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Smooth Muscle Differentiation Control Comes Full Circle: The Circular Non-Coding RNA, circActa2, Functions as a miRNA Sponge to "Fine-Tune" αSMA Expression

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Atherosclerosis is a chronic inflammatory disease that progresses to complex, unstable arterial lesions[1]. Restenosis is an acute inflammatory vascular disease and a major limitation of percutaneous angioplasty procedures. Both are characterized by de-differentiation of vascular smooth muscle cells (SMCs) resulting in neointimal hyperplasia and vessel occlusion. Differentiated SMCs are highly specialized cells whose primary role is to maintain vessel homeostasis, vessel tone, blood pressure, and blood flow distribution[3]. This function is driven through expression of SMC-specific contractile and contractile-related proteins, including smooth muscle myosin heavy chain (SMMHC/*Myh11*), smooth muscle alpha actin (αSMA/*Acta2*), SM22α (*Tagln1*), and calponin (*Cnn1*), among others. Unlike terminally differentiated cardiac and skeletal muscle, SMCs retain a significant degree of phenotypic plasticity, exhibiting the ability to undergo extensive changes in phenotype in response to specific stimuli (i.e. dedifferentiated SMC). SMC dedifferentiation is associated with a transition to a highly proliferative, inflammatory phenotype characterized by downregulation of SMC-specific genes and increased production of multiple inflammatory and matrix-associated mediators. Thus, SMCs are major contributors to vascular disease progression and defining molecular mechanisms regulating SMC phenotypic transitions is critical to define novel therapeutics for treatment of vascular disease.

Regulation of SMC differentiation is complex, involving multiple signaling pathways and transcriptional regulators. Most SMC-specific genes are under transcriptional control by the transcription factor, SRF, and its cardiac and SMC-specific cofactor, myocardin, the SRF-myocardin axis[3–5]. SRF binds the serum response element or CArG box, in which one or

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DISCLOSURES

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more are present within promoter and/or intronic regions of SMC-specific genes[3,4]. In contrast, myocardin does not directly bind DNA, but transactivates SMC-specific genes through its interaction with SRF[5]. While the SRF-myocardin axis is central to transcriptional regulation of SMC genes, additional factors and mechanisms have been identified that serve to cooperate with or fine-tune SRF-myocardin activity. For instance, SRF-myocardin cooperatively interact with other *cis*-regulatory elements and their binding factors to maintain SMC differentiation, including, but not limited to the transcription factors, Nkx3.1 and 3.2, GATA-4 and -6, SMADs, the homeodomain protein Prx1, and the LIM proteins CRP1 and CRP2[3]. Our group demonstrated that the tumor suppressor, PTEN, interacts with SRF in the nucleus and functions as an indispensable co-factor to maintain the differentiated SMC phenotype[6]. In addition, multiple signaling pathways have been identified that stimulate or maintain SMC differentiation through regulation of SMC-specific transcriptional machinery[7]. TGFβ-, RhoA-, and p38-dependent signaling have been implicated in promoting SMC differentiation through activation of transcriptional regulators interacting with the SRF-myocardin axis. The Notch intracellular domain (ICD) interacts with the transcription factor, RBPJ to control SMC differentiation thereby implicating the Notch signaling pathway in SMC differentiation control. Collectively, there are multiple parallel and/or intersecting transcriptional networks involved in maintaining the SMC differentiated phenotype.

In addition to direct transcriptional control, additional mechanisms are essential for modifying/regulating SMC contractile gene expression. Epigenetic regulation of chromatin structure of CArG-containing regions of SMC genes is essential for proper SMC differentiation control. The Owens group identified enrichment of several histone modifications involved in chromatin relaxation and gene induction, including acetylation and methylation marks on histones flanking CArG boxes in the *Myh11* and *Acta2* genes[3]. These histone modifications regulate SRF binding to SM gene CArG boxes. In addition, it has been shown that myocardin interacts with the p300 histone acetyltransferase, which mediates histone acetylation of SMC genes and SM gene expression. In contrast, overexpression of histone deacetylases (HDACs) decreases SM gene expression. Therefore, chromatin structure and specific SMC-restricted histone marks are important modifiers essential for SMC differentiation control. In addition, activity of short and long non-coding RNAs regulate SMC phenotype[8]. Several microRNAs (miRNAs) either positively or negatively regulate SMC differentiation. For instance, miR-145/143 promotes SM contractile gene expression by directly targeting transcriptional repressors of these genes (e.g. Klf4). In contrast, miR-21 and miR-221/222 promote SMC de-differentiation by targeting known growth repressors (e.g. PTEN). miRNAs that directly target SM contractile genes to promote SMC dedifferentiation have yet to be identified. While several classes of long non-coding RNAs (lncRNAs) have been described and emerging data suggest important functions for lncRNAs on SMC phenotype control, little is known of the role of the novel class of non-coding RNAs, circular (circ)RNAs, on SMC contractile protein expression.

In this issue of *Circulation Research*, Yan Sun, et. al[9]. assessed the role of the intracellular domain of the epidermal growth factor family member, neuregulin-1 (NRG-1-ICD) on SMC αSMA expression. The authors demonstrated that TGFβ induces NRG-1 expression and

cleavage thereby releasing NRG-1-ICD, which translocates to the nucleus and interacts with the transcription factor, Ikzf1. In the nucleus, the NRG-1-ICD:Ikzf1 complex binds to the first intron of the *Acta2* gene and induces formation of the circRNA, circActa2 through circularization of exon-5 to exon-9. TGFβ-mediated induction of circActa2 promotes αSMA protein expression through direct interaction with miR-548f-5p, thereby functioning as a miRNA "sponge" to decrease miR-548f-5p repression of *Acta2.* Functionally this is associated with stabilization of actin filaments and enhanced contraction (Figure). Decreased expression of circActa2 and dissociation of circActa2 and miR-548f-5p in human intimal hyperplastic lesions led the authors to conclude that the NRG-1-ICD/circActa2/miR-548f axis functions to fine-tune αSMA expression and that dysregulation of circActa2 and miR-548f expression promotes intimal lesion formation. These findings are important as they reveal a new, novel mechanism of regulating αSMA expression, which is essential for SMC function. The NRG-1-ICD/circActa2/miR-548f-5p axis, therefore, may represent a novel therapeutic target to limit vascular disease progression.

There are several novel and significant discoveries from this manuscript. First, the authors define a novel function for NRG-1 in SMCs. NRG-1 is well known for its role in cardiovascular development, largely through its paracrine effects following cleavage of the extracellular EGF domain[10]. Further, released soluble NRG-1 has been shown to suppress SMC proliferation[11]. Less is known regarding NRG-1-ICD, with the majority of information derived from studies in neurons. Here the authors demonstrate that NRG-1-ICD functions as a transcriptional activator in SMCs through a stable complex with Ikzf1 to induce expression of a non-coding circRNA that functions to suppress miRNA-mediated repression of αSMA[9]. Collectively, NRG-1 modulates SMC phenotype via release of both its intracellular domain and extracellular EGF-like domain. Release and nuclear translocation of NRG-1-ICD is mediated through proteolytic cleavage, which is similar to Notch signaling in the regulation of SMC differentiation[7]. In the case of NRG-1, TGFβ promotes proteolytic cleavage and release of NRG-1-ICD, a novel function of TGFβ in driving SMC contractile gene expression. At the present time, however, the signaling mechanism(s) mediating the effects of TGFβ on NRG-1 cleavage and NRG-1-ICD translocation and whether this effect is mediated through canonical TGFβ signaling remain unknown.

While traditionally disregarded as rare and non-functional, emerging data demonstrate that functional circRNAs harbor conserved miRNA seed sequences supporting important roles for circRNAs as miRNA "sponges" to inhibit endogenous miRNAs[12]. The role of circRNAs in heart failure has been well recognized[13]. In contrast, little is known of circRNAs in vascular biology, although two studies demonstrated an association of circRNA expression with human thoracic aortic dissection[14] and atherosclerosis[15], thereby underlying the potential clinical relevance of functional circRNAs in vascular disease. The current manuscript is the first demonstration of a functional circRNA regulating SMC differentiation control and contractile function. The studies in human intimal hyperplastic tissue provide evidence that circularization of lncRNAs may impart protection from human vascular disease. Further, while miRNAs have been shown to facilitate SM gene expression or promote SMC de-differentiation, these effects are not mediated directly on SM contractile genes. This report is the first to describe direct miRNA-mediated repression of a SM

contractile gene, which has important implications for maintenance of the contractile phenotype beyond direct transcriptional control.

Clinical Significance and Additional Questions

αSMA is a critical component of the SMC contractile machinery and essential for actin cytoskeletal dynamics necessary for physiological vessel wall homeostasis. Subtle changes in expression could have profound effects on normal vascular function and likely contributes to vascular disease progression. The findings presented here describe a novel pathway functioning to fine-tune αSMA levels and may have significant clinical impact that leads to new and novel therapeutic approaches to control the differentiated SMC phenotype. The data suggest that activation of NRG-1-ICD, induction of circActa2, or inhibition of miR548f-5p could represent novel strategies to target pathological vascular remodeling. Moving forward there are several unanswered questions. *(a)* Do similar mechanisms and unique circRNA/miRNA pairs regulate other SMC-specific contractile proteins (e.g. SMMHC/*Myh11*)? *(b)* Do other mediators promote NRG-1 induction and cleavage or is this specific to TGFβ? If specific, what mechanisms mediate NRG-1 induction? Cleavage? *(c)* Are circActa2 levels changed during intimal lesion formation? Hypertension? If so, how does this contribute to disease progression? *(d)* Induction of αSMA is observed during fibroblast-to-myofibroblast transition mediated by TGFβ. Is this pathway activated and does it play a role in chronic inflammatory, pro-fibrotic conditions? If so, targeting this axis could represent a novel therapeutic approach to limit fibroblast-to-myofibroblast transitions and myofibroblast function.

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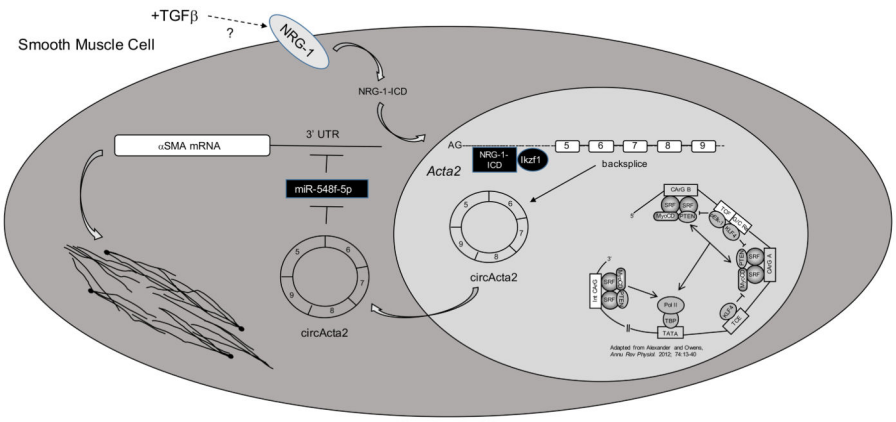

Figure.

In addition to the established direct role of SRF, myocardin, and other transcriptional activators and repressors on SMC contractile gene expression (bottom right[3]), levels of the contractile protein, αSMA, are fine-tuned through the activity of a TGFβ / NRG-1-ICD / circActa2 / miR-548f-5p axis. TGFβ stimulates NRG1 expression and cleavage, promoting nuclear translocation of NRG-1-ICD. Nuclear NRG-1-ICD recruits Ikzf1 and forms a stable transcriptional complex that interacts with the first intron of the *Acta2* gene inducing circActa2 formation. circActa2 functions as a miRNA "sponge," interacting with and repressing miR-548f-59, which targets αSMA mRNA for degradation, thereby resulting in increased αSMA levels and enhanced SMC contractile function. NRG-1, neuregulin-1; NRG-1-ICD, neuregulin-1 intracellular domain; circActa2, circular RNA Acta2; TGFβ, transforming growth factor-β; SRF, serum response factor; MyoCD, myocardin; miR-548f-5p, microRNA-548f-5p; αSMA, smooth muscle alpha actin *(Acta2).*