T-cadherin regulates vascular smooth muscle cell contractile signalling and promotes acquisition of the de-differentiated phenotype

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To my sister Simona

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Contents

ABSTRACT

Cadherins are a superfamily of intercellular adhesion molecules essential for structural maintenance of tissue cohesion, precise primary tissue segregation and regulation of regeneration processes in adult. Cadherins are widely expressed in the vasculature. Adherens junctions and desmosomes, where cadherins are the intercellular adhesion transmembrane linkers, have been demonstrated in large and small arteries *in vivo* and their participation in correct organization of vascular smooth muscle architecture is doubtless. However, knowledge on precise functional roles for cadherin in healthy or diseased vascular smooth muscle is limited.

T-cadherin is an atypical cadherin highly expressed on endothelial and smooth muscle layers of the vasculature. Dynamic T-cadherin expression on vascular smooth muscle *in vivo* has been reported in number of vascular pathologies including two major vasoproliferative disorders – atherosclerosis and restenosis. Functions and molecular mechanisms regulated by this molecule in the smooth muscle cell component of the vasculature are unknown. The primary functions of vascular smooth muscle cells (VSMC) are contraction and regulation of blood vessel tone. However, VSMC possess inherent plasticity: they can switch from mature contractile phenotype to a de-differentiated proliferative and synthetic phenotype in response to vascular injury, or local environmental cues signalling. Studies in this dissertation are aimed at establishing cellular functions for T-cadherin in VSMC contraction and phenotype plasticity and identifying mediating molecular mechanisms.

First, we found that T-cadherin modulates non-metabolic insulin signalling via Akt/mTOR, which in turn leads to alterations in VSMC contractile competence and increased matrix remodelling. T-cadherin overexpressing cells exhibited elevated constitutive levels of phosphorylated Akt^{ser473}, $GSK3\beta^{ser9}$, $S6RP^{ser235/236}$ and $IRS\text{-}1^{ser636/639}$. Contractile machinery was constitutively altered in a manner indicative of reduced intrinsic contractile competence, namely decreased phosphorylation of $MYPT1^{thr696}$ or $MYPT1^{thr853}$ and $MLC_{20}{}^{thr18/ser19}$, reduced RhoA activity and increased iNOS expression. T-cadherin overexpressing VSMC-populated collagen lattices exhibited greater compaction which was due to increased collagen fibril packing/reorganization. These cells also exhibited a state of insulin insensitivity as evidenced by attenuation of the ability of insulin to stimulate Akt/mTOR axis signalling, phosphorylation of MLC_{20} and MYPT1, compaction of free-floating lattices and collagen fibril reorganization in unreleased lattices.

Second, T-cadherin upregulation on VSMC, a phenomenon observed in VSMC-driven vascular pathologies (atherosclerosis and restenosis) promotes VSMC phenotype transition. T-cadherin upregulation in VSMC caused loss of spindle morphology, reduced/disorganized stress fibre formation, decay of SMC-differentiation marker proteins, increased levels of β-catenin and cyclin D1, and migro-proliferative behaviour. Genetic T-cadherin ablation, on the other hand, enforced differentiated phenotype. T-cadherin hyperactivates Akt axis signalling and inactivates classical downstream effector GSK3β. Ectopic adenoviral-mediated co-expression of constitutively active GSK3β restored morphological, molecular, and functional characteristics of differentiated VSMC in T-cadherin overexpressing cells, suggesting that GSK3β inactivation is essential for T-cadherin induced VSMC de-differentiation.

The studies have revealed novel cadherin-based modalities to regulate VSMC sensitivity to insulin and phenotype plasticity, which is achieved via Akt/mTOR axis hyperactivation and altered downstream effector signalling.

INTRODUCTION

1. Smooth muscle and its function

1.1. Smooth muscle

Smooth muscle is distributed in many parts of the body and has varied but highly specialized functions based on coordinated cycles of contraction and relaxation. Smooth muscle is mostly found in the hollow and tubular organs where it ensures directional movement of body fluids, or regulates organ size and shape. Organized in sheets or arrays of bundles, smooth muscle consists of uninuclear elongated, spindle shaped cells embedded and enwrapped in large amounts of extracellular matrix (ECM). In large elastic arteries ECM can constitute more than 50% of the media volume. Most of this ECM is produced and maintained by smooth muscle cells (SMC) [1].

1.2. Smooth muscle cells

The characteristic elongated spindle shape of SMC in mature smooth muscle is dictated by its principal function, which is to contract and generate mechanical output needed for the function of a particular organ. However, within the adult organism SMC expressing common set of classical marker genes may be very heterogeneous in morphology. This depends on cell location (e.g. veins vs. arteries), its embryological origin, or the organ context [2]. Smooth muscle is non-striated. Contractile apparatus of the SMC, primarily actin and myosin filaments, is attached to the cell membrane and criss-cross the entire cell body. The cell membrane is rich in ion channels, cell-to-cell and cell-to-matrix junctions, all dedicated to ensure effective communication with the environment and robust action coordination with neighbouring cells [1]. Smooth muscle function is based on cooperation between mature myocytes, as well as between myocytes and surrounding extracellular matrix. Smooth muscle function is controlled by a broad variety of stimuli – neural, mechano, hormonal, light, changes in temperature, or self SMC-induced myogenic signal propagation [1].

Vascular smooth muscle cells (VSMC) are stromal cells of the blood vessels. VSMC maintain normal blood pressure and provide structural stability and resistance to the mechanical stress in the vessels [1]. In early development VSMC in arteries and veins and pericytes in capillaries muscularize primary vessels formed by endothelial cells (EC), providing structural stability and functionality [3]. Well-coordinated cycles of smooth muscle contraction and relaxation adjust vessel diameter and regulate hemodynamics. In addition, VSMC synthesize ECM, which helps to resist the high pressure load of circulating blood and prevent physical permeability in large vessels [4].

1.3. Smooth muscle contraction

Multiple pathways exist for activation of SMC contraction. Cytosolic Ca^{2+} plays a central role in initiating and regulating contraction. The key effector in SMC contraction/relaxation signalling and mechanics is the 20 kDa myosin light chain protein (MLC_{20}) [1]. In its active state MLC_{20} activates Mg^{2+} ATPase function of myosin and enables it to bind and slide along the actin filament, eventually resulting in cell and muscle contraction [5].

Ca^{2+}-dependent MLC_{20} activation is mediated by myosin light chain kinase (MLCK), which in turn depends on the Ca^{2+}-calmodulin system. The system is activated by an increase in free cytoplasmic Ca^{2+}; this happens when extracellular stimuli open Ca^{2+} channels and extracellular or sarcoplasmic

free Ca^{2+} influx in to intracellular space. Intercellular Ca^{2+} complexes with calmodulin, activates MLCK and thereby initiates SMC contraction. Ca^{2+}-dependent SMC contraction is controlled by signals which regulate activity of numerous receptor operated Ca^{2+} channels (ROCC), located on the cell membrane or on sarcoplasmic reticulum [5]. Well known ligands such as prostaglandins, endothelin-1, norepinephrine, angiotensin II and vasopressin activate ROCC and allow Ca^{2+} influx [6]. Additional control is provided by voltage-dependent channels.

A second contraction signalling mechanism in SMC is independent of $Ca^{2+}$and is called Ca^{2+} sensitisation. Ca^{2+}-sensitisation is mediated by small Rho GTPase RhoA and Rho associated kinase (Rho-kinase) [7]. In addition to Ca^{2+} channel control, vasoconstrictors can activate the RhoA/Rho-kinase signalling pathway. This pathway regulates myosin light chain phosphatase (MLCP) and MLC_{20} activities (active MLCP directly binds to and dephosphorylates/inactivates MLC_{20}) RhoA recruits and activates Rho-kinase, which negatively regulates MLCP activity by phosphorylation and heightens SMC contraction [8]. In addition, Rho-kinase directly phosphorylates and activates MLC_{20}, further promoting SMC contractility [9].

SMC relaxation is achieved by removal of contractile stimuli and reduction of intracellular Ca^{2+} or by inhibition of RhoA/Rho-kinase signalling [5]. Vasodilators act by closing Ca^{2+} channels, activating outward Ca^{2+} pumps, and inhibiting RhoA/Rho-kinase pathway signalling. Reduction of free cytosolic Ca^{2+} activates MLCP, which dephosphorylates/ inactivates MLC_{20}, enabling the cell to switch into relaxation phase. SMC relaxation can be induced by natriuretic peptides and vasodilators adenosine, adrenomedulin, nitric oxide (NO), or insulin.

Insulin is a versatile signalling molecule and regulates multiple processes in VSMC. In addition to its "canonical" metabolic function, insulin plays an important role in maintenance of differentiated VSMC phenotype (see section 4.2.1.3.), and reducing vascular tone [10]. As a vasodilator insulin affects both Ca^{2+}-dependent, and Ca^{2+}-independent SMC contraction. Insulin regulates Ca^{2+}-dependent contractile signalling via inhibition of Ca^{2+} influx and induction of Ca^{2+} efflux [11-13]. Insulin affects Ca^{2+}-independent contractile signalling by targeting RhoA activity. Insulin signalling induces NO synthesis by activation of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS), both of which are found in SMCs [14]. NO activates guanylate cyclase, and induces production of cGMP. cGMP activates cGMP-dependent protein kinase α (PKG I α), which binds and inactivates RhoA (by phosphorylation on Ser^{188}) [15]. In addition, insulin reduces RhoA translocation to the membrane by inhibition of geranyl-geranyl transferase [16]. Insulin inhibitory pathway on SMC contraction is phosphatidylinositol-3-kinase (PI3K) and Akt signalling dependent [17,18].

1.4. Vascular smooth muscle repair and renewal (turnover)

VSMC have functional plasticity. In addition to regulation of hemodynamics and provision of structural support, VSMC fulfil vascular repair functions. Pre-existing mature VSMC are the major contributors of smooth muscle repair and vessel renewal [19]. Mature VSMC retain high degree of cellular plasticity and can switch their phenotype from quiescent-contractile to migro-proliferative and synthetic. On demand or guided by environmental cues fully differentiated VSMC can adjust their phenotype by de-differentiation in order to adopt reparative functions. VSMC undergoing de-differentiation lose contractile machinery and competence, increase ECM production and intensify matrix remodelling, become migratory, and re-enter cell cycle. Once vessel homeostasis and structural integrity is restored, de-differentiated phenotype then re-differentiates back to the

mature, contractile state [20]. In a healthy vascular wall individual VSMC de-differentiation occurs at a low event rate and ensures constant vascular wall renewal, or in case of injury, repair to damaged tissue. However, when phenotype transition controlling mechanisms fail, VSMC plasticity is detrimental. Persistent pathological VSMC-driven reparation significantly contributes to life threatening conditions like atherosclerosis, restenosis, or graft failure [19]. Despite high demand for improved cardiovascular therapeutics and intense research, mechanistic pathways controlling VSMC phenotype remain incompletely understood.

2. Cardiovascular diseases

Cardiovascular diseases (CVD) are the major cause of death worldwide [21] (Fig.1.). CVDs are heart or vasculature dysfunctions which include coronary heart diseases causing heart attacks, cerebrovascular disease causing stroke, hypertension, peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure.

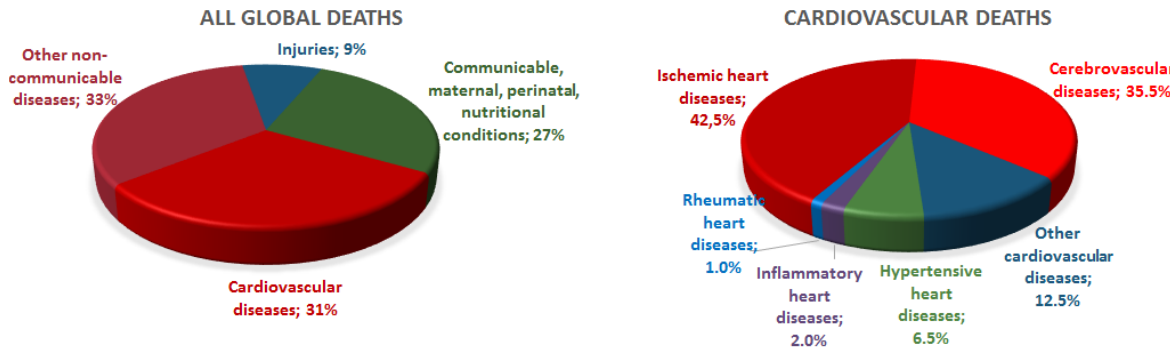

Fig.1. Global deaths rates by the cause of deaths under age of 70 years. Cardiovascular diseases are leading causes of deaths worldwide - 31% of all deaths. Ischemic heart diseases and cerebrovascular diseases have the highest mortality rates among the cardiovascular diseases. 2012 estimates by WHO [21].

The latest (2014) global status report of noncommunicable diseases published by WHO estimated 17.5 million CVD caused deaths in 2012, which represents 31% of all deaths worldwide and more than half of all deaths across the European Region. 7.4 million of all global CVD deaths were due to coronary heart disease, and 6.7 million due to the stroke together taking 80% of all CVDs [21]. Considering some global demographic and socioeconomic trends – population aging, economic growth in Asia, and endemic spread of obesity in developed countries – high cardiovascular death rates will remain an unsolved problem. It is predicted that the proportion of CVD death will only rise [22]. The introduction of statin therapy and drug-eluting stents a few decades ago offered major breakthroughs in the clinical management of CVD, however, novel, more targeted and, importantly, low cost therapeutic and early diagnostic methods are needed. About 75% of CVD deaths occur in low- and middle- income countries [21], bringing additional economic burden to the weak economies.

The highest risk factors for CVDs are behavioural: tobacco, alcohol use and bad body biochemistry (unbalanced diet, low physical activity and obesity). Higher CVD mortality rates in women suggest that important, but not yet well understood non-environmental factors play a role in cardiovascular health. Ultimately, deeper understanding of basic cardiovascular physiology and CVD mechanisms is needed. Better awareness of the major risk factors and lifestyle adjustments would certainly help to reduce the premature deaths from CVDs, however a range of diverse strategies is needed to reach

substantial result [23], and fundamental research should be one of them. – Past results show that cardiovascular research is a highly cost effective research – the returns from past cardiovascular research have been substantial, yielding 9% of internal rate of return in the UK [24], and 20.6% in Canada [25].

2.1. Vascular smooth muscle dysfunction driven cardiovascular diseases

Smooth muscle and SMC have been studied or used as model systems for decades, yet we still do not understand their biology well enough to be able to prevent or treat the smooth muscle-related pathophysiological conditions effectively. Smooth muscle disorders are numerous, and mostly non-treatable. Highest mortality rate bearing CVDs are caused by pathological vessel narrowing, which on cellular and molecular levels is largely the outcome of not yet therapeutically controllable processes and inadequate VSMC response to changes in body biochemistry.

2.1.1. Atherosclerosis and restenosis

Coronary artery diseases (CAD) leading to ischemic heart disease and stroke are the most common among cardiovascular diseases and the major causes of death worldwide (Fig.1.). CAD arises from progressing atherosclerosis, a multiplex disease caused by disbalanced lipid metabolism and maladaptive immune reaction followed by inadequate vascular cell response [26-28]. Atherosclerosis is characterised by chronic vascular wall inflammation, progressive narrowing of the vessel lumen and eventual plaque formation.

An initial step in atherosclerosis is driven by EC. Activated EC mediate immune cell adhesion to the vascular wall and monocyte infiltration to the intima where they initiate inflammation. Appearance of neointima is observed before formation of atheroma. Growth factor- and inflammatory cytokine-activated intimal VSMC proliferate rapidly and produce large amounts of ECM. In addition, intimal growth is further exacerbated by infiltration of medial VSMC which migrate to the intima in response to platelet-derived growth factor (PDGF) and other cytokine stimuli. This eventually culminates in vascular wall thickening and narrowing of the lumen [19].

Monocytes which infiltrate to the expanding intima mature to macrophages, uptake high amounts of lipids and transform into foam cells [27]. In response to inflammatory factor stimulation and cholesterol loading, VSMC also acquire macrophage-like phenotype; they start to express macrophage and mesenchymal stem cell (MSC) markers, uptake lipids by endocytosis/phagocytosis eventually turning into foam cells [29-31]. Foam cells undergo apoptosis or necrosis and form cholesterol deposits, which accumulate and form a necrotic core. Further infiltration of VSMC and immune cells and chronic inflammation leads to necrotic core growth [32]. Ultimately, progression of such plaques can lead to rupture of the vessel wall and vessel thrombosis which hinders blood supply to the heart muscle or brain, causing myocardial infraction or stroke, respectively. Atherosclerotic plaques, covered by thick VSMC and VSMC-derived ECM cap are considered stable and rarely rupture [33]. However, if the inflammatory process progresses further, fibrous cap-forming VSMC undergo apoptosis which leads then to thinning of the fibrous cap and plaque rupture [34].

Altered lipid metabolism and low density lipoprotein (LDL) pathway plays central role in atherosclerosis initiation and progression [32]. Statins, the inhibitors of hydroxymethyl glutaryl coenzyme A reductase, are the most efficient drug currently used as a mainstream therapy in atherosclerosis patients. However, statins do not cure the disease completely, and in about 20% of all patients statins are ineffective in prevention of vascular events [34,35]. Surgical interventions (e.g.

angioplasty) have similar failure rates (16-20%) [36,37] since endovascular procedures frequently lead to restenosis, which is a re-narrowing of the vessel caused primarily by excessive smooth muscle proliferation (hyperplasia) and ECM deposition [38].

Until very recently, it was widely accepted that atherosclerosis is an immune cell-driven disease, and VSMC play only a marginal role [32]. This was mostly due to unreliable lineage tracing methods and an overlooked fact of extensive phenotype plasticity in both cell lineages (hematopoietic and SMC) within the microenvironment of atherosclerotic plaque [28]. VSMC within the lesion lose most of the SMC markers and can express macrophage and mesenchymal stem cell (MSC) markers, while macrophages may begin to express *Acta2* and other SMC markers. Use of traditional immunolabeling in advanced atheromas may thus lead to misidentification of cell type. The need for revision of cell identification methods was urged by an accumulating number of studies showing that identification of cell origin in the atheroma by conventional methods might be very inaccurate. One study reported that 50% of foam cells in the advanced atheroma expressed smooth muscle (SM)-α-actin (classical SMC marker), as well as macrophage marker CD68 [31]. A study which used material derived from cross-gender bone marrow transplantation subjects found that >10% of SM-α-actin positive cells within atheroma is of hematopoietic origin [39]. A very recent study which used mouse genetic manipulations to track cell lineage showed that about 30% of all cellular mass within atherosclerotic lesions are phenotypically modulated SMCs, variously expressing SMC, macrophage or MSC markers, and that more than 80% of all of these cells would have been misidentified with conventional labelling methods [28].

The study by Shankman, Gomez et al. demonstrated the existence of transcription factor-specific guidance of SMC phenotype *in vivo*, suggesting that VSMC phenotype transition in atheroma can be manipulated in order to increase plaque stability. Krüppel-like factor 4 (KLF4) is one of the most powerful SMC phenotype transition regulating factors (see section 4.1.2.), which guides SMC to acquire a pro-inflammatory macrophage-like phenotype. KLF4 knockout results in both formation of smaller and more stable atheromas due to the enrichment of SMC-like cells which form the fibrous cap, and loss of the pro-inflammatory, macrophage-like SMC population [28].

These findings confirm the importance of VSMC phenotype transition in atherosclerosis. Previously assumed protective role of VSMC in atheroma stabilization is only one of many functions that VSMC can assume via phenotypic switching. As a large body of *in vitro* data suggests, VSMC plasticity is remarkable and can lead to acquisition of many functionally distinct phenotypes (synthetic SMC, macrophage-, MSC- chondrogenic- or osteogenic-like cells), with very different outcomes in disease progression. Moreover, VSMC phenotype transition is an environmental cue-guided, rather than spontaneous process and can be manipulated in order to reduce the degree of disease pathogenesis. To explore this option, a deeper understanding of basic molecular pathways controlling VSMC phenotype transitions, the identification of environmental cues which regulate it, and the development of therapeutic tools to manipulate VSMC phenotype transition are of crucial importance.

2.1.2. Cerebral microangiopathy

Cerebral microangiopathy or small vessel disease (SVD) is another cause of vascular dementia and strokes [40]. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by defective Notch signalling in VSMC, which occurs due to the dominant Notch3 mutation [41]. It was long thought that CADASIL occurs due to the loss of

Notch signalling, but accumulating evidence now supports that mutant Notch3 switches canonical Notch signalling to some other mode and modulates VSMC phenotype [42]. Progressing CADASIL leads to VSMC loss in small arteries, vessel stenosis, resting cerebral blood flow reduction, accumulation of ECM components (collagens and laminin) and osmiophilic material, and early death. No specific therapies are available to stop the progression of CADASIL or treat it [42].

2.1.3. Marfan syndrome

Marfan syndrome is an intrinsic monogenic disorder which targets VSMC and shifts vascular VSMC phenotype towards a more differentiated state. VSMC "over-differentiation"-caused vascular instability in Marfan syndrome illustrates well the importance of balanced VSMC phenotype plasticity for normal vascular function.

Marfan syndrome is caused by mutations in fibrilin-1, an ECM protein encoding gene. Fibrilin-1 is a structural component of connective tissue, but also a negative regulator of transforming growth factor-β (TGF-β) activity (fibrilin-1 bound TGF-β is inactive). TGF-β is a pro-differentiation cytokine in VSMC (see section 4.2.1.2.). Reduction in fibrilin-1 abundance results in excessive TGF-β signalling, which locks VSMC in a differentiated state and suppresses their reparatory and synthetic function. VSMC "over-differentiation" adds to structural and functional fragility of the vessels with increased risk of aortic aneurism formation and aortic dissections, which are the major causes of early death in Marfan syndrome [43,44].

2.1.4. Hypertension

Systemic hypertension is another circulation dysfunction, where VSMC are the targets of "upstream" pathophysiological changes. Endocrine or kidney dysfunction, aging, metabolic syndrome, or other not yet well understood processes increase vasoconstrictor signalling which manifests as a vascular smooth muscle mediated disorder [45].

Hypertension is a systemic and chronic disease characterized by high arterial pressure and increased vascular resistance. Mechanistically, hypertension is the outcome of alterations in renin-angiotensin-aldosterone or/and calcium-calmodulin systems [46]. Abnormalities in vasoconstrictor signalling enhance protein kinase C (PKC) and Rho-kinase signalling, which lead to VSMC hypertrophy (elevated contractile protein synthesis), increased VSMC contractility, vascular remodelling and extensive ECM synthesis resulting in increased vascular wall rigidity [47,48]. Constant exposure to enhanced and sustained mechanical stretch activates mitogen-activated protein kinase (MAPK) pathway and promotes VSMC proliferation [49]. In addition to dysregulation in upstream signalling, VSMC hypersensitivity to calcium or vasoconstrictor signalling is also often observed in hypertension [50], suggesting that in some cases intrinsic VSMC defects can be responsible for disease development as well.

Medial hypertrophy and arterial resistance cause permanent changes in arterial architecture and worsen high blood pressure. Untreated hypertension can progress to hypertensive heart disease, stroke, aortic aneurisms or kidney failure. It highly promotes the development and progression of other conditions such as diabetes, endothelial dysfunction or atherosclerosis [45].

2.1.5. Pulmonary arterial hypertension

Similarly to systemic hypertension, medial VSMC layer enlargement, intimal thickening and increased pulmonary artery resistance are observed in pulmonary arterial hypertension (PAH). These

anatomical changes restrict blood circulation between the lungs and the heart, which ultimately can result in right-sided heart failure. The causes of PAH can be very varied and multiplex with not yet identified molecular mechanisms or no direct relations to VSMC dysfunction. Familial PAH however is caused by intrinsic genetic VSMC defects, namely the loss-of-function mutation in bone morphogenetic protein (BMP) receptor-2. Reduced pro-differentiation BMP signalling in pulmonary artery VSMC and EC of familial PAH patients translates into weaker SMAD1-mediated p38/MAPK signalling repression, VSMC de-differentiation and hyperproliferation [51,52]. Extensive VSMC proliferation and matrix secretion causes enlargement of the intima and narrowing of the pulmonary artery.

2.1.6. Role in non-cardiovascular diseases. Cancer metastasis

Cancer does not belong to the group of CVDs. However, VSMC function might play an important role in the progression of this disease. Highly metastatic tumours often have poorly developed leaky blood vessels. This is caused by pericyte failure to differentiate and muscularize newly formed vessel [53]. It is not known whether leaky cancer vessels are the result of cancer microenvironment signalling, or the pre-existing and cancer spreading-permissive tissue dysfunction. It has been shown that the molecular signature comprising 17 genes in highly invasive tumours contained 4 pericyte differentiation marker genes (all downregulated) [54]. Leaky vessels favour physical cancer cell spreading and facilitate haemorrhage and inflammatory processes within the tumour, which further promotes tumour growth [55]. The role of VSMC phenotype switching in cancer pathogenesis is understudied, and the possible mechanistic basis of impaired tumour blood vessel maturation from the perspective of VSMC biology is not yet understood [56].

3. Vascular smooth muscle cell plasticity

3.1. Phenotype modulation, phenotype switching and phenotype transition

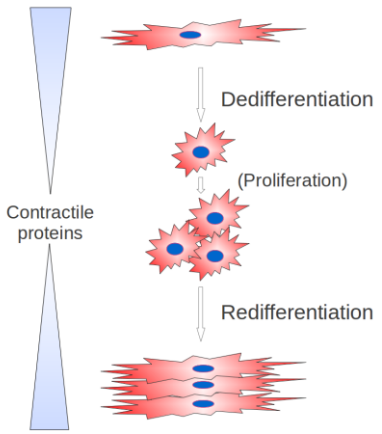

Fig.2. **VSMC phenotype plasticity**. In vascular injury, or guided by environmental cues, fully differentiated SMC de-differentiate by losing contractile apparatus (blue gradient illustrating expression intensity on the left) and re-enter cell cycle. Once tissue repair is complete or de-differentiation stimuli removed, SMC re-differentiate fully regaining contractile function.

The term “phenotypic modulation” was introduced by Julie Chamley-Campbell et al. more than 30 years ago [57] to describe the sum of morphological changes that VSMC undergo when cultivated *in vitro*. Now, together with synonymous “phenotype switching” or “phenotype transition” it envelops all functional-, structural- and molecular-level alterations that smooth muscle cells undergo in response to changing environmental cues, and it is no longer limited to *in vitro* cultures [19].

VSMC-specific marker expression is a classical and one of the most important phenotype characteristics used to describe VSMC phenotype. Decreased expression of VSMC differentiation markers (mostly vital components of VSMC contraction machinery and cell anchorage (e.g. smooth muscle SM-α-actin, SM myosin heavy chain, calponin, *h*-caldesmon)) is universally accepted as an indicator of VSMC de-differentiation. As levels of VSMC-specific marker

expression decline, expression of other proteins often involved in cell cycle progression or ECM components (e.g. collagen I, matrix-metalloproteinases (MMPs), calmodulin, cyclins) increases (Fig.2.).

SMC marker decay and morphology changes together with altered functional characteristics, increased migration and hyper-proliferation, are the indications of a full VSMC phenotype switch. It is important to note that the mentioned characteristics describe the "averaged", or "ideal" de-differentiation status. In the injured or diseased vessel, populations of de-differentiated SMC can be very diverse in the grade of de-differentiation, but also because of phenotype divergence due to either exposure to different environmental cues and/or due to their different embryologic origins (even in a healthy adult vessel smooth muscle is very heterogeneous and resembles mosaic architecture [19]). Upon exposure to different environmental cues VSMC can acquire a spectrum of phenotypes: contractile-quiescent, migro-proliferative synthetic, osteogenic-, macrophage-, or MSC-like [28].

Although VSMC phenotype switching is a major mechanism for vessel renewal and repair, alternative mechanisms within the vessel might also exist. - Multipotent vascular stem cells which can differentiate to VSMC have been found in vascular wall [58]. However, the importance and contribution of these cells for vessel renewal and repair is not yet clear [59].

3.2. Experimental models to study VSMC plasticity

Experimental restenosis of the rat carotid artery after balloon injury is the most commonly used *in vivo* model to study VSMC plasticity. Rat carotid balloon injury is made by repeatedly inflating and withdrawing balloon catheter in the carotid artery in order to mechanically remove the endothelial lining [60]. Such injury induces vessel remodelling responses – intimal SMC proliferation, matrix production and medial SMC invasion to the intimal space (all via phenotype transition), – which result in neointima formation and growth. This model offers robust reproducibility and has been used to study cellular, biochemical and molecular aspects of vessel repair, and also serves as a "proof of concept" tool for verification of *in vitro* findings [61].

The ApoE-deficient mouse (*ApoE*$^{-/-}$) is the most popular *in vivo* model to study atherosclerosis. ApoE is a major apoprotein of the chylomicron and is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. Due to the impaired clearing of plasma lipoproteins *ApoE*$^{-/-}$ mice spontaneously develop hypercholesterolemia and atherosclerosis in a very short time – 3 months if fed a normal chow. The process can be accelerated by Western-type diet chow containing high levels of fat and cholesterol. The close resemblance of atherosclerotic lesions developed by this mice to those in human atheroma together with short disease development time makes this model the most attractive *in vivo* model to study atherosclerosis [62].

Human- or animal-derived primary SMC tissue cultures have been used for decades to study SMC phenotype plasticity and contraction mechanisms. Contractile VSMC collected from human or animal vessel and cultured *in vitro* start to de-differentiate immediately due to the exposure to the high concentration of growth factors present in medium [63]. Growth factor withdrawal from the culture (serum starvation) reverts the phenotype to the differentiated state [64]. This phenomenon is very useful to study factors and conditions regulating SMC phenotype plasticity. However, primary SMC cultures have a number of inherent limitations which can lead to irreproducibility or generation of artefacts. Vascular smooth muscle is a highly heterogenic tissue, composed of different SMC subtypes derived from at least eight independent origins, each of which can respond differently to the applied stimuli and acquire diverse phenotypes [65]. High heterogeneity exists not only between different segments of the vessel, but also within the same vessel segment: therefore small inaccuracies in tissue isolation can lead to high variability in resulting primary cultures. This can be

further complicated by the variations in VSMC isolation and cultivation methods which can lead to conflicting result between different laboratories [56]. Isolation of SMC by tissue explant method preselects primarily for cells which have higher migration capacity. Isolation by enzymatic digestion is prone to fibroblast contamination and also high variability between isolates prepared on different occasions due to tissue "under-digestion" or "over-digestion" [66]. Thus, the method of isolation will markedly affect the dominance of different sub-populations in resulting cell cultures. Moreover, human VSMC isolates are susceptible to quick senescence and are often "preconditioned" by donors' age, gender or received treatments.

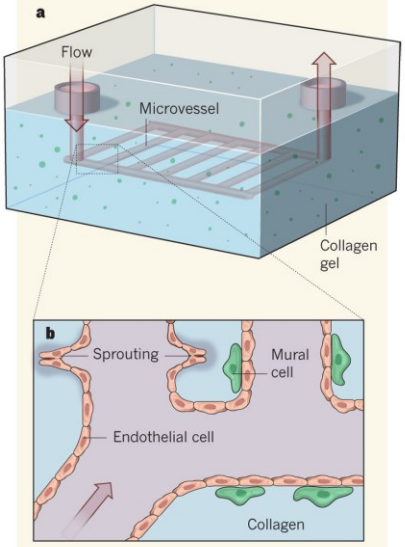

Fig.3. Living three-dimensional microvascular network – a blood vessel on a chip. The *in vitro* 3D perfusable model system used to study angiogenesis and thrombosis [68]. Additional system components (e.g. lymphocytes, signalling molecules, antibodies, inhibitors) can be introduced temporarily or constantly with the culture medium that constantly flows through the channels. Adapted from [69].

SMC phenotypic transition in the mature vessel involves the reactivation of embryonic stem cell pluripotency genes, which closely resemble SMC behaviour during vessel development [29]. Similarly like during de-differentiation, in early vasculogenesis SMC are highly migro-proliferative and exhibit intensive ECM production and remodelling [56]. Due to phenotypic similarities between SMC phenotypes observed in de-differentiation and vasculogenesis, stem cell-derived tissue culture lines primarily designed to study developmental differentiation are often used to investigate SMC phenotype transition. Unlike primary SMC cultures, stem cell-derived cell lines offer high origin purity and can be used to study SMC origin-specific phenotype plasticity aspects.

Several neural crest stem cell-derived and immortalized cell lines (e.g. Monc-I, JoMaI) are used as *in vitro* SMC differentiation models. TGF-β1 stimulation induces quick and robust differentiation toward SMC lineage in these cell lines. However, as models to study full SMC differentiation these lines are limited by the constant or temporal oncogene expression that is used as the immortalization instrument in parental stem cells. C-myc, or v-myc can interfere with response to the stimuli, or with the SMC phenotype transition program [67].

Most popular mesoderm stem cell-derived models include 10T1/2 cells and human embryonic stem cell-derived mesenchymal cells (hES-MCs). Similarly to neural crest stem cell-derived lines, mesoderm-derived models differentiate to SMC upon stimulation with TGF-β1. SMC derivatives from 10T1/2 and hES-MCs can reach higher differentiation status than Monc-I and JoMaI cells. TGF-β1-stimulated 10T1/2 and hES-MCs can acquire a functional SMC phenotype as evidenced by expression of SM myosin heavy chain (the most stringent de-differentiated SMC marker), and responsiveness to contractile stimuli [68].

Standard *in vitro* culturing eliminates many important environmental cues (e.g. three-dimensionality and (blood) flow stimulation), but is still the most commonly used method to manipulate molecular networks and study cellular aspects of SMC plasticity in the controlled environment. On the other hand, *in vivo* methods suffer from variability, limited possibilities for real-time condition control or read-out, and differences between animal and human physiology. Fast advancements in microfluidics, tissue printing and organ-on-a-chip technology are opening new ways to integrate the best parts of *in vitro* and *in vivo* models and develop easily modifiable, living tissue-like systems that

allow dynamic control and real-time analysis. Living three-dimensional microvascular networks composed of endothelial cells and mural cells (pericytes) have been used successfully to study angiogenesis and thrombosis [69,70] (Fig. 3.). Atherosclerosis on a chip is anticipated in the near future.

4. Plasticity pathways

As in every biological process, so in VSMC phenotype transition, environmental guidance is transmitted and regulated on many levels: signal generation, signal transduction, gene transcription and epigenetic (chromatin modification). Crucial regulators of VSMC phenotype transitions are emerging in all regulatory levels.

4.1. Transcriptional regulators

4.1.1. SRF and Myocardin – two major VSMC transcriptional regulators

At the transcriptional level the key element for VSMC phenotype modulation is serum response factor (SRF). SRF is an evolutionary conserved and ubiquitously expressed transcription factor, which binds to a 10 base pair CArG-box sequence ($CC[A/T]_6CC$) that is predominantly found in muscle and cytoskeletal genes [71]. SRF functions by responding to two often opposing signals – growth vs. cytoskeleton architecture – which in the context of VSMC plasticity translates into proliferation and de-differentiation vs. quiescence and contractile competence. In a simplified view, SRF integrates signals reaching it via two pathways, namely the MAPK cascade, or pro-differentiation stimuli transmitted via small Rho GTPases and actin. (Parts of Rho GTPases and actin branch signalling can be used by growth factor signalling to induce VSMC de-differenciation as well, but in different molecular contexts). SRF was first discovered as a growth inducer [72]. It later become clear that in this function SRF can be replaced by other factors [73], [74], but for organization of functional cytoskeleton and effective contractile machinery, SRF is absolutely indispensable. *Srf* knockout mouse embryos die in early development due to the inability of mesodermally fated cells to migrate properly [75] and in later developmental stages it plays an essential role in differentiation of all three muscle types [76-79]. SRF is also required for neural, craniofacial, and hematopoietic development [80-82].

SRF is widely expressed, and is not exclusive to VSMC. Therefore as a regulator *per se* it has little control on VSMC phenotype shaping. The ability of SRF to adapt to particular context and respond adequately depends on its numerous cofactors. These belong to two protein subfamilies – ternary complex factors (TCFs), and myocardin protein family (myocardin (MYOCD) and myocardin-related transcription factors (MRTFs)). By classical view, the TCFs are effectors of MAPK signalling while MRTFs and MYOCD respond to Rho GTPases-actin and pro-differentiation signalling. Depending on cofactor availability, SRF initiates the respective gene expression program, and shifts VSMC phenotype accordingly.

Among SRF cofactors, MYOCD is the most potent VSMC differentiation-driving SRF coactivator known to date [83]. MYOCD expression is cardiac and smooth muscle specific [84], it is irreplaceable in early VSMC development, and is sufficient for full VSMC differentiation – MYOCD selectively induces expression of all tested VSMC-specific genes containing CArG box, including *MYH11, Tagln, Acta2, Cnn1*, ion channel encoding genes *Kcnmb1* and *Lmod1* [56], [83]. MYOCD competes for SRF with growth signal activated cofactors and maintains VSMC quiescence [85]. It antagonizes KLF4 [86] and cyclin D1 expression [87]. MYOCD mRNA expression is downregulated in VSMC during vascular disorders [88], [89].

In contrast to MYOCD, which is cardiac- and smooth muscle- lineage specific and unambiguously a pro-differentiation factor, two other myocardin family members MRTF-A and MRTF-B (also known as MKL1 (megakaryoblastic leukemia) and MKL2, respectively) are widely expressed and controlled by actin dynamics. In spite of early hypotheses suggesting myocardin substituting roles for MRTFs, it has now emerged that MRTFs might have context-dependent roles in VSMC phenotype regulation. Data on MRTF-B is quite consistent, and strongly suggests pro-differentiation role for this factor: MRTF-B is necessary for differentiation of cardiac neural crest cells into smooth muscle [90], remodelling of branchial arch arteries, smooth muscle differentiation [91], and normal VSMC-specific gene expression [92]. However, the role of MRTF-A is still under discussion: in one study MRTF-A expression *in vitro* induced SMC differentiation from undifferentiated embryonic stem cells [93], whereas and opposingly, another study found increased MRTF-A expression in the wire-injured femoral arteries of wild-type mice and in the atherosclerotic aortic tissues of ApoE$^{-/-}$ mice [94]. Combinatorial MYOCD, MRTF-A and MRTF-B, as well as other co-factor availability or stoichiometry might be of major importance for VSMC phenotype regulation too. Widely expressed protein SCAI (suppressor of cancer cell invasion) inhibits MRTF-A and MRTF-B activity in the cell nucleus [95]. This level of MRTF regulation has been discovered and investigated in the context of cancers and to date (2015) has not been reported to function in VSMC. SCAI involvement in VSMC phenotype modulation is unknown.

4.1.2. Krüppel-like factor 4

KLF4 plays a key role in VSMC phenotype transition during development [86], after vascular injury [96] , and in PDGF- or oxidized phospholipids-treated cells *in vitro* [97,98]. KLF4 negatively regulates expression of many VSMC differentiation genes, including *MYH11, Acta2*, *Tagln* and *Cnn2*, either by binding to the G/C repressor element, which is present in most of SMC-specific gene promoters, or by competing with SRF for CArG element binding [99,100]. KLF4 also activates pro-inflammatory genes; recent ChIP-seq analyses identified >800 putative KLF4 target genes in VSMC, many of which encode atherosclerosis relevant pro-inflammatory factors [28]. KLF4 knock-out in the atherosclerosis mouse model (Apo E$^{-/-}$) favours acquisition of the synthetic VSMC phenotype, and suppressed the pro-inflammatory macrophage-like phenotype transition [28].

4.2. Signalling pathways

4.2.1. Pro-differentiation signalling pathways

4.2.1.1. Rho-actin pathway

For a long time cytoskeletal actin dynamics was seen as a passive purely structural element of the cell. It is now becoming clear that the cytoskeleton is also a powerful modality of signal transduction and gene regulation.

Posttranslational activity of MRTFs is regulated by actin dynamics [101]. MRTFs have a globular actin (G-actin) binding domain (RPEL motifs), which traps these proteins in the cytoplasm, if G-actin is available for binding. Via regulation of effector protein activity, Rho GTPases modulate G/F (fibrillar)-actin equilibrium. Control of G-actin availability by Rho GTPases regulates abundance of two MRTF states: the G-actin-bound state which is cytoplasm entrapped and inactive, and the actin-free state that is ready for nuclear translocation and action [102]. Actin polymerization triggered by RhoA liberates MRTFs from G-actin and permits their translocation to the nucleus. This type of regulation allows for rapid modulation of transcriptional co-activator availability, as it does not involve covalent protein modification and can be easily reversed.

It is important to note that MYOCD has no RPEL motif, and is thus independent of regulation by G-/F-actin [103].

In VSMC RhoA-actin-MRTF signalling can be induced by a plethora of extracellular stimuli. Activity of small GTPases is regulated by guanine exchange factors (GEFs). Thrombin, sphingosine 1 phosphate (S1P), angiotensin II, TGF-β, BMP-2, calcium, integrins, cell tension (via integrins), matrix components collagen IV and laminin have been reported to induce RhoA-actin-MRTF signalling in smooth muscle cells [104].

4.2.1.2. TGF-β-Smad

TGF-β is a multifunctional cytokine, important for blood vessel morphogenesis and stability. TGF-β signalling in VSMC induces differentiation and regulates cell interaction with extracellular matrix. TGF-β stimulates mesenchymal precursor differentiation into VSMC and the establishment of a functional vasculature in early development [105]. TGF-β is crucial for vessel muscularization in angiogenesis and regulates VSMC plasticity in mature, established vessel [106]. The importance of TGF-β signalling for vascular biology is illustrated by its involvement in many vascular pathologies including atherosclerosis, aortic aneurysms, and hypertension [107].

Three isoforms of TGF-β are known in mammalians – TGF-β1, TGF-β2, and TGF-β3. All three are secreted as latent precursors. Activation via proteolytic cleavage by endoproteases is needed for biological function [108]. To maintain the signalling of this powerful cytokine under tight control, availability of active TGF-β is additionally regulated by interaction with ECM components [109] (see section 2.1.3.).

When active, TGF-β binds to TGF-β type II receptor. This allows type II receptor heterodimerization with type I receptor. In the formed heterodimer type II receptor phosphorylates and activates type I receptor, a serine/threonine kinase. Active type I receptor recruits and phosphorylates Smad2 and Smad3. Phospho–Smad2/3 form a complex with Smad4 and translocate to the nucleus where they bind multiple Smad-binding elements (SBEs) and GC-rich sequences and function as transcription factors [110-113].

TGF-β signalling also regulates activity of TGF-β control elements (TCE) and CArG boxes [114]. CArG and TCE are often found together in many VSMC-specific marker gene promoters, including *MYH11, Acta2, Tagln, Cnn1* [115].

TCE in VSMC is controlled by KLF4 and Krüpel-like factor 5 (KLF5), which repress or activate VSMC marker expression, respectively. KLF4 acts negatively and KLF5 positively on VSMC-specific gene expression [116]. TGF-β stimulation inhibits KLF4 expression through miR-143/miR-145, and removes KLF4-mediated repression from TCE genes. In addition, KLF5 competes with KLF4, and acts synergistically with TGF-β in gene transcription initiation [116].

TGF-β can also induce SRF expression and enhance its binding activity to the CArG box [117]; Smad3, the major mediator of TGF-β induced marker gene expression in VSMC [107], can interact with SRF and MYOCD and activate CArG dependent VSMC gene promoters [113,118].

TGF-β initiates, transactivates, or cross-talks with many other pathways to regulate VSMC plasticity in a context dependent manner. The best studied crosstalk examples are Smad-dependent RhoA and Notch signalling pathways. Dominant negative RhoA blocks Smad2 and Smad3 phosphorylation and impairs their nuclear translocation, while constitutively active RhoA enhances Smad-dependent promoter activity [119]. CBF1 (Notch-regulated transcription factor) interacts with Smad2/3 and enhances their transcriptional activity [120].

4.2.1.3. PI3K/Akt/mTOR pathway

Insulin-like growth factor (IGF) and insulin signalling via classical PI3K-Akt pathway suppresses the de-differentiation program in VSMC and maintains contractile phenotype [121]. Ligand activated IGF or insulin receptor recruits insulin receptor substrate (IRS-1) and activates it by phosphorylation on tyrosine residues, which provides a docking site for PI3K. Docking in close proximity to the inner leaflet of the plasma membrane gives PI3K physical access to its substrates, inositol phospholipids. PI3K converts (3,4)-bis-phosphate to phosphatidylinositol (3,4,5)-tris-phosphate (PIP3) and enrichment of PIP3 on the inner membrane side allows Akt binding to the membrane. At the membrane Akt gets activated by phosphoinositide-dependent kinase-1 (PDK1) and mTORC2. Phosphorylated by PDK1 only (on Tyr308) Akt is partly activated and can transmit the signal to mammalian target of rapamycin complex 1 (mTORC1) [122,123] [124]. Additional Akt phosphorylation on Ser473 by mTORC2 [125] or DNA-dependent protein kinase (DNA-PK) [126] stimulates full Akt activity and enables it to phosphorylate additional substrates. Fully active Akt regulates its downstream targets in the cytoplasm and nucleus, and represses the de-differentiation program in VSMC. For instance, Foxo4 phosphorylation by Akt-promotes its nuclear export and relieves its inhibitory influence on MYOCD activity [127].

Direct PI3K/Akt pathway signalling is auto-regulated by the classical negative feedback loop, which is activated by mTORC1 through Akt. mTORC1 activates S6K, which then phosphorylates IRS-1 on serine residues and marks IRS-1 for proteosomal degradation. Reduced availability of IRS-1 weakens direct insulin and IGF signalling pathway activity [128], reducing their suppressive actions on VSMC de-differentiation [129]. mTORC1 pathway is activated in response to vascular injury [130] and can be inhibited by rapamycin [131,132] or adiponectin [133]. The latter inhibits mTORC1 through AMP-activated protein kinase (AMPK). In addition, mTORC1 inhibition stabilizes GATA-6 and favours its nuclear translocation; nuclear GATA-6 activates contractile protein promoters, suppresses cell proliferation and induces VSMC differentiation [134].

PI3K-Akt pathway can also be activated by insulin to regulate vascular tone (see section 1.3.).

4.2.1.4. Dual role of Notch

Notch signalling is a conserved intercellular signalling pathway. Notch receptors and ligands are transmembrane proteins. Signal initiation starts when the ligand expressed on one cell binds its receptor located on the neighbouring cell. Ligand binding changes receptor conformation, and makes it accessible to proteases ADAM and γ-secretase which cleave the receptor on both sides of the membrane. Cleavage by γ-secretase releases Notch intracellular domain (NICD) from the membrane, and allows its translocation to the nucleus where it forms complex with other transcription factors and initiates transcription of its target genes, of which Hes and Hey, the basic helix-loop-helix (bHLH) proteins, are best known [135]. In VSMC Notch signalling also targets *Acta2, Pdgfb* and microRNA cluster miR143/miR-145 [136-138].

In the vasculature Notch signalling can occur between EC and VSMC (mostly during angiogenesis), or through homotypic interactions between neighbouring VSMC. During development Notch coordinates many aspects of EC and VSMC interaction and positively regulates VSMC specification, differentiation and maturation [42].

In the mature vessel VSMC express Notch1, Notch2, and Notch3 receptors and a ligand Jagged-1 [139-142], but the role of Notch signalling for VSMC phenotype modulation is unclear. Some studies report Notch as a pro-differentiation pathway, the others show it as a phenotype-switch inducer: inactivation of Notch signalling by Notch1 or Hey2 deletion, or cell transfection with soluble Jagged-1 reduces neointima formation and downregulates chemotaxis of isolated cells [143], [144,145],

deletion of Jagged-1 in EC promotes intimal and medial thickening after injury [146], and upregulation of both Notch and Jagged transcripts after vascular injury was reported [147].

The fact that Notch crosstalks with both TGF-β [120] and PDGF [137], two major VSMC phenotype modulating signalling pathways standing on the opposite sides of the fence, suggests that both scenarios might be valid and the final outcome depends on particular context.

4.2.2. De-differentiation driving signalling pathways

Signalling by Ras-Raf-MEK-ERK pathway in VSMC is triggered by ubiquitous growth factors such as PDGF, epidermal growth factor (EGF) and fibroblast growth factor (FGF).

4.2.2.1. Ras-Raf-MEK-ERK pathway

Among growth factors and cytokines driving VSMC de-differentiation by MAPKs cascade, PDGF is considered to be the master [104] and an indispensable regulator of early vascular development [148,149]. Released from platelets at the site of tissue damage [150], PDGF stimulates growth and directional migration of VSMC and fibroblasts [151-153]. Further investigations *in vivo* showed that PDGF acts in VSMC mostly as a pro-migration- rather than proliferation-inducer [154,155]. PDGF is a disulphide-linked dimer. Its binding to the receptor causes receptor dimerization and activation. PDGF receptors (PDGFRs) are classical receptor tyrosine kinases (RTKs) and two known types of PDGF receptors, PDGFRα and PDGFRβ, homo- or hetero-dimerized, transmit the signal by classical growth factor regulated Ras-Raf-MEK-ERK pathway, which eventually leads to downstream phosphorylation and activation of TCF family proteins such as Elk1. This induces SRF-dependent transcription of early response growth and de-differentiation genes, and repression of smooth muscle selective markers, [104,156]. In addition, active extracellular signal regulated kinases (ERK) can phosphorylate MRTFs in the cytoplasm and prevent their translocation to the nucleus [157]. Most importantly, PDGF-BB induces pluripotency transcription factor KLF4, which modulates transcriptional programs of smooth muscle-specific genes by preventing SRF/myocardin complex to bind pro-differentiation gene promoters and activates promoters driving acquisition of the pro-inflammatory phenotype [88,158] (see section 4.1.2.).

4.2.2.2. Other signalling pathways

β-catenin signalling

Elevated β-catenin and inactive GSK3β levels have been reported after vascular injury [159,160], [161]. Rapid cadherin junction dismantling was also reported to occur in vascular smooth muscle after injury [162]: this is most likely a Wnt-independent process since Frizzled receptor expression is downregulated in response to injury in rats [163].

In vitro Wnt1 and Wnt3a induce β-catenin pathway and cyclin D1 expression in arterial VSMC [164], but the evidences for Wnt1 and 3a expression in proliferating cells or injured vasculature *in vivo* are lacking [165].

4.3. Epigenetic regulators

4.3.1. Ten-eleven-translocation 2 (TET2)

Ten-Eleven-Translocation (TET) proteins are a family of methylcytosine dioxygenases which catalyse the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and perform the first step in DNA demethylation [166]. TET2 is highly expressed in smooth muscle and out-dominates other two family members (TET1 and TET3) in coronary artery SMC [167]. TET2 has been shown to be both necessary and sufficient for SMC differentiation in genetic loss- and gain-of-function

experiments. TET2 knockdown increases KLF4 expression (see section 4.1.2.), while overexpression of TET2 protein promotes MYOCD expression and cell differentiation [168]. In an *in vivo* model of intimal hyperplasia TET2 expression was reduced in the medial layer of injured vessels compared to the uninjured. Genetic loss of TET2 in the same model resulted in stronger response to injury and decreased H3K4me3/H3K27me3 ratio at the *MYOCD, SRF, and MYH11* promoters [168]. TET2 responds to pro-differentiation signals (e.g. rapamycin treatment) with strong binding to the promoters of VSMC contractile proteins [167].

The attempts to block VSMC de-differentiation in order to prevent progression of proliferative VSMC disorders by targeting major signalling pathways has delivered mixed results. Anti-PDGF and antiplatelet therapies have been suggested and tried in restenosis patients, unfortunately with no substantial clinical value. Intravenous administration of humanized monoclonal antibody against PDGFR-β had no beneficial effects on neointimal hyperplasia after stenting [169]. Drug-eluting stents (DES) coated with rapamycin or other VSMC proliferation-blocking drugs have proved superior to bare-metal stents and revolutionised angioplasty. However, in some cases drug-eluting stenting led to early stent thrombosis [36]. High risk of thrombosis is at large due to DES cytotoxicity on endothelial cells, which impairs re-endothelialization, delays arterial healing and causes endothelial dysfunction in arterial areas distant from the stent. Endothelial dysfunction and prolonged wound healing are directly linked to increased risks of ischemia and coronary occlusion thrombosis [36]. Targeting major signalling pathways shared by many types of cells might deliver similar results as an example of rapamycin-coated stents. New, SMC-specific therapeutic strategies targeting VSMC unique factors and phenotype switching regulating signalling pathways are needed to achieve better results in treatment and prevention of SMC-linked vascular pathologies.

N-cadherin has been proposed as a target by some research studies [170,171]. N-cadherin promotes VSMC migration, survival, and regulates cell polarity establishment, which is crucial step in initiation of migration. Blocking of N-cadherin reduces neointima growth, and shows no adverse effects on endothelium [170,171].

T-cadherin could be another potential target, since its expression is upregulated during vascular pathologies and promotes vascular cell survival and proliferation [172,173], but is not necessary for basic cell functions, or vascular development (T-cadherin knock-out mice are viable and exhibit no obvious anatomical defects in the vasculature (unpublished observations)). Moreover, we have found that T-cadherin is a VSMC phenotype regulator, promoting VSMC de-differentiation (manuscript in preparation).

5. Cadherins

Cadherins are best known as Ca^{2+}-dependent intercellular adhesion receptors which mediate homotypic cell-cell cohesion to organize functional tissues [174]. Alongside with this function cadherins have “non-canonical”, adhesion-independent functions, which can be observed in a single cell.

Most of the knowledge on cadherins comes from studies on classical cadherins. However, this type of cadherins constitute only a small fraction of a large and structurally diverse cadherin superfamily (110 members at least [175,176]), strongly suggesting that the functions fulfilled by cadherins might be numerous and varied.

5.1. Classification and structure

The cadherin superfamily consists of proteins containing cadherin-like domain. All known cadherins are classified into 6 subfamilies: type I (classical, adherens junctions forming cadherins), type II, desmosomal (found in desmosomal junctions), protocadherins, 7D cadherins and atypical cadherins [177]. Classical cadherins are the best studied cadherins of the superfamily, mostly in the contexts of development and cancer. Classical cadherins are composed of three major structural domains – extracellular, single-pass transmembrane and intracellular. The extracellular (EC) domain of classical cadherins consists of five EC Ca^{2+} binding repeats. Ca^{2+} is indispensable for cadherin function: Ca^{2+} binding shapes cadherin into active conformations and enables homophilic cadherin dimerization in *trans* (between identical cadherins on adjacent cell), as well as lateral cadherin clustering in *cis* to assemble zipper-like structure. The intracellular domain contains armadillo domains – conservative sites which binds β-, or p120-catenin. β- and 120-catenins bind α-catenin and integrate adherens junctions into the cell's actin cytoskeleton [177]. Cytoskeleton-bound cadherin clusters between the adjacent cells are called adherens junctions (AJs). AJs serve structural function as physical bonds, as well as cell-cell communication/signalling hubs.

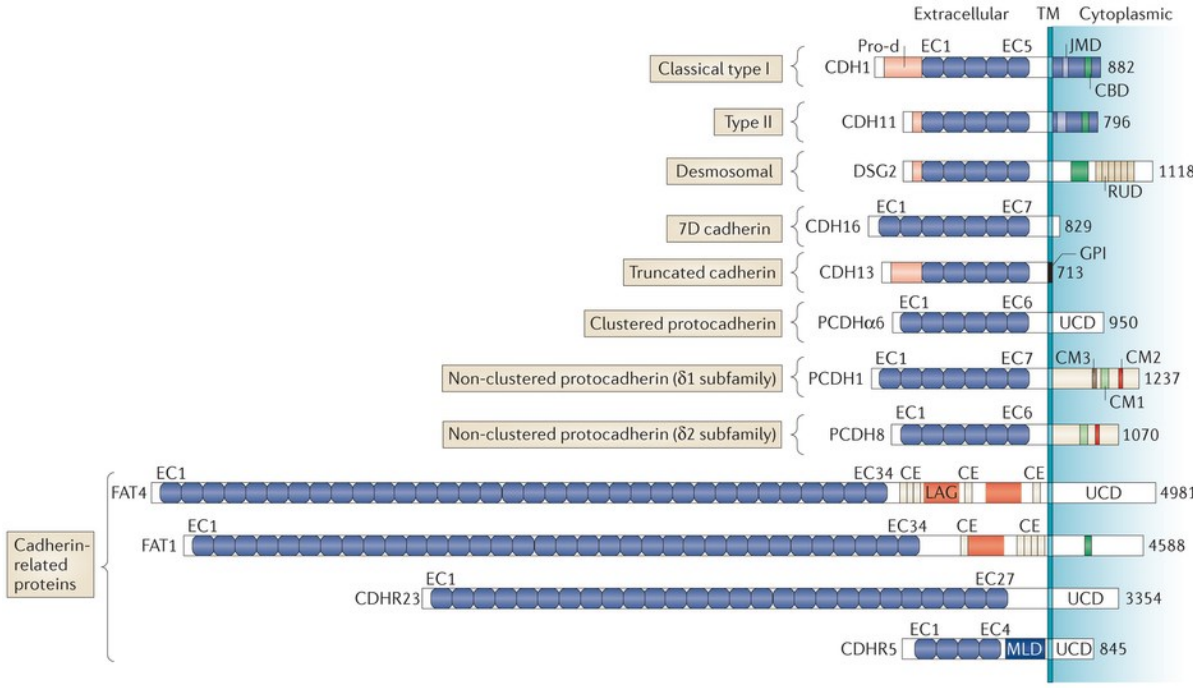

Fig.4. Schematic structural overview of representative members of the cadherin superfamily. The following protein domains are annotated: CBD (conserved β-catenin/plakoglobin-binding domain); UCD (unique cytoplasmic domain); CE (cysteine-rich EGF-repeat-like domain); CM1, CM2 and CM3 conserved motifs in the CDs of δ-PCDHs; EC1 to EC34 (extracellular cadherin repeats); GPI (glycosylphosphatidylinositol anchor); JMD (conserved juxtamembrane domain required for p120‑catenin binding); LAG (laminin-A globular domain-like domain); MLD (mucin-like domain); Pro-d (prodomain); RUD (intracellular repeated unit domain of desmosomal cadherins).Adapted from [177].

5.2. Cadherin functions

5.2.1. Adherens junctions

As a structural component of intercellular adhesion, AJs have inherent selectivity. Cadherin binding in AJs is strictly homophilic, and therefore occurs only between the cells expressing the same type of cadherins. Since different cell types express different sets of cadherins, homophilic binding ensures the selectivity and proper positioning of connected cells that is crucial for establishment of proper

tissue architecture [178]. Classical cadherins therefore are indispensable in early development, when primary tissues start to develop and segregate [174,179]. In the central nervous system adhesion complexes formed by cadherins play key roles in synapse formation and plasticity [180]. Here cadherins have dual roles. Some cadherins function as positive synapse guiding cues, while the others do negative guidance. For example, OB-cadherin signals as a positive guide [181], while T-cadherin is a negative axon guidance providing molecule [182].

5.2.2. Cell communication – polarity

Physical cell binding strength and polarity are key factors controlling differentiated cell behaviour such as polarization and migration [183,184], or stem cell fate decisions [185]. As the organizers of cell-cell contact formations cadherins play key roles in cell polarity establishment and cell guidance.

AJs are anchored to the cytoskeleton and therefore form local barriers in the cell membrane which limit free lateral floating of membrane proteins and contribute largely to establishment of membrane heterogeneity and cell polarity [186]. Cadherin binding to catenins *via* the intracellular cadherin domain immobilizes catenins to the membrane. This not only restricts the nuclear function of catenin, but also polarizes the cell biochemically since at the membrane catenins function as scaffolding proteins and recruit important elements of spatiotemporal signalling to the cell membrane [187]. In addition, classical AJs initiate tight junction formation [188], the key factor for cell polarity establishment.

Cell polarization in response to migration cues (seen as displacement of the nucleus toward the cell-cell contact and of cell migratory machinery away from it, and prepares the cell for coordinated movement) is a cadherin guided process [189]. Asymmetries in cell-cell adhesion, which can occur due to injury or the cadherin switch during the classical epithelial-to-mesenchymal transition (EMT), result in cell (re)polarization and migration. Cell division control protein 42 (Cdc42)-dependent cadherin signalling at the cell-cell border plays a primary role for cell polarization in disrupted cell monolayers [189,190]. In classical EMT, E-cadherin replacement by N-cadherin (the cadherin switch) blocks contact inhibition of locomotion (CIL) and initiates cell (re)polarisation [183]. Interestingly, although both cadherins are classical cadherins and are known to form AJs, E-cadherin re-expression in migratory cells restores CIL, while E-cadherin depletion or switch to the N-cadherin expression promotes cell polarization and induces directional migration [183]. Similar cadherin competition is observed between N-cadherin and VE-cadherin in EC [177]. VE-cadherin inhibits EC growth, migration and apoptosis by suppression of vascular endothelial growth factor receptor 2 (VEGFR2)/ERK signalling and induction of TGF-β/SMAD signalling [191], while N-cadherin functions as a pro-migratory protein [192]. VE-cadherin seems to have higher hierarchy in this context, since N-cadherin expression can be suppressed by VE-cadherin [192].

To date true mechanistic explanations for cadherin switch are lacking. Pure chemoaffinity differences between E- and N- cadherins do not explain the switch since homophilic N-cadherin ligation is stronger than that of homophilic E-cadherin ligation [193]. It has been suggested that E-cadherin replacement by N-cadherin enables epithelial cells to adhere to mesenchymal cells and invade the stroma expressing different types of cadherins [177]. Preferential OB-cadherin (osteoblast cadherin)-positive prostate cancer metastasis to the bone supports this theory [194]. Other authors suggest that the intercellular cadherin domain rather than extracellular domain is most important for CIL inactivation during the cadherin switch [183]. Earlier cadherin studies demonstrated that AJs formed by cadherins lacking β-catenin binding sites display weaker adhesions than native cadherins [195]. Since β-catenin functions as a scaffolding protein in AJs, differences in the C-terminal cadherin domains of E- and N-cadherin might result in formation of distinct multiprotein complexes and modulate intercellular adhesion properties, which might further affect cell behaviour. In addition,

homophilic adhesion-independent individual cadherin functions might be equally important in the cadherin switch. N-cadherin, for example, can activate fibroblast growth factor receptor 1 (FGFR1) signalling and induce EMT in a cell autonomous manner [196,197]. Finally, the biological context in which the cadherin switch takes place is no less important. In contrast to epithelial cells undergoing EMT, migrating VSMC display strong CIL when encountering N-cadherin expressing cells [170], which suggests complex tissue and biological context specific hierarchy between classical cadherins.

The cadherin switch phenomenon perfectly illustrates the multi-modality of cadherins and complexity of cadherin-mediated intercellular communication, suggesting the existence of a "cadherin code" which enables the cell to adopt multiple behavioural modes in order to adapt to various biological contexts.

5.2.3. Mechanotransduction

Due to the binding strength and direct connections to the cytoskeleton AJs are well suited to function as mechanotransducers (mechanosensors and mechanoresponders) [198-200]. In smooth muscle this type of communication is especially important, since well-coordinated collective cell contraction is essential for proper smooth muscle function. Upon mechanical stimulation N-cadherin strengthens connections between load-affected SMC and facilitates intracellular transition of contractile force [198,201]. Desmosomal cadherins, which mediate intercellular adhesion in desmosomes, recruit desmoplakin and intermediate filaments through plakophilins and plakoglobins, thereby rendering desmosomes tension-resistant [1]. In addition, VE-cadherin participates as an adaptor in mechanosensor complex formation between platelet endothelial cell adhesion molecule (PECAM) and VEGFR2, and facilitates activation of nuclear factor-κB [199].

5.2.4. Cell autonomous functions. Vesicle closure and cell transport

Adhesion dependent functions of N- and E-cadherin can be observed in a single cell. In a cell autonomous mode of function, classical cadherin-mediated adhesion helps to close back folding membrane ruffles and form macropinosomes [202].

5.2.5. Signal transduction

5.2.5.1. PI3K signalling

Intercellular communication and cell-matrix adhesion are vital for pro-survival stimulus and cell escape from apoptosis. In VSMC intercellular adhesion is as important as cell anchorage to the matrix for cell survival[203]. Mechanistically this is achieved via PI3K-Akt signalling: cadherin-catenin complex formation activates PI3K-Akt cascade and blocks "default" cell apoptosis by inactivation of pro-apoptotic protein Bad (Bcl-2 antagonist of cell death) and stabilization of anti-apoptotic factor Bcl-2 (B-cell lymphoma 2) [171,204-207].

5.2.5.2. β-catenin and canonical Wnt signalling

The key element in the canonical Wnt pathway is β-catenin, a multifunctional protein which can act either as a structural and scaffolding protein that stabilizes cell-cell junctions, or as a transcription coactivator in the nucleus. Cadherin-bound, cell membrane localized β-catenin functions as a structural component of the intercellular adhesome, while in the nucleus it participates actively in gene expression control [208]. Classical cadherins are therefore considered direct negative regulators of β-catenin signalling [208]. Dismantling of AJs and accumulation of free β-catenin can occur due to the initiation of developmental programs, cancerous cell transformation or tissue injury. Nuclear β-catenin translocation during developmental processes and cancer is usually initiated by Wnt signalling, while injury-induced β-catenin signalling is part of repair mechanisms.

Wnt family members are secreted proteins that coordinate cell-cell interaction during embryonic development and adult tissue homeostasis [209]. Wnt proteins bind Frizzled family receptors and activate canonical Wnt signalling pathway. Canonical Wnt pathway signalling via T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors controls morphogenesis and cell fate regulating gene expression programs [210]. In quiescent cells, β-catenin is locked in cellular junctions and free β-catenin is kept under strict control by the degradation complex consisting of scaffolding protein axin, GSK3β, casein kinase 1 (CK1), and the adenomatous polyposis coli (APC). However, in response to Wnt signalling through its receptor Frizzled, the degradation complex undergoes rearrangement and switches to the inactivate state. GSK3β becomes inactivated and can no longer phosphorylate β-catenin and direct it for degradation. Accumulating β-catenin translocates to the nucleus and initiates its target gene transcription. Most of the β-catenin/TCF/LEF target genes are cell type specific [209]. Importantly, β-catenin nuclear function can be initiated by mechanisms independent of Wnt. Free β-catenin can be stabilized by changes in cadherin abundance or cellular distribution. This can result in Wnt-independent β-catenin signalling, or heighten Wnt responses. Mutations disabling β-catenin binding to cadherins are often accompanied by higher β-catenin nuclear activity, which can lead to cancerous transformation of the cell. Recurrent cadherin-related protein Fat1 mutations together with increased β-catenin nuclear function have been reported in glioblastoma, colorectal cancer, and head and neck cancer [211].

In the vasculature β-catenin signalling is most relevant to injury repair. Elevated β-catenin and inactive GSK3β levels have been reported after vascular injury to the endothelium [159], [160], [161]. T-cadherin upregulation in endothelial cells leads to integrin-linked kinase mediated GSK3β inactivation, β-catenin stabilization, and TCF/LEF-dependent cyclin D1 induction [212]. Rapid AJs dismantling and accumulation of β-catenin has been shown to take place in vascular smooth muscle after injury [162]. MMP-9 and -12 dependent shedding of the extracellular N-cadherin domain elevates VSMC proliferation via β-catenin signalling [213].

5.2.5.4. Growth factor signalling modulation

Cadherins can interact with other types of adhesion molecules and form heterophilic interactions outside of the cadherin family [214], or even with non-adhesion molecules (e.g. growth factor receptors), and modulate their signalling. As already mentioned, N-cadherin binds FGFR1 in *cis* and prevents ligand-induced receptor internalization, which leads to sustained receptor signalling [197,215,216]. Similarly P-cadherin binding to IGF receptor 1 (IGFR1) induces ligand-independent receptor signalling and activates cytoplasmic p120-catenin. Active p120-catenin promotes cell migration [217]. Non-classical cadherin-17 interacts with α2β1 integrin heterodimer and activates β1 integrin. This leads to FAK, Ras and Jun N-terminal kinase activation, and stronger adhesion to collagen IV [218]. In squamous cell carcinoma cells T-cadherin can supress EGFR signalling, cell cycle progression and cell migration [219]. In EC, T-cadherin directly interacts with IR (insulin receptor), effectively highjack IR signalling effectors and thereby cause insulin resistance by amplifying the negative feedback look of mTORCs/S6K1-mediated serine phosphorylation and degradation of IRS-1 [220].

5.2.5.5. Cleaved cadherin signalling

Proteolytically cleaved intracellular or extracellular cadherin domains can have independent functions and act as separate signalling elements. For example, N-cadherin cleavage by ADAM10 generates a cytoplasmic fragment which strengthens β-catenin nuclear signalling [221], and MMPs cleave P-cadherin to release extracellular cadherin domain which functions as pro-migratory factor [222].

Cadherin flexibility in adhesion partner selection, combined with multi-domain organization equips these molecules with flexible specificities, and makes them adaptable for many biological contexts [188].

Table 1. **Cadherin functions**

Function	Cadherin	Reference
Adherens junction formation	Classical cadherins	174, 177, 178-182
Tight junction formation	*Classical*	181, 188
Synapse formation	*Classical, OB, T*	177, 188, 250
Cell polarity regulation	*N, E, P*	183, 189, 192
Vesicle closure	*N, E*	202
Mechanotransduction	*N, VE*	198-201
Signal transduction		
Classical PI3K pathway	*N*	171, 204-207
β-catenin/ canonical Wnt	*E, N, (other classical), T*	174, 177, 188, 211-213, 221
Growth factor signalling modulation	*N-FGFR1*	197, 214-216
	P-IGFR1	217
	Cdh17-$\alpha_2\beta_1$	218
	T-EGFR	219
	T-IR	220
Hippo pathway (activation)	*E (possibly other classical AJ forming)*	223, 224
Receptor	*T-Adiponectin*	225
	T-LDL	227

5.2.5.6. YES/YAP and Hippo pathway

Hippo pathway regulates organ growth, stem cell function, and regeneration via activity control of transcription tandem YES associated protein (YAP) and TAZ [223]. YAP/TAZ are transcription co-activators which promote cell proliferation and inhibit cell death. Hippo pathway represses YAP/TAZ activity.

E-cadherin, α-catenin and other structural AJ proteins can sequester YAP/TAZ to the junctions and repress their nuclear function directly [224], or via activation of Hippo pathway [223].

5.2.5.7. Receptor function

T-cadherin is a third adiponectin receptor which specifically binds hexameric and high molecular weight adiponectin [225]. T-cadherin binding localizes adiponectin to the endothelium and promotes revascularization [226]. T-cadherin also binds low density lipoproteins (LDL) [227] with proposed functionality as a signalling receptor for LDL that facilitates LDL-dependent mitogenic signalling in the vasculature [228,229].

5.3. Cadherins in vascular smooth muscle

VSMC express a number of cadherins. N-cadherin, T-cadherin, OB-cadherin, 6B-cadherin [230], R-cadherin, E-cadherin and Fat1-cadherin expression have thus far been reported. Although most of these cadherins have been very poorly studied in vascular smooth muscle, existing data suggests that different subsets of cadherins may be expressed by different SMC subpopulations within the vessel wall, and that mechanisms similar to cadherin switching in EMT might play important roles in SMC phenotype modulation. SMC *in vivo* are surrounded by high contents of ECM [1], and cadherins with

extracellular domains of different lengths might be needed to reach between the cells. AJs and desmosomes have been demonstrated in large and small arteries *in vivo* [231]. Cadherin involvement in organizing correct arterial tissue architecture is therefore doubtless.

5.3.1. N-cadherin

N-cadherin is a major classical cadherin in VSMC. Despite its abundance relatively little is known about N-cadherin functions in the vasculature [232]. So far two major modes of adhesion-dependent functions have been reported, namely formation of AJs and assistance in micropinosome closure. The later function is cell autonomous, but still relies on classical homophilic cadherin ligation. AJ-independent N-cadherin functions in VSMC includes pro-survival signalling, and migration initiation.

Correct intercellular adhesion is crucial for early development of the cardiovascular system. Full and endothelium-specific N-cadherin knock-out is lethal. Cardiomyocytes of N-cadherin knock-out mouse fail to form proper gap junctions, which leads to cardiac arrhythmias and embryo death at E10.5 [233,234] – the developmental stage when cardiac activity and vessel perfusion starts. Vascular EC-specific N-cadherin knock-out embryos die of severe oedema (unstable and leaky vessels) also at E10.5 [235]. In this case EC lacking N-cadherin are still able to form ordinary tubules, but fail to muscularize properly (by connecting to VSMC or pericytes) and stabilize the vessel [236,237]. This suggests that proper adhesion between EC can be formed in the absence of N-cadherin, but for the establishment of stable contacts between EC and VSMC N-cadherin is indispensable.

In adult vessel AJs formed by N-cadherin are needed for collective VSMC contraction [238] and proper regulation of arteriolar myogenic tone [232]. Upon contractile activation β-catenin is recruited to N-cadherin. This strengthens existing, or forms new AJs between adjacent cells and facilitates intracellular transition of contractile force, orchestrating collective cell contraction [198,201]; N-cadherin blockade in the rat arteriole reduces myogenic response to intravascular pressure [239].

N-cadherin also plays a part in vascular regeneration and repair. Vascular injury promotes rapid disassembly of cellular adhesions and downregulation of N-cadherin expression. In the absence of N-cadherin, β-catenin can translocate to the nucleus and induce cell proliferation [162,213]. At later stages of vessel healing (1 week after injury) N-cadherin expression is restored and upregulated. Here N-cadherin seems to function in a cell adhesion-independent mode and induces directional cell migration to the intima: in the migrating cell N-cadherin is localized to the leading cell edges (lamellipodia) or to growth factor induced dorsal membrane ruffles [202,240-242]. In the leading edge N-cadherin forms strong connective ligations between closely located membranes promoting macropinosome closure and liquid phase uptake [202], both of which are needed for cell motility and quick (migrational) cell polarity establishment [170]. N-cadherin also activates PI3K-Akt signalling to block pro-apoptotic protein Bad and inhibit cell apoptosis [171,205,206].

Pro-survival signalling can be regulated by N-cadherin in a positive manner through initiation of ligand-free FGFR signalling [197,243] in a negative manner via TCF/LEF-dependent β-catenin signalling [159].

5.3.2. E-cadherin, R-cadherin, FAT1, OB-cadherin

Expression of E-cadherin within human atherosclerotic lesions was described, although not on SMC [244]. Expression of E-cadherin on SMC *in vitro* and its downregulation in response to oxidized LDL has also been reported [245]. Studies on R-, FAT1- and E-cadherin in the context of SMC are very rare. Downregulation of R-cadherin occurs during early experimental restenosis and in association

with increased SMC proliferation [246], while FAT1 expression increases [247]. *In vitro* FAT1 stimulates SMC migration, but inhibits proliferation [247]. In SMC FAT1 localizes to both cell-cell junctions and cell free edges; cell-cell localization might limit SMC proliferation, while presence at cell free edges may signal directional migration cues during vascular remodelling [247]. *In vitro* cell collectivization experiments showed that OB-cadherin cooperates with N-cadherin and forms hybrid AJs [248]. Blocking either of OB- or N-cadherins inhibited AJ formation and cell collectivization. This type of cell collectivization was mediated via TGF-β-OB-cadherin dependent adhesive switching [248].

Contributions of E- R-, FAT1-, and OB- cadherins to SMC phenotype control have not been studied.

5.3.3. T-cadherin

T-cadherin is an atypical cadherin. In contrast to classical cadherins, T-cadherin lacks transmembrane and intracellular domains as well as the HAV motif in the extracellular cadherin repeat, all of which are crucial for stable homophilic adhesion and signal transduction by classical cadherins (Fig.5.). Nevertheless, T-cadherin is phylogenetically closest cadherin to the classical cadherins, sharing high sequence similarity [249]. Instead of intracellular domain T-cadherin is bound to the membrane via GPI-anchor, and therefore has no direct connection with the cytoskeleton and is unlikely to function as a AJs forming protein. Although GPI-anchoring does not provide connection to the cell cytoskeleton, it serves as a ligand binding site. – T-cadherin is a receptor for LDL [227] and a third receptor for adiponectin [225] (see section 5.2.5.7.). Adiponectin binding region in T-cadherin molecule is however still unknown. T-cadherin localizes adiponectin to the vasculature and activates proangiogenic revascularisation [226]. Studies to date reported functional T-cadherin involvement in nervous system, vasculature and cancer. In nervous system T-cadherin participates in neurodevelopment and synapse plasticity by guiding axon outgrowth [182] and regulating negatively inhibitory synapses [250]. In cancer T-cadherin can have tumour promoting or suppressing role, where the final outcome seems to be context dependent [251].

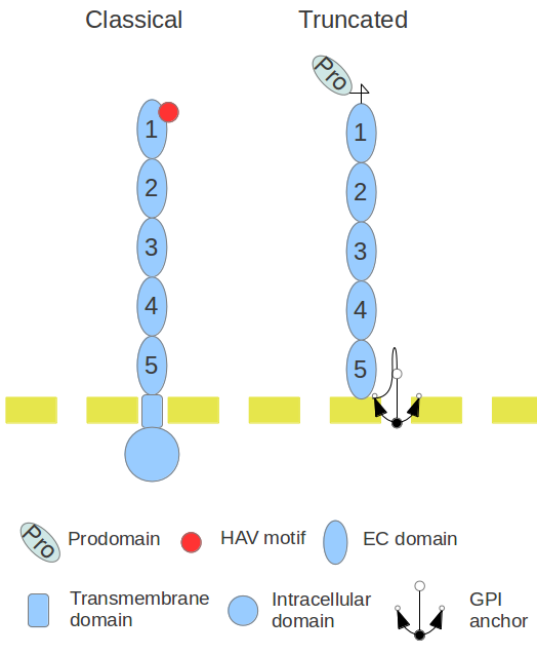

Fig.5. Atypical structure of T-cadherin. In contrast to classical cadherins (left), T-cadherin (truncated) lacks transmembrane and intracellular domains as well as the HAV motif in the first extracellular cadherin repeat, all of which are crucial for stable homophilic adhesion and signal transduction by classical cadherins.

In the vasculature T-cadherin is expressed on EC, VSMC and pericytes, but not on fibroblasts or periaortic adipose tissue [172,252]. It is also strongly expressed on cardiomyocytes [172,253], and therefore is sometimes called H-cadherin (heart cadherin), its functions in the cardiomyocte have not been studied. In EC T-cadherin is upregulated during atherosclerosis and restenosis and functions as a cell resistance factor to oxidative stress, endoplasmic reticulum stress, hyperglycemia and hyperinsulinemia [172,254-256]. It regulates cell motility, proliferation, promotes survival, angiogenesis and revascularization [173,226,251,257], and can also attenuate insulin-dependent signalling [220]. To regulate these processes in EC, T-cadherin signals via the PI3K-Akt signalling axis,

β-catenin and small RhoGTPases [212,220,258], and utilizes a variety of molecular adaptors (insulin receptor, Grp78, integrin linked kinase and integrinβ3) [220,255,259] that can be recruited to lipid raft domains of the plasma membrane where T-cad locates [260].

T-cadherin functions in vascular smooth muscle are largely unknown. As in the endothelium, on VSMC T-cadherin expression is increased at all stages of atherosclerosis and during the early (proliferative) phase of experimental restenosis. Moreover in the muscle layer its expression pattern correlates negatively with SM-α-actin expression [172,252,261,262]. *In vitro* T-cadherin expression follows cell cycle progression [263] and ectopic overexpression of T-cadherin promotes proliferation [173,264]. In response to migration stimuli T-cadherin redistributes from its resting, global cell body location to the leading edges of the cell, and plating VSMC on recombinant T-cadherin-coated substratum reduces cell spreading and cell adhesion [173].

Taken together, these data suggest that in VSMC, as in neurons, T-cadherin might have functions in homophilic interaction-dependent cellular guidance and regulation of the cellular adhesome.

Studies in this dissertation are aimed at establishing cellular functions for T-cadherin in VSMC contraction and phenotype plasticity and identifying mediating molecular mechanisms.

DISSERTATION OBJECTIVES

My dissertation comprises two studies. A very brief background together with the major objective and research plan for each of the studies are outlined below, followed thereafter by presentation of the resultant manuscripts.

1. T-cadherin modulates VSMC sensitivity to insulin

In vasculature insulins functions reach beyond the metabolic control. Insulin signalling via PI3K maintains SMC quiescence, neutralizes the de-differentiation-driving PDGF signalling, and regulates SMC contraction dynamics. Recent studies demonstrated that T-cadherin upregulation on EC promotes a state of cellular insulin resistance. T-cadherin blunts EC sensitivity to insulin by hijacking receptor and downstream effectors of the PI3K pathway of insulin. Immunohistochemical studies showed that T-cadherin expression on SMC is upregulated in pathological conditions (e.g. atherosclerosis) related to insulin resistance. Our preliminary experiments i*n vitro* confirmed these observations and showed that the phenomenon can be simulated *in vitro*: SMC culturing under conditions mimicking hyperinsulinemia or hyperglycemia leads to upregulation of T-cadherin expression. What consequences T-cadherin upregulation has on insulin signalling and smooth muscle contractile function is unknown.

OBJECTIVE

To establish whether T-cadherin affects constitutive and insulin-induced Akt/mTOR axis signalling and contractile competence of SMC.

RESEARCH PLAN

1. To investigate how alterations of T-cadherin expression impact constitutive and insulin-induced Akt/mTOR axis signalling
   1.1. Examine constitutive Akt/mTOR signalling in SMC with elevated T-cadherin expression;
   1.2. Examine insulin induced Akt/mTOR signalling in SMC with elevated T-cadherin expression;
   1.3. Define and compare IRS-1: pIRS-1$^{Ser636/639}$ ratio.
2. To investigate how elevated T-cadherin expression impacts SMC contractile competence
   2.1. Examine constitutive and insulin conditioned phosphorylation status of myosin light chain phosphatase regulatory targeting subunit MYPT1, myosin light chain MLC, and iNOS expression;
   2.2. Examine constitutive and insulin-induced activity of small Rho GTPase RhoA;
   2.3. Examine SMC behaviour in 3D-collagen gel; contractile competence and/or matrix reorganization.

The findings of this project have been published (see pages 35-46).

Frismantiene, A., Pfaff, D., Frachet, A., Coen, M., Joshi, M.B., Maslova, K., Bochaton-Piallat, M.L., Erne, P., Resink, T.J., and Philippova, M. 2014. Regulation of contractile signaling and matrix remodeling by T-cadherin in vascular smooth muscle cells: Constitutive and insulin-dependent effects. *Cell Signal* 126:1897-1908

2. T-cadherin induces VSMC phenotype switch

SMC exhibit high degree of cellular plasticity. On demand guided by environmental cues fully differentiated SMC de-differentiate and re-enter cell cycle. De-differentiating SMC lose contractile properties, start to produce ECM proteins, proliferate rapidly and gain the ability to migrate. In a healthy vascular wall individual SMC de-differentiation occurs at a low event rate and ensures constant vascular wall renewal, or in case of injury, damaged tissue repair. However, when phenotype transition controlling mechanisms fail, SMC plasticity contributes to life threatening conditions like atherosclerosis, restenosis or graft failure. Despite high demand for improved cardiovascular therapeutics and intense research, mechanistic pathways controlling SMC phenotype switch remain poorly understood.

Previous work in our laboratory showed that T-cadherin is expressed on SMC and undergoes upregulation in *vivo* during atherosclerosis and restenosis. In atherosclerotic lesions T-cadherin expression negatively correlates with SM-α-actin expression. *In vitro* proliferating SMC express higher T-cadherin levels than quiescent cells, and ectopic upregulation of T-cadherin *in vitro* causes insulin resistance and induces matrix remodelling (outcome of project 1 above). All these findings indirectly suggest that T-cadherin might play an important role in SMC phenotype plasticity. Whether and how T-cadherin regulates SMC plasticity and signalling mechanisms underlying such regulation have never been formally investigated.

OBJECTIVE

To define functional and molecular SMC characteristics regulated by T-cadherin and establish a role for this molecule in control of SMC phenotypic modulation.

RESEARCH PLAN

1. To define T-cadherin's role in SMC phenotype transition *in vitro.*
   1.1. Characterize SMC morphology;
   1.2. Investigate whether ectopic dosage of T-cadherin also results in alteration of SMC-specific marker expression;
   1.3. Investigate whether changes in T-cadherin expression affect SMC migration and proliferation.
2. To identify signalling pathway(s) utilized by T-cadherin to promote SMC phenotype transition
   2.1. Examine classical SMC phenotype transition regulating signalling pathways Ras/Raf-MEK/Erk, and p38 signalling;
   2.2. Examine GSK3β-β-catenin signalling pathway;
   2.3. Examine MRTF signalling pathway;
   2.4. Verify identified target pathway with functional assays.

The findings of this project have been published (see pages 47-61).

Frismantiene, A., Dasen, B., Pfaff, D., Erne, P., Resink, T.J., Philippova, M. 2016. T-cadherin promotes vascular smooth muscle cell dedifferentiation via a GSK3β-inactivation dependent mechanism. *Cell Signal* 28(2016):516-530.

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Regulation of contractile signaling and matrix remodeling by T-cadherin in vascular smooth muscle cells: Constitutive and insulin-dependent effects

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ABSTRACT

Expression of GPI-anchored T-cadherin (T-cad) on vascular smooth muscle cells (VSMC) is elevated in vascular disorders such as atherosclerosis and restenosis which are associated with insulin resistance. Functions for T-cad and signal transduction pathway utilization by T-cad in VSMC are unknown. The present study examines the consequences of altered T-cad expression on VSMC for constitutive and insulin-induced Akt/mTOR axis signaling and contractile competence. Using viral vectors rat (WKY and SHR) and human aortic VSMCs were variously transduced with respect to T-cad-overexpression (Tcad+-VSMC) or T-cad-deficiency (shT-VSMC) and compared with their respective control transductants (E-VSMC or shC-VSMC). Tcad+-VSMC exhibited elevated constitutive levels of phosphorylated Akt^{ser473}, $GSK3\beta^{ser9}$, $S6RP^{ser235/236}$ and $IRS\text{-}1^{ser636/639}$. Total IRS-1 levels were reduced. Contractile machinery was constitutively altered in a manner indicative of reduced intrinsic contractile competence, namely decreased phosphorylation of $MYPT1^{thr696 \text{ or } thr853}$ and MLC_{20} $^{thr18/ser19}$, reduced RhoA activity and increased iNOS expression. Tcad+-VSMC-populated collagen lattices exhibited greater compaction which was due to increased collagen fibril packing/reorganization. T-cad+-VSMC exhibited a state of insulin insensitivity as evidenced by attenuation of the ability of insulin to stimulate Akt/mTOR axis signaling, phosphorylation of MLC_{20} and MYPT1, compaction of free-floating lattices and collagen fibril reorganization in unreleased lattices. The effects of T-cad-deficiency on constitutive characteristics and insulin responsiveness of VSMC were opposite to those of T-cad-overexpression. The study reveals novel cadherin-based modalities to modulate VSMC sensitivity to insulin through Akt/mTOR axis signaling as well as vascular function and tissue architecture through the effects on contractile competence and organization of extracellular matrix.

1. Introduction

Insulin resistance is classically defined as the inability of insulin to exert its metabolic actions. In vascular tissue insulin has non-metabolic functions crucial to vascular and hemodynamic homeostasis, including preservation of endothelial-dependent vasorelaxation, reduction of endothelial cell (EC) apoptosis [1], and maintenance of the differentiated, contractile vascular smooth muscle cell (VSMC) phenotype [2]. Pathophysiological consequences of vascular insulin resistance encompass endothelial dysfunction (ED), hypertension, microvessel disease, vascular inflammation and atherogenesis [1,3]. Thus it is important to understand the cell-specific actions of insulin in vascular cells as well as the mechanisms leading to impaired insulin responsiveness in vascular cells and functional consequences thereof. Acquisition of vascular insulin resistance with its concomitant disturbance of vascular homeostasis is linked to ED, characterized by an impaired ability of insulin to induce eNOS activation [1,3]. However, controversy remains regarding whether ED arises because of the whole-body milieu typical of insulin resistant states (*e.g.* inflammation, oxidative stress, hyperinsulinemia, hyperglycemia) or whether insulin resistance in EC *per se* disturbs endothelial function [4]. VSMC-specific effects contributing to,

Abbreviations: E, empty vector control transductant; EC, endothelial cell; ECM, extracellular matrix; ED, endothelial dysfunction; Ins, insulin; IRS-1, insulin receptor substrate-1; MLC_{20}, myosin light chain 20; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase targeting subunit 1; shC, non-coding shRNA control transductant; shT, T-cadherin shRNA silenced transductant; T-cad, T-cadherin; Tcad+, T-cadherin overexpressing transductant; VSMC, vascular smooth muscle cell; VSMC-PCL, vascular smooth muscle cell-populated collagen lattice.

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0898-6568/

or arising because of, vascular insulin resistance are difficult to infer from clinical studies and *in vivo* experimental models because of confounding influences on VSMC arising secondarily to ED [4].

We previously described a novel cadherin-based mechanism for regulation of insulin sensitivity in EC [5]. GPI-anchored T-cadherin (T-cad) is constitutively expressed on EC and functions to promote survival, migration, proliferation and angiogenesis [6–8]. Central to these protective functions of T-cad in EC is its ability to stimulate signal transduction *via* the PI3K/Akt pathway [7,9]. However, T-cad upregulation on EC induces constitutive PI3K/Akt/mTOR pathway hyperactivation [9] which has deleterious consequences of persistent activation of the negative feedback loop of the insulin cascade and enhanced IRS-1 degradation [5]. Accordingly chronic T-cad upregulation engenders a state of insulin resistance in EC, manifest as blunted insulin-stimulated eNOS activation, migration and angiogenesis [5].

It has long been recognized that VSMC also constitutively express T-cad [10] but remarkably little is known regarding its functions in this cell type. Histological studies demonstrated the upregulation of T-cad in intimal VSMC during atherosclerosis, most prominently within lesions of high disease severity [11], and also during the period of active tissue reparation/restenosis following arterial injury [12]. Consistent with these immunohistological findings are *in vitro* observations that T-cad expression on VSMC is higher during proliferation than at quiescence [13] and that ectopic T-cad overexpression increases proliferation [6]. VSMC proliferation induced by some tyrosine kinase-linked receptor agonists (*e.g.* PDGF) and G-protein-coupled receptor agonists (*e.g.* Ang II, ET-1) has been associated with attenuated insulin responsiveness through agonist-promoted PI3K/Akt/mTOR axis signaling and subsequent feedback serine phosphorylation and degradation of IRS-1/IRS-2 [14–16]. Since T-cad upregulation on VSMC occurs in vascular disorders that are linked to insulin resistance and in association with increased proliferation [11,12] we questioned whether the upregulation of T-cad might also participate in the development of insulin resistance in VSMC.

Signal transduction pathway utilization by T-cad in VSMC has never been studied. The PI3K/Akt/mTOR pathway is a major intracellular target of T-cad in EC, and its constitutive hyperactivation in T-cad overexpressing EC was identified as the mechanism underlying attenuation of insulin signaling in EC [5,7,9]. Further, PI3K/Akt/mTOR signaling axis plays a central role in VSMC proliferation and contractile competence [17] and in the development of insulin resistance [18]. Therefore, we investigated the influence of altered T-cad expression on constitutive and insulin-induced Akt/mTOR pathway activity and on contractile competence in VSMC.

2. Materials and methods

2.1. Cell culture and transduction

VSMC were normally maintained in DMEM (containing 5.5 mM glucose) supplemented with 10% FCS. The isolation and characterization of VSMC from the descending thoracic aorta of 20-week old male Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats have been detailed before [19]. T-cad was stably overexpressed (Tcad +) in rat VSMC using pLVX-puro vector carrying full length human T-cad cDNA (empty pLVX-puro vector served as control (E)) [5]. For each transduction of WKY-VSMC and SHR-VSMC, 4–5 different isolates at passages 2–3 were randomly selected from our cryostocks of VSMC isolates from 8 WKY and 8 SHR animals and pooled. Human aortic VSMC (Hu-VSMC) cultures were obtained from Lonza (Basel, Switzerland) and transduced following expansion to passage 3. Hu-VSMC were transduced to either transiently or stably overexpress T-cad using Adeno-X [6] or pLVX-puro [5] vectors; respective empty vectors served as the controls (E). Additionally, stable transductants of T-cad-deficient Hu-VSMC (shT) were generated using pLKO.1-puro vector carrying human T-cad shRNA or non-target shRNA as control (shC) [5]. We used both transient adenovirus-mediated (adeno) and stable lentivirus-mediated (lenti) transduction because (1) it enabled us to exclude any artifacts related to transduction protocols and number of cell passages, and (2) lentiviral approach allows generation of stable T-cad-silenced VSMC transductants. Transient and/or stable transduction protocols were performed on at least three separate occasions for any given VSMC species or strain. Transiently transduced Hu-VSMC were used at up to 2–3 passages after transfection. Stably transduced rat-VSMC and Hu-VSMC were used at up to passages 8–10 after puromycin (3.6 μg/ml) selection.

2.2. Immunoblotting

VSMC were seeded (2×10^5 cells/well in 6-well plates), allowed overnight adherence and further cultured for 24 h in DMEM/0.1% BSA, followed by DMEM/0.1% BSA refreshment and a 1 h resting incubation period before exposure to insulin (Actrapid, Novo Nordisk, Mainz, Germany). Whole cell lysates were prepared and analyzed by immunoblotting as described [9]. Primary antibodies against the following proteins/epitopes were used: T-cad (R&D Systems Europe Ltd., Abingdon, UK), phospho (p)-Akt^{ser473}, Akt, p-GSK3β^{ser9}, GSK3β, S6RP, p-S6RP$^{ser235/236}$, IRS-1, p-IRS-1$^{ser636/639}$, and p-MLC$_{20}^{thr18/ser19}$ (Cell Signalling, New England Biolabs GMBH, Frankfurt, Germany), p-MYPT1^{thr696}, p-MYPT1^{thr853}, and β-actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany), iNOS/NOS Type II (BD Biosciences, Allschwil, Switzerland) and GAPDH (Abcam, Cambridge, UK). β-Actin, GAPDH or total Akt were variously used as loading controls. Representative blots are shown.

2.3. VSMC-populated collagen lattice compaction assay

To prepare VSMC-populated collagen lattices (VSMC-PCLs) VSMC suspensions in DMEM/0.1% BSA (4×10^5 cells/ml) were mixed 1:1 with neutralized and diluted (3 mg/ml) collagen solution (BD Biosciences), aliquoted into 48-well plates (250 μl/well, triplicate wells per condition) and allowed to polymerize. The gel–dish interface was released using a curved spatula followed by the addition of DMEM/0.1% BSA (0.5 ml/well) without or with inclusion of insulin to wells. After incubation (20 h) VSMC-PCL areas were measured morphometrically using Cell^P software (Soft Imaging System GmbH, Munich, Germany).

2.4. In-gel proliferation and viability assays

To assay proliferation freshly prepared VSMC-PCLs were overlaid with DMEM/0.1% BSA containing a 1:25 dilution of Alamar Blue solution (AbD Serotec, Düsseldorf, Germany) and absorbance (560/590 nm) was measured after 3 h (baseline) and 20 h of incubation. Live–dead assay for viability was performed at the end of the 20 h incubation period. VSMC-PCLs were washed twice with PBS, and after the addition of PBS containing calcein-AM (10 μM) (Life Technologies, Basel, Switzerland) and propidium iodide (500 nM) (Sigma-Aldrich, Buchs, Switzerland) the lattices were incubated for 10 min at 37 °C. Images of 3 randomly selected fields/gel were captured under an inverted fluorescence Olympus IX50 microscope. Proportions of calcein-AM-positive (live) and propidium iodide-positive (dead) cells were calculated after enumeration using Cell^P software.

2.5. Picrosirius Red staining and image analysis

VSMC-PCLs were incubated (20 h) either as free-floating lattices or as unreleased lattices (*i.e.* without disturbance of the gel–dish interface), washed with PBS, fixed in-plate (4% paraformaldehyde, 10 min), rinsed twice with PBS and embedded in paraffin. Sections (10 μm) were stained with Picrosirius Red to label collagen and analyzed by brightfield and circularly polarized light [20] microscopy using a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany). Three sections per gel were analyzed and images of 20 randomly selected cells per section

were captured at 100× magnification. To evaluate birefringence of collagen images captured under polarized light were analyzed using ImageJ software and the default threshold color method of analysis. For every image the area selected for analysis was held constant and color space parameters for pixel selection were set as follows: hue = 25–55, saturation = maximum, and brightness = minimum. "Yellow" pixelated area was quantified and taken as an index of reorganization of collagen into thicker fibrils.

2.6. Assay for RhoA activation

RhoA activity was determined using G-LISA™ RhoA activation assay kit (Cytoskeleton Inc., Denver, CO, USA) with triplicate measurements for every condition.

2.7. Statistical analysis

All experiments were performed on at least 3 independent occasions and unless otherwise stated the results are given as mean ± 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's *post-hoc* testing for multiple comparisons. $P < 0.05$ was considered significant.

3. Results

3.1. T-cad expression in VSMC is upregulated under conditions of hyperglycemia or hyperinsulinemia

Factors contributing to upregulation of T-cad on VSMC during atherosclerosis [11] and restenosis [12] are unknown. Since these diseases are associated with insulin resistance we queried whether alterations in T-cad expression on VSMC may occur in environmental settings mimicking insulin resistance. Indeed, rat aorta-derived VSMC cultured under conditions of hyperglycemia (10 and 25 mM glucose) or hyperinsulinemia (1, 10, 100 nM) exhibited increased T-cad protein levels as compared to controls (iso-osmolar conditions or no insulin, respectively), and the relative T-cad upregulation under both hyperglycemic and hyperinsulinemic conditions was comparable in VSMC from WKY and SHR (Fig. 1).

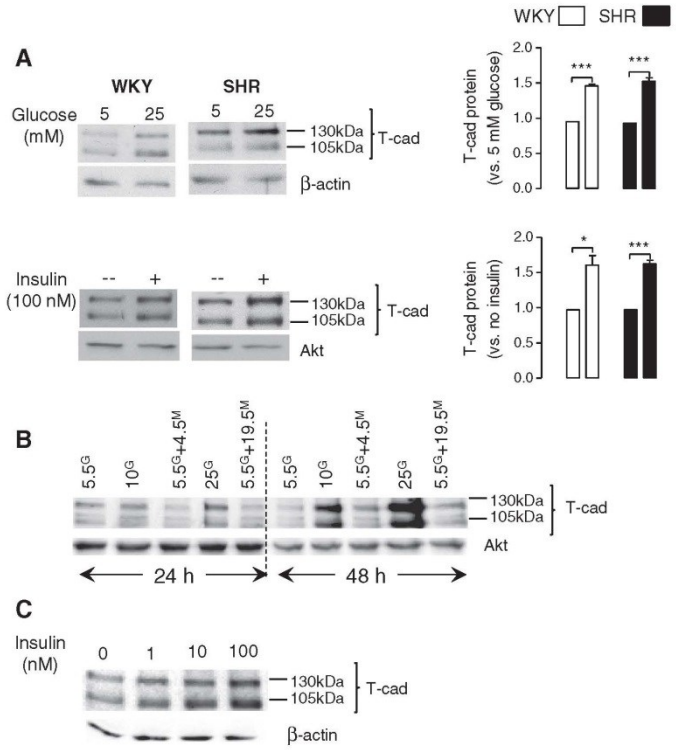


Fig. 1. Conditions of hyperglycemia and hyperinsulinemia increase T-cad protein expression in VSMC. (A) Parental WKY-VSMC and SHR-VSMC were grown to confluence in normal growth medium and then incubated for 24 h under high glucose (25 mM) conditions or with inclusion of insulin (100 nM). Hyperglycemia and hyperinsulinemia experimental series were performed independently. T-cad protein in cell lysates was determined by immunoblotting. Mature (105 kDa) and prepro (130 kDa) T-cad proteins were evaluated jointly, and data (mean ± SD, n = 3) express levels of T-cad relative to those under control conditions (5.5 mM glucose, no insulin). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (two-tailed Student's *t*-test). (B) Immunoblot for T-cad protein in lysates from parental SHR-VSMC after incubation for 24 h and 48 h under hyperglycemic (10 mM or 25 mM glucose, 10^{G} or 25^{G}) or control iso-omolar conditions (5.5 mM glucose plus 4.5 mM or 19.5 mM mannitol, $5.5^{G} + 4.5^{M}$ or 19.5^{M}) excludes that the effects of glucose are due to osmotic stress. (C) Immunoblot for T-cad protein in lysates from parental WKY-VSMC after incubation for 24 h in the presence of the indicated concentrations of insulin.

3.2. Alterations in T-cad expression on VSMC impact constitutive and insulin-induced Akt/mTOR axis signaling

Signal transduction pathway utilization by T-cad in VSMC has never been studied. Since in EC T-cad modulates Akt/mTOR pathway, and because signaling along this axis is characteristically impaired in insulin resistant states, we investigated whether modulation of T-cad expression in VSMC alters constitutive and/or insulin-induced Akt axis signaling. Experiments were performed using rat (WKY and SHR) aortic VSMC stably transduced to overexpress T-cad (Tcad+) and human (Hu) aortic VSMC either transiently transduced to overexpress (Tcad+) or stably transduced with respect to T-cad overexpression or deficiency (shT). Empty vector (E) or scrambled shRNA (shC) were used as respective controls. Transduction efficiency was estimated by Western blotting (Fig. 2A). For insulin responsiveness studies we chose 10 min as an optimal stimulation period, based on kinetic analysis of Akt phosphorylation in response to 100 nM insulin which was the highest dose used in our study (Fig. 2B).

Phosphorylation levels of key signaling effectors within the PI3K/Akt/mTOR axis were measured under baseline serum-free culture conditions and following stimulation with insulin. Comparable results were obtained with respect to T-cad overexpression for all VSMC types. At baseline the levels of p-Akt^{ser473}, p-GSK3β^{ser9} and p-S6RP$^{ser235/236}$ were higher in Tcad+-VSMC than control E-VSMC. Insulin increased phosphorylation of Akt^{ser473}, GSK3β^{ser9} and S6RP$^{ser235/236}$ in E-VSMC, while responses of Tcad+-VSMC were attenuated (Fig. 3A–C). Signaling and functional consequences of T-cad-deficiency in Hu-VSMC were qualitatively opposite to those of T-cad overexpression: shT-VSMC exhibited reduced Akt/mTOR axis signaling at basal conditions, while their relative responsiveness to insulin was greater than that of their respective control shC-VSMC (Fig. 3C).

It is to be pointed out that the experiments on the transduced WKY-VSMC, SHR-VSMC and Hu-VSMC were never performed in parallel; our study was designed to establish the effects of T-cadherin on VSMC signaling/function, and not to compare differential consequences of ectopic T-cad changes in VSMC from different strain/species origin. However, from the initial experimental series in which parental WKY- and SHR-VSMC were studied in parallel we observed a ~2-fold increase in constitutive levels of T-cad protein in SHR-VSMC (Fig. 2C). Further, our comparative analysis of parental WKY- and SHR-VSMC with respect to constitutive and insulin-induced Akt activation status (Fig. 2B) confirms the previous observations that SHR-VSMC exhibit elevated p-Akt at baseline [21] and blunted responsiveness to insulin [22]. Together, this may suggest that T-cad upregulation in SHR might be a characteristic feature of hypertensive and insulin-resistant VSMC phenotype.

3.3. T-cad modulates the levels of total and serine phosphorylated IRS-1 in VSMC

Attenuation of insulin signaling is achieved through a negative feedback loop involving Akt-dependent stimulation of mTOR complex with Raptor mTORC1 and its effector kinase S6K1 which phosphorylates IRS-1 on ser$^{636/639}$, causing IRS-1 dissociation from p85, inactivation and degradation [23]. To determine whether T-cad modulates IRS-1 expression and/or activity in VSMC, we measured the levels of total IRS-1 and p-IRS-1$^{ser636/639}$ in SHR-, WKY- and Hu-VSMC transductants. At baseline Tcad+-VSMC exhibited lower levels of total IRS-1, increased levels of p-IRS-1$^{ser636/639}$ and accordingly a higher p-IRS-1:IRS-1 ratio than E-VSMC (Fig. 4A–C). Following insulin stimulation, only E-VSMC showed a significant increase in serine phosphorylation of IRS-1, achieving

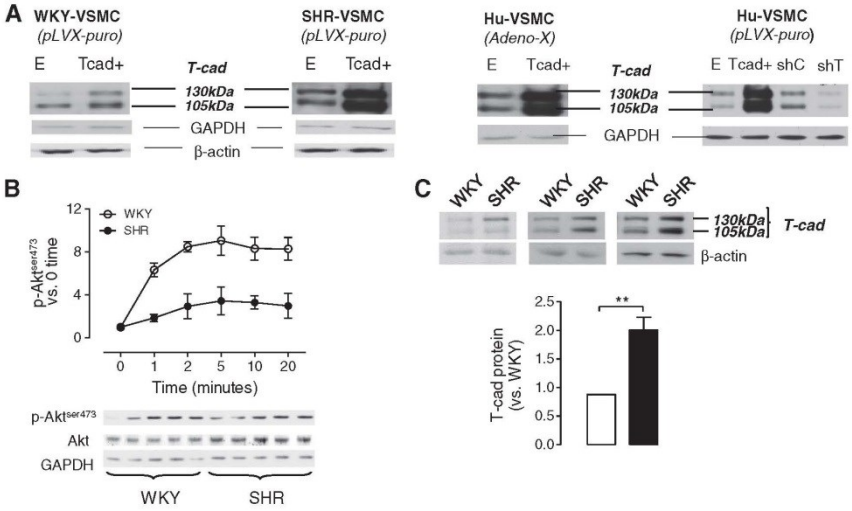


Fig. 2. (A) Viral vector-mediated alteration of T-cad expression in VSMC. T-cad was variously overexpressed in WKY-, SHR- and Hu-VSMC (Tcad+) using pLVX-puro vector (for stable transduction) or Adeno-X vector (for transient transduction) carrying full length human T-cad cDNA. Respective empty viral vectors served as controls (E). Stable transductants of T-cad-deficient Hu-VSMC (shT) were generated using pLKO.1-puro vector carrying human T-cad shRNA or non-target shRNA as control (shC). Three independent sets each of WKY-, SHR- and Hu-VSMC stable transductants and four of Hu-VSMC transient transductants were generated for the experiments described in this study. Representative immunoblots of T-cad protein (M_r of 105 kDa and 130 kDa for mature and prepro proteins) in whole cell lysates are presented. GAPDH, β-actin or Akt were variously used as internal loading controls. (B) Kinetics of insulin-induced Akt phosphorylation in parental WKY and SHR-VSMC. Parental WKY- and SHR-VSMC were grown to 70% confluence, serum-deprived for 24 h and then stimulated with insulin (100 nM) for the indicated times. Whole cell lysates were immunoblotted for p-Akt^{ser473} and total Akt or GAPDH as internal loading controls. Representative blots are shown. Insulin-induced changes in p-Akt^{ser473} levels in WKY- and SHR-VSMC (mean ± SD, n = 3) are expressed relative to those (arbitrarily set at 1.0) in the respective unstimulated VSMC. (C) T-cadherin expression in parental WKY- and SHR-VSMC. The three different sets of WKY- and SHR-VSMC used for the experiments in (B) were individually compared for T-cad protein. T-cad level in SHR-VSMC is expressed relative to WKY-VSMC (mean ± SD, n = 3). $^{**}P < 0.01$ (two-tailed Student's *t*-test).

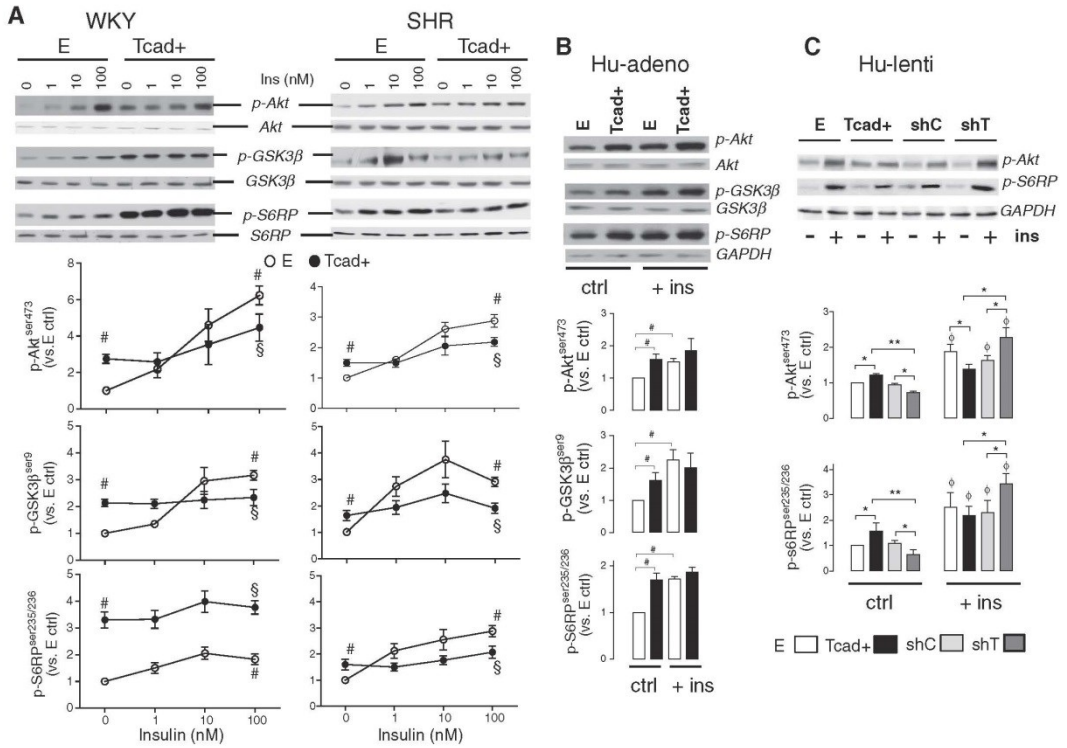


Fig. 3. T-cad alters constitutive and insulin-induced Akt axis signaling. Stable WKY-, SHR- (A), transient (B) and stable (C) Hu-VSMC transductants were stimulated (10 min) with insulin (A at 1–100 nM; B, C at 100 nM). Lysates were immunoblotted for the indicated proteins. Insulin-induced changes (mean ± SD, n = 3–4) in the levels of phospho-proteins are expressed relative to levels in unstimulated E-VSMC. (A, B) # compares E ctrl *vs.* Tcad + ctrl or E plus 100 nM insulin, and § compares E and Tcad + in the presence of 100 nM insulin (P at least <0.01; one-way ANOVA, Tukey). (C) *P < 0.05, **P < 0.01, and ϕ indicate significant effect (P at least <0.05) of insulin for any given Hu-VSMC transductant as compared to its respective ctrl (one-way ANOVA, Tukey).

the baseline level of p-IRS-1$^{ser636/639}$ and p-IRS-1:IRS-1 ratios of T-cad+-VSMC (Fig. 4A–C). As expected for the brief (10 min) duration of stimulation total IRS-1 levels remained steady. T-cad deficient Hu-VSMC exhibited decreased levels of serine phosphorylated IRS-1 concomitant with elevated levels of total IRS-1 at basal conditions and greater responsiveness to insulin than that of their respective control shC-VSMC (Fig. 4C).

3.4. T-cad influences the phosphorylation status of myosin light chain phosphatase regulatory targeting subunit MYPT1 and myosin light chain

We explored the effects of T-cad on VSMC contraction potential by investigating the iNOS expression levels and phosphorylation status of the regulatory myosin binding subunit (MYPT1) of myosin light chain phosphatase (MLCP) and of MLCP target MLC_{20}. In the T-cad+-VSMC constitutive iNOS expression was elevated and constitutive levels of p-MYPT1$^{thr696\ or\ thr853}$ and p-$MLC_{20}^{thr18/ser19}$ were lower (Fig. 5). Since iNOS expression is related to vasodilation while contraction of VSMC is accompanied by MYPT1 phosphorylation/inactivation of MLCP and concomitant MLC_{20} phosphorylation these data suggest that T-cad + upregulation might diminish intrinsic contractile competence. Insulin induced an increase in iNOS expression and phosphorylation of MYPT1 and MLC_{20} in E-VSMC transductants whereas Tcad+-VSMC transductants remained insensitive (Fig. 5). shT-VSMC exhibited elevated levels of MLC_{20} and MYPT1 phosphorylation at baseline and enhanced insulin responsiveness with respect to phosphorylation of both molecules (Fig. 5D).

Small GTPase RhoA, *via* ROKα, can phosphorylate MYPT1 and inactivate MLCP or directly phosphorylate MLC_{20} [24,25]. G-LISA assay revealed lower constitutive RhoA activity in Tcad+-VSMC and an insulin-induced increase in activity only in E-VSMC (Fig. 6A–C), which correlates with the data on MYPT1 and MLC_{20} phosphorylation status. Higher constitutive RhoA-GTP levels and a stronger response to insulin were observed in shT-VSMC (Fig. 6C).

3.5. T-cad expression on VSMC influences compaction of VSMC-populated 3D-collagen lattices

To examine whether the differential effects of T-cad overexpression or deficiency on phosphorylation status of MLC_{20} and MYPT1 might manifest as differential contractile capacities we performed the cell-populated collagen lattice (PCL) compaction assay using free-floating VSMC-PCLs. Representative phase contrast images of VSMC-PCLs after a 20 h incubation are shown in Fig. 7A. Typical data from in-gel "live–dead" and "Alamar Blue" assays on VSMC-PCLs are presented to illustrate that differences in compaction are not due to differences in cell viability and proliferation (Fig. 7B and C, respectively).

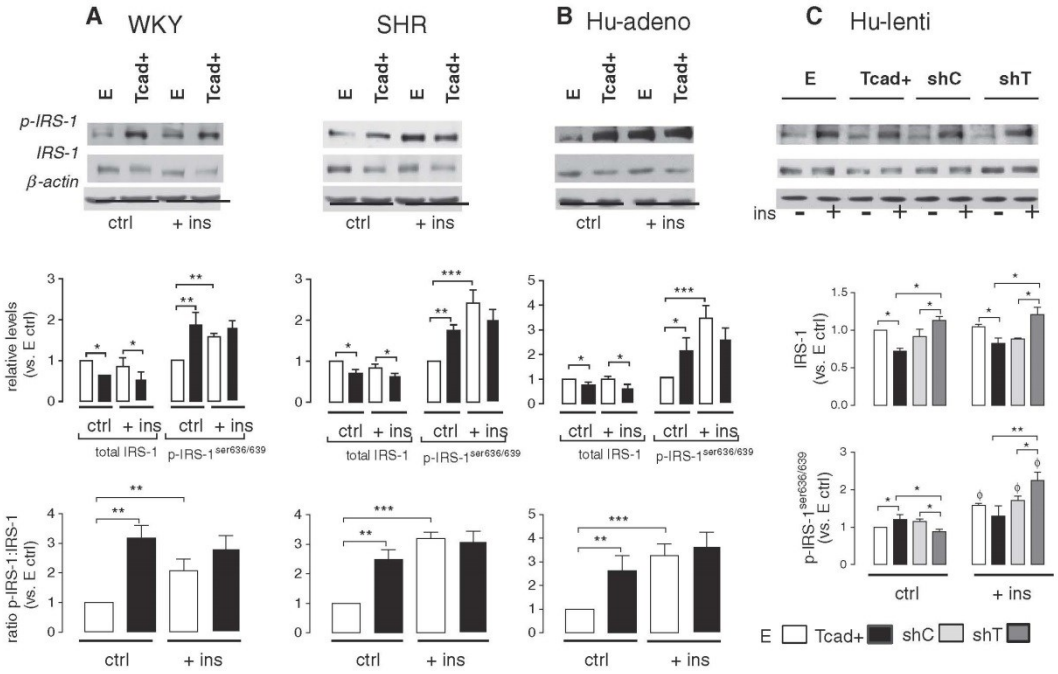


Fig. 4. T-cad modulates the levels of IRS-1 protein and IRS-1 phosphorylation. Stable WKY- and SHR- (A) VSMC transductants and transient (B) and stable (C) Hu-VSMC transductants were stimulated (10 min) with 100 nM insulin and immunoblotted for the indicated proteins. Levels of p-IRS-1$^{ser636/639}$, total IRS-1 (A–C) and ratios of total p-IRS-1$^{ser636/639}$:total IRS-1 (A, B) are expressed relative to values in unstimulated E-VSMC (E ctrl). Values are mean ± SD (n = 3). *P< 0.05, **P< 0.01, ***P< 0.001, and ϕ indicate significant (P at least <0.05) effect of insulin for any given Hu-VSMC transductant as compared to its respective ctrl (one-way ANOVA, Tukey).

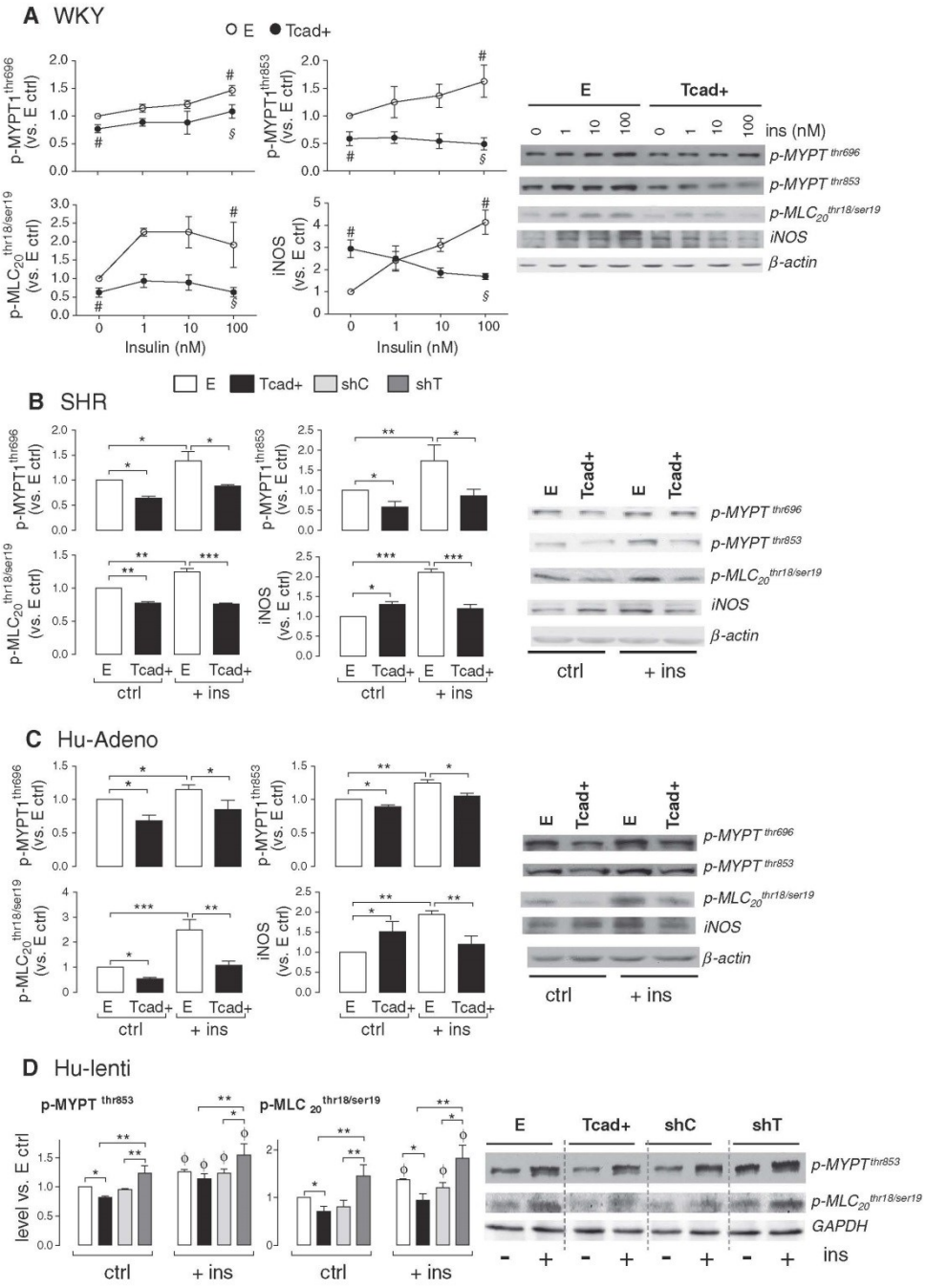
Surprisingly, given that the levels of p-MLC$_{20}$/p-MYPT1 were decreased in Tcad+-VSMC and increased in shT-VSMC, compaction (estimated as decreased lattice area under baseline conditions) was greater for T-cad+-VSMC-PCLs and smaller for shT-VSMC PCLs (Fig. 7D, E). Consistent with its effects on MLC$_{20}$ and MYPT1 phosphorylation, insulin caused compaction of E-VSMC-PCLs whereas T-cad+-VSMC-PCLs did not further compact, again demonstrating insulin resistance in T-cad-overexpressing VSMC (Fig. 7D, E). In some experiments on WKY- and SHR-VSMC the free-floating PCLs were fixed at the end of incubations, embedded in paraffin and sectioned for staining with Picrosirius Red. Brightfield imaging at low (20×) magnification revealed that E-VSMC generally remained as "single" cells whereas Tcad+-VSMC appeared more "interconnected" (Fig. 7F). This prompted us to consider whether the unexpected findings for free-floating PCL compaction might not reflect modulation of microfilament-mediated cell contraction, but rather how cell populations interact with and/or organize themselves within the 3D-matrix [26,27].

3.6. T-cad expression on VSMC affects collagen fibril organization in 3D-collagen matrices

To further investigate the effects of T-cad on collagen matrix reorganization we applied high (100×) magnification analysis after Picrosirius Red staining using both brightfield microscopy to grossly view collagen fibril organization and circularly polarized light microscopy to enable quantification of pericellular organization into thicker collagen fibrils. To exclude any possible artifacts due to lattice compaction following the release of the gel–dish interface, these experiments were performed on unreleased PCLs with in-plate fixation.

We detected prominent effects of T-cad on collagen fibril organization (Fig. 8 for rat VSMC and Fig. 9 for Hu-VSMC). Under baseline conditions collagen fibrils in Tcad+-VSMC-PCLs were visibly and quantitatively more packed and condensed than in E-VSMC-PCLs and aligned in parallel arrays, particularly in the pericellular space and zones of contact between telepodia/lamellipodia of the cells and collagen fibrils. In E-VSMC-PCLs insulin stimulated a reorganization of collagen into thicker, more packed and aligned fibrils than at baseline whereas no further fibril thickening was obvious in Tcad+-VSMC-PCLs (Figs. 8 and 9). T-cad silenced cells displayed reduced collagen fibril reorganization at basal conditions but were able to reorganize collagen in response to insulin stimulation (Fig. 9). These data exactly match the results of the compaction assay (Section 3.5) suggesting that T-cad effects on 3D-lattice geometry are indeed due to modulation of matrix remodeling and not to alterations in VSMC contractile apparatus.

Fig. 5. T-cad alters the expression of iNOS and phosphorylation status of MYPT1 and MLC. Stable WKY- (A) and SHR- (B) VSMC transductants and transient (C) and stable (D) Hu-VSMC transductants were stimulated for 10 min with insulin (A, 1–100 nM; B–D, 100 nM) and immunoblotted for the indicated proteins. Levels are expressed relative to levels in unstimulated E-VSMC (E ctrl) and values are given as mean ± SD (n = 3). (A) # compares E ctrl *vs.* Tcad+ ctrl or E plus 100 nM insulin, and § compares E and Tcad+ in the presence of 100 nM insulin (P at least <0.05; one-way ANOVA, Tukey). (B–D) *P< 0.05, **P< 0.01, ***P< 0.001 (one-way ANOVA, Tukey). (D) ϕ indicates significant effect (P at least <0.05) of insulin for any given Hu-VSMC transductant as compared to its respective ctrl (one-way ANOVA, Tukey).


A WKY
E
Tcad+
p-MYPT1thr696 (vs. E ctrl)
p-MYPT1thr853 (vs. E ctrl)
p-MLC20thr18/ser19 (vs. E ctrl)
iNOS (vs. E ctrl)
Insulin (nM)
ins (nM)
p-MYPT thr696
p-MYPT thr853
p-MLC20thr18/ser19
iNOS
β-actin
shC
shT
B SHR
ctrl
+ ins
C Hu-Adeno
D Hu-lenti
p-MYPT thr853
p-MLC 20thr18/ser19
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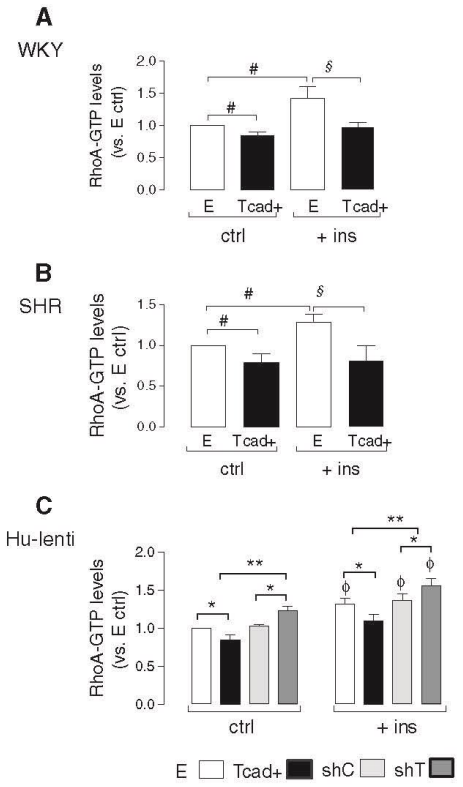

Fig. 6. T-cad alters the activity of RhoA GTPase. Stable WKY- (A), SHR- (B) and Hu-VSMC (C) transductants were stimulated (10 min) with 100 nM insulin and cell lysates analyzed for RhoA activation by G-LISA. Values for levels RhoA-GTP (mean ± SD, n = 3) are expressed relative to levels in unstimulated E-VSMC (E ctrl). (A, B) # compares E ctrl *vs.* Tcad+ ctrl or E plus 100 nM insulin, and § compares E and Tcad+ in the presence of 100 nM insulin (P at least <0.05; one-way ANOVA, Tukey). (C) *$P < 0.05$, **$P < 0.01$, and ϕ indicate significant (P at least <0.05) effect of insulin for any given Hu-VSMC transductant as compared to its respective ctrl (one-way ANOVA, Tukey).

4. Discussion

We previously reported that chronic upregulation of T-cad in EC promotes the development of insulin resistance in this cell type [5]. This study provides first evidence for a relationship between T-cad upregulation and insulin resistance also in VSMC, in as much as exposure of VSMC to components of the "whole-body" milieu of insulin-resistance states (*e.g.* hyperinsulinemia or hyperglycemia) caused upregulation of T-cad protein on cultured VSMC while ectopic overexpression of T-cad in VSMC blunted sensitivity to insulin. Although under basal growth conditions upregulation of T-cad *per se* increases constitutive Akt/mTOR signaling, reduces contractile competence and promotes collagen matrix remodeling, it significantly attenuates these signaling and functional cell responses to insulin stimulation. T-cad deficiency, on the contrary, increased sensitivity of VSMC to insulin. The ensuing discussions on the effects of T-cad expression on VSMC signaling/function are focused on T-cad upregulation since this is the *in vivo* pathological event during atherosclerosis [11] and restenosis [12].

Elevation of T-cad in VSMC cultured under hyperinsulinemic or hyperglycemic conditions is consistent with previous observations of T-cad upregulation in EC under conditions of hyperinsulinemia [5] or oxidative stress [9] and supports the view of T-cad as a marker of vascular cell activation and stress [28]. The SHR, a widely studied model of essential hypertension is hyperinsulinemic and insulin resistant [29] and aortic VSMC isolated from SHR also exhibit blunted responsiveness to insulin [22], as confirmed herein. Our data spectrum indicates that the response of SHR-VSMC, whether parental or transduced, to insulin would generally appear to be blunted when compared with the respective WKY-VSMC counterpart. The mechanisms previously suggested to underlie insulin resistance in VSMC include, *inter alia*, alterations in insulin receptor expression [30] and also growth factor and/or vasoconstrictor peptide associated alterations in signal transduction pathways/effectors that converge on, and negatively impact or uncouple, the insulin signal cascade [14–16]. We found that T-cad protein levels were higher in parental SHR-VSMC than parental WKY-VSMC. Further, following transduction T-cad protein levels increased approximately 3–5-fold in VSMC from both strains (*i.e.* levels in SHR remain higher). These data do not permit inference of a direct causative relationship between elevated T-cad expression in SHR-VSMC and their state of insulin resistance. Nevertheless, we speculate that altered T-cad expression is likely related to general changes in VSMC gene expression profiles associated with acquisition of hypertension and insulin resistance. Clinical and genetic data on T-cad expression profiles in cardiovascular and metabolic diseases are scant; however, correlations between single nucleotide polymorphisms within intron 11 and promoter regions of CDH13 gene and long-term average diastolic and systolic blood pressure phenotypes and arterial stiffness have been reported [31,32], implicating an involvement of T-cad in the progression of hypertension.

IRS proteins serve as crucial multisite docking proteins that couple insulin receptor (and IGF-1 receptor) with a complex network of downstream effector molecules [23]. They are targets for serine/threonine phosphorylation-based regulation that leads to receptor–effector uncoupling and attenuation of insulin/IGF-1-dependent signaling [23]. Kinases implicated in this negative regulation include those that are mediators of insulin signaling (*e.g.* mTOR/S6K1, Erk1/2) and those activated by pathways independent of insulin (*e.g.* AMPK, JNK, PKC) [23]. Persistent activation of PI3K/Akt signaling with sustained feedback mTOR/S6K1-dependent serine phosphorylation and degradation of IRS-1 is recognized as one of the most common mechanisms underlying the development of insulin resistance in major insulin-sensitive tissues [23] and also VSMC [14–16]. Consistent with this mechanism, we found that Tcad+-VSMC constitutively exhibited hyperactivation of Akt/mTOR/S6K1 axis signaling, increased p-IRS-1$^{ser636/639}$ and decreased total IRS-1 in association with attenuated insulin-stimulated signaling through Akt/mTOR/S6K1. Thus T-cad upregulation on VSMC in response to stress, hyperglycemia or hyperinsulinemia initiated as a compensatory response to loss of insulin sensitivity might lead to T-cad-dependent inactivation of insulin signaling, further exacerbating the progression of insulin resistance.

The role of PI3K/Akt in regulation of VSMC contractility is complex. On the one hand, studies in cultured VSMC and de-endothelialized smooth muscle strips reported that PI3K-C2α/Akt activation positively modulates membrane depolarization- and excitatory receptor agonist-induced contraction by promoting Ca^{2+}-dependent, Rho/ROK-mediated negative regulation of MLCP *via* MYPT1 phosphorylation and thereby increased MLC_{20} phosphorylation [33]. On the other hand, gain- and loss-of function studies in cultured VSMC demonstrated a role for constitutive Akt hyperactivation in induction of iNOS expression, suppression of RhoA/ROK activity and MYPT1 phosphorylation and accordingly decreased phosphorylation of MLC_{20} [34]. In agreement with the latter setting of constitutive Akt hyperactivation we found that T-cad+-VSMC exhibited increased iNOS expression, reduced RhoA activity and decreased p-MYPT1 and p-MLC_{20}, which would imply a reduced intrinsic contractile competence. These data concur with immunohistological demonstrations of increased T-cad expression

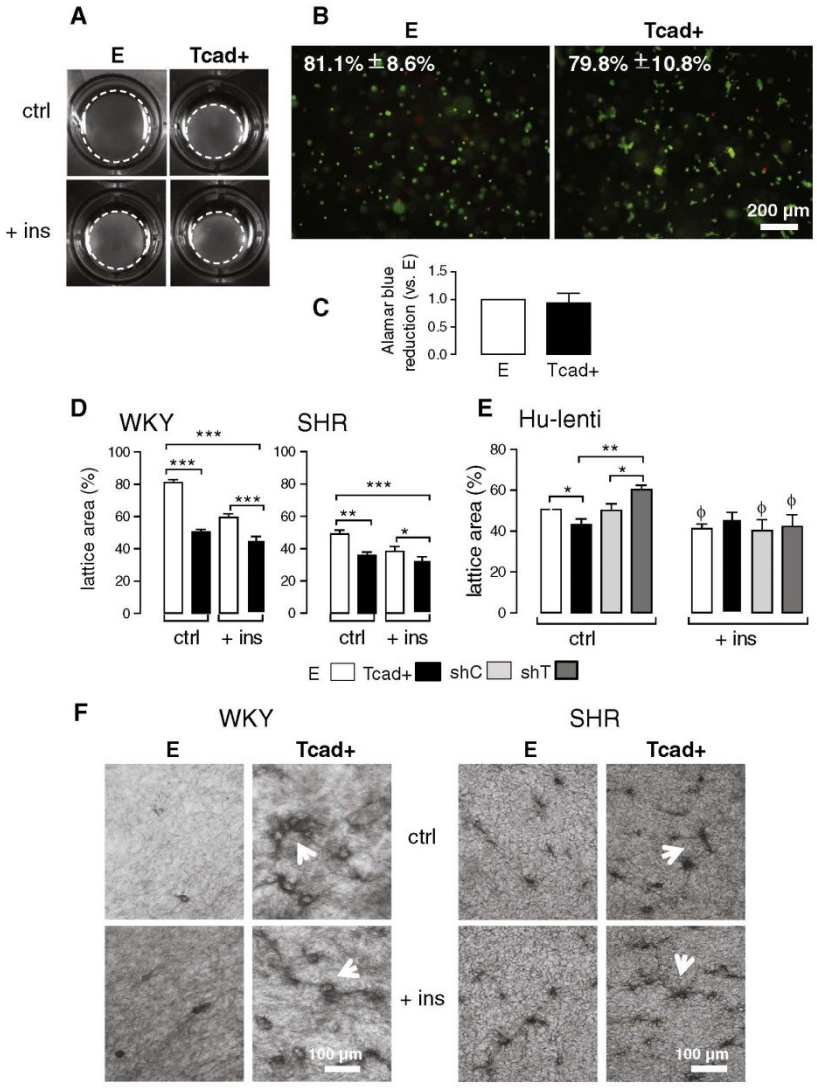

Fig. 7. T-cad influences collagen lattice compaction. Free-floating PCLs containing stable WKY-, SHR-VSMC or Hu-VSMC were cultured (20 h) under serum-free conditions without (ctrl) or with 100 nM insulin. Data from WKY-VSMC-PCLs only are shown in A–C. (A) Typical phase contrast images of lattices after 20 h incubation. (B) In-gel live–dead assay after 20 h under serum-free conditions using calcein-AM and propidium iodide to stain live and dead cells, respectively. Representative images are shown (scale bar, 200 µm for both photomicrographs) and numbers represent the proportion of viable cells (mean ± SD, n = 3). (C) Alamar Blue staining in-gel was applied to examine for any differences in proliferation. Alamar Blue reduction in Tcad+-VSMC populated lattices is expressed relative to that in control E-VSMC populated lattices (mean ± SD, n = 3). (D, E) VSMC-PCL areas were measured at the end of incubations and expressed relative to the area (100%) of the whole well. Data are mean ± SD (n = 3–4). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and ϕ indicate significant (P at least <0.05) effect of insulin for any given Hu-VSMC transductant as compared to its respective ctrl (one-way ANOVA, Tukey). (F) Following area measurements WKY- and SHR-VSMC-PCLs were fixed and paraffin-embedded. Sections were stained with Picrosirius Red and viewed under brightfield microscopy at 20× magnification. Representative images are shown (scale bar, 100 µm for all photomicrographs). Arrows indicate the more "interconnected" appearance of embedded T-cad+-VSMC.

in the vessel during atherosclerosis and restenosis in association with loss of the contractile VSMC phenotype [11,12]. Since insulin-dependent activation of PI3K/Akt pathway plays a role in the maintenance of VSMC quiescence and contractile phenotype [2], T-cad upregulation in the vessel wall under stress conditions and resulting insulin resistance may jointly contribute to VSMC phenotypic modulation and changes in contractile competence during disease progression.

Insulin is an important regulator of vascular tone, with vasodilatation properties *in vivo* that are mainly mediated *via* Akt-dependent phosphorylation and eNOS activation in EC [1,3]. Direct relaxant effects

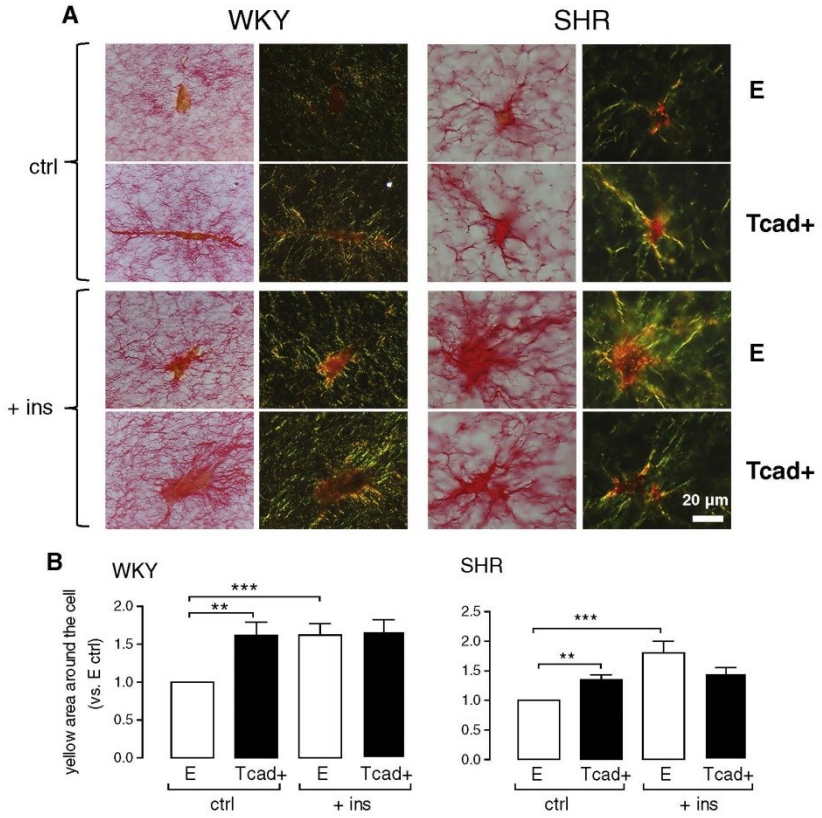

Fig. 8. T-cad overexpression promotes collagen fibril reorganization. Unreleased PCLs containing WKY- or SHR-VSMC transductants were cultured (20 h) under serum-free conditions without (ctrl) or with 100 nM insulin, then fixed in-plate and paraffin-embedded. (A) Sections were stained with Picrosirius Red and viewed at 100× magnification under brightfield (upper panels) and circularly polarized light (lower panels) microscopy. Representative images are shown. Scale bar, 20 µm for all photomicrographs. (B) Images captured under polarized light were analyzed using a standardized color thresholding method to determine the changes in collagen fibril organization. Pericellular area occupied by thicker (yellow) fibrils was quantified and data (mean ± SD, n = 3–4) are expressed relative to the value in unstimulated (ctrl) E-VSMC-PCLs. $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA, Tukey).

of insulin on contractile machinery in cultured VSMC have been described to partly involve activation of Akt, iNOS-cGK1α and inhibition of RhoA/ROKα [34–36]. Here, in control E-VSMC insulin induced increases in MLC_{20}/MYPT1 phosphorylation, iNOS expression (considered downstream of Akt) and activity of RhoA (considered upstream of MYPT1). These data suggest direct constrictor effects of insulin on VSMC and contrast with previous reports by Begum et al. [35,36]. On the other hand, our findings of a pro-contractile response of VSMC to insulin are consistent with other reports of increased MLC_{20}/MYPT1 phosphorylation in VSMC following acute [34] or chronic [37] insulin stimulation and insulin-induced constriction of endothelium-denuded arterioles [38]. Likewise, in airway SMC, which are qualitatively similar to other SMC with respect to mechanical, structural and biochemical properties [39] insulin induced phosphorylation of MYPT1 *in vitro* [40] and contraction in epithelium-denuded tracheal smooth muscle strips *ex vivo* [41]. Further, insulin may have biphasic effects on VSMC and with pro-relaxation effects being conditional and dependent upon an existing state of contraction [34,42].

VSMC-PCL contraction/compaction has been used as an *in vitro* model to mimic *in vivo* extracellular matrix (ECM) remodeling and the arterial contraction and inward remodeling that occurs in atherosclerosis and restenosis. Paradoxically, given that their balance of MLCK/MLCP activities is apparently biased toward MLCP, Tcad+-VSMC exhibited greater capacity to cause compaction of free-floating lattices under serum-free conditions. On the other hand, insulin stimulated compaction of free-floating lattices populated with E-VSMC but not those populated with Tcad+-VSMC, which is in keeping its effects on MYPT1/MLC_{20} phosphorylation status in E-VSMC (pro-contractile) and Tcad+-VSMC (negligible). Insulin-induced compaction of E-VSMC-PCLs is consistent with a previous study using rabbit aortic VSMC [43]. Additionally, insulin has been shown to enhance compaction of gels populated with other cell types including cardiac fibroblasts [44] and C2C12 myoblast cells [45].

Realignment and contraction of collagen fibrils in cell-PCL assays are thought to mimic ECM reorganization during morphogenesis and wound healing. Atherosclerosis resembles the wound healing process in several ways, including cellular hyperplasia and ECM reorganization leading to constrictive remodeling. Following injury VSMC lose their contractile phenotype and migrate out from the media into the intima where they proliferate and secrete extracellular matrix rich in collagen

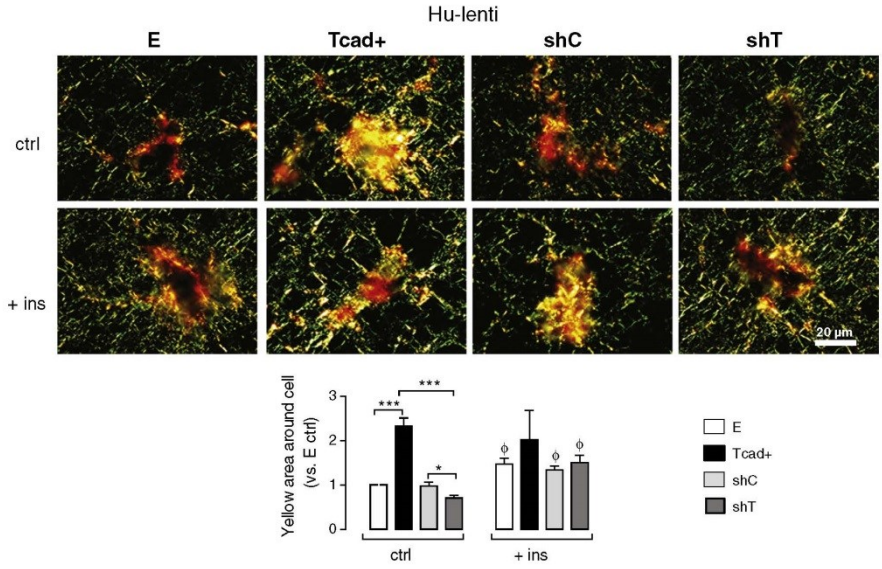

Fig. 9. Collagen fibril reorganization is differentially affected by T-cad overexpression or T-cad deficiency. Unreleased PCLs containing Hu-VSMC transductants were cultured (20 h) under serum-free conditions without (ctrl) or with 100 nM insulin, then fixed in-plate and paraffin-embedded. Sections were stained with Picrosirius Red and viewed at 100× magnification under circularly polarized light microscopy. Representative images are shown (scale bar, 20 µm for all photomicrographs). Collagen fibril organization was analyzed using a standardized color thresholding method. Pericellular area occupied by thicker (yellow) fibrils was quantified and data (mean ± SD, n = 3) are expressed relative to the value in unstimulated (ctrl) E-VSMC-PCLs. $^{*}P < 0.05$, $^{***}P < 0.001$, and ϕ indicate significant (P at least <0.05) effect of insulin for any given transductant as compared to its respective ctrl (one-way ANOVA, Tukey).

and proteoglycans, and they can participate in higher organization of newly synthesized collagen matrix to form the dense network of collagen typically seen in advanced atherosclerotic lesions [46]. VSMC are capable of regulating structure in a collagenous matrix both acutely through rapid MLCK/MLCP-controlled active cell shortening and contraction and/or chronically through reorganization of collagen fibrils [26,27]. To discriminate between these two mechanisms we performed VSMC-PCL assay using unreleased gels, which allows the determination of the influence of T-cad on collagen microarchitecture *per se*. Formation of thicker fibrils pericellularly for Tcad+-VSMC-PCLs at baseline was clearly evident under brightfield and polarizing light microscopy. These data may explain the paradox between reduced levels of MYPT1/MLC_{20} phosphorylation and increased compaction for free-floating Tcad+-VSMC-PCLs at baseline, and also serve to demonstrate that T-cad can influence 3D-lattice architecture by regulating matrix reorganization rather than by promoting microfilament-mediated contraction.

A further novel finding in our study is that insulin induced a change in collagen microarchitecture of unreleased VSMC-PCLs. This, to our knowledge, is the first demonstration of effects of insulin on matrix fibril reorganization by VSMC. The potential clinical relevance of this phenomenon is not clear; however, altered ECM structure and composition causing enhanced inward remodeling, altered vessel wall/lumen ratio and impaired vessel dilation in diabetic subjects are well documented [47] suggesting that regulation of matrix structure by insulin might be an important factor in the maintenance of healthy vascular tissue architecture.

In conclusion we have provided evidence, for the first time, that chronic T-cad upregulation on VSMC alters constitutive activity of PI3K/Akt/mTOR signaling axis which carries feedback impact for modulating responsiveness of VSMC to environmental cues (*i.e.* promotion of insulin resistance). Further, in demonstrating effects of T-cad on VSMC contractile machinery and collagen fibril organization, the study reveals novel cadherin-based modalities to modulate vascular function and geometry through effects on contractile competence and organization of ECM.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

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T-cadherin promotes vascular smooth muscle cell dedifferentiation *via* a GSK3β-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 SMCs 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 SMCs stably transduced with respect to T-cadherin upregulation (Tcad+) or T-cadherin-deficiency (shTcad) were compared with their respective control transductants (E-SMCs or shC-SMCs). Tcad+-SMCs displayed several characteristics of the dedifferentiated phenotype including loss of spindle morphology, reduced/disorganized stress fiber 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 SMCs and we investigated mTORC1/S6K1 and GSK3β axes as mediators of T-cadherin-induced dedifferentiation. Inhibition of mTORC1/S6K1 signalling by rapamycin suppressed proliferation in both E-SMCs and Tcad+-SMCs but failed to restore expression of contractile protein markers in Tcad+-SMCs. Ectopic adenoviral-mediated co-expression of constitutively active GSK3β mutant S9A in Tcad+-SMCs restored the morphological and molecular marker characteristics of differentiated SMCs and normalized rate of proliferation to that in control SMCs. In conclusion our study demonstrates that T-cadherin promotes acquisition of the dedifferentiated phenotype *via* a mechanism that is dependent on GSK3β inactivation.

1. Introduction

Vascular smooth muscle cells (SMCs) are highly specialized cells whose main function is contraction and maintenance of vascular tone. In the mature vessel SMCs 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 SMCs 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 down-regulation 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 the 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, and

Abbreviations: BSA, Bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; E, Empty vector; FCS, Foetal calf serum; GSK3β, Glycogen synthase kinase 3β; 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; *sm*MHC, Smooth muscle myosin heavy chain; Tcad+, T-cadherin upregulation; TRITC, Tetramethylrhodamine-isothiocyanate; R-SMC, Wistar–Kyoto rat aortic smooth muscle cells.

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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, 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 SMCs 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 SMCs 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 SMCs 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 SMCs 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 stages 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 SMCs have been noted [12]. Ectopic upregulation of T-cadherin in SMCs *in vitro* increased cell cycle progression [18]. Further, upregulation of T-cadherin transcript and protein expression in SMCs can be induced *in vitro* under pathological conditions (*e.g.* high glucose, oxidative stress, hyperinsulinemia) [19] that are encountered by SMCs *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 SMCs is a molecular discriminator between quiescent/contractile and dedifferentiated/proliferating SMC populations. In this study, and using arterial SMCs 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* the Akt/GSK3β pathway.

2. Materials and methods

2.1. Cell culture and transduction

The isolation and characterization of SMCs from the descending thoracic aortae of 20-week old male Wistar–Kyoto rats (R-SMCs) has been detailed [20]. Isolates of spindle porcine coronary artery SMCs (P-SMCs) were provided by Prof. M.-L. Bochaton-Piallat (University of Geneva, Geneva, Switzerland) [21]. R-SMCs and P-SMCs were maintained in Dulbecco's modified Eagle's medium (DMEM, containing 5.5 mM glucose) supplemented with 10% FCS. Human aortic SMCs (H-SMCs) 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 SMCs 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-SMCs (shTcad) were generated using pLKO.1-puro vector carrying human T-cadherin shRNA or non-target shRNA as control (shC) [22]. SMCs were transduced at passages 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 passages 8–10 after puromycin (3.6 μg/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 SMCs with adenoviral vector encoding constitutively active mutant of GSK3β (Adv-S9A) was performed as described previously [23].

2.2. Immunoblotting

SMCs 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 the 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 (*sm*MHC; clone hSMV recognizing human and porcine species), *h*-caldesmon (Sigma-Aldrich Chemie, Buchs, Switzerland), *sm*MHC (ab53219 recognizing rat species), GAPDH, lamin B1 (Abcam, Cambridge, UK), histone H3, MRTF-A, Akt, phospho (p)-Akt^{Ser473}, p-Akt^{Thr308}, GSK3β, p-GSK3β^{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, and 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 SMCs 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 48 h 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-labelled 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 μg/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

SMCs 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 SMCs was measured using the scratch wound assay [25]. Confluent monolayer cultures of SMCs 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 2 mM 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% CO_2 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

SMCs 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 analyzed by Western blot.

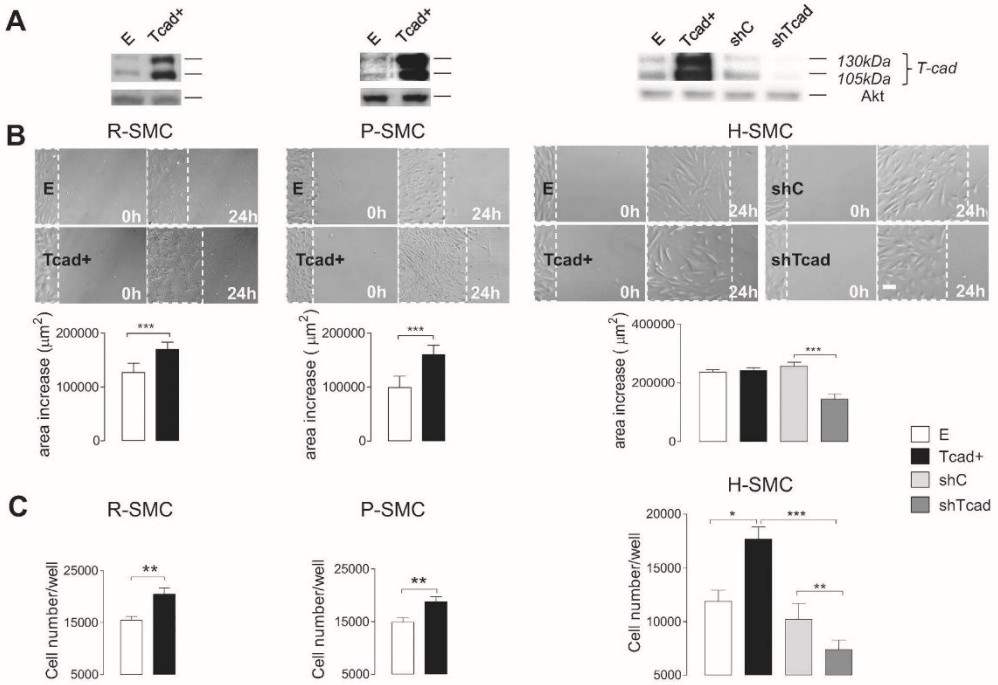

Fig. 1. T-cadherin upregulation promotes SMC migration and increases proliferation capacity. (A) Lentiviral vector-mediated alteration of T-cadherin expression in VSMCs. Stable T-cadherin upregulation in Wistar–Kyoto rat (R-SMCs), porcine (P-SMCs) and human (H-SMCs) vascular smooth muscle cells (Tcad+) was achieved using pLVX-puro vector carrying full length human T-cadherin cDNA. Respective empty viral vector transduced SMCs served as controls (E). Stable transductants of T-cadherin-silenced H-SMCs (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 24 h time points, scale bar: 100 μm. Lower panel: quantification of the area of wound repair. (C) Cell counts after 72 h culture estimated by cell enumeration. Significant differences are marked with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

2.7. Gelatin zymography

SMCs were plated into 24-well plates (45×10^3 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 48 h, centrifuged (5 min, 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 3 × Laemmli sample buffer (1:2, *v*/*v*) and subjected to electrophoresis (50 μl/lane) on 8% SDS-PAGE co-polymerized with gelatin (1 mg/ml; Sigma-Aldrich G-8150). Following electrophoresis gels were sequentially incubated in renaturing solution (2.5% *v*/*v* Triton X-100 in water) for 30 min (3 × 10min) at room temperature and then in developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 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), and 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 ± 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 SMCs at functional, morphological and molecular levels we used SMC isolates from rat (R-SMCs), porcine (P-SMCs) and human (H-SMCs) aortae stably transduced with respect to T-cadherin upregulation (Tcad+) or T-cadherin depletion (shTcad in H-SMCs) and their respective empty vector (E) or scrambled shRNA (shC) controls. Fig. 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-SMCs and P-SMCs migrated into the wound space more rapidly, respectively filling ~30% and ~60% more wound area than their respective controls after a 24 h migration period (Fig. 1B). In the case of H-SMCs, migration capacity was not affected by T-cadherin upregulation but it was significantly reduced by T-cadherin depletion, with shTcad-SMCs filling ~35% less wound area than their shC controls (Fig. 1B). Absence of effects of T-cadherin upregulation on migration capacity of H-SMCs might appear inconsistent with the findings in rat and porcine SMCs. However it is noteworthy that of the three species H-SMCs migrated the fastest; control- (E and shC) and Tcad+-H-SMCs

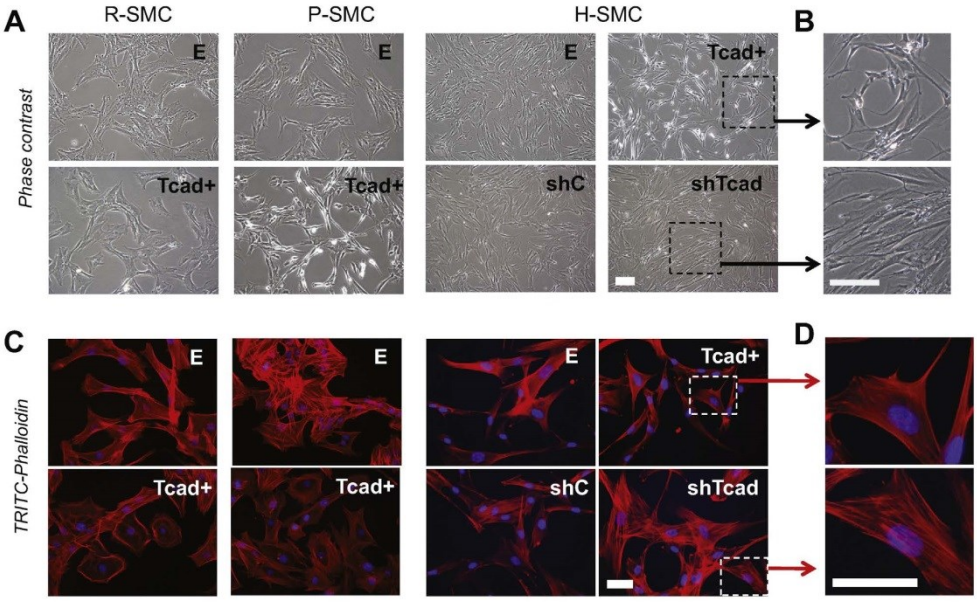

Fig. 2. T-cadherin expression affects SMC morphology and cytoskeleton organization. (A) Phase contrast micrographs of subconfluent SMC monolayers overexpressing T-cadherin (Tcad+), or depleted of T-cadherin (shTcad) and their respective controls (E and shC). Scale bar: 100 μm. (B) High magnification 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: 50 μm.

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-SMCs (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 +-SMCs 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-SMCs 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 +-SMCs and shTcad-SMCs 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 +-SMCs 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-SMCs 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 +-SMCs and shTcad-SMCs 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 SMCs 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

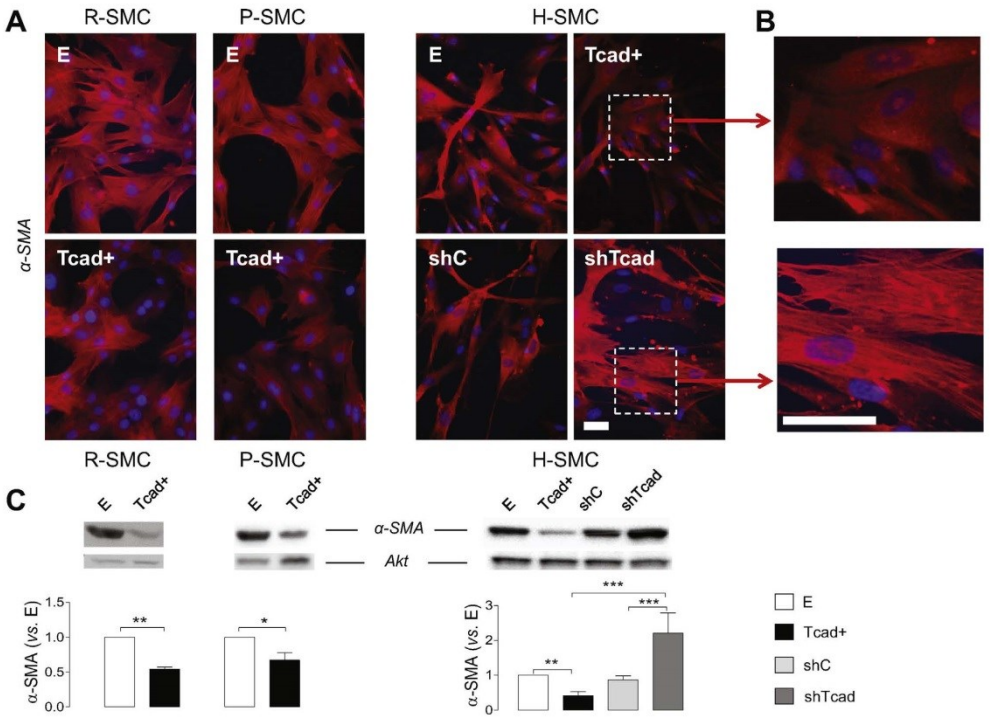

Fig. 3. T-cadherin upregulation reduces α-SMA expression and stress fiber formation. (A) Fluorescence micrographs of α-SMA-stained (red) SMC monolayers. Counterstaining of nuclei is with Hoechst 33342 (blue). (B) Enlarged areas of (A) for better visualization of fibrillar *vs.* diffuse α-SMA dominance.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

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+-SMCs but stronger in shTcad-SMCs as compared with the respective control SMC transductants (Fig. 3A). The α-SMA staining pattern in Tcad+-SMCs was frequently very diffuse with weak and/or truncated stress fibers. In shTcad-SMCs the pattern of α-SMA staining was indicative of very prominent and strong stress fibers. Higher magnification images of human Tcad+-SMCs and shTcad-SMCs 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+-SMCs and shTcad-SMCs, respectively, was confirmed by immunoblotting of whole cell lysates (Fig. 3C).

Other established SMC differentiation marker proteins, namely *sm*MHC and *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+-SMCs exhibited decreased levels of *sm*MHC and *h*-caldesmon and elevated levels of calmodulin (Fig. 4A, B). Conversely, T-cadherin-depletion resulted in increased levels of *sm*MHC and *h*-caldesmon concomitant with decreased levels of calmodulin (Fig. 4b). Reductive effects of T-cadherin upregulation on expression of α-SMA, *sm*MHC 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 T-cad+-SMCs (Fig. 4A, B) but decreased in shTcad-SMCs (Fig. 4B).

Expression of phenotype markers in SMCs 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+-SMCs and increased in shTcad-SMCs (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 T-cad+-SMCs and increased in shTcad-SMCs.

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.

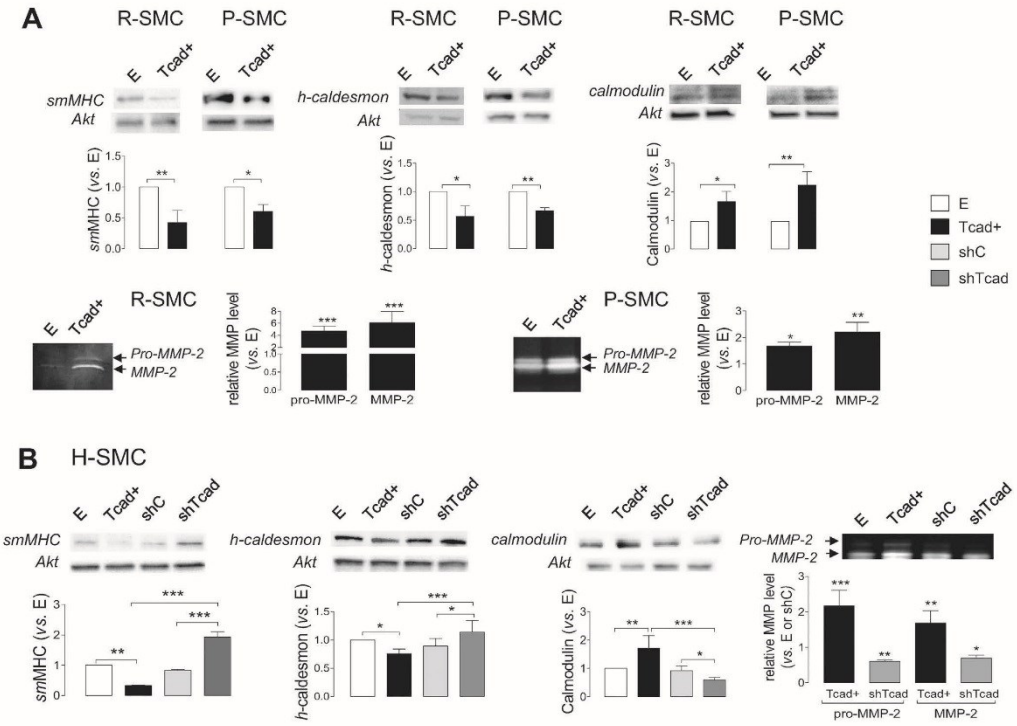

Fig. 4. T-cadherin upregulation leads to decay of SMC differentiation markers. (A) Upper panels: immunoblots of rat and porcine SMC lysates probed for *sm*MHC, *h*-caldesmon and calmodulin. Akt was used as loading control. Contractile marker protein expression levels in Tcad+-SMCs are expressed relative to those in the respective E controls. Lower panels: gelatin zymograms of conditioned media from rat and porcine SMCs. Proteolytic activities of pro- and mature MMP-2 in Tcad+-SMCs 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+-SMCs and shTcad-SMCs are expressed relative to those in their respective E and shC controls. Significant differences are marked with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

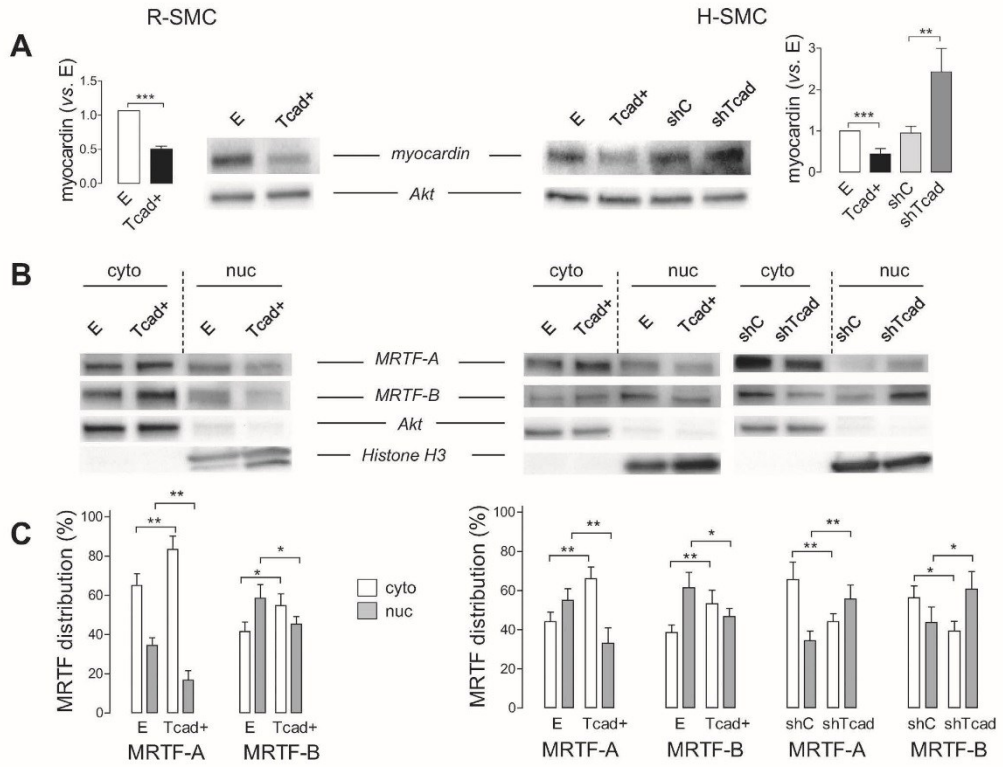

Fig. 5. T-cadherin affects myocardin expression and myocardin-related transcription factor (MRTF) subcellular distribution in R- and H-SMCs. (A) Immunoblots of crude SMC lysates probed for myocardin with Akt used as loading control. Histograms express levels of myocardin relative to the levels in control E-SMCs. (B) Cytosolic (cyto) and nuclear (nuc) fractions of SMCs 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 (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

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

Our previous studies demonstrate that T-cadherin activates the PI3K/Akt transduction pathway in SMCs [19]. In the following we further examine the activity of Akt pathway components in T-cadherin transductants. One of the major targets of Akt kinase is GSK3β 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 GSK3β is respectively elevated and decreased in T-cadherin-upregulated and T-cadherin-deficient SMCs under both serum-deprivation [19] and serum-containing (Fig. 6) culture conditions. Active (unphosphorylated) GSK3β downregulates levels of cytosolic β-catenin by phosphorylating and targeting it for degradation by the ubiquitin–proteasome system; phosphorylation of GSK3β 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 GSK3β phosphorylation, the levels of total and active β-catenin were elevated in T-cad+-SMCs (Fig. 7A, B) but reduced in shTcad-SMCs (Fig. 7B). Subcellular fractionation demonstrated higher levels of β-catenin in all fractions (membrane, cytoplasm, soluble nuclear, chromatin bound) from Tcad+-R-SMCs (Fig. 7C) and in cytoplasmic and nuclear fractions of T-cad+-H-SMCs (Fig. 7D). β-Catenin levels in the respective fractions of shTcad-SMCs 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-SMCs and Tcad+-SMCs (data not shown). Nevertheless, and in keeping with the data on proliferation potential (Fig. 1C) expression of cyclin D1 protein was increased in Tcad+-SMCs (Fig. 8A, B) and decreased in shTcad-SMCs (Fig. 8B).

Cyclin D1 expression can be regulated by mechanisms independent of β-catenin. The 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 GSK3β 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+-SMCs (Fig. 8C), but calculation of the phosphorylated to total cyclin D1

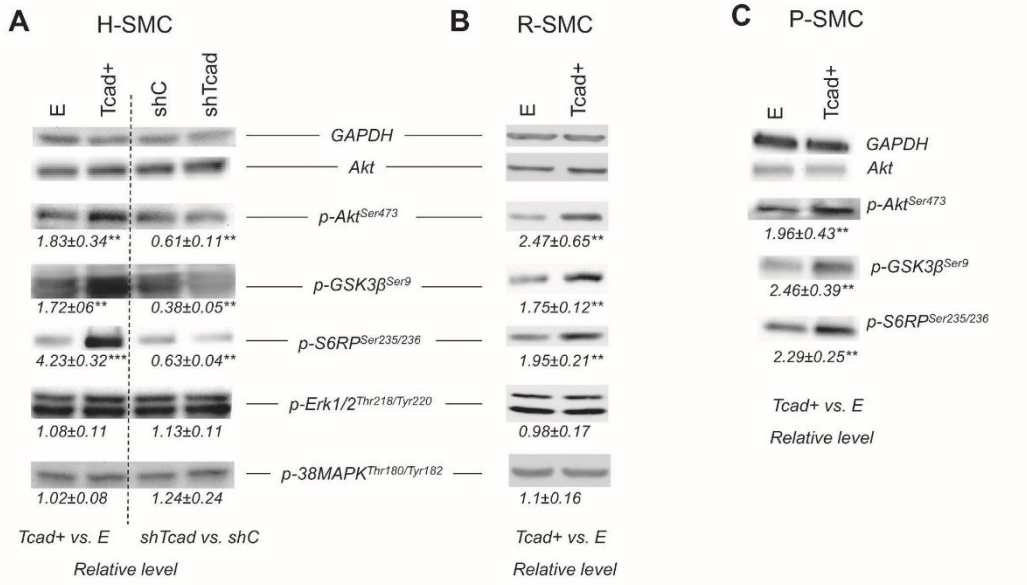

Fig. 6. T-cadherin alters classical PI3K–Akt axis signalling, but has no effect on MAPK/ERK and p38MAPK signalling. Immunoblots of crude lysates from H-SMCs (A), R-SMCs (B), and P-SMCs (C) probed for major PI3K/Akt, MAPK and p38 signalling effectors. GAPDH and Akt served as loading controls. Numeric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

signal ratios indicated significantly lower relative levels of phospho-cyclin D1^{Thr286} in Tcad+-SMCs (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-SMCs 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 receptor 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 the SMC phenotype we analyzed expression levels of SMC differentiation markers in T-cad+-SMCs after treatment with mTORC1 inhibitor rapamycin.

Hyperactivation of mTORC1/S6K1 signalling in T-cad+-SMCs 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, 8 h) decreased p-S6RP$^{Ser235/236}$/p-IRS-1$^{Ser636/639}$ and increased total IRS-1, with abundancies achieving equivalence between E-SMCs and Tcad+-SMCs (Fig. 9A, B). Rapamycin induced feedback activation of Akt signalling in both E- and Tcad+-SMCs as evidenced by elevation in levels of p-Akt^{Ser473}, p-Akt^{Thr308}, p-GSK3β^{Ser9} and β-catenin, although their relative abundancies remained significantly higher in Tcad+-SMCs. Levels of SMC contractile protein markers α-SMA, *sm*MHC and *h*-caldesmon were elevated following rapamycin treatment in E-SMCs but remained unchanged in Tcad+-SMCs. Rapamycin decreased cyclin D1 levels in E-SMCs (by ~50–60%) but was without effect in Tcad+-SMCs (Fig. 9A,B), while levels of p-cyclin D1 were decreased in both SMCs but to a lesser extent in Tcad-SMCs (Fig. 9B; reduced by ~50–60% in E-SMCs and by ~10–20% in Tcad+-SMCs). The ratio p-cyclin D1:cyclin D1 was unchanged in E-SMCs and there was a trend toward a lower ratio, albeit not significant, in Tcad+-SMCs (Fig. 9B). Rapamycin did not alter the morphological appearance of either E-SMCs or Tcad+-SMCs (Fig. 9C). Nonetheless, rapamycin inhibited proliferation of both E-SMCs and Tcad+-SMCs (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. GSK3β branch of the PI3K/Akt signalling axis mediates effects of T-cadherin on SMC differentiation

We next examined whether the GSK3β branch of the Akt pathway, or more specifically GSK3β inactivation, might mediate T-cadherin effects on SMC differentiation status. To examine this possibility we infected E- and Tcad+-R-SMCs with adenovirus expressing nonphosphorylable, constitutively active GSK3β-S9A mutant (Adv-S9A) or empty adenovirus (Adv-E) control. Equivalence of GSK3β protein expression in E- and Tcad+-Adv-S9A SMCs was controlled by immunoblotting (Fig. 10A). GSK3β-S9A mutant was without effect on mTORC1/S6K1 axis signalling in either E or Tcad+ SMCs, 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 GSK3β-S9A could override SMC dedifferentiation induced by T-cadherin upregulation. In Tcad+-Adv-S9A-SMCs, the levels of β-catenin and cyclin D1 decreased, as did expression of dedifferentiation marker calmodulin, while abundancies of myocardin and SMC contractile proteins

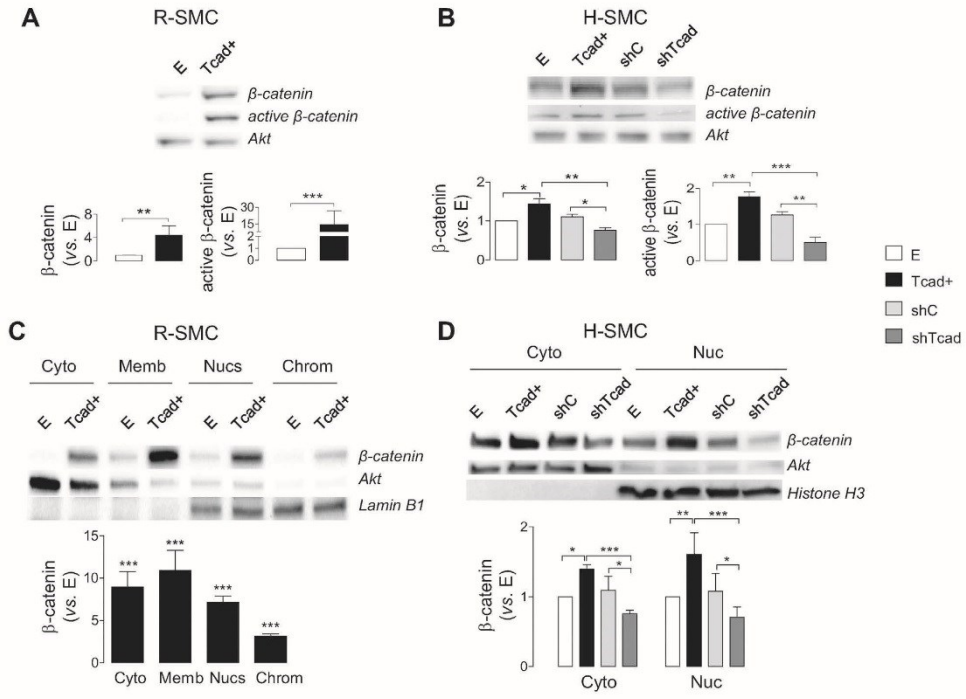

Fig. 7. T-cadherin upregulation promotes β-catenin stabilization and nuclear accumulation. Immunoblots of crude lysates from R-SMCs (A) and H-SMCs (B) probed for β-catenin and active (non-phosphorylated) β-catenin and cyclin D1. Akt was used as loading control. Levels in Tcad+-SMCs and shTcad-SMCs are expressed relative to control E-SMCs. (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-SMCs. Significant differences are marked with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

*sm*MHC, α-SMA and *h*-caldesmon increased (Fig. 10A), all reaching the respective levels of control Adv-E-SMCs. GSK3β-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 GSK3β-S9A in E-SMCs was without significant effect on any of the measured parameters. Taken together, these data support that T-cadherin-induced SMC dedifferentiation is dependent on GSK3β inactivation.

4. Discussion

This study has demonstrated that T-cadherin is capable of promoting the phenotypic switch of SMCs toward dedifferentiation. T-cadherin upregulation in SMCs 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 and MRTFA/B) and proliferation (β-catenin and cyclin D1) and functionally translated into increased proliferative and migratory capacities (Fig. 11). Accordingly, silencing of T-cadherin enforced SMC differentiation. We identified GSK3β 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 SMCs and the complex mechanisms involving the regulatory circuits of transcription factors and crosstalk of intracellular signalling pathways [1–5]. The main characteristics of differentiated SMCs 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, *sm*MHC and *h*-caldesmon [1–4]. An important role in the 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 SMCs 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 GSK3β 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

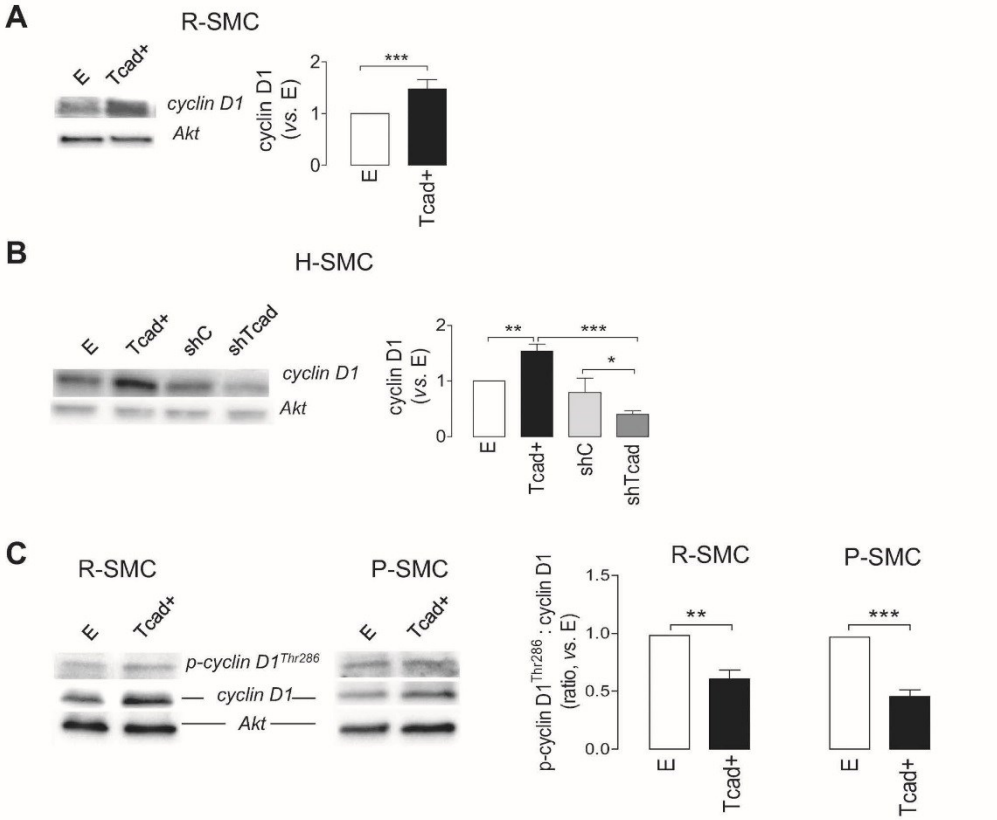

Fig. 8. T-cadherin upregulation increases cyclin D1 protein concomitant with a decrease in the ratio of phosphorylated cyclin D1 to total cyclin D1. Immunoblots of crude lysates from R-SMCs (A) and H-SMCs (B) probed for cyclin D1. Akt was used as loading control. Levels in Tcad+-SMCs, shC-SMCs or shTcad-SMCs are expressed relative to control E-SMCs. (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 of p-cyclin $D1^{Thr298}$ to cyclin D1 were calculated and expressed relative to the ratios in the control E-SMCs. Significant differences are marked with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

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 are 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 SMCs causes loss of filamentous actin/stress fibers and contractile proteins α-SMA, *sm*MHC, and *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 SMCs, 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-β1 can increase SMC proliferation concomitant with the appearance of an elongated, differentiated morphology and the expression of α-SMA [49]. Heparin can inhibit proliferation of SMCs without restoration of a differentiated morphology or expression of α-SMA [50]. *In vivo* during late embryogenesis and postnatal development SMCs exhibit a high proliferation rate that is concomitant with induction of differentiation genes [51–53]. Conversely, in advanced atherosclerotic lesions, proliferation of SMCs is very low, and so is the expression level of contractile markers [54,55]. T-cadherin upregulation induces both hyper-proliferation and

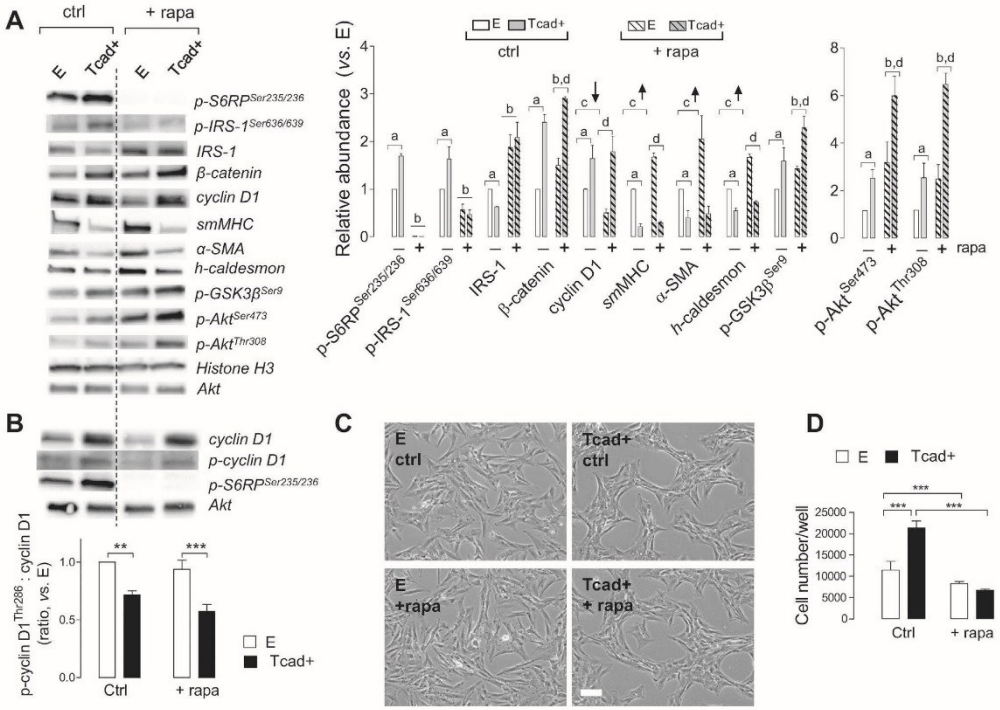

Fig. 9. Blockade of negative mTOR/S6K1 signalling feedback by rapamycin does not restore differentiation marker expression in Tcad+-SMCs. E and Tcad+ R-SMC transductants were cultured in the absence (Ctrl) or presence of 30 nM rapamycin (rapa) for 8 h (A, B) or 72 h (C, D). (A) Immunoblots from whole 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-SMCs. Letters a–d within the 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^{Thr286} (and Akt as loading control). The histogram depicts quantification of the p-cyclin D1^{Thr286} to cyclin D1 ratio expressed relative to that in control E-SMCs. (C) Phase contrast micrographs of E- and Tcad+-SMCs. Scale bar: 100 μm. (D) Cell proliferation estimated by cell enumeration. (B, D) Significant differences are marked with asterisks (*P < 0.05; **P < 0.01; ***P < 0.001).

dedifferentiation; yet our finding that rapamycin inhibits proliferation but failed to restore contractile protein marker expression in Tcad+-SMCs 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* the Akt/GSK3β 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 the 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-1β [61], PDGF/IL-1β [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 SMCs under both serum-containing culture conditions and after serum-deprivation, suggesting that 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 SMCs 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 SMCs 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+-SMCs.

Rather, our data show that the GSK3β branch of the Akt pathway, more specifically GSK3β inactivation, is the dominant mediator of T-

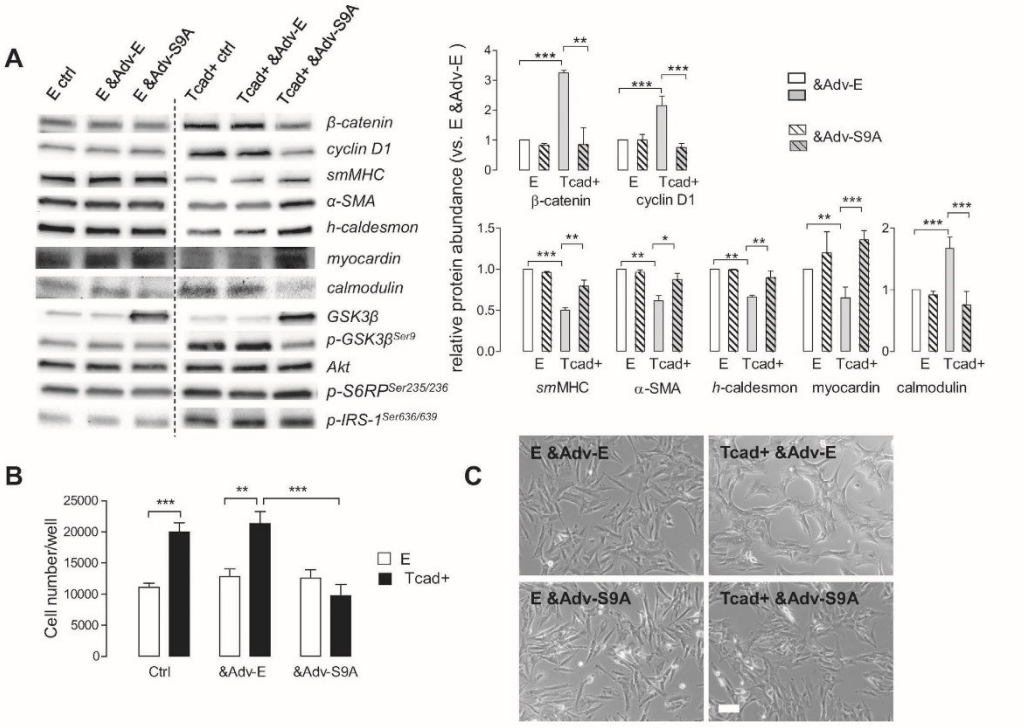

Fig. 10. Ectopic expression of constitutively active GSK3β restores differentiation marker expression and blocks hyperproliferation in Tcad+-SMCs. (A) Immunoblots of whole cell lysates of E and Tcad+-R-SMC transductants without (ctrl) or following co-transduction with an empty adenovector (&Adv-E) or adenovector encoding a constitutively active mutant of GSK3β (&Adv-S9A). GSK3β 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 SMCs are expressed relative to the levels in E &Adv-E SMCs. (B) Proliferation of control (Ctrl) and co-transduced E- and Tcad+-R-SMCs estimated by cell enumeration after 72 h 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-SMCs. Scale bar: 100 μm.

cadherin-induced SMC proliferation and dedifferentiation. This is in line with previous data demonstrating a permissive role for GSK3β in maintaining a differentiated SMC phenotype. GSK3β was identified as a positive regulator of the transcriptional activity of myocardin in SMCs, whereby GSK3β-mediated phosphorylation of myocardin increased its recruitment to target genes [65]. Further, expression of myocardin target genes in SMCs can be abrogated by pharmacological or siRNA-mediated inhibition of GSK3β as well as ectopic expression of a dominant negative, kinase inactive GSK3β mutant [65]. Inhibition of GSK3β can also influence *MYOCD* splice patterning in SMC; chemical inhibitors of GSK3β promoted expression of *MYOCD* ΔExon 11 in association with adoption of a dedifferentiated morphology, increased proliferation and repression of *MYH11* gene expression [66]. *In vivo* gene transfer of GSK3β-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 GSK3β [68]. Also monocrotaline-induced pulmonary artery remodelling *in vivo* was associated with GSK3β inactivation [69]. Our data show that the phenotype shift toward dedifferentiation caused by T-cadherin can be fully prevented by forced GSK3β activation implying a central role of the GSK3β 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-SMCs and T-cad+-SMCs 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

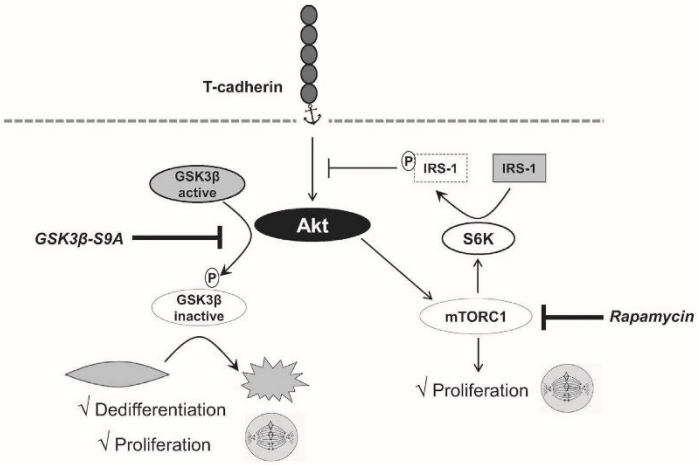

Fig. 11. A model for T-cadherin-dependent regulation of SMC phenotype. Upregulation of T-cadherin in SMCs induces proliferation and a phenotype transition toward dedifferentiation *via* distinct branches of the Akt pathway. Activation of Akt by T-cadherin upregulation leads to phosphorylation and inactivation of GSK3β promoting both proliferation and loss of differentiated contractile phenotype. These effects of T-cadherin can be ameliorated by ectopic co-expression of constitutively active GSK3β-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 GSK3β inactivation and not on the mTORC1/S6K1 branch of the Akt pathway.

over the cell body with negligible localization to intercellular junctions in resting SMCs, and it redistributes to the leading edges of migrating SMCs [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 SMCs 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 SMCs from different species (human, porcine and rat) our study identifies T-cadherin/GSK3β 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 SMCs 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 the 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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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

AF, TJR, MP and PE designed the study and experiments. AF conducted the experiments. AF, TJR and MP analyzed 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.

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CONCLUSIONS and FUTURE PERSPECTIVES

Conclusions

Collectively, the studies undertaken in this thesis contribute to a better understanding of cadherin involvement in regulation of fundamental SMC functions – contractility and ability to switch phenotype. We found that T-cadherin, an atypical member of cadherin family, regulates intrinsic VSMC contractile competence, responsiveness to insulin and promotes matrix remodelling. Moreover, our studies demonstrate that this molecule also regulates VSMC phenotype switch and promotes transition to the de-differentiated phenotype.

While VSMC de-differentiation is crucial for tissue regeneration and reparation, faulty control of transition with failure to either maintain or regain the differentiated contractile phenotype underlies development of vascular pathologies. The knowledge gained in the course of this dissertation reveals a completely novel aspect of cadherin-based cellular and molecular mechanisms that can participate in VSMC phenotype transition processes and is relevant for the search of new therapeutic tools and strategies to treat VSMC-driven vascular pathologies. Importantly, our findings position T-cadherin as a potential target molecule in prevention of adverse vessel remodelling and SMC de-differentiation. Although not presented here, our preliminary investigations on vascular tissues from T-cadherin knock-out mice failed to identify any anatomical vascular defects in these mice. This might suggest that T-cadherin functions primarily as a regeneration factor and is dispensable in vasculogenesis, and would reinforce our *in vitro* findings with T-cadherin-deficient VSMC as reported in this dissertation.

We established here that the shift in functions which T-cadherin induces in VSMC is achieved via modulation of Akt/mTOR and Akt/GSK3β signalling: hyperactivation of the classical PI3K/Akt signalling core heightens negative mTORC1/S6K feedback, and inactivates GSK3β. On one hand this leads to elevated IRS-1 degradation and blunted insulin response, and on the other hand it stabilizes cyclin D1 via GSK3β inactivation which promotes cell cycle progression and cell proliferation. Although we did not specifically address the interaction between the separate branches of PI3K/Akt signalling (mTORC1/S6K and GSK3β/cyclin D1), cooperation between them in our model is very likely, as has been shown by many other studies investigating classical PI3K/Akt signalling. Our findings suggest the existence of tight mechanistic links between cellular insulin resistance, matrix remodelling and phenotype transition.

Perspectives

The following briefly outlines some of many issues regarding the regulation of VSMC function and phenotype by T-cadherin that could be addressed in future investigations. Key topics are indicated in italicized and underlined font.

GSK3β-inactivation stabilized both cyclin D1 and β-catenin. Although β-catenin can transcriptionally regulate cyclin D1 levels, here we found that β-catenin accumulation does not link to TCF/LEF binding and activation of de novo cyclin D1 transcription. Furthermore, upregulated β-catenin did not show TCF/LEF binding, and the *functional role of β-catenin in T-cadherin-induced VSMC de-differentiation* remains unknown. Further studies are needed to address this question in VSMC.

PI3K/Akt pathway is central for T-cadherin signalling in VSMC, however, upstream partners of T-cadherin signalling remain unknown. *How T-cadherin regulates the PI3K/Akt signalling axis* and whether its activation is a primary or subsequent *event in the phenotype transition-initiating cascade* have not yet been investigated.

A further set of data obtained during the course of this dissertation shows that T-cadherin also regulates cellular adhesion: T-cadherin overexpressing VSMC display defective cell spreading and reduced cell-to-matrix adhesion together with a distinct actin cytoskeleton organization. This is consistent with our previous findings in EC and prostate cancer cells, both of which also exhibit T-cadherin-dependent alterations in morphological characteristics. T-cadherin overexpression also reduced general activity of small RhoGTPases – RhoA, Rac1, and Cdc42 in resting cells. RhoGTPases are powerful regulators of adhesion and cytoskeleton dynamics. Reduced activity of all three proteins suggests involvement of some important upstream regulator(s) in T-cadherin signalling, possibly components of the integrin adhesome. Indeed, in EC we have demonstrated physical interactions of T-cadherin with β3 integrin and integrin linked kinase. Through an ability to modulate *cell-matrix adhesion* T-cadherin might regulate different aspects of cell behaviour, including cell motility, polarity, growth, and survival. This suggest clear directions for further studies aimed to *identify immediate molecular partners in T-cadherin signalling*, as well as possible cadherin role in regulation of cell-matrix adhesion.

The research presented in this dissertation established cellular functions for T-cadherin in VSMC contraction and phenotype plasticity and identified molecular mechanisms mediating T-cadherin signalling. Most of the study, however, has been undertaken using *in vitro* models. The T-cadherin knock-out mice ($Cdh13^{-/-}$) is viable and appear anatomically normal. Validation of our findings through *use of ($Cdh13^{-/-}$) mice* would add further value to the existing data; experimentation designed to microscopically/immunohistologically study the vasculature could be performed, or *in vitro* experimentation using VSMC isolates (and eventually "rescue" experiment using vectors carrying human or murine T-cadherin). In addition, $Cdh13^{-/-}$mice *challenge experiments* (e.g. carotid artery balloon injury) or *generation of double knock-out* for T-cadherin and Apo E ($apoE^{-/-}$ /$Cdh13^{-/-}$) would be instrumental in scrutinizing the most relevant biological contexts (e.g. atherosclerosis, restenosis) for T-cadherin functions in vascular smooth muscle.

Another interesting question directly related to T-cadherin signalling in VSMC, but not addressed in this study is the *involvement of adiponectin*. T-cadherin is a third adiponectin receptor, which locates adiponectin to the vasculature. How presence of adiponectin might alter T-cadherin signalling and what consequences for VSMC functions this can have, remains to be determined.

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Abbreviations

5mC, 5 methylcytosine
5hmC, 5 hydroxymethylcytosine
AJs, adherens junctions
AMPK, AMP-activated protein kinase
APC, adenomatous polyposis coli
$ApoE^{-/-}$, ApoE-deficient mouse model
bHLH, basic helix-loop-helix
Bad, Bcl-2 associated death promoter
Bcl-2, B-cell lymphoma 2
BMP, bone morphogenetic protein
CAD, coronary artery diseases
CADASIL, Ccerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CArG-box, $CC[A/T]_6GG$ motif
Cdc42, cell division control protein 42
CIL, contact inhibition of locomotion
CK1, casein kinase 1
CVD, cardiovascular disease
DES, drug-eluting stent
EC, endothelial cell
EC, extracellular
ECM, extracellular matrix
EGF, epidermal growth factor
EMT, epithelial-to-mesenchymal transition
eNOS, endothelial nitric oxide synthase
ERK, extracellular signal regulated kinases
F-actin, fibrillary actin
FGF, fibroblast growth factor
FGFR1, FGF receptor 1
G-actin, globular actin
GEF, guanine exchange factors
hES-MCs, human embryonic stem cell-derived mesenchymal cells
IGF, insulin-like growth factor
IGFR1, Insulin-like growth factor receptor 1
iNOS, inducible nitric oxide synthase
IR, insulin receptor
IGF, insulin-like growth factor
IGFR1, IGF receptor 1
IRS-1, insulin receptor substrate
KLF4, Krüppel-like factor 4
KLF5, Krüppel-like factor 5
LDL, low density lipoprotein
MAPK, mitogen activated protein kinase(s)
MKL1/2, megakaryoblastic leukemia 1 and 2
MLC_{20}, 20 kDa myosin light chain
MLCK, myosin light chain kinase
MLCP, myosin light chain phosphatase
MMP, matrix metalloproteinase
MRTF, myocardin-related transcription factor
MSC, mesenchymal stem cell
mTOR, mammalian target of rapamycin

mTORC1/2, mTOR complex 1 or 2
MYOCD, myocardin
NCID, Notch intracellular domain
NO, nitric oxide (NO)
OB, osteoblast
PAH, pulmonary arterial hypertension
PDGF, platelet-derived growth factor
PDGFR, platelet-derived growth factor receptor
PECAM, platelet endothelial cell adhesion molecule
PI3K, phosphatidylinositol-3-kinase
PIP3, phosphatidylinositol (3,4,5)-tris-phosphate
PKC, protein kinase C
PKG I α, cGMP-dependent protein kinase alpha
ROCC, receptor operated Ca^{2+} channels
S1P, sphingosine 1 phosphate
SBE, Smad-binding element
SCAI, suppressor of cancer cell invasion
SM, smooth muscle
SMC, Smooth muscle cell
SRF, serum response factor
SVD, small vessel disease
Rho-kinase, Rho-associated kinase
TAZ, transcriptional coactivator with a PDZ-binding domain
TCE, TGF-β control elements
TCF, ternary complex factors
TCF/LEF, T-cell factor/lymphoid enhancer factor
TET, Ten-Eleven-Translocation
TGF-β, transforming growth factor-β
VSMC, vascular smooth muscle cell
VEGFR2, vascular endothelial growth factor receptor 2
YAP, YES associated protein

Curriculum Vitae

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EDUCATION

2011-2016	**Ph. D. in Cell Biology**, Universität Basel, Switzerland
Supervisor	Prof. Therese Resink
Focus	*Vascular biology, cell plasticity, regeneration*
2006-2008	**M. Sc. in Biochemistry**, Vilnius University, Lithuania
	Biotechnology, genetic engineering
2002-2006	**B. Sc. in Biochemistry**, Vilnius University, Lithuania and Bielefeld University, Germany
	Microbiology, biotechnology

SCHOLARSHIPS

2016	Universität Basel: Förderung exzellenter Nachwuchsforschender
2007	Lithuanian Research Council: Research practice scholarship
2006-2007	Vilnius University: Erasmus scholarship

WORK EXPERIENCE

2008-2011	**Junior Scientist**, Thermo Fisher Scientific, Lithuania
	Cell biology, biotechnology, molecular biology, quality control, project management
2006-2008	**Research Assistant**, Institute of Biotechnology, Lithuania
	Biotechnology, genetic engineering, microbiology

SUMMER SCHOOLS AND PRACTICES

2007	Construction, expression and purification of virus-like particles. Scientific summer practice, Institute of Biotechnology, Lithuania
2006	Methods in clinical biochemistry. Scientific summer practice, Institute for Laboratory and Transfusion Medicine, Heart and Diabetes center NRW, Germany
2005	Optimization of PCR-based techniques for rapid diagnostics of tick-borne encephalitis and Lyme disease. Scientific summer practice, Laboratory of Lithuanian AIDS Centre, Lithuania

TECHNICAL SKILLS

Tissue culture: standard culture maintenance and manipulation, lenti-viral particle production; Immuno-techniques: ELISA, WB, IP, IF, FACS; Microscopy: light and confocal, image processing; *In vivo* (mouse) model: basic skills (administration of substances, tissue collection); Molecular biology: cloning, (q)PCR; Protein analysis: basic protein purification, zymography; Computer skills: GraphPad Prism, Vector NTI, RasMol, Java Script (basics), R.

TEACHING (TUTORING)

Tutor, Introduction to Biology (Einführung in die Biologie) autumn semester 2013, Universität Basel, Faculty of Natural Sciences

INTERESTS AND LANGUAGES

Interests: regeneration biology, cell plasticity, vascular biology, tissue engineering, cell-cell, and cell-matrix communication
Foreign language competence: English – fluent; German – good; Russian – good
Other interests: Modern art, skiing, tennis

Publications

- Frismantiene, A., Pfaff, D., Dasen, B., Erne, P., Resink, T.J., and Philippova, M. 2016. T-cadherin promotes vascular smooth muscle cell dedifferentiation via a GSK3β-inactivation dependent mechanism. *Cell Signal* 28(2016):516-530.
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- A. Petuskaite, D. Pfaff, A. Frachet, M. Coen, M.B. Joshi, K. Maslova, M-L. Bochaton-Piallat, P. Erne, T.J. Resink, M. Philippova. T-cadherin upregulation in vascular smooth muscle cells promotes insulin resistance and extracellular matrix remodeling. *Proceedings, AGLA and Cardiovascular Biology, P19, page 52, 2014*
- A. Frismantiene, B. Dasen, D. Pfaff, P. Erne, Th. Resink, M. Filippova. T-cadherin promotes vascular smooth muscle cell phenotype switch by a novel cadherin-β-catenin-dependent mechanism *Cardiovascular Medicine 2015;17(online publication): Suppl 25, Abstract 153, page 61*

Conference presentations

AGLA and Cardiovascular Biology, Bern Jan 10-11, 2013

- Petuskaite A, et al. Upregulation of T-cad in smooth muscle cells; consequences for vascular function?

20th International Local Drug Delivery Meeting, Geneva February 07-09, 2013

- Petuskaite A, et al. Upregulation of T-cad in smooth muscle cells; consequences for vascular function.

AGLA and Cardiovascular Biology Meeting, Fribourg Jan 16-17, 2014

- Petuskaite A, et al. T-cadherin upregulation in smooth muscle cells modulates vascular function.

Annual Meeting of the Swiss Society for Cardiology, Interlaken Jun 11-12, 2014

- Petuskaite A, et al. T-cadherin upregulation in vascular smooth muscle cells promotes insulin resistance and extracellular matrix remodelling.

Annual Meeting of the Swiss Society for Cardiology, Zurich Jun 12-13, 2015

- Frismantiene A, et al. T-cadherin promotes vascular smooth muscle cell phenotype switch by a novel cadherin-β-catenin-dependent mechanism.

AGLA and Cardiovascular Biology Meeting, Fribourg Jan 14-15, 2016

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