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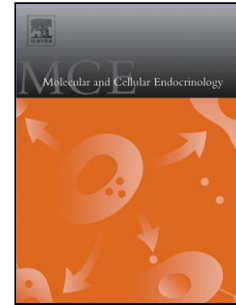
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**Cloning and characterization of the murine claudin-5 promoter**

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**SUMMARY**

Claudin-5, an integral tight junction protein component, plays a critical role in permeability of the endothelial cell barrier. Recently, we have shown that claudin-5 protein is down-regulated by the pro-inflammatory cytokine TNF alpha and its levels restored by dexamethasone treatment. In order to investigate the regulation of claudin-5 at the transcriptional level, we have cloned the murine claudin-5 promoter. The claudin-5 promoter sequence (1131 bp) showed no consensus TATA box. We identified putative transcription factor binding sites, including six full and two half-sites degenerated glucocorticoid response elements (GREs), two NFkappaB, three Sp1, one Sp2, one Ap2, as well as three E-boxes. Serially deleted promoter constructs showed high basal activity. TNF alpha significantly reduced the promoter activity and mRNA levels of claudin-5 in brain cEND and myocardial MyEND endothelial cells. Dexamethasone treatment led to a significant increase of the murine claudin-5 promoter activity and mRNA levels in cEND cells. However, no claudin-5 induction could be observed in MyEND cells in response to dexamethasone. Our studies suggest tissue-specific regulation of the claudin-5 gene via glucocorticoids and a high vulnerability of claudin-5 to TNF alpha. This could be an important mechanism in diseases accompanied by the release of proinflammatory cytokines, for example in patients with chronic heart failure or multiple sclerosis.

## INTRODUCTION

Members of the claudin protein family are major components of tight junctions, which are essential in the control of paracellular ion flux and the maintenance of cell polarity. So far, 24 members of the claudin family have been identified [1-3]. Several claudins have been described to have tissue-specific functions and are regulated at the transcriptional level to meet the demands of the particular organs [4-8]. A number of these important tight junction proteins, such as claudin-1, claudin-3, claudin-5 and -12 were reported to be expressed in endothelial tight junctions of the blood-brain-barrier (BBB), even though the presence of claudin-1 at the BBB is debatable [9-12]. Although immunostaining with anti-claudin-1 antibody was detected in brain capillary endothelial cells of wild type mice, it was reported to be still detectable in claudin-1-deficient mice. The observed claudin-1 immunoreactivity in endothelial cells of the BBB could thus be based on cross-reactivity of some claudin-1 antibody employed with claudin-3 arguing against claudin-1 expression at the BBB [12].

Claudin-5 plays an important role in maintenance of BBB function. Claudin-5 deficient mice are born alive, but die within one day after birth without any morphological abnormalities. However, the BBB integrity of these mice is severely affected, being selectively permeable to small molecules (< 800 D) [12]. The gene for claudin-5 is expressed ubiquitously, even in organs lacking epithelial tissues such as brain, skeletal muscle and spleen. Particularly high expression levels of claudin-5 were detected in lung, brain and heart vasculature [13]. At present, not much is known about the transcriptional regulation of the claudin-5 gene. Recently, it was reported that human claudin-5 is regulated by SOX18, an endothelial-specific transcription factor, leading to high expression of claudin-5 in post-confluent, differentiated human umbilical vein cells [14]. This report supports the view that claudin-5 is an endothelium-specific member of the claudin protein family [15,16]

Although our previous studies have shown in murine and human cellular models of the BBB that glucocorticoid and TNF alpha (TNF $\alpha$ ) regulation of claudin-5 contributes to the barrier function of endothelial cells [17,18], there had been no studies at the promoter level. In this study we report for the first time the complete nucleotide sequence of a 5'-flanking region of the murine claudin-5 gene along with putative transcription factor binding sites, suitable to test its regulation at the transcriptional level. In order to characterize the regulation of the claudin-5 gene we performed reporter gene analyses in murine brain (cEND) [17] and myocardial (MyEND) [19,20] microvascular endothelial cells. Furthermore, we have

examined the influence of a proinflammatory cytokine, TNF $\alpha$  and the synthetic glucocorticoid dexamethasone on claudin-5 promoter activity and mRNA expression. TNF $\alpha$  is a powerful activator of endothelial cells and has been shown to modulate the expression of cell adhesion molecules and the transmigration of inflammatory cells [21-24]. Glucocorticoids show barrier-tightening effects, that has been demonstrated for cerebral endothelial cells in vitro [17,25-27] and in vivo in the mouse [19]. Our results show differential regulation of the claudin-5 gene and mRNA expression by TNF $\alpha$  and dexamethasone in microvascular endothelial cell lines of brain and myocardial origin.

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## MATERIALS AND METHODS

### Cell culture and chemicals

Brain (cEND) and myocardial (MyEND) microvascular endothelial cell lines were isolated as previously described [17,20]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Taufkirchen, Germany) with 10 % (v/v) fetal calf serum (FCS), and 100 IU/ml penicillin and 100 mg/ml streptomycin on plates coated with collagen IV (Fluka, Taufkirchen, Germany). Cells were allowed to reach confluence, and were then transfected or stimulated with dexamethasone or TNF $\alpha$  in DMEM with 1 % (v/v) of FCS as indicated in the figure legends. In previous studies we had shown that under these cultivation conditions, the immortalized brain microvascular endothelial cell line cEND retains principal features of the BBB in vivo, i.e. high transendothelial electrical resistance and the expression of endothelial and BBB markers without the necessity of a co-cultivation system [19,28-30].

MCF-7 cells, human breast adenocarcinoma cell line, were maintained in RPMI 1640 (Sigma) supplemented with 10% FCS and antibiotics.

TNF $\alpha$  and dexamethasone were purchased from Sigma.

Gene specific oligodeoxynucleotide primers were obtained from MWG Biotech AG (Biotech (High Point, NC, USA).

### Cloning of the murine claudin-5 promoter

Genomic DNA was isolated from cEND cells using phenol/chloroform extraction. 500 ng of genomic DNA was used for PCR reaction. Gene specific oligonucleotides spanning the 5' flanking region of the murine claudin-5 gene were designed according to the published sequence from the mouse genome. Oligonucleotides were used as follows (5'-3'): AACAAAGGGAGCAGGGAGGAG, CATGGCTAAAGACTGAATGCTCAC. The PCR reaction was performed with iProof High-Fidelity DNA Polymerase (Bio-Rad Laboratories, Richmond, CA, USA) using 60°C annealing temperature. PCR-products were diluted 1: 50 and amplified in a nested PCR with following primers containing restriction sites for *Mlu*I and *Hind*III restriction enzymes (underlined): GAACGCGTAACAAAGGGAGCAGGGAG (forward) and GAAAGCTTCTCCGGTGCTCTGCG (reverse). Reaction products were analyzed by electrophoresis on 1.2% agarose gel, and a 1131 bp (-1020/+111, [chr16:18775920+18777050](#)) band was excised from gel, extracted and ligated into the *Mlu*I - *Hind*III sites of the reporter gene plasmid pGL3-basic from Promega (Promega, Madison, USA). The resulting constructs were screened by digestion with restriction endonucleases

followed by sequencing (MWG Biotech AG). Two deletions mutants, the 611 bp (-500/+111) and 379 bp (-268/+111) fragments were cloned into pGL3-basic. For cloning of the -500/+111 fragment an additional forward primer was used: GAACGCGTATGAGGTTATAATTAAGG (*Mlu*I restriction site underlined). The -268/+111 fragment was constructed by restriction digestion of the -500/+111 construct with *Bgl*III and *Hind*III. Amplified plasmids were screened by restriction digestion and the sequences were confirmed by DNA sequencing. Potential transcription factor binding sites were identified by MatInspector (<http://www.genomatix.de>) and TFSEARCH (<http://www.cbrc.jp>).

### **Transient transfection and reporter gene assay**

MCF-7 were seeded on a 6-well cell culture plate (Greiner, Frickenhausen, Germany) 24 hours before transfection at a density of  $3 \times 10^5$  cells per well. cEND and MyEND cells were seeded on a 6-well plate coated with collagen IV and grown to confluence before transfection. Transient transfection experiments using the Effectene reagent (Qiagen, Hilden, Germany) were performed as suggested by the manufacturer, using 2  $\mu$ g of promoter constructs DNA and 1  $\mu$ g of the internal control reporter pTRL-TK (Promega, Madison, WI, USA) [31] in the absence or presence of stimulators.

To assess dexamethasone and TNF $\alpha$  effects on claudin-5 promoter transactivation, after addition of the DNA-Effectene mixture, cells were incubated overnight at 37°C in 5% CO<sub>2</sub>. After this, 2 ml fresh medium containing 1% FCS and 100 nM dexamethasone and 10 nM / 100 nM TNF $\alpha$  or vehicle alone were added. After 24 hours, cells were washed once with PBS and harvested with 200  $\mu$ l lysis buffer (Promega). Thereafter, cellular extracts were prepared and analyzed for luciferase activity. Measurement of both firefly and Renilla luciferase activity was performed with the Dual-Luciferase assay kit (Promega) according to the manufacturer's instructions. Protein concentration was estimated by standard Bradford protein assay [32]. Cell lysate (20  $\mu$ l) was used for assaying the enzymatic activities, using a LB9507 luminometer with dual injector (Berthold, Bad Wildbad, Germany). Each lysate was measured twice. Promoter activities were expressed as relative light units (RLU) normalized for the protein content and the activity of Renilla luciferase in each extract. The data were calculated as the mean of three identical set-ups.

### **Quantitative real-time RT-PCR**



For real-time RT-PCR, RNA was isolated from the cEND and MyEND cells treated or untreated with dexamethasone or TNF $\alpha$  using an RNeasy kit (Qiagen). Two micrograms of RNA were used for the cDNA synthesis using iSCRIPT cDNA synthesis kit (Bio-Rad). Claudin-5 real-time RT-PCR was performed using the TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA). Assays for claudin-5 (Mm00727012\_s1) and GAPDH (Mm99999915\_g1) were obtained from Applied Biosystems. The ABI PRISM 7300 SDS software (Applied Biosystems; relative quantification study) was used to determine the cycle threshold ( $C_t$ ) for each reaction and gene expression was normalized to expression of the endogenous housekeeping gene, GAPDH, based on the  $2^{-\Delta\Delta C_t}$  method.

### **Analysis and Statistics**

Throughout the experiments, averaged values were reported as means  $\pm$  standard deviation (s.d.). Mann–Whitney  $U$ -test was performed and  $P < 0.05$  (\*) was considered statistically significant.

## RESULTS

### Cloning of the murine claudin-5 promoter

In our previous studies we have shown the regulation of claudin-5 mRNA and protein expression via glucocorticoids and TNF $\alpha$  in human hCMEC/D3 and murine brain endothelial cEND and cerebEND cells [17,18,30]. In order to investigate the role of genomic sequences upstream from the transcription start point for the murine claudin-5 mRNA, we have cloned a 1131 bp DNA fragment using claudin-5 gene specific oligonucleotides.

The murine claudin-5 gene is located on chromosome 16 and the cloned fragment has the position 18775920+18777050 (Mouse genome sequence, 7 July 2007). The promoterless pGL3-basic vector fused to a luciferase reporter gene was used to generate the reporter plasmids. This sequence of 1131 bp was subjected to computer analysis using Genomatix MatInspector (<http://www.genomatix.de>) and TFSEARCH (<http://www.cbrc.jp>) to identify conserved potential transcription factor binding sites.

The complete nucleotide sequence of 1131 bp claudin-5 gene is shown in Fig. 1 (GenBank accession no. **EU623469**). The predicted transcriptional initiation site of claudin-5, as determined by Genomatix MatInspector, is an adenine 149 bp downstream of the start codon. A number of putative transcription factor binding sites were found in this fragment. Selected putative transcription factor binding sites are shown in Fig. 1. We failed to identify a consensus TATA-box or TATA-like sequence. We were able to identify six full and two half-site degenerated glucocorticoid response elements (GREs) using a formula: HHN KGH nnn HCM NNW where H=A/C/T, W=A/T, K=T/G, M=A/C [33]. The other selected putative transcription factor binding sites were: two NF $\kappa$ B sites, three Sp1, one Sp2, one Ap2, as well as three E-boxes (Fig.1).

### Claudin-5 basal promoter activities in vitro and deletion analysis

Two 5'-deletion constructs of the -1020/+111 fragment, -500/+111 and -268/+111 were inserted into pGL3-basic vector to functionally characterize the claudin-5 promoter. All three fragments were sufficient to mediate luciferase gene expression (Fig. 2). A claudin-5 gene promoter fragment spanning positions -1020/+111 was transiently expressed in MCF-7, cEND and MyEND cells. It showed high basal activity in all three cell lines, MCF-7 cells:  $72.5 \pm 13.46$  fold; MyEND cells:  $34.87 \pm 12.1$  fold and cEND cells:  $23.02 \pm 6.18$  fold as compared to the empty pGL3-basic vector, which was set as 1 (Fig. 2B). Shorter fragments of the claudin-5 promoter showed lower activity in MCF-7 cells: transcriptional activity of -500/+111 and -268/+111 constructs was  $33.6 \pm 8.1$  fold and  $18.34 \pm 5.2$  fold, respectively. In

cEND cells the -500/+111 and -268/+111 constructs had an activity of  $23.45 \pm 5.47$  fold and  $28.63 \pm 9.17$  fold, respectively, as compared to the empty pGL3 basic vector. In MyEND cells, the -500/+111 construct showed basal activity of  $26.8 \pm 19.8$  fold and the shortest fragment, -268/+111 showed an activity of  $16.0 \pm 12.1$  fold as compared to the empty vector.

### **Effects of TNF $\alpha$ and dexamethasone on claudin-5 promoter activity**

We have previously shown that the expression of claudin-5 protein is strongly reduced after administration of 100 nM TNF $\alpha$  in human hCMEC/D3 and in murine brain endothelial cerebEND (10 nM TNF $\alpha$ ) cell lines [18,30]. In order to test whether TNF $\alpha$  treatment would affect claudin-5 expression at the transcriptional level in cEND and MyEND cells, we examined the effect of TNF $\alpha$  on claudin-5 promoter activity in cEND and MyEND cells within 24 hours of treatment (Fig. 3). Administration of 10 nM and 100 nM TNF $\alpha$  in cEND cells led to a suppression of the -1020/+111 promoter construct to  $0.82 \pm 0.21$  fold and  $0.6 \pm 0.09$  fold of untreated cells, respectively. Stimulation of MyEND cells with 10 nM TNF $\alpha$  reduced promoter activity to  $0.63 \pm 0.26$ , whereas the addition of higher concentrations of TNF $\alpha$  did not further increase this effect.

As we recently reported, glucocorticoid treatment of hCMEC/D3 cells resulted in a significant increase of human claudin-5 mRNA and protein levels [18]. In order to examine the glucocorticoid responsiveness of the murine claudin-5 promoter, we treated cEND and MyEND cells transfected with the -1020/+111 construct with 100 nM dexamethasone for 24 hours (Fig. 3). We could detect a  $2.3 \pm 0.09$  fold increase from basal levels in dexamethasone treated cEND cells. However, only a slight increase in promoter activity ( $1.14 \pm 0.18$  fold) was observed in MyEND cells as compared to the untreated control (Fig. 3).

### **Differential effects of TNF $\alpha$ and dexamethasone on claudin-5 mRNA expression**

Quantitative real-time RT-PCR analysis revealed significantly decreased claudin-5 mRNA levels in cEND and MyEND cells after 24 hours treatment with 100 nM TNF $\alpha$  (Fig. 4). In cEND cells claudin-5 mRNA level was  $0.62 \pm 0.2$  fold and in MyEND cells  $0.66 \pm 0.1$  fold as compared to the untreated control, which was set as 1. Treatment of cEND cells with 100 nM dexamethasone for 24 hours increased claudin-5 mRNA levels  $1.78 \pm 0.24$  fold over untreated cells. Dexamethasone treatment of MyEND cells led to  $1.11 \pm 0.41$  fold increase in claudin-5 mRNA expression.

## DISCUSSION

Claudin-5 is expressed in endothelial cells and plays an important role in the maintenance of the BBB [12]. To analyze the regulation of this tight junction protein at the molecular level, we cloned a 1131 bp fragment from the murine genomic DNA with claudin-5 gene specific primers. This promoter fragment showed no consensus TATA-box or TATA-like sequence. We could identify the putative transcription start point for claudin-5 mRNA and a number of putative transcription factor binding sites. We found several imperfect glucocorticoid response elements (GREs) [33] in the claudin-5 promoter sequence and two NF $\kappa$ B putative binding sites. These promoter elements might mediate the observed regulation of the claudin-5 gene by glucocorticoid and TNF $\alpha$  [34,35]. Further, we have identified three E-boxes within murine claudin-5 promoter. Snail and Slug transcription factors, which act by binding to the specific E-boxes, have been described to be direct repressors of E-cadherin, occludin, claudin-1, -3, -4 and -7 in the process of epithelial-mesenchymal transition [4,36-38]. Similar regulation might also play a role in claudin-5 expression. Recently, SOX-18 has been shown to bind directly to the human claudin-5 promoter and to regulate its expression in human umbilical vein endothelial cells (HUVEC) [14].

It will be important task for the future to analyze the nature of transcription factors binding directly to the claudin-5 promoter region *in vivo* to elucidate its regulation in detail, especially in view of the fact that claudin-5 expression, which was assumed to be exclusive for endothelial cells has recently been described in olfactory ensheathing cells [39]. The characterization of transcriptional regulators influencing claudin-5 expression might thus help elucidate the basis for tissue-specific expression of claudin-5.

Eukaryotic promoters contain a core promoter, which is responsible for a basal activity and a proximal promoter, which is responsible for the induction of gene expression. The basal promoter activity of subsequent deletion mutants was assessed in epithelial human breast adenocarcinoma MCF-7 cells and cEND and MyEND microvascular endothelial cells. Deletion of the full length fragment revealed decreased expression of the luciferase reporter gene, but the expression levels were still more than 15 times higher in promoter constructs compared to an empty vector. We conclude that the shortest fragment -267/+111 of 5' flanking sequence analyzed in this study contains most of the elements required for basal level expression. Further deletion of the -268/+111 construct would be required to completely eliminate promoter activity.

The inflammatory mediator TNF $\alpha$  plays a key role within many pathological processes e.g. multiple sclerosis [40,41]. We have previously shown that the expression of

claudins and occludin is strongly reduced after TNF $\alpha$  administration in human hCMEC/D3 and murine brain endothelial cerebEND and cEND cell lines [18,30,33]. The decrease in the tight junction protein expression was shown to reduce barrier function of the monolayers.

Here we demonstrate that the claudin-5 promoter is strongly suppressed via TNF $\alpha$ . We have used a -1020/+111 promoter construct in reporter gene studies in endothelial cell lines and observed in both, cEND and MyEND cells, decreased claudin-5 promoter activity after treatment of cells with TNF $\alpha$ . Two NF $\kappa$ B putative binding sites, which could have a role in claudin-5 regulation, were identified within the promoter sequence. Both endothelial cell lines used in experiments, express endogenously high levels of claudin-5. After treatment of the cells with TNF $\alpha$  we could observe a strong down-regulation of claudin-5 mRNA expression in cEND and MyEND cell lines as compared to the untreated control.

Comparable effects of TNF $\alpha$  have been previously reported for occludin expression [42]. Detailed analysis of the occludin promoter has shown that TNF $\alpha$  modulates occludin gene expression through the suppression of promoter activity by a number of *cis*-acting motifs, such as NF-IL6 or NF $\kappa$ B, which were identified within the promoter sequence [42]. Suppression of tight junction protein expression via TNF $\alpha$  leads to an increased permeability of the BBB [18,30,33], what in vivo might be responsible for clinical symptoms of multiple sclerosis due to an increased infiltration of the central nervous system with inflammatory cells [43].

This is in line with our previous results and suggests a vulnerability of claudin-5 to the proinflammatory cytokine. Decreased expression of claudin-5 and other tight junction proteins due to TNF $\alpha$  might be an important mechanism in disorders connected with inflammatory cytokines release, such as multiple sclerosis or chronic heart failure.

Glucocorticoids have been demonstrated to have barrier-tightening effects on cerebral endothelial cells in vitro [17,25-27] and in vivo [19]. In our previous studies we have demonstrated that occludin, another tight junction protein expressed in BBB, is a direct target gene for glucocorticoids [17].

We could identify several imperfect GREs [33] in the claudin-5 promoter sequence, which might have a role in glucocorticoid regulation of claudin-5 gene. Dexamethasone treatment of cEND and MyEND cells revealed an increase in the promoter activity and claudin-5 mRNA levels in both cell lines. However, the effect of dexamethasone was more profound in brain microvascular endothelial cEND cells, what suggests tissue-specific regulation of claudin-5 via glucocorticoids or tissue-specific activity of glucocorticoid receptor, as shown for occludin gene [19].

Induction of claudin-5 by glucocorticoids has been described in rat brain microvessel endothelial cells (BMEC, GPNT), where addition of hydrocortisone to the cultures resulted in fewer frayed junctions and a more uniform distribution of claudin-5 at the cell borders as compared with cultures without hydrocortisone treatment [26,44]. In human alveolar epithelial cells, as well as in HUVEC claudin-5 has been shown to be one of the major genes up-regulated during differentiation in culture [14,45,46]. Also expression of adherens proteins, such as E-cadherin or VE-cadherin was enhanced by glucocorticoids [47,48]. These results show strong influence of glucocorticoids on endothelial barrier properties and might contribute to a better understanding of its disorders.

Taken together, our results show regulation of claudin-5 expression via  $\text{TNF}\alpha$  and dexamethasone on a chromatin and mRNA level. Dexamethasone effects on claudin-5 expression appeared to be much stronger in microvascular endothelial cells of brain origin than in that of heart origin. The nature and DNA binding motifs of the involved transcription factors binding directly to the claudin-5 promoter mediating dexamethasone and  $\text{TNF}\alpha$  effects remains to be investigated.

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**FIGURE LEGENDS**

**Fig. 1.** Sequence of murine claudin-5 genomic DNA fragment (GenBank accession no. EU623469). Putative transcription factor binding sites are underlined or when overlapping in italic. Sequence from the first ATG codon is in lower case. GRE: glucocorticoid-responsive element; NFκB: nuclear factor kappa B; Sp1: stimulating protein 1; Sp2: stimulating protein 2; Ap2: activator protein 2, TSP: transcription start point. \* indicates the first nucleotide of subsequent deletions fragments and ^ indicates the last nucleotide of all three constructs.

**Fig. 2.** Basal transcriptional activity of murine claudin-5 promoter in MCF-7, cEND and MyEND cells. The promoterless pGL3-basic vector fused to luciferase gene was used as reporter vector. **(A)** A schematic diagram of the putative transcription factor binding sites and the 5' deletions constructs. The arrow indicates the transcriptional initiation site as determined by MatInspector (Genomatix). **(B)** MCF-7, cEND and MyEND cells were transfected with -1020/+111, -500/+111 and -268/+111 promoter constructs. 24 hours later the cells were lysed and luciferase activity was measured. Each transfection reaction in an assay was carried out in triplicate and assays were repeated three times. Values are normalized for protein content and expression of co-transfected Renilla luciferase reporter gene. Data (means ± S.D.) are expressed as fold over pGL3-basic, which was arbitrarily set as 1, (\*\*) $p < 0.001$ .

**Fig. 3.** Transcriptional activity of murine claudin-5 promoter -1020/+111 construct after treatment with TNFα and dexamethasone in cEND and MyEND cells. cEND and MyEND cells were transfected with -1020/+111 promoter construct and 24 hours later treated either with 10 nM / 100 nM TNFα or 100 nM dexamethasone (Dex). After 24 hours stimulation, cells were lysed and luciferase activity was measured. Each transfection reaction in an assay was carried out in triplicate and assays were repeated three times. Values are normalized for protein content and expression of co-transfected Renilla luciferase reporter gene. Data (means ± S.D.) are expressed as fold over pGL3-basic, which was arbitrarily set as 1.

**Fig. 4.** Effects of TNFα and dexamethasone on claudin-5 mRNA expression. cEND and MyEND cells were grown to confluence, and then maintained in differentiation medium containing 1% FCS for additional 48 hours. After that, cells were treated with 100 nM TNFα or 100 nM dexamethasone (Dex) for 24 hours followed by total RNA extraction and quantitative RT-PCR. Claudin-5 expression was normalized to GAPDH and expressed as fold over untreated cells, which was set as 1.

Figures:

Fig. 1

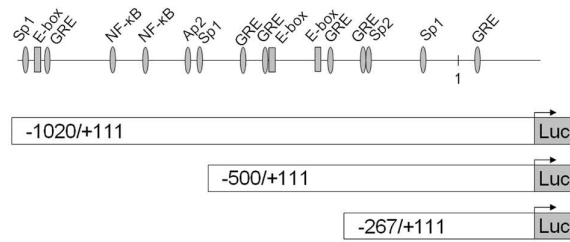
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-1020 AACAAAGGGA GCAGGGAGGA GTGTTCACTG GAAAGAGGCT GCGCCCTAG TTGGTTGTG
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-960 GGCTGTGGGG CCTGGTGCCC CCACCTGAGC CTCAGGGACC CAGTGTGTCT AACCCAGTGG
      Sp1          E-box      GRE
-900 ACCTTTCAAG AAATGGCTGG GCCATTGTGC AGAAGAATGC CCGGAAATCC CGCGGCTCCC
                                     NF-kB
-840 TCCTCCACCG AGGATGGGGG CTCTTCTCC TGGCCAGGAA ACTCCAAGTT GGCTTCCGGA
                                     NF-kB
-780 GGGTGGCCTG GGGGCTGGGG TGGCAAAGCA CCACAACAT TGTAGGATG GCAAGGCATA
      Ap2          Sp1
-720 GCCTTTCTAT GTCCCATCGT GTCTCTGCTG TCCCAGGATT CCAGTATGCT GTGTTAGGAT
-660 CACAGCCTAT CTGCTGGAGA AGACTGGACG TCGGAGAAC CTCAGGGGTT GCGCAGCATC
-600 CCAGAGGAGA GCACAGGCCC TTGCAGTGTT CACTTTGTGT CAGACCAGTG GTTCCAGGGA
                                     GRE
-540 AGGAGTGAGA ACTGTAGGCG GTGGTTGGGT AGGATCATGA ATGAGGTTAT AATTAAGGGC
                                     *
                                     GRE
-480 AAGCCCAACA CAGGCTTGCC GAGCATCTCC GCTTGGTGTG CCAAGGAAA AACGGCGGGC
-420 GTAGAGGTTT GTTTCACCTG TGCACGGGGG CTCAGAGAAC CTCCAGGCCA AGCAGTCTGC
      E-box 1/2 GRE
-360 AGAGAGGAGT TGGAAATGAT CGGGATGTAG CAGGTGGAGC TGGTGAGTGT AGGTTAGGGG
      E-box
-300 TCATGCTGCT AACAGTGGAG ACAGGGGCGC ACAGATCTCC TGTGTACCTG CCAAGATCAG
      *
-240 CCCCTACTAG GACAGAAACT GGTAGGTCT CAGACTGCGG TTCTGCAGGG TCGGGTTCGG
                                     GRE          GRE
-180 GTGAGCAGGA CTAGTCCTGC TCGCCCGTGG TTTAGTAAC TGTAGCTGTC AGGGGGTCCG
      Sp2
-120 CGATTGGCTG TGGGCCGAAG GTCTGCTCAT GGATTGGTTG AGCTAACTCG GGGGGCTGCG
-60 CCCTGGGGGC AGAGTCCGCC CCCGAGACTT CAAATAGGAC AACAGGTTAC TTCAGAACCC
      Sp1 (GC box)          1/2 GRE
  1 AGTTGGTGT AGTTAAAACC TCCTCTTCTGC TCCAGGACTG GAGGCTCCAG AGCAGAGGCA
  TSP          GRE
  61 CCAGAATCAA TTCCAGCTC CCAGCCTAAG CAGCGCAGAG AGCACCCGGA GGCCCCAAGG
      ^
121 GCCGTCGGGT GAGCATTCAg TCTTTAGCCa tgggggt

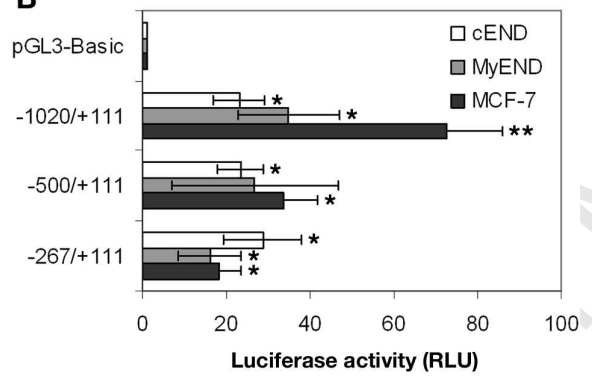
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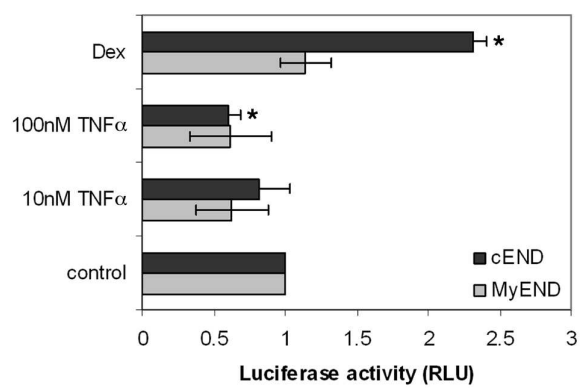
Fig. 2

A



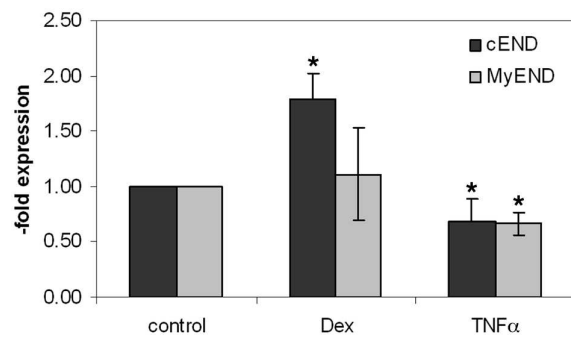
B



**Fig. 3**

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Fig. 4



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