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Fli-1 transcription Factor Affects Glomerulonephritis Development by Regulating Expression of Monocyte chemoattractant protein-1 in Endothelial Cells in the Kidney

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Abstract

Expression of transcription factor Fli-1 is implicated in the development of glomerulonephritis. Fli-1 heterozygous knockout (Fli1^{+/-}) NZM2410 mice, a murine model of lupus, had significantly improved survival and reduced glomerulonephritis. In this study, we found that infiltrated inflammatory cells were significantly decreased in the kidneys from Fli-1^{+/-} NZM2410 mice. The expression of Monocyte chemoattractant protein-1 (MCP-1) was significantly decreased in kidneys from Fli-1^{+/-} NZM2410 mice. The primary endothelial cells isolated from the kidneys of Fli-1^{+/-} NZM2410 mice produced significantly less MCP-1. In endothelial cells transfected with specific Fli-1 siRNA the production of MCP-1 was significantly reduced compared to cells transfected with negative control siRNA. By Chromatin Immunoprecipitation (ChIP) assay, we further demonstrated that Fli-1 directly binds to the promoter of the MCP-1 gene. Our data indicate that Fli-1 impacts glomerulonephritis development by regulating expression of inflammatory chemokine MCP-1 and inflammatory cell infiltration in the kidneys in the NZM2410 mice.

Keywords

Glomerulonephritis; inflammatory chemokines; infiltrations; transcription factors

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with a wide spectrum of clinical and immunological abnormalities (1–2). Glomerulonephritis is a major cause of

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Conflict of interest statement

The authors have no financial conflicts of interest.

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death in both SLE human patients and animal models (1–5). It has been well demonstrated that infiltration of inflammatory cells, including dendritic cells, macrophages, T cells and B cells, into the kidneys plays a critical role in the development of glomerulonephritis (5–7). Proinflammatory cytokines and chemokines such as MCP-1 produced in kidneys have been shown to play a critical role in infiltration of inflammatory cells into the kidneys and glomerulonephritis development (8). MCP-1, a member of the chemokine family of inflammatory mediators and also known as Chemokine (C-C motif) ligand 2 (CCL2), recruits macrophages, B cells, and T cells into the kidney and is a key mediator of glomerulonephritis in murine models of SLE (6, 8–9). MCP-1 deficient MRL/MpJ-Fas^{lpr} (MRL/*lpr*) mice, a murine model of SLE, have dramatically reduced macrophage and T cell infiltration in the kidney, decreased proteinuria, limited renal disease, and significantly prolonged survival (8). Furthermore, pharmacologically blocking MCP-1 significantly decreases renal disease in murine lupus (10). Several reports have demonstrated that following an immune complex deposition and before inflammatory cell infiltration, proinflammatory cytokine and chemokine production was upregulated in the kidneys in murine models of SLE, MRL/*lpr* mice as well as MZB/W F1 mice (6–7). Specifically within glomerular mesangium and in sub-endothelial areas, MCP-1 in MRL/*lpr* mice was up-regulated prior to inflammatory cell infiltration into kidneys (6–7). In addition, recent studies also suggest a key role for MCP-1 because its urinary excretion was increased in human lupus nephritis development (11–13).

The Fli-1 gene was first characterized in 1991 and belongs to the Ets gene family of transcription factors (14–15). Members of the Ets gene family are widely conserved in genomes of diverse organisms, including *Drosophila*, *Xenopus*, sea urchin, chicken, mouse, and human (16–18). Expression of Fli-1 was found in endothelial cells, fibroblasts and immune cells, including T cells and B cells. Several reports have demonstrated that expression of Fli-1 protein is implicated in SLE development in both human patients and murine models (19–22). In humans, overexpression of the Fli-1 gene has been detected in peripheral blood lymphocytes of SLE patients compared to normal healthy controls, and the level of Fli-1 expression paralleled clinical activity measures of SLE (19). Furthermore, Fli-1 transgenic mice developed a lupus-like disease (20). We have generated Fli-1 heterozygous knockout (Fli-1^{+/-}) MRL/*lpr* mice and NZM2410 mice with decreased expression of Fli-1 protein to study the role of Fli-1 in SLE development (21–22). Both MRL/*lpr* mice and NZM2410 mice share many clinical manifestations found in human SLE (4–5). Compared to littermate wild-type mice, we have found that both Fli-1^{+/-} MRL/*lpr* and NZM2410 mice had significantly lower serum autoantibodies, lower proteinuria, reduced pathologic renal disease and markedly prolonged survival (21–22). In this report, we found that expression of proinflammatory chemokine MCP-1 was significantly lower in the kidneys from Fli-1^{+/-} NZM2410 mice compared to wild-type littermate controls at the pre-disease stage. In addition, endothelial cells isolated from the kidneys of Fli-1^{+/-} NZM2410 mice produced significantly less MCP-1 compared to renal endothelial cells from wild-type littermate controls. We further demonstrated that Fli-1 directly binds the promoter region of MCP-1 in endothelial cells, and inhibition of expression of Fli-1 with Small interfering RNA (siRNA) resulted in significantly reduced production of MCP-1 in endothelial cells. Thus, Fli-1 transcription factor affects glomerulonephritis development by regulating expression of inflammatory chemokine in endothelial cells in the kidneys of NZM2410 mice.

2. Material and Methods

2.1. Mice

NZM2410 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Fli-1^{+/-} NZM2410, and wild-type littermates mice used in the study were generated by backcrossing

with Fli-1^{+/-} B6 mice for 12 generations as previously reported (22). All mice were housed under pathogen-free conditions at the animal facility of the Ralph H. Johnson Veterans Affairs Medical Center.

2.2. Genotyping of the mice by PCR

For genotyping of the mice, PCR was used to detect fragments of wild-type and Fli-1^{+/-} alleles as previously reported (21). The primers for PCR were as follows: Fli-1 exon IX/forward primer (positions 1156 to 1180), GACCAACGGGGAGTTCAAATGACG; Fli-1 exon IX/reverse primer (positions 1441 to 1465), GGAGGATGGGTGAGACGGGACAAAG; and Pol II/reverse primer, GGAAGTAGCCGTTATTAGTGGAGAGG. DNA was isolated from tail snips (4-week old mice) using a QIAamp Tissue kit (Qiagen, Santa Clarita, CA). PCR analyses were performed under the following conditions: 1 cycle at 95 °C for 5 min, followed by 36 repeating cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min followed by 72 °C for 7 min. A 309-bp fragment indicates the presence of the wild-type allele, and a 406-bp fragment is amplified from the mutated allele.

2.3. Cell line

Murine endothelial cell line MS1 was purchased from the American Type Culture Collection (ATCC) and maintained with DMEM medium with 5% fetal bovine serum.

2.4. Isolation of Endothelial cells from kidney

Endothelial cells were isolated from mouse kidney by immunomagnetic separation as previously described (23). Briefly, kidneys were collected from euthanized mice and homogenized using a sterile razor. Next, the kidneys were incubated with 5ml of DMEM medium with 1mg/ml collagenase A for 30 minutes at 37 °C. The samples were then placed on ice and 5ml cold EBM media (Lonza, Switzerland) was added. The samples were sieved through a 70-µm cell strainer then washed twice with EBM media. The samples were incubated in media with 10µg/ml anti-mouse CD31 biotin conjugated antibody for 30 minutes on ice (eBioscience, CA). After the incubation, cells were washed twice with EBM media. Finally, the endothelial cells were selected by magnetic separation following manufacturer's directions with anti-biotin MACSi Bead Particles (Miltenyi Biotec, Germany). Cells were cultured on T25 cm² flasks with EBM media. After 5 days in culture, or when the cells were about 80% confluent, the cells were passaged, and the purity of the culture was verified by the Di-Ac-LDL uptake assay following manufacturer's directions (Biomedical Technologies Inc. Stoughton, MA).

2.5. Western blot analysis

MS1 cells were lysed with radioimmunoprecipitation assay buffer, and immunoblots were performed as described previously (21).

2.6. Chemokine measurement

Concentrations of MCP-1 in the supernatants were determined by ELISA using a kit from eBioscience. The assays were performed using the manufacturer's instructions.

2.7. Measurement of MCP-1 expression in kidneys by Real-time PCR

Total RNA was prepared from kidneys from Fli-1^{+/-} NZM2410 mice and wild-type littermates at the age of 18 weeks. 2µg of RNA was used to synthesize cDNA with SuperScript First-Strand Synthesis System (Invitrogen, CA). Real-time PCR was performed in duplicate using Platinum SYBR Green qPCR SuperMix UDG (Bio-Rad) according to the manufacturer's instruction, with three independent RNA preparations. The primers for the

MCP-1 gene and the house keeper gene GAPDH were purchased from SABiosciences, and the cycling conditions for all genes followed instructions from the company. PCR was done using the LightCycler (Roche) and relative expression analysis was conducted using the program provided by SABiosciences.

2.8. Immunofluorescence Staining and Immunohistochemical Staining

Sections (5 μ M) cut from frozen kidneys were used for immunofluorescence staining. All sections were blocked with PBS with 10% normal rat serum and incubated with fluorescently labeled antibodies for 1 hour. The antibodies used in this study were as follows: anti-CD3, anti-CD19, anti-CD11c, anti-CD11b (BD Biosciences) and anti-neutrophil (AbD Serotec, UK). Positive cells were counted from 10 randomly selected glomeruli and tubules per section in a blinded fashion.

For immunohistochemistry staining, a frozen section was fixed with acetone for 5 min and blocked with PBS containing 10% normal goat serum. The sections were incubated with rabbit anti-MCP-1 primary antibodies, and color was revealed with VECTASTAIN ABC System from Vector Lab (Burlingame, CA). Immunofluorescence staining was used to co-localizing the MCP-1 and endothelial cells. The kidney section was stained with hamster monoclonal antibodies against MCP-1 and rat monoclonal antibodies against CD31 (Biolegend, CA) overnight at 4°C. The section was stained with Fluorescein isothiocyanate (FITC)-goat anti-hamster and rhodamine-labeled goat anti rat secondary antibodies for 1 hour (Jacksonimmunoresearch, PA). Nuclei were counterstained with DAPI. The staining was observed and photographed using a Nikon Eclipse 80i microscope equipped with a digital camera.

2.9 siRNA transfection

Specific Fli-1 siRNA and negative control siRNA were purchased from Invitrogen Inc. and transfected into the MS1 cells following the manufacturer's instruction.

2.10. Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed as we have described previously using anti-Fli-1 rabbit polyclonal antibody (24–25). The primers used for ChIP assay are listed in Table I.

2.11. Statistics

The unpaired Student's t-test was used to test for significant differences between groups. A $p < 0.05$ was considered to be statistically significant. The Mann-Whitney test was used when appropriate. Survival significance was determined via the analysis of survival curve with Prism software from GraphPad Software, Inc. (San Diego, CA)

3. Results

3.1. Significantly reduced infiltration of inflammatory cells in kidneys from Fli-1^{+/-} NZM2410 mice

We found that decreased expression of Fli-1 resulted in significant prolonged survival and reduced renal pathological scores in Fli-1^{+/-} NZM2410 mice compared to wild-type littermate controls (22). Since infiltration of inflammatory cells into kidneys plays an important role in glomerulonephritis development, we quantitated and compared the inflammatory cells in kidneys from Fli-1^{+/-} NZM2410 mice and wild-type littermates. 18 Fli-1^{+/-} NZM2410 mice and 21 wild-type littermate controls were sacrificed at the age of 34 weeks, and kidneys were removed from these mice to make frozen sections. Frozen sections were stained with specific anti-CD3, CD19, CD11b, CD11c or Ly-6B.2 (neutrophil

marker), and positive cells were counted from 10 random sections. Table II shows a statistically significant decrease in all inflammatory cells in the kidneys from Fli-1^{+/-} NZM2410 mice compared to wild-type controls. Ly-6B.2⁺ cells decreased 44% and all other inflammatory cells decreased more than 50% in the kidneys from Fli-1^{+/-} NZM2410 mice compared to wild-type controls. Notably, there was a 70% decrease in CD19⁺ cells in Fli-1^{+/-} NZM2410 mice compared to wild-type controls.

3.2. Expression of MCP-1 in kidneys from Fli-1^{+/-} NZM2410 mice was significantly decreased and Fli-1^{+/-} NZM2410 mice had decreased MCP-1 in serum

MCP-1 plays a critical role in the infiltration of inflammatory cells, and production of MCP-1 was reduced in T cells from Fli-1^{+/-} MRL/lpr mice (8–9, 21). Next, we examined if the expression of MCP-1 in the kidneys was decreased in Fli-1^{+/-} NZM2410 mice compared to wild-type littermate controls. To exclude the influence of inflammatory cells on expression of MCP-1 in kidneys, the kidneys were removed from mice at the age of 18 weeks, a pre-disease stage. Expression of MCP-1 was measured by RT PCR. Figure 1 shows more than 50% significant reduction ($p < 0.05$) of MCP-1 mRNA expression in Fli-1^{+/-} NZM2410 mice when compared to wild-type controls. Next, we measured serum MCP-1 concentrations in the Fli-1^{+/-} and wild-type NZM2410 mice. As shown in Fig. 2, serum MCP-1 levels were significantly lower in the Fli-1^{+/-} NZM2410 mice at the ages of 34 weeks compared with wild-type littermates ($p < 0.05$, Fig. 2).

3.3. Expression of MCP-1 was mainly located in glomeruli in kidneys

Next, we examined MCP-1 expression in kidney by immunohistochemical staining. The frozen sections of kidneys were removed from NZM2410 mice at the age of 34 weeks and were fixed with acetone and stained with anti-MCP-1 antibodies, strong positive staining was observed in glomeruli. There was no any staining with normal rabbit Ig (supplementary Fig. 1). Co-localization of endothelial cells and MCP-1 in kidneys was confirmed by immunofluorescence staining with MCP-1 and endothelial cells marker CD31 (supplementary Fig. 1, D–F). Pre-incubation of MCP-1 antibodies with specific antigen abolished staining, demonstrating the specificity of the immunohistochemical staining (data not shown).

3.4. Primary endothelial cells from Fli-1^{+/-} NZM2410 mice produce significantly less MCP-1

To establish a link between the strong expression of MCP-1 in the glomeruli and Fli-1, we examined Fli-1 expression levels in the 3 cell types primarily found in the glomerulus: mesangial cells, podocytes, and endothelial cells. As a result, we found that only endothelial cells express high detectable levels of Fli-1 mRNA (data not shown). Next, we isolated primary endothelial cells from kidneys of Fli-1^{+/-} NZM2410 and wild-type controls at 8 weeks of age as described in the materials and methods section. The purity of cells was verified by the Di-Ac-LDL uptake assay. Almost all of the cells showed positive incorporation of Di-Ac-LDL (data not shown). The expression of Fli-1 in unstimulated endothelial cells from Fli-1^{+/-} NZM2410 mice were significantly lower compared to the cells from wild-type littermate controls ($p < 0.05$, Fig. 3). Furthermore, a significant reduction of MCP-1 levels was observed in Fli-1^{+/-} NZM2410 mice 2 hours after stimulation with LPS when compared to wild-type controls ($p < 0.05$). In addition, there was a non-significant difference in MCP-1 levels after 6 hours of stimulation (Fig. 3).

3.5. Inhibition of Fli-1 resulted in decreased production of MCP-1 in endothelial cells

To further confirm decreased expression of Fli-1 and the results of reduced production of MCP-1 in endothelial cells, we transfected specific Fli-1 siRNA into the endothelial cell line MS1. As shown in Fig. 4a, the expression of Fli-1 protein was inhibited after transfection

with specific siRNA. Next, we measured the concentrations of MCP-1 in the supernatants from the MS1 endothelial cells and found that there was more than a 50% reduction in MCP-1 levels before stimulation from endothelial cells transfected with specific Fli-1 siRNA compared to the cells transfected with non-specific siRNA ($p<0.01$). In endothelial cells transfected with specific Fli-1 siRNA compared with cells transfected with non-specific siRNA, the concentration of MCP-1 was also significantly decreased at 2 and 6 hours after stimulation with LPS (0 hour, Fli-1 siRNA, 18.71 ± 2.07 ng/ml versus control siRNA 36.48 ± 3.90 ng/ml, $n=4$, $p<0.01$; 2 hours, Fli-1 siRNA, 23.70 ± 0.22 ng/ml versus control siRNA 45.21 ± 0.21 ng/ml, $n=4$, $p<0.01$; 6 hours, Fli-1 siRNA, 109.59 ± 9.10 ng/ml versus control siRNA 265.56 ± 13.77 ng/ml, $n=4$, $p<0.01$; Fig. 4b).

3.6. Fli-1 binds directly to the promoter region of MCP-1 gene in endothelial cells

Our results showed a reduced expression of Fli-1 resulted in decreased production of MCP-1 in endothelial cells. Therefore, we examined whether Fli-1 directly or indirectly regulates the expression of MCP-1. There are many putative Fli-1 binding sites in the promoter region of the mouse MCP-1 gene. We designed 13 pairs of primers to cover these sites, and a ChIP assay was performed to examine if Fli-1 binds to the promoter of MCP-1. The primers used are listed in Table I. After immunoprecipitation by a Fli-1 specific antibody with crosslinked protein/DNA complexes from MS1 cell lines, three Fli-1 sites were significantly enriched with specific Fli-1 antibodies as detected by PCR amplification and compared to normal rabbit IgG controls (Fig. 5). These results clearly indicate Fli-1 can directly bind to the promoter of the MCP-1 gene in endothelial cells and regulate the expression of MCP-1.

4. Discussion

We have demonstrated that decreased expression of Fli-1 significantly reduced renal pathology and markedly prolonged survival in murine models of lupus including NZM2410 mice (22). In this report, we found that one of the molecular mechanisms that expression of Fli-1 impacts on disease is regulation of the proinflammatory chemokine MCP-1 in the endothelial cells in kidneys.

Infiltration of inflammatory cells plays a critical role in lupus nephritis development and renal injury (4–8). MCP-1 recruits inflammatory cells into the kidneys in murine lupus models as clearly demonstrated by Tesch and his colleagues using MCP-1-deficient MRL/*lpr* mice (8). In their experiments, MRL/*lpr* mice lacking MCP-1 had significantly reduced inflammatory cell infiltration into the kidneys with decreased proteinuria and prolonged survival compared to wild-type controls (8). In this report, we found that Fli-1^{+/-} NZM2410 mice had significantly decreased infiltration of inflammatory cells, including CD3⁺ cells, CD19⁺ cells, Ly-6B.2⁺ and CD11b⁺ cells, in the kidneys compared to wild-type littermates (Table II). We previously reported that Fli-1^{+/-} NZM2410 had significantly reduced proteinuria and prolonged survival with markedly reduced pathological renal score (22). Hence, we demonstrated that the local expression of MCP-1 in the kidneys was significantly reduced in Fli-1^{+/-} NZM2410 mice compared to littermate controls, which likely contributed to the reduction of inflammatory cells infiltrating into kidneys (Fig. 1). The expression of MCP-1 in the kidneys from diseased NZM2410 mice was mainly located in the glomeruli area. Many reports are contradictory regarding which area in the kidney expresses MCP-1. Some reports demonstrated that the major area in the kidney in which they detected expression of MCP-1 was in tubular areas (8, 26–28). While other papers demonstrated that expression of MCP-1 was mainly in glomerular cells (6, 29–30). The specificity of MCP-1 staining in this report was demonstrated by the fact that pre-incubation of MCP-1 antibodies with the specific antigen abolished staining. To directly prove that renal endothelial cells produce MCP-1, we isolated primary endothelial cells from kidneys of NZM2410 mice and we verified the purity of these endothelial cells by specific Di-Ac-

LDL uptake assay. As shown in Fig. 4, primary endothelial cells from the kidneys of NZM2410 mice produce very high amounts of MCP-1. It has been also reported that primary endothelial cells isolated from kidneys of MRL/*lpr* mice produced large amounts of MCP-1 (31). Thus it is highly likely that many cells express MCP-1 in inflammatory kidney disease. To further define the role of Fli-1 in regulating MCP-1 production, we demonstrated the inhibition of Fli-1 protein in MS1 endothelial cells resulted in a significant decrease in the production of MCP-1. Therefore, our data clearly indicates that transcription factor Fli-1 regulates the production of MCP-1. The regulation of MCP-1 by Fli-1 is likely cell-specific since several reports have demonstrated mesangial cells also produce MCP-1 in which no detectable expression of Fli-1 was found (32–34). It has been reported that MCP-1 is produced by many cell types including endothelial cells, fibroblasts, epithelial cells, smooth muscle cells, and monocytes (9, 35). Specifically, monocyte/macrophages are reported to be the major source of MCP-1. However, in this report we have found that endothelial cells also produce high amounts of MCP-1. Elsewhere, endothelial cells have also been reported to produce significantly high amounts of MCP-1 (36–40). Despite the fact that the endothelium is composed of $1-6 \times 10^{13}$ endothelial cells and plays an important role in the trafficking of immune cells and inflammation, there is limited research on the role of endothelial cells in response to inflammatory stimulation and renal disease development (41–42). Our findings indicate that endothelial cells likely play an active role in the cell infiltration and inflammation.

Ets-1, another transcription factor in the ETS family, has been reported to regulate the expression of MCP-1 in human endothelial cells (43). It is likely that both Ets-1 and Fli-1 are involved in regulating the production of MCP-1. In addition, many other transcription factors, including AP-1, C/EBP β , PREP1/PBX2, Sp1 and EGR1, have been demonstrated to regulate expression of MCP-1 (44–50). Figure 4 shows despite a 90% decrease in expression of protein levels of Fli-1 caused by siRNA inhibition; a large amount of MCP-1 was produced in the endothelial cells. EGR-1 has been demonstrated to regulate MCP-1 expression through JNK-MKK7-c-Jun pathway (50). In our previous reports, we demonstrated that Fli-1 directly regulates the expression of EGR-1, and its expression was significantly decreased in the kidneys in Fli-1^{+/-} NZM2410 mice compared to the wild-type littermates (22, 51). Thus, Fli-1 may affect MCP-1 production in endothelial cells through regulating EGR-1 expression. In this study, we demonstrated that Fli-1 directly binds to the promoter of MCP-1 and may directly regulate the production of MCP-1 in endothelial cells.

Upregulation of proinflammatory chemokines in kidneys from murine models of lupus prior to inflammatory cell migration has been demonstrated (6–7). Increased expression of proinflammatory chemokines is likely caused by the deposition of immune complex in the glomeruli in murine models of lupus, which results in increased infiltration of inflammatory cells. Decreased expression of Fli-1 also results in a decrease in autoantibodies in the murine models of lupus, including both MRL/*lpr* and NZM2410 mice (21–22). In turn, decreased autoantibodies lead to less immune complex deposition in the kidneys of Fli-1^{+/-} NZM2410 mice, which likely contributes to the lower production of MCP-1. Decreased expression of Fli-1 in kidney endothelial cells results in decreased production of the inflammatory chemokine MCP-1 in local tissue, and leads to less recruitment of inflammatory cells into the kidney. Thus many factors contribute to the reduced disease development in Fli-1^{+/-} NZM2410 mice such as reduced autoantibody production. It is our hypothesis that Fli-1 impacts multiple factors important in disease development including B cells, T cells and endothelial cells. This is likely why Fli-1 has such a profound impact on the prolonged survival in NZM2410 mice as we have previously reported (22).

In summary, this study has demonstrated that transcription factor Fli-1 directly binds to the promoter of MCP-1 gene and regulates the production of this inflammatory chemokine in

endothelial cells in kidney in NZM2410 mice. Reduced expression of Fli-1 leads to decreased production of MCP-1 in kidneys, which contributes to reduced renal disease in NZM2410 mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations in used this paper

ChIP	Chromatin Immunoprecipitation
siRNA	Small interfering RNA
SLE	systemic lupus erythematosus

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Highlights

- Fli-1 regulates expression of MCP-1 in the endothelial cells in kidneys.
- less inflammatory cells infiltrate in the kidneys with reduced expression of Fli-1.
- Fli-1 directly binds to the promoter of the MCP-1 gene.
- Fli-1 impacts glomerulonephritis development by regulating expression of MCP-1.

Figure 1. Reduced expression of MCP-1 in kidneys from Fli-1^{+/-} NZM2410 mice compared with wild-type littermates

Total RNA was prepared from kidneys at the age of 18 weeks (n = 6 in each group). Total RNA was converted to cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). Real-time polymerase chain reaction was performed in triplicate with the appropriate primers. * $P < 0.05$.

Figure 2. Decreased MCP-1 concentrations in serum from Fli-1^{+/-} NZM2410 mice compared with wild-type littermates

The serum was collected from Fli-1^{+/-} (n=16) and wild-type littermates (n=14) NZM2410 mice at the age of 34 weeks. MCP-1 concentrations were detected by ELISA and are presented as pg/ml (mean $\pm$ SE). * $p < 0.05$.

Figure 3. Decreased production of MCP-1 in primary endothelial cells from Fli-1^{+/-} NZM2410 mice

Primary endothelial cells were isolated from both Fli-1^{+/-} and wild-type NZM2410 as described in the material and methods (n=6 in each group). MCP-1 concentrations in supernatants were measured by ELISA method before and after LPS stimulation are presented as pg/ml (mean $\pm$ SE). * $p < 0.05$.

Fig. 4a **Fig. 4b**

Figure 4. Effective inhibition of Fli-1 expression with Fli-1 SiRNA transfection in MS1 endothelial cells (A)

MS1 endothelial cells were transfected with specific Fli-1 SiRNA or negative control SiRNA from Invitrogen following instruction from the company. The proteins were extracted from the cells and equal amounts of proteins were loaded in each well and probed by an anti-Fli-1 and anti- β -actin antibody. Lane 1: Fli-1 SiRNA; lane 2: Fli-1 SiRNA; Lane 3: negative control SiRNA; Lane 4: endothelial cells without any transfection. **Fli-1 regulates the production of MCP-1 in endothelial cells (B).** The MCP-1 concentrations in the supernatants were measured by ELISA. The supernatants were collected from MS1 endothelial cells transfected with specific Fli-1 SiRNA or negative control SiRNA after stimulation with LPS as indicated in figure. Data presented are the mean $\pm$ SD. * indicates $p < 0.01$.

Figure 5. ChIP analysis of Fli-1 binding to the MCP-1 promoter

Total MS1 endothelial cells were cross-linked with formaldehyde and chromatin was isolated from cells and immunoprecipitated with specific Fli-1 antibodies or control IgG. The genomic fragments associated with immunoprecipitated DNA were amplified by RT-PCR using the primer shown in Table I. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Input represents 1% total cross-linked, reversed chromatin before immunoprecipitation. Fold changes were calculated by RT-PCR.

Table I

PCR primer sequences for mouse MCP-1 promoter ChIP assay

Primer Name	Forward Primer	Reverse Primer	Position from TSS ^a	Amplicon Length
ChIP 1	CAAATCCAACCCACAGTTTCTC	GGTGCCAAGGAGTAGCATCAC	−272 to −150	123
ChIP 2	GGGCTTTCAGATTTTATCGCTTTG	TCTGCCCTGTTTCCTTCGTG	−1143 to −1015	129
ChIP 3	TCTTGGGTACAACCTGCCAATC	AAAAGAGTGGCGCTCTCACA	−1298 to −1173	126
ChIP 4	CCATCACAATGCTGCTAAGGAC	GGGGATGCTTGGAAGAGAAC	−1463 to −1346	118
ChIP 5	AGGACAGCCAGGACTATACAGAGAA	CCATAGGTAACACAGGTGACACG	−1631 to −1508	124
ChIP 6	ACGCACAACCTACTGCCTCCA	GATCTTGCTAGTCACTGTCTCCTC	−1947 to −1813	135
ChIP 7	GCTACAGAAAGCCAAAAGAATG	CTCGTTGCTACCTCCACGAA	−2074 to −1969	106
ChIP 8	CCCTGGGGTTCTTGTGTCTC	CCACGAAGTTTTCCTCATGTT C	−2129 to −1982	148
ChIP 9	CAGAAGCCTGCCACAAAG	GAACCCAGGGAAATTGTATAGTG	−2247 to −2119	129
ChIP 10	AAATATCTCTCCCGAAGGGTCTG	TTGTCTGTTTCCCTCTCACTTCAC	−2492 to −2350	143
ChIP 11	ATGTGCAGGAAGGAAAAGAAGG	TGTAATGGGGTGAGGACTGATAGA	−2775 to −2630	146
ChIP 12	CTTCTTCTTTCTCCCACTCTGACAC	CACCCAGATGGACAATGGAAC	−2832 to −2714	119
ChIP 13	CAAACAGGAATAAGCAAGCAAGTG	CATGTGCCCTGGTCAGTGTC	−2922 to −2773	150

^aTSS, translation start site.

Table II

Decreased inflammatory cell infiltration in kidneys from Fli-1^{+/-} NZM2410 mice compared wild-type littermates ^a

Genotypes of mice	CD3+ cells ^b	Ly-6B.2+ cells	CD11b+ cells	CD19+ cells
Wild-type	44.00±7.6 *	19.57±2.5 *	58.61±10.5 *	33.59±5.6 *
Fli-1 ^{+/-}	21.81±6.0 *	11.00±1.6 *	20.18±4.6 *	8.70±2.8 *

^a Mice were sacrificed at the age of 34 weeks. The kidneys were removed from wild-type NZM2410 mice (n=21) and Fli-1^{+/-} NZM2410 mice (N=18) and frozen immediately in liquid nitrogen. Frozen sections were stained with fluorescein-conjugated anti-mouse CD3, Ly-6B.2 alloantigen, CD11b or CD19 antibodies.

^b The number was positive cells from examined 10 high power fields (HPF) and data presented are the mean number/per mouse/per 10 HPF ± SD.

* indicates $p<0.01$