WT1 regulates the expression of inhibitory chemokines during heart development

Victor Velecela¹,², Laura A. Lettice¹, You-Ying Chau¹, Joan Slight¹, Rachel L. Berry¹, Anna Thornburn¹, Quinn D. Gunst³, Maurice van den Hoff³, Manuel Reina², Fernando O. Martínez⁴, Nicholas D. Hastie¹,∗ and Ofelia M. Martínez-Estrada²,∗

¹MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, Edinburgh EH4 2XU, UK, ²Department of Cell Biology, Faculty of Biology, University of Barcelona, Barcelona 08028, Spain, ³Heart Failure Research Center, Academic Medical Center, Amsterdam 1105AZ, The Netherlands and ⁴Kennedy Rheumatology Institute, University of Oxford, Oxford, UK

Received June 3, 2013; Revised and Accepted July 23, 2013

The embryonic epicardium is an important source of cardiovascular precursor cells and paracrine factors that are required for adequate heart formation. Signaling pathways regulated by WT1 that promote heart development have started to be described; however, there is little information on signaling pathways regulated by WT1 that could act in a negative manner. Transcriptome analysis of Wt1KO epicardial cells reveals an unexpected role for WT1 in repressing the expression of interferon-regulated genes that could be involved in a negative regulation of heart morphogenesis. Here, we showed that WT1 is required to repress the expression of the chemokines Ccl5 and Cxcl10 in epicardial cells. We observed an inverse correlation of Wt1 and the expression of Cxcl10 and Ccl5 during epicardium development. Chemokine receptor analyses of hearts from Wt1gfp/¹mice demonstrate the differential expression of their chemokine receptors in GFP⁺ epicardial enriched cells and GFP⁻ cells. Functional assays demonstrate that CXCL10 and CCL5 inhibit epicardial cells migration and the proliferation of cardiomyocytes respectively. WT1 regulates the expression levels of Cxcl10 and Ccl5 in epicardial cells directly and indirectly through increasing the levels of IRF7. As epicardial cell reactivation after a myocardial damage is linked with WT1 expression, the present work has potential implications in adult heart repair.

INTRODUCTION

The epicardium is crucial for heart development by contributing to the formation of cardiovascular precursor cells and as a source of secreted factors that influence heart morphogenesis (1,2). The epicardium originates from the proepicardium and envelops all the surface of the myocardium by Day E10.5 in the mouse (1). From this early formation of the epicardium until the adult, epicardial cells undergo dynamic changes in the expression of embryonic genes that correlate with changes in the embryonic epicardial cell properties such as proliferation, plasticity and migration (3,4). Appropriate formation and function of the epicardium is essential for myocardial proliferation and coronary blood vessel development (1).

The production of mitogenic factors by embryonic epicardial cells that promote cardiomyocyte proliferation and stimulate coronary blood vessel development suggests the possibility that the same growth factors and signaling pathways which are involved in the initial heart morphogenesis could also play a role in the heart repair response that takes place following myocardial infarction (MI) (2,5). Several of these molecules have been identified, including retinoic acid (RA), erythropoietin, fibroblast growth factors (Fgfs), platelet-derived growth factor, vascular endothelial growth factor (VEGF), angiopoietin 2, Sonic hedgehog homolog (SHH) and Thymosin beta 4 (1,2). Wt1 encodes a zinc finger protein, which is essential for heart development (6,7). During embryonic heart formation, WT1 is mainly expressed in epicardial cells and epicardial-derived
cells (EPDCs) (6). Its expression has been used to trace epicardial derivatives in embryos, and recently it has been used to follow the reactivation of epicardial cells after MI (3,8–10). Interestingly, the highest levels of expression of WT1 during epicardium development correlate with the highest proliferative state, stem cell properties and migratory capacity of epicardial cells (4). There are few identified genes expressed in the embryonic epicardium that are expressed de novo and are part of a putative reparative response in ischemic heart, WT1 being one of them (3,8,9).

We have characterized epicardial-specific Wt1 KO mice and showed a defect in the formation of the coronary blood vessels associated with a reduced ventricular myocardium (7). This suggests that Wt1 KO epicardial cells are a good model to identify growth factors and signaling pathways produced by epicardial cells that are involved in coronary blood vessel and cardiomyocyte proliferation.

In order to identify new target genes regulated by WT1 during epicardial development and how these influence heart formation, we performed a microarray-based expression analysis of transcriptional changes associated with Wt1 deletion in immortalized embryonic epicardial cells. The microarray study reveals an unexpected role for WT1 in repressing the expression of chemokines in epicardial cells. We provide evidence that links the overexpression of the chemokines CXCL10 and CCL5 with chemokines in epicardial cells. We have performed microarray-based expression analysis of transcriptional changes associated with Wt1 deletion in CreER Wt1 KO mice and control Wt1 KO cells, whereas there was no change in expression levels of CreER Wt1 KO heart phenotype, in particular the chemokines CXCL10 and CCL5. CXCL10 is an angiostatic chemokine that can also regulate fibroblast cell migration (13–15).

RESULTS

Identification of novel WT1 targets in epicardial cells

The embryonic epicardium contains mixed populations of cells that are essential for heart development (11). The WT1 gene is one of the embryonic epicardial genes that are re-expressed during MI, suggesting an important role in the dynamic process that takes place during heart repair. To identify new target genes regulated by WT1 in epicardial cells and how these influence heart morphogenesis, we generated E11.5 tamoxifen-inducible Wt1KO (CreER\(^{+/+}\)/Wt1\(^{loxP/loxP}\)) and control (CreER\(^{-/-}\)/Wt1\(^{loxP/loxP}\)) epicardial cells referred to as Cre\(^{+}\) and Cre\(^{-}\) from here on. Briefly, Cre\(^{+}\) and control Cre\(^{-}\) cell lines were generated by cross-breeding Wt1\(^{E8P/+}\)/Immorto\(^{+/+}\) mice, with mice that were homozygous Wt1 conditional and heterozygous for CAGG promoter-driven CreER\(^{TM}\) (CAGG-CreER; Wt1\(^{loxP/loxP}\)). This strategy allows us to generate Cre\(^{+}\) and Cre\(^{-}\) populations of epicardial cells. These epicardial cells display cobblestone morphology typical of epicardial cells and express GFP and WT1 (Supplementary Material, Fig. S1). A quantitative real-time PCR (qRT-PCR) analysis confirms that Wt1 expression was significantly downregulated following 6 days of tamoxifen treatment of Cre\(^{+}\) cells, whereas there was no change in expression levels of Wt1 in Cre\(^{-}\) cells (Fig. 1A).

We performed microarray-based expression analysis of transcriptional changes associated with Wt1 deletion in Cre\(^{+}\) embryonic epicardial cells. A total of 47 known unique genes were found to be significantly regulated by tamoxifen, 21 upregulated and 26 downregulated. For a full list and detailed functional and regulators analysis, see Supplementary Material, Table S1. The genes most upregulated in Wt1KO were the chemokines Ccl5, Cxcl10, Ccl5, and the interferon responsive gene Irf7 (Supplementary Material, Table S1). Chemokines are pleiotropic cytokines which are able to orchestrate the attraction and release of cells. CCL5 is chemotactic on T cells, macrophages, dendritic cells, eosinophils, NK cells, mast cells and basophils, CCL7 attracts monocytes and macrophages while CXCL10, also known as interferon gamma-induced protein 10 (IP-10), attracts monocytes/macrophages, T cells, NK cells and dendritic cells (12). However, they display other functions as discussed below. Given the pleiotropic functions of these, and other genes modulated in Wt1KO such as Oas2, Ifit3, Mx2, the pathway analysis that results is a mixture of inflammation-related pathways and pathways more relevant to heart development and function, Supplementary Material, Table S1. Among the categories more relevant to our topic, genes such as Endothelin 1 (Ednl1), Vcam1, and Ly6e have been linked, for example, with the stimulation of cardiomyocytes, spreading of cardiomyocytes, morphology of the heart ventricle and contraction of microvessels. CXCL10 and EDN1 have also been linked with the differentiation of vascular endothelial cells, and while CXCL10 is responsible for the inhibition of endothelial cells, END1 regulates hypertrophy cell viability of cardiomyocytes. Regulator analysis suggests that many of the genes regulated in Wt1KO are interferon responsive and links the genes to Irf7, 3 and 1, and Stat1. However of those only Irf7 was upregulated by the knockdown, as further discussed below (Supplementary Material, Table S1).

Cxc10 and Ccl5 levels are upregulated in Wt1 KO epicardial cells

Six target genes were selected and validated successfully by a qRT-PCR, and the results are shown in Supplementary Material, Table S2. Next, we decided to focus our analysis on genes that were upregulated in Wt1KO (tamoxifen treated Cre\(^{+}\)) epicardial cells and encode soluble proteins whose increased expression could explain the Wt1 KO heart phenotype, in particular the chemokines CXCL10 and CCL5. CXCL10 is an angiostatic chemokine that can also regulate fibroblast cell migration (13–15). CCL5 expression is upregulated during the first days following cardiac ischemia and the administration of a neutralizing antibody anti-CCL5 reduced infarct size (16). CXCL10 and CCL5 were upregulated at both mRNA and protein levels in Wt1KO epicardial cells (Fig. 1B and C). A qRT-PCR demonstrated that levels of Cxc10 and Ccl5 increased around 4-fold in tamoxifen-treated Cre\(^{+}\) epicardial cells in comparison with ethanol (vehicle) treatment of Cre\(^{+}\) cells (Fig. 1B and Supplementary Material, Fig. S2). Cre\(^{-}\) cells were used also as a control for our experiments. We confirmed that tamoxifen treatment of Cre\(^{-}\) cells did not induce any change in Wt1, Cxc110 and Ccl5 mRNA expression (Fig. 1A and B). CXCL10 and CCL5 protein levels were also measured by an enzyme-linked immunosorbent assay using the conditioned media from epicardial cells (Fig. 1C). The upregulation of mRNA levels of Cxc110 and Ccl5 in Wt1KO epicardial cells is reflected by an upregulation at the protein level (Fig. 1C). In addition, chemokine protein levels
were also examined in total hearts of CreER<sup>+</sup>/Wt1loxP/gfp (<em>Cre</em><sup>+</sup>) and CreER<sup>2</sup>/Wt1loxP/gfp (<em>Cre</em><sup>2</sup>) mice in the presence of tamoxifen. Conditioned media from tamoxifen-treated <em>Cre</em><sup>+</sup> hearts also showed an upregulation of both chemokines (Fig. 1D and E). Taken together, these data clearly show that epicardial loss of <em>Wt1</em> results in increased <em>Cxcl10</em> and <em>Ccl5</em> expression.

Inverse correlation of <em>Wt1</em> versus chemokines <em>Cxcl10</em> and <em>Ccl5</em> during epicardium development

Given our findings demonstrating the upregulation of CXCL10 and CCL5 after <em>Wt1</em> deletion in epicardial cells, we sought to examine the role that <em>WT1</em> could play in modulating chemokine expression during epicardium development. To address this question, heart ventricles of <em>Wt1</em>gfp/+ mice at different stages of development were dissociated and cells sorted by fluorescence-activated cell sorting (FACS) (Fig. 2A). This approach makes it possible to analyze gene expression in freshly isolated GFP<sup>+</sup> enriched epicardial cells. As expected, qRT-PCR analysis showed high levels of <em>Wt1</em> and <em>Raldh2</em> mRNA expression in the GFP<sup>+</sup> population but no expression in the GFP<sup>−</sup> population, confirming the presence of epicardial cells and EPDCs in the GFP<sup>+</sup> population (Fig. 2B). Analysis of <em>Cxcl10</em> and <em>Ccl5</em> expression in freshly isolated GFP<sup>+</sup> epicardial cells at different stages of heart development demonstrated an inverse correlation of <em>Wt1</em> expression versus these two chemokines (Fig. 2C and D). On the other hand, we also observed a direct correlation of <em>Wt1</em> versus <em>Raldh2</em>, consistent with the role of WT1 in activating <em>Raldh2</em> gene expression in epicardial cells, Figure 2C (17). All these results indicate that WT1 functions as a negative regulator of <em>Cxcl10</em> and <em>Ccl5</em> chemokine expression during epicardium development.

CXCL10 and CCL5 receptor expression in embryonic hearts

Chemokines elicit their effects by binding to chemokine receptors (12). A few chemokine ligands and receptors have been characterized during early embryogenesis, although there is no information on their expression in the embryonic epicardium. To address whether the increase in CXCL10 and CCL5 expression could have a cell autonomous role or paracrine effect, we investigated the expression of the main receptors of these two chemokines in embryonic hearts. Heart ventricles of E13.5 <em>Wt1</em>gfp/+ mice were dissociated and cells sorted by FACS. qRT-PCR analysis demonstrated almost no expression of <em>Cxcr3</em>, one of the better known receptors of CXCL10, in freshly isolated embryonic hearts. A few other chemokine receptors were upregulated after <em>Wt1</em> deletion in embryonic hearts, but their role in modulating the expression of chemokines has not been investigated.
GFP+−enriched epicardial cells nor the GFP− population of cells compared with spleen as a positive control (Fig. 3A). We also analyzed the expression of Syndecan-4 (Sdc4) and Thr4, two recently identified CXCL10 receptors (15,18). Both were expressed in freshly isolated GFP+ epicardial cells and GFP− population of cells, indicating a possible cell autonomous and a paracrine effect of CXCL10 (Fig. 3B and C). We then measured the expression of Ccr5 and Ccr1, the best characterized receptors for CCL5 (12). Neither Ccr5 nor Ccr1 were found to be expressed in the GFP+ epicardial cells, but interestingly, they were expressed in GFP− sorted cells, suggesting a paracrine effect of CCL5 produced by epicardial cells (Fig. 3D and E). These analyses demonstrate the expression of conventional and non-conventional chemokine receptors in embryonic hearts.

**Effect of CXCL10 and CCL5 in epicardial cell migration and cardiomyocyte proliferation**

Wt1/KO and epicardial-specific Wt1KO mouse hearts exhibit impaired coronary vascular development (6,7). Deficient epicardial EMT in these mice contributes to a defect in the formation of EPDCs precursor cells that will differentiate into different components of the coronary blood vessels (7). Interestingly, CXCL10 is an angiostatic and antifibrotic chemokine that is able to inhibit blood vessel formation and fibroblast migration (13−15). Given the pattern of receptor expression, we investigated whether recombinant CXCL10 had any effect on epicardial cell migration. The migration of immortalized epicardial cells was assayed using a transwell membrane chamber and revealed that recombinant mouse CXCL10 was able to significantly inhibit cell migration even in the presence of the positive stimulus VEGF (Fig. 4A). In addition, the decrease in migration observed was not due to a change in proliferation or apoptosis/survival of immortalised epicardial cells (Fig. 4B and C).

The expression of the CCL5 receptors in the GFP− population of cells suggested a paracrine effect of the CCL5 produced by epicardial cells. In the heart, CCL5 has been shown to be present at a significantly elevated level following MI and ischaemia reperfusion (16). This suggests that a high CCL5 concentration may have a detrimental role in cardiomyocytes. We stained the GFP− cells with markers for cardiomyocyte cells (MF20), endothelial cells (CD31) and mature immune cells (CD3, CD11b, Ter119, CD45R/B220, Gr1). Characterization by FACS analysis of the GFP− population of cells revealed that this population of cells is highly enriched in cardiomyocyte-positive cells (83.4%), while endothelial and mature immune cells represent a 10 and 12.6%, respectively (data not shown). Thus, we wanted to analyze the direct effect of recombinant CCL5 on the proliferation of the non-cardiomyocyte population.
was also determined by the double staining and counting of MF20-negative and pH3-positive cells in the same experiments. No significant effect on the proliferation of MF20-negative cells was observed (Fig. 4E). In contrast, treatment of cardiomyocytes with CXCL10 had no effect on proliferation (data not shown).

These effects on both cell proliferation and migration, demonstrate that overexpression of the chemokines CXCL10 and CCL5 could contribute to the heart phenotype observed in Wt1KO embryos.

Increased Irf7 expression following Wt1 deletion in epicardial cells

We next aimed to identify the pathways through which WT1 regulates Cxcl10 and Ccl5 expression in epicardial cells. The increased transcription of a subset of interferon-regulated genes observed in tamoxifen treated Cre+ epicardial cells when compared with the controls suggests that WT1 functions as a repressor of the interferon response in epicardial cells. Irf7 is one of the nine members of the IRF family and is considered a major regulator of the type I IFN pathway (19). Mechanistic pathway analysis of our microarray data highlights Irf7 as activated, with a supportive score of +2.768 and a P-value of $4 \times 10^{-10}$. Amongst the genes regulated by IRF7, the pathway analysis identifies Cxcl10 and Ccl5 (20,21), but also Gbp4, Ifit3, Isg15, Mx2, Oas2, Usp18 all of them upregulated in Wt1KO epicardial cells (Supplementary Material, Table S1).

We confirmed the upregulation of Irf7 after 3 and 6 days of tamoxifen treatment in Wt1KO immortalized epicardial cells (Fig. 5A), as well as in freshly isolated epicardial cells from epicardial-specific Wt1KO mice (Fig. 5B). Heart sections from epicardial-specific Wt1KO mice also confirmed an increased expression of IRF7 protein in the epicardium (Fig. 5C).

Regulation of the expression of Cxcl10 and Ccl5 in immune cells by IRF7 has been well studied however the role of IRF7 in regulating the expression of these two chemokines in epicardial cells is unknown. To investigate this, Chromatin immunoprecipitation (ChIP) analyses of binding of IRF7 to the Cxcl10 and Ccl5 promoters were performed. ChIP analyses corroborated that IRF7 does interact with the Cxcl10 and Ccl5 promoters in epicardial cells (Fig. 5D). In addition, transient transfection of Cxcl10 and Ccl5 promoters in the presence of IRF7 demonstrated that IRF7 does exert an activator effect of both promoters in epicardial cells (Fig. 5E and F).

All these results demonstrate that Wt1 deletion in the embryonic epicardium is associated with an upregulation of Irf7 and indicate the importance of IRF7 in the regulation of Ccl5 and Cxcl10 expression in epicardial cells.

WT1 is a transcriptional repressor of Irf7 in epicardial cells

Next, we analyzed the molecular mechanism by which WT1 could be involved in the regulation of expression in epicardial cells of the IRF7-mediated interferon response pathway.
Analysis of the mouse *Irf7* genomic sequence [from 3 kb upstream to 1 kb downstream of the transcription start site (TSS)] revealed several putative WT1 binding sites (Fig. 6A). ChIP followed by qPCR using primers flanking the putative binding sites demonstrated the in vivo binding of WT1 to the regions A, B, D and E containing binding sites located at positions −2643 and −2592, −1330 and −1306, +354 and +688, respectively, with no binding to region C containing binding site +204 (Fig. 6B). We next cloned the *Irf7* promoter and a fragment containing the TSS downstream region upstream of a luciferase reporter. In agreement with the ChIP results, the luciferase reporter assays demonstrated that the −KTS WT1 isoform was able to repress in a dose-dependent manner both fragments (Fig. 6C and D). All together, these results indicate that WT1 is a repressor of *Irf7* in epicardial cells.

**DISCUSSION**

The main goal of this study was to identify new signals and pathways regulated by WT1 during epicardium development. We performed a microarray analysis to find genes differentially expressed after *Wt1* deletion in embryonic epicardial cells. Among the genes most upregulated in *Wt1* KO cells were the chemokines *Ccl5*, *Cxcl10*, and the interferon responsive gene *Irf7*. These results were validated in immortalized epicardial cells and embryonic hearts. We also observed an inverse correlation of *Wt1* expression and the chemokines *Ccl5* and *Cxcl10* during epicardium development. Chemokine receptor analyses demonstrate the differential expression of the receptors of these two chemokines in GFP<sup>+</sup> epicardial cells and cardiomyocytes.

**WT1 is involved in the direct regulation of Cxcl10 and Ccl5 in epicardial cells**

We also decided to explore whether WT1 could directly repress *Cxl10* and *Ccl5* in epicardial cells. Analyses of the mouse *Cxcl10* and *Ccl5* genomic sequences revealed the presence of two conserved WT1 binding sites in each promoter sequence (Fig. 7A). ChIP experiments demonstrated the in vivo binding of WT1 to the promoter region of *Cxcl10* and *Ccl5* genes (Fig. 7B). Thus, we transiently transfected the *Cxcl10* and *Ccl5* promoters in the presence of different amounts of the −KTS WT1 isoform in epicardial cells. We observed a clear repression of both promoters in a dose-dependent manner (Fig. 7C and D). These results lead us to propose a model, in which WT1 regulates the expression of the chemokines *Cxcl10* and *Ccl5* directly through the activation of their promoters and indirectly through increasing the levels of *Irf7*. 

---

**Figure 4.** Effect of CXCL10 and CCL5 on the migration of epicardial cells and the proliferation of cardiomyocytes. (A) Transwell migration assay of epicardial cells in the presence or absence of CXCL10. Epicardial cells were allowed to migrate in the presence or absence of 200 ng/ml of recombinant CXCL10 over 24 h. Migrated cells on the lower face were quantified. *P < 0.01 (n = 3). (B) BrdU proliferation colorimetric assay of epicardial cells treated for 24 h with CXCL10 shows no significant difference in proliferation. (C) Quantification of the percentage of Annexin V-positive epicardial cells by FACS analysis in the presence of recombinant CXCL10 shows no significant difference in cell death. Treatment with staurosporin was used as a positive control. (D) Quantification of the number of cardiomyocytes proliferating in the presence of 200 ng/ml of recombinant CCL5. Cardiomyocyte enriched primary culture cells were stained for the cardiomyocyte marker MF20 and the mitotic marker phospho-H3. MF20 and phospho-H3 double positive cells (D) and MF20-negative and pH3-positive cells were quantified (E). *P < 0.04 (n = 4).
Functional assays provided here demonstrate that CXCL10 and CCL5 are able to regulate the migration of epicardial cells and the proliferation of cardiomyocytes, respectively, suggesting a physiological function of these chemokines during heart morphogenesis. WT1 regulates the expression of the chemokines Cxcl10 and Ccl5 directly by repressing their promoters and indirectly though the regulation of Irf7. Overall, our study shows that WT1 is required for the repression of the expression of inhibitory chemokines Cxcl10 and Ccl5 during epicardium development.

One of the hallmarks of the embryonic epicardium is its ability to regulate myocardial growth and coronary blood vessel development through the secretion of paracrine factors (22). During the last 10 years, tremendous efforts have been made to identify these paracrine factors. However, all the strategies adopted have been focused on the identification of paracrine factors that are able to regulate heart morphogenesis in a positive manner. We have used a novel and distinct approach and have centered our research on the identification of soluble molecules upregulated in Wt1 KO epicardial cells that could influence heart formation in a negative manner. Interestingly, these paracrine factors upregulated in the Wt1 KO situation are expressed differently during the course of heart development suggesting a physiological role.

The analysis of the receptors for CXCL10 and CCL5 in embryonic Wt1 GFP+ hearts demonstrated a differential expression in GFP+ enriched epicardial cells and GFP− cells enriched in cardiomyocytes. Trl4 and Syndecan 4, non-conventional receptors for

Figure 5. WT1 downregulation induces an increase in Irf7 expression in embryonic epicardium. (A) qRT-PCR analysis of Irf7 mRNA levels in Cre+ epicardial cells treated with tamoxifen for 3 and 6 days. (B) qRT-PCR analysis of Irf7 in GFP+ epicardial cells of Gata5Cre+/Wt1loxP/gfp (Cre−) and Gata5Cre+/Wt1loxP/gfp (Cre+) mice. Wt1 downregulation induces a clear increase in the Irf7 mRNA levels. *P < 0.05 (n = 3). (C) Irf7 expression on sections from E15.5 Cre+ and Cre− hearts. Increased Irf7 expression is observed in the mutant epicardium when compared with the control (arrows). (D) Chromatin IP of Irf7 on Cxcl10 and Ccl5 promoters in immortalized epicardial cells. IRF7 binding was examined by ChIP with an IRF7 antibody and IgG as a negative control. *P < 0.05 (n = 3). (E and F) Luciferase reporter assays of mouse Cxcl10 (E) and Ccl5 (F) promoters in epicardial cells in the presence of indicated amounts of Irf7 expressing vector. Data show a dose-dependent activation of both promoters by the −KTS WT1 isoform. The graphs represent the mean values ± sem from three independent experiments.
CXCL10, were expressed in both GFP<sup>+</sup> and GFP<sup>−</sup> cells, suggesting an autocrine and paracrine role of this chemokine. However, the CCL5 receptors Ccr5 and Ccr1 were only expressed in GFP<sup>−</sup> cells suggesting a paracrine function of this chemokine.

Chemokines were first described as important mediators of immune responses (12), but more recently have been implicated in a wide variety of developmental processes, such as primordial germ cell migration, axon growth, lateral line formation, and endoderm development (23–25). In addition, chemokines have been shown to have a central role in tissue repair of several organs including the heart (26). However, the role of CCL5 and CXCL10 during epicardium development and the link with WT1 is hitherto unknown.

Our results demonstrated that CXCL10 is able to inhibit epicardial cell migration providing an additional mechanism for the observed reduction of EPDCs in the myocardium of the WT1 epicardial-specific KO mice (7). Recently, it has been demonstrated that the migration of cardiomyocytes is essential for the regeneration of the zebrafish heart (27). Given the fact that neonatal mammalian heart also possesses the ability to regenerate itself, it is possible that mammalian cardiomyocytes continue to migrate after epicardium formation up to the neonatal stage (28). The expression of the CXCL10 receptors in GFP<sup>−</sup> cells enriched in cardiomyocytes suggests that CXCL10 could be implicated in this process.

Moreover CXCL10 is an angiostatic chemokine that can also contribute to impairment in the formation of coronary blood vessels observed in epicardial-specific WT1 KO mice by inhibiting the recruitment of perivascular cells to the forming arteries.

Decreased cardiomyocyte proliferation contributes to myocardial hypoplasia and is a possible explanation for the thin myocardium observed in WT1 KO hearts (6,29). WT1 KO conditioned medium is able to inhibit the proliferation of cardiomyocytes (V.V. unpublished observation). In addition, recombinant CCL5 was able to inhibit cardiomyocyte proliferation. These results suggest that epicardial cells that express low levels of WT1 are also able to produce secreted factors that negatively influence the proliferation of cardiomyocytes.

IRF7 belongs to a growing family of transcription factors that were initially studied in the context of immune response (30). Their identified functions have expanded to include distinct roles in cell proliferation and differentiation (31,32). We
identified \textit{Irf7} as a transcriptional target of WT1 in epicardial cells and plausibly one of the main causes of the increase in chemokine levels. The increased expression of other IRF7 target genes like \textit{Gbp4}, \textit{Ifit3}, \textit{Isg15}, \textit{Mx2}, \textit{Oas2} and \textit{Usp18} defines a gene signature of interferon stimulated genes in \textit{Wt1} KO epicardial cells.

Our findings support a crucial role for WT1 in the repression of the chemokines \textit{Cxcl10} and \textit{Ccl5} expression in epicardial cells. The fact that WT1 also represses directly the expression of these two chemokines suggests that during the course of heart development the expression of these inhibitory chemokines is a precisely regulated process.

The increase in chemokines mRNA expression levels in epicardial cells at later stages of heart growth correlates with a decrease in epicardial cell migration, a decrease in the proliferation of cardiomyocytes and cessation in the formation of new coronary blood vessels. Interestingly, the mouse heart loses this regenerative potential within the first week of postnatal life, which coincides with a downregulation of WT1. Our results provide a novel mechanism of how epicardial cells control heart morphogenesis by producing inhibitory molecules (Fig. 8). Thus, heart formation requires a finely balanced equilibrium between stimulatory and inhibitory factors.

We hypothesize that \textit{Wt1} downregulation in epicardial cells during heart development contributes to the inhibition of the embryonic epicardial program and the acquisition of a mature quiescent stage. Analogous to this situation are the \textit{Wt1} KO epicardial cells that gained the signature of a ‘mature’ epicardium characterized by a shift to a more epithelial phenotype and upregulation of chemokines that are involved in the repression of the proliferation of cardiomyocytes and the migration of epicardial cells.

Heart failure constitutes one of the leading causes of death worldwide. An important concept emerging from this study is the production of soluble factors released by epicardial cells that are able to regulate heart growth in a negative manner. The observation that most of the embryonic pathways regulated by WT1 are reactivated after MI suggests that the function of WT1 in the repression of the interferon-regulated genes could be involved in heart repair. Evidence suggests that neutrophils, mononuclear cells, endothelial cells and pericytes contribute to the suppression and resolution of the inflammatory reaction (26). We speculate that the early activation of epicardial cells also contribute to the suppression of the inflammatory response following MI. The reactivation of \textit{Wt1} in epicardial cells of the ischemic area supports a new mechanism to repress interferon-related genes in inflammatory disease.

The \textit{Irf7} gene is not the only member of the IRF family that is regulated by WT1, as WT1 has been shown to bind the promoter and represses \textit{Irf8} expression in leukemia cells (33). Interestingly, ChIP-chip analysis of WT1 in embryonic kidneys also revealed the binding of WT1 to \textit{Irf3} and \textit{Irf2} (34). Our findings may have a much broader relevance than for the cardiovascular field alone. \textit{Wt1} is expressed at high levels in breast cancers patients with poorer prognosis and recently silencing of IRF7 pathways has been linked with bone breast cancer metastasis (35,36).
MATERIALS AND METHODS

Epicardial specific Wt1 knock-out mice

The epicardial-specific Wt1 KO mice (Gata5Cre+/Wt1loxP/gfp), the tamoxifen inducible Wt1KO mice (CAGG-CreER; Wt1loxP/loxP) and Wt1gfp/+ mice have been previously described (7,37,38). All animal experiments were approved by the University of Edinburgh ethical committee.

Generation of E11.5 immortalized epicardial cells

Tamoxifen-inducible Wt1 knock-out immortalized epicardial cell lines (Cre+; CreER+/Wt1loxP/gfp) and control (Cre−; CreER−/Wt1loxP/gfp) cell lines were generated by crossbreeding Wt1gfp/+Immorto+/− mice with mice that were homozygous Wt1 conditional and heterozygous for CAGG promoter-driven CreERTM (CAGG-CreER; Wt1loxP/loxP) (7,37). Briefly, heart ventricles from E11.5 CreER+/Wt1loxP/gfp /Immorto+/− and CreER−/Wt1loxP/gfp /Immorto+/− were placed in 24-well gelatine dishes, epicardial cells were allowed to attach and the ventricles removed. Cells were allowed to reach confluence and propagated at 33°C.

Isolation of FACS-sorted GFP+ epicardial cells and GFP− cells

The heart ventricles of Wt1gfp+ mice and Gata5 Cre+/Wt1loxP/gfp and Gata5 Cre−/Wt1loxP/gfp mice were dissected at different stages of development from E11.5 to E16.5. Ventricles were treated with a trypsin:versene solution, in a 1:10 ratio, in a 37°C heated shaking block at 1000 rpm. FACS sorting of GFP populations was carried out by gating against a littermate GFP-negative control. Cells were analyzed in graphs of SSC against GFP. RNA isolated from FACS-sorted GFP+ epicardial cells was reverse-transcribed using a first-strand cDNA kit for RT-PCR (Roche).

Microarray analysis of tamoxifen-inducible Wt1KO immortalized epicardial cells

Tamoxifen inducible Cre+ and Cre− epicardial cells, described above, were treated with 100 nM tamoxifen solution diluted in ethanol for 6 days. Cells were treated with equivalent amounts of ethanol as a control. Wt1 gene knock-out expression was confirmed by a qRT-PCR. RNA from Cre+ samples was extracted (Qiagen), DNase treated (Qiagen) and quality analyzed by the microfluidics-based platform Agilent 2100 Bioanalyzer. Ambion’s Illumina TotalPrep RNA Amplification Kit and 200 ng of RNA with RNA integrity number (RIN) over 8/10 were used to generate amplified biotinylated cRNA for mouse transcriptome analyses. AffymetrixGeneChip Mouse Genome 430 2.0 arrays were used for this analysis. Expression values were summarized using RMA and differences between the two sets of samples were assessed with the T test (P value 0.05, |Fold| > = 1.4). Functional analysis of the genes modulated in the dataset was done with IPA ingenuity; results for gene networks, upstream regulators, toxicological pathways,
biological pathways and canonical pathways are summarized in Supplementary Material, Table S1.

**Real time PCR analysis**

Analysis of gene expression was carried out by Taqman® quantitative real-time PCR. The expression levels of interested genes were normalized to the housekeeping gene Gapdh as an internal control (Roche). Primers used are listed in Supplementary Material, Table S3. Unless stated data are shown as the average of three biological replicates. Relative values were calculated using an internal standard curve. Values were considered significant where Student’s t-test P-value falls <0.05. Error bars are ± standard error.

**Isolation of cardiomyocytes from E11.5 CD1 mouse ventricles**

E11.5 CD1 mouse ventricles were dissected and treated with a trypsin:versene solution, in a 1:10 ratio, in a 37°C heated shaking block at 1000 rpm. After the first 10 min incubation the supernatant was replaced, and every subsequent supernatant was kept in DMEM medium containing 10% fetal calf serum. Medium containing cells was added to a cell culture plate and incubated at 37°C for 2 h. Cells that have not attached to the plate were collected, counted and 50 000 cells were added per well in a 24-well plate containing coverslips treated with 0.1% gelatin and incubated for an additional 24 h period. Then, the plate containing coverslips treated with 0.1% gelatin was added to a 96-well plate and allowed to grow overnight. Ten thousand immortalized epicardial cells were seeded per well in a 1:100 dilution for 1 min. Slides were mounted and counterstained with DAPI (Vector Laboratories).

**Ex vivo heart culture**

For ex vivo experiments, 0.8% agar solution was prepared using sterile phosphate buffered saline (PBS). Fifty microliter of the dissolved agar solution was added to a 96-well plate and allowed to set at room temperature. Ventricles were dissected from E11.5 tamoxifen inducible Cre+ (CAGG-CreErt2; Wt1loxP/GFP) and Cre− (CAGG-CreErt2; Wt1loxP/GFP) mice. Ventricles were washed in cold PBS and added to a 96-well plate containing agar. PBS was removed and 150 μl of DMEM medium containing 10% serum and 0.03% sodium azide and incubated with sections overnight at 4°C. Biotinylated anti-rabbit secondary Ab (Vector) was diluted in PBS containing 2% ovalbumin, 1% goat serum, and 2% ovalbumin in PBS for 1 h at room temperature. Endogenous Biotin was blocked using Avidin/Biotin block solutions (Vector Laboratories). Sections were washed with PBST (0.05% Tween-20 in PBS) for 5 min in between every step. Endogenous peroxidase activity was quenched by adding 20% H2O2 with 10% methanol in PBS. Sections were blocked with blocking solution containing 0.1% glycine, 2% goat serum, 2% horse serum and 2% ovalbumin in PBS for 30 min at room temperature. DAPI (Vector Laboratories) was diluted in PBS containing 0.1% glycine, 2% goat serum, 2% horse serum and 2% ovalbumin in PBS for 30 min at room temperature. Endogenous Biotin was blocked using Avidin/Biotin block solutions (Vector Laboratories) as per manufacturer’s instructions. Anti-IRF7 Ab (Santa Cruz) was diluted in PBS containing 2% ovalbumin, 1% goat serum, and 0.03% sodium azide and incubated with sections overnight at 4°C. Biotinylated anti-rabbit secondary Ab (VECTASTAIN®) was diluted in PBS containing 5% BSA, 1:400 and incubated with sections for 2 h. A VECTASTAIN® ABC kit was subsequently used (standard kit PK-4000), an ABC complex was prepared 30 min before use and incubated with sections for 20 min. Tyramide-Cyn3 was then used (TSA tyramide-cyn3 amplification kit, Perkin Elmer NEL 744) and incubated at a 1:100 dilution for 1 min. Slides were mounted and counterstained with DAPI (Vector Laboratories).

**CXCL10 and CCL5 ELISAs**

CXCL10 and CCL5 levels were measured in epicardial cell culture medium and ex vivo heart culture supernatants using CXCL10 and CCL5 ELISA kits from R&D Systems (Mouse CXCL10 ELISA DuoSet Catalog number DY466 and CCL5 catalog number DY478).

**Colorimetric BrdU proliferation assay**

Ten thousand immortalized epicardial cells were seeded per well in a 96-well plate and allowed to grow overnight. Cells were incubated for an additional 24 h period in control medium (DMEM, 10% FCS and 1% Penicillin/Streptomycin) or control medium with recombinant CXCL10. A bromodeoxyuridine (BrdU) colorimetric assay was purchased from Calbiochem® catalog number QIA58 and the assay was carried out according to the manufacturer’s protocol.

**Apoptosis assay**

Annexin V staining for FACS analysis (Annexin V-APC apoptosis kit, BD Biosciences) was used to assay apoptosis of immortalized epicardial cells. Treatment with Staurosporin (Sigma) at a concentration of 1 μg/ml for 3 h was used as a positive control for apoptosis. The assay was carried out as indicated by the manufacturer.

**Tyramide signal amplification of IRF7 staining**

Paraffin-embedded serial 7 μm sections were deparaffinized and hydrated, followed by antigen retrieval with 10 min citrate buffer, pH6, for 3 min and allowed to cool for 10 min. Sections were washed with PBST (0.05% Tween-20 in PBS) for 5 min in between every step. Endogenous peroxidase activity was quenched by adding 20% H2O2 with 10% methanol in PBS. Sections were blocked with blocking solution containing 0.1% glycine, 2% goat serum, 2% horse serum and 2% ovalbumin in PBS for 30 min at room temperature. Endogenous Biotin was blocked using Avidin/Biotin block solutions (Vector Laboratories) as per manufacturer’s instructions. Anti-IRF7 Ab (Santa Cruz) was diluted in PBS containing 2% ovalbumin, 1% goat serum, and 0.03% sodium azide and incubated with sections overnight at 4°C. Biotinylated anti-rabbit secondary Ab (VECTASTAIN®) was diluted in PBS containing 5% BSA, 1:400 and incubated with sections for 2 h. A VECTASTAIN® ABC kit was subsequently used (standard kit PK-4000), an ABC complex was prepared 30 min before use and incubated with sections for 20 min. Tyramide-Cyn3 was then used (TSA tyramide-cyn3 amplification kit, Perkin Elmer NEL 744) and incubated at a 1:100 dilution for 1 min. Slides were mounted and counterstained with DAPI (Vector Laboratories).

**Irf7 luciferase assay**

The Irf7 promoter and the fragment containing the Irf7 TSS downstream region were amplified by PCR from mouse genomic DNA and cloned into the pGL4.10 vector (Promega). Briefly, primers were designed with an extra Nhel and Xhol, Xhol and HindIII restriction sites, respectively, to facilitate cloning (Supplementary Material, Table S3). Digested PCR products were cloned into the corresponding sites upstream of the firefly luciferase in the pGL4.10 vector. The mouse Cxcl10 and Ccl5 promoter plasmids were kindly provided by Professor...
Daniel A. Muruve (University of Calgary, Canada) and Xiaojing Ma (Weill Cornell Medical College, New York), respectively (40,41). The reporter constructs (250 ng) were transfected in immortalized epicardial cells, in the presence of the indicated amounts of expression construct encoding – KTS Wt1 isoform and pUNO-miR7 expression vector (Invivogen). The total amount of transfected DNA was normalized with a neomycin containing plasmid. A renilla plasmid was also cotransfected as a control for efficiency. Twenty-four hour after transfection, firefly luciferase and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega).

**ChIP assay**

Control and Wt1 KO immortalized epicardial cells at 70% confluence were fixed with 1% formaldehyde (room temperature, 10 min) and stopped with 0.125M Glycine. Cells were immediately centrifuged and washed with ice-cold PBS. Ten milliliter lysis buffer (5 mM HEPES, 85 mM KCl, 0.5% NP-40 and protease inhibitor cocktail set III from Calbiochem) was added and incubated for 10 min on ice. The nuclei pellet was resuspended in 0.3 ml of sonication buffer per 2 x 10^6 cells (50 mM Tris – Cl, 10 mM EDTA, 1% SDS and protease inhibitor). DNA was sonicated into 200–500 bp fragments using a Diagenode Bioruptor. Chromatin lysates were centrifuged (10 min, 13000 rpm, 4°C) and supernatants were used for immunoprecipitation. Magnetic beads (Pierce® A/G) were incubated with 10 µg of the antibodies Wt1 (rabbit polyclonal Ab, C-19 X, Santa Cruz Biotechnology), 5 µg IRF7 (rabbit mAb, abcam 109255) and rabbit mlgG(DA1E Cell signaling). Immunoprecipitations with cross-linked chromatin were carried out overnight at 4°C using antibody prebound magnetic beads. Beads were then washed with a high salt wash buffer (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% TritonX-100, 0.1% Na deoxycholate) six times. Bound complexes were eluted with 0.3 ml of 50 mM Tris – HCl, 10 mM EDTA, 1% SDS and proteinase-K (Sigma, 200 µg). Immunoprecipitated and input DNA were isolated by phenol:chloroform extraction followed by ethanol precipitation. For relative quantification by real-time PCR, dilutions of input DNA were normalized with a neomycin resistance plasmid. A renilla plasmid was also cotransfected as a control for efficiency. Twenty-four hour after transfection, firefly luciferase and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**ACKNOWLEDGMENTS**

We thank Elisabeth Freyer for assistance and running the FACs facility (MRC HGU, IGMM, Edinburgh). We also thank Paul Perry and Matt Pearson (MRC HGU, IGMM, Edinburgh) for assistance with image capturing, Philippe Gautier for the bioinformatic analysis of gene promoters (MRC HGU, IGMM, Edinburgh), Craig Nicol for assistance with graphic (Fig. 8) (MRC HGU, IGMM, Edinburgh) and Ramón Muñoz-Chapuli for critical reading of the manuscript.

**REFERENCES**


