Quaking, an RNA-Binding Protein, Is a Critical Regulator of Vascular Smooth Muscle Cell Phenotype


**Rationale:** RNA-binding proteins are critical post-transcriptional regulators of RNA and can influence pre-mRNA splicing, RNA localization, and stability. The RNA-binding protein Quaking (QKI) is essential for embryonic blood vessel development. However, the role of QKI in the adult vasculature, and in particular in vascular smooth muscle cells (VSMCs), is currently unknown.

**Objective:** We sought to determine the role of QKI in regulating adult VSMC function and plasticity.

**Methods and Results:** We identified that QKI is highly expressed by neointimal VSMCs of human coronary restenotic lesions, but not in healthy vessels. In a mouse model of vascular injury, we observed reduced neointima hyperplasia in Quaking viable mice, which have decreased QKI expression. Concordantly, abrogation of QKI attenuated fibroproliferative properties of VSMCs, while potently inducing contractile apparatus protein expression, rendering noncontractile VSMCs with the capacity to contract. We identified that QKI localizes to the spliceosome, where it interacts with the myocardin pre-mRNA and regulates the splicing of alternative exon 2a. This post-transcriptional event impacts the Myocd_v3/Myocd_v1 mRNA balance and can be modulated by mutating the quaking response element in exon 2a of myocardin. Furthermore, we identified that arterial damage triggers myocardin alternative splicing and is tightly coupled with changes in the expression levels of distinct QKI isoforms.

**Conclusions:** We propose that QKI is a central regulator of VSMC phenotypic plasticity and that intervention in QKI activity can ameliorate pathogenic, fibroproliferative responses to vascular injury. *(Circ Res. 2013;113:1065-1075.)*

**Key Words:** alternative splicing • differentiation • myocardin • Qk • restenosis • RNA-binding protein Quaking • vascular injury • vascular smooth muscle cells

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RNA-binding proteins are central regulators of gene expression in both health and disease.1-2 The RNA-binding protein Quaking (QKI) is a member of the highly conserved signal transduction and activator of RNA (STAR) family of RNA-binding proteins.3 Alternative splicing of the mammalian qki transcript yields 3 protein isoforms, notably QKI-5, QKI-6, and QKI-7,2 with dimerization of QKI isoforms being required for the regulation of pre-mRNA splicing, mRNA export, and stability.2-3 QKI drives central and peripheral nervous system myelination by regulating oligodendrocyte and Schwann cell differentiation, respectively.2-3 However, a role for QKI outside the neural network is poorly understood.

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QKI has been proposed to regulate processes outside the nervous system because of its ubiquitous expression profile\(^2\) and capacity to impact the processing of many RNA species by binding to quaking response elements (qre’s).\(^5,6\) Importantly, QKI null mice are embryonic-lethal because of an inability of immature mural cells to migrate and differentiate into vascular smooth muscle cells (VSMCs) effectively, resulting in perturbed investment and stabilization of nascent vessels in the yolk sac vasculature.\(^7,9\)

In adults, VSMCs of the artery wall contract and provide vascular tone.\(^10\) However, in response to vascular injury, VSMCs can dedifferentiate from a contractile to a synthetic state.\(^10,11\) The unabated expansion of VSMCs can lead to pathophysiologic complications, such as clinical restenosis after percutaneous coronary intervention,\(^12,13\) arteriovenous shunt failure,\(^14,15\) and transplant vasculopathy.\(^16\) Generally, VSMCs are programmed to avoid this excessive reparative response, because they possess the capacity to sense and respond to extracellular and intracellular cues that trigger the reversion to the contractile phenotype.

The transcriptional coactivator Myocardin (Myocd) is a primary driver of this redifferentiation process,\(^17\) because interaction with serum response factor (SRF) at CArG-box–containing promoters\(^18–20\) activates contractile apparatus protein expression that is required for physiological VSMC functioning.\(^21,22\)

Given that QKI is a regulator of embryonic vascular development, we sought to determine the role of QKI in the adult vasculature. Here, we show that the RNA-binding protein QKI is a critical regulator of VSMC phenotype by binding to and regulating Myocd expression and alternative splicing, implicating a critical role for QKI in the regulation of physiological VSMC function and vascular repair.

### Methods

#### Human Studies

Coronary atherectomy specimens were obtained from a cohort of patients that underwent elective directional coronary atherectomy of the target vessel 3 to 6 months after percutaneous transluminal coronary angioplasty\(^23\) and immunohistochemically stained for QKI using a mouse monoclonal antihuman pan-QKI antibody (clone N147/6; UC Davis/NIH NeuroMab Facility) and smooth muscle \(\alpha\)-actin (ACTA2; clone 1A4; Sigma-Aldrich), whereas a rabbit polyclonal antibody was used to detect \(\beta\)-actin (Abcam, Cambridge, United Kingdom).

Animal Studies

Female Quaking viable (Qk\(^v\)) mice on a C57/Bl6-J background together with age- and sex-matched C57Bl6-J wild-type (WT) mice (Jackson Laboratories, Bar Harbor, ME) were sheathed with a non-constrictive cuff as previously described.\(^24\) Immunohistochemical analysis and quantitation of femoral arteries was performed as described previously.\(^25\) Incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA as a marker of DNA synthesis was studied by intraperitoneal BrdU injection (100 mg/kg) 72, 48, and 24 hours before euthanization. QKI was detected using a rabbit polyclonal antihuman QKI antibody (N20; Santa Cruz Biotechnology, CA). Transluminal wire injury of the left common carotid artery was performed in female apoE\(^2/2\) fed a Western-type diet as previously described.\(^26\) Uninjured arteries (day 0) and wire-injured arteries were harvested at days 1, 3, 7, 14, 21, and 28. All animal work was approved by the regulatory authorities of the Leiden University and was in compliance with the Dutch government guidelines.

#### VSMC Isolation and Culture

Aortic explants harvested from WT and Qk\(^v\) mice were cultured in DMEM containing 10% FCS and 0.01 \(\mu\)g/mL glutamine at 37°C and 5% CO\(_2\). The human internal thoracic (HIT6 and HIT2A) clonal cell lines were generated and cultured as previously described.\(^21,27\) The Material and Methods section in the Online Data Supplement details cloning strategies and construction of vectors used for luciferase (pMIR-REPORT), splicing, and lentiviral overexpression of Myocd. Primary mouse and human VSMCs were stably transduced using lentiviral shRNA that specifically targeted qki or using a scrambled control shRNA (MISSION library; Sigma-Aldrich). Stable transductants were selected with 3 \(\mu\)g/mL puromycin for 72 hours. Clonal HIT2A subpopulations were scored microscopically and expanded for experiments.

#### Collagen Production Assay

Primary WT and Qk\(^v\) mouseVSMCs and HIT6 VSMCs were seeded at a density of 1.5x10\(^5\) cells/cm\(^2\), extensively washed with PBS and fixed for 30 minutes using Formalin (Thermo Scientific, Waltham, MA). Cellular collagen production was assessed by staining with Sirius Red F3B dye and concentrations determined spectrometrically at 550 nm.

#### DNA Synthesis and Cellular Migration Assays

DNA synthesis and cellular migration of VSMCs derived from WT and Qk\(^v\) aortas and HIT6 VSMCs were performed as previously described.\(^28\)

#### Western Blot Analysis

Proteins were resolved by polyacrylamide electrophoresis. Primary antibodies used to detect QKI-5, QKI-6, and QKI-7 were either rabbit polyclonal (EMD Millipore, Amsterdam, the Netherlands) or mouse monoclonal antibodies that specifically target QKI-5 (N195A/16), QKI-6 (N182/17), or QKI-7 (N183/15; UC Davis/NIH NeuroMab Facility). Mouse monoclonal antibodies were used to detect ACTA2 (clone 1A4; R&D Systems, Leiden, the Netherlands), calponin (CNN1; iCP; Sigma-Aldrich), caldesmon (CALD1; clone hHCD; Sigma-Aldrich), and smoothelin (SMTN; kindly provided by Dr G. van Eys, Maastricht University Medical Center). Hemagglutinin–tagged Myocd protein was detected using a monoclonal antimouse HA.11 antibody (clone 16B12). As a reference, a mouse monoclonal antibody was used to detect \(\alpha\)-tubulin (clone DM1A; Sigma-Aldrich), whereas a rabbit polyclonal antibody was used to detect \(\beta\)-actin (Abcam, Cambridge, United Kingdom).

### Functional Assessment of VSMC Contractility

To identify single contractile cells, stably transduced sh-cont and sh-qki HIT2A VSMCs or VSMCs overexpressing enhanced green fluorescent protein (EGFP), MYOCd-v1a, MYOCd-v1b, MYOCd-v3a, or MYOCd-v3b were seeded onto deformable silicone substrates (softness 5 kPa; Excellex Biotech, Lausanne, Switzerland) in M199 culture medium containing 1% FCS. Cells were seeded onto substrates coated with 10 \(\mu\)g/mL fibronectin isolated from human plasma. Twenty-four hours after seeding, 10 random fields were photographed using phase contrast microscopy.
Results

Quantitation of mRNA and Alternative Transcript Generation

For all experiments, N defines the number of biological replicates. All image quantification was performed using Image J software, and results of Western blot and PCR quantitation are provided beneath the images.

Statistics

For all experiments, N defines the number of biological replicates. All in vitro and in vivo results were analyzed using either Student t test or ANOVA (with Tukey, Bonferroni, or Kruskal–Wallis post-test being used when appropriate). Results are expressed as mean±SE or mean ±/−SE, as indicated. Differences in probability values <0.05 were considered significant, where degree of significance is indicated as follows: *P<0.05, **P<0.01, ***P<0.001, #P<0.0001. Quantitation of semi-quantitative PCR and Western blots are provided in Online Table II.

**Figure 1.** Quaking (QKI) is abundantly expressed in vascular smooth muscle cells (VSMCs) in human restenotic lesions. A and B, Low magnification images of pan-QKI expression in healthy coronary artery (n=4 subjects) and restenotic atherectomy specimens (n=5 subjects), with positive cells indicated by red immunostaining and nuclei staining deep blue. Scale bar, 100 μm. C and D, High-magnification image of box provided in (A) and (B). E, High-magnification image of Pan-QKI expression in restenotic lesion. Arrows denote QKI-positive VSMCs. F, Double immunostain of restenotic lesion with Pan-Qki (red) and smooth muscle α-actin (ACTA2; blue), with double-positive VSMCs (purple cells indicated with arrows). G, Isotype control of human cortex specimens were used as control tissue (IT). H, Pan-QKI expression in human cortex. Scale bars, 100 μm.

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(Olympus CX41 and CellSens Entry software) and the percentage of cells inducing wrinkles was determined using ImageJ software.

**RNA Immunoprecipitation**

RNA was isolated from HITa2 VSMCs as per manufacturer instructions using the Magna-RIP kit (EMD Millipore) using either goat antirabbit IgG antibody (control) or the IP-validated goat antirabbit QKI-5 antibody (EMD Millipore).

**Quantitation of mRNA and Alternative Transcript Generation**

Generally, mouse and human cDNA was made using random primers (Invitrogen). For immunoprecipitation and alternative transcript studies, cDNA was generated using random primers (Invitrogen). qRT-PCR analysis for designated mRNA products was performed using SYBR Green master mix (Bio-Rad) in combination with primer combinations described in Online Table I.

**Statistics**

For all experiments, N defines the number of biological replicates. All image quantification was performed using Image J software, and results of Western blot and PCR quantitation are provided beneath the images. All in vitro and in vivo results were analyzed using either Student t test or ANOVA (with Tukey, Bonferroni, or Kruskal–Wallis post-test being used when appropriate). Results are expressed as mean±SE or mean ±/−SE, as indicated. Differences in probability values <0.05 were considered significant, where degree of significance is indicated as follows: *P<0.05, **P<0.01, ***P<0.001, #P<0.0001. Quantitation of semi-quantitative PCR and Western blots are provided in Online Table II.

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followed by a significant increase after 3 days and peak QKI mRNA expression 7 days postinjury (Figure 2A; n=6 mice per time point; *P<0.05, ***P<0.001), coinciding with the proliferative peak of VSMCs in vascular injury models.30 Furthermore, we also assessed the temporal expression profile of QkI-5, QkI-6, and QkI-7 mRNA after cuff placement in apoE⁻/⁻ mice (n=4 mice per time point). These studies revealed that QkI-5 and QkI-6 mRNA are markedly increased at day 7 (QkI-6; P<0.05), whereas QkI-7 mRNA levels were consistently decreased in expression (Online Figure IA). Similar to the mRNA profile, medial layer VSMCs displayed maximally increased QKI protein expression 7 days after femoral cuff placement (Figure 2B and Online Figure IB). Interestingly, QKI mRNA levels are significantly downregulated 14 days after cuff placement (Figure 2A).

Next, we sought to determine if a reduction in QKI expression could impact neointima formation after femoral
cuff placement, using WT and Qkv mice. Qkv mice possess a megabase deletion in the proximal region of chromosome 17. Quantitative analyses of QKI mRNA levels from flash-frozen aortas (n=6; Online Figure IC) and protein expression levels in VSMCs derived from WT and Qkv aortas revealed a significant decrease in expression of all 3 QKI isoforms (Figure 2C, with quantitation from 3 explant cultures provided beneath each Western blot along with statistical significance). As shown in Figure 2D, immunohistochemical analysis of WT and Qkv neointimal lesions for elastin and ACTA2 revealed significant reductions in neointimal area (Figure 2E; n=6 mice; P<0.05), intimal/medial ratio (Figure 2F; WT 0.34±0.06% versus Qkv 0.20±0.04%; n=6 mice; P<0.05), luminal stenosis (Figure 2G; WT 35±5% versus Qkv 20±4%; n=6 mice; P<0.05), ACTA2-positive cells (Figure 2H; WT 62.5±4.7% versus Qkv 46.5±4.1%; n=6 mice; P<0.05), and neointimal BrdU incorporation (Figure 2I; n=8 mice; P<0.05) 2 weeks after cuff placement. Total vessel area (Figure 2J; n=6 mice; P=0.42) of cuffed portions of WT and Qkv femoral arteries was similar. We did not observe differences in lesional apoptosis (TUNEL staining), collagen, and CD45+ leukocyte content (data not shown). Collectively, these studies demonstrate that a reduction in QKI expression in vivo decreases neointima formation after vascular injury.

**Conclusion:**

Our findings indicate that QKI expression plays a crucial role in regulating VSMC proliferation, migration, and ECM production after vascular injury. The reduced QKI expression in Qkv mice results in increased neointima formation, which is associated with reduced cell proliferation, migration, and collagen production. These observations suggest that QKI may serve as a potential therapeutic target for the treatment of vascular diseases.

**Declaration of Interests:**

We declare no competing interests.

**References:**


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QKI Expression Levels Impact VSMC Phenotype

Our identification that QKI expression levels impact fibroproliferative properties of VSMCs suggests that QKI could play a role in regulating VSMC phenotype. Therefore, we investigated if the abrogation of QKI could drive synthetic VSMCs to the contractile state. For this, we assessed the mRNA expression profile of contractile apparatus proteins in serially passed, aorta-derived VSMCs from WT and Qk' mice. As shown in Figure 4A, significantly higher levels of Acta2, smooth muscle myosin heavy chain (Myh11), and Cnn1 mRNAs were expressed in VSMCs derived from Qk' as compared with WT mice (n=6 mice; *P<0.05, **P<0.01). In contrast, the mRNA levels of smoothelin A (SmtnA) and SmtnB were unaltered (Figure 4B; n=6 mice; SmtnA, P=0.42; SmtnB, P=0.45). Western blot analyses of cellular lysates harvested from WT and Qk' aortic VSMCs revealed that a decrease in QKI expression yields increased ACTA2 levels (Figure 4B; n=5 mice; P<0.05). Interestingly, we did not observe any changes in the mRNA levels of contractile apparatus proteins harvested from snap-frozen aortas of WT and Qk' mice (data not shown). Next, we verified that decreased QKI expression is coupled with a global increase in contractile apparatus protein expression using HITC6 VSMCs. Western blot analysis of lysates harvested from sh-qkI HITC6 VSMCs showed a robust stimulation of ACTA2, CNN1, and the heavy isoform of CALD1 protein expression as compared with sh-cont HITC6 VSMCs (Figure 4C; n=3 biological replicates; quantitation provided beneath respective Western blots), whereas the levels of SMTN were unchanged (Figure 4C; n=3 biological replicates).

Further proof that QKI expression levels play a role in regulating VSMC phenotype was derived from the fact that HITA2 VSMCs, a constitutively proliferative clonal cell population that poorly expresses contractile apparatus proteins, expressed significantly higher levels of all 3 QKI isoforms as compared with HITC6 VSMCs (Figure 4D). Interestingly, the forced reduction of QKI shifted HITA2 VSMCs from a rhomboid to an elongated state (Figure 4E) and was associated with increased expression of contractile apparatus mRNAs and protein (Figure 4E and 4F, respectively). We also observed an association between QKI expression levels and VSMC morphology, because clonally expanded cuboidal VSMCs expressed significantly higher levels of QKI than spindle-shaped VSMCs (Online Figure IIA).

Moreover, we also tested if QKI levels could impact contractile function by seeding sh-cont and sh-qkI HITA2 VSMCs on a silicone elastomere substrate, where cell contractile forces induce deformations that are visible as wrinkles under a phase contrast microscope. As shown in Figure 4G, sh-cont HITA2 VSMCs were incapable of contracting the elastomere substrate, whereas 77% of sh-qkI HITA2 VSMCs acquired the capacity to contract the elastomere substrate functionally (Figure 4G; n=3 biological replicates; ***P<0.001). Collectively, these findings strongly suggest that a reduction in QKI protein levels shifts VSMCs to the contractile phenotype.
QKI Modulates Myocd Expression and Alternative Splicing

VSMC maturation is largely dependent on the interaction of SRF with the transcriptional coactivator Myocd at CArG-boxes in the promoters of contractile apparatus proteins.18,19 Because attenuation of QKI was associated with increased contractile apparatus protein expression, we investigated whether QKI levels could impact Myocd expression. We found that Myocd mRNA expression was significantly increased in both QKι and sh-qki HITC6 VSMCs (Figure 5A) as compared with WT and sh-cont VSMCs (WT 1.0±0.35 versus QKι 3.0±0.25 fold; n=4 mice; P<0.01; sh-cont 1.0±0.39 versus sh-qki 2.6±0.24 fold; n=3 transductions; P<0.05). Next, we tested whether overexpression of QKI in human VSMCs could impact Myocd mRNA expression levels. For this, we stably transduced HITC2 VSMCs with QKI-5, QKI-6, or QKI-7. These studies revealed positive and negative feedback loops among the distinct QKI isoforms (Figure 5B; left), and that QKI-6 overexpression significantly attenuates the expression of Myocd mRNA (Figure 5B; right; n=3 transductions; P<0.05).

Although previous in silico analyses identified a qre in the 3′-UTR of the Myocd mRNA,6 our assessment of the Myocd pre-mRNA uncovered 3 qre’s that are conserved between mice and human, namely a coding sequence qre (qre-1), as well as 2 3′-UTR qre’s (qre-2 and qre-3; Figure 5C). This led us to investigate whether QKI could bind to the Myocd (pre)-mRNA transcript. Therefore, we performed RNA immunoprecipitation of QKI isoforms in HITC2 using anti-QKI-5 antibody. Indeed, QKI was found to bind to the Myocd (pre)-mRNA 5.1-fold more effectively than an anti-IgG control (Figure 5D; P<0.001). However, luciferase reporter experiments revealed no regulation of QKI at the qre’s in the Myocd 3′-UTR (Online Figure IIIA and IIIB).

Therefore, we focused our attention on qre-1 in the Myocd coding region. Importantly, this qre is located at a critical junction of the Myocd pre-mRNA, namely the 3′-splice site of an alternative exon termed exon 2a (Figure 5C). Moreover, QKI has recently been shown to regulate alternative splicing events globally in myotubes by binding proximally to exon-intron boundaries.36 Interestingly, the inclusion of exon 2a introduces a premature stop codon into the mature Myocd transcript (termed Myocd_v3). The subsequent use of a downstream ATG leads to the generation of an 856-amino acid MYOCD_v3 isofrom that is greatly enriched in VSMCs as compared with cardiac or skeletal muscle.30,31

Surprisingly, HITC2 VSMCs immunostained for QKI-5, QKI-6, and QKI-7 revealed that QKI-6 colocalized with HITA2 and HITA2 sh-cont and sh-qki HITC6 VSMCs (n=3 separate transductions). D, Western blot analysis of QKI expression in HITC6 and HITA2 VSMCs (n=3 separate cultures). E, Phase-contrast images of sh-cont– and sh-qki–transduced HITA2 VSMCs (scale bar, 50 μm), with corresponding relative mRNA transcript abundance detailing contractile apparatus expression (n=3 biological replicates). F, Western blot analysis of protein expression levels in HITA2 sh-cont and HITA2 sh-qki VSMCs. Blots are representative of 4 separate transductions. G, Phase-contrast images of sh-cont and sh-qki–transduced HITA2 VSMCs seeded on fibronectin-coated, deformable elastomere substrate (5 kPa; left). Quantitation of contraction competent sh-cont– and sh-qki–transduced HITA2 VSMCs (right; scale bar, 100 μm; n=3). Data are means±SE. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Quaking (QKI) expression levels impact vascular smooth muscle cell (VSMC) phenotype. A, mRNA transcript abundance for contractile apparatus proteins in wild-type (WT) and Quaking viable (Qkι) VSMCs relative to GAPDH mRNA abundance (n=6 mice). B, Western blot analysis of smooth muscle α-actin in cellular lysates harvested from 5 separate WT and Qkι aortic explant cultures normalized to α-tubulin (TUBA1A) (n=5). Quantitation of protein expression was normalized to β-actin and is shown beneath each blot along with statistical significance. C, Western blot analysis of contractile protein expression in sh-cont and sh-qki–transduced HITC6 VSMCs (n=3 separate transductions).
Spliceosomal proteins in the cellular nucleus (Figure 5F).

Next, we tested if the abrogation of QKI protein could influence Myocd_v3 expression levels. For this, we assayed the mRNA levels of Myocd_v3 in explanted aortic VSMCs derived from WT and Quaking (Qkv) mice (Figure 5G and Online Figure IIIB). WT aortic VSMCs exclusively expressed Myocd_v1 after serial passaging (Figure 5G; quantitation shown below PCR), whereas Qkv VSMCs retained 30.9% exon 2a inclusion (Myocd_v3; n=4 biological replicates; P<0.05). This association between a reduction in QKI expression and enriched exon 2a inclusion was validated in sh-qki HITA2 VSMCs (6.8-fold versus sh-cont; n=3 transductions; P<0.05) and spindle HITA2 VSMC (3.8-fold versus cuboidal; n=3; P<0.01; Online Figure IIC and IID). To confirm that QKI expression levels impact the Myocd_v3/Myocd_v1 mRNA balance, we harvested RNA from HITC6 VSMCs cultured in the presence of serum and 3 days after serum withdrawal, which drives these cells toward a contractile phenotype. As a control, we also tested if QKI impacts the Myocd_v4/Myocd_v2 balance; however, we did not observe alternative splicing of Myocd exon 10a, which in the absence of qre proximal to this alternative exon is unlikely (Figure 5H; bottom). As shown in Figure 5H and Online Figure IIIC, proliferative sh-cont HITC6 VSMCs primarily express Myocd_v1 (lane 1; 27% Myocd_v3). In contrast, Myocd_v3 is the primary mRNA transcript expressed in sh-qki HITC6 VSMCs (lane 2; 51% Myocd_v3). Interestingly, serum withdrawal–induced ablation of QKI enhanced exon 2a inclusion in sh-cont VSMCs (lane 3; 40% Myocd_v3), while...
trigerring the virtually exclusive inclusion of exon 2a in sh-
qkl VSMCs (lane 4; 78% Myocd_v3). Given that Myocd_v1
is the primary splice variant expressed in the heart, we sought
to determine if QKI-5, QKI-6, and QKI-7 expression levels
were increased in the heart as compared with the aorta. As
shown in Online Figure IV A, QKI mRNA expression levels
are significantly higher in the heart than in the aorta, provid-
ing the interesting possibility that QKI protein levels are also
involved in regulating Myocd_v3/Myocd_v1 balance in the
healthy and diseased heart (n=6 mice).

Finally, to test the role of QKI directly in regulating exon
2a alternative splicing, we generated a splicing reporter con-
struct by replacing exon 2 of the encoded β-hemoglobin (hbb)
gene by the genomic sequence encoding myocd exon 2a along
with flanking intronic regions (2a-wt-qre). Next, we mutated
the qre from ACTAA→ACTGA (2a-mut-qre; Figure 5I). This
mutation was chosen because it maintains the presence of a
stop codon in exon 2a, while concomitantly abolishing the ca-
pacity of QKI to bind there.6 Mutation of the qre in exon 2a
led to a striking enhancement of exon 2a inclusion (Figure 5I
and 5J). Our findings strongly suggest that QKI regulates the
expression and alternative splicing of Myocd.

Myocd_v3 Expression Levels Impact VSMC
Contraction Function
To gain insight into the consequences of Myocd pre-mRNA al-
ternative splicing events, we transduced HITa2 VSMCs with
lentivirus encoding EGFP, 2 variants of MYOCD_v1 (v1a
lacks exon 2a, whereas v1b lacks ATG2 and ATG3 and, there-
fore, exclusively produces MYOCD_v1), or 2 MYOCD_v3
variants (v3a includes exon 2a, whereas in v3b the first 2 exons
are deleted from the encoded cDNA leading exclusively to the
production of MYOCD_v3; Figure 6A and see Material and
Methods section in the Online Data Supplement). As a vector
expression control, total Myocd mRNA levels were signifi-
cantly increased in all cases as compared with EGFP (prim-
er in common sequence; Online Figure VA; n=3; P<0.001),
whereas exon 2 to 5 expression levels in EGFP- and v3b-
overexpressing VSMCs were comparable because of the fact
that v3b lacks the 5’-primer site (Figure 6A and Online Figure
VB). Gel electrophoresis of these mRNA species confirmed
that EGFP-, v1a-, and v1b-transduced VSMCs exclusively
express Myocd_v1 (Figure 6B; lanes 1–3), whereas v3a-over-
expression exclusively yields Myocd_v3 (Figure 6B; lane 4).
Interestingly, transduction of VSMCs with v3b was found to
induce expression of endogenous Myocd_v3 (Figure 6B; lane 5,
upper band).

Subsequent Western blot analysis for HA-tagged MYOCD
revealed that overexpression of v3a and v3b in VSMCs in-
creased MYOCD protein expression as compared with v1a-
and v1b-overexpressing VSMCs (Figure 6C). In keeping with
literature, both MYOCD_v1 and MYOCD_v3 overexpression
potently induced ACTA2 protein expression25 (Figure 6C).

To determine if augmentation of MYOCD_v3 could in-
deed impact VSMC contractility, we seeded EGFP-, v1a-
and v1b-, v3a-, and v3b-transduced HITa2 VSMCs on the afor-
mentioned silicone elastomere substrate. Similar to sh-con-
transduced HITa2 VSMCs (Figure 4G), EGFP-transduced
VSMCs did not contract the substrate and remained pro-
liferative (Figure 6D and 6E; n=3 biological replicates).

Figure 6. Expression levels of Myocardin (Myocd) isoforms impact
vascular smooth muscle cell (VSMC) contraction competency. A, Schematic
representation of overexpressed Myocd isoforms, with primary translational start
site (ATG1), alternative translational start sites (ATG2+ATG3), and premature stop
site within exon 2a. B and C, mRNA and Western blot analysis (HA tag) for
Myocd expression after transduction with constructs described in A (n=3
separate transductions). Densitometry of Western blots is depicted underneath
(normalized to β-actin; pooled protein lysates from 3 separate transductions).
Expression levels assessed by qRT-
PCR are shown in Online Figure V.
D, Phase-contrast images of VSMCs
transduced with myocd constructs
described in (A), seeded onto deformable
silicone substrate. E, Quantitation of
contraction competence and cellular
proliferation in transduced VSMCs
(n=3; P<0.0001; ANOVA with Bonferroni
multiple comparison test; n.s. denotes
nonsignificant). F, Relative mRNA
expression levels of CArG (Cald1, Cnn1,
Myl11, Acta2) and MEF (Myl1, Srsf3,
Bop1)-induced genes (n=3 biological
replicates; und denotes undetectable).
Data are mean±SE. *P<0.05, **P<0.01,
***P<0.001.
In contrast, MYOCD_v1a and v1b overexpression induced a low level of contraction and modest proliferation, whereas MYOCD_v3a- and v3b-overexpressing VSMCs displayed both significantly greater cellular contractility and decreased cellular proliferation (Figure 6D and 6E). In keeping with literature, we observed modest, yet significant increases in the mRNA levels of contractile apparatus protein as a result of MYOCD_v3 overexpression as compared with MYOCD_v1 overexpression (Figure 6F; n=3 biological replicates). Collectively, these studies suggest that the expression levels of the distinct MYOCD isoforms can indeed influence VSMC contractile function.

Vascular Injury Triggers Myocd Alternative Splicing

It is well established that arterial injury induces VSMC dedifferentiation. To test if this response to arterial injury is associated with a change in the expression levels of Myocd splice variants, apoE−/− mice on a Western-type diet underwent wire injury of the left carotid artery. At days 0 (uninjured), 1, 3, 7, 14, 21, and 28, the injured carotid arteries were harvested and mRNA transcript levels of QkI-5, QkI-6, QkI-7, total Myocd, and Myocd_v3/Myocd_v1 mRNA balance were determined (n=4 mice per time point). In keeping with the QkI mRNA expression profile observed after femoral cuff placement in WT and apoE−/− mice (Figure 2A and Online Figure IA), wire injury of the carotid artery resulted in an initial decrease in QkI mRNA expression levels, followed by an increase in qkI mRNA expression levels, with, in particular, a 1.85-fold increase in QkI-6 mRNA levels after 7 days (Figure 7A; P<0.05). Concordantly, Myocd expression was 2.7-fold reduced 7 days after arterial injury and was coupled with a striking shift in expression of Myocd_v3 to Myocd_v1 expression (n=4 mice per time point; 2 mice per time point shown on gel; P<0.05; Figure 7B). This coexpression of both Myocd_v3 and Myocd_v1 persisted until 21 days post–wire injury, whereas by 28 days the expression of Myocd in carotid arteries had once again reverted to the almost exclusive expression of Myocd_v3 (Figure 7B). These findings strongly suggest that QKI-induced alternative splicing of the Myocd pre-mRNA is functionally relevant and generates MYOCID isoforms that are required for both physiological and pathophysiologic situations in the artery wall.

Discussion

The current study demonstrates that QKI expression levels play a central role in the determination of VSMC phenotype. We have identified that QKI is poorly expressed in VSMCs of healthy coronary artery specimens, suggesting that the RNA-binding properties of QKI are repressed in contractile VSMCs. In contrast, our in vivo studies indicate that QKI expression is strongly induced in VSMCs in response to vascular injury, indicating a potential role for QKI in guiding VSMC dedifferentiation, enabling the VSMC to aid in the repair of the damaged portion of the artery wall. Importantly, the unabated proliferation of synthetic VSMCs can lead to rapid luminal narrowing. To this end, the observed decrease in neointima formation in Qk−/− mice after femoral cuff placement could be the direct result of a decreased capacity to activate gene expression profiles that VSMCs require to proliferate, migrate, and produce ECM production.

At present, little is known regarding a role for RNA-binding proteins in regulating VSMC biology in either healthy or diseased situations. HuR, a RNA-binding protein that targets AU-rich regions in 3′-UTRs, has been found to be highly expressed in VSMCs of neointimal lesions where it stabilizes mRNAs encoding cell cycle proteins. In contrast to HuR, recent studies have identified that the majority of qre’s in (pre-)mRNAs are localized to intronic regions, whereas recent studies by Hall et al identified that the depletion of QKI in C2C12 myoblasts critically regulates alternative splicing events in these cells. In keeping with this
notion, we identified that QKI is a critical post-transcriptional regulator of the Myocd pre-mRNA (Figure 7C). This event profoundly impacts MYOCD isoform production, as exon 2a exclusion (Myocd_v1) leads to the generation of a 935-amino acid MYOCD_v1 isoform, whereas exon 2a inclusion (Myocd_v3) leads to the production of a 856-amino acid MYOCD_v3 isoform. Although both isoforms serve as cotranscriptional activators of SRF, the N-terminal portion of MYOCD_v1 confers the protein with the capacity to interact with the myocyte enhancing factor 2 (MEF2) family of transcription factors and encodes a RPEL domain responsible for the interaction with G-actin.

Evidence that the QKI-mediated alternative splicing of the Myocd pre-mRNA is physiologically relevant can be derived from the fact that QKI is virtually absent in VSMCs of the healthy artery wall, where Myocd is highly expressed and almost exclusively present as Myocd_v3 splice variant (Figure 7C). It is well established that injury to the artery wall triggers VSMC dedifferentiation. Importantly, Firulli et al.20 have previously identified that this event is tightly coupled with the dynamic modulation of QKI expression, and that the differential expression of the distinct QKI isoforms induces the enrichment of the Myocd_v1 splice variant in proliferative VSMCs. Here, we hypothesize that alterations in the Myocd splice variant balance are part of the VSMC-mediated response to vascular injury, possibly enhancing the expression of MEF2 target genes (Figure 7C). As such, a reduction in Myocd expression coupled with a decreased interaction with SRF could be required to activate gene expression profiles that drive arterial repair. Importantly, our data also suggest that VSMCs preferentially express MYOCD_v3 protein, because VSMCs expressing equivalent levels of the Myocd_v1 and Myocd_v3 splice variants expressed markedly more MYOCD_v3 protein, indicating that QKI-mediated alternative splicing of Myocd exon 2a plays a role in regulating MYOCD protein abundance.

SMCs also serve critical roles in the physiological functioning of the intestines, testis, uterus, and urinary bladder. Interestingly, QKI is abundantly expressed in these tissues, providing the interesting possibility that, similar to the artery wall, alterations in QKI expression levels could play a regulatory role in pathological complications in these tissues, such as uterine leiomyoma formation, epididymal hyperplasia, and bladder dysfunction.

In conclusion, our findings imply that QKI is not exclusively associated with neural development and disease, but that QKI is in fact a critical regulator of the functional plasticity that is required for VSMC function under both physiological and pathophysiological conditions.

Sources of Funding
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Disclosures
None.

References
Vascular injury induces the expression of the RNA-binding protein HuR. This process requires myocardin to trigger a gene expression profile that restores physiological VSMC functioning.

**Novelty and Significance**

**What Is Known?**

- In response to vascular injury, vascular smooth muscle cells (VSMCs) of the artery wall aid in the repair of the damaged artery by adopting a proliferative phenotype.
- To avoid an excessive reparative response, VSMCs must revert to the contractile phenotype.
- This process requires myocardin to trigger a gene expression profile that restores physiological VSMC functioning.

**What New Information Does This Article Contribute?**

- Vascular injury induces the expression of the RNA-binding protein Quaking (QKI) in VSMCs.
- QKI regulates VSMC phenotype by modulating the expression and alternative splicing of the myocardin pre-mRNA.
- Targeted reduction of QKI drives VSMCs to the contractile phenotype in vitro and reduces injury-induced neointima formation in vivo.

Despite the fact that RNA-binding proteins mediate alternative splicing events, their role in the vasculature, and in particular the VSMC-mediated response to vascular injury, is poorly understood. Here, we report that the RNA-binding protein QKI influences VSMC phenotype by post-transcriptionally regulating myocardin activity. By regulating a key splicing event in the myocardin pre-mRNA, we propose that QKI expression levels determine whether VSMCs will participate in vessel repair after injury or maintain the vascular tone.
Quaking, an RNA-Binding Protein, Is a Critical Regulator of Vascular Smooth Muscle Cell Phenotype


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http://circres.ahajournals.org/subscriptions/
## Online Table I: Primer sets used for PCR experiments

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Online Table II: Statistical analysis of PCR and Western blot data.

Figure 2: QKI expression levels influence neointima formation after vascular injury.

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<td>0.41 ± 0.05</td>
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Figure 4: QKI expression levels impact VSMC phenotype.

| Figure 4B | ACTA2 | 2.38 ± 0.31 | n=5, p<0.05 |

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Figure 5: QKI regulates the expression and alternative splicing of the myocardin pre-mRNA.

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Online Table II: QKI gene dosage determines VSMC morphology and phenotype.

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Online Material and Methods

Luciferase vector construction

An approximately 1100 base pair portion of the myocd 3′-UTR was cloned from HITC6 cDNA into the luciferase reporter vector pMIR-REPORT (termed pMIR-qre2+3), as well as cDNAs encoding 100 base pair portions of the myocd 3′-UTR for qre-2 and qre-3 (pMIR-qre-2 and pMIR-qre-3). For this, primers were designed containing SpeI and HindIII linker sequences; forward: 5′-CTCAACTAGTCTCTACCACAAAACGTTGCCT-3′, reverse: 5′-CTCAAGCTTACGATAAGCAAGTAGCTGAG-3′, respectively. Sh-cont or sh-qkl transduced HEK293T cells were transfected with either pMIR-qre2+3, pMIR-qre-2 or pMIR-qre-3. pLV-Renilla was co-transfected as a normalization control and luciferase/renilla expression determined.

Minigene construction and mutagenesis

The minigene splicing reporter was constructed by subcloning myocd exon2a and partially flanking introns, amplified from human genomic DNA, between the Apal and BglII sites of pDUP51 splicing reporter (kindly provided by Dr. M. Ares Jr., UCSC) as previously described. Due to the presence of an Apal restriction site within the proposed myocd gDNA insert, the vector was first cut with Apal according to the manufacturer’s instructions and blunted by T4 DNA polymerase (New England Biolabs). Subsequently, BglII digestion was performed and the insert amplified using the forward 5′-TATTAAGATATCCGTCTCCCTGCTACT-3′ (EcoRV restriction site underlined) and reverse 5′-TATTAAGATACAGCCGACAGTGTTGG-3′ (BamHI restriction site underlined) primers and ligated into pDUP51.

A site-directed mutation in exon 2a was made using the QuikChange II site directed mutagenesis kit (Stratagene) following manufacturer’s instructions. For this, the forward A-to-G myocd_2a 5′-CTTTATTTTTTTTGCAAGCTAGTACAGTACAGCACG-3′ and reverse A-to-G myocd_2a 5′-GTGGCTGCTGCTAGCTGAGTACAGTC-TGCCAAAATAAAAG-3′ primers were used. The pDUP-2a-wt-qre and pDUP-2a-mut-qre inserts were Sanger sequenced starting at the CMV promoter to validate the exon 2a mutation. The reporter was transiently expressed in HEK293T cells, and the mRNAs were assayed for exon 2a inclusion by PCR.

Production of myocardin-generating lentiviral vectors

The effect of forced human myocardin (myocd) gene expression on the properties of human VSMCs was studied using self-inactivating (SIN) lentiviral vectors (LVs). To this end, four different SIN-LVs were generated, namely LV.hCMV-IE.hMYOCD41+907-HA.WHPRE (encoding Myocd_v3a), LV.hCMV-IE.hMYOCD907-HA.WHPRE (encoding Myocd_v3b), LV.hCMV-IE.hMYOCD986-HA.WHPRE (encoding Myocd_v1a) and LV.hCMV-IE.hMYOCD986.M80V.M97V-HA.WHPRE (encoding Myocd_v1b). All SIN-LVs include the myocd exon 10a and are extended at their C-terminus within an epitope derived from the human influenza A virus hemagglutinin (HA) protein. Myocd_v3a and Myocd_v3b both direct the synthesis of the long smooth muscle-enriched isoform of myocardin. Myocd_v1a additionally codes for a 41-amino acid protein with a MEF2 binding domain, while Myocd_v1b codes for the long cardiac muscle-enriched isoform of myocardin but can also generate the long smooth
muscle-enriched isoform of myocardin through internal initiation. In Myocd_v1b, the ATG codons corresponding to amino acid positions 80 and 97 have been mutated to GTG to prevent the synthesis of smooth muscle-like myocardin isoforms. Transgene expression in these SIN-LVs and the control vector (see below) is driven by the human cytomegalovirus *immediate-early* gene promoter and boosted by the presence of the woodchuck hepatitis virus posttranscriptional regulatory element immediately downstream of the myocardin-coding sequence. For the nucleotide sequence of the shuttle plasmids used to generate *myocd_v3a*, *myocd_v3b*, *myocd_v1a* (GenBank accession number EF186078) and *myocd_v1b* (GenBank accession numbers pending for italicized constructs). As a negative control for these experiments, we used a SIN-LV directing the synthesis of the *Aequorea victoria* enhanced green fluorescent protein (EGFP). This control vector was generated with the aid of the previously described shuttle plasmid pLV-CMV-IRES-EGFP. All plasmids used for SIN-LV production were purified with the aid of the JETSTAR 2.0. Plasmid Maxiprep Kit (Genomed, Löhne, Germany).

Vesicular stomatitis virus G-protein-pseudotyped SIN human immunodeficiency virus type 1 vectors were produced in 175-cm² culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) seeded with 10⁵ 293T cells per cm² in DMEM containing 10% FBS. The next day, the producer cells in each flask were transfected with a total of 35 μg of DNA at a 2:1:1 molar ratio of 1) one of the SIN-LV shuttle plasmids, 2) psPAX2 (Addgene, Cambridge, MA) and 3) pLP/VSVG (Invitrogen) using 3 μg of polyethyleneimine (Polysciences Europe, Eppelheim, Germany) per μg of DNA as transfection agent. After 16h, the transfection medium in each flask was replaced by 15 mL of DMEM containing 5% FBS and 25 mmol/L HEPES-NaOH (pH 7.4). At 40-48 h after the start of the transfection, the culture fluid was collected and freed of cellular debris by centrifugation at RT for 10 min at 3,500× g and filtration of the resulting supernatant through a 0.45-μm pore size polyethersulfone filter with a diameter of 33 mm (Millex-HP; Merck Millipore, Billerica, MA). To concentrate the SIN-LV particles, 5 mL of 20% (w/v) sucrose in PBS was carefully layered under 30 mL of the cleared culture medium, which was then centrifuged for 2h at 15,000 rpm and 4°C in an SW28 rotor (Beckman Coulter, Fullerton, CA). Next, the supernatant in each centrifuge tube was discarded and the pellet containing the SIN-LV particles was suspended in 500 μL of PBS-1% BSA by gentle rocking overnight at 4°C. The resulting SIN-LV suspension was stored in 50-100 μL aliquots at -80°C.

The gene transfer activity of the SIN-LV stocks was determined by immunostaining of formaldehyde-fixed and Triton X-100-permeabilized adult human mesenchymal stem cells (hMSCs) that had been exposed to different vector doses with mouse monoclonal antibody HA.11 (Covance, Rotterdam, the Netherlands). This monoclonal antibody is directed against the HA tag present at the C-terminus of the different myocardin proteins. As secondary antibodies, we used Alexa Fluor 568-conjugated goat anti-mouse IgG(H+L) antibodies (Life Technologies Europe, Bleiswijk, the Netherlands).
REFERENCES


Online Figure I: Expression levels of qkI transcripts are affected by injury to the vessel wall. (A) Temporal mRNA expression profile of QkI-5, QkI-6, QkI-7 in response to femoral artery placement of a perivascular cuff in 3 apoE^-/- mice per timepoint. Values shown are relative to uninjured control and are normalized to Gapdh (n=3). (B) High magnification images of femoral arteries displayed in Figure 2 illustrating the spatiotemporal expression profile of QKI protein after perivascular cuff placement in WT mice (n=6 mice; scale bars, 10 µm; IT, isotype control). C) Relative change in QkI-5, -6 and -7 mRNA expression levels in flash-frozen aortas of WT and Qk^- mice (n=6). Data are normalized to Gapdh and are relative to QkI-5 mRNA expression levels. Data are mean ± s.e. *p<0.05, **p<0.01.
Online figure II: QKI gene dosage determines VSMC morphology and phenotype. (A) Phase-contrast images of isolated and clonally subcultured cuboidal and spindle sh-qk1 HITA2 VSMCs. Western blot analysis of QKI and contractile apparatus protein expression in whole cell lysates harvested from cuboidal and spindle sh-qk1 HITA2 VSMCs (n=3). Quantitation of protein expression in contractile VSMCs is relative to cuboidal VSMCs and is normalized to ACTB (n=3). (B) Schematic depicting primer sets used to assess Myocd splice variants. Light grey boxes indicate alternative exons of Myocd. (C-D) PCR analysis of Myocd exon 2-5 in HITA2 kd-cuboidal and HITA2 kd-spindle VSMCs (C) and HITA2 sh-cont and HITA2 sh-qk1 VSMCs (D). Top panel: The approximately 300 bp fragment illustrates inclusion of exon 2a. Quantitation provided is indicative of percent exon 2a inclusion and is normalized to Gapdh. Cuboidal vs. contractile, n=3; sh-cont vs. sh-qk1, n=4. *p<0.05. **p<0.01.
Online Figure III: Mechanisms of modulation of myocd pre-mRNA by QKI. (A) Schematic depicting the coding region of the myocardin locus, including the 5- and 3-UTR regions (white boxes), exonic regions (black boxes), and intronic regions (lines between exons). The qre in the myocd 3'-UTR are defined as qre-2 and qre-3 (which are 643 and 2085 base pairs downstream of the translational stop codon in exon 14), which are also found in the luciferase constructs (bottom right). qre-1 is found in the intronic region between exon 2 and exon 3, and is located on the 3'-splice site of exon 2a (top left). (B) Luciferase/Renilla relative expression levels of pMIR-REPORT-qre2, qre3 and qre-2+3 constructs as depicted in (A) 24h after transient transfection into three separate sh-cont and sh-qkl transduced HEK293 cultures (n=3 separate transfection with aforementioned luciferase constructs). (C) Quantitation of percent exon 2a inclusion in sh-cont and sh-qkl HITC6 VSMCs cultured in the presence (day 0) and absence (day 8) of serum. Data are mean ± s.e. n.s indicates non-significant, *p<0.05, **p<0.01.
Online Figure IV: qkl is abundantly expressed in the heart. (A) Qkl and Myocd mRNA expression levels were assessed quantitatively in the aorta (designated a) and heart (designated h) of 6 WT mice. 40-Ct values are depicted due to large differences in Gapdh expression levels in aorta versus heart (n=6 mice, **p<0.01, ***p<0.001).
Online Figure V

(A-C) Relative mRNA abundance of myocd exon 10-13 (A) and myocd exon 2-5 (B) in human VSMCs overexpressing EGFP, v1a, v1b, v3a or v3b (C). N=3. Data are mean ± s.e.

Online Figure V: Quantitative assessment of myocd mRNA expression in VSMCs overexpressing distinct Myocd isoforms.