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

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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/+ mice 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 Wt1KO mice and showed a defect in the formation of the coronary blood vessels associated with a reduced ventricular myocardium (7). This suggests that Wt1KO 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 the deletion of Wt1 and importantly the Wt1KO heart phenotype. Finally, we describe the molecular mechanism by which WT1 regulates chemokine expression in epicardial cells through the direct transcriptional repression of Cxcl10 and Ccl5 and indirectly through the regulation of the Irf7 gene, also supported by the microarray analysis.

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<sup>+</sup>/Wt1<sup>loxP/loxP</sup>) and control (CreER<sup>−</sup> / Wt1<sup>loxP/loxP</sup>) epicardial cells referred to as Cre<sup>+</sup> and Cre<sup>−</sup> from here on. Briefly, Cre<sup>−</sup> and control Cre<sup>−</sup> cell lines were generated by cross-breeding Wt1<sup>floplflopl</sup>/Immorto<sup>−/−</sup> mice, with mice that were homozygous Wt1<sup>−/−</sup> and heterozygous for CAGG promoter-driven CreER<sup>TM</sup> (CAGG-CreER; Wt1<sup>loxP/loxP</sup>). This strategy allows us to generate Cre<sup>+</sup> and Cre<sup>−</sup> 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<sup>+</sup> cells, whereas there was no change in expression levels of Wt1 in Cre<sup>−</sup> cells (Fig. 1A).

We performed microarray-based expression analysis of transcriptional changes associated with Wt1 deletion in Cre<sup>+</sup> 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, Ccl7, 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, Ms2, 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 (Edn1), 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<sup>+</sup>) 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 angiotrophic chemokine that can also regulate fibroblast cell migration (13–15). CCL5 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 Supplementary Material, Fig. S2). A qRT-PCR demonstrated that levels of Cxcl10 and Ccl5 were upregulated in both Wt1KO and control cells (Fig. 1A and B). CXCL10 and CCL5 were also measured by an enzyme-linked immunoassay using the conditioned media from epicardial cells (Fig. 1C). The upregulation of mRNA levels of Cxcl10 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+
/wt1loxP/gfp (Cre+) and CreER2/wt1loxP/gfp (Cre2) mice in the presence of tamoxifen. Conditioned media from tamoxifen-treated Cre+
hearts also showed an upregulation of both chemokines (Fig.1D and E).

Taken together, these data clearly show that epicardial loss of Wt1 results in increased Cxcl10 and Ccl5 expression.

Inverse correlation of Wt1 versus chemokines Cxcl10 and Ccl5 during epicardium development

Given our findings demonstrating the upregulation of CXCL10 and CCL5 after Wt1 deletion in epicardial cells, we sought to examine the role that WT1 could play in modulating chemokine expression during epicardium development. To address this question, heart ventricles of Wt1gfp/+ 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+ enriched epicardial cells. As expected, qRT-PCR analysis showed high levels of Wt1 and Raldh2 mRNA expression in the GFP+ population but no expression in the GFP− population, confirming the presence of epicardial cells and EPDCs in the GFP+ population (Fig. 2B). Analysis of Cxcl10 and Ccl5 expression in freshly isolated GFP+ epicardial cells at different stages of heart development demonstrated an inverse correlation of Wt1 expression versus these two chemokines (Fig. 2C and D). On the other hand, we also observed a direct correlation of Wt1 versus Raldh2, consistent with the role of WT1 in activating Raldh2 gene expression in epicardial cells, Figure 2C (17). All these results indicate that WT1 functions as a negative regulator of Cxcl10 and Ccl5 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 Wt1gfp/+ mice were dissociated and cells sorted by FACS. qRT-PCR analysis demonstrated almost no expression of Cxcr3, one of the better known receptors of CXCL10, in freshly isolated
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 Tlr4, 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

Wt1KO 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 ischemia 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 cardiomyocyte proliferation. Since we used a primary enriched cardiomyocyte culture (Supplementary Material, Fig. S3), cells were stained for the cardiomyocyte marker MF20 and the mitotic marker phospho-H3, and cells that were positive for both markers were counted. We found a significant decrease of 21% in the number of proliferating cardiomyocytes (Fig. 4D) after treatment with CCL5. The 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 Wt1 KO 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.

Figure 3. Analysis of the expression of chemokine receptors in Wt1gfp/+ embryonic hearts. (A–E) qRT-PCR analysis of expression of chemokine receptors in the GFP sorted cells isolated from of Wt1gfp/+ embryonic hearts at E13.5 days and in the spleen as a positive control. The analyses demonstrate the expression of nonconventional CXCL10 receptors, Sdc4 and Tlr4, in both GFP+ and GFP− cells, while the conventional receptors for CCL5, Ccr1 and Ccr5 were expressed in GFP− cells.
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.

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

We also decided to explore whether WT1 could directly repress Cxcl10 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.

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 Cxcl10 and Ccl5 during epicardium development. Chemokine receptor analyses demonstrate the differential expression of the receptors of these two chemokines in GFP+ epicardial cells and cardiomyocytes.
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 through 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. 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^gr^ hearts demonstrated a differential expression in GFP^+^-enriched epicardial cells and GFP^-cells enriched in cardiomyocytes. Trl4 and Syndecan 4, non-conventional receptors for

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**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 isomorf. 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 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 Gbp4, Ifit3, Isg15, Mx2, Oas2 and Usp18 defines a gene signature of interferon stimulated genes in Wt1 KO epicardial cells. Our findings support a crucial role for WT1 in the repression of the chemokines Cxcl10 and 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 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 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 Wt1 in epicardial cells of the ischemic area supports a new mechanism to repress interferon-related genes in inflammatory disease.

The 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 Irf8 expression in leukemia cells (33). Interestingly, ChIP-chip analysis of WT1 in embryonic kidneys also revealed the binding of WT1 to Irf3 and Irf2 (34). Our findings may have a much broader relevance than for the cardiovascular field alone. 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).
Epicardial specific \textit{Wt1} knock-out mice

The epicardial-specific \textit{Wt1} knockout mice (\textit{Gata5Cre}+/\textit{Wt1loxP/gfp}), the tamoxifen inducible \textit{Wt1}KO mice (\textit{CAGG-CreER}; \textit{Wt1}loxP/loxP) and \textit{Wt1}gfp/+ 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 \textit{Wt1} knock-out immortalized epicardial cell lines (\textit{Cre}+) (\textit{CreER}+/\textit{Wt1loxP/gfp}) and control (\textit{Cre}−) (\textit{CreER}−/\textit{Wt1loxP/gfp}) cell lines were generated by crossbreeding \textit{Wt1} conditional and heterozygous \textit{Wt1} immortal /Immorto+− mice with mice that were homozygous \textit{Wt1} conditional and heterozygous for CAGG promoter-driven \textit{CreERTM} (\textit{CAGG-CreER}; \textit{Wt1}loxP/loxP) (7,37). Briefly, heart ventricles from E11.5 \textit{CreER}+/\textit{Wt1loxP/gfp} /Immorto+− and \textit{CreER}−/\textit{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 \textit{Wt1}gfp+ mice and \textit{Gata5} \textit{Cre}+/\textit{Wt1oxP/gfp} and \textit{Gata5} \textit{Cre}−/\textit{Wt1oxP/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 \textit{Wt1}KO immortalized epicardial cells

Tamoxifen inducible \textit{Cre}+ and \textit{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. \textit{Wt1} gene knock-out expression was confirmed by a qRT-PCR. RNA from \textit{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 (\textit{P} value 0.05, \textit{|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 at 37°C overnight (39). The supernatant was disposed of, the trypsin:versene solution was added to a cell culture plate and incubated in DMEM medium containing 10% fetal calf serum. Medium was then replaced and every subsequent supernatant was kept 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 at 37°C overnight.

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-CreER<sup>+</sup>; Wt<sup>loxP/GFP</sup>) and Cre<sup>+</sup> (CAGG-CreER<sup>+</sup>; Wt<sup>loxP/GFP</sup>) mice. Ventricles were washed in cold PBS and added to a 96-well plate containing set agar. PBS was removed and 150 μl of DMEM medium containing 10% FCS and 100 nm tamoxifen was added. Samples were incubated at 37°C. Conditioned media were collected on Day 6 and stored at -20°C until analyzed.

Transwell migration assay of immortalized epicardial cells
Immortalized epicardial cells were serum-deprived in DMEM medium containing 0.2% BSA for 24 h. Tryptsinised cells were seeded on gelatine coated 12-well transwell membranes with 8 μm pores at a density of 8 × 10<sup>4</sup> cells per well and allowed to migrate toward control medium, containing 20% FCS and 10 ng/ml VEGF, or control medium with 200 ng/ml CXCL10 overnight at 37°C. Transwell membranes were fixed with 4% paraformaldehyde and stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) containing mounting medium. Images were acquired with Zeiss Axiovert microscope.

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<sup>®</sup> 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 mM 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% H<sub>2</sub>O<sub>2</sub> with 10% methanol in PBS. Sections were blocked with blocking solution containing 0.1 mg glycine, 2% goat serum, 2% horse serum and 2% ovalbumin in PBS for 1 h 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 (VECTOR<sup>®</sup>) was diluted in PBS containing 5% BSA, 1:400 and incubated with sections for 2 h. A VECTASTAIN<sup>®</sup> 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 XhoI, XhoI 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. Murome (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-ctMRF7 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 0.125 M glycine. Cells were immediately centrifuged and washed with ice-cold PBS. Ten millilitre 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 × 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 were used to construct standard curves. PCRs were run in 0.3 ml of sonication buffer for 30 cycles.

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