by elevated levels of canonical Wnt ligands but did associate with increased levels of TG2 [48]. Hence, in the absence of canonical Wnt ligands, TG2 may function as an unconventional activator of β -catenin signaling [48, 50] to drive the phenotypic switch of VSMCs and the hyperplastic response in the blood vessel wall, and may therefore also act as an essential link between β -catenin and growth factor signaling in VSMC proliferation.

In this report, we have shown that genetic deletion of TG2 attenuates PDGF-induced neointima formation and the activation of both PDGFR/Akt1 and β -catenin signaling cascades in VSMCs in vitro and in vivo. The proposed concept of the PDGFR/ β -catenin integration by extracellular TG2 is important for understanding the complex intracellular networks controlling the phenotypic switch of VSMCs and neointima formation in blood vessels. This integration may operate through enhanced physical association (bridging) of the PDGFR and LRP5/6 signaling receptors mediated by TG2 on the surface of VSMCs. Alternatively, or in addition, inside the cells Akt1 may serve as a principal convergence point for integrating the PDGF/PDGFR and the LRP5/6/ β -catenin cascades. Indeed, upon PDGFR activation in VSMCs, the Akt1 kinase can potentially activate β -catenin both through its direct phosphorylation at Ser522 [60, 61] and by inhibiting its attenuator GSK3 β [62, 63]. Our new finding that TG2 is essential for PDGF-dependent Akt1 activation in VSMCs in vitro and in blood vessels is in agreement with its demonstrated ability to activate Akt1 in other cell types [34–36]. Identifi-

cation of extracellular TG2 as a potent dual activator of two major neointima-relevant signaling pathways in VSMCs identifies this protein as an attractive therapeutic target for blocking intimal hyperplasia.

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Disclosure Statement

This work was prepared while Maria Nurminskaya was employed at the University of Maryland. The opinions expressed in this article are the author's own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

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