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GATA4-Twist1 signalling in disturbed flow-induced atherosclerosis

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Abstract

Endothelial cell (EC) dysfunction (enhanced inflammation, proliferation and permeability) is the initial trigger for atherosclerosis. Atherosclerosis shows preferential development near branches and bends exposed to disturbed blood flow. By contrast, sites that are exposed to non-disturbed blood flow, are atheroprotected. Disturbed flow promotes atherosclerosis by promoting EC dysfunction. Blood flow controls EC function through transcriptional and post-transcriptional mechanisms that are incompletely understood. We identified the developmental transcription factors Twist1 and GATA4 as being enriched in EC at disturbed flow, atheroprone regions of the porcine aorta in a microarray study. Further work using the porcine and murine aortae demonstrated that Twist1 and GATA4 expression was enhanced at the atheroprone, disturbed flow sites *in vivo*. Using controlled *in vitro* flow systems, the expression of Twist1 and GATA4 was enhanced under disturbed compared to non-disturbed flow in cultured cells. Disturbed flow promoted Twist1 expression through a GATA4-mediated transcriptional mechanism as revealed by a series of *in vivo* and *in vitro* studies. GATA4-Twist1 signalling promoted EC proliferation, inflammation, permeability and EndoMT under disturbed flow, leading to atherosclerosis development, as shown in a combination of *in vitro* and *in vivo* studies using GATA4 and Twist1-specific siRNA and EC-specific GATA4 and Twist1 KO mice. We revealed that GATA4-Twist1-Snail signalling triggers EC dysfunction and atherosclerosis, this work could lead to the development of novel anti-atherosclerosis therapeutics.

Introduction

Atherosclerosis is a chronic, inflammatory disease of the vasculature that gives rise to heart attacks and stroke. Endothelial cells (EC) that line the vasculature play a central role in mediating vascular health, and EC dysfunction is the initial trigger for atherosclerosis. ECs are in direct contact with the flowing blood that exerts a mechanical drag known as shear stress. Despite the contribution of systemic risk factors (age, obesity, high cholesterol, age, smoking), atherosclerosis shows a non-random distribution in the vasculature. Atherosclerotic plaques show a preferential development at bends and

branch points; these vascular sites are exposed to disturbed blood flow, which generates multidirectional, low wall shear stress. Whereas, sites that are exposed to non-disturbed flow, that exerts uniform, high shear stress are atheroprotected (Kwak et al., 2014, Suo et al., 2007, Dai et al., 2004, Passerini et al., 2004). Disturbed flow induces atherosclerosis development by promoting EC dysfunction (enhanced inflammation and proliferation) (Dai et al., 2004, Passerini et al., 2004, Schober et al., 2014, Guo et al., 2007). As a result of enhanced proliferation, ECs lose contact with neighbouring cells and become more permeable to inflammatory cells and molecules (LDL), leading to atherosclerotic lesion formation (Cancel et al., 2011). Blood flow promotes EC dysfunction through modulating endothelial gene expression by molecular mechanisms that are not fully understood (Passerini et al., 2004, Cuhlman et al., 2011, Senbanerjee et al., 2003, Dunn et al., 2014, Ni et al., 2010).

To further characterize the molecular mechanisms that control focal atherogenesis, we conducted a microarray study which revealed that the transcription factors Twist1 and GATA4 showed enhanced gene expression in EC at disturbed flow, atheroprone regions of the porcine aorta (Serbanovic-Canic et al., 2017). Both of these transcription factors control major developmental processes, Twist1 controls gastrulation during embryogenesis (Thisse et al., 1988), whereas GATA4 is a master regulator of cardiac development (Kuo et al., 1997). Although the role of Twist1 and GATA4 in embryonic development is well characterized, their potential function in ECs and vascular disease is entirely unknown.

The role of GATA4 and Twist1 in embryonic development.

GATA4 controls heart development.

GATA4 is a zinc finger transcription factor that belongs to the GATA family, containing GATA1-GATA6 proteins. GATA proteins play non-redundant roles and display tissue-specific expression; GATA1-3 are more closely related in sequence and are predominantly expressed in the hematopoietic system, whereas GATA4-6 are more closely related and are expressed in the cardiovascular system and the gonads (Zhou et al. 2012). The process of mammalian heart development begins with looping of the early heart tube (consisting of cardiomyocytes and endothelial cells), which then becomes divided into four chambers that are separated by endocardial cushion-mediated valve formation. This process is controlled by a tightly regulated cardiac transcriptional network. GATA4 is regarded as a master cardiac transcription factor as it acts upstream of the transcriptional network controlling heart development, including myocyte enhancer factor (Mef)2c, NK2 homeobox 5 (Nkx2.5), serum response factor (Srf) and Hand2 (Schlesinger et al. 2011; McFadden et al. 2000; E. M. Zeisberg et al.2007). Grepin et al. established the dominant role of GATA4 in cardiac development by revealing that GATA4 overexpression in pluripotent P19 embryonic carcinoma cells committed the cells to

undergo differentiation to the cardiac lineage and gave rise to beating cardiomyocytes (Grepin et al. 1995). Genetic inhibition of GATA4 restricted beating cardiomyocyte differentiation (Grepin et al. 1995), thus indicating that GATA4 expression is required to drive cardiomyocyte differentiation. Interestingly, when overexpressed in embryonic stem cells, GATA4 induces extraembryonic endoderm, not cardiomyocytes (Fujikura et al., 2002). However, under defined culture conditions (serum-free media), GATA4 is able to efficiently promote cardiogenesis in embryonic stem cell derivatives (Turbendian et al., 2013). Mice lacking GATA4 die before the onset of heart development due to defects in the extraembryonic endoderm (Kuo et al., 1997; Molkentin et al., 1997), while rescue experiments providing the null embryos with wild-type extraembryonic endoderm showed that GATA4 is required for cardiogenesis and proepicardium development (Watt et al., 2004).

Twist1 plays a major role in multiple stages of embryonic development. Twist1 is a transcription factor that belongs to the basic Helix-Loop-Helix (bHLH) family of transcriptional regulators. Twist1 was originally discovered in Drosophila, as one of the genes essential for mesoderm specification (Thisse et al, 1987). Drosophila embryos lacking the Twist1 gene died during embryogenesis with a “twisted” appearance (Thisse et al, 1987). In mouse, Twist1 plays a crucial role during multiple stages of embryonic development, from controlling mesodermal differentiation during gastrulation (around embryonic day (E) 6, up to controlling valve formation at E 16 (Reviewed in Qin et al. 2012).

Twist1 also acts as a master regulator of the osteoblast differentiation program by controlling the activity of downstream pathways that are central to osteoblast differentiation and development, including fibroblast growth factor (FGF) and bone morphogenic protein (BMP) signalling by controlling FGFR2, FGF8, FGF10, and BMP4 transcription (Bialek et al. 2004; Connerney et al. 2008; Loebel et al. 2002; Rice et al. 2010).

GATA4 and Twist1 promote heart valve formation through endothelial-to mesenchymal transition.
GATA4 and Twist1 exert overlapping functions during atrioventricular valve development by inducing endothelial-to-mesenchymal transition (EndoMT), which describes a process in which mature endothelial cells adopt a mesenchymal phenotype. Markwald et al discovered EndoMT as a critical process during heart valve development, where it was observed that cells of the endocardium acquire a more mesenchymal identity and participate to the formation of the mesenchymal heart cushion, the precursor of the cardiac valves (Markwald et al. 1975). Ultimately, EndoMT cells separate from the monolayer and penetrate the underlying extracellular matrix (a process referred to as

delamination) and become highly migratory and invasive (Lim & Thiery 2012). Therefore, enhancements in cell proliferation, permeability, migration, and invasion are all functional outputs of cells undergoing EndoMT. GATA4 and Twist1 are downstream targets (through direct transcriptional activation or through post-translational modifications) of EndoMT promoting pathways, i.e. TGFβ, BMP, Notch and Wnt signalling (Lamouille et al. 2014; Daoud et al. 2014; Lin & Xu 2008; Reinhold et al. 2006; George et al. 2015). Interestingly, Twist1 and GATA4 can also act as positive regulators of these EndoMT promoting pathways (Chen et al.2014; O'Rourke et al. 2002; Moskowitz et al. 2011). *In vivo* studies have shown that endothelial-specific deletion of Twist1 or GATA4 blocks the EndoMT programme and subsequently heart valve formation (Rivera-Feliciano et al. 2006). Specifically, the EndoMT-promoting mechanism of Twist1 and GATA4 involves the transcriptional activation of mesenchymal genes Snail, Slug, α-Sma, and Cdh2 (Rivera-Feliciano et al. 2006; Lamouille et al. 2014; Hong et al. 2011; Chakraborty et al. 2010).

Do GATA4 and Twist1 play a role in the adult vasculature and in disturbed flow-induced atherosclerosis?

In the disease context, GATA4 and Twist1 have been shown to drive cancer progression and survival by mediating metastasis and proliferation (Yang et al. 2004, Wang et al. 2003, Chia et al. 2015, Takagi et al. 2014). However, the role of GATA4 and Twist1 in the adult vasculature and in atherosclerosis is completely unknown. We recently demonstrated for the first time that disturbed flow promotes GATA4-dependent induction of Twist1 in EC. Studies using the zebrafish embryo revealed that Twist was expressed in early embryonic vasculature where it promoted angiogenic sprouting by inducing EC proliferation and migration. In adult mammalian arteries, GATA4 and Twist1 were expressed preferentially at atheroprone sites exposed to disturbed flow where GATA4-Twist1 signalling promoted the development of atherosclerosis by inducing EC dysfunction (Proliferation, permeability and inflammation) (Mahmoud et al., 2016).

GATA4 and Twist1 expression is enhanced by atheroprone, disturbed flow.

To investigate the expression of flow-mediated genes, we conducted a microarray study on ECs isolated from either disturbed blood flow sites (inner curvature) or non-disturbed flow (outer curvature) of the porcine aorta (Serbanovic-Canic et al., 2017). The study revealed that hundreds of genes showed differential expression under flow, this gene set included Twist1 and GATA4, which were identified as being enriched at the atheroprone, disturbed flow site. This observation was validated by qPCR studies of an independent cohort of pigs (Figure 1 A-C). Similarly, en face staining of the murine aortic endothelium demonstrated elevated levels of Twist1 and GATA4 protein levels at the inner curvature of the aortic arch (disturbed flow) compared to the outer curvature (non-disturbed flow) (Figure 1 D-F). These observations revealed that the expression of GATA4 and Twist1 is driven by atheroprone, disturbed flow in the vasculature.

To directly study the effects of disturbed flow on Twist1 and GATA4 expression we used *in vitro* models of flow. Using two complementary systems, an orbital plate or a parallel plate system (Warboys et al., 2014, Dardik et al., 2005) ECs were exposed to either low, oscillating shear stress (mimicking disturbed flow *in vivo*) or high, unidirectional shear stress (mimicking non-disturbed flow *in vivo*) and it was observed using both systems that low shear stress induced the expression of Twist1 and GATA4 mRNA and protein. To conclude, these data showed that disturbed flow drives the expression of GATA4 and Twist1 *in vitro* and *in vivo* (Mahmoud et al., 2016).

GATA4 and Twist1 function as part of the same pathway during valve formation in embryonic development (Lim et al., 2012), thus we hypothesized that GATA4 and Twist1 may be acting in a pathway in response to disturbed flow in vascular ECs. We tested this hypothesis *in vitro* using a combination of gene silencing and chromatin immunoprecipitation experiments, which revealed that GATA4 acted upstream of Twist1 in ECs exposed to disturbed flow, where it promoted Twist1 expression through a transcriptional mechanism. To validate these observations *in vivo*, we assessed the effects of EC-specific genetic deletion of GATA4 ($Gata^{cKO}$) on endothelial expression of Twist1 in the murine aorta. *En face* staining demonstrated that the expression of Twist1 at the disturbed flow area was reduced in $Gata^{cKO}$ compared to control $Gata4^{flox/flox}$ mice, revealing that GATA4 positively regulates Twist1 in atheroprone, disturbed flow sites *in vivo* (Mahmoud et al., 2016).

GATA4-Twist1 signalling promotes atherosclerosis by driving EC dysfunction. Disturbed flow promotes EC dysfunction and subsequently atherosclerosis, since GATA4-Twist1 signalling was elevated in ECs at atheroprone, disturbed flow sites, we assessed whether GATA4-Twist1 signalling promoted disturbed flow-induced EC dysfunction (inflammation, proliferation, and permeability) and atherogenesis. This was tested using a combination of complementary approaches consisting of *in vitro* studies using cultured ECs and exposing them to flow using well-controlled systems (orbital plate and parallel plate systems) following gene silencing of Twist1 and GATA4 using siRNA, and *in vivo* studies using mice with an endothelial-specific deletion for GATA4 and Twist1. The results from these studies revealed that GATA4 and Twist1 promoted EC proliferation, permeability and inflammation under disturbed flow, reflecting that GATA4-Twist1 signalling promotes disturbed flow-induced EC dysfunction. The mechanisms by which GATA4-Twist1 signalling could be mediating EC proliferation involved the regulation of cell cycle genes such as Cyclins D1, G2, and CDK4 (Mahmoud et al., 2016). This observation is consistent with other studies, that have demonstrated the role of GATA4 and Twist1 in driving proliferation and cell cycle regulation in other contexts (Gitelmann et al., 1997, Chakraborty et al., 2010). Another mechanism by which GATA4-

Twist1 signalling drives EC dysfunction was through the induction of EndoMT (Mahmoud et al 2016, Mahmoud et al 2017).

We next investigated whether GATA4-Twist1 signalling promoted atherosclerosis since it promoted EC dysfunction under disturbed flow. Using a PCSK9-adenoassociated virus (AAV) followed by high fat diet feeding (6 weeks), we induced hypercholesterolemia in $Gata4^{cKO}$ and $Twist1^{cKO}$ mice (Figure 2 A). Interestingly, atherosclerotic lesion formation was suppressed in mice lacking the GATA4 and Twist1 gene, compared to control mice, revealing that GATA4-Twist1 signalling mediated atherogenesis (Figure 2 B). To conclude, GATA4-Twist1 signalling drives EC proliferation, permeability, inflammation (EC dysfunction) and atherogenesis in response to disturbed flow (Mahmoud et al., 2016).

EndoMT and atherosclerosis.

Recent studies established the contribution of EndoMT to atherosclerosis (Moonen et al., 2015, Chen et al., 2015, Evrard et al., 2016). EndoMT has notably been linked to plaque progression since the extent of EndoMT observed in human plaques correlates strongly with the severity of the disease (Chen et al., 2015, Evrard et al., 2016). Furthermore, endothelial lineage tracing studies in the mouse established the occurrence of EndoMT during plaque development (Chen et al., 2015; Evrard et al., 2016). These elegant studies revealed that a large proportion of aortic EC delaminate and migrate into the atherosclerotic lesions and may promote plaque growth by increasing inflammation. EndoMT process has also been linked to disease initiation as Chen et al. demonstrated that inhibition of FGF, an antagonist of EndoMT, increases lesion initiation in a mouse model of atherosclerosis.

As Twist1 and GATA4 are regulators of EndoMT during embryonic development, we investigated whether the mechanism for GATA4-Twist1 driven EC dysfunction leading to atherosclerosis involves EndoMT. A series of studies revealed that disturbed flow induced EndoMT in cultured EC and that GATA4 and Twist1 are positive regulators of this process. Further studies revealed that GATA4-Twist1 signalling directly activated the transcription factor Snail, a key driver of EndoMT, through a transcriptional mechanism (Mahmoud et al., 2017). In addition, we found that GATA4-Twist1-mediated activation of Snail contributed to proliferation and permeability under disturbed flow. Collectively, our observations reveal that GATA4-Twist1 signalling promotes EC dysfunction under disturbed flow via induction of EndoMT (Mahmoud et al., 2016, Mahmoud et al., 2017). Our study gives a new insight into the mechanisms controlling EC dysfunction in response to disturbed flow and, in accordance with Chen's studies, suggests that EndoMT plays a crucial role in the initiation of atherosclerosis.

Summary
The role of GATA4 and Twist1 in embryonic development is well established, whereas the role of GATA4 and Twist1 in adult arteries was previously unknown. Here we reveal that the developmental GATA4-Twist1 signalling is regulated by mechanical forces in adult ECs, where atheroprone disturbed flow induced GATA4 expression in ECs, which in turn transcriptionally activated Twist1 expression. GATA4 and Twist1 have been shown to be regulated by mechanical forces in other contexts, GATA4 has been shown to be activated in cardiac cells in response to mechanical loading of the heart ventricles (Tenhunen et al. 2004) and Twist1 to be regulated by mechanical forces exerted by tissue deformations during Drosophila embryonic development (Desprat et al., 2008), and in response to matrix stiffness in tumour cells (Wei et al., 2015). We show that disturbed flow-driven GATA4-Twist1 signalling, promoted EC proliferation, permeability, and inflammation, key characteristics of dysfunctional ECs. As a result of inducing EC dysfunction, GATA4-Twist1 signalling promoted the development of atherosclerosis (Figure 3). We propose that the mechanism by which GATA4-Twist1 signalling promoted EC dysfunction and atherosclerosis is in part, mediated by the initiation of EndoMT, a process that has been recently linked with the progression of atherosclerosis (Moonen et al., 2015, Chen et al., 2015, Evrard et al., 2016). These observations have implications for the development of new therapies to prevent the initiation or progression of atherosclerosis by targeting EndoMT.

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