T-cadherin promotes vascular smooth muscle cell dedifferentiation via a GSK3 β -inactivation dependent mechanism

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ABSTRACT

Participation of the cadherin superfamily of adhesion molecules in smooth muscle cell (SMC) phenotype modulation is poorly understood. Immunohistochemical analyses of arterial lesions indirectly suggest upregulated expression of atypical glycosylphosphatidylinositol-anchored T-cadherin on vascular SMC as a molecular indicator of the dedifferentiated/proliferative phenotype. This study investigated the role of T-cadherin in SMC phenotypic modulation. Morphological, molecular and functional SMC-signature characteristics of rat, porcine and human arterial SMC stably transduced with respect to T-cadherin upregulation (Tcad+) or T-cadherin-deficiency (shTcad) were compared with their respective control transductants (E-SMC or shC-SMC). Tcad+-SMC displayed several characteristics of the dedifferentiated phenotype including loss of spindle morphology, reduced/disorganized stress fibre formation, decay of SMC-differentiation markers (smooth muscle α -actin, smooth muscle myosin heavy chain, *h*-caldesmon), gain of SMC-dedifferentiation marker calmodulin, reduced levels of myocardin, nuclear-to-cytoplasmic redistribution of the myocardin related transcription factors MRTFA/B and increased proliferative and migratory capacities. T-cadherin depletion enforced features of the differentiated SMC phenotype. PI3K/Akt is a major signal pathway utilized by T-cadherin in SMC and we investigated mTORC1/S6K1 and GSK3 β axes as mediators of T-cadherin-induced dedifferentiation. Inhibition of mTORC1/S6K1 signalling by rapamycin suppressed proliferation in both E-SMC and Tcad+-SMC but failed to restore expression of contractile protein markers in Tcad+-SMC. Ectopic adenoviral-mediated co-expression of constitutively active GSK3 β mutant S9A in Tcad+-SMC restored the morphological and molecular marker characteristics of differentiated SMC and normalized rate of proliferation to that in control SMC. In conclusion our study demonstrates that T-cadherin promotes acquisition of the dedifferentiated phenotype *via* a mechanism that is dependent on GSK3 β inactivation.

Keywords: T-cadherin, vascular smooth muscle cells, phenotype modulation, SMC plasticity, GSK3 β

1. INTRODUCTION

Vascular smooth muscle cells (SMC)¹ are highly specialized cells whose main function is contraction and maintenance of vascular tone. In the mature vessel SMC exhibit a differentiated phenotype characterized by the expression of a unique repertoire of contractile proteins, filaments and signalling molecules necessary for the regulation of the smooth muscle contractility. Unlike the majority of cells in the adult organism which are terminally differentiated, vascular SMC retain inherent plasticity even in the mature vessel and can undergo reversible changes in phenotype in response to changes in local environmental cues [1-5]. Loss of the differentiated phenotype, manifested as downregulation of contractile markers and acquisition of synthetic, migratory and proliferative properties, is important during vascular development and for reparation after vascular injury or remodelling in response to altered blood flow. However, deregulated SMC phenotype switching and failure to maintain/regain the differentiated state is a pivotal detrimental contributor to development of several vasculopathies such as atherosclerosis, post-angioplasty restenosis, hypertension and cancer. Although the concept of SMC phenotypic modulation was formulated decades ago, the molecular mechanisms that control critical transitions in SMC phenotype have still not been fully elucidated. Areas of active research with respect to SMC phenotype switching include epigenetic control mechanisms, transcriptional regulation of differentiation marker genes, embryonic stem cell pluripotency, signal transduction, cell-cell and cell-matrix interactions. A remarkably overlooked field of research in SMC (patho)biology concerns the cadherins.

Cadherins are a superfamily of cell surface adhesion molecules that mediate calcium-dependent homophilic cell-cell interactions. Disruption of cadherin function and/or inappropriate “cadherin switching” has significant implications in many disease states including cancer and disorders of the central nervous system, skin and heart, *inter alia* [6, 7]. Cadherins are also capable of heterophilic interactions with many extracellular and intracellular proteins and are involved in regulating cell polarity, migration, proliferation,

¹ **Abbreviations:** BSA, Bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; E, Empty vector; FCS, Fetal calf serum; GSK3 β , Glycogen synthase kinase 3 β ; HRP, Horseradish peroxidase; H-SMC, Human aortic smooth muscle cells; IRS, Insulin receptor substrate; MMP, Matrix metalloproteinase; MRTF, Myocardin-related transcription factors; mTOR, Mammalian target of rapamycin; PBS, Phosphate buffered saline; P-SMC, Porcine aortic smooth muscle cells; S6K1, p70 ribosomal protein S6 kinase 1; S6RP, 40S ribosomal protein S6; shC, lentivector carrying scrambled shRNA; shTcad, T-cadherin deficiency; SMC, Smooth muscle cell; *smMHC*, Smooth muscle myosin heavy chain; Tcad+, T-cadherin upregulation; TRITC, Tetramethylrhodamine-isothiocyanate; R-SMC, Wistar Kyoto rat aortic smooth muscle cells.

survival, phenotype and differentiation [6]. Abnormalities in these processes are characteristic of obstructive vasculopathies; however, only few studies have attempted to understand the involvement of cadherins in this context (reviewed in [8-10]).

Cadherins currently identified in SMC include the classical N-, R-, E- 6B- and OB-cadherins, the protocadherin FAT1, and the atypical T-cadherin. T-cadherin lacks transmembrane and cytoplasmic domains and is anchored to the membrane *via* a glycosylphosphatidylinositol moiety. Given these biomolecular characteristics, T-cadherin is unlikely to support strong homophilic intercellular adhesion and rather functions as a signalling receptor involved in integration of environmental cues in developmental and tissue remodelling processes such as neuronal guidance, angiogenesis and cancer (reviewed in [11]). T-cadherin is ubiquitously expressed in SMC of healthy vessels [12]; however, its function there is still not clear and studies published to date are mostly descriptive. T-cadherin gene expression was reported to be lower in porcine athero-prone coronary arteries than in athero-resistant mammary arteries [13], suggesting a negative association of vascular T-cadherin expression with predisposition to atherosclerosis. However, a growing number of immunohistochemical and genetic studies support a positive association of T-cadherin expression with obstructive vasculopathies. T-cadherin upregulation on SMC was observed during the early phase of reparative proliferation in the model of experimental restenosis [14], in cardiac allograft vasculopathy (i.e. with remodelling like restenosis) and ischemic cardiomyopathy (i.e. atherosclerotic vessels) in a rat allograft model [15], and on intimal SMC in human atherosclerotic lesions at all stages of atherosclerosis [12, 16] (although another study demonstrated strong T-cadherin expression within stages I-II and II –III of human aortic and coronary lesions with progression toward reduced expression at stage V-VI [17]). In lesional arterial tissues from human atherosclerosis and experimental restenosis patterns of inverse staining intensities for T-cadherin and smooth muscle α -actin (α -SMA) and of positive staining intensities for T-cadherin and PCNA on SMC have been noted [12]. Ectopic upregulation of T-cadherin in SMC *in vitro* increased cell cycle progression [18]. Further, upregulation of T-cadherin transcript and protein expression in SMC can be induced *in vitro* under pathological conditions (e.g. high glucose, oxidative stress, hyperinsulinemia) [19] that are encountered by SMC *in vivo* and induce phenotype switching *in vitro*.

The foregoing investigations, taken together with our recent data showing a role for T-cadherin in regulation of insulin effects on SMC contractile competence and matrix remodelling [19], prompted the hypothesis that T-cadherin on SMC is a molecular discriminator between quiescent/contractile and dedifferentiated/proliferating SMC populations. In this study, and using arterial SMC from human, rat and porcine origins, we demonstrate that T-cadherin upregulation prompts phenotype shifting toward

dedifferentiation and directly modulates activity of major signalling checkpoints controlling SMC plasticity *via* Akt/GSK3 β pathway.

2. MATERIALS and METHODS

2.1. Cell culture and transduction

The isolation and characterization of SMC from the descending thoracic aortae of 20-week old male Wistar Kyoto rats (R-SMC) has been detailed [20]. Isolates of spindle porcine coronary artery SMC (P-SMC) were provided by Prof. M.-L. Bochaton-Piallat (University of Geneva, Geneva, Switzerland) [21]. R-SMC and P-SMC were maintained in Dulbecco's modified Eagle's medium (DMEM, containing 5.5mM glucose) supplemented with 10% FCS. Human aortic SMC (H-SMC) were obtained from Lonza (Basel, Switzerland) and normally cultured in DMEM supplemented with 5% FCS and smooth muscle cell growth supplement (SMGS; Thermo Scientific-Life Technologies, Zug, Switzerland). T-cadherin was stably overexpressed (Tcad+) in rat, porcine and human SMC using pLVX-puro vector carrying full length human T-cadherin cDNA (empty pLVX-puro vector served as control (E)) [22]. Stable transductants of T-cadherin-deficient H-SMC (shTcad) were generated using pLKO.1-puro vector carrying human T-cadherin shRNA or non-target shRNA as control (shC) [22]. SMC were transduced at passage 3-4, transduction protocols were performed on at least four separate occasions for any given SMC species, and stably transduced cells were used at up to passage 8-10 after puromycin (3.6 μ g/ml) selection. Within any experimental series and for any given SMC species, transductant sets at the same passage were compared. Expression of T-cadherin was routinely monitored by immunoblotting. Transduction of SMC with adenoviral vector encoding constitutively active mutant of GSK3 β (Adv-S9A) was performed as described previously [23].

2.2. Immunoblotting

SMC were plated (2×10^5 cells/well into 6-well dishes) and cultured for 48 h. Whole cell lysates were prepared and analyzed by immunoblotting as described [24]. Primary antibodies against following proteins/epitopes were used: T-cadherin (R&D Systems Europe Ltd., Abingdon, UK), smooth muscle α -actin (α -SMA, clone 1A4), smooth muscle myosin heavy chain (smMHC; clone hSMV recognizing human and porcine species), *h*-caldesmon (Sigma-Aldrich Chemie, Buchs, Switzerland), smMHC (ab53219 recognizing rat species), GAPDH, lamin B1 (Abcam, Cambridge, UK), histone H3, MRTF-A, Akt, phospho (p)-Akt^{Ser473}, p-

Akt^{Thr308}, GSK3 β , p-GSK3 β ^{Ser9}, S6RP, p-S6RP^{Ser235/236}, p-44/42 MAPK (Erk1/2)^{Thr218/Tyr220}, p-p38 MAPK^{Thr180/Tyr182}, cyclin D1, insulin receptor substrate 1 (IRS-1), p-IRS-1^{Ser636/639} (Cell Signalling Technologies, New England Biolabs GMBH, Frankfurt, Germany), MRTF-B, myocardin (Santa Cruz Biotechnology Inc., Heidelberg, Germany), β -catenin (BD Biosciences, Allschwil, Switzerland), active β -catenin (Upstate Cell Signalling Solutions, Lake Placid, NY, USA), calmodulin, p-cyclin D1^{Thr286} (Thermo Scientific Life technologies, Lubio-Bioscience GmbH, Luzern, Switzerland). Secondary HRP-conjugated anti-species specific IgGs were from Southern Biotechnology (BioReba AG, Reinach, Switzerland). GAPDH, Akt, histone H3 or lamin B1 were variously used as loading controls. Representative blots following signal detection using the Bio-Rad Molecular Imager Gel Doc XR+ system (Bio-Rad Laboratories, Hercules, CA, USA) are shown. Densitometric quantification of signals was performed using Image J (NIH, Bethesda, MD, USA) or Image Lab (Bio-Rad) software.

2.3. Phase contrast and immunofluorescence microscopy

Gross morphology of live SMC cultures grown to subconfluency in standard tissue culture dishes was examined by phase contrast microscopy under an Olympus IX50 microscope (Olympus Switzerland, Volketswil, Switzerland). For immunofluorescence microscopy SMC were plated into 24-well plates (25×10^3 cells/well) containing round 10-mm glass coverslips precoated with 0.5% gelatin. Following culture for 48h cells were washed with PBS, fixed in 4% ice-cold paraformaldehyde, permeabilized and blocked with 0.3% Triton X-100/10% BSA. To investigate actin cytoskeletal organization cells were stained either for α -SMA by sequential incubation with primary anti- α -SMA mAb and secondary anti-mouse Alexa Fluor® 546-labeled IgG (Invitrogen, Basel, Switzerland), or for fibrous/filamentous actin (F-actin) by incubation with tetramethylrhodamine-isothiocyanate (TRITC)-conjugated phalloidin (Sigma-Aldrich, diluted to 0.5 μ g/ml in FCS). Counterstaining of nuclei was achieved with Hoechst 33342 (Sigma-Aldrich). Preparations were mounted on slides using FluorSave reagent (Calbiochem, Darmstadt, Germany). Images were acquired under an Olympus BX63 fluorescent microscope, equipped with a DP80 camera and CellSens software (all from Olympus).

2.4. Cell proliferation

SMC were seeded in quadruplicates into 96-well plates (5×10^3 cells/well) and allowed to adhere overnight. Medium was refreshed (standard growth medium) and cell proliferation was assayed after 72 h further culture by cell enumeration after enzymatic dissociation (0.25% trypsin/1 mM EDTA) using the particle counter Beckman Coulter Z1. Cell enumeration was performed also at time 0 (i.e. after overnight adherence and medium refreshment) to control for equivalence of starting cell numbers.

2.5. Cell migration

Migration of SMC was measured using the scratch wound assay [25]. Confluent monolayer cultures of SMC grown on collagen-I (50 µg/ml; BD Biosciences, Basel, Switzerland) pre-coated 24-well plates were scratch-wounded, gently rinsed with PBS and further cultured in fresh growth medium with inclusion of 2mM hydroxyurea (Sigma-Aldrich) to inhibit proliferation. Phase contrast images were taken at 0 and 24 h time points using an Olympus IX-81 inverted time-lapse microscope equipped with a digital camera (Olympus, Tokyo, Japan) and CellSens software (Olympus Switzerland) within a humidified incubation chamber with 5% CO₂ at 37°C. Each experiment contained 3 parallel wells for every experimental condition. Three different fields of observation at the initial wound front (time 0) were randomly selected and set. The wound area newly occupied by migrating cells after 24 h was measured using CellSens software (Soft Imaging System GmbH, Munich, Germany). Representative images are shown.

2.6. Cell fractionation

SMC were cultured to subconfluency in standard growth medium. Subcellular fractionation generating cytoplasmic, membrane, soluble nuclear and chromatin fractions was performed using Pierce™ Subcellular Protein Fractionation Kit for cultured cells (Thermo Scientific-Life Technologies). Alternatively, cell fractionation generating cytoplasmic and nuclear fractions was performed using 101Bio (Medibena, Vienna, Austria) Cytoplasmic & Nuclear Protein Extraction Kit. Total protein concentration of each resulting fraction was determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific-Life Technologies). Relative abundance of certain proteins (β-catenin, active β-catenin, Akt, lamin B1, MRTF-A, MRTF-B, GAPDH, histone H3) in each fraction was analysed by Western Blot.

2.7. Gelatin zymography

SMC were plated into 24-well plates (45×10³ cells/well) with standard culture medium, allowed overnight adherence and washed twice with warm PBS before addition of DMEM/0.1% BSA (250 µl/well). Conditioned medium was collected after 24 and 48h, centrifuged (5min, 3000 rpm) and stored frozen (-20°C) until use. Equivalence of cell numbers was controlled by cell enumeration after medium collection. Medium samples were mixed with reducing agent-free 3xLaemmli sample buffer (1:2, v/v) and subjected to electrophoresis (50 µl/lane) on 8% SDS-PAGE co-polymerized with gelatin (1mg/ml; Sigma-Aldrich G-8150). Following electrophoresis gels were sequentially incubated in renaturing solution (2.5% v/v Triton X-100 in water) for 30min (3x10min) at room temperature and then in developing buffer (50mM Tris, 0.2M NaCl, 5mM CaCl₂, 0.02% Brij 35) overnight at 37°C.

Gels were stained with 0.5% (w/v) Coomassie Blue R-250, and destained with 50% methanol/10% acetic acid solution. Areas of substrate digestion appear as clear bands against the stained background. Images were acquired using Gel Doc XR+ imager (BioRad), areas of enzymatic activity were quantified by densitometry with Image Lab analysis software (BioRad).

2.8. Statistical analysis

All experimental series were performed on at least 3 independent occasions and unless otherwise stated results are given as mean $\pm$ SD. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. P-values were calculated by two tailed Student's t-test for single comparisons and by one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* testing for multiple comparisons. A 2-tailed probability value <0.05 was considered as threshold of significance.

3. RESULTS

3.1. T-cadherin upregulation promotes SMC migration and increases proliferation capacity

T-cadherin upregulation is observed in several vasculopathies associated with SMC phenotype modulation. To study consequences of T-cadherin upregulation in SMC at functional, morphological and molecular levels we used SMC isolates from rat (R-SMC), porcine (P-SMC) and human (H-SMC) aortae stably transduced with respect to T-cadherin upregulation (Tcad⁺) or T-cadherin depletion (shTcad in H-SMC) and their respective empty vector (E) or scrambled shRNA (shC) controls. Figure 1A depicts representative immunostainings for T-cadherin in the transductants.

Increased migration and proliferation capacities represent two major functional hallmarks of SMC phenotype switch toward dedifferentiation. Cell migration was examined by scratch-wound assay on a substratum of collagen I. T-cadherin overexpressing R-SMC and P-SMC migrated into the wound space more rapidly, respectively filling ~30% and ~60% more wound area than their respective controls after a 24h migration period (Fig. 1B). In the case of H-SMC, migration capacity was not affected by T-cadherin upregulation but it was significantly reduced by T-cadherin depletion, with shTcad-SMC filling ~35% less wound area than their shC controls (Fig. 1B). Absence of effects of T-cadherin upregulation on migration capacity of H-SMC might appear inconsistent with the findings in rat and porcine SMC. However it is noteworthy that of the three species H-SMC migrated the fastest; control- (E and shC) and Tcad⁺-H-SMCs filled ~300 000 μm^2 of the wound area, whereas even the most

migratory of rat and porcine SMCs (i.e. T-cad⁺ transductants) filled only ~160 000 μm^2 (Fig. 1B). Thus effects of T-cadherin on H-SMC migration could have been masked due their high inherent migratory capacity. Proliferation rates of the T-cadherin-overexpressing stable human, rat and porcine SMC transductants were increased ~1.5-fold above those of the respective E controls, while T-cadherin depletion reduced SMC proliferation potential to ~40% of that in control shC-SMC (Fig. 1C).

3.2. *T-cadherin alters SMC morphology and cytoskeleton organization*

Important parameters for the definition of SMC phenotypes are cell morphology and myofilament arrangement: the morphologies at opposite extremes of the SMC phenotype continuum, namely differentiated/contractile and dedifferentiated/synthetic, are profoundly distinct (elongated/spindle and epitheloid/rhomboid, respectively), as are the myofilament arrangements (rich ordered array and irregular/disordered, respectively). An examination of subconfluent cultures of the SMC transductants under phase contrast microscopy revealed alterations in cell morphologies following upregulation or depletion of T-cadherin expression (Fig. 2A). Compared with control transductants, Tcad⁺-SMC exhibited a less fusiform and more disorganized, multilayer pattern of growth; the cells also appeared to be poorly spread with prominent thin intercellular filopodial-like extensions. In contrast, for shTcad-SMC the pattern of fusiform growth with elongated and spread cells aligned in parallel rows as seen in control E or shC cultures was much more pronounced (Fig. 2A). Higher magnification images of Tcad⁺-SMC and shTcad-SMC are presented (Fig. 2B) to more clearly illustrate their distinct patterns of growth. Alterations in SMC morphology were accompanied by changes in the actin cytoskeleton organization as evaluated by TRITC-phalloidin–staining of F-actin in sparse cultures plated on a gelatin substratum (Fig. 2C). Compared with control transductants Tcad⁺-SMC were generally smaller and more rhomboid in shape, exhibited decreased stress fiber formation with prominent redistribution of filamentous actin toward the cell periphery and disassembled/disrupted appearance of the actin fibers. Conversely, shTcad-SMC appeared larger and exhibited more robust array of stress fibers with actin filament bundles being well-organized and oriented parallel to the major cell axis. Higher magnification images of human Tcad⁺-SMC and shTcad-SMC are presented (Fig. 2D) to underscore the differential effects of T-cadherin upregulation and depletion on filamentous actin organization.

3.3. *T-cadherin upregulation leads to decay of SMC differentiation markers*

The most important phenotypic characteristic of differentiated SMC is their expression of a cytocontractile proteome that is necessary for the principal function of these cells, namely contraction. Immunofluorescence microscopy studies demonstrated that the T-cadherin

associated alterations in cytocontractile fibers as revealed by TRITC-phalloidin staining (above) were attributable to alterations in filaments formed by α -SMA, the main protein marker of the contractile SMC phenotype (Fig. 3). The intensity of α -SMA staining was weaker in the Tcad⁺-SMC but stronger in shTcad-SMC as compared with the respective control SMC transductants (Fig. 3A). The α -SMA staining pattern in Tcad⁺-SMC was frequently very diffuse with weak and/or truncated stress fibers. In shTcad-SMC the pattern of α -SMA staining was indicative of very prominent and strong stress fibres. Higher magnification images of human Tcad⁺-SMC and shTcad-SMC are presented to underscore the differential effects of T-cadherin upregulation and depletion on α -SMA filament organization (Fig. 3B). Reduction and elevation α -SMA protein levels in Tcad⁺-SMC and shTcad-SMC, respectively, was confirmed by immunoblotting of whole cell lysates (Fig. 3C).

Other established SMC differentiation marker proteins, namely *smMHC*, *h-caldesmon* [1-4] and dedifferentiation marker protein calmodulin [26], were also affected by changes in T-cadherin expression. Compared with their respective control E transductants Tcad⁺-SMC exhibited decreased levels of *smMHC* and *h-caldesmon* and elevated levels of calmodulin (Fig. 4A, B). Conversely, T-cadherin-depletion resulted in increased levels of *smMHC* and *h-caldesmon* concomitant with decreased levels of calmodulin (Fig. 4b). Reductive effects of T-cadherin upregulation on expression of α -SMA, *smMHC* and *h-caldesmon* were evident also under serum-free culture conditions (Supplementary Fig. S1).

SMC phenotype switching is associated with changes in synthesis of matrix-degrading proteinases [27]. Gelatin zymography of conditioned media from SMC cultures showed that levels of pro-MMP-2 and cleaved active MMP-2 were elevated in Tcad⁺-SMC (Fig. 4A, B) but decreased in shTcad-SMC (Fig. 4B).

Expression of phenotype markers in SMC are under control of transcriptional coactivator myocardin and myocardin-related transcription factors A (MRTF-A) and B (MRTF-B). Immunoblot analysis of whole cell lysates demonstrated that levels of myocardin protein were decreased in Tcad⁺-SMC and increased in shTcad-SMC (Fig. 5A). T-cadherin expression had no effect on total cellular levels of either MRTF-A or MRTF-B (not shown), but significantly modulated their cellular distribution as determined by immunoblotting of nuclear and cytoplasmic fractions of rat and human SMC transductants (Fig. 5B and C). Compared with the respective control transductants, proportions of both MRTF-A and MRTF-B located in the nuclear fraction were reduced in Tcad⁺-SMC and increased in shTcad-SMC.

Collectively, the above-described consequences of gain and loss of T-cadherin expression on functional, morphological and molecular parameters that classically define SMC

phenotype provide clear evidence for a shift toward greater dedifferentiation following T-cadherin upregulation whereas depletion of T-cadherin reinforces the differentiated phenotype. In the following we investigate intracellular signalling pathways underlying effects of T-cadherin on SMC phenotype.

3.4. *T-cadherin modulates PI3K/Akt axis signalling in SMC*

Our previous studies demonstrate that T-cadherin activates the PI3K/Akt transduction pathway in SMC [19]. In the following we further examine activity of Akt pathway components in T-cadherin transductants. One of the major targets of Akt kinase is GSK3 β which is active in the unphosphorylated state and is inactivated by Akt phosphorylation. Constitutive activity of the PI3K/Akt pathway measured as the phosphorylation status of Akt and GSK3 β is respectively elevated and decreased in T-cadherin-upregulated and T-cadherin-deficient SMC under both serum-deprivation [19] and serum-containing (Fig. 6) culture conditions. Active (unphosphorylated) GSK3 β downregulates levels of cytosolic β -catenin by phosphorylating and targeting it for degradation by the ubiquitin-proteasome system; phosphorylation of GSK3 β by Akt leads to its inhibition, thus stabilizing β -catenin protein [28]. In accordance with the differential effects of T-cadherin upregulation and deficiency on GSK3 β phosphorylation, the levels of total and active β -catenin were elevated in T-cad+-SMC (Fig. 7A, B) but reduced in shTcad-SMC (Fig. 7B). Subcellular fractionation demonstrated higher levels of β -catenin in all fractions (membrane, cytoplasm, soluble nuclear, chromatin bound) from Tcad+-R-SMC (Fig. 7C) and in cytoplasmic and nuclear fractions of T-cad+-H-SMC (Fig. 7D). β -catenin levels in the respective fractions of shTcad-SMC were decreased (Fig. 7D).

Akt pathway controls SMC proliferation through regulation of cyclin D1 which is required for progression through the G1 phase of the cell cycle. Cyclin D1 is a classical target gene for β -catenin/TCF-LEF-induced transcription [29, 30]. However, quantitative RT-PCR analysis revealed no effect of T-cadherin on cyclin D1 mRNA (data not shown). Furthermore, TCF/LEF reporter assay also did not detect differences between E-SMC and Tcad+-SMC (data not shown). Nevertheless, and in keeping with the data on proliferation potential (Fig. 1C) expression of cyclin D1 protein was increased in Tcad+-SMC (Fig. 8A, B) and decreased in shTcad-SMC (Fig. 8B).

Cyclin D1 expression can be regulated by mechanisms independent of β -catenin. Absence of effects of T-cad on cyclin D1 mRNA would exclude other promoter activity regulatory mechanisms (e.g. direct repression by myocardin [31]). Our findings of altered cyclin D1 protein concomitant with unchanged transcript expression would rather suggest a mechanism of protein stability. Importantly, and like β -catenin, cyclin D1 is a substrate for

GSK3 β which phosphorylates cyclin D1 on Thr286 and facilitates its proteasomal degradation [32]. Immunoblotting analysis of rat and porcine SMC transductants revealed slightly stronger levels of cyclin D1 phosphorylation on Thr286 in Tcad+-SMC (Fig. 8C), but calculation of the phosphorylated to total cyclin D1 signal ratios indicated significantly lower relative levels of phospho-cyclin D1^{Thr286} in Tcad+-SMC (Fig. 8C).

Apart from Akt signalling axis, SMC proliferation can be strongly regulated by mitogen-activated protein kinases (MAPKs). However, neither Erk1/2 nor p38MAPK in H- or R-SMC were sensitive to alterations in T-cadherin expression (Fig. 6).

3.5. *mTORC1/S6K1 branch of the PI3K/Akt pathway is not the major mediator for T-cadherin effects on SMC phenotype*

We have previously demonstrated that the hyperactivation of Akt signalling by T-cadherin induces the mTORC1/S6K1-dependent negative feedback loop resulting in Ser636/639 phosphorylation and degradation of insulin receptors substrate 1 (IRS-1) and causing cellular insensitivity to insulin [19, 22]. To examine whether the mTORC1/S6K1 branch of the Akt pathway is also involved in T-cadherin-dependent regulation of SMC phenotype we analysed expression levels of SMC differentiation markers in Tcad+-SMC after treatment with mTORC1 inhibitor rapamycin.

Hyperactivation of mTORC1/S6K1 signalling in Tcad+-SMC under control conditions (2.5% serum and absence of rapamycin) was evident from the elevated phosphorylation status of S6K1 substrates S6RP and IRS-1 concomitant with a decrease in levels of total IRS-1 (Fig. 9A, B). Rapamycin treatment (30 nM, 8h) decreased p-S6RP^{Ser235/236}/p-IRS-1^{Ser636/639} and increased total IRS-1, with abundancies achieving equivalence between E-SMC and Tcad+-SMC (Fig. 9A, B). Rapamycin induced feedback activation of Akt signalling in both E- and Tcad+-SMC as evidenced by elevation in levels of p-Akt^{Ser473}, p-Akt^{Thr308}, p-GSK3 β ^{Ser9} and β -catenin, although their relative abundancies remained significantly higher in Tcad+-SMC. Levels of SMC contractile protein markers α -SMA, *sm*MHC and *h*-caldesmon were elevated following rapamycin treatment in E-SMC but remained unchanged in Tcad+-SMC. Rapamycin decreased cyclin D1 levels in E-SMC (by ~50-60%) but was without effect in Tcad+-SMC (Fig. 9A,B), while levels of p-cyclin D1 were decreased in both SMC but to a lesser extent in Tcad-SMC (Fig. 9B; reduced by ~50-60% in E-SMC and by ~ 10-20% in Tcad+-SMC). The ratio p-cyclin D1:cyclin D1 was unchanged in E-SMC and there was a trend toward a lower ratio, albeit not significant, in Tcad+-SMC (Fig. 9B). Rapamycin did not alter the morphological appearance of either E-SMC or Tcad+-SMC (Fig. 9C). Nonetheless, rapamycin inhibited proliferation of both E-SMC and Tcad+-SMC (Fig. 9D). These data demonstrate that T-cadherin-induced downregulation of SMC contractile proteins,

upregulation of cyclin D1 and morphological alterations are mediated by mechanisms distinct from the mTOR/S6K1 branch of the Akt pathway.

3.6. *GSK3 β branch of the PI3K/Akt signalling axis mediates effects of T-cadherin on SMC differentiation*

We next examined whether the GSK3 β branch of the Akt pathway, or more specifically GSK3 β inactivation, might mediate T-cadherin effects on SMC differentiation status. To examine this possibility we infected E- and Tcad⁺-R-SMC with adenovirus expressing nonphosphorylatable, constitutively active GSK3 β -S9A mutant (Adv-S9A) or empty adenovirus (Adv-E) control. Equivalence of GSK3 β protein expression in E- and Tcad⁺-Adv-S9A SMC was controlled by immunoblotting (Fig. 10A). GSK3 β -S9A mutant was without effect on mTORC1/S6K1 axis signalling in either E or Tcad⁺ SMC, based on the unchanged (vs. respective controls) phosphorylation status of S6K1 targets S6RP^{Ser 235/236} and IRS-1^{Ser636/639} (Fig. 10A). However, we found that the ectopic expression of GSK3 β -S9A could override SMC dedifferentiation induced by T-cadherin upregulation. In Tcad⁺-Adv-S9A-SMC, the levels of β -catenin and cyclin D1 decreased, as did expression of dedifferentiation marker calmodulin, while abundancies of myocardin and SMC contractile proteins *smMHC*, α -SMA and *h-caldesmon* increased (Fig. 10A), all reaching the respective levels of control Adv-E-SMC. GSK3 β -S9A mutant also eliminated functional effects of T-cadherin overexpression in SMC: proliferation potential in Tcad⁺-Adv-S9A cells was decreased as compared to Tcad⁺-Adv-E control (Fig. 10B), and the spread/fusiform morphology was restored (Fig. 10C). Expression of GSK3 β -S9A in E-SMC was without significant effect on any of the measured parameters. Taken together, these data support that T-cadherin-induced SMC dedifferentiation is dependent on GSK3 β inactivation.

4. DISCUSSION

This study has demonstrated that T-cadherin is capable of promoting the phenotypic switch of SMC toward dedifferentiation. T-cadherin upregulation in SMC resulted in loss of spindle morphology, reduced stress fiber formation and downregulation of SMC differentiation markers. These changes were associated with modulation of levels, activity and/or cellular localization of major signalling molecules controlling SMC plasticity (myocardin, MRTFA/B) and proliferation (β -catenin, cyclin D1) and functionally translated into increased proliferative and migratory capacities (Fig. 11). Accordingly, silencing of T-cadherin enforced SMC differentiation. We identified GSK3 β inactivation as the major downstream mechanism underlying T-cadherin-induced loss of SMC contractile phenotype.

There has been significant progress in understanding the hallmarks of the phenotypic modulation of SMC and the complex mechanisms involving the regulatory circuits of transcription factors and crosstalk of intracellular signalling pathways [1-5]. The main characteristics of differentiated SMC are the presence of a rich array of myofilaments and expression of a cytocontractile proteome that is necessary for the principal function of these cells, namely contraction. Established SMC contractile marker proteins include α -SMA, *smMHC* and *h-caldesmon* [1-4]. An important role in control of their expression is played by myocardin and the myocardin-related transcription factors A and B which function as coactivators for serum response factor (SRF)-dependent transcription of genes encoding SMC-specific cytoskeletal and contractile proteins [33-35]. Myocardin, which is constitutively localized to the nucleus [36] is a critical factor for establishment and maintenance of SMC differentiation [36, 37]. *In vivo* studies have consistently shown reduced myocardin expression under conditions favoring SMC dedifferentiation (e.g. arterial injury [38-40]). *In vitro* ablation of myocardin in SMCs leads to acquisition of a dedifferentiated morphology and decreased contractile protein expression [41]. Cellular abundance of myocardin protein ultimately represents an index of transcriptional, post-transcriptional and post-translational activities involved in regulation of SMC differentiation status. Expression of MYOCD gene in SMC has been reported to be regulated by transcription factor (e.g. SRF, TEAD, NFAT, FOXO) or homeodomain protein (e.g. Nkx2.5, Prx1) binding to the MYOCD promoter, and to be responsive to a Rho-ROCK, ERK, Akt and GSK3 β signal pathway activities [42, 43]. A variety of myocardin binding proteins can regulate stability of myocardin protein through either protection against (e.g. FHL2 [44], UBR5 [45]) or promotion of (CHIP [46]) proteasomal degradation. In contrast to the nuclear location of myocardin the MRTFs are mostly located in the cytoplasm; in response to RhoA activation and subsequent actin polymerization, which reduces the level of monomeric G-actin to which cytoplasmic MRTFs is sequestered, MRTFs translocate to the nucleus where they activate SRF-dependent genes promoting differentiation and cytoskeletal organization [47]. MRTF protein stability may also be regulated through ubiquitin-mediated proteasomal degradation [44].

All the above-described indices of SMC differentiation status are modulated by T-cadherin. T-cadherin overexpression in SMC causes loss of filamentous actin/stress fibers and contractile proteins α -SMA, *smMHC*, *h-caldesmon*, gain of SMC dedifferentiation marker calmodulin, downregulation of myocardin protein level and nuclear-to-cytoplasmic redistribution of transcription factors MRTF-A and B. Functionally these molecular events translated into acquisition of a typical dedifferentiated SMC phenotype evident from the loss of the "spindle" morphology, increase in proliferation and migration rates, and also gain of matrix remodelling capacity manifested by increased expression of MMP-2 [27]. Taken together with our previous data on reduced RhoA-GTPase activity and decreased

phosphorylation status of the regulatory myosin binding subunit MYPT1 of myosin light chain phosphatase (MLCP) and of MLCP target MLC20 [19] in T-cadherin overexpressing SMC, our study unequivocally identifies T-cadherin as a factor promoting SMC dedifferentiation and phenotypic plasticity. The data are in line with immunohistological findings of inverse staining intensities for T-cadherin and α -SMA in human aorta and upregulation of T-cadherin protein under pathological conditions characterized by loss of SMC differentiation status such as atherosclerosis, restenosis and allograft vasculopathies [12, 14, 15].

Although loss of differentiation status indeed often accompanies onset of proliferative activity during abnormal vascular tissue remodelling, it is recognized that these two processes are not necessarily tightly coupled or mutually exclusive [4, 48]. For example, *in vitro* TGF- β 1 can increase SMC proliferation concomitant with appearance of an elongated, differentiated morphology and the expression of α -SMA [49]. Heparin can inhibit proliferation of SMC without restoration of a differentiated morphology or expression of α -SMA [50]. *In vivo* during late embryogenesis and postnatal development SMC exhibit a high proliferation rate that is concomitant with induction of differentiation genes [51-53]. Conversely, in advanced atherosclerotic lesions, proliferation of SMC is very low, and so is the expression level of contractile markers [54, 55]. T-cadherin upregulation induces both hyper-proliferation and dedifferentiation; yet our finding that rapamycin inhibits proliferation but failed to restore contractile protein marker expression in Tcad⁺-SMC presents another example for uncoupling of proliferation and dedifferentiation, in as much as cessation of proliferation alone is not sufficient to promote SMC differentiation.

Of special interest is the question of intracellular signalling pathways mediating T-cadherin effects on SMC phenotype. Previous studies demonstrated that T-cadherin can activate two branches of the Akt signalling axis. On the one hand, in endothelial cells T-cadherin upregulation *per se* induces proliferation, survival and angiogenesis *via* Akt/GSK3 β pathway [24, 56, 57]. On the other hand, in endothelial and smooth muscle cells hyperactivation of Akt induces negative feedback signalling through the mTORC1/S6K1 which leads to serine phosphorylation and degradation of IRS-1 and loss of cellular sensitivity to insulin with respect to its effects on angiogenesis, contractile function and matrix remodelling [19, 22].

With respect to SMC differentiation, it is evident that PI3K/Akt pathway may function either suppressively or permissively, depending on the stimuli and preferential activation of pathway branches. PI3K/Akt activation induced by PDGF [58-60], PDGF/IL-1 β [61], PDGF/IL-1 β [61] and PTEN inactivation [62] or transfection with constitutively active Akt [61] suppressed SMC contractile protein expression, whereas PI3K inhibition or expression of dominant negative Akt restored expression of contractile marker proteins [61, 62]. In

contrast, induction of PI3K/Akt by IGF-1 promotes a differentiated state which can be prevented by inhibition of PI3K [59, 60], or enforced by rapamycin which prevents mTORC1/S6K1-mediated phosphorylation and proteasomal degradation of the adaptor IRS-1 thereby amplifying IGF-1 activation of PI3K/Akt [63, 64]. Opposite effects of Akt activation by different stimuli on SMC differentiation might be explained by the observation that rapamycin selectively activates only the Akt2 isoform which is required for SMC differentiation, but not Akt1 which seems to oppose contractile protein expression [63]. We find elevated Akt pathway signalling and decreased contractile protein marker expression in T-cadherin overexpressing SMC under both serum-containing culture conditions and after serum-deprivation, suggesting effects of T-cadherin on SMC differentiation do not directly depend on the presence of serum growth factors. Moreover, and although T-cadherin is able to hyperactivate Akt/mTORC1/S6K1 in SMC causing cellular insulin insensitivity [19], our data demonstrate that mTORC1/S6K1 is not the major signalling branch downstream of Akt for T-cadherin-dependent phenotype switching: inhibition of mTORC1 by rapamycin inhibited proliferation of SMC independently of whether T-cadherin was upregulated or not, but failed to induce either re-expression of SMC contractile protein markers or a differentiated morphology in Tcad⁺-SMC.

Rather, our data show that the GSK3 β branch of the Akt pathway, more specifically GSK3 β inactivation, is the dominant mediator of T-cadherin-induced SMC proliferation and dedifferentiation. This is in line with previous data demonstrating a permissive role for GSK3 β in maintaining a differentiated SMC phenotype. GSK3 β was identified as a positive regulator of the transcriptional activity of myocardin in SMC, whereby GSK3 β -mediated phosphorylation of myocardin increased its recruitment to target genes [65]. Further, expression of myocardin target genes in SMC can be abrogated by pharmacological or siRNA-mediated inhibition of GSK3 β as well as ectopic expression of dominant negative, kinase inactive GSK3 β mutant [65]. Inhibition of GSK3 β can also influence *MYOCD* splice patterning in SMC; chemical inhibitors of GSK3 β promoted expression of *MYOCD* Δ Exon 11 in association with adoption of a dedifferentiated morphology, increased proliferation and repression of *MYH11* gene expression [66]. *In vivo* gene transfer of GSK3 β -S9A inhibited SMC proliferation and reduced neointima formation after balloon injury in rats [67]. Inhibition of SMC proliferation *in vitro* and injury-induced vascular remodelling *in vivo* by the testosterone analogue dehydroepiandrosterone was demonstrated to depend on its inhibition of Akt signalling and concomitant activation of GSK3 β [68]. Also monocrotaline-induced pulmonary artery remodelling *in vivo* was associated with GSK3 β inactivation [69]. Our data show that the phenotype shift toward dedifferentiation caused by T-cadherin can be fully prevented by forced GSK3 β activation implying a central role of the GSK3 β branch in T-cadherin effects on SMC plasticity.

An unresolved issue concerns how GPI-anchored T-cadherin activates the intracellular signalling that leads to SMC dedifferentiation. For example, does T-cadherin act as a sensor responding to extracellular ligands, are homophilic interactions involved and/or does T-cadherin transmit signals inwardly by coupling to signalling adaptors? Currently, we can only notionally address these questions. Since adiponectin is a recognized extracellular ligand for T-cadherin [70], we might speculate on involvement of T-cadherin-adiponectin binding. However, we believe this to be unlikely in the context of SMC-phenotype switching since (1) SMC-dedifferentiation following T-cadherin upregulation was evident under both serum-containing and serum-free (i.e. adiponectin-free) culture conditions, (2) *in vitro*, adiponectin promotes SMC differentiation [71] and inhibits SMC proliferation [72], and (3) adiponectin-deficient mice exhibit increased neointima formation after vascular cuff injury [73]. Because shTcad-SMC and T-cad⁺-SMC display opposing phenotypes (differentiated and dedifferentiated, respectively) a more probable determinant of T-cadherin regulated SMC phenotype is the level of T-cadherin expression *per se*. The classical cadherins generally activate intracellular signalling through interactions with a variety of signalling molecules after *trans*-ectodomain homophilic interactions and ensuing cell-cell contact zone extension [74]. Although T-cadherin is capable of homophilic ligation [74], it lacks transmembrane and cytosolic domains to enable either direct cooperation with cellular signalling effectors or formation of strong *trans*-dimer complexes at intercellular contacts. Cellular localization of T-cadherin is also distinct from that of the classical cadherins: T-cadherin localizes to lipid raft domains [75], it is mostly expressed over the cell body with negligible localization to intercellular junctions in resting SMC, and it redistributes to the leading edges of migrating SMC [76]. Furthermore, T-cadherin effects on SMC differentiation status are evident also in low-density cultures where *trans*-intercellular junctions are minimal. We suggest that T-cadherin-activated inward signal transmission in SMC likely involves homophilic *cis*-interactions in combination with heterophilic *cis*-interactions with effector molecules within the lipid raft signalling platform. Demonstrating such interactions poses an exciting future challenge.

5. Conclusions

Collectively, based on the evidence from morphological, molecular and behavioural analyses in SMC from different species (human, porcine and rat) our study identifies T-cadherin/GSK3 β signalling as a novel molecular circuit controlling vascular SMC phenotype. Importantly, enforcement of SMC differentiation by genetic ablation of T-cadherin implies that although T-cadherin is ubiquitously expressed by SMC in healthy vessels, it is not essential for mature SMC contractile function and may rather play a role in the maintenance of SMC plasticity. The study is relevant for development of specific targeted therapeutic strategies for

pathological SMC-driven reparation in cardiovascular diseases, or for harnessing phenotype-modulation for tissue regeneration/engineering purposes.

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Author contributions

AF, TJR, MP and PE designed the study and experiments. AF conducted the experiments. AF, TJR and MP analysed data; BD and DP generated lentiviral vectors and viral particles. While each author contributed to the writing of the manuscript, AF, MP and TJR are largely responsible for its writing.

REFERENCES

- [1] D. Gomez, G.K. Owens, Smooth muscle cell phenotypic switching in atherosclerosis, *Cardiovasc Res*, 95 (2012) 156-164.
- [2] J.M. Miano, Vascular smooth muscle cell differentiation-2010, *J Biomed Res*, 24 (2010) 169-180.
- [3] S.S. Rensen, P.A. Doevendans, G.J. van Eys, Regulation and characteristics of vascular smooth muscle cell phenotypic diversity, *Neth Heart J*, 15 (2007) 100-108.
- [4] E.M. Rzucidlo, K.A. Martin, R.J. Powell, Regulation of vascular smooth muscle cell differentiation, *J Vasc Surg*, 45 Suppl A (2007) A25-32.
- [5] N. Shi, S.Y. Chen, Smooth Muscle Cell Differentiation: Model Systems, Regulatory Mechanisms, and Vascular Diseases, *Journal of cellular physiology*, DOI 10.1002/jcp.25208(2015).
- [6] M.J. Wheelock, Y. Shintani, M. Maeda, Y. Fukumoto, K.R. Johnson, Cadherin switching, *J Cell Sci*, 121 (2008) 727-735.
- [7] M.P. Stemmler, Cadherins in development and cancer, *Mol Biosyst*, 4 (2008) 835-850.
- [8] T.J. Resink, M. Philippova, M.B. Joshi, E. Kyriakakis, P. Erne, Cadherins and cardiovascular disease, *Swiss Med Wkly*, 139 (2009) 122-134.
- [9] E.P. Moiseeva, Adhesion receptors of vascular smooth muscle cells and their functions, *Cardiovasc Res*, 52 (2001) 372-386.
- [10] Z. Sun, A.R. Parrish, M.A. Hill, G.A. Meininger, N-cadherin, a vascular smooth muscle cell-cell adhesion molecule: function and signaling for vasomotor control, *Microcirculation*, 21 (2014) 208-218.
- [11] M. Philippova, M.B. Joshi, E. Kyriakakis, D. Pfaff, P. Erne, T.J. Resink, A guide and guard: the many faces of T-cadherin, *Cell Signal*, 21 (2009) 1035-1044.
- [12] D. Ivanov, M. Philippova, J. Antropova, F. Gubaeva, O. Iljinskaya, E. Tararak, V. Bochkov, P. Erne, T. Resink, V. Tkachuk, Expression of cell adhesion molecule T-cadherin in the human vasculature, *Histochem Cell Biol*, 115 (2001) 231-242.
- [13] M. Qin, Z. Zeng, J. Zheng, P.K. Shah, S.M. Schwartz, L.D. Adams, B.G. Sharifi, Suppression subtractive hybridization identifies distinctive expression markers for coronary and internal mammary arteries, *Arterioscler Thromb Vasc Biol*, 23 (2003) 425-433.
- [14] E. Kudrjashova, P. Bashtrikov, V. Bochkov, Y. Parfyonova, V. Tkachuk, J. Antropova, O. Iljinskaya, E. Tararak, P. Erne, D. Ivanov, M. Philippova, T.J. Resink, Expression of adhesion molecule T-cadherin is increased during neointima formation in experimental restenosis, *Histochem Cell Biol*, 118 (2002) 281-290.
- [15] S. Collot-Teixeira, J.L. McGregor, K. Morser, L. Chalabreysse, C. McDermott-Roe, C. Cerutti, A. Guzman, J.B. Michel, P. Boissonnat, L. Sebbag, F. Thivolet-Bejui, G. Bricca, J.F.

- Obadia, A. Roussoulieres, T-cadherin expression in cardiac allograft vasculopathy: bench to bedside translational investigation, *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation*, 29 (2010) 792-799.
- [16] T. Takeuchi, Y. Adachi, Y. Ohtsuki, M. Furihata, Adiponectin receptors, with special focus on the role of the third receptor, T-cadherin, in vascular disease, *Medical molecular morphology*, 40 (2007) 115-120.
- [17] C.G. Kostopoulos, S.G. Spiroglou, J.N. Varakis, E. Apostolakis, H.H. Papadaki, Adiponectin/T-cadherin and apelin/APJ expression in human arteries and periadventitial fat: implication of local adipokine signaling in atherosclerosis?, *Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology*, 23 (2014) 131-138.
- [18] D. Ivanov, M. Philippova, V. Tkachuk, P. Erne, T. Resink, Cell adhesion molecule T-cadherin regulates vascular cell adhesion, phenotype and motility, *Exp Cell Res*, 293 (2004) 207-218.
- [19] A. Frismantiene, D. Pfaff, A. Frachet, M. Coen, M.B. Joshi, K. Maslova, M.L. Bochaton-Piallat, P. Erne, T.J. Resink, M. Philippova, Regulation of contractile signaling and matrix remodeling by T-cadherin in vascular smooth muscle cells: constitutive and insulin-dependent effects, *Cell Signal*, 26 (2014) 1897-1908.
- [20] T. Scott-Burden, T.J. Resink, U. Baur, M. Burgin, F.R. Buhler, Epidermal growth factor responsiveness in smooth muscle cells from hypertensive and normotensive rats, *Hypertension*, 13 (1989) 295-304.
- [21] H. Hao, P. Ropraz, V. Verin, E. Camenzind, A. Geinoz, M.S. Pepper, G. Gabbiani, M.L. Bochaton-Piallat, Heterogeneity of smooth muscle cell populations cultured from pig coronary artery, *Arterioscler Thromb Vasc Biol*, 22 (2002) 1093-1099.
- [22] M. Philippova, M.B. Joshi, D. Pfaff, E. Kyriakakis, K. Maslova, P. Erne, T.J. Resink, T-cadherin attenuates insulin-dependent signalling, eNOS activation, and angiogenesis in vascular endothelial cells, *Cardiovasc Res*, 93 (2012) 498-507.
- [23] M.B. Joshi, D. Ivanov, M. Philippova, P. Erne, T.J. Resink, Integrin-linked kinase is an essential mediator for T-cadherin-dependent signaling via Akt and GSK3beta in endothelial cells, *FASEB J*, 21 (2007) 3083-3095.
- [24] M.B. Joshi, M. Philippova, D. Ivanov, R. Allenspach, P. Erne, T.J. Resink, T-cadherin protects endothelial cells from oxidative stress-induced apoptosis, *FASEB J*, 19 (2005) 1737-1739.
- [25] C.C. Liang, A.Y. Park, J.L. Guan, In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro, *Nature protocols*, 2 (2007) 329-333.
- [26] M. Coen, G. Marchetti, P.M. Palagi, C. Zerbinati, G. Guastella, T. Gagliano, F. Bernardi, F. Mascoli, M.L. Bochaton-Piallat, Calmodulin expression distinguishes the smooth muscle cell population of human carotid plaque, *Am J Pathol*, 183 (2013) 996-1009.

- [27] A.C. Newby, Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates, *Cardiovasc Res*, 69 (2006) 614-624.
- [28] S. Frame, P. Cohen, GSK3 takes centre stage more than 20 years after its discovery, *Biochem J*, 359 (2001) 1-16.
- [29] H. Quaschnick, S.C. Slater, C.A. Beeching, M. Boehm, G.B. Sala-Newby, S.J. George, Regulation of smooth muscle cell proliferation by beta-catenin/T-cell factor signaling involves modulation of cyclin D1 and p21 expression, *Circ Res*, 99 (2006) 1329-1337.
- [30] C. Mill, S.J. George, Wnt signalling in smooth muscle cells and its role in cardiovascular disorders, *Cardiovasc Res*, 95 (2012) 233-240.
- [31] X. Long, R.D. Bell, W.T. Gerthoffer, B.V. Zlokovic, J.M. Miano, Myocardin is sufficient for a smooth muscle-like contractile phenotype, *Arterioscler Thromb Vasc Biol*, 28 (2008) 1505-1510.
- [32] J.A. Diehl, M. Cheng, M.F. Roussel, C.J. Sherr, Glycogen synthase kinase-3 β regulates cyclin D1 proteolysis and subcellular localization, *Genes & development*, 12 (1998) 3499-3511.
- [33] J.M. Miano, X. Long, K. Fujiwara, Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus, *American journal of physiology. Cell physiology*, 292 (2007) C70-81.
- [34] D.Z. Wang, E.N. Olson, Control of smooth muscle development by the myocardin family of transcriptional coactivators, *Current opinion in genetics & development*, 14 (2004) 558-566.
- [35] M.S. Parmacek, Myocardin-related transcription factors: critical coactivators regulating cardiovascular development and adaptation, *Circ Res*, 100 (2007) 633-644.
- [36] D. Wang, P.S. Chang, Z. Wang, L. Sutherland, J.A. Richardson, E. Small, P.A. Krieg, E.N. Olson, Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor, *Cell*, 105 (2001) 851-862.
- [37] J. Chen, C.M. Kitchen, J.W. Streb, J.M. Miano, Myocardin: a component of a molecular switch for smooth muscle differentiation, *Journal of molecular and cellular cardiology*, 34 (2002) 1345-1356.
- [38] J.A. Hendrix, B.R. Wamhoff, O.G. McDonald, S. Sinha, T. Yoshida, G.K. Owens, 5' CArG degeneracy in smooth muscle α -actin is required for injury-induced gene suppression in vivo, *The Journal of clinical investigation*, 115 (2005) 418-427.
- [39] D.L. Tharp, B.R. Wamhoff, H. Wulff, G. Raman, A. Cheong, D.K. Bowles, Local delivery of the KCa3.1 blocker, TRAM-34, prevents acute angioplasty-induced coronary smooth muscle phenotypic modulation and limits stenosis, *Arterioscler Thromb Vasc Biol*, 28 (2008) 1084-1089.

- [40] A. Talasila, H. Yu, M. Ackers-Johnson, M. Bot, T. van Berkel, M.R. Bennett, I. Bot, S. Sinha, Myocardin regulates vascular response to injury through miR-24/-29a and platelet-derived growth factor receptor-beta, *Arterioscler Thromb Vasc Biol*, 33 (2013) 2355-2365.
- [41] J. Huang, L. Cheng, J. Li, M. Chen, D. Zhou, M.M. Lu, A. Proweller, J.A. Epstein, M.S. Parmacek, Myocardin regulates expression of contractile genes in smooth muscle cells and is required for closure of the ductus arteriosus in mice, *The Journal of clinical investigation*, 118 (2008) 515-525.
- [42] J.M. Miano, Myocardin in biology and disease, *J Biomed Res*, 29 (2015) 3-19.
- [43] X.L. Zheng, Myocardin and smooth muscle differentiation, *Archives of biochemistry and biophysics*, 543 (2014) 48-56.
- [44] J.S. Hinson, M.D. Medlin, J.M. Taylor, C.P. Mack, Regulation of myocardin factor protein stability by the LIM-only protein FHL2, *American journal of physiology. Heart and circulatory physiology*, 295 (2008) H1067-H1075.
- [45] G. Hu, X. Wang, D.N. Saunders, M. Henderson, A.J. Russell, B.P. Herring, J. Zhou, Modulation of myocardin function by the ubiquitin E3 ligase UBR5, *J Biol Chem*, 285 (2010) 11800-11809.
- [46] P. Xie, Y. Fan, H. Zhang, Y. Zhang, M. She, D. Gu, C. Patterson, H. Li, CHIP represses myocardin-induced smooth muscle cell differentiation via ubiquitin-mediated proteasomal degradation, *Mol Cell Biol*, 29 (2009) 2398-2408.
- [47] T. Minami, K. Kuwahara, Y. Nakagawa, M. Takaoka, H. Kinoshita, K. Nakao, Y. Kuwabara, Y. Yamada, C. Yamada, J. Shibata, S. Usami, S. Yasuno, T. Nishikimi, K. Ueshima, M. Sata, H. Nakano, T. Seno, Y. Kawahito, K. Sobue, A. Kimura, R. Nagai, K. Nakao, Reciprocal expression of MRTF-A and myocardin is crucial for pathological vascular remodelling in mice, *The EMBO journal*, 31 (2012) 4428-4440.
- [48] M.R. Alexander, G.K. Owens, Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease, *Annu Rev Physiol*, 74 (2012) 13-40.
- [49] A. Orlandi, P. Ropraz, G. Gabbiani, Proliferative activity and alpha-smooth muscle actin expression in cultured rat aortic smooth muscle cells are differently modulated by transforming growth factor-beta 1 and heparin, *Exp Cell Res*, 214 (1994) 528-536.
- [50] A.W. Clowes, M.M. Clowes, O. Kocher, P. Ropraz, C. Chaponnier, G. Gabbiani, Arterial smooth muscle cells in vivo: relationship between actin isoform expression and mitogenesis and their modulation by heparin, *J Cell Biol*, 107 (1988) 1939-1945.
- [51] G.K. Owens, M.S. Kumar, B.R. Wamhoff, Molecular regulation of vascular smooth muscle cell differentiation in development and disease, *Physiol Rev*, 84 (2004) 767-801.

- [52] C.L. Cook, M.C. Weiser, P.E. Schwartz, C.L. Jones, R.A. Majack, Developmentally timed expression of an embryonic growth phenotype in vascular smooth muscle cells, *Circ Res*, 74 (1994) 189-196.
- [53] S.H. Lee, J.E. Hungerford, C.D. Little, M.L. Iruela-Arispe, Proliferation and differentiation of smooth muscle cell precursors occurs simultaneously during the development of the vessel wall, *Developmental dynamics : an official publication of the American Association of Anatomists*, 209 (1997) 342-352.
- [54] E.R. O'Brien, C.E. Alpers, D.K. Stewart, M. Ferguson, N. Tran, D. Gordon, E.P. Benditt, T. Hinohara, J.B. Simpson, S.M. Schwartz, Proliferation in primary and restenotic coronary atherectomy tissue. Implications for antiproliferative therapy, *Circ Res*, 73 (1993) 223-231.
- [55] J.N. Wilcox, Analysis of local gene expression in human atherosclerotic plaques, *J Vasc Surg*, 15 (1992) 913-916.
- [56] M. Philippova, A. Banfi, D. Ivanov, R. Gianni-Barrera, R. Allenspach, P. Erne, T. Resink, Atypical GPI-anchored T-cadherin stimulates angiogenesis in vitro and in vivo, *Arterioscler Thromb Vasc Biol*, 26 (2006) 2222-2230.
- [57] M. Philippova, D. Ivanov, M.B. Joshi, E. Kyriakakis, K. Rupp, T. Afonyushkin, V. Bochkov, P. Erne, T.J. Resink, Identification of proteins associating with glycosylphosphatidylinositol- anchored T-cadherin on the surface of vascular endothelial cells: role for Grp78/BiP in T-cadherin-dependent cell survival, *Mol Cell Biol*, 28 (2008) 4004-4017.
- [58] N. Kaplan-Albuquerque, C. Garat, C. Desseva, P.L. Jones, R.A. Nemenoff, Platelet-derived growth factor-BB-mediated activation of Akt suppresses smooth muscle-specific gene expression through inhibition of mitogen-activated protein kinase and redistribution of serum response factor, *J Biol Chem*, 278 (2003) 39830-39838.
- [59] K. Hayashi, H. Saga, Y. Chimori, K. Kimura, Y. Yamanaka, K. Sobue, Differentiated phenotype of smooth muscle cells depends on signaling pathways through insulin-like growth factors and phosphatidylinositol 3-kinase, *J Biol Chem*, 273 (1998) 28860-28867.
- [60] K. Hayashi, M. Takahashi, K. Kimura, W. Nishida, H. Saga, K. Sobue, Changes in the balance of phosphoinositide 3-kinase/protein kinase B (Akt) and the mitogen-activated protein kinases (ERK/p38MAPK) determine a phenotype of visceral and vascular smooth muscle cells, *J Cell Biol*, 145 (1999) 727-740.
- [61] C.N. Chen, Y.S. Li, Y.T. Yeh, P.L. Lee, S. Usami, S. Chien, J.J. Chiu, Synergistic roles of platelet-derived growth factor-BB and interleukin-1beta in phenotypic modulation of human aortic smooth muscle cells, *Proc Natl Acad Sci U S A*, 103 (2006) 2665-2670.
- [62] S.B. Furgeson, P.A. Simpson, I. Park, V. VanPutten, H. Horita, C.D. Kontos, R.A. Nemenoff, M.C.M. Weiser-Evans, Inactivation of the tumour suppressor, PTEN, in smooth

muscle promotes a pro-inflammatory phenotype and enhances neointima formation, *Cardiovasc Res*, 86 (2010) 274-282.

[63] K.A. Martin, B.L. Merenick, M. Ding, K.M. Fetalvero, E.M. Rzucidlo, C.D. Kozul, D.J. Brown, H.Y. Chiu, M. Shyu, B.L. Drapeau, R.J. Wagner, R.J. Powell, Rapamycin promotes vascular smooth muscle cell differentiation through insulin receptor substrate-1/phosphatidylinositol 3-kinase/Akt2 feedback signaling, *J Biol Chem*, 282 (2007) 36112-36120.

[64] K.A. Martin, E.M. Rzucidlo, B.L. Merenick, D.C. Fingar, D.J. Brown, R.J. Wagner, R.J. Powell, The mTOR/p70 S6K1 pathway regulates vascular smooth muscle cell differentiation, *American journal of physiology. Cell physiology*, 286 (2004) C507-517.

[65] Y.X. Zhou, Z. Shi, P. Singh, H. Yin, Y.N. Yu, L. Li, M.P. Walsh, Y. Gui, X.L. Zheng, Potential Role of Glycogen Synthase Kinase-3beta in Regulation of Myocardin Activity in Human Vascular Smooth Muscle Cells, *Journal of cellular physiology*, DOI 10.1002/jcp.25084(2015).

[66] R.M. Ilagan, C.W. Genheimer, S.F. Quinlan, K.I. Guthrie, N. Sangha, S. Ramachandrannair, R.W. Kelley, S.C. Presnell, J. Basu, J.W. Ludlow, Smooth muscle phenotypic diversity is mediated through alterations in myocardin gene splicing, *Journal of cellular physiology*, 226 (2011) 2702-2711.

[67] K.W. Park, H.M. Yang, S.W. Youn, H.J. Yang, I.H. Chae, B.H. Oh, M.M. Lee, Y.B. Park, Y.S. Choi, H.S. Kim, K. Walsh, Constitutively active glycogen synthase kinase-3beta gene transfer sustains apoptosis, inhibits proliferation of vascular smooth muscle cells, and reduces neointima formation after balloon injury in rats, *Arterioscler Thromb Vasc Biol*, 23 (2003) 1364-1369.

[68] S. Bonnet, R. Paulin, G. Sutendra, P. Dromparis, M. Roy, K.O. Watson, J. Nagendran, A. Haromy, J.R. Dyck, E.D. Michelakis, Dehydroepiandrosterone reverses systemic vascular remodeling through the inhibition of the Akt/GSK3- β /NFAT axis, *Circulation*, 120 (2009) 1231-1240.

[69] P. Sklepikiewicz, R.T. Schermuly, X. Tian, H.A. Ghofrani, N. Weissmann, D. Sedding, T. Kashour, W. Seeger, F. Grimminger, S.S. Pullamsetti, Glycogen synthase kinase 3beta contributes to proliferation of arterial smooth muscle cells in pulmonary hypertension, *PloS one*, 6 (2011) e18883.

[70] C. Hug, J. Wang, N.S. Ahmad, J.S. Bogan, T.S. Tsao, H.F. Lodish, T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin, *Proc Natl Acad Sci U S A*, 101 (2004) 10308-10313.

[71] M. Ding, Y. Xie, R.J. Wagner, Y. Jin, A.C. Carrao, L.S. Liu, A.K. Guzman, R.J. Powell, J. Hwa, E.M. Rzucidlo, K.A. Martin, Adiponectin induces vascular smooth muscle cell

differentiation via repression of mammalian target of rapamycin complex 1 and FoxO4, *Arterioscler Thromb Vasc Biol*, 31 (2011) 1403-1410.

[72] W. Zhang, C. Shu, Q. Li, M. Li, X. Li, Adiponectin affects vascular smooth muscle cell proliferation and apoptosis through modulation of the mitofusin-2-mediated Ras-Raf-Erk1/2 signaling pathway, *Molecular medicine reports*, 12 (2015) 4703-4707.

[73] O.J. Harrison, F. Bahna, P.S. Katsamba, X.S. Jin, J. Brasch, J. Vendome, G. Ahlsen, K.J. Carroll, S.R. Price, B. Honig, L. Shapiro, Two-step adhesive binding by classical cadherins, *Nat Struct Mol Biol*, 17 (2010) 348-U121.

[74] A.S. Yap, E.M. Kovacs, Direct cadherin-activated cell signaling: a view from the plasma membrane, *Journal of Cell Biology*, 160 (2003) 11-16.

[75] M.P. Philippova, V.N. Bochkov, D.V. Stambolsky, V.A. Tkachuk, T.J. Resink, T-cadherin and signal-transducing molecules co-localize in caveolin-rich membrane domains of vascular smooth muscle cells, *FEBS Lett*, 429 (1998) 207-210.

[76] M. Philippova, D. Ivanov, V. Tkachuk, P. Erne, T.J. Resink, Polarisation of T-cadherin to the leading edge of migrating vascular cells in vitro: a function in vascular cell motility?, *Histochem Cell Biol*, 120 (2003) 353-360.

FIGURE LEGENDS

Figure 1. T-cadherin upregulation promotes SMC migration and increases proliferation capacity. (A) Lentiviral vector-mediated alteration of T-cadherin expression in VSMC. Stable T-cadherin upregulation in Wistar-Kyoto rat (R-SMC), porcine (P-SMC) and human (H-SMC) vascular smooth muscle cells (Tcad+) was achieved using pLVX-puro vector carrying full length human T-cadherin cDNA. Respective empty viral vector transduced SMC served as controls (E). Stable transductants of T-cadherin-silenced H-SMC (shTcad) were generated using pLKO.1-puro vector carrying human T-cadherin shRNA or non-target shRNA as control (shC). Representative immunoblots of T-cadherin protein (Mr of 105 kDa and 130 kDa for mature and prepro proteins) in whole cell lysates are presented. Akt was used as internal loading control. (B) Scratch-wound migration assay on collagen-I substratum. Upper panel: monolayer micrographs taken at 0 and 24h time points, scale bar: 100µm. Lower panel: quantification of the area of wound repair. (C) Cell counts after 72h culture estimated by cell enumeration. Significant differences are marked with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 2. T-cadherin expression affects SMC morphology and cytoskeleton organization. (A) Phase contrast micrographs of subconfluent SMC monolayers overexpressing T-cadherin (Tcad+), or depleted of T-cadherin (shTcad) and their respective controls (E and shC). Scale bar: 100µm.. (B) High magnification images illustrating morphological distinction between Tcad+ and shTcad. (C) Fluorescence micrographs of TRITC-Phalloidin (red) stained SMC cultures. Counterstaining of nuclei was achieved using Hoechst 33342 (blue). (D) Enlarged areas of (C) accentuating differences in fibrillar actin morphology and distribution. Scale bar: 50µm.

Figure 3. T-cadherin upregulation reduces α-SMA expression and stress fiber formation. (A) Fluorescence micrographs of α-SMA-stained (red) SMC monolayers. Counterstaining of nuclei is with Hoechst 33342 (blue). (B) Enlarged areas of (A) for better visualisation of fibrillar vs. diffuse α-SMA dominance. Scale bar: 50µm. (C) α-SMA expression analysed in whole cell lysates by immunoblotting. Akt was used as loading control. Levels of α-SMA expression in Tcad+-SMC and shTcad-SMC are expressed relative to control E-SMC. Significant differences are marked with asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Figure 4. T-cadherin upregulation leads to decay of SMC differentiation markers. (A) Upper panels: immunoblots of rat and porcine SMC lysates probed for *smMHC*, *h-caldesmon*

and calmodulin. Akt was used as loading control. Contractile marker protein expression levels in Tcad⁺-SMC are expressed relative to those in the respective E controls. Lower panels: gelatin zymograms of conditioned media from rat and porcine SMC. Proteolytic activities of pro- and mature MMP-2 in Tcad⁺-SMC are expressed relative to respective E controls. (B) Immunoblots of human SMC lysates probed for *sm*MHC, *h*-caldesmon and calmodulin, and gelatin zymogram (far right) of conditioned medium from these cells. Akt was used to control for loading in immunoblots. Contractile marker protein expression levels and gelatinase activities in Tcad⁺-SMC and shTcad-SMC are expressed relative to those in their respective E and shC controls. Significant differences are marked with asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Figure 5. T-cadherin affects myocardin expression and myocardin-related transcription factor (MRTF) subcellular distribution in R- and H-SMC. (A) Immunoblots of crude SMC lysates probed for myocardin with Akt used as loading control. Histograms express levels of myocardin relative to the levels in control E-SMC. (B) Cytosolic (cyto) and nuclear (nuc) fractions of SMC probed for MRTF-A and MRTF-B. Akt and histone H3 were used as fraction and loading controls for immunoblotting of subcellular fractions. (C) Densitometry analysis of data in (B) expressed as percentage distribution between nuclear and cytosolic fractions. Significant differences are marked with asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Figure 6. T-cadherin alters classical PI3K-Akt axis signalling, but has no effect on MAPK/ERK and p38MAPK signalling. Immunoblots of crude lysates from H-SMC (A), R-SMC (B), and P-SMC (C) probed for major PI3K/Akt, MAPK and p38 signalling effectors. GAPDH and Akt served as loading controls. Numeric values express densitometry-quantified band intensities in Tcad⁺ or shTcad SMC relative to those in their respective (E or shC) control SMC. Asterisks indicate statistical significance between E and Tcad⁺ and between shC and shTcad. ** $P < 0.01$; *** $P < 0.001$.

Figure 7. T-cadherin upregulation promotes β -catenin stabilization and nuclear accumulation. Immunoblots of crude lysates from R-SMCs (A) and H-SMC (B) probed for β -catenin and active (non-phosphorylated) β -catenin and cyclin D1. Akt was used as loading control. Levels in Tcad⁺-SMC and shTcad-SMC are expressed relative to control E-SMC. (C) Immunoblots of R-SMC subcellular fractions – cytoplasmic (Cyto), membrane (Memb), nuclear soluble (Nucs) and chromatin bound (Chrom) – probed for β -catenin. Akt or lamin B1 served as controls for fraction separation and loading. (D) Immunoblots of H-SMC subcellular fractions – cytoplasmic (Cyto) and nuclear (Nuc) – probed for β -catenin. Akt, lamin b1, or

histone H3 served as controls for fraction separation and loading. Histograms present densitometry-quantified levels of target proteins expressed relative to respective levels in control E-SMC. Significant differences are marked with asterisks (* P <0.05; ** P <0.01; *** P <0.001).

Figure 8. T-cadherin upregulation increases cyclin D1 protein concomitant with a decrease in the ratio of phosphorylated cyclin D1 to total cyclin D1. Immunoblots of crude lysates from R-SMCs (A) and H-SMC (B) probed for cyclin D1. Akt was used as loading control. Levels in Tcad⁺-SMC, shC-SMC or shTcad-SMC are expressed relative to control E-SMC. (C) Whole cell lysates of E and Tcad⁺ R-SMC and P-SMC transductants were immunoblotted for total cyclin D1 and p-cyclin D1^{Thr298} (and Akt as loading control). Detection of signals for p-cyclin D1^{Thr298} necessitated that blots be developed for a much longer period than blots probed for cyclin D1. Typical blots are shown. Signal intensity ratios p-cyclin D1^{Thr298} to cyclin D1 were calculated and expressed relative to the ratios in the control E-SMC. Significant differences are marked with asterisks (* P <0.05; ** P <0.01; *** P <0.001).

Figure 9. Blockade of negative mTOR/S6K1 signalling feedback by rapamycin does not restore differentiation marker expression in Tcad⁺-SMC. E and Tcad⁺ R-SMC transductants were cultured in the absence (Ctrl) or presence of 30nM rapamycin (rapa) for 8h (A, B) or 72h (C, D). (A) Immunoblots from whole cell lysates probed for the indicated proteins. Akt or Histone H3 were used as internal loading controls. The histogram depicts densitometry-quantified levels of indicated proteins expressed relative to respective levels in control E-SMC. Letters a-d within histogram indicate significance differences (P at least < 0.05). a: significant difference between E and Tcad⁺ under control conditions; b: significant effect of rapamycin treatment on E and Tcad⁺. c: significant effect of rapamycin in E only, arrows depict direction (increase or decrease) of change; d: significant difference between E and Tcad⁺ in the presence of rapamycin. (B) Immunoblots from whole cell lysates probed in parallel for total cyclin D1 and p-cyclin D1^{Thr298} (and Akt as loading control). The histogram depicts quantification of the p-cyclin D1^{Thr298} to cyclin D1 ratio expressed relative to that in control E-SMC. (C) Phase contrast micrographs of E- and Tcad⁺-SMC. Scale bar: 100µm. (D) Cell proliferation estimated by cell enumeration. (B, D) Significant differences are marked with asterisks (* P <0.05; ** P <0.01; *** P <0.001).

Figure 10. Ectopic expression of constitutively active GSK3β restores differentiation marker expression and blocks hyperproliferation in Tcad⁺-SMC. (A) Immunoblots of whole cell lysates of E and Tcad⁺ R-SMC transductants without (ctrl) or following co-

transduction with empty adenovector (&Adv-E) or adenovector encoding constitutively active mutant of GSK3 β (&Adv-S9A). GSK3 β was used to control for transduction efficiency and Akt served as loading control. Data for densitometry-quantified levels of the indicated proteins in the ctrl and the co-transduced SMC are expressed relative to the levels in E &Adv-E SMC. (B) Proliferation of control (Ctrl) and co-transduced E- and Tcad+-R-SMC estimated by cell enumeration after 72h culture. Significant differences are marked with asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (C) Phase contrast micrographs of subconfluent monolayers of the co-transduced E- and Tcad+-R-SMC. Scale bar: 100 μ m.

Figure 11. A model for T-cadherin-dependent regulation of SMC phenotype.

Upregulation of T-cadherin in SMC induces proliferation and a phenotype transition toward dedifferentiation *via* distinct branches of the Akt pathway. Activation of Akt by T-cadherin upregulation leads to phosphorylation and inactivation of GSK3 β promoting both proliferation and loss of differentiated contractile phenotype. These effects of T-cadherin can be ameliorated by ectopic co-expression of constitutively active GSK3 β -S9A mutant. T-cadherin-dependent hyperactivation of Akt signalling axis also leads to stimulation of the mTORC1/S6K1 negative feedback loop resulting in IRS-1 degradation. Inhibition of mTORC1 by rapamycin prevents T-cadherin induced proliferation but not loss of contractile markers. Thus, T-cadherin-dependent acquisition of the dedifferentiated phenotype is dependent on GSK3 β inactivation and not on the mTORC1/S6K1 branch of the Akt pathway.

Figure 1

Figure 2

[illegible]

A

R-SMC P-SMC R-SMC P-SMC R-SMC P-SMC

smMHC Akt E Tcad+ E Tcad+ E Tcad+ E Tcad+ E Tcad+ E Tcad+

h-caldesmon Akt E Tcad+ E Tcad+ E Tcad+ E Tcad+

calmodulin Akt E Tcad+ E Tcad+ E Tcad+ E Tcad+

smMHC (vs. E)

h-caldesmon (vs. E)

Calmodulin (vs. E)

relative MMP level (vs. E)

pro-MMP-2 MMP-2

relative MMP level (vs. E)

pro-MMP-2 MMP-2

Legend: E (white), Tcad+ (black), shC (light gray), shTcad (dark gray)

B

H-SMC

E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad

smMHC Akt E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad

h-caldesmon Akt E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad

calmodulin Akt E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad

Pro-MMP-2 MMP-2 E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad E Tcad+ shC shTcad

smMHC (vs. E)

h-caldesmon (vs. E)

Calmodulin (vs. E)

relative MMP level (vs. E or shC)

pro-MMP-2 MMP-2

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11