**INTRODUCTION**

Ecto-5′-nucleotidase (CD73) is a glycosyl-phosphatidylinositol-linked plasma membrane glycoprotein that is expressed on multiple cell types and in different tissues (Misumi et al., 1990; Resta et al., 1993; Knapp et al., 2012). CD73 catalyzes the conversion of extracellular AMP to adenosine (Resta et al., 1998; Zimmermann et al., 2012). Adenosine controls numerous physiological responses by activating one of four subtypes of G-protein–coupled receptors (Antonioli et al., 2013a; Chen et al., 2013). By virtue of its role as the major extracellular source of adenosine, CD73 has emerged as an important regulator of tissue homeostasis and pathophysiologic responses related to immunity, inflammation, pain, ischemia, tissue fibrosis, and cancer (Colgan et al., 2006; Żylika, 2011; Beavis et al., 2012; Antonioli et al., 2013b; Roberts et al., 2013). The only human disease linked to mutations in NT5E (the CD73-encoding gene) is calcification of joints and arteries (CALJA), an adult-onset condition characterized by joint pain (St Hilaire et al., 2011). The mechanism of how loss of CD73 function is related to the pathogenesis of this disease is not known, although the disease-associated CD73 isoforms appear to have trafficking defects (Fausther et al., 2014).

In addition to its direct genetic link to a human disease, there is evidence for the involvement of CD73 in cancer. For example, there is a positive correlation in triple-negative breast cancer between CD73 transcript levels and poor prognosis, as well as to resistance to chemotherapy (Loi et al., 2013). The findings in human breast cancer correlate with mouse models that have demonstrated

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**ABSTRACT**

Ecto-5′-nucleotidase (CD73), encoded by NT5E, is the major enzymatic source of extracellular adenosine. CD73 controls numerous pathophysiological responses and is a potential disease target, but its regulation is poorly understood. We examined NT5E regulation by alternative splicing. Genomic database analysis of human transcripts led us to identify NT5E-2, a novel splice variant that was expressed at low abundance in normal human tissues but was significantly up-regulated in cirrhosis and hepatocellular carcinoma (HCC). NT5E-2 encodes a shorter CD73 isoform we named CD73S. The presence of CD73S protein, which lacks 50 amino acids, was detected in HCC using an isoform-specific antibody. A noncanonical mouse mRNA, similar to human CD73S, was observed, but the corresponding protein was undetectable. The two human isoforms exhibited functional differences, such that ectopic expression of canonical CD73 (CD73L) in human HepG2 cells was associated with decreased expression of the proliferation marker Ki67, whereas CD73S expression did not have an effect on Ki67 expression. CD73S was glycosylated, catalytically inactive, unable to dimerize, and complexed intracellularly with the endoplasmic reticulum chaperone calnexin. Furthermore, CD73S complexed with CD73L and promoted proteasome-dependent CD73L degradation. The findings reveal species-specific CD73 regulation, with potential significance to cancer, fibrosis, and other diseases characterized by changes in CD73 expression and function.

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This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-06-1167) on October 8, 2014. Address correspondence to: Natasha Snider (nsnider@umich.edu). Abbreviations used: CD73L, long protein isoform of CD73; CD73S, short protein isoform of CD73; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NAFLD, nonalcoholic fatty liver disease.

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RESULTS

Human NT5E is regulated by alternative splicing in cirrhosis and hepatocellular carcinoma

Using the Ensembl genome browser (Flicek et al., 2014), we compared the alternative splicing pattern of human NT5E to nine other vertebrate species. Whereas human NT5E encodes five transcripts, all other species, except for cow, are predicted to have one NT5E transcript (Figure 1A). Aside from NT5E-001, which encodes the full-length canonical CD73 protein, NT5E-201 (Figure 1B) is the only other human transcript annotated by the Collaborative Consensus Coding Sequence (CCDS) project (Harte et al., 2012) as being highly likely to encode a protein. Therefore we focused our subsequent analysis on comparing these two transcripts, and we refer to NT5E-1 and NT5E-2, respectively. The only difference between the two transcripts is the absence of exon 7 in NT5E-2 (Figure 1C).

We found that the two transcripts had comparable relative distribution profiles across 14 normal human tissues (Figure 2A), but NT5E-2 was expressed at significantly lower levels than NT5E-1 (Figure 2B). We also evaluated the relative expression of these two isoforms in nine human cancer cell lines (Figure 2C) and found that the two hepatocellular carcinoma (HCC) cell lines Huh7 and HepG2 expressed among the highest levels of the two transcripts relative to the other tested cell lines (AsPC-1, MCF7, NCI-H118, HT-29, K-562, CCRF-CEM, and MOLT-4). Therefore we focused our subsequent analysis on NT5E expression in normal and diseased livers. Because alternative splicing of genes is known to be altered in disease states (David and Manley, 2010; Singh and Cooper, 2012), we evaluated the expression of NT5E-2 in chronic human liver diseases, including HCV, NAFLD, and HCC. Whereas expression of NT5E-2 did not

limited tumor growth and metastasis upon ablation of CD73 function (Stagg et al., 2010, 2011; Wang et al., 2011; Allard et al., 2014). The apparent tumor-promoting roles of CD73 appear to be linked to its ability to generate adenosine, which acts as a potent suppressor of antitumor T-cell responses (Zhang, 2010; Beavis et al., 2012; Sitkovsky et al., 2014). However, nonenzymatic CD73 functions, which are poorly understood, may also be involved (Mikhailov et al., 2008; Terp et al., 2013).

Aside from its well-known roles in cancer and immunity, CD73 has recently emerged as a novel regulator of disease pathology in multiple liver disease models in mice. Specifically, CD73 promotes drug-induced hepatocellular injury and Mallory–Denk body inclusion formation (Snider et al., 2013), fibrosis (Peng et al., 2008; Fausther et al., 2012), and steatosis (Peng et al., 2009). In contrast to the animal models, dramatic decreases in NT5E mRNA are observed in human hepatitis C (HCV) and nonalcoholic fatty liver disease (NAFLD) livers (Snider et al., 2013), which suggests potential species-specific differences in CD73 regulation and/or function.

These observations, in combination with the fact that relatively little is known regarding CD73 regulation, prompted us to address the potential existence of alternative splice variants that may encode CD73 with different functions. Because alternative splicing is known to be a major driver for species-specific gene regulation (Barbosa-Morais et al., 2012; Merkin et al., 2012), understanding the major differences between CD73 in preclinical models and humans should help facilitate the translation of basic findings into the clinic. In the present study, we report on the identification of CD73S, a functionally distinct isoform of CD73 that is generated by alternative splicing of NT5E in the setting of human liver cirrhosis and hepatocellular carcinoma.

FIGURE 1: The human NT5E gene is regulated by alternative splicing. (A) Species comparison of the number of NT5E predicted transcripts using the Ensembl database. (B) Human NT5E has five splice variants, all predicted to be protein coding (bp, base pairs; aa, amino acids), but only two are annotated by CCDS. (C) The CCDS-validated transcripts NT5E-001 (NT5E-1) and NT5E-201 (NT5E-2), which differ with respect to the presence of exon 7 (arrow) in NT5E-1, are shown schematically.
Relative to normal livers (Figure 3B). Because up-regulation of NT5E-2 was also observed in the adjacent nontumor tissue, which is frequently cirrhotic, we analyzed NT5E expression in liver biopsies from patients with confirmed HCV-related cirrhosis (in the absence of HCC) and found that NT5E-2 was elevated in these livers (Figure 3C) to an extent similar to what is seen in HCC (Figure 3B), thereby indicating that NT5E-2 up-regulation occurs before HCC development in the context of cirrhosis.

Alternative splicing of NT5E generates CD73S, a functionally distinct CD73 protein

The NT5E-2 transcript is predicted to encode a shorter protein, which we termed CD73S (S, short; Figure 3D). NT5E-1 encodes canonical CD73, which we refer to as CD73L (L, long). Human CD73S lacks amino acids 404–453, encoded by the missing exon 7 and located in the C-terminus. The C-terminus contains the substrate-binding domain and the interface important for forming the functional CD73 homodimer expressed at the cell surface (Knapp et al., 2012). Based on this, CD73S is predicted to have compromised activity as an AMPase because it lacks a critical catalytic residue (Phe-417). Furthermore, the missing secondary structure (three β-strands and two α-helices; Figure 3D) suggests that this protein would have different folding and dimerization properties.

To assess the presence of CD73S in HCC, we generated a rabbit antibody directed to the peptide ERNNGIHV representing the unique junction present in CD73S but not CD73L (Figure 3E). DNA encoding Flag epitope-tagged versions of CD73L and CD73S or control vector was transfected into HEK293T cells, and the presence of both proteins was assessed by a Flag immunoblot (Figure 3F). This validated that NT5E-2 encodes the shorter (∼67 kDa) CD73S compared with CD73L (∼72 kDa) protein. Furthermore, the rabbit anti-CD73S antibody selectively recognized CD73S in the overexpression system (Figure 3F, top) and detected an ∼67-kDa protein in HCC but not normal livers (Figure 3G, arrow), although the antibody also exhibited nonspecific binding to other proteins in normal and HCC tissue lysates. Blotting with preimmune rabbit serum did not reveal differences between normal and HCC livers (Supplemental Figure S1). In agreement with the mRNA results (Figure 3B), both the HCC tumors and adjacent livers expressed the 67-kDa protein (Figure 3G), which we predict is CD73S.

We also assessed the expression of a putative mouse protein (Uniprot ID Q8C8G9) that shares some sequence similarity to human CD73S (Figure 4A). Despite robust mRNA detection upon overexpression of the corresponding cDNA in primary mouse hepatocytes, we did not detect any protein (Figure 4, B and C). Using
FIGURE 3: NT5E-2 is up-regulated in cirrhosis, and HCC and encodes a shorter CD73 protein (CD73S), which is functionally distinct from canonical CD73 (CD73L). (A–C) Relative expression of NT5E-1 and NT5E-2 mRNA in HCC cell lines (A), tumors and adjacent nontumor tissue from HCC surgical specimen obtained from six patients (B), and biopsies from patients with HCV-associated cirrhosis of the liver (C; clinical information on the human HCC and cirrhosis samples provided in Supplemental Table S2). CXCL10 is included as a positive control for HCV cirrhosis samples (Brownell and Polyak, 2013). (D) Protein sequence alignment of the C-termini of CD73L (NP_002517) and CD73S (NP_001191742). The 50 residues (404–453) missing in CD73S form three β-strands and two α-helices and include a catalytic residue (Phe-417). (E) Sequence of the synthetic peptide (ERNNGIHV) used to generate rabbit anti-CD73S antibodies. (F) Detection of total Flag-CD73S and Flag-CD73L protein (bottom) and validation of the CD73S antibody reactivity in Flag immunoprecipitates of transfected HEK293T cell lysates. CD73S and CD73L have predicted molecular weight of 58 and 63 kDa, respectively, but migrate at ~67 and ~72 kDa because of glycosylation and the Flag tag. (G) CD73S immunoblot of total tissue lysates from two normal human livers (1, 2) and six HCC paired tumors (T) and adjacent uninvolved liver (L) tissues (HCC 1–6; same as those used in B).
CD73S is a dimerization-deficient, endoplasmic reticulum–associated glycoprotein that lacks 5′-nucleotidase activity. To understand the reason behind the functional differences between CD73S and CD73L in the HepG2 cells, we analyzed the molecular characteristics of CD73S. As predicted from the protein sequence, we found that CD73S completely lacks 5′-nucleotidase activity (Figure 5C). The glycosylation sites on CD73, Asn-311/333/403 (Chen et al., 2009), are present in both isoforms and, consistent with this, treatment with peptide-N-glycosidase F resulted in deglycosylation of both isoforms (Figure 5D). Of note, upon expression in primary mouse hepatocytes, human CD73L exhibited plasma membrane and intracellular distribution, whereas human CD73S was primarily intracellular (Figure 5E). Another major difference between CD73L and CD73S was the lack of disulfide-linked homodimer a different cell host (BHK-21 cells), addition of a proteasome inhibitor and analysis of the cell culture medium also did not reveal protein expression in the cells or the culture medium (unpublished data), suggesting that CD73S is a human-specific variant.

To determine whether CD73S and CD73L exhibit functional differences, we transfected each isoform into HepG2 cells and monitored the expression of the cell proliferation marker Ki67. The cells expressing CD73L had a significant decrease in Ki67 staining, whereas CD73S expression did not significantly alter Ki67 expression relative to neighboring untransfected cells (Figure 5, A and B). Therefore CD73L and CD73S exhibit functional differences with respect to their ability to modulate the proliferation potential of cultured human HCC cells.

CD73S is a dimerization-deficient, endoplasmic reticulum–associated glycoprotein that lacks 5′-nucleotidase activity. To understand the reason behind the functional differences between CD73S and CD73L in the HepG2 cells, we analyzed the molecular characteristics of CD73S. As predicted from the protein sequence, we found that CD73S completely lacks 5′-nucleotidase activity (Figure 5C). The glycosylation sites on CD73, Asn-311/333/403 (Chen et al., 2009), are present in both isoforms and, consistent with this, treatment with peptide-N-glycosidase F resulted in deglycosylation of both isoforms (Figure 5D). Of note, upon expression in primary mouse hepatocytes, human CD73L exhibited plasma membrane and intracellular distribution, whereas human CD73S was primarily intracellular (Figure 5E). Another major difference between CD73L and CD73S was the lack of disulfide-linked homodimer
FIGURE 5: Molecular and functional characteristics of CD73S. (A) Coimmunofluorescence analysis of the proliferation marker Ki67 (red) and Flag-CD73S/L (green) in transfected HepG2 cells. Blue, 4′,6-diamidino-2-phenylindole (DAPI); bar, 20 μm. (B) Quantification of Ki67 immunofluorescence staining in CD73-expressing cells relative to neighboring untransfected cells (30 CD73+ cells and 150 CD73− untransfected cells were counted per condition). ****p < 0.0001, unpaired t test. (C) Measurement of 5′-nucleotidase activity in transfected HEK293T cell lysates. Samples expressing equal protein levels (bottom) were analyzed in triplicate. (D) Treatment of CD73-transfected HEK293T cell lysates with peptide-N-glycosidase F results in similar deglycosylation of both isoforms. (E) Immunofluorescence-based localization of Flag-CD73L or Flag-CD73S in primary mouse hepatocytes. Blue, DAPI; bar, 20 μm. (F) Flag immunoblot of Flag-CD73L− and -CD73S−expressing HEK293T total cell lysates analyzed under reducing (+β-mercaptoethanol, βME) or nonreducing (−βME) conditions, showing that CD73S does not dimerize. (G) Coomassie-stained gel of Flag immunoprecipitates of the nonreduced samples shown in F. Asterisk denotes a unique protein present in CD73S immunoprecipitates, identified by mass spectrometry as calnexin. (H) Biochemical validation that CD73S coimmunoprecipitates with calnexin.
CD73S negatively regulates CD73L activity and protein expression in a proteasome-dependent manner

To assess whether there is cross-regulation between the two human CD73 isoforms, we transfected equal amounts of DNA encoding Flag-CD73L cDNA together with empty vector or Flag-CD73S. This resulted in a >50% decrease in the 5′-nucleotidase activity of CD73L, which correlated with decreased CD73L protein expression (Figure 6A). This effect was specific to CD73S, since control overexpression of excess Flag-GAPDH did not result in the same decrease in CD73L protein levels (Supplemental Figure S2B), and the effect was observed regardless of the epitope tag, since GFP-CD73L was similarly

CD73S negatively regulates CD73L expression in a proteasome-dependent manner.

(A) Measurement of 5′-nucleotidase activity in lysates from HEK293T cells transfected with equal amount of Flag-CD73L cDNA together with empty vector or Flag-CD73S. ****p < 0.0001, unpaired t test. (B) Measurement of CD73L (endogenous) and CD73S (transfected) mRNA in Huh-7 cells, showing that CD73S does not affect CD73L mRNA. (C) Determination of the effect of increasing levels of CD73S on CD73L dimer formation, which is detected under nonreducing conditions (−βME). (D) CD73 immunoblot of CD73-transfected HEK-293T lysates (±MG132). The same membrane under two exposure times from a triplicate experiment is shown. (E) Quantification of blot from D, showing that MG132 restores CD73L protein levels. ***p < 0.001, unpaired t test. (F) Reciprocal communoprecipitation of CD73S/L-transfected HEK293T lysates, demonstrating that CD73S complexes with CD73L. Anti-V5 antibody was used as a negative control.

formation in the latter, as determined by immunoblot and Coomassie stain of Flag-CD73–transfected cell lysates and Flag immunoprecipitates under reducing and nonreducing conditions (Figure 5, F and G). The Coomassie stain further revealed the presence of an ~90-kDa protein that coimmunoprecipitated with CD73S but not CD73L (Figure 5G, asterisk). This protein was identified by mass spectrometry (not shown) as the endoplasmic reticulum (ER) chaperone calnexin, which was validated biochemically (Figure 5H). Coimmuno-fluorescence staining of CD73S and another ER marker protein, PDI, also revealed colocalization (Supplemental Figure S2A). These data demonstrate that CD73S has molecular and enzymatic properties that are significantly different from those of CD73L.
down-regulated in the presence of CD73S (Supplemental Figure S2C). Furthermore, the inhibitory effect of CD73S was not limited to the ectonucleotidase CD73L, since CD73S also down-regulated the expression of the cytosolic 5’-nucleotidase IA in a co-overexpression system (Supplemental Figure S2D).

The inhibitory effect of CD73S on CD73L expression did not involve decreased mRNA, since the levels of endogenous CD73L mRNA in the presence of CD73S overexpression were unchanged relative to control levels (Figure 6B); similar results were obtained when both isoforms were co-overexpressed (unpublished data). In addition, the presence of increasing levels of CD73S protein did not significantly affect the expression of the CD73L dimer (Figure 6C), which is typically found at the cell surface, suggesting that CD73S inhibits CD73L protein expression most likely during processing in the ER. To that end, addition of MG132 restored CD73L protein in the presence of CD73S (Figure 6, D and E), indicating that CD73S promotes proteasome-dependent degradation of CD73L.

Reciprocal coimmunoprecipitation of the two isoforms revealed that a fraction of CD73L is associated in a complex with CD73S (Figure 6F), indicating that CD73L degradation may result from an interaction between the two CD73 isoforms. Collectively, these data demonstrate that CD73S functions as a negative regulator of CD73L protein expression, and possibly cytoplasmic nucleotidases as well.

DISCUSSION

Alternative splicing as a mechanism for species-specific CD73 regulation

We demonstrate that alternative splicing is a novel mode of human CD73 regulation. Alternative splicing of pre-mRNA is a mechanism by which multiple mRNA and protein isoforms are generated from a limited set of genes, which promotes functional complexity of organisms (Matera and Wang, 2014). Important protein properties, including intracellular localization, posttranslational modifications, turnover, enzymatic activity, and the shaping of signaling networks, are dictated by alternative splicing; it is estimated that >90% of multiexon human genes are subject to alternative splicing regulation (Barbosa-Morais et al., 2012; Merkin et al., 2012; Singh and Cooper, 2012). Cross-species comparisons using RNA-Seq revealed that, unlike tissue-specific gene expression, which is largely conserved, alternative splicing differs significantly between species (Barbosa-Morais et al., 2012). Therefore alternative splicing of human NT5E may contribute to some of the previously reported differences between human and mouse CD73. For example, CD73 is abundantly expressed on afferent lymphatic vessels in humans but not in mice (Aldars et al., 2011), whereas the opposite pattern has been observed in regulatory T-cells (Toth et al., 2013). Given the important biological functions of CD73 and its potential as a disease target, better understanding of species-specific CD73 regulation could facilitate translation of preclinical findings to the clinic.

Alternative splicing as a mechanism for CD73 regulation in human diseases

Our findings show that NT5E-2 mRNA and its protein product, CD73S, are not abundant in normal human tissues but are induced in HCV-associated cirrhosis and HCC. Pre-mRNA splicing factors and events are known to be deregulated in human cancers (David and Manley, 2010; Singh and Cooper, 2012; De Craene and Berx, 2013), including HCC (Chan et al., 2014; Qi et al., 2014). For example, increased activities of the splicing factors CUGBP1, hnRNP H, hnRNP A1, hnRNP A2B1, and SF2/ASF are believed to contribute to a switch in insulin receptor (IR) isoform expression in HCC (Chettouh et al., 2013). IR-B, which mediates the metabolic effects of insulin, is the predominant isoform in normal adult liver. However, during hepatocyte neoplastic transformation, IR expression switches to the IR-A isoform, which promotes cell proliferation (Chettouh et al., 2013). Another example is the expression of a metastasis-promoting, N-terminally truncated isoform of carboxypeptidase E (CPE), which is generated by alternative splicing of the CPE gene in HCC and other cancers (Lee et al., 2011). Nothing is known regarding the involvement of CD73 in progression to cirrhosis and HCC development. Studies in mouse models demonstrated a promoting role of CD73 in hepatocellular injury (Snider et al., 2013), fibrosis (Peng et al., 2008; Fausther et al., 2012), and steatosis (Peng et al., 2009), with the last two likely involving adenosine receptor signaling. However, NT5E-1 is dramatically decreased in HCV and NAFLD (Snider et al., 2013) as well as HCC (this study). In light of the functional differences between CD73S and CD73L, any association between NT5E expression and human disease outcomes should be carefully interpreted if the data were generated using microarray platforms (e.g., Affymetrix) that do not distinguish between NT5E-1 and NT5E-2 expression.

Regulation of CD73L protein levels via CD73S-CD73L complex formation

The association of CD73 with the ER chaperone calnexin, combined with its intracellular localization, suggests that CD73S may be recognized by the ER quality control system as a misfolded glycoprotein (Vembar and Brodsky, 2008). Furthermore, the ability of CD73S to complex with CD73L and promote CD73L degradation in a proteasome-dependent manner provides a novel mechanism for regulation of CD73 protein levels. It remains to be determined whether NT5E-2 mRNA and CD73S protein induction is associated with CD73 decreases observed in other cancers, such as malignant melanoma (Wang et al., 2012), in which methylation-dependent transcriptional silencing of NT5E appears to be involved. However, given the growing appreciation for epigenetic mechanisms in alternative splicing regulation (Luco et al., 2011), these two pathways may converge to regulate CD73 expression in human cancers, as well as in other diseases.

CD73S as a potential negative regulator of proapoptotic and antiproliferative functions of adenosine in HCC

The inverse association between CD73L expression and Ki67 positivity in HepG2 cells suggests that CD73L may function to limit hepatoma cell proliferation. This may involve adenosine because of the known proapoptotic and antiproliferative functions of adenosine or adenosine A3 receptor agonists in these cells (Hermes et al., 2007; Cohen et al., 2011; Tamura et al., 2012). Given that CD73S promotes CD73L down-regulation, one potential function of CD73S in HCC may be to limit the antiproliferative and proapoptotic function of CD73L-generated adenosine (Figure 7). The effects of adenosine in cancer are complex and can result in different outcomes, depending upon which receptor is activated (Antonioli et al., 2013a). In general, activation of the adenosine receptors A1, A2A, and A2B promotes cancer cell proliferation, whereas activation of the A3 receptor blocks it (Antonioli et al., 2013a). Furthermore, activation of A3 receptor promotes apoptosis of cancer cells, and the adenosine A3 receptor agonist CF102 has antitumor effects in the liver (Cohen et al., 2011). Additional studies will be necessary to assess whether NT5E-2 mRNA and CD73S protein levels correlate with liver disease progression and the ultimate development of HCC.
the surgery to remove the tumor(s). The normal control livers that were used for immunoblotting were obtained from the National Disease Research Interchange, and the control livers that were used for qPCR analysis were from biopsies and previously described (Snider et al., 2013).

Cell culture, transfections, and immunofluorescence analysis

The human HCC cell line Huh7 was obtained from the Japanese Collection of Research Bioresources (JCRB), and all other cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured according to ATCC recommendations. Embryonic kidney (HEK293T) and HCC (HepG2) human cells were cultured in DMEM and EMEM, respectively, supplemented with 10% fetal bovine serum. The cell lines were transfected with CD73S and/or CD73L plasmid DNA using Lipofectamine LTX (Invitrogen). Primary mouse hepatocytes were isolated and transfected for 24 h as described (Snider et al., 2011). MG132 (10 μg/ml) was added for 12–14 h after the transfection. For the immunofluorescence analyses, cells were fixed, stained, and imaged as described (Snider et al., 2011).

MATERIALS AND METHODS

Antibodies and reagents

We used mouse anti-Flag M2 clone (Sigma-Aldrich, St. Louis, MO), rabbit anti-turboGFP (Origene, Rockville, MD), mouse anti-V5 (Invitrogen, Carlsbad, CA), rabbit anti-CD73 (Novus, Littleton, CO), and rabbit anti-calnexin, -PDI, and -Ki67 (Cell Signaling, Danvers MA). Rabbit anti-CD73S antibody was generated by Abbiotec (San Diego, CA) using a 90-d immunization protocol with the peptide C-ERNNGIHV conjugated to KLH, followed by immunoglobulin G purification using affinity chromatography. cDNAs encoding human myc-DDK (Flag)-CD73L, -cN-IA, and turboGFP-tagged CD73L in pCMV6-Entry vector were purchased from Origene. cDNAs encoding myc-DDK tagged CD73S and mouse AK047143 in pCMV6-Entry vector was synthesized by Blue Heron Biotechnology, Bothell, WA.

Quantitative PCR

RNA extraction, quantitative PCR (qPCR), and data analysis were performed as described (Snider et al., 2011) using sequence-specific primers (Supplemental Table S1).

Human RNA and tissues

Normal human tissues RNA panels were obtained from Clontech (Mountain View, CA). Diseased human livers were from biopsies (HCV, NAFLD) or surgical specimens (HCC) that were collected at the University of Michigan under an approved human subjects protocol and are described in Supplemental Table S2. None of the HCC patients received pharmacologic therapy before CD73 enzyme activity, immunoprecipitation, and immunoblotting

CD73 enzymatic activity was assessed biochemically by its ability to catalyze the enzymatic hydrolysis of 5'-inosine monophosphate (5'-IMP) to form inosine as described (Snider et al., 2013). Immunoprecipitation of control and CD73-expressing cell lysates was performed using the designated antibodies conjugated to Protein G-Dyna beads (Invitrogen, Carlsbad, CA) for 3 h at 4°C. Immunoblotting was performed as described (Snider et al., 2013).

Data analysis

Statistical analysis was done using Prism 6 (GraphPad Software). Photoshop (CS2; Adobe) was used for densitometry and quantification of the Ki67 fluorescence (5000-pixel areas were measured for each cell).

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