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FRET-ting about RhoA signalling in heart and vasculature - a new tool in our cardiovascular toolbox --Manuscript Draft--

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Commentary on ‘A RhoA-FRET Biosensor Mouse for Intravital Imaging in Normal Tissue Homeostasis and Disease Contexts’ by Nobis M, Herrmann D *et al.*, Cell Rep, 2017. ¹

Rho small GTPases are a family of signalling proteins that play a role in a multitude of actin-dependent and independent cellular functions including cell adhesion, migration and cytokinesis². They cycle between a GTP-bound active state and a GDP-loaded inactive state. In the active state, they can bind and regulate a large number of different proteins through which they influence many different pathways. Research in the cardiovascular field has largely focused on two Rho small GTPases, RhoA and Rac1, and has shown that they are essential for the development of the heart and vasculature, but also linked them to a number of cardiovascular pathologies including cardiac hypertrophy, atherosclerosis and heart failure development^{3,4}. It is therefore important to improve our understanding of the pattern and regulation of Rho GTPase activity in the cardiovascular system.

RhoA is required in early heart development for heart tube fusion⁵ and at later stages for the establishment of the conduction system⁶ as shown in chick embryos. Furthermore, regulation of RhoA activity is essential for different aspects of vasculogenesis, including the differentiation of coronary smooth muscle cells from proepicardial cells⁷. Aside these known roles, a detailed understanding of the temporal and spatial activation of RhoA during development and the mechanisms involved is lacking and a more comprehensive appreciation of the cardiovascular developmental roles of RhoA and its potential involvement in congenital heart disease is required.

Similar to the developing embryo, tight regulation of RhoA signalling in the adult heart and vasculature is vital. On one hand, RhoA signalling is required to maintain and remodel cell-cell contacts in endothelial cells and cardiomyocytes, though direct *in vivo* evidence of the latter is missing to date^{8,9}. Involved in mechanosensing, RhoA can reinforce junctions to insure mechanical integrity of the heart muscle and vasculature, but can also disrupt contacts for example to increase vascular permeability¹⁰. Furthermore, RhoA controls vascular tone through its effector Rho kinase (ROCK)⁴. On the other hand, RhoA/ROCK signalling has well established connections to a number of cardiovascular pathologies. Chronically increased RhoA activity leads to enhanced vasoconstriction and hypertension¹¹, it promotes endothelial dysfunction and vascular remodelling associated with arteriosclerosis⁴ and it is involved in the development of aortic aneurism and restenosis⁴. RhoA function in the myocardium is less well understood, but there is good evidence that RhoA participates in actin organisation, gene regulation and impacts both cardiomyocyte death and survival¹². Consequently, RhoA signalling has been linked with cardiac hypertrophy and increased infarct size and fibrosis after ischemic injury. Other heart-related RhoA functions include a recently identified role for RhoA-mediated transcriptional regulation in the progression of calcific aortic valve stenosis¹³. In addition, RhoA signalling also influences inflammatory events in both heart and

vasculature, such as the recruitment of immune cells into arteriosclerotic lesions⁴ and accumulation of macrophages in fibrotic areas of hypertrophic hearts, a process associated with development of systolic dysfunction¹⁴. Given this multitude of physiological and pathological functions ascribed to RhoA signalling, it is clear that activation and suppression of the pathway must be carefully balanced. Therefore, a detailed understanding of spatial and temporal regulation of RhoA signalling will help develop treatments that target abnormal RhoA activity in disease.

Förster Resonance Energy Transfer (FRET) probes have been employed successfully to investigate the activation pattern of a variety of signalling proteins including RhoA. These probes are based on the concept that active, but not inactive signalling proteins will bind to their effector proteins. Coupled to compatible fluorescence proteins, interaction between signalling and effector protein will bring the fluorescent moieties in close enough proximity to permit energy transfer and emission from the donor fluorophore will excite the acceptor fluorophore. Most studies using FRET relied on transiently overexpressing the probe or at best generating stable expressing cell lines, therefore limiting their use to *in vitro* studies and a few specific *in vivo* applications. Recent years however, have seen the development of genetically engineered biosensor animal models that will permit the investigation of signalling in any type of tissue and in its native environment, which is particularly important for signalling events that are regulated by mechanical cues.

After previously developing a Rac1-FRET mouse¹⁵, researchers under the lead of Kurt Anderson and Paul Timpson now engineered a RhoA-FRET biosensor mouse and investigated RhoA signalling in diverse cell types and contexts¹. Nobis, Herrmann, Warren and colleagues generated RhoA-OFF mice where expression of the RhoA-FRET biosensor is induced by Cre recombinase. They used this system to observe mechanosensing of osteocytes in compressed calvaria bone demonstrating directional RhoA activation in cell protrusions relatively perpendicular to the force direction. Given the constant mechanical forces applied to cells in the beating heart, investigating the role of RhoA mechanosensing in the heart should prove highly informative, especially in the context of altered mechanical tissue properties such as after scarring following myocardial infarction and heart remodelling during cardiac hypertrophy. Next, the paper showed expression of the probe in swarming neutrophils and demonstrated correlation between elevated RhoA activity and increased neutrophil activation level. In cardiovascular disease, this sensor could be utilised to understand the activation pattern of immune cells in acute conditions such as myocardial infarction or chronic settings such as the hypertrophic failing heart and arteriosclerotic plaques. For instance in the latter, RhoA activity in immune cells is likely to be differentially regulated in stable and unstable plaques and throughout plaque progression and remodelling and thus might be a target for drugs aiming at stabilising plaques. Lastly, the authors follow RhoA activity in breast cancer and pancreatic cancer progression and use intravital imaging through optical windows to assess the effect of anti-cancer drugs on RhoA activity in real time, an approach that will permit optimising of treatment regimens in future preclinical *in vivo* studies. Given the ambivalent roles of RhoA in the cardiovascular system, fine-tuning of any treatment targeting RhoA signalling in heart and vasculature will be of particular importance. Statins, for example, have been shown to target RhoA pathways which has been largely correlated with beneficial effects of the treatment in atherosclerosis, but excessive inhibition seems to compromise vascular homeostasis¹⁶.

This study thus provides a valuable new tool to investigate a key signalling and mechanosensing pathway in the cardiovascular system. When combined with novel imaging techniques, which now

allow high speed, high resolution imaging of the beating heart¹⁷, this new mouse model could significantly expand our understanding of RhoA signalling in cardiovascular development and function and its dysregulation in disease states. Ultimately, this knowledge will permit the development of new tailored drug treatments to counteract imbalanced RhoA function. The promise of targeting RhoA has already been hinted through the use of statins to treat atherosclerosis, where treatment effects have been partially ascribed to the inhibition of RhoA/ROCK signalling or pathways governed by it¹⁶. The RhoA-FRET mouse will now allow real-time imaging of RhoA activity *in vivo* following drug treatment to confirm effects of non-specific drugs such as statins and specific agents such as ROCK inhibitors on RhoA function and help optimise drug scheduling before clinical studies commence.

Biographical sketches

Dr Susann Bruche is a postdoctoral researcher at the University of Oxford and a Junior Research Fellow at Wolfson College. After previously working on adherens junction signalling, she now investigates the role of alternative splicing in the regulation of epicardial activation and heart repair after myocardial infarction. Susann's academic achievements have been acknowledged with prestigious scholarships from organisations including the German National Merit Foundation and the British Heart Foundation.

Prof. Manuela Zaccolo, MD. Dr Zaccolo graduated in Medicine at the University of Torino, Italy. After training in molecular biology and protein engineering at the LMB, Cambridge, UK, she worked at the University of Padova, Italy where she developed novel imaging methods for the study of intracellular signalling in real-time. This work led to the development of the first genetically encoded FRET-based reporter for intracellular detection of cAMP and led to the direct demonstration of the compartmentalised nature of cAMP signalling in cardiac myocytes. She subsequently moved to the University of Glasgow and, more recently, to the University of Oxford, where she is currently the Director of the Burdon Sanderson Cardiac Science Centre in the Department of Physiology, Anatomy and Genetics. Dr Zaccolo is currently exploring the regulatory principles by which intracellular cyclic nucleotides signalling networks achieve the plasticity and context-sensitivity necessary for a cardiac myocyte to function. She combines real-time FRET imaging, genetic, biochemical and mathematical approaches to define a detailed and quantitative map of cyclic nucleotides signalling subdomains and to establish how local signalling is altered in disease. Her ultimate goal is to develop precision medicine strategies to target individual subcellular pools, rather than global intracellular cyclic nucleotide levels, to achieve greater therapeutic efficacy and specificity.

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