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Mechanosensitive transcriptional regulation of gene expression in smooth muscle

Implications for health and disease

FATIMA DAOUD | DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE
FACULTY OF MEDICINE | LUND UNIVERSITY

وَفِي أَنْفُسِكُمْ أَفَلَا تُبْصِرُونَ
الذَّارِيَاتِ : ٢١

And within yourselves. Do you not see? (51:21)



Mechanosensitive transcriptional regulation of gene expression in smooth muscle

Implications for health and disease

Fatima Daoud



LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 15th of June at 09.00 in Segerfalksalen, BMC A10, Lund, Sweden

Faculty opponent

Dr. Helle Jørgensen

Division of Cardiovascular Medicine

University of Cambridge

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| Abstract | | |
| <p>Smooth muscle participates in forming the wall of hollow organs, tracts, and blood vessels. Therefore, it plays a critical role in mediating vital functions in the body including regulation of blood pressure. In response to changes in the surrounding microenvironment, smooth muscle cells can modulate their phenotype and dedifferentiate from being quiescent contractile to a more proliferative and migratory phenotype. The mechanical force represents a principle part of the microenvironment cues. Smooth muscle senses the mechanical force and converts it into a biochemical signal that affects gene expression eventually. Until now, there are two known families of mechanosensitive transcriptional regulators; myocardin-related transcription factors (MRTFs); and the downstream effectors of the Hippo signaling pathway, Yes-associated proteins (YAP), and closely related transcriptional coactivator with PDZ-binding motif (TAZ). However, the role of these mechanosensitive transcriptional regulators and their contribution to smooth muscle homeostasis and disease are not well understood.</p> <p>This work demonstrated the lethal impact of inducible deletion of YAP/TAZ in adult smooth muscle and showed that YAP and TAZ are critical for short- and long-term mechanotransduction. Arteries that lack YAP/TAZ have impaired agonist- and pressure-induced contraction which resulted in multiple vascular lesions under hypertensive setting. These findings suggest a protective role of YAP/TAZ against arterial damage caused by hypertension which is well-known risk factor for cardiovascular diseases. In addition to hypertension, atherosclerosis is also considered a risk factor for cardiovascular diseases. We investigated the role of MRTFA in foam cell formation which is a hallmark of atherosclerosis. We found that MRTFA stimulates lipid accumulation in human vascular cells by multiple mechanisms including, increased pinocytosis, decreased lipid efflux, and upregulation of LDL receptor. These changes collectively participate in converting smooth muscle cells into foam cells. We also examined the relationship between MRTFA and unfolded protein response. We noticed tight negative correlation between smooth muscle markers and unfolded protein response in three well-recognized examples of phenotypic modulation <i>in vivo</i>. We also induced the unfolded protein response chemically <i>in vitro</i> and we observed that MRTFA could ameliorate the induction of unfolded protein response.</p> <p>In summary, these results demonstrated essential roles of YAP/TAZ and MRTFA for smooth muscle structure and function, providing new insights into their physiological functions, as well as their role in disease development and progression, in particular the vascular diseases. However, further studies are needed to propose new potential therapeutic targets against diseases with underlying smooth muscle modulation.</p> | | |
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Mechanosensitive transcriptional regulation of gene expression in smooth muscle

Implications for health and disease

Fatima Daoud



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The eight-point star in the Islamic pattern represents perfection. This pattern and similarly our knowledge regards human body are incomplete. This thesis aims to fill in the knowledge gap by participating in adding small pieces to the pattern.

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MADE IN SWEDEN 

To mom, dad, and Areen

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List of papers

This thesis is based on the following papers:

- I. **Daoud, F.**, Holmberg, J., Alajbegovic, A., Grossi, M., Rippe, C., Swärd, K., & Albinsson, S. 2021. Inducible Deletion of YAP and TAZ in Adult Mouse Smooth Muscle Causes Rapid and Lethal Colonic Pseudo-Obstruction. *Cell Mol Gastroenterol Hepatol*, 11(2): 623-637.
- II. **Daoud, F.**, Arevalo Martinez, M., Holmberg, J., Alajbegovic, A., Ali, N., Rippe, C., Sward, K., & Albinsson, S. 2022. YAP and TAZ in Vascular Smooth Muscle Confer Protection Against Hypertensive Vasculopathy. *Arterioscler Thromb Vasc Biol: ATVB*AHA121317365.
- III. Zhu, B., **Daoud, F.**, Zeng, S., Matic, L., Hedin, U., Uvelius, B., Rippe, C., Albinsson, S., & Sward, K. 2020. Antagonistic relationship between the unfolded protein response and myocardin-driven transcription in smooth muscle. *J Cell Physiol*, 235(10): 7370-7382.
- IV. Alajbegovic, A., Holmberg, J., **Daoud, F.**, Rippe, C., Kalliokoski, G., Ekman, M., Daudi, S., Ragnarsson, S., Swärd, K., & Albinsson, S. 2021. MRTFA overexpression promotes conversion of human coronary artery smooth muscle cells into lipid-laden foam cells. *Vascul Pharmacol*: 106837.

Popular summary

There are three types of muscle tissues in the body, heart, skeletal, and smooth muscle. Smooth muscle lines the hollow organs (stomach, intestine, bladder, and uterus). It also covers tracts and passages (urinary tracts, respiratory tracts, and vascular walls). Smooth muscle mediates vital functions in the body, including propelling food through the gastrointestinal tract, childbirth, and urination. It is also involved in airway and blood pressure regulation. Therefore, the smooth muscle is continuously exposed to mechanical stimuli and it senses increased stretch via particular proteins on its cell membrane. This stretch is then translated into a molecular signal that spreads throughout the cell and reaches the nucleus. As a response, specific genes are chosen to be either inhibited or activated. This thesis focuses on two families of proteins that can translocate to the nucleus in response to mechanical stimuli. They are called YAP/TAZ and MRTFs. However, their importance for smooth muscle function and contribution to vascular disease are still largely unknown.

To examine the role of YAP/TAZ in smooth muscle, we used a unique mouse model where we deleted these two genes in smooth muscle only. We found that YAP/TAZ play a critical role in maintaining the normal structure and function of smooth muscle in the adult mice. After YAP/TAZ deletion, the smooth muscle became weak and lost significant contractile ability throughout the mouse's body. The colon manifested this as inability to eliminate the undigested material (feces), which resulted in feces accumulation inside the colon, intestinal dilatation, and serious sickness.

Hypertension is a major risk factor for cardiovascular diseases, the leading cause of death in the developed countries. Hypertension means elevated blood pressure (increased stretch), which is sensed primarily by the vascular smooth muscle. We were interested in examining the role of YAP/TAZ as mechanical messengers in the hypertensive setting. Therefore, we deleted YAP/TAZ in the smooth muscle of hypertensive mice. The blood vessels of these mice could not tolerate the elevated blood pressure, and we observed multiple lesions in the small vessels. This indicates that YAP/TAZ play an essential role in preventing vascular complications associated with hypertension.

Similar to hypertension, atherosclerosis is also considered a major risk factor for cardiovascular diseases. In atherosclerosis, there is a buildup of fat, inflammatory

cells, and smooth muscle on the vessel wall, leading to progressive narrowing. We examined the role of MRTFA (a member of MRTFs) in atherosclerosis and found that MRTFA encouraged smooth muscles to collect lipid inside the cells, creating a foam-like appearance (foam cells) which is a hallmark of atherosclerosis.

Lastly, when the cell cannot handle excessive protein production, dysfunctional proteins start to accumulate inside the cells. This will trigger certain adaptive cascades trying to rescue the cell. However, if these attempts fail, the cell will undergo programmed death. This response underlies several disease processes, and we found that MRTFA participate in mitigating it.

In conclusion, we found that smooth muscle cells use YAP/TAZ as messengers to respond to the surrounding mechanical stimuli, and it seems that a balanced activity of YAP/TAZ and MRTFs is essential to maintain the normal structure and function of smooth muscle. Any disturbance in YAP/TAZ or MRTFs levels could result in disease development. Understanding the potential triggers and molecular mechanisms of YAP/TAZ and MRTFs regulation will probably shed light on new therapeutic targets in the future.

Populärvetenskaplig sammanfattning

Det finns tre typer av muskelvävnad: hjärtmuskel, skelettmuskel och glatt muskel. Den glatta muskulaturen täcker ihåliga organ (mage, tarmar, urinblåsa och livmoder). Glatt muskel täcker också urinvägar, luftvägar och blodkärl. Den glatta muskulaturen fyller flera viktiga funktioner i kroppen. Bland annat krävs glatt muskulatur för att föra fram mat genom mag- och tarmkanalen, vid förlösning och för urinering. Därtill reglerar glatt muskel både luftvägars och blodkärls diameter, där det senare är viktigt för exempelvis blodtrycksreglering. Förmågan att känna av och svara på mekaniska stimuli verkar vara en central funktion hos den glatta muskulaturen. Mekanisk sträckning kan översättas till molekylära signaler in i cellen och vidare in till cellkärnan vilket i sin tur aktiverar eller bromsar specifika gener. Den aktuella avhandlingen fokuserar på två proteinfamiljer som är känsliga för mekaniska stimuli; YAP/TAZ och MRTF. Båda är viktiga för att översätta mekaniska stimuli till gensvar men hur de funktionellt påverkar den glatta muskulaturens funktion och deras roll i hjärt- och kärlsjukdomar är inte klarlagt.

För att undersöka vilken roll YAP/TAZ spelar i glatt muskel har vi använt en unik djurmodell (mus) där vi kan ta bort generna för YAP och TAZ specifikt i den glatta muskulaturen. Vi ser att YAP/TAZ är avgörande för att upprätthålla den glatta muskulaturens struktur och funktion hos vuxna möss. Om generna för YAP och TAZ avlägsnas blir den glatta muskulaturen svag och förlorar sin kontraktile förmåga. Extra tydligt visas detta i tjocktarmen med svår förstoppning som följd.

Högt blodtryck (hypertension) och åderförfettning (ateroskleros) tillhör idag de vanligaste dödsorsakerna i den utvecklade världen. Hypertension leder till ökad sträckning av blodkärlen som ibland annat registreras av blodkärlens glatta muskulatur. Vi har undersökt vilken roll YAP/TAZ spelar som förmedlare av mekaniska stimuli i samband med hypertension och kan visa att möss som saknar YAP/TAZ inte kan hantera ökat blodtryck med bland annat svåra skador på mindre blodkärl som följd. Det antyder att YAP/TAZ spelar en avgörande roll i att förhindra uppkomst av kärlskador i samband med hypertension.

Ateroskleros karakteriseras av inlagring av fett, ökat antal inflammatoriska celler och glatta muskelceller i blodkärlens vägg vilket leder till förträngningar i kärnen. Vi har undersökt vilken roll MRTFA (en medlem i MRTF-familjen) spelar för uppkomst av ateroskleros och upptäckte att MRTFA driver glatta muskelceller att

ta upp fett och därigenom bilda så kallade skumceller, ett av flera kännetecken för ateroskleros.

Slutligen behandlar den här avhandlingen glatta muskelcellers svar på en patologisk ökning av proteinproduktion i cellen. Vid abnorm produktion av proteiner eller ackumulering av defekta proteiner i cellen sätts flera processer igång som ett försök att rädda cellen. Om dessa processer inte fungerar begår cellen självmord (apoptos). Cellens förmåga att svara på ökad stress kan kopplas till flera sjukdomstillstånd och vi har undersökt vilken roll MRTFA spelar i dessa processer.

Sammantaget visar vi att glatta muskelceller använder YAP/TAZ och MRTFA som budbärare för att förmedla mekaniska stimuli utanför cellen till gensvar inne i cellen. Mycket tyder på att rätt nivå av YAP/TAZ och MRTF är avgörande för att upprätthålla den glatta muskulaturens struktur och funktion där avvikande nivåer kan resultera i olika sjukdomar. En bättre förståelse för de mekanismer och processer som YAP/TAZ och MRTF reglerar kommer förhoppningsvis att bidra till att vidga framtida behandlingsalternativ.

نحو الجدار الداخلي للوعاء الدموي، الأمر الذي يُؤدّي إلى تضيق تدريجي في مجرى الدم. لذلك لقد فُمنّا في هذه الرسالة بِفحص دور MRTFA (عضو في MRTFs) في تصلب الشرايين وتبيّن أنّ وجود MRTFA قد حفّز العضلة الملساء على جمع الدُهون داخلها وخلق مظهر يُشبه الرغوة (خلايا رغوية) وهي سمة مميزة لتصلب الشرايين.

وفي البحث الأخير، فُمنّا بدراسة علاقة MRTFA مع محاولات الخلية لإعادة التوازن بعد تراكم البروتينات الغير القادرة على التشكّل بصورة صحيحة داخل الخلية الملساء. حيث أنّ تكّس هذه البروتينات في داخل الخلية مرتبط بنشأة و تطور عدة أمراض لذلك تحاول الخلية تشغيل بعض التسلسلات التكوينية التي من شأنها إنقاذ الخلية. لكنها إذا فشلت فإنّها تؤدي إلى الموت المُبرمج. لقد ساهم MRTFA في تخفيف حدّة هذه الاستجابة.

خُلاصةً، لقد وجدنا أنّ العضلة الملساء تستخدم YAP/TAZ و MRTFs كمراسلين للمُحفّزات الميكانيكية المُحيطة وأنّ النشاط المُتوازن لهما ضروري للحفاظ على الشكل الطبيعي ووظيفة العضلة الملساء. وأنّ أي اضطراب في مُستويات YAP/TAZ أو MRTFs قد يؤدي إلى المرض. إنّ هذه النتائج تُساهم في فهم الآليات الجزيئية التنظيمية لكلّ من YAP/TAZ و MRTFs التي قد تُساهم مستقبلاً في اكتشاف علاجاتٍ دوائية جديدة خصوصاً ضد أمراض القلب والأوعية الدموية.

مُلخَص الرِّسَالَة

هناك ثلاثة أنواع من أنسجة العضلات في الجسم، عضلة القلب والعضلات الهيكلية والعضلات الملساء. تُبْطِن العضلة الملساء الأعضاء المُجَوِّفة كالمعدة والأمعاء والمثانة والرَّحِم، و تُعْطِي المسالك والأوعية كالمجرى البولي والتنفسى والأوعية الدموية مما يجعلها مهمة في الكثير من العمليات الحيوية في الجسم كالهضم والولادة والتبول، كما أنها تقوم في تنظيم مجرى الهواء وضغط الدم و تدفِّقه. بسبب طبيعة هذه الوظائف، تتعرَّض العضلة الملساء إلى مُحَفِّزَات ميكانيكِيَّة بِاسْتِمْرَار وتُسْتَشْعِرُ هذا كَتَمَدُّدِ عِبْرَ بروتينات مُحدَّدة على غشاء الخلية يتم ترجمتها إلى إشارة جزيئية تنتشر داخل الخلية حتى تصل إلى النواة. في النواة -واستجابةً لذلك المُحفِّز- يتم اختيار جينات مُعَيَّنة إمَّا لِتُنْبِطِهَا أو لِتَنْشِطِهَا. تَمَرِّكُزُ هذه الأطروحة على عائلَتَيْنِ من البروتينات الحساسة للمُدخَلَات الميكانيكِيَّة YAP/TAZ و MRTFs. هذه البروتينات لديها القُدرة على الانتقال من داخل الخلية إلى النواة بعد التعرُّض لمُحفِّز ميكانيكِي. لكنَّ وظيفة YAP/TAZ و MRTFs في العضلة الملساء وطبيعة مساهمتهم في أمراض الأوعية الدموية لا تزال غير معروفة إلى حدِّ كبير.

من أجل تقييم دور YAP/TAZ في العضلة الملساء، طَوَّرْنَا نموذجًا فريدًا من الفئران المعدلة جينيًا حيثُ فُمنَّا بِحَذْفِ هَذَيْنِ الجينَيْنِ في العضلة الملساء فقط. وجدنا أنَّ YAP/TAZ يلعبان دورًا مهمًّا في الحفاظ على البنية الطبيعية للعضلة الملساء في الفئران البالغة وفقدانها أدى إلى صَعْفِ العضلة وفقدانها القُدرة على الانقباض بشكلٍ ملحوظ. هذا التأثير كان واضحًا في أماكن مُتعدِّدة من جسم الفأر لكنَّه كان أكثر وُضوحًا في القولون حيث فقدت هذه الفئران القُدرة على دفع الفضلات خارج الجسم. الأمر الذي أدى إلى تراكم البراز داخل الأمعاء الغليظة وتوسُّعها. وكان ذلك سببًا مباشرًا للمرض الشديد للفئران وموتها.

وقد كُنَّا مُهْتَمِّينَ أيضًا بفحص دور YAP/TAZ كَوَسِيطَيْنِ ناقِلَيْنِ للحركة الميكانيكِيَّة في بيئة ارتفاع ضغط الدم. ولذلك فُمنَّا بِحَذْفِ YAP/TAZ في العضلة الملساء لفئرانٍ تُعاني من ارتفاع ضغط الدم. لم تستطع الأوعية الدموية لهذه الفئران تحمُّل ارتفاع ضغط الدم، ولاحظنا تغيُّراتٍ مَرَضِيَّة (آفات) مُتعدِّدة في الأوعية الدموية الصغيرة. يُشِيرُ هذا إلى أنَّ YAP / TAZ يلعبان دورًا أساسيًا في الحماية من التأثير السلبي لارتفاع ضغط الدم على الأوعية الدموية. ويُعدُّ ارتفاع ضغط الدم عامِلَ حَظَرٍ رئيسي لأمراض القلب والأوعية الدموية وسببًا رئيسيًا للوفاة في البلدان المتقدمة.

على غرار ارتفاع ضغط الدم يُعْتَبَرُ تَصَلُّبُ الشرايين أحدَ عَوَامِلِ الخطر الرئيسية لأمراض القلب والأوعية الدموية. ويتَّصِفُ تَصَلُّبُ الشرايين بِتَرَاكُمِ الدُّهُونِ والخلايا المَنَاعِيَّةِ وهجرة العضلة الملساء

List of Abbreviations

ACE- angiotensin-converting enzyme
ACTA2- smooth muscle alpha-actin
ACTG1- smooth muscle gamma-actin
ARBs- inhibitors of angiotensin II receptor blockers
CALD1- caldesmon
CNN1- calponin
DAG- diacylglycerol
ECM- extracellular matrix
ER- endoplasmic reticulum
ERT2- estrogen receptor
ETS- erythroblast transformation specific
GDP- guanosine diphosphate
GFP- green fluorescent protein
GI- gastrointestinal
GO- gene ontology
GPCRs- G-protein coupled receptors
GTEx- Genotype-Tissue Expression portal
GTP- guanosine triphosphate
HRP- horseradish peroxidase
IP3- inositol (1,4,5) trisphosphate
IPA- Ingenuity Pathway Analysis
KLF4- Krüppel-like factor 4
LDL- low-density lipoprotein
MLC- myosin light chain

MLC- myosin regulatory light chain
MLCK- myosin light chain kinase
MLCP- myosin phosphatase
MRTF- myocardin-related transcription factor
MYH11- smooth muscle myosin heavy chain
PIP2- phosphatidylinositol 4,5-bisphosphate
RISC- RNA-induced silencing complex
RNAi- RNA interference
ROCK- Rho-kinase
RT-qPCR- reverse transcription quantitative polymerase chain reaction
SDS- sodium dodecyl sulfate
shRNA- short hairpin RNA
siRNA- small interfering RNA
SM-MHC- smooth muscle myosin heavy chain
SRE- serum response element
SRF- serum response factor
TAZ- transcriptional coactivator with PDZ-binding motif
tdTomato- tandem dimer tomato protein which has a bright red color
TEAD- TEA-domain transcription factors
TN- tunicamycin
TPM1- tropomyosin
UPR- unfolded protein response
YAP- Yes-associated proteins

Prologue

How does the cell perceive and convert the mechanical stimulus into a biochemical signal, and how does this signal propagate from the cell membrane to the nucleus? Despite extensive research, several pieces of the puzzle are still missing. Last year, Ardem Patapoutian was awarded The Nobel Prize in Physiology or Medicine for the discovery of a mechanosensitive ion channel. It was given the name Piezo1, after the Greek word for pressure (*i; píesi*). This highlights the significance of this field and the possibility of identifying new mechanosensitive proteins. Studying mechanotransduction has the utmost significance in smooth muscle, as it lines the hollow organs and mediates vital physiological functions by sensing and responding to stretch. Moreover, the vascular smooth muscle senses the pressure and responds by adjusting vessel radius, hence regulating blood pressure and flow.

Adaptation to mechanical stimuli likely involves induction or inhibition of gene transcription. Therefore, throughout this thesis, I have studied two families of mechanosensitive transcriptional cofactors in smooth muscle, YAP/TAZ and myocardin-related transcription factors (MRTFs). The function of YAP/TAZ in adult smooth muscle *in vivo* has never been explored before, and for that we developed a mouse model with inducible YAP/TAZ deletion specifically in smooth muscle. We examined the impact of YAP/TAZ deletion on visceral and vascular smooth muscle contractility and gene expression. We also investigated the role of YAP/TAZ in vascular mechanotransduction and adaptation to hypertension. Hypertension is considered a major risk factor for cardiovascular diseases, which are the leading cause of death worldwide. Mechanical injury is also linked directly to atherosclerosis development in small and large arteries¹. A hallmark of atherosclerosis is the presence of foam cells; therefore, we investigated the role of the mechanosensitive cofactor MRTFA in foam cell formation. Lastly, we also studied the relation between MRTFA and endoplasmic reticulum stress, which has a crucial role in maintaining tissue hemostasis and has been connected to the pathogenesis of several diseases.

In conclusion, this thesis focuses on mechanosensitive transcriptional regulation of smooth muscle, aiming to provide new insight into their physiological and pathological roles and, in the long term, to propose new therapeutic targets against vascular disease.

Introduction

Smooth muscle cell

Anatomy and histology

There are three distinct types of muscle tissue across the body: cardiac, skeletal, and smooth muscle. As the name implies, the cardiac muscle is heart-specific and functions as a pump distributing blood to the systemic and pulmonary circulations. Skeletal muscle is connected to bone and is responsible for voluntary movements. The diaphragm is also considered a skeletal muscle and has a crucial role in respiration. Smooth muscles line hollow organs (stomach, intestine, bladder, and uterus), and cover tracts and passages (urinary tracts, respiratory tracts, and vascular walls). Smooth muscle modulates numerous physiological functions in the body, including propelling food through the gastrointestinal (GI) tract, parturition, and micturition. It is also involved in regulation of airway diameter and blood pressure. Alongside the differences in anatomical location and physiological function, muscle tissues also vary in structure, rate and duration of contraction, and underlying regulatory machinery.

Smooth muscle is classified into single- or multi-unit depending on the degree of intercellular communication. The single-unit smooth muscle has prominent gap junctions between the cells, allowing ions and small molecules to diffuse freely and form a syncytium. Therefore, these cells have a high degree of coordination, and upon contraction, they contract together as one unit. The single-unit smooth muscle is present predominantly in GI and urinary tracts, uterus, and some blood vessels. However, the size of the functional unit can vary from small to big, like in blood vessels and GI tract, respectively. On the other hand, multi-unit smooth muscle like in the iris and ciliary body has less intercellular coupling and is capable of more fine movement control.

Smooth muscle fibers have different alignments between organs to fit their function. For example, in the bladder, the smooth muscle fibers (detrusor muscle) are arranged in two layers and have variable directions across the bladder². The muscle layer in the colon (muscularis mucosae) is composed of an outer longitudinal and inner circular layer. The vascular smooth muscle cells are arranged in circular

orientation in the arterial wall (tunica media), and the muscle to elastin ratio is increased towards smaller branches.

Microscopically, smooth muscle lacks sarcomeres and striation of actin and myosin as found in skeletal muscle. The actin filaments in smooth muscle are attached to specialized locations called dense bodies (cytoplasm) and dense bands (cell membrane). They are analogous to the Z-disc of the skeletal muscle and are predominantly affluent in α -actinin. Dense bodies and bands also serve as binding sites for intermediate filaments like vimentin and desmin. The myosin filaments intersperse between actin filaments, and they have side-polar arrangements that allow for extensive shortening of smooth muscle upon contraction³. The combination of actin, thin filaments (smooth muscle alpha-actin, α -actin, *ACTA2*; and gamma-actin, γ -actin, *ACTG1*); myosin, thick filaments (smooth muscle myosin heavy chain, SM-MHC, *MYH11*; and myosin light chain, MLC); and actin-binding proteins (calponin, *CNN1*; caldesmon, *CALDI*; tropomyosin, *TPMI*) is commonly referred to as the contractile machinery of smooth muscle.

Contraction

The resting membrane potential of smooth muscle generally ranges between -50 and -60 mV. This can be changed in response to mechanical stimulation, neural, and hormonal factors. Moreover, interstitial cells of Cajal in the intestine can produce spontaneous electrical activity (slow waves) that propagate and spread across some smooth muscle cells. If the stimulus is strong enough, it crosses the threshold and fires an action potential, hence initiating contraction. There are different forms of action potential in smooth muscle cell: a simple spike, a spike followed by a plateau, and spikes on top of slow waves. Unlike skeletal muscle, the depolarization of smooth muscle is primarily due to the opening of L-type voltage-gated Ca^{2+} channels and the repolarization is due to the opening of voltage-gated K^{+} channels.

Regardless of the source, all stimuli generally result in increased intracellular calcium. Four Ca^{2+} ions bind to calmodulin, and the resultant complex binds to and activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin regulatory light chain (MLC), leading to conformational changes of the myosin head allowing attachment to actin filaments and initiation of cross bridge cycling. This cycle continues as long as MLC is phosphorylated despite returning of the intracellular Ca^{2+} to near basal levels. The contraction ends once the MLC is dephosphorylated by myosin phosphatase (MLCP). Thus, smooth muscle contraction relies on the phosphorylation status of MLC, which in turn reflects the activity of MLCK and MLCP. Some agonists can potentiate contraction by inhibiting MLCP, a phenomenon referred to as Ca^{2+} -sensitization.

G-protein coupled receptors (GPCRs) are seven-transmembrane helical receptors. A wide diversity of ligands, including drugs, bind GPCRs and influence smooth

muscle contraction, growth, proliferation, and differentiation⁴. The GPCR is bound to a heterotrimeric G protein complex (α , β , and γ). Upon ligand binding, GPCRs undergo conformational changes that activate the α -subunit, which exchanges guanosine diphosphate (GDP) with guanosine triphosphate (GTP) and dissociates from the β - and γ -subunits. The subsequent events depend on the subclass of the α -subunit. G_q activates phospholipase C, which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol (1,4,5) trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ stimulates the release of Ca²⁺ from the sarcoplasmic reticulum, whereas DAG activates PKC. PKC phosphorylates CPI-17, which has an inhibitory effect on myosin phosphatase, thus causing Ca²⁺ sensitization. Vasopressin, angiotensin II, serotonin, acetylcholine (M₃), and adrenergic agonists (including e.g. noradrenaline, cirazoline) are linked to the G_q subunit. On the other hand, $G_{\alpha12/13}$ stimulates Rho-kinase (ROCK) via the small GTPase RhoA. ROCK phosphorylates myosin phosphatase target subunit 1 (MYPT1) and possibly CPI-17 leading to inhibition of MLCP⁵⁻⁹. Thromboxane A2 primarily stimulates $G_{\alpha12/13}$, whereas vasopressin and angiotensin II can activate both G_q and $G_{\alpha12/13}$ ^{7,10,11}. Taken together, PKC and ROCK play an important role in Ca²⁺ sensitization.

Transcriptional regulation: SRF, Myocardin, MRTFs, YAP/TAZ

Smooth muscle has fascinating transcriptional regulatory mechanisms that fine-tune protein expression to execute critical functions throughout the body. There is a set of genes commonly referred to as smooth muscle markers that includes contractile machinery genes (*MYH11* and *ACTA2*), intermediate filaments (*DES*), and ion channels (*KCNMB1*). These genes are important for the differentiated state and contractile function of smooth muscle.

The transcription factor, serum response factor (SRF) regulates many of the smooth muscle genes that are involved in growth and differentiation. Smooth muscle-specific deletion of SRF results in lethal intestinal phenotype, due to loss of smooth muscle contraction and peristalsis. Whereas, vascular smooth muscle deletion of SRF leads to a prominent reduction in contractility and downregulation of smooth muscle markers (Ganesh et al., preprint)^{12,13}. SRF binds as a dimer to a specific 10 bp-DNA consensus sequence, CC (A/T)₆GG (shortly known as a CA₆G box) within the serum response element (SRE)¹⁴⁻¹⁶. This binding is highly influenced by the presence of cell-specific cofactors, since SRF lacks tissue specificity and can induce disparate gene programs in the same cell^{14,17,18}.

As the name implies, SRF responds to serum and mitogens and stimulates the growth and proliferation of smooth muscle. SRE is found in the promoter regions of immediate early genes such as *FOS* and *EGR1*. Additionally, the promoter region of these genes also contains another important binding site adjacent to SRE which is designed for erythroblast transformation specific (ETS) transcription factor family. Therefore, it seems that the cooperative function between SRF and ternary

complex factor (TCF), a member of ETS-family, is responsible for the transcription of the immediate early genes and smooth muscle growth¹⁹⁻²¹.

To induce smooth muscle markers expression, SRF binds to an important transcriptional cofactor in smooth muscle which is myocardin^{22, 23}. Myocardin is selectively expressed in cardiomyocytes and differentiated smooth muscle *in vivo*^{23, 24}. When fibroblasts are transfected with myocardin, they start to express smooth muscle specific genes and change their phenotype to smooth muscle-like cells²⁵. Moreover, mice deficient in myocardin die by embryonic day 10.5 and display no signs of vascular smooth muscle differentiation²². The SRF-myocardin complex recognizes CARG boxes found in the promoters and/or introns of smooth muscle markers, suggesting a cooperative interaction to induce smooth muscle-specific gene program²⁶. Moreover, these promoter regions lack the ETS-binding site, hence TCF cannot bind. In conclusion, smooth muscle cells select SRF cofactors to meet cellular demand depending on the surrounding extracellular cues, placing TCF and myocardin under competition to govern gene expression²⁷.

Similar to myocardin, myocardin-related transcription factors (MRTFs) A and B (MRFTA) and (MRTFB) induce the expression of smooth muscle markers via SRF^{28, 29}. However, there are some differences between myocardin and MRTFs: 1) MRFTA and MRTFB are widely expressed in the body and not restricted to cardiac and smooth muscle cells; 2) it has been shown that MRTFs can shuttle between the cytoplasm and the nucleus, whereas myocardin is primarily present in the nucleus. The two RPEL motifs in MRTFs bind unpolymerized actin with high affinity retaining MRTFs in the cytoplasm³⁰ (Figure 1). Subsequently, when the pool of unpolymerized actin shifts towards more filamentous actin formation, this stimulates nuclear localization of MRTFs³¹ (Figure 1).

Emerging contributors to transcriptional regulation of smooth muscle are the transcriptional coactivators YAP/TAZ, the downstream effectors of Hippo signaling pathway. The Hippo signaling pathway is highly conserved in mammals and was initially discovered in *Drosophila* two decades ago³². It is composed of a cascade of kinases, MST/SAV and LATS/MOB1 that eventually phosphorylate YAP/TAZ (Figure 1)³³. Phosphorylation of YAP/TAZ by Hippo kinases leads to either degradation or cytoplasmic sequestration. When YAP/TAZ are unphosphorylated, they translocate to the nucleus and exert their biological functions primarily via TEA-domain transcription factors (TEAD) (Figure 1)³⁴. YAP/TAZ regulate cell proliferation, apoptosis, and differentiation, hence playing critical roles in organ size control, tissue homeostasis, and tumorigenesis³³. A wide variety of stimuli can affect YAP/TAZ function, such as substrate stiffness, GPCRs, cell polarity, and cell-cell interactions. Like MRTFs, YAP/TAZ are also affected by actin dynamics, either through the Hippo pathway or independently³⁵(Figure 1). Genetic deletion of YAP/TAZ has revealed their importance in the development of multiple organs like heart, vasculature, GI-tract, and kidneys.

Considering that both YAP/TAZ and MRTFs are affected by actin polymerization, crosstalk between them has been suggested. Kim et al. showed that PPXY motif of MRTFs physically interacts with the WW domain of YAP. This interaction further potentiates YAP-TEAD dependent transcription³⁶. Moreover, it has been demonstrated the YAP-TEAD reporter activity is affected by depletion of MRTFs and SRF in fibroblast and vice versa, suggesting a mutual dependence between YAP-TEAD and MRTFs-SRF³⁷. Some genes have been recognized as targets for both YAP and MRTFs. For example, *CYR61*, a classic target of YAP-TEAD complex, has been also identified as MRTFs-SRF target and its promoter contains binding sites for both^{38,39}. In bladder smooth muscle, MRTFs and YAP are equally important in controlling the expression of nexilin, which is an actin-binding protein⁴⁰. Taken together, several lines of evidence propose the crosstalk between the two pathways; however more studies in smooth muscle are needed.

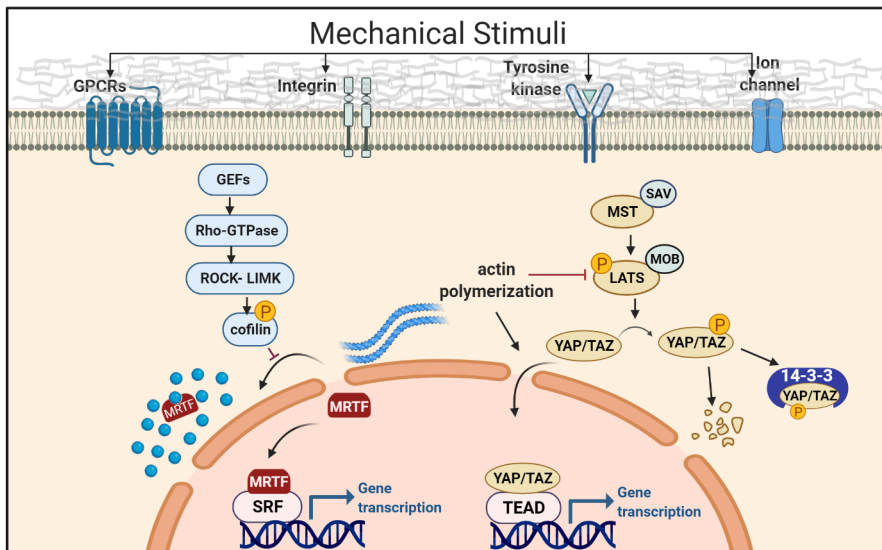


Figure 1. Schematic illustration represents the pathways that are involved in responding to mechanical stimuli in smooth muscle. The mechanical stimuli are transmitted through cell membrane proteins such as GPCRs, integrin, tyrosine kinase, and ion channel. This will result in actin polymerization that promotes MRTF and YAP/TAZ nuclear localization. Actin polymerization is largely regulated by Rho pathway to the left. To the right the Hippo pathway is demonstrated which regulates YAP/TAZ activity.

Mechanotransduction

The ultimate function of the circulatory system in the body is to deliver nutrients, hormones, and oxygen to the tissues, as well as to get rid of waste products and carbon dioxide. To a great extent, blood flow is determined locally to match the tissue demand, considering that the demand can vary significantly between one tissue and another. Applying the Poiseuille equation (Equation 1) to the circulation dynamics results in that blood flow (Q) is proportional to the fourth power of the vessel radius (r) and the difference in pressure along the blood vessel (ΔP). It is also inversely proportional to the blood viscosity (η) and vessel's length (l). According to this equation, blood flow is determined mainly by the vessel radius, and any minor change in the radius can cause a dramatic change in blood flow.

$$Q = \frac{\pi \Delta P r^4}{8 \eta l} \quad (\text{Equation 1})$$

Vascular smooth muscle can adjust the vessel radius by contraction and relaxation, hence it plays a vital role in blood flow regulation. Vessel radius is controlled systemically by various neurotransmitters (norepinephrine, epinephrine), and hormones (angiotensin II, serotonin). It is also affected by the local environment, including tissue metabolites (P_{CO_2}), endothelial vasoactive agents (nitric oxide, endothelin) and mechanical stimuli (myogenic response). These local factors usually override the systemic regulations leading to variable flow in different vascular beds. Lastly, besides the involvement in local regulation of blood flow, the vascular smooth muscle of small resistance arteries and arterioles contributes to the resistance of the vascular system and thereby to the arterial blood pressure.

This section will be dedicated to vascular smooth muscle mechanotransduction. A process refers to the ability of the vascular smooth muscle to sense mechanical stimuli and translate these signals into appropriate physiological response (Figure 1).

Focal adhesions

Focal adhesions refer to the connections between the extracellular matrix (ECM) and intracellular actin cytoskeleton. They are primarily made up of integrins, which are heterodimer proteins (α , β) that span the plasma membrane and have both intracellular and extracellular domains. They are considered mechanosensors that transmit the change in mechanical cues to the intracellular compartment and initiate signal transduction (Figure 1)⁴¹. Focal adhesions are highly dynamic and composed of over 50 proteins with variable structural and catalytic activity. Although integrins *per se* do not have intrinsic catalytic activity, they mediate their function by

recruiting other cytoplasmic proteins, like zyxin, vinculin, paxillin, and focal adhesion kinases⁴²⁻⁴⁴. Eventually, the signals can modulate cytoskeleton organization, smooth muscle proliferation and differentiation, and cell motility⁴⁵.

Actin dynamics

Smooth muscle expresses four actin isoforms. Two of them are primarily smooth muscle specific (α - and γ -SM actin), whereas the other two are ubiquitously expressed (β - and γ -NM actin)⁴⁶. α -SM-actin is the predominant isoform in vascular smooth muscle, whereas γ -SM-actin is the predominant isoform in the GI tract^{47, 48}. In the smooth muscle cells, α - and γ -SM actin participate in forming the contractile machinery, while β - and γ -NM actin are part of the cytoskeleton. Actin mediates multiple functions in the cell, such as migration, adhesion, providing structure, contraction, and involvement in gene regulation⁴⁹.

Actin is present in two forms in the cells, globular (G-actin) and filamentous (F-actin). The first step of F-actin formation is called nucleation, where three ATP-bound G-actin molecules assemble to form a seed. This process can be assisted by nucleation initiation protein Arp2/3 complex⁵⁰. ATP-bound G-actin binds to the (+) end and causes elongation of the filaments (polymerization). On the (-) end, G-actin is released, causing shortening of the filament (depolymerization). Several actin binding proteins are involved in the regulation of actin dynamics including profilin, which accelerates polymerization, and cofilin, which accelerates depolymerization⁵¹.

When the vascular smooth muscle is subjected to stretch, it activates ROCK, leading to subsequent phosphorylation of LIM kinase. LIM kinase inhibits cofilin, therefore stabilizes F-actin (Figure 1)⁵². Moreover, RhoA activates mammalian diaphanous homolog mDia, which binds to profilin and enhances F-actin formation^{53, 54}. Actin polymerization in response to stretch has been observed in portal veins subjected to stretch *ex vivo* (method section), as well as pressurized arteries^{52, 55}. Actin dynamics can be manipulated experimentally by various compounds, such as jasplakinolide which stabilizes F-actin, whereas latrunculin B and cytochalasin D inhibit F-actin assembly⁵⁶.

Myogenic response

When vascular smooth muscle senses an increase in intraluminal pressure and the accompanying stretch, it responds by contraction within seconds. This phenomenon was discovered by William Bayliss in 1902 and later referred to as myogenic response⁵⁷. The myogenic response is most prominent in arterioles but can also be detected in small arteries and veins⁵⁸. It is an inherent characteristic of the vascular smooth muscle that occurs independently from any neural or hormonal influences.

It aims to maintain organ perfusion within the normal range and protect the organ from injury. This autoregulation of blood flow is highly important in organs sensitive to ischemia or hypoxia, such as the brain, heart, and kidneys. Moreover, myogenic response in the small arteries and arterioles can potentially contribute to blood pressure regulation.

Extensive research has been directed towards understanding the underlying molecular mechanisms of the myogenic response. Stretch-sensitive nonselective cation channels, GPCRs, integrins, L-type voltage-gated Ca^{2+} channels, and actin cytoskeleton reorganization have been proposed to be involved in mechanosensing⁵⁹⁻⁶². Most of these mechanisms will result in an increase of intracellular Ca^{2+} concentration and activation of myosin-actin cross bridge cycling. A sustained myogenic response is commonly referred to as myogenic tone and involves Ca^{2+} sensitization through activation of ROCK⁶³.

Shear stress

In contrast to the myogenic response, which leads to decreased vessel radius, shear stress causes vascular smooth muscle relaxation and dilatation. An increase in laminar flow (high shear stress) will stimulate endothelium to synthesize and produce nitric oxide, and other vasodilators, which cause vasodilation. Studies have suggested the involvement of specific endothelial proteins in sensing the change in blood flow, such as vascular endothelial cell cadherin (VE-cadherin), endothelial cell adhesion molecule (PECAM), and vascular endothelial growth factor receptor2 (VEGFR2)^{64, 65}.

Phenotypic modulation

Under specific environmental cues and in response to hormonal stimuli, smooth muscle cells can alter their gene expression, morphology, and function in a process referred to as phenotypic modulation. In adult tissue, smooth muscle cells are normally quiescent, contractile, and spindle shaped (Figure 2). However, in response to stimuli, such as vascular injury, they can dedifferentiate to a migratory, proliferative synthetic phenotype with rhomboid shape (Figure 2). Phenotypic modulation represents a broad phenotype spectrum rather than dichotomous states between contractile and synthetic phenotypes. In the contractile phenotype, gene expression is directed towards the synthesis of protein involved in the contractile machinery, cytoskeleton, signaling molecules, and ion channels. The cells in the synthetic phenotype produce more ECM proteins, such as collagen, elastin, and proteoglycans, and nonmuscle myosin heavy chain isoform⁶⁶. This is in stark contrast to cardiac and skeletal muscle cells, which are terminally differentiated.

The phenotypic modulation can occur in response to certain physiological stimuli, like the uterine smooth muscle, which undergoes dramatic changes during gestation and after delivery⁶⁷. Experimentally, isolating smooth muscle cells from intact tissues and culturing them *in vitro* result in marked reduction of smooth muscle markers and shifting the cells towards more synthetic phenotype. Phenotypic switching can start as an adaptation process but then progress to disease. Phenotypic switching has been implicated in the pathogenesis of several diseases such as hypertension, atherosclerosis, neointimal formation, chronic intestinal pseudo-obstruction⁶⁸, and bladder outlet obstruction⁶⁹ (Figure 3).

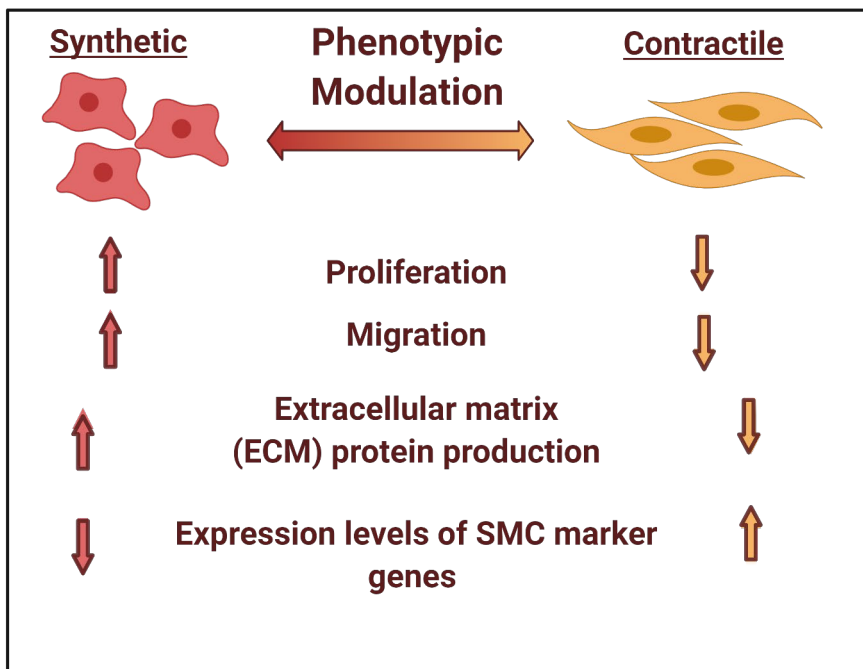


Figure 2. Characteristics of phenotypic modulation. Smooth muscle has remarkable plasticity to switch between a contractile and a synthetic phenotype. The contractile phenotype is characterized by low level of proliferation, migration, extracellular matrix production, and high expression of smooth muscle markers. The synthetic phenotype represents the other extreme end with opposite characteristics.

Phenotypic modulation in disease

Hypertension

Vascular smooth muscle reacts to increased pressure in two ways, acutely by the myogenic response and chronically by vascular remodeling. Vascular remodeling includes structural changes like hypertrophy and proliferation of vascular smooth muscle, and ECM reorganization. These adaptations aim to reduce wall tension, hence wall stress according to the law of Laplace, which states that wall tension is proportional to intraluminal pressure and radius (Equation 2). Wall stress is the force per cross sectional area unit (Equation 3). Vascular remodeling can be classified into inward and outward remodeling depending on whether the diameter is reduced or increased, respectively. It is also classified depending on the cross sectional area into hypertrophic (decreased), eutrophic (unchanged), and hypertrophic (increased).

$$\text{Wall tension (T)} = \text{Pressure (P)} \times \text{radius (r)} \quad (\text{Equation 2})$$

$$\text{Wall stress } (\sigma) = \text{wall tension} / \text{wall thickness} \quad (\text{Equation 3})$$

$$\sigma = P \times r/w \quad (2 \text{ and } 3)$$

Eutrophic inward remodeling of small resistance arteries has been associated with essential hypertension⁷⁰. Several studies have indicated that mechanical stress *per se* is a direct trigger for remodeling; and the vessels that are protected from high blood pressure do not show any signs of remodeling⁷¹⁻⁷³. In addition, accompanying changes in vasoactivity and response to neuro-hormonal factors also play an important role in remodeling⁷⁴. Vascular remodeling seems to have an adaptive nature initially, but can eventually have a detrimental impact; which may start a vicious cycle and exaggerate hypertension.

According to American Heart Association (2017), hypertension is defined as increased systolic blood pressure to 130 mmHg or above and/or diastolic 80 mmHg or above. Hypertension is a risk factor for atherosclerosis, heart failure, ischemic stroke, and kidney failure⁷⁵⁻⁷⁸. Currently, the major lines of treatment, beside life style modifications, are diuretics, angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs), and calcium channel blockers. Alongside their control of blood pressure, ACE and ARBS are able to attenuate vascular remodeling⁷⁹. Despite the current available treatment options, antihypertensive medications can only reduce the risk of heart failure by 50% and stroke by 30 to 40%⁸⁰. Moreover, many patients suffer from resistant hypertension where they take three antihypertensive medications of different classes without reaching the desired blood pressure target. These limitations highlight the need for more research to discover new potential targets.

Atherosclerosis

Atherosclerosis is a chronic disease characterized by the deposition of lipid, smooth muscle and inflammatory cells in the vessel wall. Risk factors include dyslipidemia, hypertension, diabetes, smoking, and genetic predisposition. Atherosclerosis has been connected to low shear stress and altered flow, due to the recurrent observations at the branching points, bends, and bifurcations⁸¹⁻⁸³. Atherosclerosis can start early in the childhood and develop progressively with age. It starts with fatty streaks formation that are composed of lipid-laden cells and are associated with variable intimal thickening⁸⁴. Smooth muscle cells dedifferentiate, migrate from the media towards the intima, and produce ECM. Some studies also showed the contribution of the hematopoietic stem in lesion development⁸⁵. Over time, lesions accumulate more modified low-density lipoprotein (LDL), inflammatory cells, and foam cells. Together with connective tissue, smooth muscle forms a fibrous cap which is believed to have a protective role against plaque rupture⁸⁶. As the lesion grows, blood cannot supply the core and results in a necrotic core, which marks the advanced plaque⁸⁷. Atherosclerosis is generally asymptomatic unless the plaque causes 70 or 80% narrowing of luminal diameter or it ruptures resulting in subsequent thrombosis and vessel occlusion⁸⁸. This occlusion can manifest as myocardial infarction, stroke, and sudden death.

The impact of vascular smooth muscle contribution to atherosclerosis pathogenesis is still controversial. Classically, it is believed that vascular smooth muscle has a protective role against plaques rupture by forming the fibrous cap^{86, 89-91}. However, recent lineage-tracing experiments demonstrate that 70% of foam cells have smooth muscle origin^{92, 93}. Shankman et al. revealed a critical role of Krüppel-like factor 4 (Klf4) in vascular smooth muscle phenotypic switching and involvement in plaque formation⁹⁴. However, the molecular mechanisms that underlie switching of smooth muscle to foam cell are still largely undefined.

Restenosis and neointimal formation

Coronary angioplasty refers to the technique that is used to open clogged coronary arteries by inflating a balloon with or without stent. Previously, the stents were made of bare metal, which resulted in activation of smooth muscle cells and gradual re-narrowing of the vessel lumen after the procedure (restenosis). The underlying pathogenesis of restenosis includes, smooth muscle proliferation and migration from the media to the intima, extracellular matrix reorganization, and inflammation. If left untreated, this progressive narrowing eventually results in recurring angina or myocardial infarction. To decrease incidence of in-stent restenosis, drugs with antiproliferative and immunosuppression activities were added to the stents⁹⁵⁻⁹⁷. This resulted in 75% reduction of restenosis incidence compared with bare metal

stent^{98,99}. Despite this significant reduction, restenosis is still considered as a major challenge along with thrombosis and complete vessel occlusion¹⁰⁰.

Neointimal formation has also been observed in venous grafts restenosis and occlusion. The saphenous vein graft is commonly used to anastomose aorta with coronary arteries to bypass the atherosclerotic lesions and restore blood flow. Currently, most patients receive one or two arterial grafts in addition to saphenous vein graft. Left internal mammary artery graft is the most common arterial graft and it has higher rate of patency and higher long-term survival in comparison to venous grafts¹⁰¹. After the surgery, the patients receive drug combination of antiplatelet, anticoagulants and lipid lowering agents to prevent graft stenosis and occlusion.

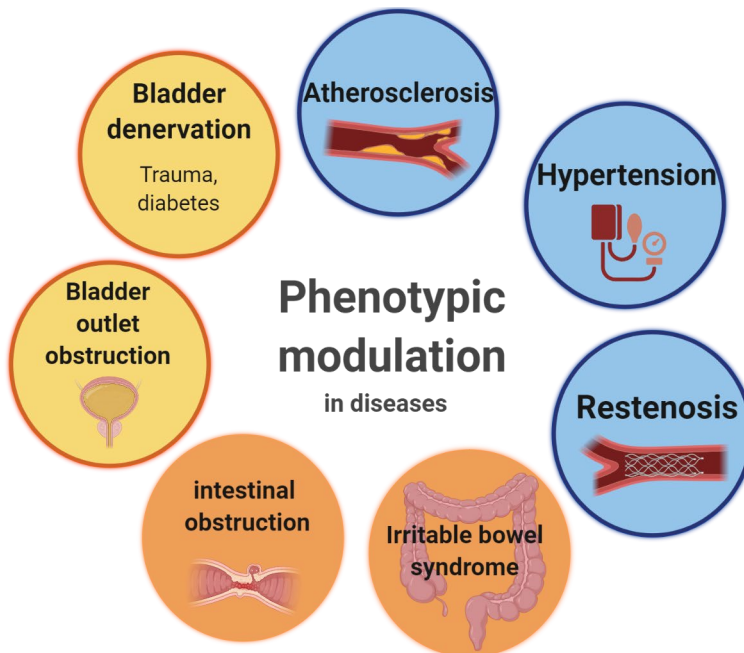


Figure 3. Diseases that are associated with smooth muscle phenotypic modulation. Smooth muscle phenotypic modulation participates in the pathogenesis of multiple disease in various systems, like vascular, gastrointestinal, and urinary.

Aims of the study

In general, this thesis aimed to shed the light on mechanosensitive transcriptional regulation of smooth muscle, in particular the role of YAP/TAZ and MRTFs, and to provide new insight into their physiological functions and role in disease development.

The specific aims were:

Paper I: To investigate the role of YAP/TAZ in adult smooth muscle cells *in vivo*, and to examine the consequences of YAP/TAZ deletion in colonic smooth muscle.

Paper II: To assess the importance of YAP/TAZ in vascular smooth muscle homeostasis and mechanotransduction, as well as to evaluate the function of YAP/TAZ in hypertension-induced vascular disease.

Paper III: To study the relationship between the unfolded protein response and expression of contractile smooth muscle markers, and to identify the major molecular mechanisms mediate this relationship.

Paper IV: To investigate the role of MRTFA for lipid accumulation and foam cell formation in smooth muscle.

Materials and Methods

Animal models

Animal experiments are performed under strict ethical regulations. As researchers, we consider the 3Rs principle (Replace, Refine, and Reduce) throughout the procedures, to minimize suffering for the animals. The animal experiments in papers I and II were approved by Malmö/Lund animal ethics committee (M6-15, M61-16, and M2990-20). Animal experiments were performed in mice since we can delete specific genes of interest using Cre-Lox recombination system.

Inducible smooth muscle-specific YAP/TAZ Knockout mouse

We have purchased the double floxed YAP/TAZ mouse from The Jackson Laboratory¹⁰². In this mouse model, the second exons of YAP (*Yap1*) and TAZ (*Wwtr1*) are flanked on both sides by a 34 bp-DNA sequence called the *loxP* site (floxed) (Figure 4). The females of double floxed YAP/TAZ mice were intercrossed with male mice that express Cre recombinase under the control of *Myh11* promoter¹⁰³. The Cre recombinase is also fused to a triple mutant form of the human estrogen receptor (ERT2) (Figure 4). Therefore, Cre is retained in the cytoplasm and can only translocate to the nucleus upon administration of 4-hydroxytamoxifen, but not estradiol. In the nucleus, two Cre proteins recognize and bind each *loxP* site. This results in recombination of the two *loxP* sites, and deletion of gene of interest (here: *Yap1* and *Wwtr1*). However, this system is imperfect, and some studies have reported tamoxifen-independent Cre activity^{104,105}. To overcome this and to control for possible adverse effect of tamoxifen, we have used tamoxifen-treated, Cre-negative, floxed mice as controls^{105,106}. Because the CreER^{T2} protein is regulated by *Myh11* promoter, deletion of YAP/TAZ will occur only in smooth muscle cells of the offspring after tamoxifen treatment (Figure 4). In conclusion, CreER^{T2}-*loxP* system allowed us to delete YAP and TAZ in specific tissue at a chosen time point.

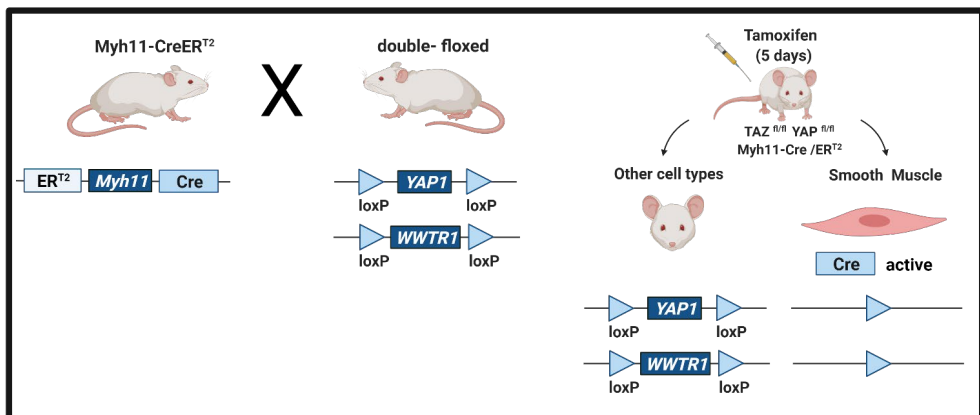


Figure 4. Breeding of inducible smooth muscle-specific YAP/TAZ knockouts. To the left, a mouse expresses Cre recombinase under the control of myosin heavy chain promoter which is regulated by mutated estrogen receptor. This mouse is crossed with double floxed *Yap1* and *Wwtr1* mouse. The offspring to the right are injected tamoxifen to induce the deletion of YAP/TAZ only in smooth muscle.

ROSA^{mT/mG} Cre reporter mouse

The development of Cre reporter mice allows researchers to localize Cre recombinase activity and enables lineage tracing studies. We purchased the reporter mouse: Gt(ROSA)26Sor^{tm4(CTB-tdTomato,-EGFP)}, commonly named ROSA^{mT/mG}, from The Jackson Laboratory¹⁰⁷. We bred this mouse to YAP/TAZ double-floxed, *Myh11*-CreER^{T2}. The offspring ubiquitously expresses membrane-bound, tandem dimer tomato protein which has a bright red color (tdTomato/ mT). In the smooth muscle cells after tamoxifen injection, *Myh11*-CreER^{T2} excises the tdTomato, and the red fluorescent is now replaced by a green signal from the enhanced, membrane-bound, green fluorescent protein (EGFP/ mG). All other cells that lack Cre activity will continue to express tdTomato, hence appear red (Figure 5). By using this model, we could distinguish the smooth muscle cells and their derivatives.

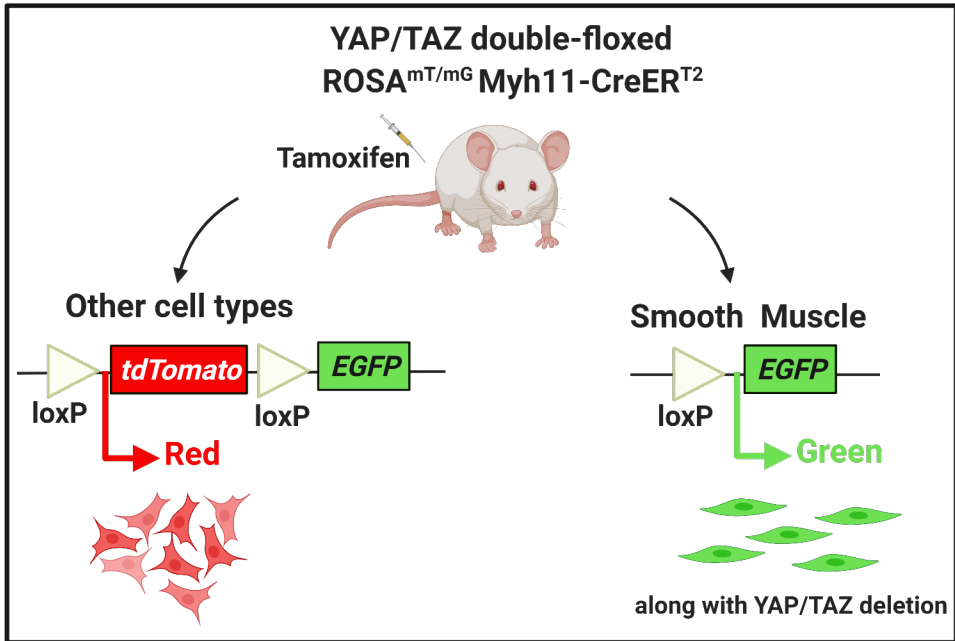


Figure 5. ROSA mT/mG Cre reporter mouse. This mouse model expresses tdTomato protein with red fluorescence in all the cells. After tamoxifen injection and only in the smooth muscle the red fluorescence is replaced by green fluorescence due to the expression of green fluorescent protein.

Angiotensin II-induced hypertension model

Several animal models have been developed to study hypertension, and they vary in the underlying approach. The general categories include genetically modified/selected animals, pharmacologically-induced, environmentally-induced, and surgically-induced hypertension. These models simulate primary or secondary hypertension in humans and provide valuable tools to understand the pathogenesis of hypertension.

Renin-angiotensin system plays a critical role in essential hypertension, and several successful antihypertensive medications target the renin-angiotensin pathway at different levels. Angiotensin II is a potent vasoconstrictor, and an infusion of 200-1000 ng/kg/min angiotensin II successfully induces hypertension in mice. The onset of elevated blood pressure depends on dose, i.e., higher doses are able to increase blood pressure faster than lower doses^{108,109}. In paper II, under isoflurane anesthesia, we subcutaneously implanted osmotic minipumps that secrete angiotensin II (≈ 700 ng/kg/min) in 4 to 6-week-old mice. Blood pressure measurements were recorded using a tail-cuff system after proper training.

Cell culture

As mentioned above, there are considerable advantages in performing *in vivo* experiments. However, *in vitro* experiments are still important tools to study cell biology and examine molecular mechanisms in more controlled environment. In papers III and IV, we have used human bladder and coronary artery smooth muscle cells. Bladder smooth muscle cells were obtained from patients who underwent cystectomies after their informed consent, whereas coronary artery smooth muscle cells were purchased from Gibco (Life Technologies). Bladder smooth muscle cells were cultivated in DMEM/Ham's F-12 medium with L-glutamine, 10% fetal bovine serum, and antibiotics. Coronary artery smooth muscle cells were kept in a medium designed specifically for them and supplemented with smooth muscle growth supplements and antibiotics. Cell culture plates were kept in 5% CO₂ and 95% air humidified incubator at 37°C. The culture medium was changed every other day, and cells were only used between passages 3 to 8.

Modification of gene expression

Recombinant adenoviruses

To understand the biological role of a specific protein, we perform gain of function or loss of function studies using overexpression and silencing of gene of interest. There are different methods to introduce genetic material to the cell and the recombinant adenoviruses have emerged as efficient vectors for transduction of primary cell. The genome of these viruses has been modified to ensure safety by deleting genes responsible for replication. The recombinant adenoviruses have high infectious ability for mammalian cells and minimal toxicity. After cell entry, the viral DNA is transported to the nucleus. However, it remains epichromosomally, which means it will not get incorporated into the host genome, and the cells will eventually lose the virus through division. The virus uses the transcriptional machinery of the host cell to transcribe the target gene (overexpression) or the short hairpin (shRNA) against it (knockdown). We always carry out titration experiments for new targets to decide the best tolerated and most efficient multiplicity of infection for that particular target. Transduction efficacy could be assessed by measuring expression level of gene of interest or by counting the transduced cells if the viral genome was designed to include fluorescent reporter like green fluorescent protein.

shRNA and siRNA

As mentioned above, the recombinant virus can carry a specific shRNA sequence. shRNA is an artificially occurring RNA that is widely used to suppress the expression of its target. This sequence is usually under the control of the U6

promoter. In the nucleus, RNA polymerase III transcribes the sequence which forms complementary base pairing within itself creating a hairpin structure, hence the name. This shRNA molecule gets exported to the cytoplasm by Exportin-5 for further processing. The dicer ribonuclease cuts the loop off in the cytoplasm, leaving a duplex of 21-nucleotide long molecule called small interfering RNA (siRNA). Then siRNA molecule binds to argonaute protein, and one strand remains bound while the other strand is released and degraded. These strands are called guide and passenger strands, respectively. Argonaute and guide strand combination recruits other proteins and forms RNA-induced silencing complex (RISC). The guide strand directs the RISC to find a perfect complementary mRNA. Once bound, the mRNA is cleaved by argonaute and then degraded. This overall process is usually referred to as RNA interference (RNAi) and plays a critical role in controlling gene expression. siRNA can also be delivered to the cells via oligofectamine. In comparison to shRNA, siRNA has a shorter half-life and usually requires multiple administrations to maintain suppression of the target gene. Moreover, siRNA has been associated with a higher off-target effect and increased risk of cellular toxicity.

GapmeRs

GapmeRs mediate their mRNA silencing function independently of RISC. GapmeRs are designed as 16-nucleotides long antisense oligonucleotides. They are composed of central DNA segment flanked by RNA analogs with unique conformation that provides higher melting temperature upon binding to their complementary strand. The GapmeRs have high degree of stability and resistance to enzymatic degradation, which allow their unassisted uptake by the cells. However, a higher dose is usually required. Once the GapmeR binds its targeted mRNA, the mRNA will be degraded by ribonucleases H, which naturally recognizes any RNA-DNA duplex. Therefore, GapmeRs result in strand-specific silencing without any RISC associated off-target activity.

Organ culture

The portal vein model

This model has been developed in our laboratory to evaluate the smooth muscle mechanosensing in an intact vessel. In the animal models of hypertension, vascular smooth muscle cells are exposed to both humoral and mechanical factors, whereas in this model, we primarily examine the influence of mechanical stretch on vascular smooth muscle. The portal vein smooth muscle cells have predominantly longitudinal orientation; therefore, attaching a weight to one end of a hanging preparation will stretch the cells along their axis, similar to the pressure effect on

arterial smooth muscle cells, which have circumferential orientation. Malmqvist and Arner demonstrated that partial ligation of the portal vein resulted in increased transmural pressure, smooth muscle cells growth, and increased contractile force¹¹⁰. These findings were reproduced using stretched portal vein *ex vivo*¹¹¹. Moreover, several contractile and cytoskeletal proteins maintained their expression in the stretched portal vein compared to the unstretched control^{52, 112}.

In paper II, the portal vein model was used to examine the role of YAP/TAZ in the stretch-induced expression of smooth muscle markers. After dissection and cleaning from surrounding tissue, a 0.3 g gold weight was attached to one end of the portal vein. This load stretches the smooth muscle to the optimal length for force development. The other end of the portal vein was attached to a hook and used to hold the portal vein in the tube. The control was left without the load. The portal veins were incubated in DMEM Ham's F12 supplemented with 2% of dialyzed fetal bovine serum, 1% antibiotics, and 10 nM insulin and kept in 5% CO₂, humidified incubator at 37°C. After 24h, samples were snap-frozen in liquid nitrogen.

Human left internal mammary artery

Left-over pieces of left internal mammary arteries were collected from patients who underwent coronary artery bypass surgery, after their informed consent. Samples were kept in calcium-free N-HEPES buffer and microdissected from the surrounding tissue and fat under the microscope. Then the samples were incubated in serum-free DMEM-Ham's F12 medium with antibiotics. The cell culture incubator was set to 37 °C in 5% CO₂. Samples were kept for a week, and the cell culture medium was changed every other day. After one week, samples were fixed in 4% formaldehyde for 24h and then transferred to 20% sucrose and frozen in optimal cutting temperature medium for further analysis. This model was used in paper IV and gave our findings a translational impact.

Gene expression measurement

Next-generation RNA-Sequencing

In the last two decades, enormous advancement has been made in sequencing technology, and nowadays we can get a comprehensive overview of cellular transcriptomic at a reasonable price and within short time. We performed RNA sequencing on different tissues (colon, bladder, and aorta) from wild-type and YAP/TAZ knockout mice for papers I and II. After tissue homogenization, total RNA was extracted, and RNA concentration and quality were determined. Library preparation was done according to the TruSeq Illumina protocol. Briefly, mRNAs

are selected by oligo (dT) coupled to magnetic beads and broken down into small RNA fragments (200-300 nucleotides). Then, these small fragments are primed and reverse transcribed to cDNA. This process is also repeated to create another complementary strand. Afterward, these small dsDNA undergo several modifications such as end repair, overhang removal, and polyadenylation to be ready for adapter ligation. These adapters contain sequencing binding sites, complementary regions to flow cells oligos, and indices. The next step after library preparation is clustering, where each fragment is isothermally amplified. The flow cell is covered by two types of oligos which are complementary to the adapters. Therefore, fragments hybridize into these coated oligos and are amplified by bridge amplification.

Sequencing starts by pairing the tethered fragments with specific fluorescently labeled nucleotides. Each nucleotide is tagged with different fluorescence; hence after each addition, the flow cell gets excited by a light source, and a distinct fluorescence is emitted. In the sequencing step, some parameters are modifiable and can improve the quality and discovery coverage, like the number of reads and whether the sequencing is paired or single. For our experiments, we did paired read where both the forward and reverse primers were used as template and the total reads number was ≈ 30 million. These reads were aligned and compared to the reference genome sequence (Mouse GRCh38) from the Ensemble database using the HISAT2 software. The alignments were assembled into full transcripts, and the expression level of each gene and transcript was quantified using the StringTie software. Finally, differentially gene expression was calculated using DESeq2.

mRNA quantification (RT-qPCR)

While RNA sequencing measures the expression level of all transcripts and genes in the sample, reverse transcription (RT) quantitative polymerase chain reaction (RT-qPCR) only measures a specific gene in each reaction. One-step RT- qPCR, where the starting material is RNA, was used throughout the papers I-IV. The master mix is composed of deoxy-nucleotides, HotStarTaq Plus DNA Polymerase, salts buffer, SYBR green fluorescent dye, forward and reverse primers for the desired targets, and reverse transcriptase enzyme. RNA template and corresponding master mix were added to a 96-well plate. StepOnePlus™ Real-Time PCR Systems from applied biosystem was used. The protocol starts with 10 minutes incubation at 50°C; this allows reverse transcriptase to convert the RNA template to cDNA. The HotStarTaq Plus DNA Polymerase is designed to be inactive at room temperature, and to activate it, a 5-minutes incubation time at 95°C is required. This also will lead to inactivation of reverse transcriptase. This technology allows to carry both reactions in the same tube sequentially and decreases the formation of misprimed products and primer-dimers. After these steps, the machine is set to perform a 2-step cycle 40 times. Each cycle consists of a denaturation step at 95°C for 10 seconds

and an annealing/extension step at 60°C for 30 seconds. In the denaturation step, each dsDNA is separated into two single strand DNA which allows the primer to attach to its complementary region (annealing). DNA polymerase starts to elongate the strand by adding complementary nucleotides. Ideally, this will result in a doubled amount of target DNA. The master mix contains SYBR green dye, which preferentially binds the dsDNA and emits a green fluorescent signal. This signal is detected continuously by the machine providing real-time quantification. At the end of the experiment, a melt curve is usually included to verify target identity and specificity. The temperature is set to 60°C and gradually increased to 95°C while the SYBR green fluorescent signal is monitored. As the temperature increases, SYBR green dissociates from the dsDNA, and the signal is reduced dramatically. The melt curve is generated by plotting the SYBR green fluorescent signal over different temperatures. The presence of multiple peaks suggests the formation of primer-dimers or nonspecific products. The fold changes are calculated by the $2^{-\Delta\Delta C_t}$ method, C_t refers to the cycle number where the fluorescent signal crosses the threshold and exceeds the background, and a lower C_t value reflects higher abundance of the target in the sample.

Protein quantification (Western blotting)

The cell carries most biological processes through proteins; therefore, it is crucial to measure protein expression, considering that the mRNA level does not always correlate with the protein level. Western blotting is a widely used technique to identify proteins depending on their molecular weights. This method was used in papers I-IV according to the following protocol: cells or disrupted tissues are collected in Laemmli buffer (2% SDS, 10% glycerol, 60 mM Tris-HCl, pH 6.8) containing protease and phosphatase inhibitors. Sodium dodecyl sulfate (SDS) is a detergent that denatures proteins by breaking the non-covalent bonds. It also distributes negative charges throughout the protein at a constant ratio, preserving the charge-to-mass ratio. The samples also undergo sonication, boiling, and treatment with mercaptoethanol to denature the protein and break the disulfide bonds. These steps collectively result in denatured, linear, and negatively charged proteins. Protein concentration is measured using the modified Lowry method, and equal amounts of proteins are loaded into polyacrylamide gel. The gel is subjected to an electrical field that forces proteins to migrate towards a positively charged electrode (Electrophoresis). Smaller proteins travel faster than larger proteins because of the sieving effect of the gel matrix.

To make it more feasible, proteins are transferred to nitrocellulose membrane either by wet or semi-dry method. Afterward, the membrane is stained with Revert™ Total Protein Stain (Licor), and image is captured by LI-COR Odyssey Fc instrument. As the name indicated, it stains all proteins in the samples and is considered a preferential way for normalization. The unoccupied protein-binding sites on the

membrane are blocked with 1% casein to prevent unspecific binding. Then, the membrane is incubated with the selected primary antibody overnight at 4°C. The secondary antibody recognizes and binds the IgG portion of the primary antibody. Detection depends on whether the secondary antibody is conjugated to fluorescent dye or horseradish peroxidase (HRP). The latter technique is called chemiluminescence, since it is based on light emission after chemical reaction. HRP- linked antibody requires the addition of substrate, whereas membrane with fluorescent antibody is ready for imaging. The amount of HRP substrate and incubation time are two variables that can affect the results. There is a risk of signal saturation with HRP antibodies for high-abundance proteins and failure to detect differences. The signal from fluorescent antibody has longer half-time, wider range of detection, and there is a possibility of multiplexing by using secondary antibodies from different species. However, within the linear range of detection, chemiluminescence has 10-100 times higher sensitivity than the fluorescent detection. Images are acquired by LI-COR Odyssey Fc instrument, and the signals are measured using Image Studio software (LI-COR). The signal from the target protein is normalized to the signal from the total protein.

Physiological studies

Wire myography

Mulvany and Halpern developed this technique in the late 1970s. The aim was to measure contractile response in small resistance arteries *in vitro*. In our lab, we have 610 and 620 models of Multi Myograph System (Danish Myo Technology), and this method was used in papers I and II. Colon rings were mounted using pins, whereas the tail artery was mounted by passing two tungsten wires inside the lumen. Each preparation is connected to a force transducer that transmits the generated force to the computer software and to a micrometer that controls the internal diameter of the preparation (Figure 6).

The colon and tail arteries were preloaded to 2 and 5 mN, respectively. This load corresponds to the optimal length for force development and is predetermined by the length-tension relationship curves. The preparations were let to equilibrate in HEPES-buffered solution at 37 °C. Two rounds of stimulation with 60 mM potassium chloride were done. Potassium chloride induces membrane depolarization and activation of voltage-operated Ca²⁺ channels, hence bypassing GPCR pathway. We also generated dose-response curves for some agonists by adding compounds in a cumulative manner. The force is integrated over the stimulation period and normalized to the length of the preparation.

Wire myography can also be used to assess the passive properties of the tissue. The internal circumference is adjusted under the microscope and set to a point where the

passive force is approximately zero. Increments in internal circumference are made in the presence of Ca^{2+} HEPES-buffered solution. After equilibration, preparations are stimulated with 60 mM potassium chloride for 7 minutes (total force), then washed with nominally Ca^{2+} -free HEPES-buffered solution, and passive force is recorded. The active force is the result of the subtraction of passive force from the total force. The passive force can be plotted against internal circumference, giving the length-tension relationship curve.

Pressure myography

Following an increase in intraluminal pressure, small resistance arteries contract in a process referred to as the myogenic response. In paper II, the pressure myography system (Living Systems Instrumentation) was used to examine myogenic response in the mesenteric arteries. Different vascular beds can be used, and we chose mesenteric artery because they are easily accessible and have a strong and reproducible myogenic contraction. The experimental setup includes mounting of the second or third-order mesenteric artery between two glass cannulae in a bath chamber (Figure 6). The chamber is filled with Ca^{2+} containing HEPES-buffered solution, and the temperature is maintained at 37 °C throughout the experiment. The intraluminal pressure of the preparation is determined by a pressure servo controller that is connected to peristaltic pumps on both sides. The mesenteric artery is allowed to equilibrate at 45 mmHg for 40 minutes and then stimulated with potassium chloride to assess the viability of preparation. After that, the pressure is increased stepwise from 20 to 120 mmHg while monitoring the vessel diameter. The vessel diameter is recorded by a microscope, camera with edge detection technology, and computer software. After replacing the solution with Ca^{2+} -free HEPES-buffered solution with EGTA (Ca^{2+} chelating agent), similar increments in pressure are repeated, and the passive vessel diameter is recorded. The myogenic contraction was calculated by subtracting the active diameter from the passive and dividing it by the passive diameter. The pressure myography can also be used to examine the influence of different pharmacological compounds on contractility, and the myogenic response.

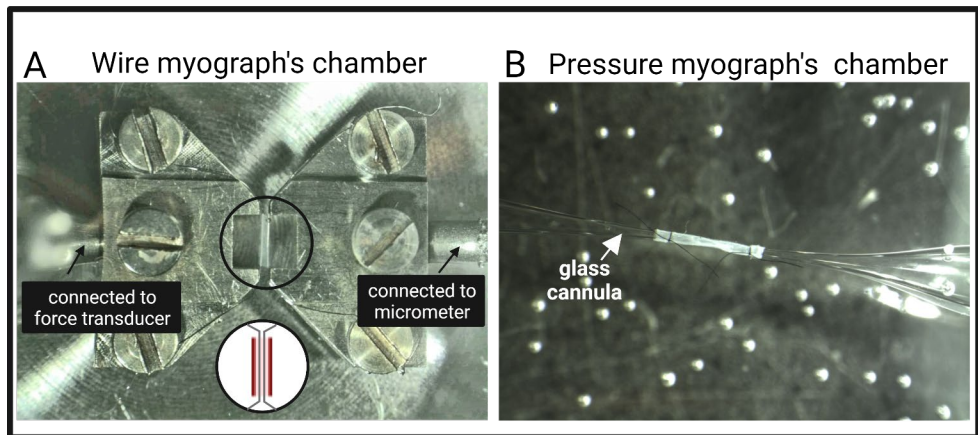


Figure 6. Wire myograph chamber to the and pressure myograph chamber. To the left, two wires are passing the lumen of the tail artery and then connected to four screws on both sides. The wire on one side is connected to micrometer to adjust the internal circumference, while the other one is connected to force transducer. To the right is the pressure myograph chamber, where a piece of mesenteric artery is mounted and tied to two glass cannulae.

Results and Discussion

The following section summarizes the findings in papers I-IV. Readers are referred to the original papers for more details.

Mechanosensitive transcriptional regulators are essential to maintain smooth muscle homeostasis (Papers I- III)

Despite the extensive research on smooth muscle mechanosensors and proposed involvement of integrins, tyrosine kinase, GPCR, and stretch-sensitive nonselective cation channels, the mechanosensitive transcriptional regulators are largely unexplored. There are some important questions that need to be answered: how do smooth muscle cells convert mechanical inputs and formulate appropriate responses; what are the consequences of impaired mechanosensitive transcriptional regulation?

This thesis focuses on two mechanosensitive transcriptional cofactors in smooth muscle, YAP/TAZ and MRTFs. It addresses the impact of lacking YAP/TAZ in smooth muscle cells and highlights their protective role against hypertensive vasculopathy. It also examines the relationship between MRTFA and unfolded protein response (UPR), and the role of MRTFA in foam cell formation.

Role of YAP/TAZ in visceral and vascular smooth muscle cells

Deletion of YAP and TAZ in adult smooth muscle leads to lethality within two weeks

YAP and TAZ are known regulators of cell proliferation, apoptosis, and organ size determination during development¹¹³. They are also widely investigated in embryogenesis and tumorigenesis¹¹³. Different knockout models have been developed to examine the role of YAP/TAZ. Conventional deletion of YAP led to early embryonic lethality¹¹⁴. However, TAZ knockout mice reached postnatal life despite developing of multiple cysts in the kidney at the early stage of embryogenesis^{114, 115}. Interestingly, embryos deficient in both YAP and TAZ died before the morula stage (16- 32 cells)¹¹⁶. After development of Cre-Lox system,

researchers were able to delete a gene of interest in specific tissue depending on the promoter that is linked to Cre gene. For example, Wang et al. used SM22 α Cre recombinase to constitutively delete YAP in mouse cardiomyocytes and vascular smooth muscle cells. This resulted in perinatal lethality due to various degrees of cardiac abnormalities, and altered structure of blood vessels¹¹⁷. It is also possible to determine the time of knockout induction if the Cre recombinase is fused with, for example, a mutated estrogen receptor that can be activated by giving the mice tamoxifen injection. In conclusion, using genetically modified animal allow us to study YAP/TAZ in specific tissues and at specific time points.

To examine YAP/TAZ role in adult smooth muscle cells, we crossed double floxed mice for YAP and TAZ with mice expressing Cre recombinase directed by smooth muscle myosin heavy chain promoter (*Myh11*). After knockout induction, YAP/TAZ KO (Y/T KO) mice showed signs of reduced liquid food intake and decreased defecation. After twelve days of the first tamoxifen injection, Y/T KO mice became critically sick and reached the ethical endpoint criteria. Necropsy revealed signs of distended large intestine with profound fecal impaction. Of interest, the inducible smooth-muscle specific single knockout of either YAP or TAZ did not show any GI phenotype and had normal life expectancy, suggesting that YAP and TAZ can compensate for each other and losing both YAP and TAZ is incompatible with life both during embryogenesis and postnatally.

Reduced Expression of Smooth Muscle Markers in Y/T KO

Using the inducible smooth muscle-specific Y/T KO model, deletion of YAP/TAZ likely took place in all smooth muscle cells throughout the mouse body. However, the most drastic and prominent changes were observed in the large intestine, which could be explained by the higher demand on smooth muscle to contract and to propel the hard feces due to progressive water reabsorption in the distal GI tract. To understand the impact of YAP/TAZ loss on gene expression, we performed RNA sequencing analyses on colon, aorta, and bladder from control and Y/T KO mice. In this thesis, I present a new analysis of the combined data from the colon and aorta, whereas the individual datasets were analyzed separately in papers I and II. Using the STRING database, cellular component Gene Ontology (GO) analysis of the downregulated genes from both datasets showed enrichment of genes related to smooth muscle contractile fibers, myosin complex and filament, actin filaments, and stress fibers (Figure 7A). Moreover, the enriched pathways were directly related to smooth muscle contraction and extracellular matrix organization (Figure 7B). Overlapping the significantly downregulated genes in the colon and the aorta pointed out several smooth markers, such as *Acta2*, *Myh11*, *Myl9*, *Tpm1*, *Tagln*, and *Synn* (Figure 7C). As mentioned earlier, SRF and myocardin play important roles in maintaining smooth muscle contractile phenotype. The expression of SRF and *Myocd* was reduced in colon and aorta, respectively (Figure 7D). Interestingly, similar to Y/T KO the smooth muscle-specific SRF knockout mice also display

signs of colonic pseudo-obstruction^{12, 13}. In a good agreement with our results, Wang et al. also demonstrated a downregulation of various smooth muscle markers in the aorta of Y/T KO using the same mouse model¹¹⁸.

Master regulators that are likely to mediate the effect of YAP/TAZ deletion were predicted using Ingenuity Pathway Analysis (IPA) on RNA sequencing data from the aorta. This prediction was accompanied by giving a Z- score for each master regulator; which was calculated depending on the fold changes of the related/ target genes. The Z-score magnitude and charge reflects the degree of activation or inhibition. After sorting the master regulators based on their Z- score and fold change, *Pparg* and its coactivators *Ppargc1a* and *Ppargc1b* were among the most activated master regulators. PPAR γ is a nuclear receptor that is activated by naturally occurring fatty acids, and some antidiabetic medications. Upon ligand binding, PPAR γ heterodimerizes with the retinoid X receptor and controls adipogenesis, glucose metabolism, and inflammation¹¹⁹. An upregulation of PPAR γ protein level was observed in Y/T KO aortic lysate, particularly in the nuclear fraction, suggesting an increased activation of PPAR γ . Interestingly, it has been shown that PPAR γ antagonizes the contractile phenotype of vascular smooth muscle both *in vitro* and *in vivo*, and upregulation of PPAR γ was associated with decreased expression of *Myocd*^{120, 121}.

In vitro studies, demonstrated that YAP silencing caused an upregulation of smooth muscle markers and attenuated proliferation, shifting the smooth muscle towards a contractile phenotype and YAP overexpression resulted in a synthetic phenotype^{122, 123}. This discrepancy can be due to differences between the *in vitro* and *in vivo* environment, which are especially important for a mechanosensitive factor like YAP, which has been demonstrated to be influenced by cell geometry and ECM. Moreover, we and others have noticed a redundancy between YAP and TAZ, hence deleting one of them could be compensated from the other¹¹⁸. In summary, YAP and TAZ play essential roles in preserving the expression of contractile smooth muscle markers in adult smooth muscle *in vivo*.

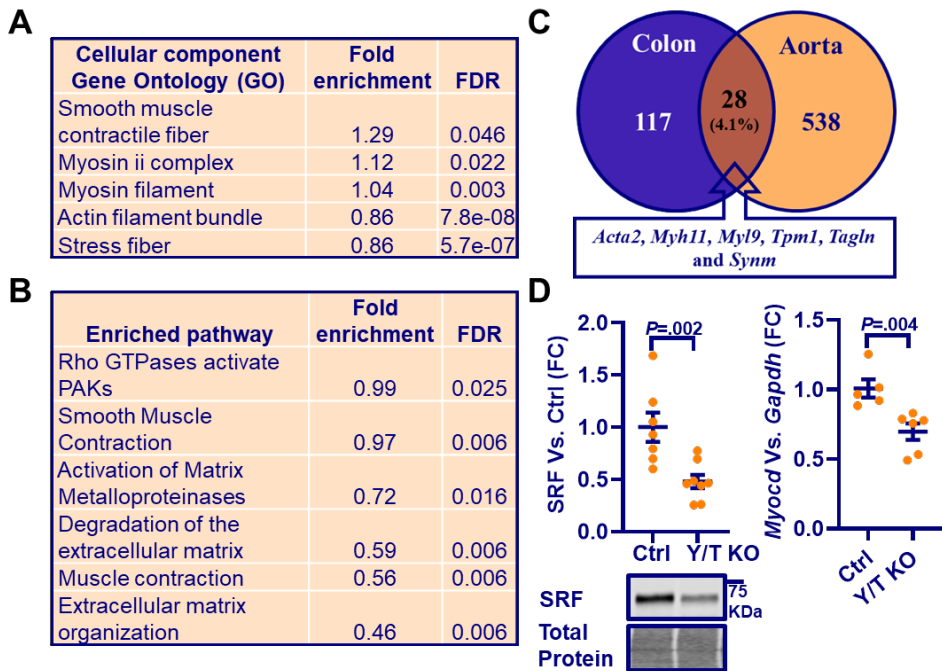


Figure 7. YAP/TAZ deletion results in a reduction of smooth muscle markers and regulators of smooth muscle differentiation. Gene ontology analysis of downregulated genes from colonic and aortic RNA sequencing data, cellular component (A), and pathways (B). Venn diagram of significantly downregulated genes from colon and aorta (C). Western blot analysis of SRF expression in colon and *Myocd* mRNA expression in the aorta (D). FDR indicates false discovery rate. Errors bars represent the standard error of the mean. Panel D is adapted from paper I and II.

Role of MRTFA in the bladder and human coronary artery smooth muscle cells

Reciprocal antagonism between unfolded protein response and smooth muscle marker gene program.

In response to the surrounding microenvironment, smooth muscle cells can modulate their gene expression to a less differentiated phenotype with higher capability of proliferation and migration. It has been noticed that the modulated smooth cells have prominent large endoplasmic reticulum (ER)^{124, 125}. The ER is involved in folding and assembly of secreted proteins. When the load on the ER exceeds its capacity, it activates the unfolded protein response (UPR) to restore its homeostasis. This response includes synthesis of chaperone proteins, translation suspension, and degradation of misfolded proteins. If these attempts fail, the cell usually induces apoptosis.

To examine the relationship between UPR and smooth muscle markers, we performed Spearman correlation analyses on three datasets of well-recognized examples of phenotypic modulation *in vivo*: 1) bladder denervation, which involves cryofreezing of the pelvic ganglion of female rats and subsequent growth of urinary bladder (GSE104540)¹²⁶, 2) bladder outlet obstruction, results from partial ligation of urethra that leads to growth of urinary bladder (GSE47080)¹²⁷, 3) carotid artery balloon injury, which includes expansion and endothelium denudation of common carotid artery followed by neointimal formation¹²⁸. The analyses revealed tight negative correlations between smooth muscle markers (*Myh11*, *Myocd*, *Kcnmb1*, *Synpo2*) and UPR genes (*Sdf2l1*, *Calr*, *Manf*, *Pdia4*), suggesting antagonism between the two programs (Figure 8A).

Tunicamycin (TN) is a naturally occurring antibiotic that inhibits N-glycosylation and interferes with protein folding, thus results in accumulation of misfolded proteins and ER stress. We treated human bladder smooth muscle cells with tunicamycin which resulted in an induction of UPR as expected. This was accompanied by suppression of smooth muscle markers. (Figure 8B). We also were able to reproduce the results using human coronary artery and dithiothreitol. Dithiothreitol is another commonly used ER stress inducer which blocks disulfide-bond formation (paper III). These results led us to speculate if this is a two-way relationship, rather than one-way. Therefore, we transduced the cells with viruses that overexpress myocardin and MRTFA (*MKLI*) with or without ER stressors and checked the mRNA expression of various UPR markers. For example, protein disulfide isomerase family A member 4 (*PDIA4*) is an ER resident protein that facilitates forming and rearrangement of disulfide bond, hence helps in protein folding and tertiary structure stabilization¹²⁹. Interestingly, the basal level of *PDIA4* was reduced in the presence of myocardin and MRTFA (Figure 8C). Moreover, the induction of *PDIA4* after treatment with TN was attenuated in the presence of myocardin and MRTFA (Figure 8D). Complementary to this, Huang et al. demonstrated an upregulation of UPR after deletion of myocardin in mouse aortic smooth muscle cells¹³⁰.

It seems that smooth muscle has high adaptive properties, and due to accumulation of misfolded proteins, it directs the transcriptional machinery towards the UPR at the expense of contractile machinery aiming to restore homeostasis. However, if the ER stress persists, the ER induces the proapoptotic pathways which lead to smooth muscle death. This has been observed in advanced atherosclerotic lesions, where apoptosis of smooth muscle in fibrous cap occurs and leads to detrimental effect on plaque stability^{131, 132}. Therefore, targeting ER stress as a therapeutic option should be investigated cautiously, considering the disease status.

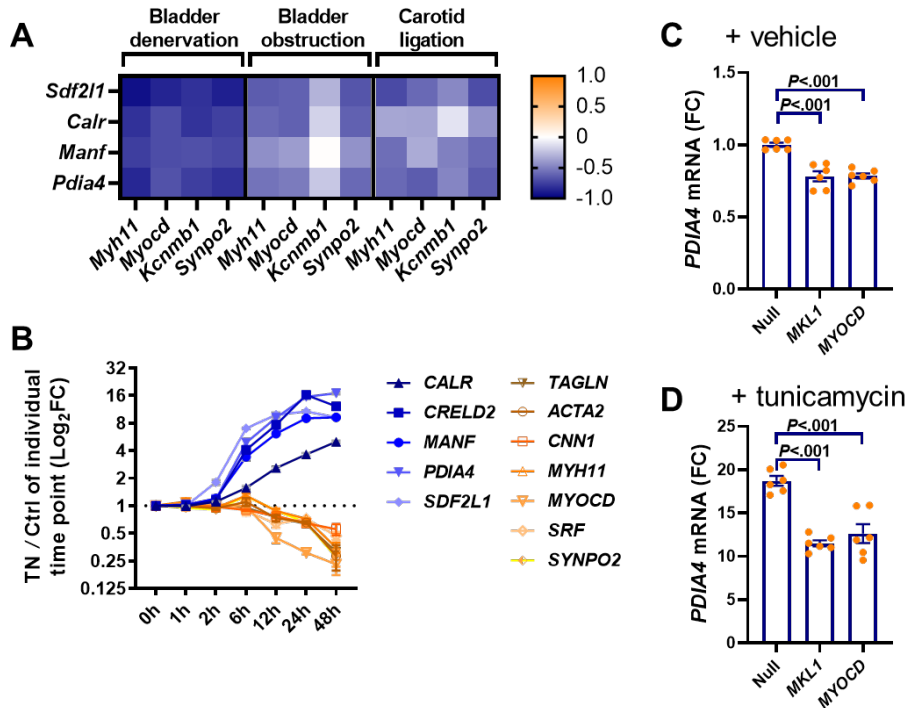


Figure 8. Reciprocal antagonism between unfolded protein response and smooth muscle marker gene program. Heat map of Spearman correlation coefficient (r) for selected genes from microarray datasets (A). Time course of tunicamycin treatment of bladder smooth muscle cells, unfolded protein response genes in blue and smooth muscle markers in yellow-brown (B). *PDIA4* mRNA expression after vehicle treatment and overexpression of *MKL1* and *MYOCD* in bladder smooth muscle cells (C). *PDIA4* mRNA expression after treatment of tunicamycin and overexpression of *MKL1* and *MYOCD* in bladder smooth muscle cells (D). TN indicates tunicamycin. Errors bars represent the standard error of the mean. This figure is adapted from paper III.

YAP/TAZ deletion leads to decreased contractility and impaired mechanotransduction

Marked reduction in spontaneous and agonist-induced contraction in visceral and vascular smooth muscle of Y/T KO mice

To test the impact of YAP/TAZ loss on smooth muscle contractility, colonic rings and segments of the tail artery were mounted in wire myography (more explanation in methods section). A marked decreased in spontaneous contraction, and contractile response to potassium chloride and carbachol, an acetylcholine agonist, were observed in Y/T KO colonic rings (Figure 9A). We also stimulated the tail artery with vasopressin, serotonin, cirazoline hydrochloride (α_1 -receptor agonist), and U46619 (thromboxane A₂ receptor agonist). This resulted in a weaker contraction in Y/T KO for all the agonists at one concentration or more, giving

vasopressin dose-response curve as an example here (Figure 9B). On the other hand, there was no difference between control and Y/T KO arteries in response to potassium chloride. This could be explained by the short lifespan of the knockout and less prominent arterial pathology eleven days post tamoxifen injection.

The impaired contractility of Y/T KO in the colon and arteries seems to be due to multiple different yet related mechanisms, which include (a) reduction of several smooth markers expression in both tissues, (b) reduced expression of *myocd* and SRF in the vasculature and colon, respectively, (c) low expression of relevant receptors like vasopressin 1a receptor (*Avpr1a*) and serotonin receptor 2A (*Htr2a*) in arteries and muscarinic receptors M2 and M3 in the colon, (d) reduced expression of ROCK1 in arteries.

Reduced stretch-induced smooth muscle-specific gene expression in Y/T KO portal veins

A popular method to study the effect of stretch on smooth cells is to culture them on flexible membranes that can be stretched to various degrees. However, this method is entirely different from the 3D environment of the cells *in vivo*. In the portal vein model, the smooth muscle cells are in their natural environment, including attachments to the surrounding cells and extracellular matrix. As described earlier in the method section, the portal vein is stretched by a gold weight resembling optimal load *in vivo*. To study the possible contribution of YAP/TAZ in this process, we subjected portal veins from control and Y/T KO to stretch and compared them to unstretched condition. The stretched portal veins from Y/T KO failed to induce smooth muscle markers to a similar level as the control, suggesting a crucial role of YAP/TAZ in stretch sensing and activation of smooth muscle marker transcription.

Decreased myogenic response in mesenteric arteries of Y/T KO mice

As suggested from the results of the portal vein model, YAP and TAZ play an essential role in mediating long term mechanotransduction in vascular smooth muscle. To determine if YAP/TAZ is also required for short term mechanotransduction, we evaluated the myogenic response where sensing pressure is fundamental. Small mesenteric arteries from control or Y/T KO were mounted in a pressure myograph (more explanation in methods section). As expected, the systematic increase in pressure above 45 mmHg resulted in a myogenic response in control arteries, whereas this response was four times weaker in Y/T KO. It has been shown that angiotensin II can enhance the myogenic response and we therefore tested the myogenic response after stimulation with a single dose of 100 nM angiotensin II^{133, 134}. The difference between control and Y/T KO was maintained in the presence of angiotensin II despite potentiation of both curves.

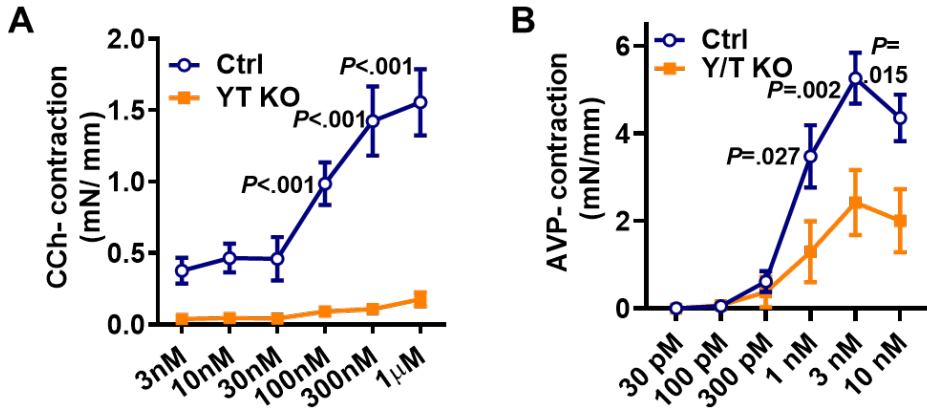


Figure 9. Reduction in smooth muscle contraction of Y/T KO mice. Carbachol induced contraction in colonic rings; the force was integrated over 2 minutes for each concentration and normalized to preparation's length (A, $n \geq 4$). Dose-response curve for vasopressin in caudal arteries, the force was integrated over 7 minutes for each concentration and normalized to preparation's length (B, $n \geq 7$). CCh indicates carbachol and AVP, vasopressin. Errors bars represent standard error of the mean. This figure is adapted from paper I and II.

As suggested from the RNA-sequencing analyses, multiple genes that are involved in focal adhesions, actin dynamics, and GPCR-dependent signaling were dysregulated in Y/T KO. This could explain mechanosensing impairment in Y/T KO arteries.

Altered expression of mechanosensitive transcription cofactors is associated with disease development (Papers II and IV)

Protective role of YAP/TAZ in established hypertension

The results presented above implies that the vascular smooth muscle cells utilize YAP/TAZ as translators of the surrounding mechanical stimuli to induce changes in gene expression. Hypertension, which results in increased tension in the vascular wall, is a major risk factor for cardiovascular diseases. We aimed to investigate the role of YAP/TAZ in established hypertension to provide new insights regarding the molecular mechanisms involved in the pathogenesis, and potentially discover new therapeutic targets. To establish hypertension, we implanted mini-osmotic pumps that secrete angiotensin II in the mice two weeks before the first tamoxifen injection. Both control and Y/T KO mice were euthanized nine or eleven days after first tamoxifen injection. Blood pressure measurements revealed that Y/T KO mice were able to maintain angiotensin II-induced hypertension despite the reduced myogenic

response and agonist-mediated contraction. This is likely due to: 1) smooth muscle-independent effects of angiotensin II, such as increased water retention and plasma volume, 2) initiation of angiotensin II infusion two weeks before knockout induction, and/or 3) maintained contractile response to angiotensin II stimulation in Y/T KO arteries. Similarly, Crowley et al. demonstrated that angiotensin II-induced hypertension and subsequent cardiac hypertrophy were mainly mediated by angiotensin II receptors on the kidneys rather than the vasculature¹³⁵.

During microdissection of mesenteric arteries, we observed multiple sites of vascular lesions in all hypertensive Y/T KO mice. However, only one of the hypertensive controls had a single lesion. No lesions were detected in normotensive control or normotensive Y/T KO mice, suggesting that the combined effect of losing YAP/TAZ and high blood pressure is required for the resultant phenotype. Histological examination of the lesions revealed signs of neointimal formation and adventitial hyperplasia. Using lineage tracing, we were able to conclude that cells in the neointima were derived originally from smooth muscle (Figure 10A). Although the adventitia was heavily infiltrated with cells which have high rate of proliferation as detected by Ki67 marker, lineage tracing analysis revealed that they were most likely not derived from smooth muscle cells. A large number of the adventitial cells were positive for Mac2, which is a macrophage marker associated with inflammation and fibrosis (Figure 10B). Moreover, we observed degradation of both the internal and external elastic laminae in the vascular lesions (Figure 10C). Several studies have shown that elastin degradation is involved in the pathogenesis of neointimal formation, and intact elastic laminae is a negative regulator of smooth muscle phenotypic switching and act as a barrier against cell migration¹³⁶⁻¹³⁸.

In conclusion, Y/T KO arteries have decreased contractile response to various agonists and pressure, increased compliance, impaired stretch-induced gene expression, and a decreased expression of mediators of smooth muscle contraction and contractile differentiation. These effects collectively result in increased wall stress with an inability to adapt to the increased wall stress by reducing vessel diameter. Prolonged excessive wall stress likely leads to vascular lesion development, characterized by smooth muscle migration to the intima, ECM reorganization, elastin degradation, and inflammation.

A similar phenotype was reported previously from our lab for mice deficient of the smooth muscle-enriched miRNA, miR 143/145. These miRNAs are important in maintaining contractile phenotype of smooth muscle, and mice deficient of miR 143/145 demonstrated loss of myogenic response and stretch-induced contractile differentiation¹³⁹⁻¹⁴¹. When miR 143/145 knockout mice were subjected to angiotensin II-induced hypertension, they developed vascular lesions in small mesenteric arteries. This is consistent with earlier studies in humans, which have shown that mutations in contractile machinery were associated with vascular wall abnormality including aneurysm formation^{142,143}. Collectively, a substantial body of

evidence points towards a crucial role of smooth muscle mechanotransduction for vascular wall integrity and adaptation to hypertension.

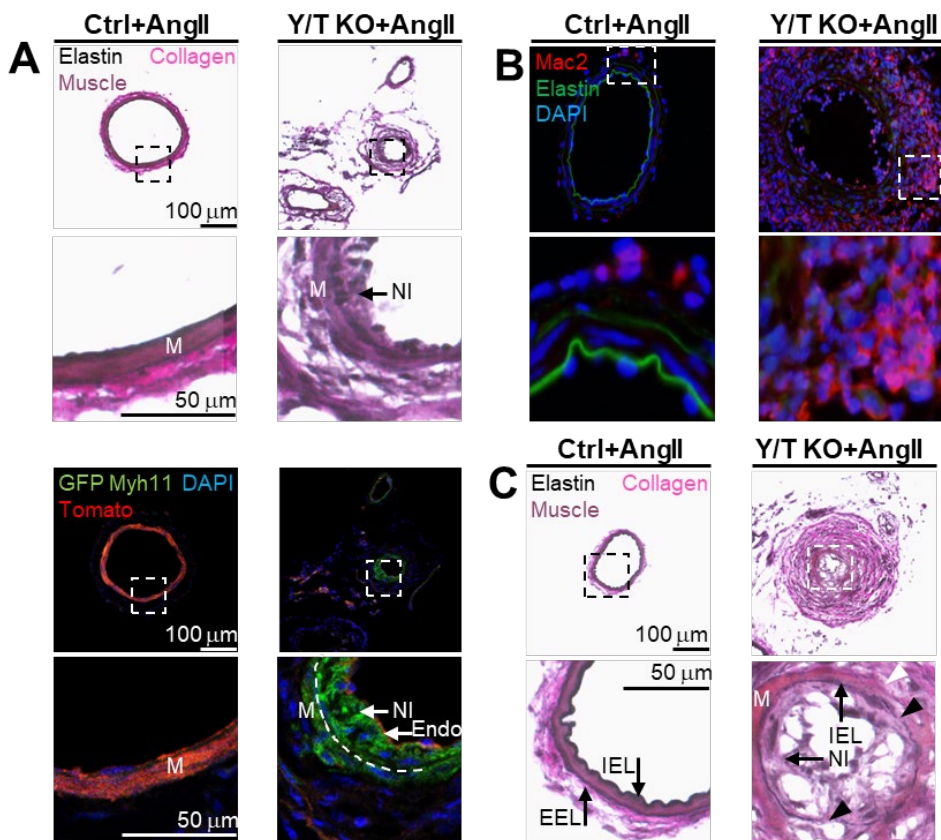


Figure 10. Vascular lesion development in mesenteric arteries of hypertensive Y/T KO mice. Cryosections of $YAP^{fl/fl}/TAZ^{fl/fl}$ Cre/ERT2 ROSA^{mt/mGAL} and corresponding control were stained with Verhoeff Van Gieson staining to visualize elastin (top two rows, A). The same sections were pictured by fluorescence microscope (bottom two rows, A), all cells appear red before Cre recombination and only smooth muscle cells change to green after tamoxifen injections. The dotted line in A marks the location of the elastic lamina. Mac2, macrophage marker; and DAPI nuclear staining of mesenteric arteries (B). Verhoeff Van Gieson staining of hypertensive control and Y/T KO, the black heads point to site of internal elastic lamina degradation, and the white head points to site of external elastic lamina degradation (C). M indicates media, NI, neointima; IEL, internal elastic lamina; EEL, external elastic lamina. This figure is adapted from paper II.

MRTFA promotes lipid accumulation in human vascular smooth muscle cells without induction of macrophage-like phenotype

Hyperlipidemia, hypertension, diabetes, smoking, and genetic predisposition are known risk factors for atherosclerosis. Several lines of evidence from clinical data and experimental models suggest that hypertension enhances the development and

progression of atherosclerosis^{144, 145}. A recent study from Minami et al. suggested involvement of MRTFA in atherosclerotic lesion development. They crossed ApoE^{-/-} mice with MRTFA knockout and fed the mice high cholesterol diet. These mice showed a remarkable reduction in size of aortic atherosclerotic lesions in comparison to ApoE^{-/-} mice with normal MRTFA expression¹⁴⁶. Several mechanisms linking hypertension and atherosclerosis have been proposed, such as mechanical injury, endothelium dysfunction, oxidative stress, and inflammatory mediators¹⁴⁷. However, we hypothesize that smooth muscle MRTFA could be involved in connecting hypertension and atherosclerosis. We test this hypothesis by examining the role of MRTFA in lipid accumulation and foam cell formation. This provides the foundation for future investigations in intact human arteries and animal models.

Human coronary artery smooth muscle cells were transduced with adenovirus that expresses MRTFA. Overexpression of MRTFA resulted in dramatic increase in intracellular lipid accumulation compared to control cells (Figure 11A). It is believed that the modified LDL particles play a crucial role in atherogenesis¹⁴⁸. Therefore, we treated the cells with fluorescently labeled oxidized LDL particles. MRTFA overexpression caused a 4-fold increase in oxidized LDL accumulation. This accumulation was more prominent in cells with strong GFP-positive nucleus i.e. highly transduced cells. To test if physiological levels of MRTFA can regulate lipid accumulation, we treated smooth muscle cells with isoxazole, which is known to activate MRTFA and enhance its nuclear localization. The effect of isoxazole on lipid accumulation was comparable to MRTFA overexpression. To test if MRTFA expression is relevant in a clinical setting, human arteries with evident neointimal hyperplasia were analyzed for MRTFA expression using immunofluorescence. Interestingly, MRTFA expression was significantly increased in the neointima compared to the adjacent media of the same artery suggesting that MRTFA may be involved in the pathogenesis of human vascular disease.

CCG1423 and CCG203971 are well-recognized MRTF inhibitors; several mechanisms were proposed for how these compounds work, such as disrupting actin filaments and interfering with MRTF translocation to the nucleus. Treating coronary artery smooth muscle cells that overexpress MRTFA with oxidized LDL and CCG compounds resulted in 60% reduction in lipid accumulation. Furthermore, CCG203971 was able to reduce lipid accumulation in cells treated with oxidized LDL without MRTFA overexpression, supporting the previous result that endogenous level of MRTFA contributes to the observed effect. Interestingly, Hinohara et al. discovered a single nucleotide polymorphism (SNP) in *MKLI* (MRTFA) promoter associated with a higher risk of coronary artery syndrome¹⁴⁹. This SNP led to an increased expression of MRTFA¹⁴⁹. Taken together, these results strongly suggest the involvement of MRTFA in atherosclerosis development.

It has been shown that mouse vascular smooth muscle cells transdifferentiate to macrophage-like phenotype upon cholesterol loading^{94, 150}. To determine whether

MRTFA-induced lipid accumulation is associated with phenotypic modulation towards macrophage-like cells, we examined the mRNA level of macrophage marker (*ABCA1*)¹⁵¹ and smooth muscle marker (*ACTA2*) (Figure 11B). Indeed, MRTFA *per se* induced the expression of smooth muscle markers, but it did not affect the expression of macrophage markers. Oxidized LDL with or without MRTFA overexpression caused upregulation of *ABCA1*, suggesting that the expression of macrophage markers in smooth muscle cells is an effect of the surrounding lipid environment and not MRTFA *per se*. Functionally, the phagocytic activity was increased by elevated lipid levels but not by overexpression of MRTFA, which again argues against MRTFA-induced transdifferentiation of smooth muscle cells to a macrophage-like phenotype.

Next, we sought to explore other molecular mechanisms that potentially explain MRTFA-induced lipid uptake and foam cell formation. The LDL receptor mediates uptake of native and minimally modified LDL, whereas extensively modified LDL is taken by scavenger receptors. Overexpression of MRTFA led to upregulation of the expression of LDL receptor, but not the scavenger receptors. We found two CARG-like boxes in the promoter of LDL receptor and the promoter reporter activity of the LDL receptor was increased after MRTFA overexpression. Moreover, downregulation of LDL receptor expression resulted in decreased MRTFA-induced lipid accumulation in smooth muscle. We also treated the cells with different inhibitors of fluid-phase pinocytosis, which represents an alternative route of lipid uptake. These inhibitors resulted in attenuation of lipid accumulation after MRTFA overexpression. Lastly, since lipid accumulation can also result from impaired efflux, we added fluorescently labeled cholesterol and measured the fluorescence intensity in the cells and medium. A reduction in cholesterol efflux after MRTFA overexpression was observed. In summary, lipid uptake after MRTFA overexpression can be due to, a decrease in cholesterol efflux, increased fluid-phase pinocytosis, and higher expression of LDL receptor.

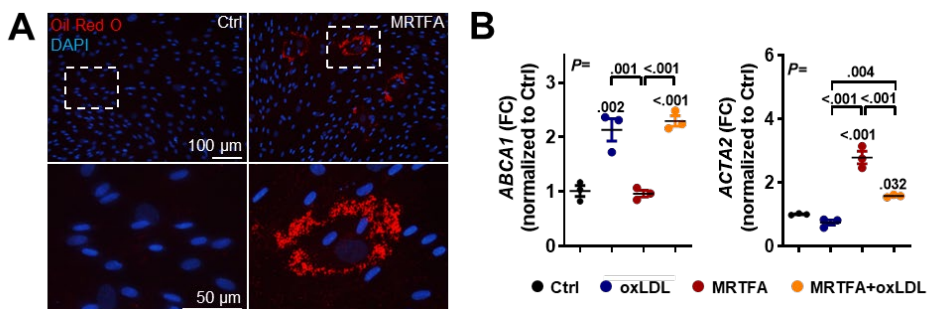


Figure 11. MRTFA promotes lipid accumulation in human vascular smooth muscle cells without transformation to a macrophage-like phenotype. Cells were incubated in 5% fetal bovine serum and stained with oil red to visualize lipid and DAPI as nuclear staining (A). mRNA expression of *ABCA1* and *ACTA2* normalized to the control group. Errors bars represent the standard error of the mean. This figure is adapted from paper IV

Correlation analyses suggest extensive crosstalk between YAP/TAZ and MRTFs in smooth muscle gene transcription

YAP/TAZ and MRTFs are both actin-sensitive transcriptional coactivators which translocate to the nucleus upon increased stretch^{30, 152}. Esnault et al. found that TEAD motifs were associated with MRTFs-SRF binding sites¹⁵³. Moreover, some studies also demonstrated that several genes are under regulation of both SRF and TEAD³⁷⁻⁴⁰. The previous studies were performed mainly in cells other than smooth muscle cells. To explore if similar crosstalk between YAP-TEAD and MRTFs-SRF is present in smooth muscle, we used Genotype-Tissue Expression portal (GTEx) to correlate the expression of YAP/TAZ-TEAD, MRTFs-SRF, selected smooth muscle markers. This resulted in striking, consistent, and positive Spearman correlation coefficients throughout the dataset from the transverse colon (Figure 12A). This correlation was largely maintained in the coronary artery except for *TEAD1*, which was only significant with *YAP1*, *WWTR1*, and *MYOCD* (Figure 12B). It has been shown that *Tead1* is crucial in cardiogenesis and vascular development and its deletion results in embryonic lethality due to hypoplastic cardiac and vascular wall¹⁵⁴. This likely indicates a higher importance of *TEAD1* in the embryogenic stage and the possibility of different regulations in the adult/human tissue. Similar to the data from the Y/T KO mice that demonstrated an upregulation of PPAR γ after YAP/TAZ deletion. Here, we can notice a negative correlation between PPAR γ on one side, and YAP/TAZ and smooth muscle markers on the other side, and this correlation seems to be more negative the colon (Figure 12 A and B).

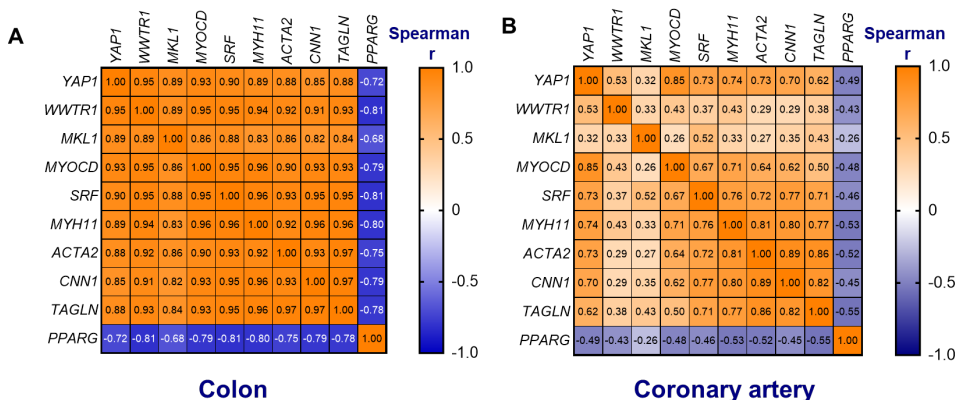


Figure 12. Heat map of Spearman correlation coefficient (r) between selected genes. Data were obtained from Genotype-Tissue Expression portal (GTEx) transverse colon ($n=404$) and coronary artery ($n=238$). Panel B is adapted from paper II.

Conclusion

Smooth muscle cells utilize YAP/TAZ and MRTFs to respond to the surrounding mechanical stimuli. These transcriptional cofactors are affected by actin dynamics and shuttle between the cytoplasm and the nucleus to drive or inhibit gene expression. A balanced activity/expression of YAP/TAZ and MRTFs appears to be essential to maintain smooth muscle homeostasis. Either downregulation or overexpression of these factors can result in dramatic influence in the gene transcription, and contribute to disease development. Studying the potential triggers and molecular mechanisms of YAP/TAZ and MRTFs regulation will increase our understanding of vascular disease development and potentially provide new therapeutic targets.

- Inducible smooth muscle-specific deletion of YAP/TAZ in adult mice results in a lethal phenotype within two weeks of induction, primarily due to intestinal pseudo-obstruction. This is accompanied by a marked reduction of contractility in colonic smooth muscle and a complete loss of peristalsis, which can be explained by a reduced expression of several contractile smooth muscle markers and muscarinic receptors.
- YAP and TAZ are critical for short- and long- term mechanotransduction and deletion of YAP/TAZ results in impaired agonist- and pressure-induced contraction. Small arteries that lack YAP/TAZ cannot withstand elevated pressure, hence develop vascular lesions.
- A mutual antagonism exists between the smooth muscle marker program driven by myocardin and MRTFA and the unfolded protein response.
- MRTFA causes lipid accumulation and transformation of human vascular smooth muscle cells into foam cells thorough multiple processes including, increased expression of LDL receptor, increased pinocytosis, and decreased lipid efflux.

Future perspective

The short life expectancy of Y/T KO mice due to the severe gastrointestinal phenotype prevents long-term investigation. However, our collaborator Professor Joseph Miano and coworkers have developed a Cre recombinase mouse model with preferential activity in vascular smooth muscle. They exchanged the promoter of Cre recombinase from *Myh11* to integrin 8 (*Itga8*). *Itga8* has higher expression in the vascular smooth muscle than the visceral. Thus, using the *Itga8*-Cre mouse will allow us to explore the role of YAP/TAZ in the vasculature without any secondary influence from the gastrointestinal phenotype. Mouse models of chronic vascular diseases like hypertension, atherosclerosis, and aneurysm commonly require more than two weeks, and this model is therefore of great value. Another advantage of using the *Itga8*-Cre mouse is the ability to examine both genders, which is not possible with *Myh11*-Cre since the Cre-gene is integrated into the Y-chromosome.

Deletion of YAP/TAZ *in vivo* provided invaluable information. However, the complexity of the system and simultaneous changes of multiple parameters challenge the interpretations of the underlying molecular mechanisms. In a more controlled environment like cell culture, it is possible to manipulate gene expression of YAP/TAZ by overexpressing or silencing, which will allow us to study the direct influence of YAP/TAZ. Moreover, we can easily generate time course experiments to study YAP/TAZ effect on proliferation, apoptosis, differentiation, and migration. Loss of function, gain of function, and actin polymerization studies can lead to discovery and validation of YAP/TAZ signature genes in smooth muscle. We also can examine the possible redundancy between YAP and TAZ and distinguish the unique and shared functions.

One of our results suggested an upregulation of PPAR γ and other transcription factors involved in adipogenesis in Y/T KO smooth muscle. However, we could not detect any signs of lipid accumulation in Y/T KO arteries two weeks after the knockout induction. It would be interesting to cross the *Itga8*-Cre YAP/TAZ double floxed mice with apolipoprotein E-deficient (ApoE^{-/-}) mice and evaluate lipid accumulation and atherosclerotic lesion development in these mice. Moreover, it is also possible to manipulate YAP/TAZ gene expression *in vitro* and examine the impact of YAP/TAZ on adipogenic differentiation.

We demonstrated the involvement of MRTFA in lipid accumulation and foam cell formation. It would be intriguing to draw a link between mechanical stimuli,

MRTFA and lipid accumulation. This can be done by culturing human coronary artery smooth muscle cells on flexible membranes, which can be stretched to various degrees. Then, we can either overexpress or downregulate MRTFA and treat the cells with different forms of lipids and perform immunocytochemistry, RT-PCR, and western blotting. Another approach is to use the portal vein model, where the portal vein is either stretched or not, followed by combinations of MRTFA and lipid treatments.

Lastly, depending on our correlation analyses using human data from GTEx portal, we showed a positive correlation between YAP/TAZ and smooth muscle markers and negative correlation between YAP/TAZ and PPAR γ both in colonic and coronary artery smooth muscle. This suggests similar regulatory mechanisms in humans, providing a translational impact for our findings. However, these correlations should be validated using human material either directly from patients who undergo cardiac surgeries or post mortem.

There are still some questions regarding the relation between the defective mechanotransduction and disease development that need to be investigated. More research directed towards the role of YAP/TAZ and MRTFs in smooth muscle would provide valuable answers to these questions.

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Dear Future, I am ready!

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Mechanosensitive transcriptional regulation of gene expression in smooth muscle



How does the cell perceive and convert the mechanical stimulus into a biochemical signal, and how does this signal propagate from the cell membrane to the nucleus? Despite extensive research, several pieces of the puzzle are still missing. Studying the molecular mechanisms that participate in translating the mechanical stimuli has the utmost significance in smooth muscle, as it lines the hollow organs and mediates vital physiological functions by sensing and responding to stretch. This thesis proposed that YAP/TAZ and MRTFs have their perfect place in the puzzle.

