

# Transactivation of MCP-1/CCL2 by beta-catenin/TCF-4 in human breast cancer cells

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## Abstract

The loss of E-cadherin expression and the translocation of  $\beta$ -catenin to the nucleus are frequently associated with the metastatic conversion of epithelial cells. In the nucleus,  $\beta$ -catenin binds to the TCF/LEF-1 (T-cell factor/ lymphoid enhancer factor) transcription factor family resulting in the activation of several genes, some of them having important implications in tumour progression. In our study, we investigated the potential regulation of monocyte chemotactic protein-1 (MCP-1/CCL2) expression by the  $\beta$ -catenin/TCF pathway. This CC-chemokine has been implicated in tumour progression events such as angiogenesis or tumour associated macrophage (TAM) infiltration. We thus demonstrated that MCP-1 expression correlates with the reorganization of the E-cadherin/ $\beta$ -catenin complexes. Indeed, MCP-1 was expressed by invasive breast cancer cells (MDA-MB- 231, BT549 and Hs578T), which do not express E-cadherin but was not produced by noninvasive breast cancer cell lines (MCF7 and T47D) expressing high level of E-cadherin. In addition, the MCP-1 promoter was activated in BT549 breast cancer cells transfected with  $\beta$ -catenin and TCF-4 cDNAs. The MCP-1 mRNA level was similarly upregulated. Moreover, we showed that MCP-1 mRNA was downregulated after transfection with a siRNA against  $\beta$ -catenin in both BT549 and Hs578T cells. Our results therefore identify MCP-1 as a target of the  $\beta$ -catenin/TCF/LEF pathway in breast tumour cells, a regulation which could play a key role in breast tumour progression.

**MESH Keywords** Breast Neoplasms ; genetics ; pathology ; Cadherins ; biosynthesis ; Carcinoma ; genetics ; pathology ; Cell Transformation, Neoplastic ; Chemokine CCL2 ; biosynthesis ; Disease Progression ; Down-Regulation ; Humans ; Neovascularization, Pathologic ; Promoter Regions, Genetic ; TCF Transcription Factors ; physiology ; Transcriptional Activation ; Tumor Cells, Cultured ; Up-Regulation ; beta Catenin ; physiology

**Author Keywords** MCP-1 ;  $\beta$ -catenin ; breast cancer

Chemokines constitute a superfamily of proinflammatory cytokines that are implicated in tumour progression, modulating not only host tumour-specific immunological response but also angiogenesis or tumour cell invasion and proliferation.<sup>1-3</sup> Monocyte chemotactic protein-1 (MCP-1) or CCL2 is a CC-chemokine that specifically attracts monocytes and memory T cells. It has been particularly implicated in processes characterized by mononuclear cell infiltration including breast cancer.<sup>4,5</sup> In some models, MCP-1 has been shown to display an antitumoral effect.<sup>6,7</sup> Nevertheless, a large number of studies demonstrate that MCP-1 facilitates tumour progression through its ability to recruit stromal cells including tumour associated macrophages (TAMs) and endothelial cells. A pro-angiogenic role of MCP-1 has been demonstrated in many systems, facilitating tumour growth and dissemination.<sup>8,9,11,12</sup> Moreover, a direct effect of MCP-1 on tumour cell migration in vitro has also been described.<sup>13</sup> Recently, Lebrecht et al.<sup>14</sup> have correlated an increased MCP-1 serum level with an advanced breast tumour stage and the presence of lymph node metastasis. In the same way, Amann et al.<sup>15</sup> reported a significant association between MCP-1 urinary levels and tumour stage and grade. They suggested a use of its urinary level as prognosis marker in bladder cancer. The correlation between MCP-1 expression and a poor prognosis was also demonstrated for the squamous cell carcinoma of the oesophagus.<sup>16</sup> In tumours, MCP-1 is produced both by peri-tumoral stromal cells and by tumour cells themselves, but an overexpression of MCP-1 in invasive tumour cells has been documented in several human carcinomas including breast, gastric and oesophageal carcinomas.<sup>10,17,18</sup> If the functional implication of MCP-1 in tumour progression is therefore largely documented, the mechanisms regulating MCP-1 expression in tumour cells are still poorly understood.

A major mechanism involved in the regulation of tumour-promoting genes by tumour cells is the loss of E-cadherin mediated cell-cell adhesion.<sup>19,20</sup> E-cadherin is a transmembrane glycoprotein found in adherens junctions implicated in the maintenance of a polarized and cohesive epithelium. In normal conditions, the intracellular domain of the E-cadherin is linked to the actin cytoskeleton via cytoplasmic linkers: the catenins ( $\alpha$ -,  $\beta$ - and  $\gamma$ -catenin). In pathological processes including cancer progression,  $\beta$ -catenin can accumulate in the cytoplasm and then translocate to the nucleus where it binds transcription factors of the TCF/LEF family (T Cell Factor/Lymphoid Enhanced Factor), leading to the activation of specific genes.<sup>21,23</sup> The accumulation of cytoplasmic  $\beta$ -catenin and its translocation to the nucleus has been associated with epithelial cell migration and invasion in various physiological and pathological processes including

tumour progression. 21, 23–26 Several  $\beta$ -catenin/TCF target genes have now been identified. They pertain to various families of proteins including matrix metalloproteases, cytoskeletal proteins or angiogenic cytokines and chemokines (IL-8, MMP-7, MMP-14, CD44, cyclin D1, c-myc, claudin-1, vimentin, MMP26, VEGF...), some of them having important implications in cancer progression. 27–36

These independent data from the literature therefore suggest that a reorganisation of E-cadherin/ $\beta$ -catenin and an overexpression of MCP-1 in tumour cells are 2 events associated with cancer progression. This prompted us to investigate the potential regulation of MCP-1 by the  $\beta$ -catenin/TCF pathway. We thus here document the overexpression of MCP-1 mRNA and the transactivation of the MCP-1 promoter following the activation of the  $\beta$ -catenin/TCF pathway.

## Material and methods

### Cell lines and culture conditions

Human breast carcinoma cell lines (MCF7, T47D, MDA-MB-231, BT549 and Hs578T) were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's Modified Eagle's Medium (Life Technologies, Grand Island, NY) supplemented with 10% FCS, 2 mM of L-glutamine and 100 U/ml of penicillin and streptomycin (Life Technologies).

### RT-PCR analysis

Total RNA was extracted using the High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany). Ten nanograms of RNA was then reverse transcribed into cDNA and amplified using the GeneAmp ThermoStable rTth enzyme (Perkin Elmer Life Sciences, Boston, MA). The following reverse (R) and forward (F) primers were used: MCP-1R 5'-AATGGTCTTGAAGATCAC-AGCTTC-3', MCP-1F 5'-TAGCAGCCACCTTCATTCCCC-AAG-3', 28SR 5'-GATTCTGACTTAGAGGCGTTCAGT-3', 28SF 5'-GTTACCCACTAATAGGGAACGTGA-3', E-cadherinR 5'-CTGTCACCTTCAGCCATCCTGTTT-3', E-cadherinF 5'-CCC-ATCAGCTGCCAGAAAATGAA-3'. Amplification cycles were as follow : 30 cycles at 94°C 15 sec, 60°C 20 sec, 72°C 10 sec for MCP-1 detection, 16 cycles at 94°C 15 sec, 68°C 20 sec, 72°C 10 sec for 28S detection and 35 cycles at 94°C 15 sec, 68°C 20 sec and 72°C 10 sec for E-cadherin detection. Each experiment was performed at least 3 times.

### Detection of MCP-1 by ELISA

MCP-1 secretion was measured by ELISA in conditioned media of the different breast cancer cell lines.  $3 \times 10^5$  cells of each cell lines were seeded in 6-well plates containing 2 ml of DMEM 10% FCS per well. The MCP-1 ELISA was performed on 48 hr conditioned media according to the manufacturer's instructions (DuoSet human MCP-1 kit, R&D Systems, Minneapolis, MN). Results are expressed in pg/ml of conditioned medium as means  $\pm$  SEM of 3 independent experiments.

### Western blot analysis

Total protein extracts from the different cell lines were made in Tris 50 mM, pH 7.4, NaCl 150 mM, Nonidet P-40 1%, Triton X-100 1%, Deoxycholic acid 1%, SDS 0.1% and Iodoacetamid 5 mM supplemented with a cocktail of proteases inhibitors (Roche Diagnostics, Mannheim, Germany). Ten micrograms of protein samples were separated by electrophoresis on 12% SDS-polyacrylamide gels and then transferred on a PVDF membrane (Perkin-Elmer Life Sciences). Immunoblotting was performed with a monoclonal anti E-cadherin or  $\beta$ -catenin antibody (Transduction Laboratories, BD Bioscience, San Diego, CA) followed by an incubation with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Dako, Glostrup, Denmark). Signals were visualized using the Western Lightning Chemiluminescence Reagent kit (Perkin-Elmer Life Sciences). In order to normalize the signals, the blots were then incubated, after extensive washing, with a rabbit anti-actin antibody (Sigma Chemical Co., St. Louis, MO) followed by a horseradish peroxidase-conjugated swine anti-rabbit secondary antibody (Dako). Actin signals were also visualized using the Western Lightning Chemiluminescence Reagent kit (Perkin-Elmer Life Sciences).

### Immunofluorescence

Confluent cell monolayers on glass coverslips were fixed in 2.5% paraformaldehyde in PBS for 30 min and then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. The coverslips were then saturated for 30 min with 3% bovine serum albumin in PBS. After intermediate washes in PBS, monolayers were incubated for 1 hr with a monoclonal antibody to  $\beta$ -catenin (Transduction Laboratories, BD Bioscience, San Diego, CA). Cells were then exposed to a tetramethylrhodamine isothiocyanate-conjugated antibody (Dako). The coverslips were then mounted with Aquapolymount antifading solution (Polysciences, Warrington, PA) onto glass slides and observed under a MRC 600 confocal laser scanning microscope (Bio-Rad, Richmond, CA).

### Plasmids

The human MCP-1 promoter construct was generated by sub-cloning a 3.8 kb fragment of the 5' promoter region (GI access number: 516772) into the firefly luciferase reporter plasmid pGL-3 (Promega, Madison, WI). This fragment was isolated from the pCAT $\Delta$ 3.8k plasmid previously described. This human MCP-1 promoter contains a consensus  $\beta$ -catenin/TCF binding site (5'-CTTTGTA-3') located

1,399 bp upstream of the transcription initiation start. We also constructed a reporter vector containing a mutated  $\beta$ -catenin/TCF binding site (5'-CTTTGGC-3') using the Quick Change site directed mutagenesis kit (Stratagene, LaJolla, CA) according to the manufacturer's instructions. The mutation was verified by sequencing. Such a two nucleotide substitution in the  $\beta$ -catenin/TCF binding site has previously been described to dramatically reduce  $\beta$ -catenin/TCF signal transduction. 38,39 The TCF-4 cDNA cloned into pCDNA3zeo expression vector and the  $\Delta$ NTCF-4 expression vector (which lacks the  $\beta$ -catenin binding domain) were kindly provided by Dr. H. Clevers (University Hospital, Utrecht, The Netherlands). The  $\beta$ -catenin expression vector (in pCDNA3) expresses a mutated form of  $\beta$ -catenin less susceptible to degradation and was kindly provided by Dr. K. Orford and Dr. S. Byers (Lombardi Cancer Center, Georgetown University, Washington DC).

### **Transient transfections of $\beta$ -catenin and TCF-4**

Transient transfections were performed with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) on  $2 \times 10^5$  BT549 cells plated in 6-well-plates. Twenty-four hours after plating, transfection was carried on as recommended by the manufacturer by adding in each well a mixture containing 500  $\mu$ l of serum-free medium, 3  $\mu$ l of Lipofectamine 2000 and 1  $\mu$ g of the  $\beta$ -catenin expression vector + 1  $\mu$ g of pCDNA3zeo vector or 1  $\mu$ g of the TCF-4 expression vector + 1  $\mu$ g of pCDNA3 vector or 1  $\mu$ g of both  $\beta$ -catenin and TCF-4 expression vectors. Controls were generated by transfecting the cells with 1  $\mu$ g of each corresponding empty vectors. RNA extraction was performed for MCP-1 RT-PCR analysis.

### **Luciferase reporter assays**

Transient transfection experiments were performed using Fugene-6 transfection reagent (Roche Diagnostics) on  $5 \times 10^4$  MCF7, BT549 or Hs578T cells seeded in a 24-well plate half an hour before the addition of the DNA/Fugene mixture. Each well was incubated with a mixture containing 20  $\mu$ l of serum-free DMEM, 0.6  $\mu$ l of Fugene, 0.15  $\mu$ g of the firefly promoter-luciferase reporter plasmid containing either the wild-type MCP-1 human promoter (WT MCP-1) or the MCP-1 promoter in which the putative  $\beta$ -catenin/TCF binding site was mutated (mut MCP-1), 0.15  $\mu$ g of the  $\beta$ -catenin expression vector (or the corresponding empty vector), 0.15  $\mu$ g of the TCF-4 expression vector (or the corresponding empty vector) and 0.8 ng of the renilla luciferase expression vector phRG-TK (Promega). To compare the effect of wild-type TCF-4 vs.  $\Delta$ NTCF-4 on the activation of the MCP-1 promoter, each well was incubated with a mixture containing 20  $\mu$ l of serum-free DMEM, 0.6  $\mu$ l of Fugene, 0.05  $\mu$ g of the wild-type MCP-1 promoter and 0.4  $\mu$ g of TCF-4 or  $\Delta$ NTCF-4 expression vector (or the corresponding empty vector as control) and 0.8 ng of the renilla luciferase vector phRG-TK (Promega). Twenty-four hours after transfection, the cells were lysed in 100  $\mu$ l of passive lysis buffer and the luciferase activity was determined with a luminometer using the Dual Luciferase Assay System (Promega) on 20  $\mu$ l of lysate. For each experiment, the firefly luciferase activity was normalized to the activity of the renilla luciferase used as internal control. For  $\beta$ -catenin/TCF-4 induction experiments, results were expressed as fold induction determined by normalizing each firefly luciferase value to the renilla luciferase internal control value and by dividing these normalized values with the mean normalized value of the corresponding reporter construct transfected with the empty expression vectors. Each experiment was performed at least 3 times in triplicate. Data are expressed as means  $\pm$  SEM.

### **Transfection of small interfering RNA**

A 19-nt specific sequence was selected in the coding sequence of  $\beta$ -catenin (Genbank accession number: X87838) to generate 21-nt sense and 21-nt antisense strands of the type (19N)TT (N, any nucleotide). The sense and antisense strands were then annealed to obtain duplexes with identical 3' overhangs. The sequence was submitted to a BLAST search against the human genome to ensure the specificity of the siRNA to the targeted sequence. A corresponding scramble duplex which does not recognize any sequence in the human genome was used as control. The 19-nt specific sequence for the  $\beta$ -catenin siRNA is as follow: 5'-GAAACGGCTTTTCAGTTGAG-3'. The 19-nt specific sequence for the control siRNA is as follow: 5'-GCTTATGGTGACAGA-TGTT-3'. For transfection of the siRNA duplexes,  $7 \times 10^3$  BT549 cells and  $1 \times 10^6$  Hs578T cells were plated in 6-well plates in 2 ml per well of culture medium. Twenty-four hours after plating, the cells were transfected by phosphate calcium precipitation by adding in each well 200  $\mu$ l of a mixture containing the siRNA duplexes (50 nM), 140 mM NaCl, 0.75 mM  $\text{Na}_2\text{HPO}_4$ , 6 mM glucose, 5 mM KCl, 25 mM HEPES and 125 mM  $\text{CaCl}_2$ . Twenty-four hours after transfection, the cells were extensively washed with PBS and incubated for 48 hr in culture medium before they were harvested for RT-PCR, Western blotting or Elisa analyses.

## **Results**

### **MCP-1 is expressed by invasive, E-cadherin-negative breast cancer cell lines**

In order to establish a potential relationship between a reorganization of E-cadherin/ $\beta$ -catenin complexes and MCP-1 expression, we first examined the expression of MCP-1 in a set of breast tumour cell lines previously characterized for their in vitro invasive properties and for their expression of E-cadherin. 41-43 Examining MCP-1 mRNA expression by RT-PCR analysis, we observed that invasive, E-cadherin-negative cell lines (MDA-MB-231, BT549 and Hs578T) expressed high levels of MCP-1, whereas noninvasive, E-cadherin-positive cell lines (MCF7 and T47D) did not express MCP-1 mRNA (Fig. 1a). This selective expression of MCP-1 by invasive, E-cadherin-negative breast cancer cell lines was confirmed at the protein level by ELISA (Fig. 1b). Looking at  $\beta$ -catenin

expression by Western blotting, we found it expressed in all cell lines regardless of E-cadherin expression (Fig. 2a ). However, the subcellular localization of  $\beta$ -catenin was different among these cell lines. Indeed, as we previously showed, the nuclear activity of  $\beta$ -catenin revealed by the TOP-FLASH/FOP-FLASH reporter system, correlating with a nuclear localization of  $\beta$ -catenin, was higher in E-cadherin-negative cell lines than in E-cadherin-positive cell lines which displayed a membrane-associated staining for  $\beta$ -catenin<sup>34</sup> . Such a different localization of  $\beta$ -catenin in E-cadherin-negative, MCP-1-positive cell lines is also illustrated here (Fig. 2b ). Indeed,  $\beta$ -catenin was mainly localized in the cytoplasm and in the nucleus of E-cadherin-negative, MCP-1-positive cells, whereas it was mainly found at the cell membrane in E-cadherin-positive, MCP-1-negative cell lines. These observations prompted us to examine the potential regulation of MCP-1 by the  $\beta$ -catenin/TCF pathway.

### **MCP-1 is upregulated by the $\beta$ -catenin/TCF pathway**

In order to show a direct effect of the  $\beta$ -catenin/TCF pathway on MCP-1 expression, we used a luciferase reporter plasmid containing the human MCP-1 promoter or the same promoter in which the consensus  $\beta$ -catenin/TCF binding site was mutated (CTTTGTA $\rightarrow$ CTTTGGC). Cotransfection experiments were performed in MCF7, BT549 and Hs578T cells using the wild-type or the mutated human MCP-1 promoter cotransfected with expression vectors encoding  $\beta$ -catenin and/or a member of the TCF/LEF family, TCF-4. In the E-cadherin-positive and MCP-1-negative MCF7 cell line, no activation of the human MCP-1 promoter was observed (Fig. 3a ). In contrast, cotransfection of  $\beta$ -catenin and TCF-4 in invasive, E-cadherin-negative BT549 and Hs578T cells led, respectively, to a 2.3- and 2.8-fold activation of the wild-type MCP-1 promoter (Fig. 3b,c ). The use of the mutated MCP-1 promoter gave the same results than those obtained with the wild-type promoter in all 3 cell lines. Revealing a similarity between the regulation of the MCP-1 promoter and the MCP-1 mRNA, we also observed an increased expression of MCP-1 at the mRNA level in BT549 cells cotransfected with  $\beta$ -catenin and TCF-4 (between 2.3- and 3.5-fold induction) or transfected with TCF-4 alone (between 2.2- and 2.9-fold induction) (Fig. 4a,b ).

### **$\Delta$ NTCF-4 does not activate the MCP-1 promoter**

As shown in Figures 3 and 4 , an effect of TCF-4 alone was found on MCP-1 expression. In order to examine the contribution of  $\beta$ -catenin in this regulation, we used a deleted form of TCF-4 lacking the NH<sub>2</sub>-terminal 30 amino acids ( $\Delta$ NTCF-4) encompassing the  $\beta$ -catenin/TCF binding domain which cannot bind  $\beta$ -catenin. This mutant TCF-4 was unable to transactivate the MCP-1 promoter, suggesting that  $\beta$ -catenin binding is essential for this regulation. Indeed, the MCP-1 promoter activity was 2.9-fold enhanced in BT549 cells and 2.7-fold in Hs578T transfected with wild-type TCF-4, while the promoter activity in the presence of  $\Delta$ NTCF-4 was similar to that of the promoter cotransfected with the empty vector used as control (Fig. 5 ).

### **$\beta$ -catenin siRNA downregulates MCP-1 expression**

In order to clearly demonstrate the implication of  $\beta$ -catenin in MCP-1 regulation, we used RNA silencing strategy to downregulate endogenous  $\beta$ -catenin expression in human BT549 and Hs578T breast cancer cell lines. Transfection of a siRNA against  $\beta$ -catenin downregulated its expression in BT549 and Hs578T cells at the protein level (Fig. 6a ). Using RT-PCR analyses, we also showed that the  $\beta$ -catenin siRNA downregulates MCP-1 mRNA expression in BT549 and Hs578T cells compared to the control siRNA (3.7 fold decreased in BT549 and Hs578T cells) (Fig. 6b ). Such a decrease of MCP-1 expression following  $\beta$ -catenin siRNA transfection was also observed at the protein level by Elisa analyses (Fig. 6c ).

## **Discussion**

In our study, we first demonstrated that MCP-1 is selectively expressed in breast tumour cell lines known to display high in vitro invasive properties and reorganized E-cadherin/ $\beta$ -catenin complexes. Even though the correlation between a reorganisation of E-cadherin/ $\beta$ -catenin complexes and MCP-1 expression has never been reported before, the large amount of data from the literature, associating E-cadherin/ $\beta$ -catenin complexes reorganization and the expression of an invasive phenotype<sup>20,22,40-44</sup> strengthen our findings. Furthermore, our data are in agreement with data reporting MCP-1 expression in a variety of human tumour cell lines including gastric<sup>10</sup> , ovarian<sup>45</sup> , breast<sup>9</sup> , melanoma,<sup>46</sup> pancreatic<sup>7</sup> and neuroblastoma cancer cell lines.<sup>47</sup> Similarly, in vivo, MCP-1 was expressed by tumour cells of different types including ovarian,<sup>45,48</sup> gastric,<sup>10</sup> pancreatic,<sup>7</sup> oesophageal<sup>16,18</sup> and also breast carcinoma cells.<sup>8,9,7</sup> More particularly in accordance with our results showing a selective expression of MCP-1 in invasive breast tumour cell lines, Valkovic et al. <sup>17</sup> have shown that MCP-1 overexpression in tumour breast carcinoma cells correlates with the histological grade and a low level of differentiation. Similarly, the expression of other chemokines, such as IL-8/CXCL8 (Interleukin-8) and SDF-1/CXCL12 (Stromal Cell Derived Factor-1), has also been associated with the metastatic and invasive phenotype of breast carcinoma cells, melanoma cells,<sup>51</sup> gastric carcinoma cells,<sup>52,53</sup> prostate cancer cells<sup>54</sup> and bladder cancer cells. Together with our results, these data suggest that the surexpression of MCP-1 is restricted to tumour cells which display invasive properties, a loss of E-cadherin expression at the cell membrane and a nuclear translocation of  $\beta$ -catenin.

In addition to the correlation between E-cadherin/ $\beta$ -catenin redistribution and MCP-1 expression, we also showed that MCP-1 promoter activity and MCP-1 mRNA expression were upregulated after cotransfection of  $\beta$ -catenin and TCF-4. Even though the sequence

of the MCP-1 promoter revealed a putative  $\beta$ -catenin/ TCF binding site, mutation of this site did not abrogate the regulation of the MCP-1 promoter. The implication of other  $\beta$ -catenin/ TCF binding sites similar in sequence cannot be excluded. Alternatively, other  $\beta$ -catenin/TCF target genes could activate the MCP-1 promoter. Furthermore, the fact that MCP-1 regulation following  $\beta$ -catenin/TCF-4 transfections was observed only in invasive, E-cadherin-negative BT549 and Hs578T cells but not in MCF7 cells underlines the influence of the cellular context on the  $\beta$ -catenin/TCF transcriptional regulation. This indeed emphasizes the importance of the levels of endogenous  $\beta$ -catenin and TCF/LEF factors but also of other transcriptional activators or repressors that can directly or indirectly interact with this complex. Indeed, an increasing number of TCF-bound compressors (Groucho, CtBP,...) have been identified.<sup>21,56-58</sup> Also, a synergy between  $\beta$ -catenin-TCF/LEF complexes and other transcription factors (including AP-1 or PEA-3) have been described in the promoter regulation of several  $\beta$ -catenin/TCF targets genes including MMP-7 or the  $\gamma$ 2 chain of laminin-5.<sup>35,59</sup> The human MCP-1 promoter has accordingly been shown to be activated by several transcription factors including NF $\kappa$ B, Sp1 or AP-1.<sup>37,60-62</sup> Also emphasizing the importance of the cellular context, we observed that the transfection of TCF-4 alone led to an upregulation of the MCP-1 promoter activity in the BT549 and Hs578T cells in which  $\beta$ -catenin is abundantly found in the nucleus.<sup>34</sup> This endogenous level of nuclear  $\beta$ -catenin might thus be sufficient to activate the MCP-1 promoter in the presence of TCF-4. In agreement with our results, Levy et al.<sup>32</sup> have shown that, in hepatoma cells containing a constitutively activated  $\beta$ -catenin allele, exogenous wild-type TCF-4 is sufficient to activate the IL-8 promoter. In contrast, the deleted form of TCF-4 which lacks the  $\beta$ -catenin binding domain ( $\Delta$ NTCF-4) was unable to activate this promoter. Accordingly, our findings showing that  $\Delta$ NTCF-4 does not transactivate the MCP-1 promoter clearly implicate  $\beta$ -catenin in this regulation. The decreased expression of MCP-1 mRNA and protein observed following transfection of a  $\beta$ -catenin siRNA in BT549 and Hs578T also emphasizes the implication of the  $\beta$ -catenin/TCF pathway in MCP-1 regulation. Therefore, if the cellular context obviously modulates the  $\beta$ -catenin/TCF regulation of the human MCP-1 promoter, our data clearly indicate that MCP-1 can be a target of the  $\beta$ -catenin/TCF pathway. Because of the major roles of MCP-1 in tumour progression, such a positive regulation of MCP-1 by the  $\beta$ -catenin/TCF pathway have obvious implications for tumour progression. Accordingly, many targets of the  $\beta$ -catenin pathway identified so far have been shown to play a key role in tumour progression by affecting tumour cell proliferation, tumour cell invasive properties or tumour cell survival. More particularly in agreement with our observations, other chemokines, including IL-8/CXCL8, another angiogenic chemokine, and MCP-3/CCL7, have been shown to be regulated by the  $\beta$ -catenin/TCF pathway.<sup>32,63</sup> The proangiogenic vascular endothelial growth factor (VEGF) has also been shown to be regulated by the  $\beta$ -catenin/TCF pathway.

In conclusion, our results demonstrate that MCP-1 expression is restricted to invasive breast cancer cell lines and correlates with the reorganization of the E-cadherin/ $\beta$ -catenin complexes in these cells. We also show that MCP-1 is a target gene of the  $\beta$ -catenin/TCF pathway. Considering the various stimulatory roles of MCP-1 on tumour progression, such a regulation of MCP-1 by the  $\beta$ -catenin/TCF pathway might play an important role in breast cancer progression.

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## References:

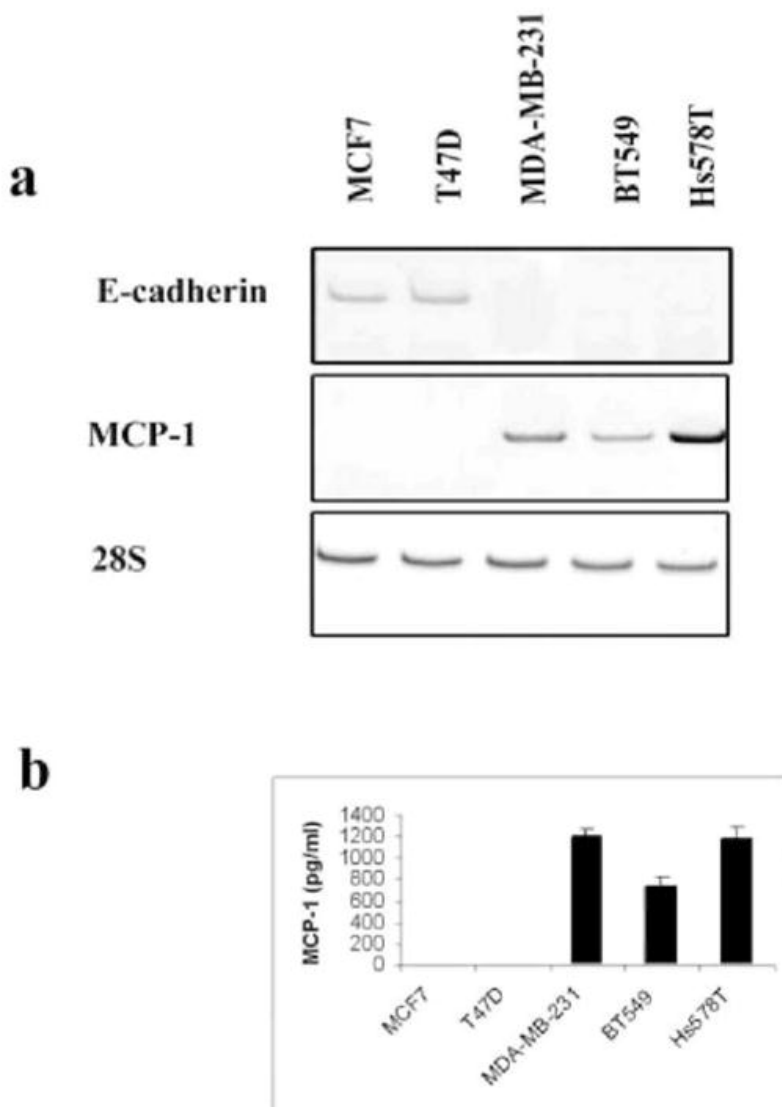
1. Baggiolini M, Dewald B, Moser B. Human chemokines: an update. *Annu Rev Immunol*. 1997; 15: 675 - 705
2. Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol*. 2000; 18: 217 - 42
3. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001; 410: 50 - 6
4. Daly C, Rollins BJ. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. *Microcirculation*. 2003; 10: 247 - 57
5. Yu JL, Rak JW. Host microenvironment in breast cancer development: inflammatory and immune cells in tumour angiogenesis and arteriogenesis. *Breast Cancer Res*. 2003; 5: 83 - 8
6. Huang S, Singh RK, Xie K, Gutman M, Berry KK, Bucana CD, Fidler IJ, Bar-Eli M. Expression of the JE/MCP-1 gene suppresses metastatic potential in murine colon carcinoma cells. *Cancer Immunol Immunother*. 1994; 39: 231 - 8
7. Monti P, Leone BE, Marchesi F, Balzano G, Zerbi A, Scaltrini F, Pasquali C, Calori G, Pessi F, Sperti C, Di CV, Allavena P. The CC chemokine MCP-1/CCL2 in pancreatic cancer progression: regulation of expression and potential mechanisms of antimalignant activity. *Cancer Res*. 2003; 63: 7451 - 61
8. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, Koike M, Inadera H, Matsushima K. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res*. 2000; 6: 3282 - 9
9. Saji H, Koike M, Yamori T, Saji S, Seiki M, Matsushima K, Toi M. Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. *Cancer*. 2001; 92: 1085 - 91

- 10 . Ohta M , Kitadai Y , Tanaka S , Yoshihara M , Yasui W , Mukaida N , Haruma K , Chayama K . Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human gastric carcinomas . *Int J Oncol* . 2003 ; 22 : 773 - 8
- 11 . Nakashima E , Mukaida N , Kubota Y , Kuno K , Yasumoto K , Ichimura F , Nakanishi I , Miyasaka M , Matsushima K . Human MCAF gene transfer enhances the metastatic capacity of a mouse cachectic adenocarcinoma cell line in vivo . *Pharm Res* . 1995 ; 12 : 1598 - 604
- 12 . Salcedo R , Ponce ML , Young HA , Wasserman K , Ward JM , Kleinman HK , Oppenheim JJ , Murphy WJ . Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression . *Blood* . 2000 ; 96 : 34 - 40
- 13 . Youngs SJ , Ali SA , Taub DD , Rees RC . Chemokines induce migrational responses in human breast carcinoma cell lines . *Int J Cancer* . 1997 ; 71 : 257 - 66
- 14 . Lebrecht A , Grimm C , Lantzsch T , Ludwig E , Hefler L , Ulbrich E , Koelbl H . Monocyte chemoattractant protein-1 serum levels in patients with breast cancer . *Tumour Biol* . 2004 ; 25 : 14 - 7
- 15 . Amann B , Perabo FG , Wirger A , Hugenschmidt H , Schultze-Seemann W . Urinary levels of monocyte chemo-attractant protein-1 correlate with tumour stage and grade in patients with bladder cancer . *Br J Urol* . 1998 ; 82 : 118 - 21
- 16 . Koide N , Nishio A , Sato T , Sugiyama A , Miyagawa S . Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus . *Am J Gastroenterol* . 2004 ; 99 : 1667 - 74
- 17 . Valkovic T , Lucin K , Krstulja M , Dobi-Babic R , Jonjic N . Expression of monocyte chemotactic protein-1 in human invasive ductal breast cancer . *Pathol Res Pract* . 1998 ; 194 : 335 - 40
- 18 . Ohta M , Kitadai Y , Tanaka S , Yoshihara M , Yasui W , Mukaida N , Haruma K , Chayama K . Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas . *Int J Cancer* . 2002 ; 102 : 220 - 4
- 19 . Behrens J . Cadherins and catenins: role in signal transduction and tumor progression . *Cancer Metastasis Rev* . 1999 ; 18 : 15 - 30
- 20 . Van Aken E , De Wever O , Correia da Rocha AS , Mareel M . Defective E-cadherin/catenin complexes in human cancer . *Virchows Arch* . 2001 ; 439 : 725 - 18
- 21 . Hecht A , Kemler R . Curbing the nuclear activities of beta-catenin: control over Wnt target gene expression . *EMBO Rep* . 2000 ; 1 : 24 - 8
- 22 . Wijnhoven BP , Dinjens WN , Pignatelli M . E-cadherin-catenin cell-cell adhesion complex and human cancer . *Br J Surg* . 2000 ; 87 : 992 - 1005
- 23 . Gottardi CJ , Gumbiner BM . Adhesion signaling: how beta-catenin interacts with its partners . *Curr Biol* . 2001 ; 11 : R792 - 94
- 24 . Conacci-Sorrell M , Zhurinsky J , Ben Ze'ev A . The cadherin-catenin adhesion system in signaling and cancer . *J Clin Invest* . 2002 ; 109 : 987 - 91
- 25 . Giles RH , van Es JH , Clevers H . Caught up in a Wnt storm: Wnt signaling in cancer . *Biochim Biophys Acta* . 2003 ; 1653 : 1 - 24
- 26 . Jechlinger M , Grunert S , Tamir IH , Janda E , Ludemann S , Waerner T , Seither P , Weith A , Beug H , Kraut N . Expression profiling of epithelial plasticity in tumor progression . *Oncogene* . 2003 ; 22 : 7155 - 69
- 27 . He TC , Sparks AB , Rago C , Hermeking H , Zavel L , da Costa LT , Morin PJ , Vogelstein B , Kinzler KW . Identification of c-MYC as a target of the APC pathway . *Science* . 1998 ; 281 : 1509 - 12
- 28 . Brabletz T , Jung A , Dag S , Hlubek F , Kirchner T . beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer . *Am J Pathol* . 1999 ; 155 : 1033 - 8
- 29 . Wielenga VJ , Smits R , Korinek V , Smit L , Kielman M , Fodde R , Clevers H , Pals ST . Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway . *Am J Pathol* . 1999 ; 154 : 515 - 23
- 30 . Miwa N , Furuse M , Tsukita S , Niikawa N , Nakamura Y , Furukawa Y . Involvement of claudin-1 in the beta-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers . *Oncol Res* . 2000 ; 12 : 469 - 76
- 31 . Marchenko GN , Ratnikov BI , Rozanov DV , Godzik A , Deryugina EI , Strongin AY . Characterization of matrix metalloproteinase-26, a novel metalloproteinase widely expressed in cancer cells of epithelial origin . *Biochem J* . 2001 ; 356 : 705 - 18
- 32 . Levy L , Neuveut C , Renard CA , Chameau P , Branchereau S , Gauthier F , Van Nhieu JT , Cherqui D , Petit-Bertron AF , Mathieu D , Buendia MA . Transcriptional activation of interleukin-8 by Beta-catenin-Tcf4 . *J Biol Chem* . 2002 ; 277 : 42386 - 93
- 33 . Easwaran V , Lee SH , Inge L , Guo L , Goldbeck C , Garrett E , Wiesmann M , Garcia PD , Fuller JH , Chan V , Randazzo F , Gundel R . Beta-Catenin regulates vascular endothelial growth factor expression in colon cancer . *Cancer Res* . 2003 ; 63 : 3145 - 53
- 34 . Gilles C , Polette M , Mestdagt M , Nawrocki-Raby B , Ruggeri P , Birembaut P , Foidart JM . Transactivation of vimentin by beta-catenin in human breast cancer cells . *Cancer Res* . 2003 ; 63 : 2658 - 64
- 35 . Hlubek F , Spaderna S , Jung A , Kirchner T , Brabletz T . Beta-catenin activates a coordinated expression of the preinvasive factors laminin-5 gamma2 chain and MT1-MMP in colorectal carcinomas . *Int J Cancer* . 2004 ; 108 : 321 - 6
- 36 . Marchenko ND , Marchenko GN , Weinreb RN , Lindsey JD , Kyshtoobayeva A , Crawford HC , Strongin AY . Beta-catenin regulates the gene of MMP-26, a novel metalloproteinase expressed both in carcinomas and normal epithelial cells . *Int J Biochem Cell Biol* . 2004 ; 36 : 942 - 56
- 37 . Ueda A , Okuda K , Ohno S , Shirai A , Igarashi T , Matsunaga K , Fukushima J , Kawamoto S , Ishigatsubo Y , Okubo T . NF-kappa B and Spl regulate transcription of the human monocyte chemoattractant protein-1 gene . *J Immunol* . 1994 ; 153 : 2052 - 63
- 38 . Korinek V , Barker N , Morin PJ , van Wichen D , de Weger R , Kinzler KW , Vogelstein B , Clevers H . Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC<sup>-/-</sup> colon carcinoma . *Science* . 1997 ; 275 : 1784 - 7
- 39 . van de WM , Cavallo R , Dooijes D , van Beest M , van Es J , Loureiro J , Ypma A , Hursh D , Jones T , Bejsovec A , Peifer M , Mortin M . Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF . *Cell* . 1997 ; 88 : 789 - 99
- 40 . Orford K , Crockett C , Jensen JP , Weissman AM , Byers SW . Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin . *J Biol Chem* . 1997 ; 272 : 24735 - 8
- 41 . Thompson EW , Paik S , Brunner N , Sommers CL , Zugmaier G , Clarke R , Shima TB , Torri J , Donahue S , Lippman ME . Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines . *J Cell Physiol* . 1992 ; 150 : 534 - 44
- 42 . Sommers CL , Byers SW , Thompson EW , Torri JA , Gelmann EP . Differentiation state and invasiveness of human breast cancer cell lines . *Breast Cancer Res Treat* . 1994 ; 31 : 325 - 35
- 43 . Nawrocki RB , Polette M , Gilles C , Clavel C , Strumane K , Matos M , Zahm JM , Van Roy F , Bonnet N , Birembaut P . Quantitative cell dispersion analysis: new test to measure tumor cell aggressiveness . *Int J Cancer* . 2001 ; 93 : 644 - 52
- 44 . Sommers CL , Thompson EW , Torri JA , Kemler R , Gelmann EP , Byers SW . Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities . *Cell Growth Diff* . 1991 ; 2 : 365 - 72
- 45 . Negus RP , Stamp GW , Relf MG , Burke F , Malik ST , Bernasconi S , Allavena P , Sozzani S , Mantovani A , Balkwill FR . The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer . *J Clin Invest* . 1995 ; 95 : 2391 - 6
- 46 . Nesbit M , Schaidt H , Miller TH , Herlyn M . Low-level monocyte chemoattractant protein-1 stimulation of monocytes leads to tumor formation in nontumorigenic melanoma cells . *J Immunol* . 2001 ; 166 : 6483 - 90
- 47 . Metelitsa LS , Wu HW , Wang H , Yang Y , Warsi Z , Asgharzadeh S , Groshen S , Wilson SB , Seeger RC . Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2 . *J Exp Med* . 2004 ; 199 : 1213 - 21
- 48 . Negus RP , Stamp GW , Hadley J , Balkwill FR . Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines . *Am J Pathol* . 1997 ; 150 : 1723 - 34
- 49 . De Larco JE , Wurtz BR , Rosner KA , Erickson SA , Gamache DE , Manivel JC , Furent LT . A potential role for interleukin-8 in the metastatic phenotype of breast carcinoma cells . *Am J Pathol* . 2001 ; 158 : 639 - 46
- 50 . Fernandes AZ , Prasad A , Band H , Klosel R , Ganju RK . Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells . *Oncogene* . 2004 ; 23 : 157 - 67
- 51 . Singh RK , Gutman M , Radinsky R , Bucana CD , Fidler IJ . Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice . *Cancer Res* . 1994 ; 54 : 3242 - 7

- 52. Kitadai Y, Haruma K, Mukaida N, Ohmoto Y, Matsutani N, Yasui W, Yamamoto S, Sumii K, Kajiyama G, Fidler IJ, Tahara E. Regulation of disease-progression genes in human gastric carcinoma cells by interleukin 8. *Clin Cancer Res*. 2000; 6: 2735 - 40
- 53. Saito S, Kitayama J, Jin ZX, Tsuno N, Kaisaki S, Seto Y, Nagawa H. Beta-chemokine, macrophage inflammatory protein-1beta (MIP1beta), is highly expressed in diffuse type human gastric cancers. *J Exp Clin Cancer Res*. 2003; 22: 453 - 9
- 54. Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CP. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res*. 2000; 6: 2104 - 19
- 55. Inoue K, Slaton JW, Kim SJ, Perrotte P, Eve BY, Bar-Eli M, Radinsky R, Dinney CP. Interleukin 8 expression regulates tumorigenicity and metastasis in human bladder cancer. *Cancer Res*. 2000; 60: 2290 - 9
- 56. Cavallo RA, Cox RT, Moline MM, Roose J, Polevoy GA, Clevers H, Peifer M, Bejsovec A. Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature*. 1998; 395: 604 - 8
- 57. Levanon D, Goldstein RE, Bernstein Y, Tang H, Goldenberg D, Stifani S, Paroush Z, Groner Y. Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *Proc Natl Acad Sci USA*. 1998; 95: 11590 - 5
- 58. Barolo S, Stone T, Bang AG, Posakony JW. Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes Dev*. 2002; 16: 1964 - 76
- 59. Crawford HC, Fingleton BM, Rudolph-Owen LA, Goss KJ, Rubinfeld B, Polakis P, Matrisian LM. The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. *Oncogene*. 1999; 18: 2883 - 91
- 60. Ueda A, Ishigatsubo Y, Okubo T, Yoshimura T. Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappaB sites and NF-kappaB/Rel subunit specificity. *J Biol Chem*. 1997; 272: 31092 - 9
- 61. Wang N, Verna L, Hardy S, Forsayeth J, Zhu Y, Stemerman MB. Adenovirus-mediated overexpression of c-Jun and c-Fos induces intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 in human endothelial cells. *Arterioscler Thromb Vasc Biol*. 1999; 19: 2078 - 84
- 62. Kanda N, Watanabe S. 17Beta-estradiol inhibits MCP-1 production in human keratinocytes. *J Invest Dermatol*. 2003; 120: 1058 - 66
- 63. Fujita M, Furukawa Y, Nagasawa Y, Ogawa M, Nakamura Y. Down-regulation of monocyte chemotactic protein-3 by activated betacatenin. *Cancer Res*. 2000; 60: 6683 - 7

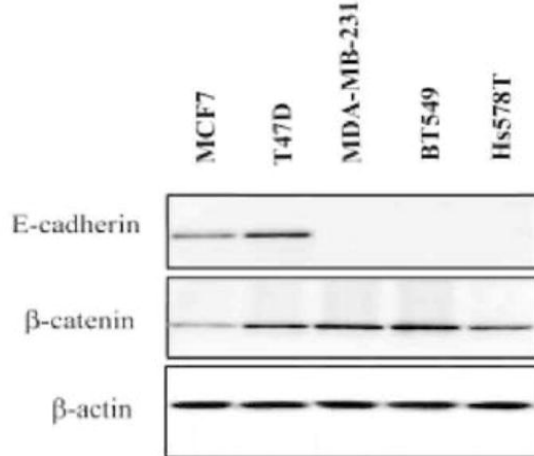
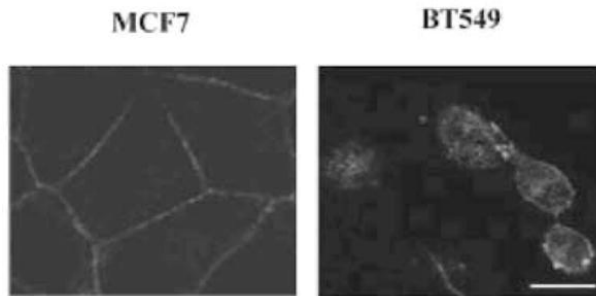
### FIGURE 1

MCP-1 expression is restricted to invasive, E-cadherin-negative breast tumour cell lines, (a) RT-PCR analysis of MCP-1 and E-cadherin in different human breast cancer cell lines (MCF7, T47D, MDA-MB-231, BT549 and Hs578T). The RT-PCR of the 28S mRNA is shown as a control. Each experiment was performed at least 3 times and 1 representative experiment is shown. (b) ELISA analysis of MCP-1 in 48 hr conditioned media obtained from the different breast cancer cell lines.



**FIGURE 2**

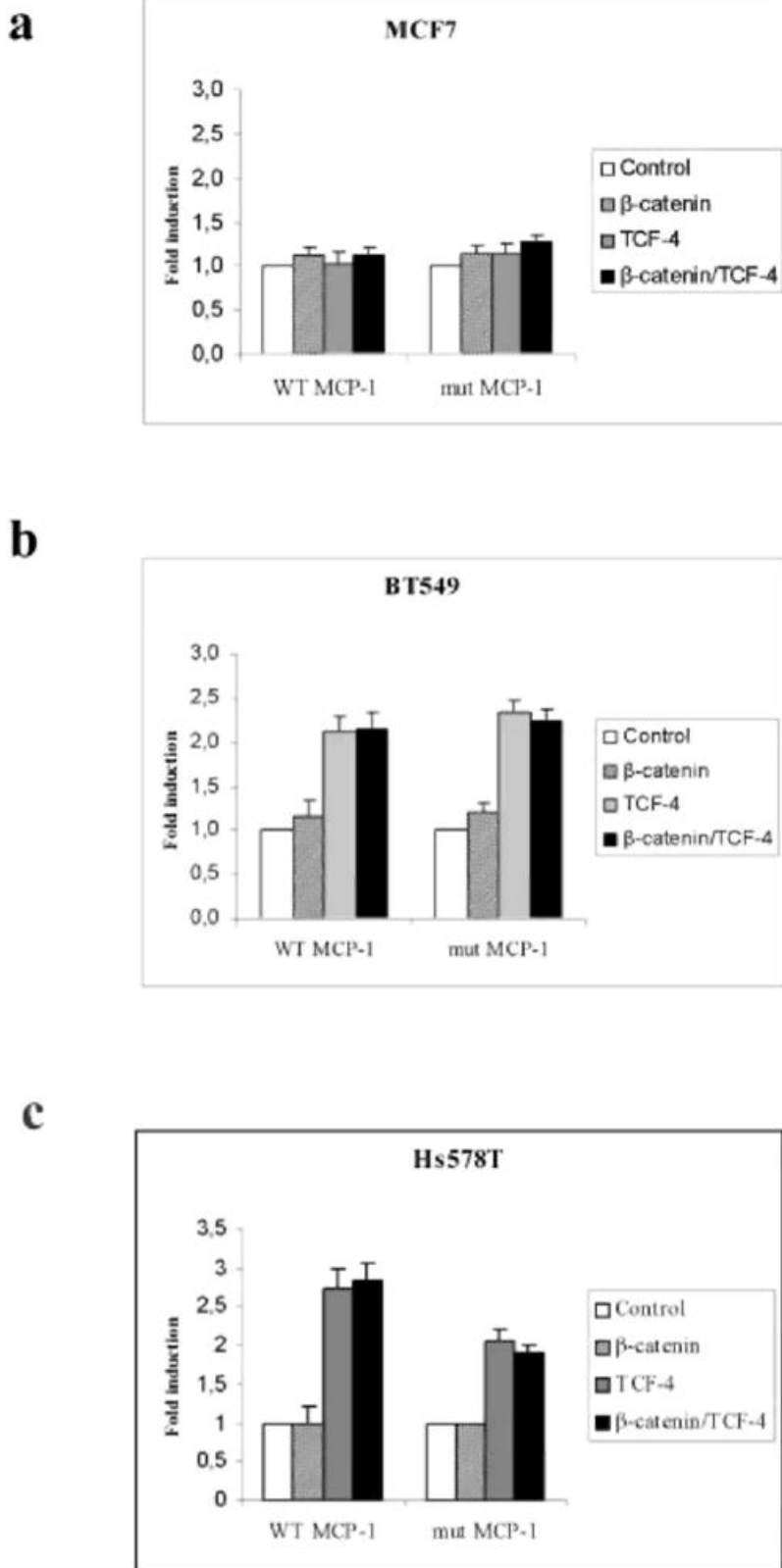
$\beta$ -catenin localization in the different breast cancer cell lines. (a) Western blot analysis of E-cadherin and  $\beta$ -catenin expression in human breast cancer cell lines (MCF7, T47D, MDA-MB-231, BT549 and Hs578T).  $\beta$ -actin level is shown as a control. (b) Confocal analysis of  $\beta$ -catenin localization in noninvasive, E-cadherin- positive, MCP-1-negative MCF7 breast cell line and in invasive, E-cadherin-negative, MCP-1-positive BT549 breast cancer cells. Bar = 8 $\mu$ M.

**a****b**



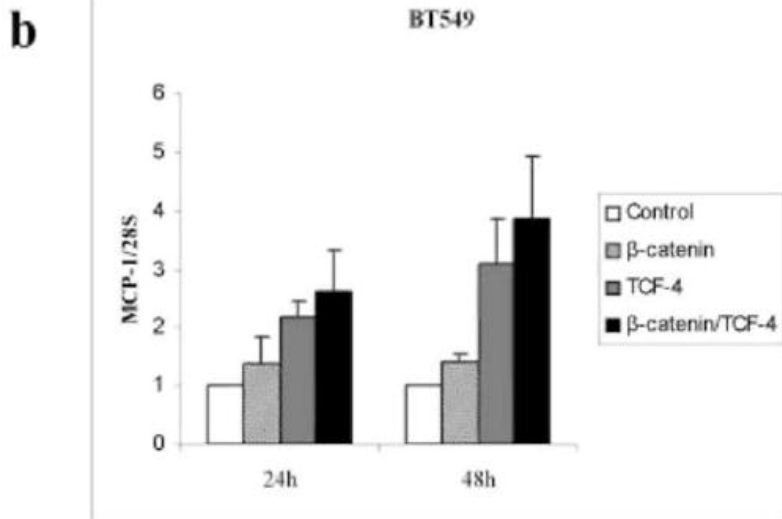
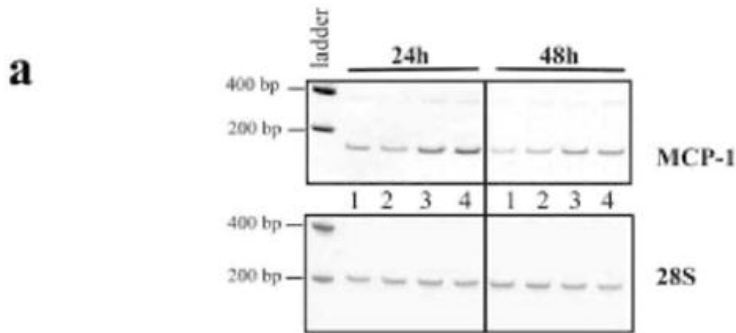
**FIGURE 3**

$\beta$ -catenin and TCF-4 transactivate the MCP-1 promoter. Luciferase activity of the wild-type (WT MCP-1) or the mutated (mut MCP-1) MCP-1 promoter cotransfected with the empty expression vectors (Control), the  $\beta$ -catenin expression vector ( $\beta$ -catenin), the TCF-4 expression vector (TCF-4) or the  $\beta$ -catenin and the TCF-4 expression vectors ( $\beta$ -catenin/TCF-4) in MCF7 (a), BT549 (b) and Hs578T cells (c). Results are expressed as fold induction relative to the cotransfection of the reporter plasmid (WT MCP-1 or mut MCP-1) with the empty expression vectors.



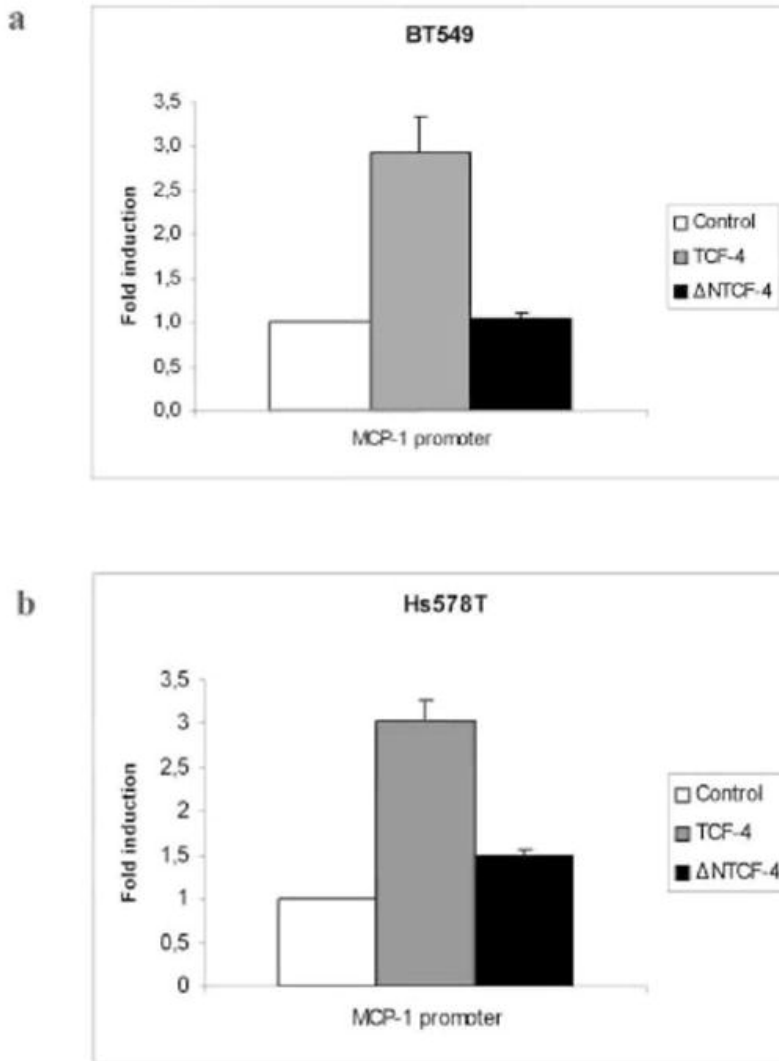
**FIGURE 4**

$\beta$ -catenin and TCF-4 upregulate MCP-1 mRNA in BT549 cells. (a) Representative RT-PCR analysis of MCP-1 and 28S expression in BT549 cells transfected with the  $\beta$ -catenin expression vector + pCDNA3zeo vector (lane 2), the TCF-4 expression vector + pCDNA3 vector (lane 3) or both  $\beta$ -catenin and TCF-4 expression vectors (lane 4). Controls were generated by transfecting the cells with the corresponding empty vectors (pcDNA3 + pcDNA3 zeo) (lane 1). (b) Quantification of the RT-PCR analyses of MCP-1 normalized with the 28S values in 3 independent transfection experiments as described in (a). Data are presented as fold of induction relative to the control condition and expressed as means  $\pm$  SEM.



**FIGURE 5**

$\Delta$ NTCF-4 does not activate the MCP-1 promoter. Luciferase activity of the wild-type MCP-1 promoter cotransfected with the wild-type TCF-4 or the  $\Delta$ NTCF-4 (which lacks the  $\beta$ -catenin binding domain) expression vector or the corresponding empty vector (Control) in BT549 (a) and Hs578T cells (b).



**FIGURE 6**

$\beta$ -catenin siRNA downregulates MCP-1 expression. (a) Western blotting analysis of  $\beta$ -catenin expression in BT549 and Hs578T cells transfected with the siRNA against  $\beta$ -catenin (lane 2) or the control siRNA (lane 1).  $\beta$ -actin level is shown as a control. (b1) Representative RT-PCR analysis of MCP-1 and 28S expression in BT549 and Hs578T cells transfected with the siRNA against  $\beta$ -catenin (lane 2) or the control siRNA (lane 1). (b2) Quantification of the RT-PCR analyses of MCP-1 normalized with the 28S values in 3 independent siRNA transfection experiments as described in (b1). Data are expressed as means  $\pm$  SEM. (c) ELISA analyses of MCP-1 in conditioned media obtained from 3 independent experiments on Hs578T and BT549 cells transfected with  $\beta$ -catenin siRNA or the scrambled siRNA used as control. Data are expressed as means  $\pm$  SEM.

