Human stem cells contain substantial amounts of the xenograft N-glycolyamineuraminic acid (Neu5Gc), although the levels of Neu5Gc are low or undetectable in human body fluids and most other human tissues. The lack of Neu5Gc in human tissues has been previously explained by the loss of hydroxylase activity of the human CMP-N-acetylneuraminic acid hydroxylase (CMAH) protein caused by a genetic error in the human Cmah gene. We thus wanted to investigate whether the human redundant Cmah gene could still function in stem cell-specific processes. In this study, we show that CMAH gene expression is significantly upregulated in the adult stem cell populations studied, both of hematopoietic and mesenchymal origin, and identify CMAH as a novel stem cell marker. The CMAH content co-occurs with higher levels of Neu5Gc within stem cells as measured by mass spectrometric profiling. It seems that despite being enzymatically inactive, human CMAH may upregulate the Neu5Gc content of cells by enhancing Neu5Gc uptake from exogenous sources. Furthermore, exposure to exogenous Neu5Gc caused rapid phosphorylation of β-catenin in both CMAH overexpressing cells and bone marrow-derived mesenchymal stem cells, thereby inactivating Wnt/β-catenin signaling. The data demonstrate the first molecular evidence for xenograft Neu5Gc-induced alteration of crucial stem cell-specific signaling systems for the maintenance of self renewal. These results add further emphasis to the crucial need for completely xenofree culturing conditions for human stem cells. Stem Cells 2010;28:258–267
Reiske iron-sulfur-binding region that has been concluded to be essential for the enzyme activity [11, 12]. The Cmah gene deletion is unique to humans and is one of the few protein-level differences between man and apes. The Cmah mutation has been dated to have occurred approximately 3 million years ago [13–14] and a mechanistic model for the Ato-mediated replacement event for the missing exon has been proposed [15].

As a consequence of the CMAH mutation, human cells exhibit increased levels of Neu5Ac on the surface compared to other mammals [10]. The level of Neu5Gc, a xenograft antigen in humans, is generally very low in human tissues and cells. Neu5Gc can, conversely, be taken up by human cells from an environment containing animal products [16] or even from dietary sources such as red meat and milk products [17]. It is well established that humans react immunologically against free Neu5Gc or Neu5Gc glycoconjugates by producing high titers of circulating antibodies (Hanganutzu-Dechert antibody) [18–20]. It has been established that Neu5Gc glycans are taken up from the extracellular environment by pinocytic/endoctytic pathways and incorporated in human cells by a salvage pathway [16, 17, 21].

We, among others, have previously shown that human stem cells contain higher amounts of the xenograft Neu5Gc than other human cells, also specifically detected on the cell surface with a Neu5Gc-specific antibody [22, 23]. The Neu5Gc content of human embryonic (ESC) and mesenchymal stem/stroma cells (MSC) is substantially reversible by culturing cells in xeno-free conditions [23]. It is also known that cancer cells efficiently take up more exogenous Neu5Gc than other human cells [17]. In a previous study characterizing the global gene expression profile of primitive adult hematopoietic stem cells (HSC), we found CMAH expression to be sevenfold higher in primitive CD133+ HSCs compared to CD133- cells (Supplemental Table 2 [24]). This led us to further investigate CMAH expression in adult human stem cells since it seems pointless to transcriptionally regulate a nonfunctional gene.

In this study we show that Cmah gene expression is significantly upregulated in adult human stem cells, both HSCs and MSCs, making CMAH a novel stem cell marker. Furthermore, we show that the stem cell-specific CMAH mRNA is translated to protein. Interestingly, we show that higher cellular CMAH expression can be correlated with higher levels of cellular Neu5Gc, which indeed raises questions about novel functions for CMAH. We also show that CMAH overexpression in cells endogenously almost devoid of CMAH produce a stem cell-like phenotype with an activated intracellular Wnt/β-catenin signaling system. Finally, we demonstrate the first molecular evidence for xenograft Neu5Gc-induced alteration of crucial stem cell-specific signaling systems for the maintenance of self-renewal and stemness. Exposure to exogenous Neu5Gc causes rapid phosphorylation of β-catenin in both CMAH overexpressing cells and bone marrow (BM)-derived MSCs, thereby inactivating Wnt pathway and, as a consequence, possibly forcing stem cells to lose pluripotency.

**Materials and Methods**

Reagents and Antibodies

All cell culture reagents were purchased from Gibco Invitrogen (Paisley, U.K., http://www.invitrogen.com) if not mentioned otherwise. The used antibodies are listed in Supplement 1 with the supporting information.

**Cells and Cell Culture**

Human umbilical cord blood (HUCB) was obtained via the Finnish Cord Blood Bank. All donors gave informed consent, and the study protocol was accepted by the ethical review board of the Helsinki University Central Hospital and the Finnish Red Cross Blood Service. Mononuclear cells (MNC) were isolated from HUCB by Ficoll-Hypaque density gradient (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com). CD34+ and CD133+ cells were enriched as described [25]. HUCB lineage negative (Lin-) cells were selected from MNCs through negative selection using Stem-Sep Human Progenitor Enrichment Cocktail (StemCell Technologies, Vancouver, Canada, http://www.stemcell.com) and MACS affinity columns (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). HUCB CD8+ cells were selected using CD8 MicroBeads (Miltenyi Biotec) according to the manufacturer’s instructions. When necessary, bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) was replaced by human serum albumin (HSA) (Albunin SPR, Sanquin, Amsterdam, The Netherlands, http://www.sanquin.nl) for animal reagent-free isolation. The non-enriched cell pools (CD34-, CD133-, Lin-, CD8-) from the same units were also collected and used as control cells. Commercially available frozen HUCB MNC and human BM MNC (Cambrex, Walkersville, MD, http://www.cambrex.com) were also used and thawed according to the supplier’s instructions.

Human embryonic kidney 293 (HEK-293) (ATCC) and human hepatoma HuH-7 cells were cultured in Dulbecco’s modified Eagle’s and RPMI 1640 media, respectively, supplemented with heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. HUCB-derived MSC line 177A was derived and cultured as described [26]. Human adult BM-derived MSCs (lines 85 and 105) were derived and cultured in standard cell culture medium (α-MEM) supplemented with FCS as described [27]. All cells were cultured in a humidified 5% CO₂ atmosphere at 37°C.

**RNA Isolation, Reverse Transcription Polymerase Chain Reaction, and Quantitative Real-Time RT-PCR**

Total RNA was isolated by using the RNasy mini kit (Qiagen, Hilden, Germany, http://www1.qiagen.com). HeLa cell total RNA was purchased from Clontech (Palo Alto, CA, http://www.clontech.com). For RT-PCR, 5 μg of total RNA was reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). A RT-control sample omitting the reverse transcriptase was prepared for each sample and water served as no template controls. The reverse transcription polymerase chain reaction (RT-PCR) was performed in a 25-μl final volume composed of 0.5-1 units of DyNAzyme EXT DNA polymerase (Finnzymes, Espoo, Finland, http://www.finnzymes.com), one μl of DyNAzyme EXT Buffer (with 15 mM MgCl₂), 0.2 mM dNTPs, 0.2 μM of each primer, 2% DMSO, and 1.0-2.0 μl template amplification. These conditions were used for 25-45 cycles. The PCR products were analyzed by agarose gel electrophoresis. Quantitative real-time RT-PCR (qRT-PCR) of CMAH expression was performed using TaqMan reagents and the thermal cycling conditions described earlier [24]. Standard curves for expression of detected genes were generated by serial dilution of cDNA from HUCB MNCs. The relative mRNA expression level of CMAH was normalized against the level of GAPDH expression in the same RNA preparation. TaqMan gene primer and probe mixture for CMAH and GAPDH were Hs00186003_m1 and Hs99999905_m1, respectively (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). Changes in fluorescence were monitored using the ABI PRISM 7,900 Sequence Detection System, and raw data were analyzed with Sequence Detection System 1.1 Software (Applied Biosystems) [24].

**Cloning and Sequence Analysis of Human CMAH cDNA**

Full-length CMAH cDNA was cloned from HUCB MNC (cbCMAH) and HeLa cells (wtCMAH). Cloning details are...
presented in Supplement 2 with the supporting information. The purified PCR products were ligated into pGEM-T easy vector (Promega, Madison, WI, http://www.promega.com). Several clones from each ligation were sequenced. Sequencing was accomplished using automated sequencing (ABI Prism Automated Fluorescence Sequencer) with plasmid- and gene-specific primers. Sequence assembly was accomplished using programs available at www.csc.fi. Sequence alignments, phylogenetic trees, and homology comparisons were done with ClustalW and BLAST programs.

**Fusion Protein Gene Constructs**

Fusion protein constructs with either an N-terminal green fluorescent protein (GFP)- or FLAG-tag were prepared for cbCMAH, wtCMAH, and mouse-specific CMAH (mCMAH) by subcloning into a modified version of (with necessary frameshift) pFLAG-CMV-2 (Sigma Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) and pEGFP-C2/pEGFP-C1 (Clontech). Specific subcloning details are presented in Supplement 3 with the supporting information. In the final constructs, the FLAG- and GFP-tags are located immediately upstream of the CMAH coding regions, thus producing N-terminally tagged proteins. The sequence and correct reading frame for all constructs were verified by automated sequencing.

**Transient Transfections**

For transfections, 3–7 × 10^5 HEK-293 cells were plated per well in six-well plates (Nunc, Rochester, NY, http://www.nuncbrand.com) 24 hours before transfection. Five µg of plasmid was transiently transfected per well with standard calcium phosphate transfection [28]. For immunocytochemistry, the cells were grown on glass cover slips coated with 5 ng/ml fibronectin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). The cells were used 48 hours after transfection.

**Sialic Acid Stimulations and Uptake of Biotinylated Sialic Acids**

Cells used for sialic acid stimulations were first washed twice in warm phosphate buffered solution (PBS) and then stimulated with 3 mM synthetic Neu5Ac (Calbiochem, San Diego, CA, http://www.emdbiosciences.com) and Neu5Gc (R&S PharmChem Co., Hangzhou, Zhejiang, China, http://www.rspharmchem.com/) monosaccharides in serum-free cell culture media in a humidified 5% CO₂ atmosphere at 37°C. The pH of the media was controlled by observing the color (pH indicator) of the media. Buffering capacity was concluded to be sufficient since the pH of the media was normalized shortly after sialic acid additions. Incorporation of sialic acids was studied by using 5 µg biotinylated polyacrylamide-conjugated Neu5Ac and Neu5Gc (Lectinity Inc, Moscow, Russia, http://www.lectinity.com/) per 10 µl serum-free cell culture media supplemented with 1% HSA (Albumin SPR). Human BM MSCs were cultured in Neu5Gc-free environment 1 to 3 weeks before the experiments as described [23]. The experiments were stopped by removing the cells on ice, quickly removing the media, and washing once with cold PBS before either lysing or fixing the cells.

**Immunocytochemistry and Microscopy**

The cells were washed once in PBS and fixed with 4% PFA for 20 minutes at room temperature and washed with PBS. When necessary, the cells were permeabilized with 0.25% Triton X-100 in PBS for 5 minutes at room temperature and washed with PBS. Blocking of unspecific binding was accomplished with 2 to 4% normal goat serum (NGS), 10% BSA, or 10% HSA in PBS for 30 minutes at room temperature or +37°C. After blocking, the cells were incubated with the primary antibody diluted in 2% NGS or 3% BSA/HSA for 1 hour at room temperature and washed with PBS. Conjugated secondary antibodies were applied to the cells for 1 hour at room temperature protected from direct light. Cover slips were mounted with Vectorshield mounting medium (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) with or without DAPI. The slides were analyzed with a Zeiss Axioskop two plus fluorescence microscope and a Leica TCS SP2 confocal microscope with a Ar-Kr laser. Images were taken at 0.5 to 15 µm intervals with the confocal microscope. The images were processed with Leica LCS Lite software. The final microscope figures were produced with Adobe Photoshop 5.0 and CoreIDRAW 12 software.

**Cell Extracts, Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE), and Western Blotting**

Whole-cell extracts were prepared by lysing the cells in cold PBS with 1% Triton X-100 and 1 µg/ml aprotinin, 1 mM vanadate and 1 mM Pefablock (Roche Applied Science, Basel, Switzerland, http://www.roche-applied-science.com). The homogenate was centrifuged at 16,000 × g for 10 minutes at +4°C. The supernatant was collected and quickly frozen. Subcellular fractions of proteomic samples were prepared with the Qproteome cell compartment kit (Qiagen, Hilden, Germany, http://www1.qiagen.com/) according to the manufacturer’s instructions. The cellular fraction one (1) contain most cytosolic proteins, fraction two (2) membrane proteins (plasma membrane, proteins from membranes of organelles), fraction three (3) all soluble and membrane bound nuclear proteins and fraction four (4) all residual, mainly cytoskeletal proteins. The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com) for both isolation protocols. 4–20 µg of protein was denatured by boiling for 10 minutes in Laemmli sample buffer, then loaded and run on a 10% SDS-polyacrylamide gel. The gel was transferred to nitrocellulose membrane and blocked in 5% milk in Na-phosphate buffered saline (Na-phosphate buffered saline (Na-phosphate 11.3 mM, NaCl 0.14 M) with 0.05% Tween-20 overnight at +4°C and then incubated in the primary antibody for 1 to 2 hours at room temperature. After washes, the membrane was incubated in HRP-conjugated secondary antibodies for 1 hour at room temperature. The immunoblot was developed with an enhanced chemiluminescence method (ECL, Amersham Biosciences, Piscataway, NJ, http://www.amersham.com).

**Mass Spectrometry**

The cellular content of the xenocarbohydrate N-glycolyneuraminic acid (Neu5Gc) was analyzed by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) as described in Supplement 4 with the supporting information.

## Results

### Expression of CMAH mRNA in Adult Human Stem Cells

To determine whether the increased CMAH mRNA content we had previously discovered in HUCB [11,33, mCMAH [24] would be common to other adult human stem cells, qRT-PCR was performed. The qRT-PCR analysis revealed a clear overexpression of CMAH mRNA in HUCB HSC populations compared with unsellected MNCs (Fig. 1A). CMAH mRNA expression levels were 9.5-, 5.9-, and fivefold higher in HUCB CD133+, CD34+, and Lin- HSCs compared with unsellected MNCs, respectively. To get a broader overview of CMAH mRNA expression, a cDNA panel was prepared from a variety of adult human stem cells. The results are presented in Figure 1B. Full-length CMAH cDNA could successfully be amplified from HUCB MNCs (2 units tested), BM MNCs, HUCB HSCs (CD133+ and Lin- cells), and from MSCs of both BM (BM MSC) and HUCB (HUCB MSC 177A). The non-stem cell samples, human embryonic kidney 293 cells (HEK-293) and human hematopoietic HuH-7 cells, expressed CMAH mRNA in only trace amounts (Fig. 1A, 1B). The CMAH mRNA

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expression profile was strikingly stem cell-specific, and both human HSCs and MSCs exhibited a clear upregulated CMAH mRNA expression in human stem cell sample. We thus had to utilize a subcloning strategy concerning human CMAH cDNA, the cbCMAH cDNA was also miss-spliced, and thus a truncated N-terminus of the cbCMAH protein is produced as compared with mouse and monkey. The cbCMAH clone is 100% homologous to the deduced open-reading frame of cloned human lymphoblastoid CMAH cDNA, here named wt2CMAH, accession number AF074480 [11]. Surprisingly, the cbCMAH is somewhat different from the other previously published human HeLa cell CMAH cDNA, here named wtCMAH [12], with 98% protein homology. A 200 bp deletion in the region encoding the C-terminus of the protein produces a frame-shift yielding a 20 aa longer C-terminus in the cbCMAH as compared with the predicted protein from human HeLa cell CMAH cDNA [12] (Fig. 2B). ClustalW alignment with other mammalian CMAH proteins revealed the cbCMAH CMAH protein to have a homologous C-terminus with monkey CMAH proteins (data not shown) and an overall protein homology of 99%, 98%, and 86% to Pan troglodytes, Gorilla gorilla, and Mus musculus CMAH, respectively (Fig. 2A). An in silico predicted CMAH protein from the human genome sequence, here named GenomeCMAH, is 99% homologous to cbCMAH. Another in silico predicted CMAH protein (accession number CAI20561) was found in the GenBank with 99% sequence homology to cbCMAH.

Surprisingly, we were not able to clone the full-length CMAH cDNA from HeLa cells (wtCMAH) as described [12]. All HeLa cell CMAH clones sharing identical 3' end sequence to the CMAH cDNA contained one or several introns more upstream [12]. The HeLa CMAH clones are most likely immature, unspliced CMAH prem-RNA transcripts. We thus had to use a subcloning strategy with modified primers to produce a fusion protein construct of the wtCMAH variant. The 5' end of the cbCMAH cDNA nucleotide sequence was analyzed carefully for the presence of the 92 bp sequence corresponding to exon six of the mouse and monkey Cmah genes [11, 12], the crucial region for the enzymatic activity for the resulting protein. Consistent with previous observations concerning human CMAH cDNA, the cbCMAH cDNA was also missing this fragment (Fig. 2C), and thus a truncated N-terminus of the protein is produced as compared with mouse and monkey. The cloned human cbCMAH cannot thus encode an enzymatically active protein. The 5' end sequence and translation in all three frames is presented in Figure 2C.

**Cellular Localization of the CMAH Fusion Proteins**

To determine the subcellular localization of CMAH in more detail, an overexpression strategy with CMAH fusion protein constructs was utilized. This was also needed for immunocytochemistry, since the commercially available polyclonal CMAH antibody would not be applicable. CMAH fusion protein constructs were transiently transfected in HEK-293 cells almost devoid of endogenous CMAH mRNA expression (Fig. 1A). Transfection efficiency was estimated to be 10 to 20% (data not shown). Western blot analysis confirmed that tagged CMAH fusion proteins of expected sizes were produced (Fig. 3A). The cellular distribution of the CMAH fusion proteins was studied further by preparing subcellular fractions of transfected cells. Surprisingly, the CMAH fusion proteins were mainly localized in a fraction containing essentially cytoskeletal proteins (Fig. 3B, left panel). CMAH fusion proteins were also localized in the fractions containing cytosolic and membrane proteins. Minor amounts of CMAH fusion proteins were detected in the fraction containing nuclear proteins. To compare the authenticity of the cbCMAH fusion protein with human endogenous CMAH, cell lysates were also blotted...
with the polyclonal CMAH antibody (Fig. 3B, right panel). A similar blotting pattern is detected with pFLAGcbCMAH transfected cells as with adult human stem cell lysates (Fig. 1C), and there is substantially more of the major band detected in the pFLAGcbCMAH transfected cells than in the untransfected control cell lysates (Fig. 3B, right panel). The cellular distribution of the CMAH fusion proteins was also studied with immunocytochemistry. Tag-specific immunostaining was not detected in nonpermeabilized transfected cells. In permeabilized cells, the CMAH fusion proteins localize mainly in the cytoplasm in small droplet-like structures (Fig. 3C, 3D). Occasionally the tagged CMAH fusion proteins were found to aggregate in large cytoplasmic clusters (arrow in Fig. 3D). The CMAH fusion proteins were not seen to be colocalized with the endoplasmic reticulum specific protein calnexin, the Golgi apparatus cisternae specific protein GOLGA1, or the peroxisome membrane protein PMP70 (data not shown). However, a partial colocalization was seen with the lysosome-associated membrane protein LAMP1 (Fig. 4). Taken together, the results from immunocytochemistry and Western blot analysis indicate that the CMAH fusion proteins localize in cytoplasmic droplet-like structures distributed evenly and outside the endoplasmic reticulum, Golgi, and peroxisomes. No major differences were seen in the cellular distribution between the different CMAH proteins (cbCMAH versus wtCMAH versus mCMAH). Some CMAH fusion proteins seem to be localized in membranes and nucleus, but in very small amounts.

Potential Link Between Human CMAH Expression and Neu5Gc Uptake

To determine whether the increased CMAH expression would have an effect on Neu5Gc binding and uptake into the cells, transiently transfected HEK-293 cells overexpressing cbCMAH were exposed to biotinylated Neu5Gc for short times. Uptake of polyacrylamide (PAA) biotinylated Neu5Gc conjugate was evident already after a 5-minute exposure as visualized by avidin immunostaining (Fig. 5A, 5B). We could, however, see no difference in uptake efficiency between untransfected HEK-293 cells and cells overexpressing cbCMAH in this system (Fig. 5B, arrowheads). The rapid uptake mechanism of biotinylated Neu5Gc-PAA was verified in human BM MSCs cultured in Neu5Gc-free environment [23]. BM MSCs efficiently and quickly ingested biotinylated Neu5Gc-PAA (Fig. 5C, 5D). Identical results were seen with biotinylated Neu5Ac-PAA (data not shown). Several strategies were also undertaken to investigate a possible physical interaction of CMAH with Neu5Gc, but none of the methods could confirm a direct interaction between CMAH and Neu5Gc (data not shown).

Effect of CMAH Expression and Neu5Gc Exposure on Wnt/β-Catenin Signaling

Since the CMAH gene exhibited an evident human adult stem cell-specific expression pattern, we pursued our investigations further to reveal potential novel roles for CMAH in stem cell-
The level of phosphorylated Akt remained unaltered, and α-tubulin immunoblotting verified even amounts of loaded protein.

To verify the results of the overexpression studies in adult stem cells, human BM MSCs were cultured for 3 weeks in a Neu5Gc-free environment and stimulated with either Neu5Gc or Neu5Ac. A dynamic phosphorylation of β-catenin was observed after short Neu5Gc stimulation with dephosphorylation to control levels at 30 minutes (Fig. 6C). Phosphorylation of β-catenin was also observed after Neu5Ac stimulation, but in a more constitutive manner. The levels of total β-catenin remained generally unaltered.

**Mass Spectrometric Analysis of Stem Cell Neu5Gc Glycans**

To verify the lack of enzymatic activity of stem cell CMAH and resulting endogenous Neu5Gc production, MALDI-TOF mass spectrometry was used to detect sialylated N-glycan structures. The identification of Neu5Gc was based on detection of sialic acid residues with 16 Da larger mass addtion than the human-type sialic acid, Neu5Ac. The Neu5Gc indicator signals m/z 1,946 and 2,237 were present in human CD133+ cells, but were significantly lower in the corresponding CD133- control leucocyte population (Fig. 7A, 7B). Since the CD133+ isolation protocol requires buffers containing 0.5% BSA, a known binder of glycosylated components and therefore also a Neu5Gc contamination source, we also analyzed CD133+ cells isolated with animal material controlled reagents by substituting BSA with 0.5% HSA. The Neu5Gc indicator signals m/z 1,946 and 2,237 were absent in the CD133+ cells isolated in buffers containing HSA (Fig. 7D) as compared with the BSA isolated CD133+ cells (Fig. 7C). These findings further support that 1) human stem cell CMAH is enzymatically inactive, 2) Neu5Gc cannot be produced endogenously, but 3) it can be incorporated from exogenous sources. In this case, the exogenous sources of Neu5Gc glycans are present during the isolation process for approximately 1 hour, indicating rapid Neu5Gc glycan binding mechanisms, most likely also resulting in cellular uptake. We also wanted to rule out the magnetic beads as possible binders or sources of exogenous Neu5Gc glycans, and therefore analyzed HUCB CD8+ and CD8- T-cells isolated with a similar magnetic bead strategy with buffer containing 0.5% BSA. The Neu5Gc indicator signals m/z 1,946 and 2,237 were absent in both cell populations (Fig. 7E, 7F). The magnetic beads are therefore unlikely sources of exogenous Neu5Gc. The absence of the studied Neu5Gc indicator signals in the CD8+ T-cells and CD8- cells (Fig. 7 E, 7F), as compared with the clear indicator signals found in the isolated CD133+ HSCs (Fig. 7A, 7C), further support a rapid and stem cell-specific binding of extracellular Neu5Gc glycans.

**DISCUSSION**

There is currently firm evidence demonstrating that CMAH is the sole enzyme responsible for the intracellular production of Neu5Gc [21, 29]. Thus, it is now generally accepted that the Neu5Gc present in human cells originates from mainly extracellular sources. However, other intracellular routes for the biosynthesis of Neu5Gc in human tissues are currently under discussion, and N-glycolylglucosamine-6-phosphate, derived from glycolyl-CoA by the action of glucosamine-6-phosphate-N-acetyltransferase, is suggested to be a potential precursor of Neu5Gc [30]. It has also been established that the human Cmah gene mutation makes the encoded enzyme catalytically inactive [11, 12]. We initially found a clear stem cell-linked transcriptional upregulation of the CMAH gene, a human gene thought to be nonfunctional. Since the cloned human stem cell-specific

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**Figure 3.** CMP-N-acetylneuraminic acid hydroxylase (CMAH) fusion protein production in HEK-293 cells. (A): Western blot analysis of CMAH fusion protein overexpression on transfected HEK-293 cells using a monoclonal mouse anti-FLAG M2 antibody. (B): Left panel: Western blot analysis of the subcellular distribution of FLAG-fused human cord blood-specific CMAH (pFLAGcbCMAH) fusion protein. Right panel: Western blot analysis of cytosolic cell lysates of untransfected and pFLAGcbCMAH-transfected HEK-293 cells using a polyclonal CMAH antibody. (C): Representative microscopic pictures (40× objective) of HEK-293 cells transfected with green fluorescent protein (GFP)-fused human cord blood-specific CMAH (pEGFPcbCMAH) and (D) mouse CMAH (pEGFPMmCMAH). Arrow indicates large cytoplasmic clusters of fusion protein. Abbreviations: CMAH, CMP-N-acetylneuraminic acid hydroxylase; cb, human cord blood-specific; GFP, green fluorescent protein; m, mouse.

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**Figure 4.** Western blot analysis of cytosolic cell lysates of untransfected and pFLAGcbCMAH-transfected HEK-293 cells using a polyclonal CMAH antibody. (A): Representative microscopic pictures (40× objective) of HEK-293 cells transfected with green fluorescent protein (GFP)-fused human cord blood-specific CMAH (pEGFPcbCMAH) and (D) mouse CMAH (pEGFPMmCMAH). Arrow indicates large cytoplasmic clusters of fusion protein. Abbreviations: CMAH, CMP-N-acetylneuraminic acid hydroxylase; cb, human cord blood-specific; GFP, green fluorescent protein; m, mouse.
CMAH. cbCMAH cDNA was identical on a nucleotide level to one of the cloned human CMAH cDNAs described earlier to encode the enzymatically inactive protein [11], the enzymatic activity of cbCMAH was not studied. Furthermore, mass spectrometric analysis demonstrated that human stem cells are devoid of any endogenous synthesis of Neu5Gc, consistent with the enzymatic inactivity of stem cell-specific CMAH (Fig. 7D). Based on our initial results, we found that the interesting questions were rather to investigate why a human gene thought to be nonfunctional would be transcriptionally regulated in stem cells; why and how stem cells incorporate Neu5Gc from the extracellular milieu; how CMAH, with its clear stem cell-linked overexpression, could be linked to the higher Neu5Gc contents in the same cell populations; and, finally, if xenoantigen Neu5Gc exposure, present in large quantities in animal-derived reagents, could affect important signaling systems in stem cells affecting self renewal and stemness.

We found a clear correlation between CMAH mRNA expression and human multipotent stem cells. We could also verify that the stem cell-specific CMAH mRNA is translated to protein and that endogenous CMAH protein levels are higher in adult human stem cells. On the contrary to what has previously been thought, this indicates that human CMAH can be functional and can possess novel roles not involving hydroxylase.

**Figure 4.** Colocalization of CMP-N-acetylneuraminic acid hydroxylase (CMAH) with lysosomal protein LAMP-1 studied with confocal microscopy (40× objective). (A): HEK-293 cell control LAMP-1 staining of untransfected cells. (B): Human cord blood-specific CMAH (pFLAGcbCMAH) transfected HEK-293 cells stained with monoclonal anti-FLAG M2 antibody (red) and anti-LAMP-1 antibody (green). (C): Human HeLa cell-specific CMAH (pFLAGwtCMAH) with truncated C-terminus transfected HEK-293 cells stained with monoclonal anti-FLAG M2 antibody (red) and anti-LAMP-1 antibody (green). A partial colocalization to lysosomes can be seen with both human CMAH fusion protein variants. Abbreviations: CMAH, CMP-N-acetylneuraminic acid hydroxylase; cb, human cord blood-specific; wt, human HeLa cell-specific.

**Figure 5.** Cellular uptake of exogenous biotinylated (red fluorescence, Avidin Texas Red staining) polyvalent polyacrylamide-conjugated (PAA) Neu5Gc. (A): pFLAGcbCMAH transfected HEK-293 cells, control situation (B): pFLAGcbCMAH transfected HEK-293 cells, 5 minute biotin-PAA-Neu5Gc exposure. Ingested biotinylated Neu5Gc is evident in both CMP-N-acetylneuraminic acid hydroxylase (CMAH) overexpressing cells and control cells (arrowheads) after short exposure. Bone marrow-derived mesenchymal stem cells (BM MSC 85) cultured in human AB Rh- serum for 1 week before biotin-PAA-Neu5Gc exposure for 5 minutes (C) and 60 minutes (D). Representative microscopic pictures were taken with 40× objective. Abbreviations: Neu5Gc, N-glycolyneuraminic acid; cb, human cord blood-specific; PAA, polyvalent polyacrylamide; CMAH, CMP-N-acetylneuraminic acid hydroxylase; BM MSC, bone marrow-derived mesenchymal stem cells.
able to find any physical interaction between human CMAH and Neu5Gc in our studies, but possibilities remain for indirect mechanisms by, for example, an unknown intermediate player affected by CMAH levels and capable of binding Neu5Gc. Interestingly, the levels of Neu5Gc and CMAH hydroxylase activity were not always in line when simultaneously studied during different developmental stages in the pig small intestine [31], which also might indicate other functions for the CMAH protein.

The subcellular localization of the overexpressed CMAH proteins correlate well with previous studies, where mouse CMAH has been concluded to localize mainly to the cytoplasm in a small membrane-associated fraction [33]. Conflicting results were seen in the Neu5Gc uptake experiments, since not only the CMAH overexpressing cells were able to ingest biotinylated Neu5Gc attached to a polyacrylamide carrier (Fig. 5A, 5B). It should be pointed out that the used biotinylated polyvalent polyacrylamide-conjugated sialic acids do not represent the natural forms of either sialic acid and might not reflect actual mechanisms for sialic acid binding and uptake in human cells.

Wnt signaling is established to be crucial in stem cells for the decisions of stemness, proliferation, and differentiation [34–39], but activation of Wnt signaling has also been associated with cancer [40, 41]. The Wnts comprise a large family of highly conserved growth factors, and their intracellular signaling pathways are mostly dependent on the canonical, β-catenin-dependent pathways, although β-catenin independent pathways also exist [42]. The defining event in canonical Wnt signaling is the cytoplasmic accumulation of β-catenin and its subsequent nuclear translocation and activity. In this study we introduce an evident activation of the Wnt/β-catenin signaling system when overexpressing human CMAH in human non-stem cells. Overexpression of human stem cell-specific CMAH resulted in accumulated nuclear β-catenin levels and correspondingly decreased levels of phosphorylated β-catenin. Interestingly, we observed that exogenous Neu5Gc exposure caused a rapid phosphorylation of β-catenin in both cbCMAH overexpressing cells and in BM MSCs (Fig. 6B, 6C). This effect, although transient, still might quickly translate the Wnt inhibition signal further to the nucleus and have effects on the self-renewal capacity of the cells. The effect of exogenous Neu5Gc monosaccharide on β-catenin signaling represents a novel pathway of regulation of cellular signaling by a monosaccharide. Although the mechanism remains to be elucidated, similar phenomena have been reported previously. Effects on β-catenin expression and neuronal differentiation have been observed when human ESCs are fed with the sialic acid precursor analog (Ac5)ManNTGc, whereas the thioglycolyl (TGC) structure resembles the glycolyl group in Neu5Gc [43]. Recently, alkyl analogs of ManNAC have been shown to have a strong effect on the differentiation of PC12 cells into neurons, including phosphorylation and nuclear localization of erk1/2 [44]. Earlier observations together with the present data indicate that sialic acids can regulate cellular signaling in an unexpected manner.

**CONCLUSIONS**

We have previously shown that stem cells possess characteristic glycosylation patterns giving rise to unique glycome profiles, which can be used to identify stem cells in different differentiation stages [45, 46]. In this study we present additional data on the significance of glycosylation in stem cells. We show that the human supposedly nonfunctional gene and protein CMAH is upregulated in human adult stem cells, in which a fast binding of exogenous Neu5Gc also is seen. We show that exposure of stem cells to Neu5Gc, already known to be present in large...
amounts in animal-derived cell culture reagents such as FCS, might affect the stemness of stem cells. These results pinpoint the crucial need for totally xenofree culturing and expansion conditions for human therapeutic stem cells.

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**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

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