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Research Article

TGF-β transcriptionally activates the gene encoding the high-affinity adenosine transporter CNT2 in rat liver parenchymal cells

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Abstract. The nucleoside transporter CNT2 is the highest-affinity adenosine transporter identified so far. Recent evidence suggests that CNT2 has functions other than salvage (*i.e.* modulation of purinergic responses). Here we identified TGF-β1 as a potent inducer of CNT2 protein expression in liver parenchymal cells. By contrast, CNT1, which is a target of multifunctional cytokines involved in liver cell proliferation, does not respond to TGF-β1 treatment. Cloning of a murine CNT2 gene sequence with promoter-like activity enabled us to demonstrate that this cytokine exerts this effect by transcriptionally activating the CNT2-encoding gene in a JNK-dependent manner. The evidence that CNT2 is not a target of multifunctional cytokines involved in hepatocyte proliferation, but instead, of a cytokine that plays major roles in differentiation and apoptosis, further supports the view that the main physiological role of this transporter protein is not nucleoside salvage.

Keywords. TGF-β, nucleoside transporters, liver, apoptosis, CNT2, adenosine.

Introduction

Nucleoside salvage depends on the ability of cells to take up extracellular nucleosides and nucleobases, *via* plasma membrane proteins that mediate either concentrative or facilitative substrate uptake, even in those cells showing endogenous nucleotide biosynthetic capacity. SLC28 and SLC29 genes encode two types of membrane transporters, concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs). In principle, CNTs are assumed to be high-affinity, partially selective (at least CNT1 and CNT2, which show preference for pyrimidine and purine nucleosides, respectively) nucleoside transporters, whereas plasma membrane ENTs show broad selectivity and low affinity for substrates. ENT2 is also a nucleobase transporter. Most of the biochemical, biophysical and physiological properties of these membrane proteins have been recently reviewed [1–4].

Despite comprehensive knowledge of the functional properties and selectivity profiles of these nucleoside carriers, it is still not known why a single cell type expresses a complex pattern of nucleoside transporters, particularly if this cell type shows high endogenous purine and pyrimidine nucleotide biosynthesis, as liver parenchymal cells do. Rat and human hepatocytes express at least CNT1, CNT2, ENT1 and ENT2 [5–8]. Maintenance of the fully differentiated hepatic phenotype is associated with the expression of SLC28 genes (encoding CNTs),

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whereas transformation (*i.e.* hepatocarcinogenesis) is characterized by the loss of CNT expression and, at least in hepatoma cell lines, overexpression of SLC29 genes (particularly ENT1) [5, 9, 10]. Actually, incubation of rat fetal hepatocytes with agents known to promote differentiation, such as dexamethasone and thyroid hormone, induce a dramatic increase in CNT2 mRNA amounts [11]. Overall this could be interpreted as meaning that highly proliferating cells do not require CNT function, which is consistent with observations of murine bone marrow macrophages, which show that proliferation is indeed associated with ENT1 up-regulation. However, macrophage activation with IFN-γ is followed by increased CNT1- and CNT2-related functional activities, through a JAK/STAT-independent mechanism that seems to rely upon the transcriptional activation of both genes [12, 13]. Induction of apoptosis in macrophages treated either by LPS or TNF-α also results in CNT1 and, particularly, CNT2 up-regulation, a finding that suggests that CNTs may play physiological roles other than nucleoside salvage [14].

CNT2 is the highest-affinity adenosine transporter identified so far [15, 16]. CNT2 shows an apparent K_m value for adenosine in the very low micromolar range, a value which is close to reported plasma adenosine concentrations, particularly under conditions, such as those associated with hypoxia and oxidative stress, in which the levels of this nucleoside are known to increase dramatically [17–19]. Although the contribution of CNT2 to modulating extracellular adenosine concentrations has not yet been established, CNT2-related function is under purinergic control in rat hepatocytes and hepatoma cells *via* A1 receptors, by means of a mechanism that involves K_{ATP} channels and is dependent on cells' energy status [20]. Moreover, although CNT2 protein and mRNA amounts increase during liver regeneration early after partial hepatectomy in rats [6, 10], CNT2 is not (although CNT1 is) a target of selected multifunctional cytokines involved in hepatocyte priming for proliferation, such as TNF-α and interleukin-6 [21]. This suggests that CNT2 up-regulation is not indeed related to growth-promoting agents, but rather to other regulatory cytokines that control liver regeneration.

The possibility that the CNT2-encoding gene is a target of the multifunctional cytokine TGF-β1, one of the most potent inducers of apoptosis in normal hepatocytes and a modulator of both pro-apoptotic and survival signals in proliferating hepatocytes [22–24], was addressed in this study by monitoring the effect of this cytokine on CNT2-related activity and expression in FAO rat hepatoma cells.

Materials and methods

Cell lines, culture conditions and treatments. Cell culture reagents were purchased from Invitrogen. The JNK1/2/3 inhibitor SP600125 was from Biotrend (Köln, Germany), and [^{3}H]guanosine was from Amersham. Dexamethasone and TGF-β1 were purchased from Sigma (Saint Louis, MO).

The rat hepatoma cell lines FAO and C2, purchased from ECACC (European Collection of Cell Cultures), were routinely cultured in Coon's F12 medium supplemented with 10% and 5% calf serum, respectively, 2 mM glutamine and a mixture of antibiotics (100 U penicillin G/ml, 0.1 mg streptomycin/ml and 0.25 μg fungizone/ml). The rat hepatoma cell line FAO, which is derived from the H4IIEC3 cell line Reuber H35, is well differentiated and expresses numerous liver-specific enzymes and transcription factors, whereas the C2 cell line, a derivative of FAO, has undifferentiated phenotypes [25]. The CHO-K1 cells, purchased from American Type Culture Collection (ATCC), were routinely cultured in minimum essential medium (MEM)-Eagle supplemented with 4% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine and a mixture of nonessential amino acids and antibiotics.

Measurement of nucleoside uptake rates. Uptake measurements used as a substrate 1 μM guanosine in the presence of either 137 mM NaCl or 137 mM choline chloride. The uptake medium also contained 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM HEPES (4–2-hydroxyethyl-1-piperazineethanesulfonic acid) (pH 7.4). Incubation was stopped after 1 min (initial velocity conditions) by washing of the monolayers twice in 2 ml of cold buffer composed of 137 mM NaCl and 10 mM tris(hydroxymethyl) aminomethane-HEPES (pH 7.4). Cells were then dissolved in 100 μl 100mM NaOH, 0.5% Triton X-100. Aliquots were sampled for protein determination, according to Bradford (Bio-Rad Laboratories, Madrid, Spain), and for radioactivity measurements.

Western blot analysis. For Western blot analysis of rat CNT2 (rCNT2), cell extracts were obtained by washing cell monolayers in phosphate-buffered saline (PBS) at the desired times, and then scraped off in a lysis buffer containing 0.3 M sucrose, 25 mM imidazole (pH 7.2) and 1 mM EDTA, supplemented with a mixture of protease inhibitors (Complete MINI, Roche). The previously characterized anti-CNT2 antibody [26] was used at a 1:1000 dilution.

Reverse transcription and quantitative real-time PCR analysis. Total RNA (1 μg) was used for cDNA synthesis, by the TaqMan Reverse Transcription System (Applied Biosystems, Foster City, CA), as described by the manufacturer. The primers and probes used to amplify cDNA by real-time RT-PCR were designed by Primer Express software and the sequences employed are the following: glyceraldehyde-3-phosphate dehydrogenase (GAPDH): fw: 30–50 (gatggtgaaggtcggtgtcaa), rv: 115–

91 (caaatgtccacttgtcacaagagaa), probe: 70–89 (ccgcctg-gtcaccagggctgc); rCNT2: fw: 785–803 (tgcgggaatctg-catgtt), rv: 854–836 (ctccagctcaccgcactgt), probe: 805–831 (atcctcatcctctttgcctgctccaaa). PCR amplification of cDNAs was monitored in real time with the TaqMan Universal Master Mix (Applied Biosystems), using 150 nM probe and 700 nM of each primer, in the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The relative quantification of gene expression was performed, as described by the manufacturer, using rat GAPDH as an internal control. The threshold cycle (C_T) is defined as the cycle number at which fluorescence corresponding to the amplified PCR product is detected. The results are given as PCR arbitrary units after normalizing the mRNA levels of these genes to GAPDH expression levels.

Annexin-V assays. Cells were seeded in triplicate in 6-cm dishes. Apoptosis was assessed by Annexin-V-fluorescein-5-isothiocyanate (FITC) binding (Bender MedSystems). Attached cells were collected after treatment with TGF-β1, washed in PBS and resuspended in 1 ml of binding buffer containing 0.5 μg/ml Annexin-V-FITC and 5 μg/ml propidium iodide. Cells were incubated in the dark for 1 h at room temperature. Triplicate dishes for each treated sample were analyzed with EPICS-XL flow cytometer (Coulter). Propidium iodide-positive cells were considered the necrotic population, and only propidium iodide-negative and Annexin-V-FITC-positive cells were considered the apoptotic population.

Isolation of mouse CNT2 cDNA. The cDNA for the mouse homologue of rCNT2 (mCNT2) was obtained by rapid amplification of cDNA ends (RACE), using as template 500 ng of a cDNA library from murine spleen (‘Marathon-Ready cDNA kit’, BD Biosciences, Clontech) and the adaptor primer AP1 (provided in the kit) in combination with gene-specific primers synthesized on the basis of rat cDNA sequence (GenBank™/EBI accession no. RNU25055): 5′-RACE antisense primer, 5′-AGAGGATTCCTGCCATACAG-3′, complementary to nucleotides 2011–2030 of rCNT2; 3′-RACE sense primer, 5′-ATGACTGGAGGCTTTGCTAC-3′, nucleotides 1283–1302 of rCNT2. The amplification reaction was performed using the ‘Advantage cDNA polymerase mix’ (Clontech) and the cycling conditions were as follows: 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 68 °C for 4 min, followed by 68 °C for 7 min. The 5′- and 3′-RACE products were cloned into a pCR® II vector with a TOPO TA®Cloning kit (Invitrogen). The resulting clones were differentially screened by colony hybridization and sequenced using standard vector-specific oligonucleotide primers. The cDNAs isolated from two overlapping 5′-RACE and 3′-RACE clones, exhibiting great homology with rCNT1 and rCNT2, were used as template to synthesize the sense primer 5′-GCGAATTCCTGTGCATCCTCA CTGACAT-3′ and the antisense primer 5′-GCTCTAGACCTCACATC-CAC TCCTACAC-3′ to further amplify the full-length mCNT2 cDNA (2653 bp). The cycling conditions were similar to those described for the RACE amplifications: 94 °C for 5 min, 30 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 4 min, followed by 72 °C for 7 min. The full-length mCNT2 cDNA was further confirmed by sequencing.

Cloning of the 5′-flanking region of the mCNT2 gene. The mCNT2 whole length cDNA was labeled with [γ-^{32}P]dCTP and used as hybridization probe to screen a murine (129SvJ/6) genomic bacterial artificial chromosome library constructed in pBeloBAC11 (Genome Systems). From eight retrieved positive clones, only one (named BAC17K12) also hybridized with probes generated against the 5′ and 3′-mCNT2 UTRs. The BAC DNA corresponding to this clone (13.7 kb) was purified and used for the genomic cloning of the murine CNT2 5′-flanking region. Based on the restriction map for the mCNT2 cDNA and the partially sequenced introns (regions around the splicing areas), we looked for low-frequency restriction enzymes with sites near the 5′-upstream sequence. Thus, to get the longer 5′-flanking region of the gene, we digested the DNA in BAC17K12 with *Eco*RI, which statistically cuts every 5 kb and had a restriction site within the 2nd intron of CNT2. The digested DNA fragments were separated by agarose gel (0.8%) electrophoresis and processed by Southern hybridization with a [γ-^{32}P]dCTP-labeled 81pbs probe directed to the first exon of the mCNT2 cDNA (sense primer, 5′-CTGTG-CATCCTCACTGACATCAGACT-3′; antisense primer, 5′-AAGACTCCTGGAGAGCTACCGATCTT-3′). The size of the hybridized restriction fragment (1908 bp) was detected by autoradiography, using a 5′-[γ-^{32}P]dATP-labeled ‘1-kb DNA Ladder’ (Gibco).
As a preliminary approach to figure out where the transcription start site of the mCNT2 gene is located, we performed 5′-RACE using as template 500 ng of a cDNA library from murine spleen (Marathon-Ready™ cDNA Amplification kit, BD Biosciences, Clontech). An mCNT2-specific reverse primer, synthesized on the basis of the mCNT2 cDNA sequence (5′-AAGACTCCT-GGAGAGCTACCGATCTT-3′, complementary to the first exon), was used in combination with the adaptor-specific primer AP1 (provided in the kit). The amplification reaction was performed using the ‘Advantage cDNA polymerase mix’ (Clontech) and the cycling conditions were as follows: 94 °C for 1 min, 5 cycles of 94 °C for 30 s and 72 °C for 2 min, 5 cycles of 94 °C for 30 s and 70 °C for 2 min, 35 cycles of 94 °C for 20 s and 64 °C for 2 min followed by 72 °C for 7 min. The products amplified were cloned into a pCR® II vector with a TOPO TA® Cloning kit (Invitrogen) and 13 randomly chosen positive

clones were sequenced using the 'ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit' (PE Applied Biosystems) and aligned with the genomic clone by means of the software described above.

The target *Eco*RI DNA fragment containing part of the mCNT2 5′-flanking region was subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA) cloning vector and sequenced in triplicate in both orientations, by means of standard vector-specific and gene-specific oligonucleotide primers and the 'ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit' (PE Applied Biosystems) (Unitat de Seqüenciació dels Serveis Científico-Tècnics, University of Barcelona). Sequence comparison was performed by Clustal W Multiple Sequence Alignment Results (BCM Search Launcher) and Gene Doc software. Analyses of the 5′-flanking sequence data were also aided by computer software. Potential promoter regions and transcription start sites (TSS) were identified with programs such as FunSiteP, Promoter Scan and TSSG/TSSW.

Throughout the manuscript, nucleotides are numbered according to their position relative to the translation start site of the mCNT2 gene. Thus, a negative sign refers to nucleotides upstream from the ATG codon (exon II).

DNA transfections and reporter gene assays. Firefly (*Photinus pyralis*) luciferase reporter vectors containing varying lengths of the murine CNT2 5′-flanking region were constructed in the promoter-less pGL3Basic luciferase reporter vector (Promega, Madison, WI) using standard techniques. All the reporter gene constructs were numbered in relation to the translation start site of the mCNT2 gene.

The fragment containing the longest mCNT2 5′-flanking region obtained (–1908/+1) was excised from pBluescript II KS$^+$ with Kpn*I*/Pvu*II* and was directionally subcloned into the Kpn*I*/Sma*I* site of the pGL3-Basic reporter vector. Various deletions of the (–1908/+1) fragment (construct 1: –1450/+1, construct 2: –986/+1, construct 3: –410/+1) were also generated by excision of the pBluescript II KS$^+$ clone with different restriction enzymes and subsequent insertion into the multicloning site of the pGL3-Basic reporter vector. Thus, construct 1 was excised from pBluescript II KS$^+$ at the Ssp*I*/Pvu*II* site, subcloned into the Sma*I* site of pGL3-Basic and subsequently screened for orientation by PCR, using standard-vector oligonucleotide primers (RVprimer3/clockwise: 5′-CTAGCAAAATAGGCTGTCCC-3′ and GLprimer2/counter clockwise: 5′-CTTTATGTTTTTGGCGTCTTCCA-3′) (Promega) and gene-specific primers. In a similar way, construct 2 was excised at the Ban*I*/Pvu*II* site of pBluescript II KS$^+$ and directionally inserted into the Acc*65I*/Sma*I* site of pGL3-Basic. Construct 3 was excised at the Mun*I*/Pvu*II* site of pBluescript II KS$^+$, blunt-ended with Klenow DNA polymerase, subcloned into the Sma*I* site of pGL3-Basic and screened for orientation as previously described.

For transient transfection, 10^5 cells/well were seeded in 24-well dishes and transiently transfected with 300 ng of Firefly (*P. pyralis*) reported DNA using Fugene6 reagent (Roche, Laval, Quebec, PQ, Canada), as recommended by the manufacturer. Each reporter DNA was cotransfected in triplicate with 30 ng of the *Renilla reniformis* luciferase reporter pRL-TK (Promega), as an internal control of transfection efficiency. The promoter-less pGL3Basic luciferase reporter vector was also routinely transfected as a negative control. Cells were then incubated by culturing for 6–7 h. For functional analysis of the basal promoter activity, the medium was replaced with the medium used for cell culture before transfection and cell lysates were prepared 30 h after transfection. For analysis of the regulation of the promoter by dexamethasone (100 nM) or TGF-β1 (1 ng/ml), transfected cells were maintained for 12 h in serum-free medium supplemented with 2% BSA, before the pertinent additions. Then cell lysates were harvested and functional assays were carried out using the Dual-Luciferase Reporter Assay System (Promega). Both *Photinus* and *Renilla* luciferase activities were measured simultaneously in a microplate TD-20/20 luminometer (Promega). To determine whether Jun kinases (JNK) are implicated in the transcriptional response triggered by TGF-β1, the JNK1/2/3 inhibitor SP600125 was added at a final concentration of 20 µM 30 min before growth factor addition.

Results

Regulation of CNT2 expression by TGF-β1. The putative effect of TGF-β1 on CNT2 expression was analyzed in FAO cells, by combining different experimental approaches and comparing this response with the effect of a previously characterized positive effector of CNT2 function, dexamethasone [11].

Actually, treatment of FAO cells with 100 nM dexamethasone resulted in a significant increase in CNT2-related transport activity (Fig. 1a), which corroborated previous data in which the incubation of primary cultures of rat fetal hepatocytes with dexamethasone exerted a similar response, associated in that particular case with an increase in CNT2 mRNA amounts [11]. Under the same culture conditions, treatment of FAO cells with 1ng/ml TGF-β1 for 24 h increased CNT2-related biological activity by threefold (Fig. 1b). As shown in Figure 2a, both dexamethasone and TGF-β1 exerted this up-regulation on CNT2-related activity by a mechanism which resulted in a significant increase in CNT2 protein amounts in crude cell extracts. As previously shown for dexamethasone [11] the response of CNT2 to TGF-β1 was associated with a significant increase in CNT2 mRNA levels, here

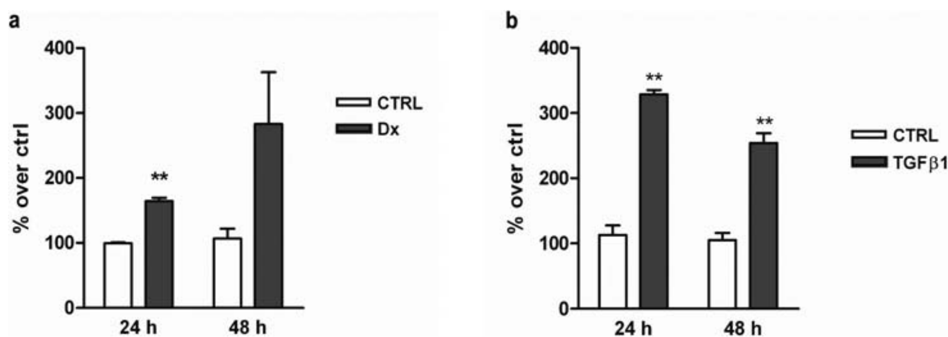

Figure 1. Effect of dexamethasone (Dx) and TGF-β1 on CNT2-related transport activity. Na^{+} dependent guanosine uptake was monitored after 24 and 48 h incubation of FAO cells with 100 nM dexamethasone (*a*) or 1 ng/mL TGF-β1 (*b*). Data are the mean ± SE of quadruplicate measurements made in three independent cultures and are expressed as the percentage change above basal values. Statistical significance was established by Student's *t*-test (** $p < 0.01$).

measured by quantitative real time RT-PCR (Fig. 2b). Under these conditions, the addition of this cytokine also resulted in an induction of programmed cell death, although the percentage of apoptotic cells was much higher at longer incubation times (48 h after treatment) than at the time point at which CNT2 protein and related functional activity were measured (24 h) (Fig. 3). Treatment of FAO cells with TGF-β1, under the same conditions in which the up-regulation of CNT2 was observed, did not elicit any change in CNT1 protein expression (not shown).

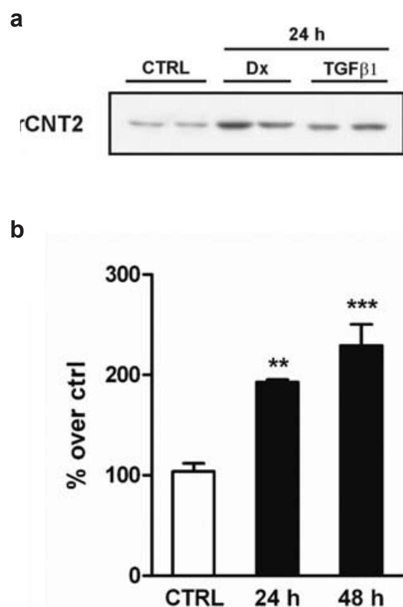

Figure 2. Regulation of CNT2 expression in rat liver parenchymal cells. (*a*) Effect of dexamethasone (Dx) and TGF-β1 on the amount of CNT2 protein. FAO cells, at 65–70% confluence, were incubated for 24 h either in absence or presence of 100 nM dexamethasone or 1 ng/mL TGF-β1. CNT2 protein expression was monitored by Western blot. Equal amounts of protein (20 µg) were loaded to allow direct comparisons. A representative immunoblot is shown. (*b*) Effect of TGF-β1 on CNT2 mRNA expression in FAO cells. mRNA levels of CNT2 transporter were determined by real-time RT-PCR analysis in FAO cells treated with 1 ng/mL TGF-β1 at the indicated times (24 and 48 h). Values (expressed as a percentage of mRNA content in control cells) are the mean ± SE of three different experiments. Statistical significance was established by Student's *t*-test (** $p < 0.01$, *** $p < 0.001$).

Evidence for transcriptional activation of the CNT2 encoding gene by TGF-β1. The constitutive transcriptional activity of the cloned 5′-flanking region of the mCNT2 gene was measured in transiently transfected cell lines, showing different levels of endogenous CNT2 transport activity and expression. As shown in Figure 4, CNT2 protein was identified by Western blot in FAO and C2 cells, with the latter showing much lower amounts than the former. This was consistent with their CNT2-related transport activity (not shown, but higher in FAO than in C2 cells) and their known degree of differentiation, with FAO cells having a greater degree of differentiation than their C2 counterpart. Our antibody, raised against the rat ortholog, did not identify any CNT2 protein in CHO cells. Although this could be explained by the fact that this cell line was derived from hamster and

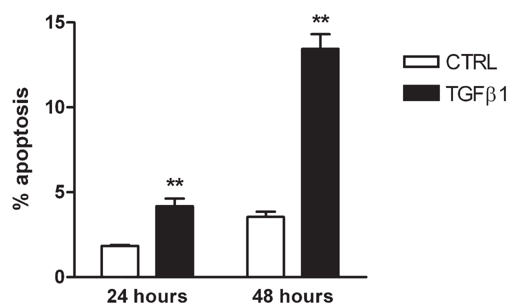

Figure 3. Apoptosis induction by TGF-β1 in FAO cells. Cell cultures treated with 1 ng/mL TGF-β1 at the indicated times (24 and 48 h) were analyzed for apoptosis by annexin V and PI staining by flow cytometry. Results were derived from triplicate estimations made in three independent experiments. Statistical significance was established by Student's *t*-test (** $p < 0.01$).

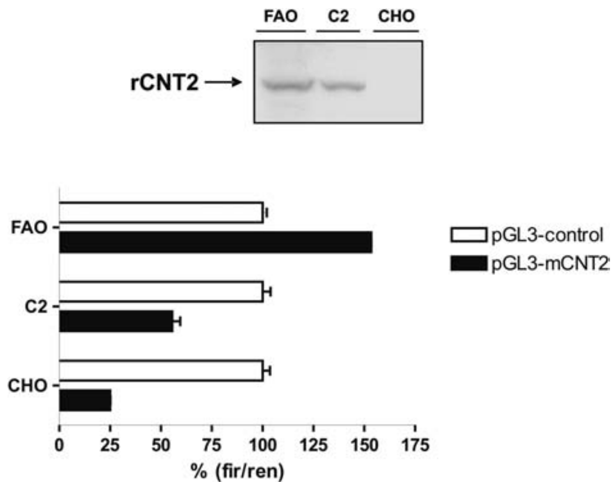

Figure 4. Functional analysis of the 5′-flanking region of mCNT2 gene. Transcriptional activity of the putative mCNT2 promoter was measured in CHO and liver parenchymal cells (FAO and C2 cell lines) showing different levels of endogenous CNT2 expression (upper panel). Equal amounts of protein were loaded (40 μg) to allow direct comparisons of CNT2 levels. A representative Western blot is shown. Cells were transiently transfected with 1 μg pGL3-control (firefly luciferase expression vector with SV40 promoter and enhancer elements) or with 1.9-kb mCNT2 promoter sequence upstream of firefly luciferase gene. An internal *Renilla* control plasmid (pRL-TK) was included in all transfections for a dual luciferase assay. The relative luciferase activity was obtained by normalizing the firefly luciferase activity to the *Renilla* luciferase activity (fir/ren). The luciferase activity in the cell lysates was measured 30 h after transfection (lower panel). The corrected luciferase activity was evaluated as a percentage of the activity from pGL3-control (100%). Data are represented as means ± SE from three independent experiments.

the antibody used was raised against a rat antigenic sequence, the putative lack of expression of CNT2 in this particular cell type would also corroborate the lack of CNT2-related transport activity in CHO cells. Constitutive transcriptional activity of the putative mCNT2 promoter in these three cell lines revealed significant differences between them, once normalized, thus avoiding putative variability in transfection efficiency (Fig. 4). Transcriptional activity was much higher in FAO than in C2 cells (nearly three times greater), whereas the constitutive transcriptional activity of the promoter in CHO cells was six times lower than in the rat hepatoma FAO cell line (Fig. 2b).

Since the whole cloned fragment corresponding to the 5′-flanking region of the mCNT2 gene showed significant transcriptional activity and, based upon the bioinformatics analysis, seemed to correspond to a genomic sequence included in the putative promoter of the mCNT2 encoding gene, we further analyzed this sequence, using a variety of available software (not shown), by scanning for putative transcription factor binding sites. To approach the minimal 5′-flanking sequence in our fragment responsible for transcriptional activity, several deleted sequences were generated from the cloned 1908-bp 5′-flanking region (sequence A) (Fig. 5). Three constructs were obtained by serial deletions (of about 450–500 bp each), thus yielding 1450, 922 and 410 bp 5′-flanking sequences (constructs B, C and D, respectively). All these constructs were checked for constitutive transcriptional activity by transiently transfecting them into FAO cells. Construct B showed a significantly higher constitutive transcriptional activity (about 50% more) than the original promoter sequence (A), which suggests that some trans-inhibitory elements occur at the deleted upstream sequence (between –1450 and –1908 bp). Constructs C and D showed no constitutive transcriptional activity (Fig. 5) and were not further used in this study. Transfection of the promoter-less pGL3Basic luciferase reporter vector did not result in any significant luciferase activity (not shown).

Since the increase in CNT2-related activity and protein after dexamethasone and TGF-β1 treatments was associated with increased mRNA amounts ([11] for dexamethasone; this work for TGF-β1), we then investigated how these two agents were able to modulate the transcriptional activity associated with the cloned fragments A and B. TGF-β1 transcriptionally activated the putative mCNT2 promoter region cloned here, when transiently transfected in FAO cells (Fig. 6a). Interestingly, this response was completely abolished when construct B, lacking the upper 5′ region (450 bp) of the cloned sequence, was used for gene transcription analysis (Fig. 5), thus suggesting that a particular transcription-binding sequence at this point of the CNT2 promoter is essential for TGF-β1 transcriptional activation of the CNT2-encoding gene. Dexamethasone similarly up-regulated this gene, although in this particular case both the full-length promoter (A) and construct B retained their ability to be transcriptionally activated following dexamethasone treatment (Fig. 6b).

As a first approach to the putative signal transduction pathway involved in the transcriptional activation of the CNT2-encoding gene, we monitored how a specific inhibitor of Jun Kinase, SP600125, modified the TGF-β1-triggered response. As shown in Figure 7a, pre-treatment of FAO cells for 30 min with 20 μM inhibitor, prior to the addition of the cytokine, resulted in complete inhibition of the increase in CNT2-related transport activity, without significantly altering basal uptake rates. This effect might be the direct result of the inhibition of the transcriptional activation of the CNT2-encoding gene, as deduced from the fact that treatment with the inhibitor also blocked the effect of TGF-β1 on the transcriptional activity associated with the mCNT2 5′-flanking region, when transfected in FAO cells (Fig. 7b). Pharmacological inhibitors of ERK and p38 kinase did not block the TGF-β1-triggered response (not shown).

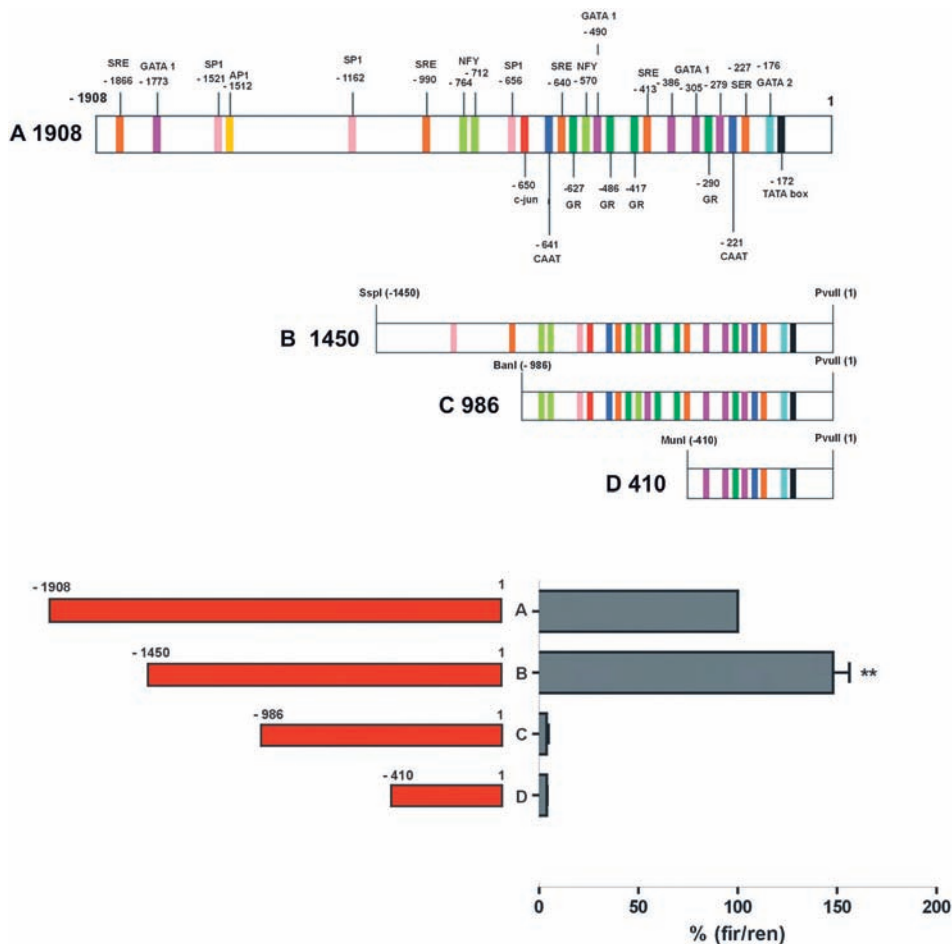

Figure 5. Deletion analysis of the 5′-flanking region of mCNT2 gene. Different constructs with different deletions were generated from the putative mCNT2 promoter (*a*, 1908 pb). For each construct, the length of the fragment and the name of the construct (*b–d*) are shown. Luciferase activity of the deleted constructs was analyzed in FAO cells as describe in the Materials and methods. The relative luciferase activity was obtained by normalizing the firefly luciferase activity to the *Renilla* luciferase activity (fir/ren). The corrected luciferase activity was evaluated as a percentage of the activity from construct A (1908 pb). The putative transcriptional factor binding sites identified by a bioinformatics approach are also shown. Data are represented as means ± SE from five independent experiments. Statistical significance was established by Student's *t*-test (** $p < 0.01$).

Discussion

This study demonstrates that the gene encoding the high-affinity adenosine transporter CNT2 is a target of TGF-β1 action on FAO cells, a rat hepatoma cell line showing selected features of differentiated hepatocytes. The reported changes in CNT2-related functional activity, CNT2 protein and its corresponding mRNA levels, suggested that this cytokine up-regulates CNT2 mostly by transcriptional activation of the CNT2-encoding gene. This was indeed demonstrated by analyzing how TGF-β1 modulated the transcriptional activity of the 5′-flanking region of the mCNT2 gene when transfected into FAO cells, a region which, according to molecular and bioinformatics analysis, might belong to the putative mCNT2 gene promoter. This effect was specific, since the other member of the CNT-related gene family expressed in FAO cells, CNT1, did not change its expression after treatment with this cytokine. Previous work showed that CNT1, but not CNT2, is a target of multifunctional cytokines involved in hepatocyte priming for proliferation, such as TNF-α and IL-6, which up-regulate CNT1 by a mechanism that appears to be dependent on the PI3 kinase and MAP kinase signaling pathways, respectively [21]. Pharmacological evidence provided in this study suggests that the transcriptional activation of the CNT2-encoding gene, triggered by TGF-β1 and leading to an increase in its corresponding transporter functional activity, is dependent upon the JNK pathway. Overall, these observations support the view that, although both CNT transporters, CNT1 and CNT2, are known to be up-regulated during the early phases of liver growth after partial hepatectomy in rats [6, 10], their effectors, signal transduction pathways and very probably their physiological

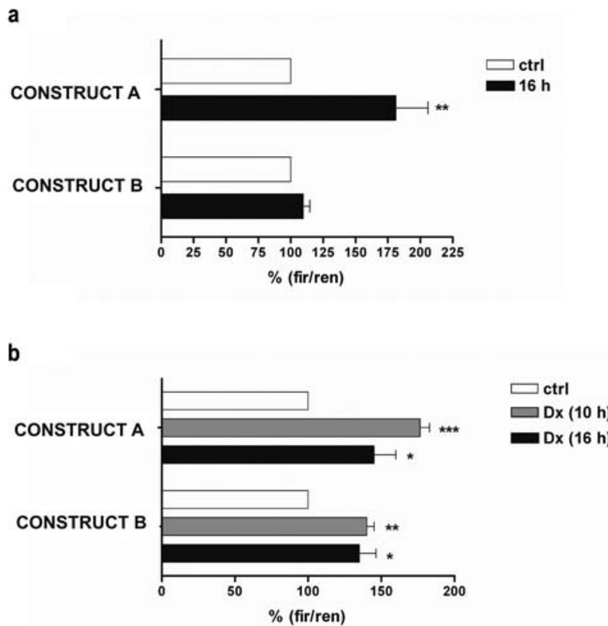

Figure 6. Transcriptional regulation of CNT2 transporter in rat liver parenchymal cells. Transactivation of luciferase reporter constructs by TGF-β1 and dexamethasone (Dx) was analyzed in FAO cells. Culture cells were transiently co-transfected with putative mCNT2 promoter (construct A, 1908 pb) or construct B (1450 pb) and pRL-TK internal control. At 24 h after transfection, cells were treated with 100 nM dexamethasone (*b*) or 1 ng/mL TGF-β1 (*a*) at the indicated times. Luciferase activity was corrected to the internal *Renilla* control (fir/ren) and expressed as a percentage of the activity from control (non-treated cells) of each construct. Data are represented as means ± SE of three independent experiments. Statistical significance was established by Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

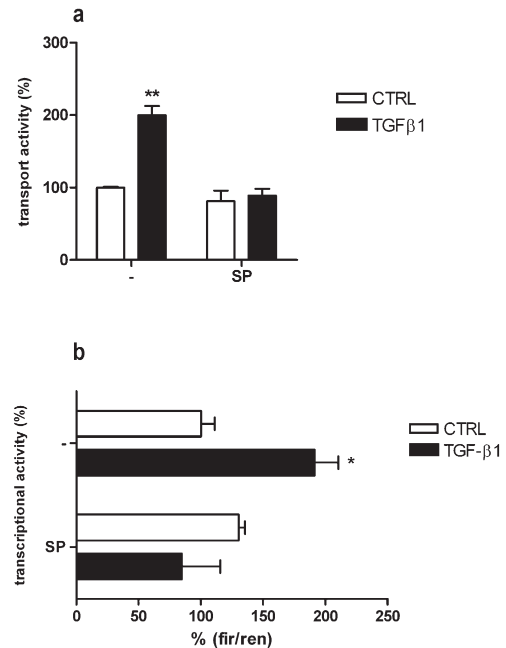

Figure 7. Involvement of JNK pathway in CNT2 regulation by TGF-β1 in rat liver parenchymal cells. (*a*) Effect of JNK inhibitor SP600125 treatment in TGF-β1 induction of CNT2 transport activity. FAO cells were pre-incubated for 30 min with the JNK inhibitor SP600125 (20 μM). Then, cultured cells were treated either in the absence or in the presence of 1 ng/mL TGF-β1 for 24 h and Na^+-dependent guanosine uptake was monitored. Results were derived from quadruplicate estimations made in four independent experiments. Data (mean ± SE) are shown as the percentage change above control values (non-treated cells). Statistical significance was assessed by Student's *t*-test (** $p < 0.01$). (*b*) The JNK inhibitor SP600125 treatment abolishes the transactivation of mCNT2 promoter. FAO cells were transiently co-transfected with mCNT2 promoter (1908 pb) and pRL-TK internal control. At 24 h after transfection, cells were pre-incubated for 30 min with the JNK inhibitor SP600125 (20 μM). Luciferase activity was measured 16 h after treatment with 1 ng/mL TGF-β1 and normalized to the internal *Renilla* control (fir/ren). Data are represented as means ± SE from three independent experiments and expressed as a percentage of the activity from control (non-treated cells). Statistical significance was established by Student's *t*-test (* $p < 0.05$).

roles might be different. Both CNT1 and CNT2 were recently identified in human hepatocytes, their expression being a feature of differentiated cells. (Fernandez-Veledo et al., submitted).

The rationale for the CNT2-encoding gene (SLC28A2, in humans) being a JNK-dependent TGF-β1 target is still obscure. TGF-β1 is known to be a major pro-apoptotic agent in normal hepatocytes [27, 28], and activates JNK *via* the TGF-β1 receptor type II-adapter protein Daxx [29]. However, this signaling pathway may indeed be anti-apoptotic in embryonic mice, as deduced from the observation that Daxx knockout embryos show widespread apoptosis [30]. Indeed, studies by Fabregat and colleagues have established that TGF-β1 might play variable roles in rat hepatocytes, depending upon whether cells are proliferating (*i.e.* fetal and regenerating rat hepatocytes) or exposed to agents that trigger survival signals [31–34]. In rat fetal hepatocytes, TGF-β1 activates both pro-apoptotic and anti-apoptotic signals, such as the PI3-K/Akt pathway. Indeed, inhibition of this signal transduction pathway in rat fetal hepatocytes sensitizes cells to TGF-β1-triggered apoptosis [24]. This might explain why published data on the effects of this cytokine that use hepatoma-derived cell lines are somehow contradictory (reviewed in [35]). Nevertheless, in our study, TGF-β1 has been shown to promote apoptosis in FAO cells, as was shown elsewhere in normal hepatocytes. Although this cytokine inhibits growth and promotes programmed cell death, it might also induce differentiation in fetal hepatocytes in the presence of survival signals, such as EGF [36–38]. To some extent this confirms the similar effect on CNT2 expression triggered by glucocorticoids, shown elsewhere in rat fetal hepatocytes (along with thyroid hormone) [11] and, recently, in a rat enterocyte cell model, IEC6 [39]. This study demonstrates that the effect of dexamethasone on CNT2 expression is initiated, as shown for TGF-β1, at the transcriptional level. The final molecular

events responsible for the transcriptional activation of the CNT2-encoding gene will require further research, but the maintenance of the dexamethasone-triggered effect, when using the upstream 5′-deleted construct 1, corroborates a putative role of the tandem GR-binding motifs at –280, –417, –486 and –627 from the translation initiation site in mediating glucocorticoid action. However, the lack of effect of TGF-β1 when using this construct is consistent with a requirement for the SP1 binding domain at the deleted 5′ promoter fragment in the transcriptional effect triggered by this cytokine, as reported elsewhere for other genes under TGF-β1 regulation [40–43].

Although we considered the effect triggered by glucocorticoids on CNT2 expression in intestinal cell models, exclusively on the basis of differentiation and promotion of the presumably absorptive function of CNT2 at the brush border apical membrane [39], this transporter protein does not appear to be exclusively located at the apical side in all epithelia. Indeed, it is mostly found in basolateral membranes in rat hepatocytes [44]. Thus, other physiological roles beyond absorption need to be evaluated. A putative, although still speculative, link between CNT2 function and apoptosis could be deduced from CNT2 being a high-affinity adenosine transporter that could contribute to modulation of purinergic signals by regulating extracellular adenosine levels. Although no direct evidence for this is available yet, we do know that CNT2 mRNA levels are markedly down-regulated in the cerebral cortex of sleep-deprived rat brains, a situation in which extracellular adenosine levels are known to increase [45]. Moreover, as mentioned above, CNT2-related nucleoside uptake is rapidly enhanced by A1R activation in rat hepatocytes and FAO cells, *via* K_{ATP} channels, in a manner probably dependent upon cell energy status. In rat hepatocytes, both A1R and CNT2 are expressed at the same plasma membrane domains and co-localize in FAO cells [20]. Adenosine exerts anti-apoptotic effects, some of them *via* A1R activation, in a variety of cell systems (for review, see [46]), including macrophages [47]. Interestingly, murine bone marrow macrophages dramatically up-regulate CNT2-related functional activity and mRNA levels when they are treated with the pro-apoptotic agents LPS and TNF-α [14]. Moreover, it has recently been shown that inosine, a nucleoside which, like adenosine, is a high-affinity substrate for CNT2, has differential requirements for adenosine receptors to exert their protective effects *in vivo*. [48].

The possibility that pro-apoptotic agents promote the induction of CNT2 as a sort of compensatory mechanism to equilibrate both pro- and anti-apoptotic signals is an exciting hypothesis. Adenosine and adenosine receptors would be the putative link between these two processes. Indeed, loss of CNT2 protein has been reported in chemically induced rat hepatocarcinomas [9]. Whether this loss of expression is merely the consequence of the progressively less differentiated state of hepatocytes or does indeed somehow contribute to hepatocarcinogenesis itself will require further analysis.

TGF-β1 has also been reported to modulate the function of a few other transporter proteins. This cytokine transcriptionally up-regulates the expression of the adenine nucleotide translocator 1, a key determinant of the aerobic capacity of the mitochondrial ox-phos system in astrocytes following CNS injury [41]. Whether a metabolic imbalance can then determine CNT2 functional activity, as we show for the purinergic activation of this transporter in FAO cells and rat hepatocytes, is still to be determined. Nevertheless, we have recently found out that CNT2 function in the intestinal cell line IEC6 is functionally coupled to the activation of the AMP-dependent kinase [49], an effect which is dependent upon the uptake of extracellular adenosine via CNT2 and its subsequent phosphorylation into AMP. Apparently, the same role for extracellular adenosine applies to FAO cells [49]. This finding provides first evidence linking CNT2 function and energy balance.

The possible pharmacological relevance of CNT2 should also be discussed in the context of these findings. In fact, finding physiological agents that up-regulate CNT2 function in liver parenchymal cells is more important than basic physiological knowledge, since CNT2 is the highest-affinity transporter identified so far for ribavirin [50], a nucleoside-derived drug currently used in the treatment of hepatitis [51, 52].

In conclusion, this study reports the cloning of a basic unit of the murine CNT2-encoding gene, with promoter-like activity, and demonstrates that CNT2 is a target of TGF-β1 action, in a JNK-dependent manner, by transcriptional activation of this gene. The physiological relevance of this finding is still to be determined, but highlights a putative role for CNT2 beyond nucleoside salvage.

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1 Baldwin, S. A., Beal, P. R., Yao, S. Y., King, A. E., Cass, C. E. and Young, J. D. (2004) The equilibrative nucleoside transporter family, SLC29. Pflugers Arch. 447, 735–743.
2 Gray, J. H., Owen, R. P. and Giacomini, K. M. (2004) The concentrative nucleoside transporter family, SLC28. Pflugers Arch. 447, 728–734.
3 Pastor-Anglada, M., Casado, F. J., Valdes, R., Mata, J., Garcia-Manteiga, J. and Molina, M. (2001) Complex regulation of nucleoside transporter expression in epithelial and immune system cells. Mol. Membr. Biol. 18, 81–85.
4 Pastor-Anglada, M., Molina-Arcas, M., Casado, F. J., Bellosillo, B., Colomer, D. and Gil, J. (2004) Nucleoside transporters in chronic lymphocytic leukaemia. Leukemia 18, 385–393.
5 del Santo, B., Valdes, R., Mata, J., Felipe, A., Casado, F. J. and Pastor-Anglada, M. (1998) Differential expression and regula-

tion of nucleoside transport systems in rat liver parenchymal and hepatoma cells. Hepatology 28, 1504–1511.

6 Felipe, A., Ferrer-Martinez, A., Casado, F. J. and Pastor-Anglada, M. (1997) Expression of sodium-dependent purine nucleoside carrier (SPNT) mRNA correlates with nucleoside transport activity in rat liver. Biochem. Biophys. Res. Commun. 233, 572–575.

7 Gomez-Angelats, M., del Santo, B., Mercader, J., Ferrer-Martinez, A., Felipe, A., Casado, F. J. and Pastor-Anglada, M. (1996) Hormonal regulation of concentrative nucleoside transport in liver parenchymal cells. Biochem. J. 313, 915–920.

8 Mercader, J., Gomez-Angelats, M., del Santo, B., Casado, F. J., Felipe, A. and Pastor-Anglada, M. (1996) Nucleoside uptake in rat liver parenchymal cells. Biochem. J. 317, 835–842.

9 Dragan, Y., Valdes, R., Gomez-Angelats, M., Felipe, A., Casado, F. J, Pitot, H. and Pastor-Anglada, M. (2000) Selective loss of nucleoside carrier expression in rat hepatocarcinomas. Hepatology 32, 239–246.

10 Felipe, A., Valdes, R., Santo, B., Lloberas, J., Casado, J. and Pastor-Anglada, M. (1998) Na^+-dependent nucleoside transport in liver: two different isoforms from the same gene family are expressed in liver cells. Biochem. J. 330, 997–1001.

11 del Santo, B., Tarafa, G., Felipe, A., Casado, F. J. and Pastor-Anglada, M. (2001) Developmental regulation of the concentrative nucleoside transporters CNT1 and CNT2 in rat liver. J. Hepatol. 34, 873–880.

12 Soler, C., Garcia-Manteiga, J., Valdes, R., Xaus, J., Comalada, M., Casado, F. J., Pastor-Anglada, M., Celada, A. and Felipe, A. (2001) Macrophages require different nucleoside transport systems for proliferation and activation. FASEB J. 15, 1979–1988.

13 Soler, C., Felipe, A., Garcia-Manteiga, J., Serra, M., Guillen-Gomez, E., Casado, F. J., MacLeod, C., Modolell, M., Pastor-Anglada, M. and Celada, A. (2003) Interferon-gamma regulates nucleoside transport systems in macrophages through signal transduction and activator of transduction factor 1 (STAT1)-dependent and -independent signalling pathways. Biochem. J. 375, 777–783.

14 Soler, C., Valdes, R., Garcia-Manteiga, J., Xaus, J., Comalada, M., Casado, F. J., Modolell, M., Nicholson, B., MacLeod, C., Felipe, A., Celada, A. and Pastor-Anglada, M. (2001) Lipopolysaccharide-induced apoptosis of macrophages determines the up-regulation of concentrative nucleoside transporters Cnt1 and Cnt2 through tumor necrosis factor-alpha-dependent and -independent mechanisms. J. Biol. Chem. 276, 30043–30049.

15 Wang, J., Su, S. F., Dresser, M. J., Schaner, M. E., Washington, C. B. and Giacomini, K. M. (1997) Na(+)-dependent purine nucleoside transporter from human kidney: cloning and functional characterization. Am. J. Physiol 273, F1058-F1065.

16 Ward, J. L., Sherali, A., Mo, Z. P. and Tse, C. M. (2000) Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine. J. Biol. Chem. 275, 8375–8381.

17 Chen, Y. F., Li, P. L. and Zou, A. P. (2001) Oxidative stress enhances the production and actions of adenosine in the kidney. Am. J. Physiol Regul. Integr. Comp. Physiol. 281, R1808-R1816.

18 Decking, U. K., Schlieper, G., Kroll, K. and Schrader, J. (1997) Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. Circ. Res. 81, 154–164.

19 Rego, A. C., Santos, M. S. and Oliveira, C. R. (1997) Adenosine triphosphate degradation products after oxidative stress and metabolic dysfunction in cultured retinal cells. J. Neurochem. 69, 1228–1235.

20 Duflot, S., Riera, B., Fernandez-Veledo, S., Casado, V., Norman, R. I., Casado, F. J., Lluis, C., Franco, R. and Pastor-Anglada, M. (2004) ATP-sensitive K(+) channels regulate the concentrative adenosine transporter CNT2 following activation by A(1) adenosine receptors. Mol. Cell. Biol. 24, 2710–2719.

21 Fernandez-Veledo, S., Valdes, R., Wallenius, V., Casado, F. J. and Pastor-Anglada, M. (2004) Up-regulation of the high-affinity pyrimidine-preferring nucleoside transporter concentrative nucleoside transporter 1 by tumor necrosis factor-alpha and interleukin-6 in liver parenchymal cells. J. Hepatol. 41, 538–544.

22 Gressner, A. M., Lahme, B., Mannherz, H. G. and Polzar, B. (1997) TGF-beta-mediated hepatocellular apoptosis by rat and human hepatoma cells and primary rat hepatocytes. J. Hepatol. 26, 1079–1092.

23 Lamboley, C., Bringuier, A. F. and Feldmann, G. (2000) Induction of apoptosis in normal cultured rat hepatocytes and in Hep3B, a human hepatoma cell line. Cell Biol. Toxicol. 16, 185–200.

24 Valdes, F., Murillo, M. M., Valverde, A. M., Herrera, B., Sanchez, A., Benito, M., Fernandez, M. and Fabregat, I. (2004) Transforming growth factor-beta activates both pro-apoptotic and survival signals in fetal rat hepatocytes. Exp. Cell Res. 292, 209–218.

25 Tanaka, F., Hori, N. and Sato, K. (2002) Identification of differentially expressed genes in rat hepatoma cell lines using subtraction and microarray. J. Biochem. (Tokyo) 131, 39–44.

26 Valdes, R., Ortega, M. A., Casado, F. J., Felipe, A., Gil, A., Sanchez-Pozo, A. and Pastor-Anglada, M. (2000) Nutritional regulation of nucleoside transporter expression in rat small intestine. Gastroenterology 119, 1623–1630.

27 McMahon, J. B., Richards, W. L., del Campo, A. A., Song, M. K. and Thorgeirsson, S. S. (1986) Differential effects of transforming growth factor-beta on proliferation of normal and malignant rat liver epithelial cells in culture. Cancer Res. 46, 4665–4671.

28 Sanderson, N., Factor, V., Nagy, P., Kopp, J., Kondaiah, P., Wakefield, L., Roberts, A. B., Sporn, M. B. and Thorgeirsson, S. S. (1995) Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. Proc. Natl. Acad. Sci. USA 92, 2572–2576.

29 Perlman, R., Schiemann, W. P., Brooks, M. W., Lodish, H. F. and Weinberg, R. A. (2001) TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nat. Cell Biol. 3, 708–714.

30 Michaelson, J. S., Bader, D., Kuo, F., Kozak, C. and Leder, P. (1999) Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. Genes Dev. 13, 1918–1923.

31 Herrera, B., Alvarez, A. M., Beltran, J., Valdes, F., Fabregat, I. and Fernandez, M. (2004) Resistance to TGF-beta-induced apoptosis in regenerating hepatocytes. J. Cell Physiol. 201, 385–392.

32 Murillo, M. M., del Castillo, G., Sanchez, A., Fernandez, M. and Fabregat, I. (2005) Involvement of EGF receptor and c-Src in the survival signals induced by TGF-beta1 in hepatocytes. Oncogene 24, 4580–4587.

33 Sanchez, A., Alvarez, A. M., Lopez Pedrosa, J. M., Roncero, C., Benito, M. and Fabregat, I. (1999) Apoptotic response to TGF-beta in fetal hepatocytes depends upon their state of differentiation. Exp. Cell Res. 252, 281–291.

34 Valdes, F., Alvarez, A. M., Locascio, A., Vega, S., Herrera, B., Fernandez, M., Benito, M., Nieto, M. A. and Fabregat, I. (2002) The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes. Mol. Cancer Res. 1, 68–78.

35 Schuster, N. and Krieglstein, K. (2002) Mechanisms of TGF-beta-mediated apoptosis. Cell Tissue Res. 307, 1–14.

36 Pagan, R., Sanchez, A., Martin, I., Llobera, M., Fabregat, I. and Vilaro, S. (1999) Effects of growth and differentiation factors on the epithelial-mesenchymal transition in cultured neonatal rat hepatocytes. J. Hepatol. 31, 895–904.

37 Sanchez, A., Alvarez, A. M., Benito, M. and Fabregat, I. (1995) Transforming growth factor beta modulates growth and differentiation of fetal hepatocytes in primary culture. J. Cell Physiol. 165, 398–405.
38 Sanchez, A., Pagan, R., Alvarez, A. M., Roncero, C., Vilaro, S., Benito, M. and Fabregat, I. (1998) Transforming growth factor-beta (TGF-beta) and EGF promote cord-like structures that indicate terminal differentiation of fetal hepatocytes in primary culture. Exp. Cell Res. 242, 27–37.
39 Aymerich, I., Pastor-Anglada, M. and Casado, F. J. (2004) Long term endocrine regulation of nucleoside transporters in rat intestinal epithelial cells. J. Gen. Physiol. 124, 505–512.
40 Greenwel, P., Inagaki, Y., Hu, W., Walsh, M. and Ramirez, F. (1997) Sp1 is required for the early response of alpha2(I) collagen to transforming growth factor-beta1. J. Biol. Chem. 272, 19738–19745.
41 Law, A. K., Gupta, D., Levy, S., Wallace, D. C., McKeon, R. J. and Buck, C. R. (2004) TGF-beta1 induction of the adenine nucleotide translocator 1 in astrocytes occurs through Smads and Sp1 transcription factors. BMC. Neurosci. 5, 1.
42 Sanchez-Elsner, T., Ramirez, J. R., Sanz-Rodriguez, F., Varela, E., Bernabeu, C. and Botella, L. M. (2004) A cross-talk between hypoxia and TGF-beta orchestrates erythropoietin gene regulation through SP1 and Smads. J. Mol. Biol. 336, 9–24.
43 Zhang, W., Ou, J., Inagaki, Y., Greenwel, P. and Ramirez, F. (2000) Synergistic cooperation between Sp1 and Smad3/Smad4 mediates transforming growth factor beta1 stimulation of alpha 2(I)-collagen (COL1A2) transcription. J. Biol. Chem. 275, 39237–39245.
44 Duflot, S., Calvo, M., Casado, F. J., Enrich, C. and Pastor-Anglada, M. (2002) Concentrative nucleoside transporter (rCNT1) is targeted to the apical membrane through the hepatic transcytotic pathway. Exp. Cell Res. 281, 77–85.
45 Guillen-Gomez, E., Calbet, M., Casado, J., de Lecea, L., Soriano, E., Pastor-Anglada, M. and Burgaya, F. (2004) Distribution of CNT2 and ENT1 transcripts in rat brain: selective decrease of CNT2 mRNA in the cerebral cortex of sleep-deprived rats. J. Neurochem. 90, 883–893.
46 Merighi, S., Mirandola, P., Varani, K., Gessi, S., Leung, E., Baraldi, P. G., Tabrizi, M. A. and Borea, P. A. (2003) A glance at adenosine receptors: novel target for antitumor therapy. Pharmacol. Ther. 100, 31–48.
47 Virag, L. and Szabo, C. (2001) Purines inhibit poly(ADP-ribose) polymerase activation and modulate oxidant-induced cell death. FASEB J. 15, 99–107.
48 Gomez, G. and Sitkovsky, M. V. (2003) Differential requirement for A2a and A3 adenosine receptors for the protective effect of inosine *in vivo*. Blood 102, 4472–4478.
49 Aymerich, I., Foufelle, F., Ferre, P., Casado, F. J. and Pastor-Anglada, M. (2006) Extracellular adenosine activates AMP-dependent protein kinase (AMPK). J. Cell Sci. 119, 1612–1621.
50 Patil, S. D., Ngo, L. Y., Glue, P. and Unadkat, J. D. (1998) Intestinal absorption of ribavirin is preferentially mediated by the Na^+-nucleoside purine (N1) transporter. Pharm. Res. 15, 950–952.
51 Azzaroli, F., Accogli, E., Nigro, G., Trere, D., Giovanelli, S., Miracolo, A., Lodato, F., Montagnani, M., Tame, M., Colecchia, A., Mwangemi, C., Festi, D., Roda, E., Derenzini, M. and Mazzella, G. (2004) Interferon plus ribavirin and interferon alone in preventing hepatocellular carcinoma: a prospective study on patients with HCV related cirrhosis. World, J. Gastroenterol. 10, 3099–3102.
52 Mangia, A., Ricci, G. L., Persico, M., Minerva, N., Carretta, V., Bacca, D., Cela, M., Piattelli, M., Annese, M., Maio, G., Conte, D., Guadagnino, V., Pazienza, V., Festi, D., Spirito, F. and Andriulli, A. (2005) A randomized controlled trial of pegylated interferon alpha-2a (40 KD) or interferon alpha-2a plus ribavirin and amantadine *vs* interferon alpha-2a and ribavirin in treatment-naive patients with chronic hepatitis C. J. Viral Hepat. 12, 292–299.

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