Cis-regulatory functions of overlapping HIF-1alpha/E-box/AP-1-like sequences of CD164

Jingqun Tang1,3, Zhaohui Luo2, Guangqian Zhou4, Chao Song3, Fenglei Yu1, Juanjuan Xiang2,3* and Gang Li5,6*

Abstract

Background: CD164 (also known as MGC-24v or endolyn) is a sialomucin which has been suggested to participate in regulating the proliferation, cell adhesion and differentiation of hematopoietic stem and progenitor cells. CD164 is also involved in the development of cancer. The functions of cis-regulatory elements of CD164 remain relatively unknown.

Methods: In this study, we investigated the function of cis-regulatory elements within the promoter of CD164. We fused the 5′-flanking region of CD164 to a luciferase reporter vector. The minimal promoter region was confirmed by luciferase reporter assay. Using in silico analysis, we found the presence of one HIF-1alpha (HIF-1A) motif (5′-RCGTG-3′) overlapping E-box (CACGTG) and two AP-1-like binding sites (CGCTGTCCC, GTCTGTTG), one of which is also overlapped with HIF-1alpha sequence. Dual-luciferase assay was performed to examine the transcriptional activity of AP-1 and HIF-1alpha of CD164 promoter. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to measure CD164 expression. Chromatin Immunoprecipitation was used to confirm the binding of HIF-1alpha and CD164.

Results: Co-transfection of c-jun, HIF-1alpha and minimal promoter region construct demonstrated that c-jun and HIF-1alpha bound the CD164 promoter and promoted CD164 expression. Hypoxia treatment also led to the up-regulation of CD164 expression. The mutation of overlapping sequences resulted in the reduced expression of CD164 induced by HIF-1alpha. Chromatin Immunoprecipitation demonstrated that the HIF-1alpha bound the minimal promoter region.

Conclusions: Determination of the optimal promoter region and transcription factors governing CD164 expression is useful in understanding CD164 functions. These results suggest that cis-regulatory elements of CD164 overlapping HIF-1alpha/E-box/AP-1-like sequences may play important regulatory roles.
the promoter region, regulating cell growth and differentiation. Our attention has recently turned to an investigation of the cis-regulatory elements of CD164. Determining the optimal promoter region and transcription factors governing CD164 expression is important in understanding CD164 functions.

In the present study, we proceeded to identify CD164 promoter and performed the functional analysis of cis-regulatory element on CD164 expression.

### Methods

1. **Prediction of promoter**
   
   Promoter 2.0 Prediction Server was employed for prediction of promoter. As CD164 first exon begins on 10418 (AL359711 CD164 gDNA), ATGtcgcggc, the 3192 bp sequence 5' upstream of TSS were analyzed by the this method.

2. **Construction of plasmids**
   
   Human c-jun expression plasmid pCMV-2/c-jun, which contains the full-length coding region of human c-jun cDNA cloned in pCMV-2 vector and pCMV-2 empty vector, were gifts from Dr. Gavin P. Collett (Department of Surgery, The Cancer Centre, Queen’s University Belfast, UK). Vector plasmids pcDNA3.1-HIF-1A(1-826) and pcDNA3.1 were kindly donated by Dr. Zhou (Department of Orthopaedics and Traumatology, The University of Hong Kong). Firefly luciferase reporter plasmid pGL3-Basic was purchased from Promega. For construction of 5'-deletions of CD164 promoter reporter plasmids, human genomic DNA was isolated using the FlexiGene DNA Kit (Qiagen), the CD164 promoter candidate fragments were amplified by polymerase chain reaction (PCR) from genomic DNA, and PGL3P4.2-4 fragment was synthesized by two complementary strand oligonucleotides. Fragments were cloned into the pCR2.1-TOPO vector (Invitrogen). Cloning of different fragments to pGL3-Basic (Promega), which is a vector carrying the firefly luciferase gene were performed after the restrictive enzyme cut. The restrictive endonucleases used for the cloning are shown in Table 1. The restriction endonucleases which were used predominantly were Sac I and Xholl, except P1 fragment, because of an internal SacI site existing in fragments between P1 and P1.5. P1 segment was obtained by using the Kpnl and Xholl sites in pCR2.1-TOPO vector. Finally, all plasmids were confirmed by sequencing. The oligonucleotides used for PCR primers are shown in Table 1.

3. **Identification and mutation of AP-1-like binding sites in CD164 promoter**
   
   Sequence homology to the AP-1 consensus sequence (TGACTCA) was identified using TFBIND, which is a software programme for searching transcription factor binding sites. Mutation of AP-1-like sites and Ebox sites was performed by Stratagene’s QuikChange II Site Directed Mutagenesis Kit.

   Primers for distal AP-1 mutation are as follows: -

   - forward: 5'-CAG GGG CCT CTC ACG CTG TCC CTG CGC GCT CCC G-3';
   - reverse: 5'-CGG GAG CGC GCA GGG ACA GCG TGA GAG GCC CCT G-3'.

   Primers for proximal AP-1 mutation were as follows: -

   - forward: 5'-CAG GGG ATT GAG GGG TCT GTT GAG CGT TGC GAG CCT TAG-3';
   - reverse: 5'-CTA AGG CTC GCA ACG CTC AAC AGA CCC CTC AAT CCC CTG-3'.

   Primers for Ebox mutation: -

   - forward: 5'-CCG AAA ACA GGG GCC TCT AGA TTG ACC CCT GGC GCC TCC CGC GGG-3';
   - reverse: 5'-CCC GCG GGA GCG CGC AGG GGT CAA TCT AGA GGC CCC TGT TTT CGG-3';

   Primers for Ebox deletion: -

### Table 1 The oligonucleotides used for PCR primers

| Forward | Reverse | Restriction sites | Amplified DNA Fragment Length (bps) |
|---------|---------|-------------------|-------------------------------------|
| P1      | 5'-aagggatctcctctagcctca-3' | 5'-cgtgccctcagcgcctggtctg-3' | Kpnl Xholl 1796 |
| P2      | 5'-aagggatctcctctagcctca-3' | 5'-cgtgccctcagcgcctggtctg-3' | Sacl Xholl 1453 |
| P3      | 5'-agttagatgctgctctctttta-3' | 5'-cgtgccctcagcgcctggtctg-3' | Sacl Xholl 1024 |
| P4      | 5'-gcacagtcgctctgaggggcc-3' | 5'-cgtgccctcagcgcctggtctg-3' | Sacl Xholl 1024 |
| P5      | 5'-taggtctcctcttgatcctc-3' | 5'-cgtgccctcagcgcctggtctg-3' | Sacl Xholl 197 |
| P6      | 5'-gaagccagggctctcctac-3' | 5'-cgtgccctcagcgcctggtctg-3' | Sacl Xholl 139 |
| P7      | 5'-cggaggagcgctagctgctc-3' | 5'-cgtgccctcagcgcctggtctg-3' | Sacl Xholl 100 |
| P8      | 5'-cggaggagcgctagctgctc-3' | 5'-cgtgccctcagcgcctggtctg-3' | Sacl Xholl 179 |

References:

Tang et al. BMC Molecular Biology 2011, 12:44
http://www.biomedcentral.com/1471-2199/12/44
Page 2 of 10
forward: 5'-GCA CGC CGA AAA CAG GGG CCT
CTA CCC CTG CGC GCT CCC GCG GGG-3';
reverse: 5'-CCC CGC GGG AGC GCG CAG GGG
TAG AGG CCC CTG TTT TCG GCG TGC-3'.

4. Luciferase activity assay
Firefly (Photinus pyralis) luciferase is widely used as a reporter of promoter activities by cloning interested promoters to the upstream of the firefly luciferase coding gene. Nowadays, the dual-reporter assays, where the activities of firefly and Renilla (Renilla reniformis, also known as sea pansy) luciferases are measured sequentially from a single sample, are commonly used to improve experimental accuracy. The activity of the experimental reporter (firefly) is normalized by the activity of the internal control (Renilla). Cells were transfected with constructed reporter vectors simultaneously with phRL vector (Promega) containing cDNA coding Renilla luciferase; 2 ng of the latter was applied to each well in a 24-well plate. 48 hrs after transfection, cells were harvested with passive Lysis buffer, a component of the Dual-Luciferase Reporter (DLR™) Assay System (Promega), according to the manufacturer’s instructions. An appropriate volume of cell lysate was added to a well of the F96 MicroWell™ Plates (NUNC, Roskilde, Denmark), followed by 25 μl of LARII. Firefly luciferase activities were measured with a luminometer (Tecan, Theale, UK). Renilla luciferase activities were also measured. Relative luciferase activity was represented by the ratio of firefly luciferase activity to renilla luciferase activity. Each experimental group included 3 repeats and data were shown as means.

5. Cell culture
In this work, HCT116 cells (cell line catalogues: ECACC 91091005) were exclusively used to study the role of CD164 in cancer development. All the cell lines were obtained from the European Collection of Cell Cultures (ECACC) and cultured in a CO2 incubator (Sanyo®). HCT116 and HT29 cell lines were cultured in DMEM and SW480, K562 in RPMI-1640 medium supplemented with penicillin G (100 U/mL; Sigma®), streptomycin (100 mg/mL; Sigma®) and 10% fetal calf serum (Gibco®). Cells were grown at 37°C in a humidified atmosphere of 5% CO2 and were routinely subcultured using 0.25% (v/v) trypsin-EDTA solution. For testing promoters’ activity under hypoxia, cells were transiently transfected with pGL3P4 plasmids and cultured in DMEM without serum for 24 hours; then the cells were transferred into an Invivo2 400™ Hypoxia workstation (Ruskinn Technology Ltd) for different time courses.

6. Western blot
Protein used for western blotting was extracted with mRIPA buffer containing protease inhibitors (mRIPA, 50 mM Tris, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, Aprotinin 10 μg/ml, Leupeptin 10 μg/ml, PMSF 1 mM). Proteins were quantified using BCA™ Protein Assay Kit (Pierce, Northumberland, UK). The Western Blot system was set up using a Biorad Bis-Tris Gel system, according to the manufacturer’s instructions (Biorad, Hertfordshire, UK). Rabbit-anti-human HIF-1α antibody (Millipore) was prepared in 3% blocking buffer at a dilution of 1:1000. The primary antibody was incubated with the membrane at 4°C overnight followed by a brief wash and incubation with secondary antibody for 1 hour at room temperature. Finally, peroxide and luminol solutions 1:1 (Pierce, UK) were added to cover the blot surface for five minutes at room temperature and the membrane was placed in a developing cassette.

7. Chromatin precipitation
Chromatin immunoprecipitation (ChIP) assay was performed using the Chromatin Immunoprecipitation Assay kit (EZ ChIP, Millipore) according to the manufacturer’s instructions. Briefly, HCT116 cells were transfected with pCDNA3.1-HIF-1A. Forty-eight hours after transfection, the cells were then cross-linked by 1% formaldehyde for 10 min. The formaldehyde was quenched using 2 M glycine for 5 min. at room temperature before harvest. Cells were collected by centrifuging in PBS containing protease inhibitors and were lysed in SDS-lysis buffer. Soluble chromatin was prepared after sonication to an average DNA length of 200 to 1000 bp. Fragmented chromatin was immunoprecipitated overnight at 4°C on a rotating platform, using antibodies against HIF-1α (Millipore) together with Protein A/G Plus-Agarose. The agarose beads were washed, chromatin extracted and protein-DNA cross-links reversed. DNA was purified and was analyzed by PCR using the specific primers (5’-GCACAGTCGCTTTGAGGCC-3’; 5’-CGTGTCCCTCAGCGCTGGCTTC-3’). Normal immunoglobulin G (IgG) was used as a negative control. Anti-RNA Polymerase II was used as positive control. The total input was the supernatant from the no-antibody control.

8. Quantitative real-time polymerase chain reaction (qRT-PCR)
The total RNAs were isolated from cells with Trizol® reagent (Invitrogen) and possible genomic DNA contamination were eliminated by treatment with RNase-free DNase 1 (Sigma) for 1 hr. The extracted RNAs were immediately reverse-transcribed using the SMART™ MMLV RT Reverse Transcriptase CDNA
Kit (Clontech). Real-time PCR was carried out on the iQ™5 Multicolor Real-Time PCR detection System (Bio-Rad) using the following specific primers: -

CD164 (5'-GTGCTGTCCGCGACAAGA-3'; 5'-TGTGAACAATAGCTCTCATC-3'); GAPDH (5'-CGAGATCCCTCAAAATCAA-3'; 5'-GGTGCTAAGCAGTGTTG-3').

The PCR primer pairs were designed with Primer3 software. Primer specificity has been validated by BLAST analysis. Real-time PCR was performed using SYBR Green Master Mix (an asymmetrical cyanine dye by Promega) with Mg²⁺ concentration 6 mM, template cDNA and 0.5 μM of each primer, with RNA-negative and water controls. The qPCR cycle was 98°C for 2 min., 40 cycles of 95°C for 15 sec., 60°C for 30 sec. Final melt-curve analysis (60°-95°C) was included. The standard curve was produced with slopes at approximately -3.32 (~100% efficiency); PCR quantification used ΔΔct method for the CD164 against the GAPDH for normalization. The data are representative of the means of three experiments.

Results

Identification of CD164 promoter

Promoter 2.0 Prediction Server [14] was employed for predicting the CD164 promoter region. Promoter 2.0 predicts transcription start sites of vertebrate PolII promoters in DNA sequences. It can also be used to predict transcription factors that bind to the sequences in promoter regions, based on the theorems that are common to neural networks and genetic algorithms (http://www.cbs.dtu.dk/services/Promoter/). As first exon of the CD164 gene begins on 10418 [GenBank: AL359711], atgtcgcggc, a 3192 bp sequence at the 5' upstream of the transcription start site (TSS) was analyzed by Promoter 2.0 Prediction. The sequence of the putative promoter region of the gene is embedded at position -800 bp upstream of the TSS (Table 2). To determine the minimal promoter region, the 1.7 kb genomic sequence was subcloned in pGL3 basic upstream of the luciferase reporter gene. These 5'-deleted constructs pGL3 P1 (-1796/1), pGL3 P2 (-1153/1), pGL3 P3 (-1024/1), pGL3 P4 (-258/1), pGL3 P5 (-197/1), pGL3 P6 (-139/1), pGL3 7 (-135/-96) and pGL3 8 (-100/1) were transfected to HCT116, as shown in Figure 1B. The primers amplifying the corresponding fragments were as shown in Methods. Renilla luciferase was used for normalization of firefly luciferase expression. The highest activity appeared in P3, in which the length of promoter is from -1024 upstream of the TSS (Figure 1B). P4 showed a lower level of activation than P3. However, balancing their length, P4 was recognized as the minimal promoter region for the CD164 gene (Figure 1C). Deletion of the region from P3 to P6 resulted in a 75% reduction in basal promoter activity. These data suggested the existence of a minimal promoter region within the sequence -258/-1. P3 activity was analyzed in different cell lines including K562, HCT116, HT29 and SW480, of which HCT116 showed the highest P4 activity (Figure 1D).

In silico prediction of minimal promoter regions

The putative minimal promoter regions P4 (-258 upstream of the TSS) were analyzed with TFBIND (http://tfbind.hgc.jp/), a software program for searching transcription factor binding sites [15]. The P4 sequence contains two AP-1-like binding sites which are located at -119 bps (A, distal) and -54 bps (B, proximal) upstream of the TSS, respectively (Figure 2B). The results are shown in Table 3.

Effects of C-Jun and HIF-1alpha on the P4 minimal promoter activity

C-Jun is a proto-oncogene. Its expression has been studied in many tumour types [16]. C-Jun, in combination with c-FOS, forms the AP-1 early response transcription factor. In order to determine the effect of the potential effects of C-Jun on minimal promoter P4 activity, we co-transfected HCT116 cells and SW480 cells with pCMV-c-jun or pCMV-2 empty vector and reporter vector pGL3 P4 (-258/1). The C-Jun was able to increase the minimal promoter activity by about 50% in both cell lines (Figure 2A). The upregulation of CD164 by C-Jun at mRNA level was observed using real-time PCR (Figure 2A). To investigate whether the AP-1-like motifs play roles in CD164 gene expression, the wild-type pGL3 P4 construct or its mutants (P4AP-1 Amu, P4AP-1 Bmu, P4AP-1 Both mu pGL3 constructs) as shown in Figure 2B, were transfected into HCT116 cells. In HCT116 cells, the activities of P4AP-1 Amu and P4AP-1, Both mu mutants, were reduced by about 40% compared to pGL3P4 wild-type (Figure 2B). In contrast, there was no significant change of activity in P4AP-1 Bmu (p > 0.05 Student’s t-test) compared to pGL3P4 wild-type. Significant difference (p < 0.05 Student’s t-test) between P4AP-1 Amu and P4AP-1 Bmu showed the location of different responses to endogenous transcription factors. The distal AP-1-like motif which

| Position | Score | Likelihood       |
|----------|-------|------------------|
| 800      | 1.107 | Highly likely prediction |
| 2100     | 0.607 | Marginal prediction |
| 2800     | 0.588 | Marginal prediction |

Table 2 Prediction of the CD164 promoter using Promoter 2.0 Prediction Server

The sequence of the putative promoter region of the gene is embedded at position -800 bp upstream of TSS
overlapped with HIF-1alpha binding sequence in the promoter was essential for CD164 transcription. The responses of AP-1-like motif to exogenous C-Jun were tested by co-transfected pCMV-C-Jun construct and the mutants. The mutants, especially P4AP-1 Amu and P4AP-1 Bmu, resulted in decreased activities compared to the wild-type. These data suggested that the AP-1-like motif is one of the main elements in the CD164 gene promoter. P4AP-1 Bmu, after treatment with exogenous C-Jun showed significantly different promoter activity, compared to the control. It seemed that AP-1-like site in -54 (proximal) was more sensitive to exogenous C-Jun than endogenous stimulatory factors.

TFBIND also identified several other transcription factors which contain putative binding sites for transcription factors, several of which exactly match or are very similar to those already known, like the AP-1-like sites described above. It also revealed the presence of one HIF-1alpha motif (5'-RCGTG-3') overlapping E-box (CACGTG) and distal AP-1-like binding sites (Figure 3A). To investigate the contribution of this E-box to the activity of the P4 region, constructs with mutation the E-box in the promoter region were subcloned into the pGL3-basic plasmids and used to transfect the HCT116 cells. For the normalized luciferase activity, a significant decrease of luciferase activity was found in the P4 containing E-box mutation compared to the HCT116 cells.
Figure 2 Effects of c-jun expression on the transcriptional activation of CD164 promoter. (A) SW480 and HCT116 cells were transiently cotransfected with the pGL3P4 (0.8 μg/μl) and pCMV-c-jun (0.8 μg/μl), luciferase activity of cell lysates was measured and normalized to renilla activity as internal control (left). Results represented the mean ± SD of three independent experiments. Exogenous c-jun significantly enhanced the CD164 expression, *p < 0.01, Student’s t-test. The Real-time PCR were performed to investigate the upregulated expression CD164 at mRNA level in HCT116 (right). (B) Mutational analysis of AP-1-like sites on CD164 promoter fragment P4. Schematic representation of the pGL3P4 regions containing AP-1-like binding sequence and its mutants: pGL3P4, a wild-type construct containing 258 bps of the 5′-flanking sequence upstream of TSS; P4AP-1 Amu, a mutated pGL3P4 plasmid containing a mutated AP-1-like motif (CGCTGTCCC) at -119; P4AP-1 Bmu, a mutated pGL3P4 plasmid containing a mutated AP-1-like motif (GTCTGTTG) at -54; P4AP-1 Both mu, a mutated pGL3P4 plasmid containing two mutated AP-1-like motifs (CGCTGTCCC, GTCTGTTG) at both of -119 and -54. pGL3P4 and its mutants were transfected in HCT 116 cells respectively and assayed for luciferase activity. The relative activities of wild-type P4 and AP-1-like mutated P4 mutants were normalized with renilla expression. P4AP-1 Amu and P4AP-1 Both mu resulted in significant decreases in luciferase activity when compared to pGL3P4, whereas no significant change of luciferase activity was observed in P4AP-1 Bmu.

Table 3 Prediction of AP-1-like transcription factor binding sites on the P4 promoter

| Access | ID   | Score  | Location | Strand | Consensus Sequence | Signal Sequence |
|--------|------|--------|----------|--------|-------------------|-----------------|
| M00174 | V$AP1_Q6 | 0.818353 | -119     | (+)    | NNTGACTCANN       | CGTGACCCCTG     |
| M00174 | V$AP1_Q6 | 0.863106 | -54      | (+)    | NNTGACTCANN       | GTGACTGAGC      |

AP-1-like binding sites (distal and proximal) were found
transfected with wild-type P4 (p < 0.001, student T-Test, Figure 3B). To determine whether HIF-1alpha mediates the increased activity of pGL3P4 in HCT116 cells, co-transfection of a HIF-1alpha plasmid with pGL3P4 was performed. Co-transfection of a HIF-1alpha expressing plasmid with pGL3P4 resulted in a significant increase in the promoter activity (p < 0.01, Student’s t-test, Figure 3B). To investigate the effect of HIF-1alpha through HIF-1alpha/E-box overlapping sequence, the pcDNA3.1-HIF-1alpha was co-transfection with PGL3P4 containing E-box mutation. For the normalized luciferase activity, a significant decrease of luciferase activity was found in the co-transfection with PGL3P4 containing E-box mutation compared to the HCT116 cells co-transfected with wild-type PGL3P4 (p < 0.001, student T-Test, Figure 3B). It demonstrated that HIF-1alpha/E-box/AP-1-like sequences is crucial cis-regulatory elements of CD164 expression.

Hypoxia exists in the microenvironment of solid tumours and stem cells. In the present study, in vitro hypoxia treatment was carried out to examine if hypoxia mediates CD164 expression. The promoter activity was determined under hypoxic conditions and PGL3P4 plasmids were transiently transfected into the HCT116 cells. The cells were cultured in DMEM without serum for 24 hours, then transferred into gas-exchange chambers maintained at 37°C with 1% pO2.

Figure 4A shows that, compared to normal oxygen conditions, 2 hours of treatment of hypoxic conditions appear to depress pGL3P4 activity, although there is no significant difference. At 8 hours of hypoxic treatment, the activity of pGL3P4 was significantly higher than that of the normal oxygen level group (p < 0.05 Student’s t-test). The upregulation of CD164 by HIF-1alpha at mRNA level was observed using real-time PCR (Figure 4C). The result suggested that hypoxia might be one of the factors upregulating CD164 gene expression in HCT116 cells. HIF-1alpha levels were therefore tested using western blot in the experiments described above. Figure 4B shows that HIF-1alpha protein expression was increased in a time-dependent manner in the hypoxic chamber.

**Binding of HIF-1alpha to PGL3P4 minimal promoter sequence**

In order to confirm the binding of HIF-1alpha to the promoter of CD164, Chromatin immunoprecipitation (ChIP) assay was performed. We transfected HIF-1alpha plasmid to the HCT116 cells. The anti-HIF-1alpha was used to precipitate protein/chromatin complexes from sonicated samples. The complexes were then processed to DNA amplification. As shown in Figure 4D, it demonstrated the binding of HIF-1alpha to the minimal region of promoter of CD164.

**Discussion**

A cis-regulatory element is a region of DNA or RNA near a gene required for proper spatiotemporal expression of that gene, often containing binding sites for transcription factors. The mechanisms by which CD164 expression is activated have been defined in this study. The different lengths of 5’-deleted mutant constructs of the CD164 showed different luciferase activities. The luciferase activities reached their peak in P2 which was 1024 bps upstream of the transcription start site and was 70-fold greater than that of the control group. The luciferase activity assays also indicated that the CD164 promoter had relative cell specificity, as its ability to activate luciferase was different in HCT116, K562, HT29 and SW480 cells. The PGL3P4 promoter had a proximal region of approximate 258 bases that increased the promoter activity by more than 50-fold and was recognized as the most effective promoter when compared to their sizes.

From the *in silico* analysis, the overlapping HIF-1alpha/E-box/AP-1-like sequences was found in the minimal region of CD164 promoter. C-jun and hypoxia upregulated the expression of CD164. Mutation of the overlapping sites resulted in reduced expression of CD164. Two AP-1-like sites (distal site and proximal site) on this proximal promoter region, of which distal site located in the overlapping sites, were identified by the *in silico* analysis. The consensus AP-1 (activator protein 1) recognition sequence is TGACTCA but many variations of this sequence (AP-1-like sites) were found in the promoter regions of target genes, including tyrosine hydroxylase [17], prodrophin [18], proenkephalin [19] and glial fibrillary acidic protein [20]. Two putative AP-1-like sites in the CD164 gene PGL3P4 segment were found in the present study by bioinformatic analyses. The AP-1 transcription factor is a dimeric complex, and correlates with multi-stage tumor development, including tumor cell proliferation [21,22]; apoptosis [23,24]; tumor invasion and angiogenesis [25,26]. The main AP-1 proteins in mammalian cells are Jun and FOS. Endogenous and exogenous transcription factors influence the CD164 expression by binding to AP-1-like sites. In the condition without exogenous factors, the distal AP-1-like site, located at -119, which was supposed to bind endogenous factors including c-jun, may play a major role, as mutation of this site induced a 40% decrease of CD164 basal expression in HCT116 cells, whereas no significant effect was found by mutating the proximal AP-1-like site. Therefore, the distal AP-1-like site which is overlapped with HIF-1alpha/E-BOX sequence plays a more important role than the proximal AP-1-like site, which is located at -54 for endogenous factors. However, the proximal AP-1-like
Figure 3 The functions of overlapping E-box, HIF-1alpha and AP-1-like sequences within CD164 P4 promoter. (A) Schematic presentation of the overlapping HIF-1α/E-box/AP-1-like in the minimal promoter region of CD164. (B) Mutation analysis of the putative E-box on CD164 promoter P4 region. Constructs with mutation of the E-box in the promoter region were subcloned into the pGL3-basic plasmid and the HCT116 cells were transfected. Significant decrease of luciferase activity was found in the E-box mutation P4 mutant (p < 0.001, Student’s t-test). Co-transfection of a HIF-1alpha expressing plasmid with pGL3P4 resulted in a significant increase in the promoter activity (p < 0.01, Student’s t-test). To investigate the effect of HIF-1alpha through HIF-1alpha/E-box overlapping sequence, the pcDNA3.1- HIF-1alpha was co-transfection with PGL3P4 containing E-box mutation. For the normalized luciferase activity, a significant decrease of luciferase activity was found in the co-transfection with PGL3P4 containing E-box mutation compared to the HCT116 cells co-transfected with wild-type PGL3P4 (p < 0.001, student T-Test).
site was sensitive to exogenous c-jun. It is likely that the CD164 expression in HCT116 cells is also controlled by both exogenous and endogenous factors through AP-1-like sites. CD164 may be a target gene in the AP-1 pathway and plays crucial roles in tumor development.

A number of studies have suggested that normal stem cells reside in “niches,” which support and maintain the undifferentiated phenotype of the stem cells. These niches may be hypoxic and hypoxia may play roles in maintaining the stem cell phenotype. Under hypoxia, HIFs regulate a variety of pro-angiogenic and pro-glycolysis pathways. In solid cancers, regions of hypoxia are commonly present throughout the tissue. In this study, hypoxia treatment and the transfection of HIF-1alpha induced the expression of CD164. Our results and previous studies showed that eight hours of treatment in a hypoxia chamber resulted in the highest level of HIF-1alpha expression. The ChIP showed the binding of HIF-1alpha to the CD164 promoter. DNA released from the precipitated complexes was amplified by PCR. The PCR products were separated by agarose gel electrophoresis. Lane 1: IgG control; Lane 2: anti-RNA polymerase II; Lane 3: HIF-1alpha antibody pull-down; Lane 3:Input

Conclusions
In conclusion, the results presented here have confirmed cis-Regulatory functions of overlapping HIF-1alpha/E-box/AP-1-like sequences of minimal promoter region of CD164. The research performed in this paper represents
the combination of in silico biology and molecular biology. The elucidation of the cis-regulatory elements is useful in understanding CD164 functions in cancer cells.

Acknowledgements
We acknowledge the support from Overseas Research Students Awards Scheme to Jinquuan Tang at Queen's University Belfast, UK (2005-2008).

Author details
1Department of Cardiothoracic Surgery, Xiangya Second Hospital, Central South University, 139 Renmin Zhong Road, Changsha, Hunan, 410011, P.R. China. 2Cancer Research Institute, Key Laboratory of Carcinogenesis and Cancer Invasion of Ministry of Education, Key Laboratory of Carcinogenesis of Ministry of Health, Central South University, 110 Xiangya Road, Changsha, Hunan, 410078, P.R. China. 3Centre for Cancer Research and Cell Biology, School of Biomedical Sciences, Queen's University Belfast, Belfast, BT9 7BL, Northern Ireland, UK. 4School of Medicine, Shenzhen University, Shenzhen, P. R. China. 5The Li Ka Shing Institute of Health Sciences, Department of Orthopaedics & Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, NT, Hong Kong. 6 Stem Cell and Regeneration Program, School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, P.R. China.

Authors’ contributions
The work presented here was carried out in collaboration between all authors. JT, ZL, JX and GZ carried out the experiments. GL, JT, JX and GZ made contributions to design, analyze and interpret data. JX, FY and JT have been involved in drafting the manuscript. All the authors have given final approval of the manuscript to be published.

Competing interests
The authors declare that they have no competing interests.

Received: 9 August 2011 Accepted: 14 October 2011 Published: 14 October 2011

References
1. Zhou GQ, Zhang Y, Ferguson DJ, Chen S, Rasmuson-Lestander A, Campbell FC, Watt SM. The Drosophila ortholog of the endolysosomal membrane protein, endolyn, regulates cell proliferation. Journal of cellular biochemistry 2006, 99(5):1380-1396.
2. Zannettino AC, Buhring HJ, Niuata S, Watt SM, Benton MA, Simmons PJ. The sialomucin CD164 (MGC-24v) is an adherent glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells that serves as a potent negative regulator of hematopoiesis. Blood 1998, 92(8):2613-2628.
3. Chan JY, Lee-Prudhoe JE, Jorgensen B, Ihike G, Doyonnas R, Zannettino AC, Buckle VJ, Ward CJ, Simmons PJ, Watt SM. Relationship between novel isoforms, functionally important domains, and subcellular distribution of CD164/endolyn. J Biol Chem 2001, 276(3):2139-2152.
4. Kurosawa N, Kanemitsu Y, Matsui T, Shimada K, Ishihama H, Muramatsu T. The sialomucin CD164 on CD34(+) and erythroid subsets, is located on human hematopoietic progenitors and bone marrow stromal cells that human glial fibrillary acidic protein gene that directs astrocyte-specific transcription. Genes Dev 1993, 7(15):181-192.
5. Ihike G, Gray SR, Luo JY. Endolyn is a mucin-type I membrane protein targeted to lysosomes by its cytoplasmic tail. Biochem J 2000, 345 Pt 2:287-296.
6. Watt SM, Chan JY. CD164—a novel sialomucin on CD34+ cells. Leuk Lymphoma 2000, 37(1-2):1-25.
7. Watt SM, Buhring HJ, Rappold I, Chan JY, Lee-Prudhoe J, Jones T, Zannettino AC, Simmons PJ, Doyonnas R, Sheer D, et al. CD164, a novel sialomucin on CD34(+) and erythroid subsets, is located on human chromosome 6q21. Blood 1998, 92(3):849-866.
8. Lee YN, Kang JS, Krauss RS. Identification of a role for the sialomucin CD164 in myogenic differentiation by signal sequence trapping in yeast. Molecular and cellular biology 2001, 21(22):7706-7716.
9. Vadav VG, Hua SB, Preeh DM, Pauling MH, Lin YH, Zhu L, Lawrence DM, Foda HD, Zucker S. CXCR4 and CXCL12 (SDF-1) in prostate cancer: inhibitory effects of human single chain Fv antibodies. Clin Cancer Res 2004, 10(16):5630-5639.
10. Masuzawa Y, Miyachi T, Hamaoune M, Ando S, Yoshida J, Takao S, Shimazu H, Adachi M, Muramatsu T. A novel core protein as well as polymorphic epithelial mucin carry peanut agglutinin binding sites in human gastric carcinoma cells: sequence analysis and examination of gene expression. Journal of biochemistry 1992, 112(5):609-615.
11. Havens AM, Jung Y, Sun YX, Wang J, Shah RB, Buhring HJ, Pienta KJ, Taichman RS. The role of sialomucin CD164 (MGC-24v or endolyn) in prostate cancer metastasis. BMC Cancer 2006, 6:195.
12. Coustan-Smith E, Song G, Clark C, Key L, Liu P, Mehpooopya M, Stow P, Su X, Shurtleff S, Pui CH, et al. New markers for minimal residual disease detection in acute lymphoblastic leukemia. Blood 2011, 117(23):6267-6276.
13. Matsui T, Kurosawa N, Hibi K, Aikyama S, Kasiy Y, Sakamoto J, Ito K, Nakao A, Muramatsu T. The ratio of splicing variants of MGC-24/CD164, a sialomucin, correlates with the metastatic potential of colorectal carcinomas. Journal of biochemistry 2000, 127(8):1103-1107.
14. Knudsen S. Promoter2.0: for the recognition of Pol promoter sequences. Bioinformatics 1999, 15(5):356-361.
15. Tsunoda T, Takagi T. Estimating transcription factor binding sites on DNA. Bioinformatics 1999, 15(7-8):622-630.
16. Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. Nat Rev Cancer 2003, 3(11):859-868.
17. Cambi F, Fung B, Chikarashi D. S’ flanking DNA sequences direct cell-specific expression of rat tyrosine hydroxylase. J Neurochem 1989, 53(5):1656-1659.
18. Douglass J, McMurray CT, Garrett JE, Adelman JP, Calavetta L. Characterization of the rat prodynorphin gene. Mol Endocrinol 1989, 3(20):2070-2078.
19. Comb M, Binberg NC, Seasholtz A, Herbert E, Goodman HM. A cyclic AMP- and phorbol ester-inducible DNA element. Nature 1986, 323(6066):353-356.
20. Masood K, Besnard F, Su Y, Brenner M. Analysis of a segment of the human gial fibrillary acidic protein gene that directs astrocyte-specific transcription. J Neurosci 1993, 13(1):160-166.
21. Liu Y, Ludes-Meyer J, Zhang Y, Munoz-Medellin D, Kim HT, Lu C, Ge G, Schiff R, Hilsenbeck SG, Osborne CK, et al. Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth. Oncogene 2002, 21(50):7680-7689.
22. Park YG, Nesterova M, Agrawal S, Cho-Chung YS. Dual blockade of cyclic AMP response element (CRE) and AP-1-directed transcription by CRE-transcription factor decoy oligonucleotide. gene-specific inhibition of tumor growth. J Biol Chem 1999, 274(3):1573-1580.
23. Eferl R, Ricci R, Kermer L, Zren R, David JP, Rath M, Wagner EF. Liver tumor development. c-Jun antagonizes the proapoptotic activity of p53. Cell 2003, 112(2):181-192.
24. Shaalan E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol 2002, 4(5):E131-136.
25. Schreiber M, Kolbbs A, Piu F, Szabowski A, Mohle-Steinlein U, Tian J, Karin M, Angel P, Wagner EF. Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev 1999, 13(5):607-619.
26. Hu E, Mueller E, Oliviero S, Papaiouannou VE, Johnson R, Spiegelman BM. Targeted disruption of the c-fos gene demonstrates c-fos-dependent and -independent pathways for gene expression stimulated by growth factors or oncogenes. Endo J 1994, 13(1):300-310.