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NARRATIVE REVIEW

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Mechanism and function of CEACAM1 splice isoforms

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Abstract

Background: Alternative splicing is a fundamental mechanism in the post-transcriptional regulation of genes. The multifunctional transmembrane glycoprotein receptor carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) undergoes extensive alternative splicing to allow for tunable functions in cell signalling, adhesion and modulation of immune and metabolic responses. Splice isoforms that differ in their ectodomain and short or long cytoplasmic tail (CEACAM1-S/CEACAM1-L) have distinct functional roles. The mechanisms that regulate CEACAM1 RNA splicing remain elusive.

Methods: This narrative review summarizes the current knowledge of the mechanism and function of CEACAM1 splice isoforms. Historical perspectives address the biological significance of the glycosylated Ig domains, the variable exon 7, and phosphorylation events that dictate its signal transduction pathways. The use of small antisense molecules to target mis-spliced variable exon 7 is discussed.

Results: The Ig variable-like N domain mediates cell adhesion and immune checkpoint inhibitory functions. Gly and Tyr residues in the transmembrane (TM) domain are essential for dimerization. Calmodulin, Calcium/Calmodulin-dependent protein kinase II delta (CamK2D), Actin and Annexin A2 are binding partners of CEACAM1-S. Homology studies of the muCEACAM1-S and huCEACAM1-S TM predict differences in their signal transduction pathways. Hypoxia-inducible factor 1-α (HIF-1-α) induces alternative splicing to produce CEACAM1-S under limited oxygen conditions. Antisense small molecules directed to exon 7 may correct faulty expression of the short and long cytoplasmic tail splicing isoforms.

Conclusion: More pre-clinical and clinical studies are needed to elucidate the precise mechanisms by which CEACAM1 RNA splicing may be exploited to develop targeted interventions towards novel therapeutic strategies.

KEYWORDS

alternative splicing, CEACAM1, exons, inflammation, ischemia-reperfusion injury, phosphorodiamidate morpholinos

1 | INTRODUCTION

Carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1) is a multifunctional transmembrane glycoprotein that is widely expressed in different cell types like epithelial, vascular, immune and cancer cells. As a member of the immunoglobulin (IgG) superfamily, CEACAM1 plays an essential role in physiological activities in normal cells. It exists in soluble form in body fluids[1] and as a single-pass transmembrane protein, in which the extracellular domains confer cis or trans homotypic interactions at or between cell surfaces, with two divergent cytoplasmic domains that confer intracellular signalling.

CEACAM1 belongs to the carcinoembryonic antigen (CEA) gene family, which includes 28 genes/pseudogenes clustered on human chromosome 19q13.2. It is considered the prototype owing to the high degree of conservation across various species, for example, rat, mouse and human.[2] Its gene structure comprises 9 distinct exons that undergo extensive alternative splicing to generate variable ectodomain and cytoplasmic isoforms of CEACAM1 (Figure 1). The signalling function of CEACAM1 is determined by exon skipping/insertion of exon 7 in its cytoplasmic domain. By convention, the number of ectodomains and the presence or absence of the variable exon 7, responsible for generating the short or long cytoplasmic tail isoform (CEACAM1-S/CEACAM1-L), form the naming basis of the various spliced isoforms (Figure 2).

The history of CEACAM1 is complex, with cumulative evidence from pre-clinical as well as clinical data showing dual functions as both a promoter of cancer progression with poor prognosis[3] and a potential tumour suppressor.[4] During further decades of study, other functions have been attributed to splice isoforms of CEACAM1, including metastasis formation in melanoma,[5] functions in cytoskeleton rearrangement,[6] migration,[7] angiogenesis,[8] inflammation[9] and metabolism.[10] Despite this wealth of knowledge about possible functions of CEACAM1-L, much less is understood about CEACAM1-S given its lack of sequence conservation and phosphorylation between mouse and man.

A thorough understanding of the mechanisms that generate CEACAM1 splice isoforms may eventually help to explain these functional differences. In this review, we aim to highlight the current knowledge of CEACAM1 splice isoforms, exploring the regulatory mechanisms that control their expression. New avenues of therapeutic research offer the potential to influence the splicing of human CEACAM1 and improve health outcomes. Decorticating the control of CEACAM1 will better help to understand how it fine-tunes cellular responses and maintains homeostasis in biological systems.

2 | HISTORICAL PERSPECTIVE OF RNA SPLICING

To fully appreciate the history of CEACAM1 alternative splicing, it is important to remember the seismic shifts that occurred in the field of molecular biology during the 1950s–1970s. The work of the 1965 recipient of the

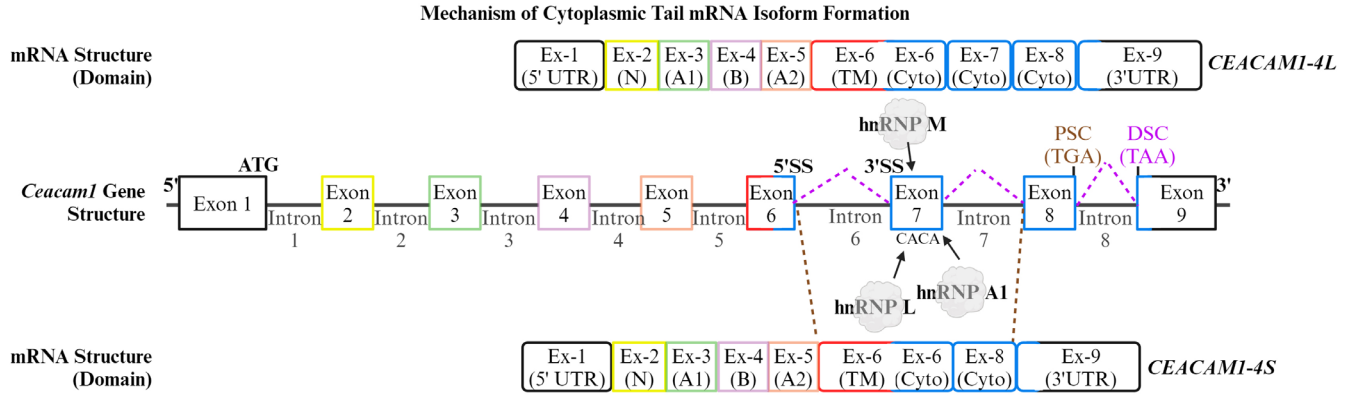

FIGURE 1 Schematic representation of human *CEACAM1* gene structure colour-coded at the RNA and domain. CEACAM1 mRNA is comprised of 9 exons (open boxes) and 8 introns (lines). The start codon is denoted by the ATG sequence, and the proximal and distal stop codons are denoted by PSC and DSC, respectively. Alternative splicing of exon 7 to generate CEACAM1-L mRNA is dependent on the interaction of hnRNP M at the 5′ end of exon 7.[27] By contrast, formation of CEACAM1-S mRNA depends on the interaction of hnRNP L with a centrally located CACA domain and hnRNP A1 at the distal end of exon 7. The use of the PSC generates CEACAM1-S (brown), whereas utilization of the DSC (purple) generates the CEACAM1-L mRNA. Dashed lines represent alternative inclusion or exclusion of exon 7 to generate the two cytoplasmic variants. The upstream exon/intron boundary contains the 5'SS (splice site) and 3'SS. N, A1, B and A2 refer to the Ig domains,[2] while Ex- is exon, UTR is untranslated region, TM is the transmembrane domain, and Cyto refers to the cytoplasmic domain. Created with Biorender.com.

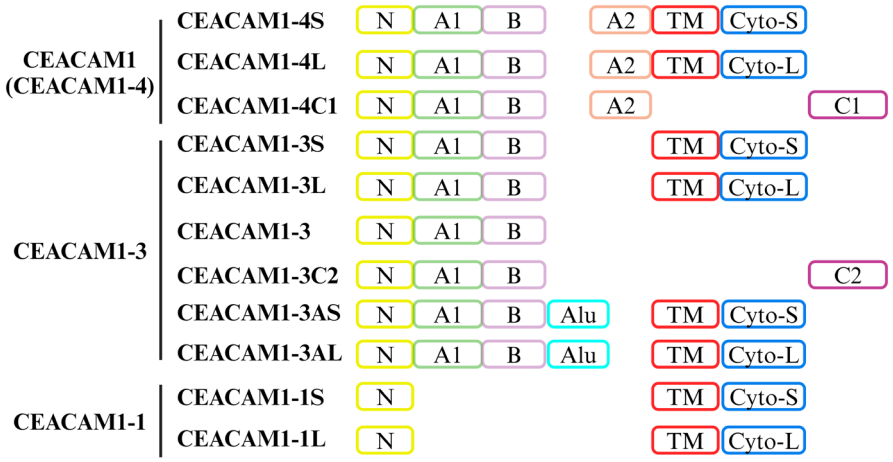

FIGURE 2 Splice variants of human CEACAM1. The number of extracellular Ig domains and length of the cytoplasmic C-termini (Cyto), short (S) or long (L), establishes the name of the various human CEACAM1 splice isoforms. N, A1, B and A2 refer to the Ig domains and TM the transmembrane domain. C1–C2 indicates different termini generated by alternative splicing and Alu refers to the inclusion of in frame Alu sequences found within the coding region. Created with Biorender.com.

Nobel Prize in Physiology or Medicine, French biochemist Jacques Monod,[11] and others,[12] established that a stretch of DNA codes for a complex set of regulatory sections that influence the precise control of gene expression to maintain proper cellular function. The prevailing view at that time, supported undoubtly by Monod's famous assertion that anything true for *E. coli* must also be true for elephants,[13,14] was that all organisms contained the same gene structure as bacteria.

It was not until the spring of 1977; however, that electron microscopic analysis provided evidence that our understanding of the central dogma of biology (one gene->one protein) was misguided. Photomicrographs showed the first evidence that adenovirus mRNAs were undergoing RNA splicing events, and in fact, evidence of 15 different mRNAs (one per primary transcript) emerging from a single large adenovirus pre-mRNA portended the future of differentially regulated splicing studies.[15–18] In quick succession, evidence for other animal virus RNAs and cellular RNAs emerged, culminating in the discovery of the first genomic sequence undergoing RNA splicing for the mouse β-globin gene.[19] In the decades that followed, intense interest focused on the surprising RNA chemistry involved in splicing (transesterification mediated by RNA:RNA interactions),[20] and on the assembly of the mega-Dalton RNA:protein spliceosome complex.[20,21] With the aid of various small nuclear RNAs and processing factors, the spliceosome was demonstrated to assemble in a dynamic stepwise process that ultimately served to link exons in mRNAs.[22] Five recognized processes take place during gene expression: (1) capping, where the 5′ triphosphate of the pre-messenger RNA (pre-mRNA) is cleaved and a guanosine monophosphate is added and methylated to produce m7GpppN; (2) editing, where individual RNA residues are converted to alternative bases, such as adenosine to inosine, resulting in mRNAs that encode different protein products; (3) splicing, where intervening sequences are removed and exons are ligated together by the spliceosome; (4) 3′ end formation, which includes pre-mRNA synthesis and cleavage of the poly(A) tail; and (5) degradation.[23]

3 | HISTORICAL PERSPECTIVE OF CEACAM1 CYTOPLASMIC SPLICING VARIANTS

Following the discovery that novel variants of CEACAM1 [then known as biliary glycoproteins-BGP] were produced by alternative splicing,[24] the CEACAM1-L isoform was shown to undergo homo- and heterotypic cell adhesion activities in a Ca(++)-independent mechanism.[25] Meanwhile, an unknown function in signal transduction was attributed to serine, threonine and tyrosine residue phosphorylation in the cytoplasmic tail of CEACAM1.[26] As confusion persisted over the function of CEACAM1, reflected by different names attributed to the protein which were later unified under a common system for the splice variants in different species,[2] missing was the evidence of how the primary transcript of *CEACAM1-S* differed from *CEACAM1-L*. Understanding how the variable exon 7 was regulated would not come for two more decades,[27] but the discovery itself of exon 7 formed the lynchpin in shedding new light on how to explain the functional differences in the cytoplasmic isoforms.[28] The unlikely story of how S.M. Najjar, a postdoctoral researcher at the National Institutes of Health (NIH) in the early 1990's made the

pivotal discovery that propelled the RNA CEACAM1 field forward is noteworthy and replicated here by permission.

Najjar recalled, 'I propose that you clone the cDNA of pp120/HA4 [later called CEACAM1], stably transfect it, and test if it is a substrate of the insulin receptor tyrosine kinase,' Simeon I. Taylor told me in one of our first meetings after I joined his lab in the Diabetes Branch. 'But you have published several papers on the issue!', I responded. 'Yes, but it remains a controversial issue'. Najjar continued, 'I am not sure if I was flattered to learn that he had full confidence that I could resolve this 'controversial issue,' even though at that time, my knowledge in molecular biology was restricted to the difference between transcription and translation.'

A year later, Najjar recounts, 'I had cloned the cDNA, carried out manual sequencing, stably transfected NIH 3T3 cells, performed in vivo phosphorylation using 1mCi of γ-P^{32}ATP/plate. I observed no phosphorylation by the co-transfected insulin receptor in response to insulin. My jaw dropped. Senior scientists who had worked around the controversy met around my almost-perfect gel. We made the disappointing conclusion that pp120 is not phosphorylated by the insulin receptor.'

'A few days later, as I was having dinner with friends from Ronald G. Crystal's lab at the NIH, I learned about the cystic fibrosis transmembrane conductance regulator (CFTR) gene being alternatively spliced. I rushed through a literature search. That is when I discovered the world of "alternatively spliced genes". Going over my earlier manual sequencing showed that some of the clones had skipped a 53 bp-sequence only to match the rest of the sequence of the other clones. I had used one of these "skipped ones" [the CEACAM1-S] in my stable transfection.' 'You would need to learn how to do genomic DNA cloning to test the hypothesis,' Simeon told me as I rushed to his office proposing the hypothesis that the pp120 gene is alternatively spliced. Najjar then described her efforts: 'I did it with the speed of light. Indeed, the 53 bp-sequence corresponded to an exon 7 that is alternatively spliced. If absent, the protein would lack all potential tyrosine phosphorylation sites. Re-subcloning the long clone that included that sequence and repeating stable transfection and phosphorylation finally confirmed that pp120 [the CEACAM1-L] is indeed phosphorylated by the co-transfected insulin receptor in response to insulin.'

Najjar reflects, 'As soon as I solved this controversy, I found myself dipped into another one: my studies were done in non-polarized transfected NIH-3T3 cells. But, in vivo, how could a protein that is mostly expressed at the bile canalicular domain be phosphorylated by the insulin receptor in response to insulin that is transported via the portal vein? Moreover, what is the functional correlation?' She continues, 'since pp120 is expressed in the liver but not in skeletal muscle or adipose tissue (the other classical insulin target tissues) and since insulin clearance primarily occurs in the liver, let us study whether pp120 plays a role in insulin clearance,' I said enthusiastically to Simeon. 'No way,' he objected unwilling to start dealing with another obvious controversy. 'You can test this when you have your own lab.'

'The world of CEA opened to me when I realized that the translated protein sequence of pp120 matches that of BGPs and C-CAMs. I joined the team that changed the name to CEACAM1.'[2] In one of the earlier CEA meetings in Sweden, the late Prof Obrink asked, 'How could CEACAM1 promote insulin clearance in hepatocytes if it is expressed on the bile canalicular domain?' 'It is possible that the long isoform is expressed on the sinusoidal domain, while the short isoform is more dominantly expressed on the bile canalicular domain', I responded. He looked at me with his investigative gaze. 'Have you tested this hypothesis?' he asked. 'No, I am not currently equipped to investigate it at the cellular level,' I responded.

Najjar recalls, 'A few years later, I generated a mouse with liver-specific inactivation of CEACAM1 by overexpressing a dominant-negative non-phosphorylatable S503A mutant of the long isoform.[29] As expected, the L-SACC1 mouse exhibited primary impaired insulin clearance that led to secondary insulin resistance. The paper got published in *Nature Genetics* 2002.[30] In the same year and in 2004, Prof. Obrink published two papers in *J. Cell Sci* showing that the short isoform of CEACAM1 is exclusively expressed on the bile canalicular domain of hepatocytes (using electron microscopy) and on the apical domain of transfected polarized MDCK cells while the long isoform can be expressed on both domains and that its cytoplasmic domain targets its localization on the lateral domain.'[31,32] That hinted that the S503A mutant in the L-SACC1 mouse was redistributed to the bile canalicular domain away from the sinusoidal circulation that would carry insulin to hepatocytes for degradation.

Najjar further reflects, 'The truth is that even after many years of focusing on CEACAM1, the controversy persists, and my hypothesis has polarized my scientific world just like CEACAM1 has a polarized expression in hepatocytes: how can reduced insulin clearance cause insulin resistance while reduced insulin clearance is commonly believed to aid increased insulin secretion in its compensation for insulin resistance that emerges from obesity? Testing this hypothesis in the clinical setting is challenged by the fact that in the Western World, insulin resistance is associated with overt obesity that masks the identification of the root cause of the problem in humans: insulin resistance or impaired insulin clearance, both depending on altered rapid binding of insulin

to its receptor?'. Nevertheless, this story highlights how serendipity in science coupled with the persistence and curiosity of the experimentalist promotes impactful scientific discovery. That CEACAM1 is alternatively spliced has opened new doors in many laboratories: for instance, the differential role of CEACAM1-L and -S in immune responses,[33,34] in tumour suppression[35] and in metabolic regulation[36] among other actions.

4 | ECTODOMAIN SPLICE VARIANTS OF CEACAM1

The identification of the N, A1, B and A2 ectodomains of CEACAM1 came from early studies of a human leukocyte cDNA library that contained three cDNA clones (W211, W233 and W239) that, when compared to genomic sequences, showed extensive evidence of alternative splicing.[24] Over the decades since, several CEACAM1 mRNA isoforms have been identified with human, mouse and rats being the most prominent ones studied (Figure 3), some with associated distinct functions or in particular disease states. All isoforms include the Ig variable-like N domain, which, contrary to the constant C2-like domains A1, B and A2, is devoid of cysteine (C) residues normally involved in disulfide bridging. The N domain contains 2 binding sites connecting these homophilic molecules in antiparallel trans interactions, followed by a conformational change to cis interactions involving all N and C2-like domains.[37] The N domain is essential for CEACAM1-mediated cell adhesion functions and for association with T-cell immunoglobulin domain and mucin domain-containing protein 3 (TIM-3) and programmed death-1 (PD-1) as a regulator of immune checkpoint inhibitory functions and as bacterial and viral pathogen receptors.[38–40] The longest CEACAM1 isoform encompasses, apart from its N domain, 3 C2-like Ig domains (termed A1, B and A2), a transmembrane domain, and a long cytoplasmic domain (CEACAM1-4L), which can also be substituted by a shorter cytoplasmic domain (CEACAM1-4S). Both the 4L and 4S isoforms are co-expressed in many cell types and tissues with characteristic signalling features through the inclusion of the ITIM/ITSM motifs either responding to tyrosine kinase-mediated Tyrosine phosphorylation[41] or their dephosphorylation through SHP-1 and SHP-2 Tyrosine phosphatase activities.[42,43]

Functional complexity related to these isoforms can be underscored by these few examples. For instance, L isoform predominance in colorectal cancer cells injected into a mouse model inhibited the development of primary colon tumours;[44] however, increased expression of the S isoform with L dominance in recurring colon tumours

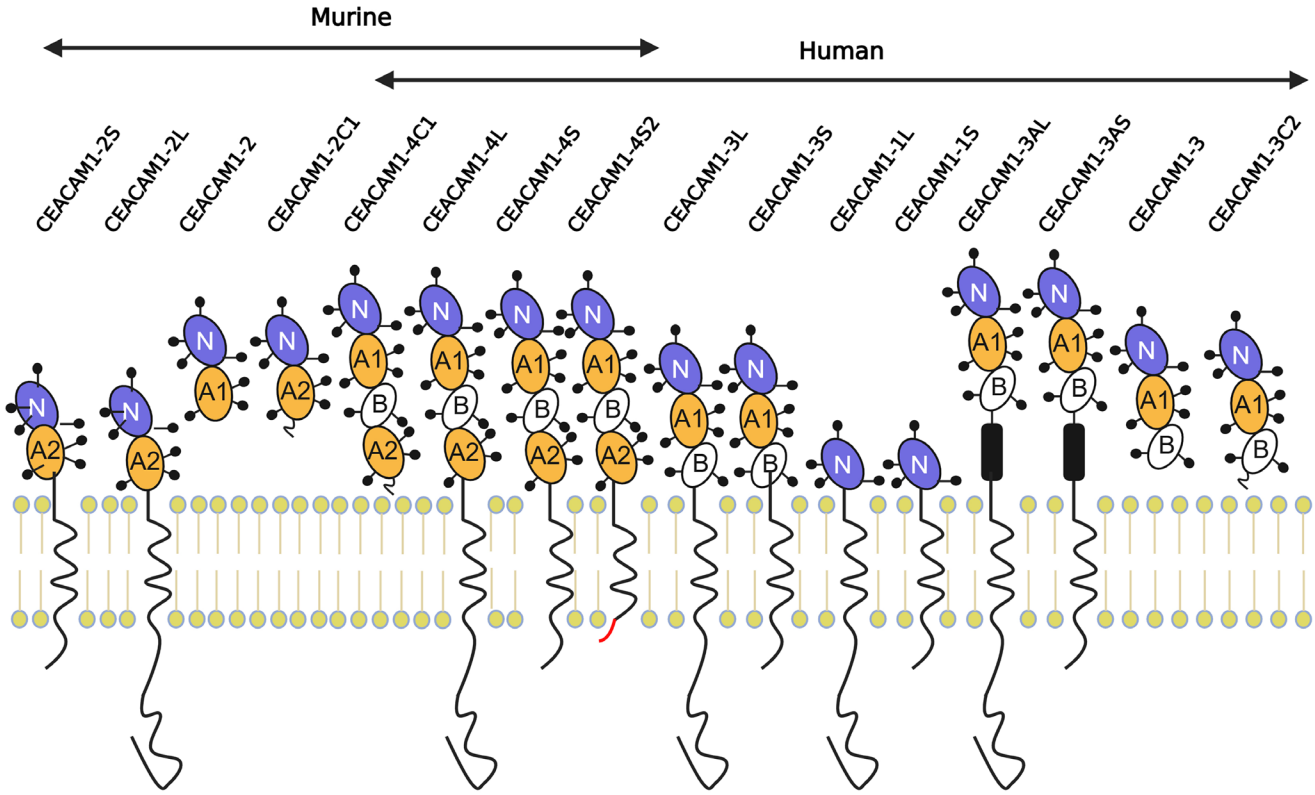

FIGURE 3 Human and murine alternatively spliced CEACAM1 isoforms.

after partial hepatectomy is associated with a worse patient prognosis.[45] A specific function for the membrane-bound CEACAM1-S isoform has been highlighted[46] where its expression through alternative splicing can be favoured: this depends on hypoxia-inducible factor 1 alpha (HIF-1α) of the splicing factor polypyrimidine tract binding protein 1 (PTBP1) in hepatocytes during hypoxic stress. The result is increased hepatocyte protection under cold stress. This condition is capital in human liver transplantation and thus, CEACAM1 may serve as a biomarker for graft selection.[47] These few examples highlight the complexity of CEACAM1 isoform functions, which remain to be ascertained.

Whereas the 4L and 4S variants are expressed in early melanoma tumours, shorter CEACAM1 isoforms denoted as CEACAM1-3L and -3S, both lacking the A2 domain, have been identified in melanoma patients with advanced-stage cancer. The 86% CEACAM1-3S increased expression in advanced melanoma qualifies it as a potential disease progression biomarker.[48] CEACAM1-3S and -3L isoforms are also upregulated in human bronchial cells in response to interferons alpha, beta and gamma and to the TLR3 agonist poly I:C.[49] The number of membrane-associated CEACAM1 isoforms in mouse tissues is limited to those containing 4 and 2 Ig domains attached via a transmembrane area to either L or S tails. No specific functions have been identified yet for their A1 and B domains.

The relevance of the A2 domain expressed in certain CEACAM1 isoforms has been studied through an A2-domain-specific monoclonal antibody, TEC-11.[50] First detected in human bile, CEACAM1 mRNAs encompassing the A2 domain were also found in certain bodily fluids, in serum and patients with obstructive jaundice as secreted variants. In this study, the A2 domain was deemed to express Lex-specific moieties, a carbohydrate structure often involved in cell adhesion and recognition processes, although recognition functions through this CEACAM1 carbohydrate remain to be clarified.

Other less abundant membrane-associated CEACAM1 isoforms have been isolated. In rat intestines and hepatocytes, a different short-tailed CEACAM1-4S2 variant (C-CAM3), resulting from the inclusion of the intron separating the transmembrane exon 6 from the conserved exon 7, translates into a 6-amino acid cytoplasmic extension, different from the S version.[51] In addition, Barnett et al. (1993) have described 2 CEACAM1 smaller isoforms (CEACAM1-1S and -1L, BGPx and x′), including only the N domain with either intracytoplasmic S and L tails, exclusively found in colorectal tumours and not present in normal tissue.[52] These authors have also identified CEACAM1 3 Ig domain-containing isoforms in which the A2 domain is absent but replaced with inverted and truncated human Alu sequences (CEACAM1-3AL and -3AS); discrete expression patterns and functions for these isoforms have not yet been assigned.

In addition to the membrane bound CEACAM1 proteins, secreted isoforms are present in many different human and murine tissues or bodily fluids (e.g. urine, bile, blood, serum, seminal fluid, etc.). All these splicing isoforms result from stop codons positioned ahead of CEACAM1 exon 6, which comprises the transmembrane domain. These secreted isoforms encompass either 4 or 3 Ig domains in human and rat tissues[52,53] but only 2 Ig domains in mouse tissues, as observed with the membrane-bound versions. The 4-domain secreted CEACAM1 isoform CEACAM1-4C1 is the only one common in human and mouse tissues.[54] Soluble CEACAM1 isoforms are important in induced angiogenesis of endothelial cells,[55] and serve as a platform for cholesterol crystallization active in gallstone formation.[56] They are abundant in obstructive jaundice[57] and interfere with membrane-bound CEACAM1-mediated homophilic adhesion,[58] in addition to binding to host pathogens such as Mouse Hepatitis Viral infections.[59] Therefore, the multiple alternatively spliced CEACAM1 isoforms contribute to various expression patterns active in many possible functions and disease states.

5 | MECHANISMS CONTROLLING CYTOPLASMIC DOMAIN SPLICE VARIANTS OF CEACAM1

In a series of pivotal studies uncovering the mechanism of CEACAM1 RNA splicing, the Shively group used a linker scanning mutagenesis approach to identify the specific elements that directed the alternative splicing of the two cytoplasmic tail variants.[27] This technique involves systematically replacing short stretches of RNA with a neutral sequence.[60] The City of Hope group mapped the structure of exon 7 to three functional domains (Figure 4, red text). Nucleotide sequence 1–20 (relative human exon 7) was shown to code for an exonic splicing element that enhances the inclusion of exon 7 in the mature mRNA transcript. By contrast, nucleotide sequence 20–50 codes for two distinct exonic splicing silencers that function by repressing splicing factors, such as heterogeneous nuclear ribonucleoproteins (hnRNPs). This provided the clue to identify three proteins (hnRNP L, hnRNP A1 and hnRNP M) that bind to exon 7 regulatory elements and affect splicing (Figure 4, red text). A combination of RNA co-immunoprecipitation, overexpression and RNA interference studies helped map the location of hnRNP binding. Our data showed that hnRNP M interacts with the

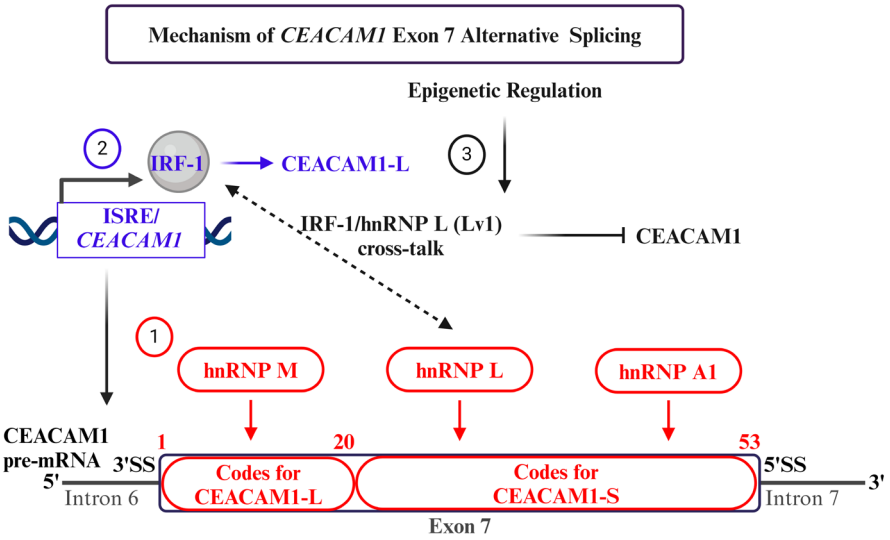

FIGURE 4 Mechanism of *CEACAM1* exon 7 alternative splicing. Summary of three studies that identified the regulatory mechanisms that control the production of the CEACAM1 isoforms in a breast cancer model. Study 1 (red colour) elucidated the exon 7 *cis* and *trans*-elements that dictated its alternative splicing.[27] RNA co-immunoprecipitation studies further identified the hnRNPs responsible for exon skipping and inclusion events. Study 2 (purple colour) showed that Interferon Response Factor 1 (IRF-1) targeted *CEACAM1's* interferon-stimulating response factor (ISRE) promoter sequence to induce CEACAM1-L.[66] Mouse xenografts models showed that the tumour microenvironment induces splice-switching programs on the invasive front, altering the expression from CEACAM1-S to CEACAM1-L. Study 3 (black colour) revealed a model of how the microenvironment promotes chromatin remodelling to silence CEACAM1 through interactions with IRF-1 and variant 1 of hnRNP L (Lv1).[66] Created with Biorender.com.

5′ end of exon 7 to help form the *CEACAM1-L* mRNA. By contrast, hnRNP L was mapped to a centrally located CACA cis-element (nucleotides 29–32) that preferentially induced the formation of *CEACAM1-S* mRNA. Similarly, hnRNP A1 was found to interact with the distal 3′-end of exon 7 and led to the formation of *CEACAM1-S* mRNA. Notably, cellular stress caused by osmotic shock promoted the cytoplasmic accumulation of nuclear hnRNP A1, causing dysregulated production of CEACAM1-L.

In the next study, the interplay between CEACAM1 transcription and splicing was investigated as a key aspect of understanding its gene regulation. By then, the Shively group had shown evidence for an interferon-stimulating response element (ISRE) in the CEACAM1 promoter, targeted by IFN-γ.[61] This cytokine was well-known to promote the expression of interferon regulatory factor-1 (IRF-1) in the transcriptional activation of various genes like IL-4 gene during Th2 polarization.[62] This clue led to the hypothesis that IRF-1 may target the CEACAM1 ISRE in *trans*, influencing the regulation of Type II immune responses. Mutation studies of the ISRE indeed demonstrated that IRF-1 was necessary to produce the CEACAM1-L isoform (Figure 4, blue text).[63] Notably, this study provided the impetus to understanding how the inflammatory microenvironment induces *CEACAM1* alternative splicing pathways during cancer development. Tumour growth (for 70 days) was investigated using breast cancer cells in a mouse xenograft model. Analyses of the splicing isoforms showed that expression of CEACAM1-L but not CEACAM1-S was more intense at the invasive front of the breast carcinoma tissue, providing the evidence that RNA switching programs were indeed responsive to changes in the microenvironment.[63]

During the course of these studies, it became evident that transcription complexes were involved in crosstalk interactions with the spliceosome machinery. One study linked the large, multiprotein complex, Mediator, to gene-specific transcription factors, RNA polymerase II enzyme and the RNA splicing factor, hnRNP L.[64] Another provided evidence for the 'recruitment model' where splicing regulators were brought in close proximity to the promoter.[65] These studies hinted that normal coordination of RNA transcription and splicing may be disrupted in cancer, leading to chromatin remodelling as a mechanism of gene silencing. The Shively group provided the evidence in support of this hypothesis when it was shown that transcription complexes containing IRF-1 interacted with spliceosome complexes containing hnRNP L (Lv1), to silence *CEACAM1* gene expression (Figure 4, black text).[66] These series of studies unlocked the complex interplay between transcriptional processes and splicing regulation and showed how exon 7 functions to direct CEACAM1's response to cellular and inflammatory signals.

6 | MOUSE VERSUS HUMAN CEACAM1-S

The cytoplasmic domains of human and mouse CEACAM1-S differ significantly (Figure 5), suggesting the possibility that their functions have diverged during evolution. Homology analyses of the transmembrane (TM) domain and exon 7 nucleotide sequence from each species reveal that 1 nucleotide is necessary to lengthen the TM domain of huCEACAM1 to generate CEACAM1-L by alternative splicing (Figure 5A). In this alignment, the start of the TM domain for murine and human CEACAM1 is the same, but the end of the TM domains likely differs. For muCEACAM1, the TM end conforms to a traditional double basic residue, namely Arg-Lys (RK in the figure). However, for huCEACAM1, the double basic residues are replaced with Gly-Lys (GK in the figure). There is strong evidence that actin interacts with F454[67] and Calcium/Calmodulin-dependent protein kinase II delta (CamK2D) phosphorylates T457[68] on huCEACAM1-S (highlighted residues in Figure 5B). Thus, it is likely that the TM domain ends at H453 for huCEACAM1-S, otherwise these interactions would either not occur or require the TM-cytoplasmic domain boundary to be flexible. If we are correct in the assignment of the start of the two CEACAM1-S cytoplasmic domains, then the entire domain is only nine residues for mouse compared to 12 residues for man. In addition, muCEACAM1-S lacks both the actin-binding F454 and the CamK2D phosphorylation site T457 found in huCEACAM1. These considerations lead to a startling conclusion: muCEACAM1-S and huCEACAM1-S must differ in their signalling.

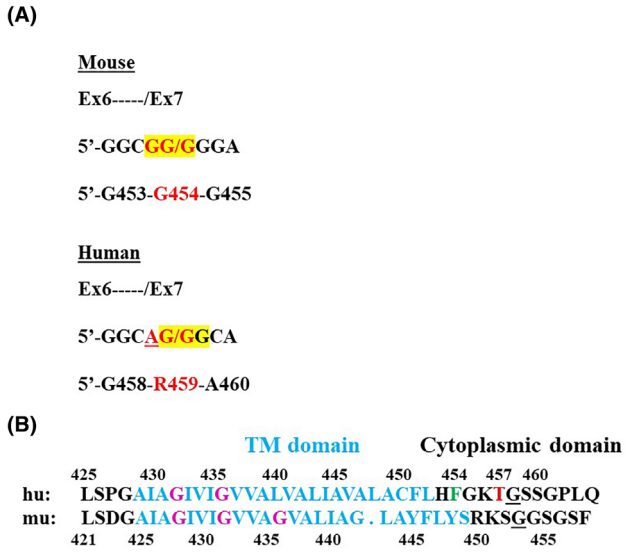

FIGURE 5 Sequence alignment of the transmembrane and cytoplasmic domains of human and murine CEACAM1-S. (A) Mouse vs. human sequence alignment of the 3′ end of exon (ex) 6 and the 5′ end of exon 7. Dash represents the exon/intron boundary. Nucleotides colours match the coded amino sequence. The underline A in human represents a single event that conserved the GGG triplet (yellow highlight) in mouse and human. (B) The last four residues of their extracellular domains are followed by their putative transmembrane (TM) domains (blue), with key Gly residues in the TM domain shown in magenta and key residues in the cytoplasmic domain shown in green (F454) and red (T457). The mRNA splice site leading to CEACAM1-L occurs in the middle of the underlined Gly's codon.

In terms of phosphorylation, CHO cells transfected with rat CEACAM1-S (then called C-CAM-S) exhibited phosphorylation on CEACAM1-S only after Phorbol 12-myristate 13-acetate (PMA) treatment,[69] a Protein kinase C (PKC)-activating drug. Thus, the sequence RKSG (underlined Ser was phosphorylated) in muCEACAM1-S was shown to be a substrate for PKC. Subsequently, we were able to show that T457 in huCEACAM1 was phosphorylated by CamK2D and not PKC.[68] Further studies on synthetic peptides from the cytoplasmic domains of mouse, rat and human CEACAM1-S showed that CaM, another binding partner of CEACAM1-S, was able to bind the mouse and rat peptides, but not the human peptides.[70]

The presence of actin binding residue in F454 in huCEACAM1-S versus its absence in muCEACAM1-S is intriguing. In an early study by Sadekova et al.,[71] CEACAM1-L was shown to be associated with the cytoskeleton, but the key amino acids were not identified. That observation prompted us to test the binding of CEACAM1-S cytoplasmic domain peptides to actin in a yeast two-hybrid assay to determine if the same was true for the short cytoplasmic domain isoform. This study identified F454 as the key actin binding amino acid, a result that was verified by cell-based studies with F454A mutants of huCEACAM1-S.[72] Strikingly, transfection of wild-type huCEACAM1-S into MCF7 cells enabled lumen formation in a 3D-culture system, whereas the F454A mutation failed, similarly to wild-type MCF7 cells.

These studies also showed that the T457A mutation blocked lumen formation, indicating that phosphorylation of this residue plus F454 was critical for actin binding and lumen formation. When MCF7 cells were transfected with muCEACAM1-S, no lumen formation was observed in the 3D-culture system (unpublished), emphasizing the difference in function between the two species. However, when MCF7 cells transfected with huCEACAM1-S were implanted in murine mammary fat pads, functional mammary glands were observed, engorged with milk.[72] Thus, we conclude that human mammary cells require huCEACAM1 to make functional mammary glands and that muCEACAM1 cannot perform the same function in human cells. This implies that while muCEACAM1-S functions properly in murine mammary epithelial cells,

substitution with huCEACAM1 may not. This remains to be tested together with identifying the evolutionary pressures that resulted in these changes to the amino acid sequences and their signalling molecules.

Another common feature of CEACAM1 is its ability to bind calmodulin (CaM).[70,73] CaM binding to the rat isoforms (called C-CAM) was first demonstrated by Obrink and coworkers[74] and later shown for huCEACAM1-S.[75] Since F454 is unique to muCEACAM1-S, we asked if this residue was directly involved in CaM binding and found, that while the wild-type peptide bound CaM, the mutant peptide F454A bound CaM more tightly.[75] This result suggested that the order of binding to huCEACAM1 was CaM first, followed by actin. This order fits the general proposal that CEACAM1 cis--s, thought to be devoid of signalling, are disrupted by CaM binding,[70] a required step to active signalling in monomeric CEACAM1 or to form trans-dimers from one cell to another. Indeed, this was shown in an elegant study by Gray-Owen and coworkers using huCEACAM1-L.

Since CEACAM1-S also form cis-dimers,[76] it is reasonable to assume they are also disrupted by Ca^{2+} stimulated CaM binding. In that work, they also addressed the question of the driving force for dimerization, long thought to reside solely in the extracellular domains of CEACAM1.[77] In fact, Beauchemin and coworkers[44] provided evidence that the TM domain was key to dimerization, similar to a study by Hixon and coworkers,[78] who showed a role for key Gly and Tyr residues in the TM domain of rat CEACAM1-S. In fact, the sequence motif Gly-X-X-X-Gly is known to be a general dimerization motif that involves many cell surface receptors.[79] When this motif was mutated (G432L, G436L) in huCEACAM1-L (Figure 5B, magenta-highlighted Gly residues), dimerization was completely abrogated.[76] Notably this motif is found in both species, and in muCEACAM1, it occurs twice. Putting together the sequence of events in disruption of huCEACAM1-S dimers, we initially proposed the following order of binding: CaM→CaMK2D→actin, a sequence that incorporates the documented ability of CaM2D to polymerize actin at the cell surface.[75] Whether or not the exact same sequence occurs in muCEACAM1-S remains to be demonstrated.

Annexin A2 (AnxA2) is yet another binding partner of huCEACAM1-S that was first identified by immunoprecipitation, GST pull downs and surface plasmon resonance binding assays.[80] We showed that phosphopeptide mimic T454E was key to binding tetrameric AnxA22P112 (AIIt), an important partner for lumen formation in CEACAM1-S–transfected MCF7 cells grown in 3D-culture. NMR structural studies using a 15N-labelled peptide from AIIt identified the key residues in P11 that bound the cytoplasmic domain of huCEACAM1-S and that F454 and phospho-T457 were in the binding site. Taken together, we have revised our model for conversion of cis- to trans-dimers as a 6-step process (Figure 6).

Although the conversion of cis-dimers to monomers and/or to trans-dimers is thought to be an important function for CEACAM1, CEACAM1 is also known to co-associate with a variety of cell surface receptors, including the TCR,[81] BCR[82] and TLR4.[83] Most of these interactions were postulated for monomeric CEACAM1-L whereas the recruitment of SHP1/2 to its ITIMs[43] was responsible for its inhibitory signalling. However, the consequence of co-association of CEACAM1-S with other receptors is not well investigated. In fact, CEACAM1-S is often assigned the role of hetero-dimerization with CEACAM1-L,[38] thus modulating its inhibitory effects. On the contrary, it is possible that the hetero-disruption of homo–cis-dimers for CEACAM1-S occurs with other receptors. An example may be the recent finding that CEACAM1 associates with the fatty acid transporter CD36.[84] Although the liver is unusual in that it expresses both isoforms of CEACAM1, they are expressed on different locations, CEACAM1-L on the sinusoid and CEACAM1-S on the bile canaliculi surface. Based on our current state of knowledge, the

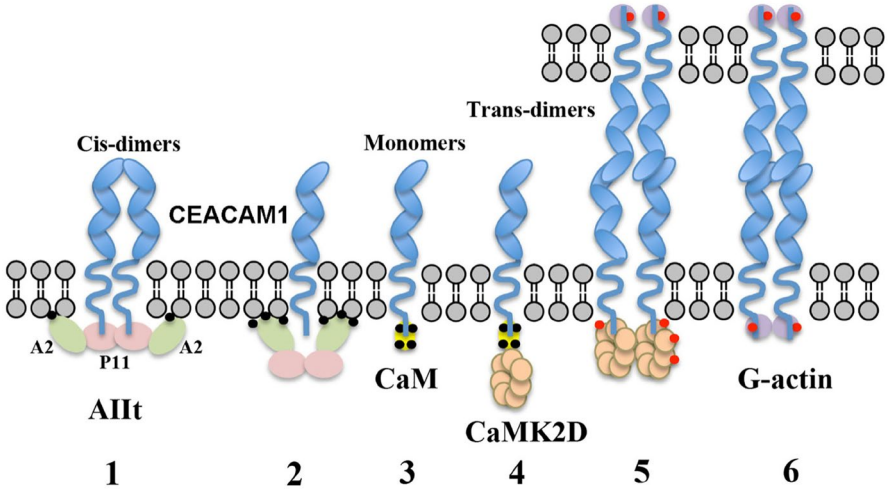

FIGURE 6 Stepwise binding of AIIt, CaM, CaMK2D and Actin to huCEACAM1-S. 1. P11 in AIIt binds to cis dimers. 2. AnxA binds phospholipids disrupting Cis-dimers. 3. CaM stabilizes monomers. 4. CaM recruits CaM2D. 5. CaMK2D auto phosphorylates subunits and T457 on CEACAM1-S. 6. G-actin recruited to F454/phospho-T457. Trans-dimers stabilized.

interaction of CEACAM1-S with CD36 may occur at the level of TMs or cytoskeleton. Hetero-dimerization at the level of the TM is a real possibility in that CD36 has two transmembrane domains that are brought together by the above mentioned GlyXXXGly motif, and since the CEACAM1 forms cis-dimers using the same motif, the two receptors may exchange TMs when occurring together in lipid rafts. Thus, the two sets of TMs interact forming CEACAM1-CD36 heterodimers. Further interactions at the level of the cytoskeleton are also possible and may explain why CEACAM1-S interacts with β-catenin[85] even though it does not have a β-catenin binding motif in its cytoplasmic domain like CEACAM1-L.[86,87]

7 | MECHANISM CONTROLLING CEACAM1 SPLICING DURING STERILE INFLAMMATION

More recently, the Kupiec lab at UCLA has begun to decipher how various CEACAM1-expressing cell types, including neutrophils, T-cells and hepatocytes, respond to sterile inflammation and liver transplantation injury. The emerging picture is that each cell type is equipped with unique mechanisms to manage cellular stress that depend on CEACAM1 splicing isoforms. The first study showed that donor liver CEACAM1 null mutation worsened liver transplantation outcomes by enhancing reactive oxygen species expression and High Mobility Group Box 1 (HMGB1) translocation during extended (18 h) cold storage.[47] Damage-associated molecular patterns (DAMPs), such as HMGB1, are well-known positive feedback triggers of the ischemia–reperfusion injury (IRI) cascade.[88] This pathological event occurs during organ transplantation, heart attacks, strokes or major surgeries where blood supply is temporarily clamped or obstructed. Inevitably, the sudden return of oxygen and nutrients triggers an inflammatory response and oxidative stress, exacerbating injury to the affected tissues.[89] Notably, the liver flush from cold-stored CEACAM1-deficient grafts showed enhanced leakage of hepatic DAMPs.[47] This led to a pronounced inflammatory response with increased mRNA levels coding for *MCP1*, *CXCL2* and *CXCL10* when cultured with bone marrow–derived macrophages. The mechanism of CEACAM1 signalling was attributed to its function in regulating ASK1, a redox-sensitive upstream activator of the JNK and p38 arms of the MAPK pathway.[90] In the clinical arm of the study, 60 human liver transplant recipients were retrospectively studied to determine if low CEACAM1 expression levels correlated with worse post-liver transplantation levels. Indeed, cold-stressed human liver grafts with low CEACAM1 experienced significantly higher post-liver transplantation enzyme levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and more frequent incidence of early allograft dysfunction. Although this study identified CEACAM1 as a biomarker for donor graft quality in mouse and human liver transplantation, it left unanswered the genetic regulatory mechanisms by which hepatic CEACAM1 controls ischemia reperfusion stress during sterile inflammation.[91]

This did not become clear until a series of experiments showed that only adenoviruses expressing CEACAM1-S isoform-specific particles rescued hypoxia-triggered cellular stress in murine hepatocytes through Ask1/p-p38 signalling.[46] Transcription factor binding site studies led to the identification of hypoxia-inducible factor-1α (HIF-1α), a transcription factor that undergoes stabilization during low oxygen levels, as a candidate that may influence Ceacam1 expression under ischemic stress. Chromatin immunoprecipitation studies demonstrated that the polypyrimidine tract-binding protein-1 (Ptbp1) promoter was targeted by HIF-1α. Ultimately, this interaction resulted in the preferential induction of CEACAM1-S splicing and improved posttransplant outcomes.[92] A retrospective analysis of 46 human donor liver grafts revealed a positive correlation between human *CEACAM1-S* and *HIF1A* expression. Post-liver transplantation outcomes showed reduced gene expression for *TIMP1*, *MCP1*, *CXCL10* cytokines and immune activation markers *IL17A*, and total bilirubin levels and incidence of delayed complications from biliary anastomosis.[46]

Follow-up studies from the UCLA group focused on the contribution of CEACAM1-L isoform in neutrophils[93] and T cells.[94] Although neutrophils' role as main villains in peri-transplant tissue injury had been well-documented,[95] new data emerged that neutrophil extracellular traps (NETs) may be regulated by CEACAM1.[96] In the experimental arm, ablation of recipient-derived neutrophil CEACAM1-L in a mouse liver transplantation model aggravated hepatic IRI by promoting NETs. The mechanism for regulation of NETosis was attributed to cross-linking of S1P, a bioactive signalling lipid, to its cognate ligands S1PR1–5 during neutrophil activation.[97,98] In the clinical arm, a retrospective analysis of 55 study subjects showed high levels of neutrophil CEACAM1-L/cathepsin G correlated with improved liver transplantation function, depressed innate/adaptive immune activation and lower incidence of early allograft dysfunction, as compared with low expressing CEACAM1-L/cathepsin G individuals.[93] The T cell study followed-up on observations by others that CEACAM1-L serves as a heterophilic ligand that regulates TIM-3 tolerance and exhaustion.[39] Kojima et al. showed that T cell–specific CEACAM1-L enhanced TIM-3 expression on CD4 + T cells, and this, in turn, caused liver-associated CEACAM1-L to suppress NF-κB in resident

Kupffer cells and alleviate liver transplantation injury.[94] Once again, analyses of human liver biopsies showed that high levels of *CEACAM1-L* expression correlated with improved outcomes, suppressed acute liver injury and higher prognostic T-cell homeostasis in human liver transplantation. These studies cumulatively show that exploiting RNA splicing pathways that control CEACAM1 isoform expression may offer new therapeutic avenues that have yet to be exploited.

8 | TARGETING CEACAM1 EXON 7 FOR THERAPEUTIC BENEFIT

Because of CEACAM1's different roles in cell signalling, adhesion and modulation of immune responses, the mis-splicing of CEACAM1 could have serious implications for human health. Small antisense molecules have emerged as promising therapeutic tools for modulating protein expression pathways linked to human diseases.[99] Some are naturally occurring like microRNAs and ribozymes. Others, like short activating RNAs, locked nucleic acids, Gapmers and phosphorodiamidate morpholino (PMOs), are synthetically designed and optimized for potency, selectivity and pharmacokinetic properties. Antisense-based technologies use nucleotide hybridization to achieve specificity, stability and efficiency in the inhibition of translation or the alteration of RNA splicing programs. Many clinical trials using antisense-based technologies have recently targeted muscular,[100] immune-related and metabolic disorders.[101] Notably, the foundation for targeting CEACAM1 using PMOs for therapeutic benefit has already been shown in several studies. Dery et al. (2018) showed that targeting the variable exon 7 of CEACAM1 in breast cancer MDA-MB-468 cells disrupted IRF-1-directed alternative splicing leading to the production of the short cytoplasmic isoform.[66] In another study, Dery et al. (2023) showed that targeting exon 7 with PMOs induced the short cytoplasmic isoform, following warm and cold storage stress in primary cultured mouse hepatocytes.[46]

To demonstrate how PMOs may be used in future clinically relevant CEACAM1 studies, a proof-of-principle comprehensive search of exon 7 single nucleotide polymorphisms (SNPs) was conducted using the UCSC Genome Browser (ver. GRCh37/hg19; https://genome.ucsc.edu; Table 1). SNPs are genetic variations that can affect human

TABLE 1 CEACAM1 exon 7 SNPs identified within location 5′-chr19:43,015,780-43,015,727-3′.

	Identifier	Allele	Chromosome/ Location	Phenotype Variant	From start Exon 7	ERE[a] 1–3	Predicted Faulty Variant
1	rs2041459216	C > T	19:43,015,780	Transcript	0	1	CEACAM1-S
2	rs2041459145	C > T	19:43,015,779	Missense	1	1	CEACAM1-S
3	rs767345563	G > A	19:43,015,774	Intron	6	1	CEACAM1-S
4	rs763395302	C > T	19:43,015,773	Missense	7	1	CEACAM1-S
5	rs2041458946	G > C	19:43,015,770	Missense	10	1	CEACAM1-S
6	rs773721151	G > A	19:43,015,767	Transcript	13	1	CEACAM1-S
7	rs765768246	C > G,T	19:43,015,766	Missense	14	1	CEACAM1-S
8	rs762437836	C > A	19:43,015,764	Missense	15	1	CEACAM1-S
9	rs2041458686	A > T	19:43015762	Missense	18	1	CEACAM1-S
10	rs1129707	G > C	19:43,015,761	Missense	19	1	CEACAM1-S
11	rs1009548482	A > T	19:43,015,760	Transcript	20	1	CEACAM1-S
12	rs776721332	C > T	19:43,015,755	Transcript	25	2	CEACAM1-L
13	rs755483328	T > C	19:43,015,754	Coding	26	2	CEACAM1-L
14	rs1334572095	C > A,T	19:43,015,753	Coding	27	2	CEACAM1-L
15	rs1342001042	T > C	19:43,015,751	Missense	29	2	CEACAM1-L
16	rs2041458076	T > G	19:43,015,747	Missense	33	3	CEACAM1-L
17	rs1450491513	G > A	19:43,015,746	Transcript	34	3	CEACAM1-L
18	rs367681999	G > A	19:43,015,732	Intron	48	3	CEACAM1-L
19	rs1358955990	T > A	19:43,015,730	Missense	50	3	CEACAM1-L
20	rs760851492	T > A,C	19:43,015,728	Missense	52	3	CEACAM1-L
21	rs2147769362	C > T	19:43015727	Splice donor	53	3	CEACAM1-L

[a]Based on mutational analyses[27] of CEACAM1, where exon responsive elements (EREs) were identified: ERE-1 is nt 1–20, ERE-2 is nt 16–35, and ERE-3 is nt 31–50.

health and diseases by altering the susceptibility of an individual to cancer, cardiovascular diseases, diabetes and autoimmune disorders.[102] Restricting our search to the q arm of chromosome 19, 21 SNPs were identified within the exon 7 gene loci. Missense mutations (52.4%, 11/21) were by far the most common, followed by transcript mutations (23.8%, 5/21), coding mutations (9.5%, 2/21) and intron and splice donor mutations (14.3%; 3/21). SNPs were then cross-referenced to previous mapping studies of exon 7 in cancer cells to predict which CEACAM1 isoform may be produced in human patients (Table 1). Dery et al. (2011) demonstrated that mutations occurring within the first 20 nucleotides of exon 7 influenced splice-switching programs to generate CEACAM1-L, instead of CEACAM1-S observed in wild-type comparison groups. Similarly, mutations in the remaining nucleotides of exon 7 led to the production of CEACAM1-L instead of CEACAM1-S under similar conditions.[27] These findings suggest that the location of these SNPs could lead to improper expression of either the long or short cytoplasmic isoform in these human subjects (Table 1). Although antisense-based technologies are still in their early stages, future studies would benefit from exploring whether targeting exon derived SNPs can modulate the correct splice isoform to improve disease progression in humans.

9 | CHALLENGES, FUTURE DIRECTIONS AND CONCLUSIONS

In summary, despite decades of studies characterizing the pleiotropic functions of CEACAM1, many scientific questions remain unanswered. Mechanistic insight is still needed to understand how the various ectodomain isoforms are formed through regulation of alternative splicing pathways. What are the key elements required for regulating exons 2 through 5? How do the serine/arginine-rich proteins and the hnRNP family of RNA splicing factors interact with the splicing machinery to influence exon inclusion/exclusion. Future studies should investigate whether exon 7 SNPs (or other exons) change intron-exon boundaries leading to variations in exon length, ultimately causing disease phenotypes. Finally, polyadenylation and 3′ untranslated region (3′UTR) processing of the *CEACAM1* gene remain unaddressed. These processes play important functions in mRNA stability and translation efficiency. Understanding how the 3′UTR is regulated can help clarify the *CEACAM1* mRNA half-life, ultimately shedding light on how protein levels increase in response to inflammatory conditions and decrease in response to homeostatic regulation.[46]

Despite the extensive work detailed in this review on the mechanistic regulation of CEACAM1 splice isoform's role in immunity, cellular differentiation and metabolism, many questions remain unclear. For example, what are the molecular switches that control the tumour suppressor and promoter cancer functions of CEACAM1 and are these controls executed simultaneously? It is also interesting to speculate on how CEACAM1-S exerts its anti-inflammatory regulation during liver transplantation. One untested explanation may be that during inflammation and injury, hepatocyte integrity is compromised, and this leads to the disruption of the tight junctions that normally isolate the bile canaliculi from the blood. Perhaps CEACAM1-S on the bile canaliculi interacts with CEACAM1 on the T cells through heterophilic binding to regulate T cell TIM-3 homeostasis. It remains to be determined whether hepatic CEACAM1-S promotes immune tolerance by engaging CEACAM1 on other immune cells (e.g. neutrophils and macrophages) to prevent an overactive immune response, which could otherwise lead to further tissue damage and fibrosis during liver injury. The recent observation that CEACAM1 interacts with immune checkpoint PD-1 may serve as the mechanism that promotes self-tolerance though more studies will be required to fully understand how this may influence other cell types during the immune response.[103] Future studies will also be needed to reconcile this with previous studies that showed that CEACAM1-S promotes T cell activation and leads to increased activation-induced cell death in a cancer context.[104] The answer to these questions, and more, will require extensive and intensive experimentation. Nevertheless, it is obvious that understanding the precise mechanisms by which CEACAM1 splice isoforms modulate cellular interactions during injury will be crucial for developing targeted therapies to manage human diseases and improve patient outcomes.

AUTHOR CONTRIBUTIONS

K.J.D. and J.W.K.-W. were involved in conceptualization; K.J.D., S.M.N., N.B. and J.E.S. were involved in writing—original draft; K.J.D., S.M.N., N.B., J.E.S. and J.W.K.-W. were involved in writing—review and editing; J.W.K.-W was involved in project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings presented in Table 1 are openly available in UCSC Genome Browser (ver. GRCh37/hg19).

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