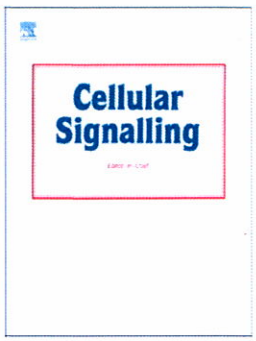

T-cadherin promotes vascular smooth muscle cell dedifferentiation *via* a GSK3β-inactivation dependent mechanism

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2.5. Cell migration

Migration of SMC was measured using the scratch wound assay [25]. Confluent monolayer cultures of SMC grown on collagen-I (50 µg/ml; BD Biosciences, Basel, Switzerland) pre-coated 24-well plates were scratch-wounded, gently rinsed with PBS and further cultured in fresh growth medium with inclusion of 2mM hydroxyurea (Sigma-Aldrich) to inhibit proliferation. Phase contrast images were taken at 0 and 24 h time points using an Olympus IX-81 inverted time-lapse microscope equipped with a digital camera (Olympus, Tokyo, Japan) and CellSens software (Olympus Switzerland) within a humidified incubation chamber with 5% 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.

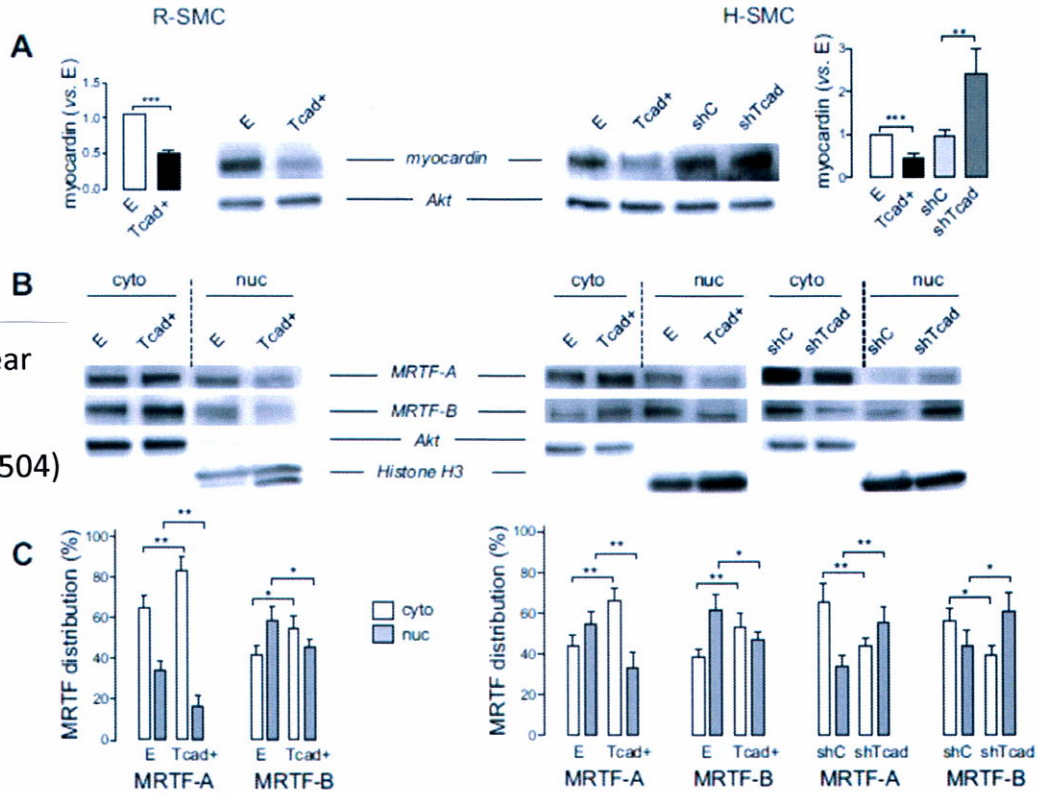
2.6. Cell fractionation

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

2.7. Gelatin zymography

SMC were plated into 24-well plates (45×10^3 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 48h, centrifuged (5min, 3000 rpm) and stored frozen (-20˚C) until use. Equivalence of cell numbers was controlled by cell enumeration after medium collection. Medium samples were mixed with reducing agent-free 3xLaemmli sample buffer (1:2, v/v) and subjected to electrophoresis (50 µl/lane) on 8% SDS-PAGE co-polymerized with gelatin (1mg/ml; Sigma-Aldrich G-8150). Following electrophoresis gels were sequentially incubated in renaturing solution (2.5% v/v Triton X-100 in water) for 30min (3x10min) at room temperature and then in developing buffer (50mM Tris, 0.2M NaCl, 5mM CaCl2, 0.02% Brij 35) overnight at 37˚C.

Figure 5



Cytoplasmic and nuclear protein extracted by 101Bio kit (Cat.#: P504)