The role of hyaluronan and its CD44 receptor in inflammation and cancer

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**Abstract**


Hyaluronan, an important extra-cellular matrix molecule, was thought to be interstitial connecting glue decades ago. However, recent evidence has revealed that hyaluronan and its binding proteins also play crucial roles in various pathophysiological conditions in humans, including inflammation and infection.

Study I focused on dengue virus infection and found that elevated serum hyaluronan levels during early infection phase was an independent predictor for occurrence of warning signs, and thus severe dengue. High circulating levels of the viral non-structural protein 1 (NS1) correlated with high concentrations of serum hyaluronan. NS1 exposure decreased the expression of CD44 in differentiating endothelial cells impairing the integrity of vessel-like structures and promoted the synthesis of hyaluronan in dermal fibroblasts and endothelial cells in synergy with dengue-induced pro-inflammatory mediators. Perturbed hyaluronan-CD44 interactions enhanced endothelial permeability through modulation of VE-cadherin and cytoskeleton re-organization, and exacerbated the NS1-induced disruption of endothelial integrity. Study II reports a negative correlation between the expression of genes encoding hyaluronan synthase HAS2, its natural antisense transcript HAS2-AS, the chromatin modulating factor HMGA2 and transforming growth factor-β (TGFβ), and survival of patients with invasive breast cancer. TGFβ induction of Hmga2, Has2as and Has2 in mouse mammary epithelial cells, and synthesis of hyaluronan were accompanied with activation of Akt and Erk1/2 MAP-kinase signaling and were required for breast cancer cell motility. Importantly, the hyaluronan receptor Cd44, but not Hmmr, was required for TGFβ-mediated epithelial-mesenchymal transition phenotype. Has2as was found to contribute to the maintenance of stem cell factors and breast cancer stemness. Study III explored the physical interaction between the inhibitor of the apoptosis-stimulating protein of p53 (iASPP) and the hyaluronan receptor CD44. The CD44 standard isoform (CD44s), but not the variant isoform, bound to iASPP via the ankyrin-binding domain in CD44s. iASPP was required for hyaluronan-induced CD44-dependent migration and adhesion of fibroblasts. CD44 altered the sub-cellular localization of the iASPP-p53 complex; thus, ablation of CD44 promoted translocation of iASPP from the nucleus to the cytoplasm, resulting in increased formation of a cytoplasmic iASPP-p53 complex in fibroblasts. Overexpression of iASPP decreased the level of intracellular reactive oxygen species, while overexpression of CD44 increased. Knock-down of CD44s, in the presence of p53, led to increased cell growth and cell density of fibroblasts by suppression of p27 and p53.

In summary, we investigated the interaction of hyaluronan and its transmembranous receptor, CD44, as well as the modulation of hyaluronan synthesis, in several different pathophysiological conditions.

**Keywords:** CD44, Dengue, Hyaluronan, HAS2, iASPP, TGFbeta

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To my family......
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

AUC        area under curve
BSA        bovine serum albumin
CCHF       Crimean-Congo hemorrhagic fever
CD44       cluster of differentiation 44
CD44s      standard isoform of CD44
CSCs       cancer stem cells
DF         dengue fever
Dengue fever cases classification based on WHO 1997 definitions
  DHF       dengue hemorrhagic fever
  DSS       dengue shock syndrome
  DVI       dengue virus infection
ECM        extracellular matrix
EMT        epithelial-mesenchymal transition
ERK1/2     extracellular signal–regulated kinases 1/2
ERM        ezrin, radixin, and moesin
FGF        fibroblast growth factor
GAGs       glycosaminoglycans
GFs        growth factors
HA         hyaluronan
HAS        hyaluronan synthase
HAS2-AS1   homo sapiens HAS2 natural antisense RNA
Has2as      natural antisence for Has2
HMG        non-histone high mobility group proteins
Hmga2      High mobility group (Hmg) a protein 2
HMW        high molecular weight
HYAL       Hyaluronidase
JNK        c-Jun N-terminal kinase
LMW        low molecular weight
lncRNA     long non-coding RNA
LPS  lipopolysaccharide
MAP  mitogen-associated protein
miRNA microRNA
NF-kB nuclear factor kappa-light-chain-enhancer of activated B
NO nitric oxide
PDGF-BB platelet-derived growth factor-BB
PG Proteoglycan
PI3K phosphatidylinositol 3’-kinase
RA rheumatoid arthritis
RHAMM hyaluronan mediated motility receptor
ROS reactive oxygen species
SMAD receptor-activated mothers against decapentaplegic hom-
Src proto-oncogene tyrosine-protein kinase
TF transcriptional factor
TGFβ transforming growth factor β
TNFα tumor necrosis factor alpha
TβRI Type I serine/threonine kinase receptors
TβRII Type II serine/threonine kinase receptors
VEGF vascular endothelial growth factor
WHO World Health Organization
WS warning signs
Abstract

Hyaluronan is a significant extracellular matrix (ECM) molecule that was initially thought to be a molecule that fills the interstitial space. But, recent research has shown that hyaluronan plays a crucial role in regulating tissue homeostasis, such as vascular integrity. The biosynthesis of hyaluronan and its interaction with the main receptor, CD44, which is found on lymphocytes, is disrupted in various pathological conditions, including inflammation and infection, which significantly affects cellular functions. We have studied the importance of hyaluronan-CD44 signalling in different biological and pathological contexts, such as dengue virus (DV) infection, inflammation, and cancer.

In our first study, we investigated whether high levels of hyaluronan in the blood could be used as a biomarker to predict the severity of dengue fever in the early stages of infection. Our analysis showed that high levels of hyaluronan in the blood were strongly correlated with high levels of the viral non-structural protein 1 (NS1), indicating that hyaluronan could be used as a tool to predict disease progression and identify patients who need to be hospitalized. When exposed to the NS1 protein, we observed the formation of pro-inflammatory hyaluronan matrices, reduced expression of CD44, rearranged VE-cadherin expression, and increased permeability of microvascular endothelial cell cultures. These changes suggest a mechanism linking NS1-induced hyaluronan production to vascular leakage.

We conducted a second study to investigate how TGFβ induces the expression of hyaluronan synthase HAS2. Upon analyzing the TCGA database, we discovered a negative correlation between the survival rate of patients with invasive breast cancer and the expression of genes that encode HAS2, HAS2-AS, HMGA2, and TGFβ. Our analysis revealed that HAS2-AS and HMGA2 play a crucial role in the TGFβ-mediated induction of HAS2-synthesized hyaluronan, which activates CD44 and triggers downstream signaling of AKT and ERK1/2. Thus, HAS2-AS and HMGA2 are implicated in breast cancer stemness and TGFβ- and HAS2-induced breast cancer EMT.

The third study explored the physical interaction between the inhibitor of the apoptosis-stimulating protein of p53 (iASPP) and the hyaluronan receptor CD44. The CD44 standard isoform (CD44s), but not variant (CD44v)
isoforms, bound to iASPP via the ankyrin-binding domain in CD44s. iASPP was required for hyaluronan-induced CD44-dependent migration and adhesion of fibroblasts. CD44 altered the sub-cellular localization of the iASPP-p53 complex; thus, ablation of CD44 promoted translocation of iASPP from the nucleus to the cytoplasm, resulting in increased formation of a cytoplasmic iASPP-p53 complex in fibroblasts. Overexpression of iASPP decreased the level of intracellular reactive oxygen species (ROS), while overexpression of CD44 increased. Knock-down of CD44s, in the presence of p53, led to increased cell growth and cell density of fibroblasts by suppression of p27 and p53.

In summary, we have investigated the interaction of hyaluronan and its transmembranous receptor, CD44, as well as the modulation of hyaluronan synthesis and the underlying mechanisms in different pathophysiological conditions.
1 Introduction

1.1 Dengue virus infection

1.1.1 Dengue – an epidemiologically expanding mosquito-borne disease

Dengue virus infection (DVI) is a global public health threat affecting at least 3.6 billion people living in more than 125 countries worldwide, especially in the tropical and subtropical areas [1]. It is estimated that the number of annual infection cases of dengue virus globally is increased up to 390 million. Among the patients with DVI, about 96 million (25%) will develop clinical symptoms and signs [2]. About 20,000 deaths are related to DVI in the world every year [3]. There were two outbreaks of DVI in Taiwan in 2014 and 2015 [4].

1.2 Clinical manifestation and immune response of DVI

DVI is a systemic and dynamic disease with wide manifestations clinically, ranged from mild dengue fever without warning signs, patients with warning signs (WS), to severe dengue and even fatal cases [5]. WS includes one or more of the following symptoms and signs: (1) abdominal pain or tenderness; (2) persistent vomiting; (3) clinical fluid accumulation; (4) mucosal bleeding; (5) lethargy or restlessness; (6) liver enlargement > 2 cm; (7) increase in hematocrit concurrent with rapid decrease in platelet count. After 4-10 days of incubation, the disease begins abruptly and is followed by three phases, i.e. febrile phase, critical phase, and then recovery phase [5]. There are a lot of inflammatory mechanisms involved in the course of DVI, including over-production of cytokines and chemokines, such as TNFα and TGFβ, complement system activation, antibody-dependent enhancement during the secondary infection caused by another serotype, and autoantibody formation [6]. As shown in Figure 1, clinicians categorize DVI patients into patients without warning signs, patients with warning signs, and those presented as severe dengue. The recommended management strategy by WHO differ based on the classification of DVI patients [5].
1.3 Dengue virus therapy and prevention

No specific and effective anti-dengue viral drugs are currently available. In addition, there are a lot of limitations of the commercial vaccine applied primarily for children [7]. The main therapeutic strategy is basically supportive care. The main idea to manage vascular leakage and depletion of intra-vascular volume is to infuse large amount of intra-venous fluid quickly when vascular leakage occurs. However, infusion of fluid should be done very carefully, since vascular leakage may improve soon usually 24-48 hours after it started. Therefore, an overdose of fluid supply will lead to fluid accumulation and compromise the respiration even during the recovery phase. This rapid-on and rapid-off phenomenon of vascular leakage suggests that the vascular leakage is associated with functional disturbance rather than destructive effects on the endothelial cells [5]. New treatment strategies, as well as biomarkers, are important to be prepared for the outbreaks and to provide appropriate cares.

1.4 Dengue virus non-structural protein 1 (NS1)

When the host cells are hijacked by dengue, virus genome replication and also viral protein production will take place. Among 7 non-structural proteins, NS1 is found to be harmful by itself, and also lead to a detrimental immune
Hyaluronan is both secreted from the infected cells during virus replication, or GPI-anchored on the cell membranes. Since NS1 is known to have effects in cells, we explored its role in hyaluronan turnover [9].

Hyaluronan is an important glycosaminoglycan (GAG) located mainly, but not only, in the extracellular matrix (ECM) [10]. In the human body, hyaluronan is abundant in the skin, vitreous of the eye, synovial fluid, and skeletal tissues [11]. It plays crucial physiological and pathological roles, such as hydration, lubrication of joints, wound healing, and involvement in tumor progression. Whereas the role of hyaluronan in cancer progression is well established, less is known about the role of hyaluronan in the infectious diseases i.e. whether it has a protective or pro-inflammatory role.

1.5 Hyaluronan
1.5.1 Biosynthesis of hyaluronan
Hyaluronan is a non-sulfated GAG comprised of repeating disaccharide units of glucuronic acid (GlcUA) and N-acetyl glucosamine (GlcNAc). There are three isoforms of hyaluronan synthases in human which are responsible for the biosynthesis of hyaluronan, hyaluronan synthase (HAS) 1, 2, and 3. These different enzymes are integral membrane proteins which synthesize hyaluronan at the inner face of the plasma membrane. Hyaluronan is extruded through the membrane during synthesis and newly synthesized molecules which are still bound to their synthases, form pericellular matrices. Hyaluronan has an important role in the maintaining of the tissue structures, but has also ascribed signaling roles through its interactions with cell surface receptors including CD44. Different hyaluronan synthases produce hyaluronan chains with different chain lengths [12]; interestingly, hyaluronan with different chain lengths play different roles in the physiological or pathological conditions, including cancer, inflammation and infection. Of note, HAS2 knockout mice die during embryogenesis because of severe abnormalities of cardiac and vascular formation. In contrast, mice with HAS1 or HAS3 depleted are viable.

In physiological conditions, HAS1 is less active to produce hyaluronan than HAS2 and HAS3 [13]. However, upon viral infections, HAS1 expression was also upregulated (Table 1). Aberrant hyaluronan synthesis and catabolism results in progression of organ fibrosis, impairment of tissue function and advances of malignancy. Growth factors, such as transforming growth factor β (TGFβ), platelet derived growth factor BB (PDGF-BB), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) as well as cytokines, such as tumor necrosis factor α (TNFα), interferon γ (INFγ), and interleukin (IL)-1β are potent stimulators of of hyaluronan production during rapid tissue remodeling. There are evidences that nuclear factor kappa-light-chain-
enhancer of activated B cells (NF-κB) can bind to the HAS2-AS and HAS2 promoters [14, 15]. Other transcriptional factors and signaling pathways are also involved in the regulation of HAS gene expressions in a cell-type- and disease-specific manner.

Hyaluronidases (HYALs) could also play an important role in the overall biological effects of hyaluronan. Hyaluronan is enzymatically degraded by hyaluronidases, including HYAL1, HYAL2, HYAL3 and PH-20 [11]. HYALs lower the amount of hyaluronan, however, they also produce hyaluronan fragments of different lengths. Fragmented hyaluronan exhibits different functions than full length hyaluronan. Thus, whereas HMW hyaluronan is anti-inflammatory, LMW hyaluronan promotes inflammation. However, the ability of HA to function as either a pro- or anti-inflammatory molecule is not only dependent on the size, but also on the microenvironment, localization, and availability of specific binding proteins [10].

Among HYALs, HYAL1 is abundant in plasma. However, HYAL1 primarily work intra-cellularly within an acidic environment [10, 12]. HYAL2 contains a glycosylphosphatidylinositol (GPI) linker and is bound to the plasma membrane. HYAL2 cleaves high-molecular-weight hyaluronan and generates fragments. A major part of the HYAL2-fragmented hyaluronan was released into the supernatants [16] in vitro. Generally speaking, the mechanism of cleavage of hyaluronan into fragments in vivo is quite complex, involving not only the HYALs, reactive oxygen species (ROS) and nitrite oxide, but also hyaluronan binding proteins (such as CD44, RHAMM and KIAA1199) and possibly the conformational change of hyaluronan itself [10]. Recently, for the purpose of adjuvant therapy of pancreatic cancer, recombinant human PH-20 (rHuPH-20) with high purity [17] has been introduced for a phase III clinical trial. Pegylated rHuPH-20 can be injected subcutaneously and show higher concentration in the bloodstream compared to the traditional formulation [18].
**Table 1. Known relationship between viruses and hyaluronan in human**

<table>
<thead>
<tr>
<th>Family</th>
<th>Virus</th>
<th>Link to hyaluronan</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retroviridae</td>
<td>Human immunodeficiency virus (HIV)</td>
<td>1. A synthetic peptide corresponding to the immunosuppressive domain of HIV-1 gp41 upregulates the expression of HAS1 in peptide-treated PBMCs 2. Knockdown of HAS2 by siRNA enhances HIV-1 replication in CD4+/CCR5+/CXCR4+ TZM-bl HeLa cells</td>
<td>1,[19] 2.[20]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low serum hyaluronic acid levels associated with spontaneous HBsAg clearance</td>
<td>[21]</td>
</tr>
<tr>
<td>Hepadnaviridae</td>
<td>Hepatitis B virus (HBV)</td>
<td>Hyaluronan increase, the severity of cirrhosis increases in HCV patients</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyaluronan increase, the manifestation of dengue virus infection get more severe</td>
<td>[23-25]</td>
</tr>
<tr>
<td></td>
<td>Hepatitis C virus (HCV)</td>
<td>Detection of E-6 antigen (HPV-16) and H11B2C2 antigen (hyaluronan binding protein) in cervical cancer tissues</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>Dengue virus</td>
<td>Among patients with Crimean-Congo hemorrhagic fever, hyaluronan is a diagnostic and prognostic marker</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>Human papilloma viruses (HPVs)</td>
<td>Hyaluronan production in synoviocytes as a consequence of EBV Infections. HAS1 activation by EBV and synthetic double- and single-stranded viral RNA analogs. EBV-treated fibroblast-like synoviocytes significantly increase hyaluronan production and release. Real time RT-PCR data show that HAS1 mRNA levels are significantly elevated in virus-treated cells, whereas mRNA levels for the genes HAS2 and HAS3 remain unchanged</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>Kaposi sarcoma-associated herpesvirus (KSHV)</td>
<td>Increased levels of HAS1 expression and hyaluronan</td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td>Respiratory syncytial virus (RSV)</td>
<td>Pre-existing RSV infection amplified bronchoalveolar lavage fluid levels of hyaluronan and airway hyperresponsiveness</td>
<td>[30]</td>
</tr>
</tbody>
</table>
1.5.2 Possible mechanisms for elevation of circulating hyaluronan levels

At least three possible mechanisms are proposed to be attributed to the increase of hyaluronan in the circulation. First, an impaired clearance or uptake by the lymphatic endothelial cells in the liver or degradation can contribute to this phenomenon. Second, the shedding of endothelial glycocalyx layer from injured endothelial cells can contribute. Third, hyaluronan can be over-produced in tissues. Clinically, we observed rapidly and robustly increased serum hyaluronan levels in patients with dengue virus infection (DVI). Our first goal was to elucidate the mechanism(s) behind this observation. The second goal was to elucidate the role of the increased circulating hyaluronan. The third goal was to explore therapeutic options to reverse the effects of sky-high levels of serum hyaluronan.

1.5.3 Hyaluronan may be a diagnostic and prognostic marker

For patients, plasma circulating hyaluronan concentrations have been shown to be markers of several different illnesses (Table 2), including rheumatoid arthritis, angiopathy of diabetes mellitus, acetaminophen-induced hepatotoxicity, viral hepatitis-related liver cirrhosis, parasitic hepatic fibrosis and several kinds of acute and chronic infectious diseases. Sepsis originating from bacteria revealed increased plasma hyaluronan levels, compared to healthy controls. Patients with bancroftian filariasis (infected by a parasite, *Wuchereria bancrofti*, which is associated with elephantiasis and microfilaraemia) also presented increased serum levels of hyaluronan, especially among those patients with active filarial infection. Within the viral etiology, there are several epidemiological studies to show that plasma hyaluronan increase in patients with dengue virus infection (DVI) compared to healthy controls or patients with other febrile illnesses. In addition, patients with Crimean-Congo hemorrhagic fever (CCHF) also presented with increased serum levels of hyaluronan. However, the mechanisms involved in this phenomenon are still unclear.

Patients with acetaminophen-induced hepatotoxicity showed very high levels of hyaluronan, i.e. more than 10 µg/ml in serum. Viral infections, such as dengue virus and CCHF, are also characterized by high serum levels of hyaluronan, i.e. several µg/ml, as are bacterial sepsis (several hundred ng/ml) and parasite infections (about one hundred ng/ml).

There are trends that “the higher levels of the circulating hyaluronan, the more severe or even mortality of the patients” for diabetic patients with angiopathy, acetaminophen-induced hepatotoxicity, dengue fever, CCHF, and septic shock patients. However, the predictive roles of circulating hyaluronan for the diseases’ severity and progress (to show a cut-off value as the
prognostic markers) of diabetic patients with angiopathy, acetaminophen-induced hepatotoxicity, dengue virus infection, and septic shock are still unclear.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Epidemiologic findings</th>
<th>Circulating hyaluronan levels</th>
<th>Possible mechanism</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver cirrhosis</td>
<td>Hyaluronan increase, the severity of cirrhosis increase in HCV patients</td>
<td>Mild cirrhosis: 28 ng/ml</td>
<td>ND</td>
<td>[22, 31, 32]</td>
</tr>
<tr>
<td>Rheumatoid arthritis (RA)</td>
<td>Patients with RA have higher level of plasma hyaluronan</td>
<td>Plasma level: 144 ng/ml</td>
<td>Hyaluronan from articular source</td>
<td>[33]</td>
</tr>
</tbody>
</table>
| Sepsis / septic shock         | Sepsis / septic shock (prognostic marker)                                              | Septic shock: 468 ng/ml (survival: 218 ng/ml; fatal: 687 ng/ml) | 1. increased HA synthesis  
2. Increased HA lymphatic outflow  
3. EGL shedding                  | [34]       |
| Peritoneal dialysis (PD)-related peritonitis | Dialysates HA peaked on Day 1; peritonitis serum HA                                      | Patients with peritonitis serum: 157 – 150 ng/ml; stable PD patients: 77.8 ng/ml | ND                                                                                 | [35]       |
| Dengue virus infection        | The higher the hyaluronan increase, the more severe manifestation of the dengue virus infection | Acute phase: 935 ng/ml (DHF)  
Critical phase: 3230 ng/ml (severe plasma leakage)  
Acute phase: 7316 ng/ml (DSS) | Impaired function of sinusoidal endothelial cells in liver, and endothelial EGL damage and shedding | [23-25] |
| CCHF                          | Crimean-Congo hemorrhagic fever (Diagnostic & Prognostic markers)                       | Survivor: 1471 ng/ml; fatal: 3018 ng/ml| Sinusoidal endothelial damage in liver                                               | [27]       |
| Parasite infection            | Patients with *bancroftian filariasis* (Diagnostic & Prognostic markers)                | Serum: 25-160 ng/ml                  | 1. Tissue damage of lymphatic tissue (worm) and EGL shedding  
2. Impaired degradation     | [36]       |
<table>
<thead>
<tr>
<th>Condition</th>
<th>Prognostic marker</th>
<th>Marker Levels</th>
<th>Notes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>A prognostic DM complications marker</td>
<td>DM: 83.6 ng/ml; healthy controls: 41.7 ng/ml</td>
<td>1. DM &amp; hyperglycemia -&gt; impair vessels and to be repaired Then over-production of hyaluronan when repairing</td>
<td>[37]</td>
</tr>
<tr>
<td>Intoxication</td>
<td>Paracetamol-induced hepatotoxicity (Prognostic marker)</td>
<td>Serum HA: 24 – 50967 ng/ml (median = 6777 ng/ml); healthy controls: 21 ng/ml</td>
<td>1. Increased production and release from damaged cells 2. Reduced clearance (by the sinusoidal cells)</td>
<td>[38]</td>
</tr>
</tbody>
</table>

DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; EGL, endothelial glycocalyx layer; ND: not determined, PD, peritoneal dialysis
1.6 Hyaluronan and CD44 interaction in cancer diseases

1.6.1 Hyaluronan – CD44 signaling

The tumor microenvironment (TME) plays a crucial role in cancer progression and metastasis, consisting of various cellular components such as tumor cells, stroma cells, and extracellular matrix (ECM). The ECM, a complex structure with diverse biochemical and biomechanical properties, serves as a reservoir for signaling molecules and influences cellular responses. It forms structures like the basement membrane and interstitial matrix, providing mechanical support for tissues and directly regulating cellular behavior. This dynamic reciprocity between cells and the ECM impacts cellular fates, including proliferation, differentiation, and migration. Hyaluronan is a prevalent component of the ECM in various tissues and fluids, playing a significant role in cellular behavior under both physiological and pathological conditions, including chronic inflammation and cancer. The molecule's negative charge at neutral pH makes it ideal for retaining water and acting as a lubricant, supporting tissue architecture. In addition, this linear molecule also possesses signaling properties when interacting with cell surface receptors.

There are several hyaluronan-binding proteins, both expressed at the cell surface and extracellularly. The most well studied are CD44 or RHAMM (also known as CD168) on chondrocytes, hematopoietic cells, epithelial and endothelial cells. We have focused our studies on hyaluronan-engaged CD44 signaling. CD44 glycoprotein isoforms are adhesion proteins that are encoded by a single gene and subjected to alternative splicing. CD44 is a signaling hub, acts as a docking place for metalloproteinases, is a co-receptor for growth factors, and is subjected to sequential proteolytic two-step cleavage releasing its cytoplasmic part which modulates transcription of CD44 and other genes (REF). CD44 cleavage occurs under physiological conditions in similarity with other cell surface receptors such as TβRI [39, 40]. In tumor tissues, the cleavage of CD44 increases most likely due to its increased levels and accumulation of MMPs and other sheddases, as well as growth factor/ cytokines and fragmented hyaluronan stimulation; both immunological and malignant diseases promote the shedding of CD44.

CD44 is a lymphocyte-homing receptor which, through its interactions with hyaluronan, leads the activated lymphocytes to inflamed tissues, and maintain tissue structure through cell-matrix and cell-cell interactions. Furthermore, CD44 modulates cancer cell invasion and adhesion to endothelium [41-45]. CD44 interacts with members of the ERM (ezrin, radixin, moesin) family, which have important functions in cytoskeletal organization and regulation of apoptosis. Hyaluronan-activated CD44 is critical in modulating the response
of fibroblasts to platelet-derived growth factor (PDGF)-BB-induced cell migration [46]. The role of CD44-HA interaction in infectious diseases differs from pathogens to pathogens [47]. Previous work in our laboratory has shown that CD44 is crucial for angiogenesis and tubular structure formation, under in vivo-like conditions [42]. Deficiency of CD44 disrupts angiogenesis in vivo, and this effect results from the loss of CD44 expression in endothelial cells. Blocking hyaluronan binding by an anti-CD44 antibody causes impaired neovascularization in mice since new vessels were unable to assemble endothelium-lined tubes adequately [48].

Hyaluronan also plays a pivotal role in maintaining tissue structure and modulating cellular functions such as adhesion, migration and proliferation through its interaction with various cell-surface receptors [49]. This multifunctionality extends to the regulation of cancer resistance mechanisms in certain species, such as the naked mole rat, where it helps to induce cyclin-dependent kinase inhibitors [50]. The synthesis of mammalian hyaluronan is orchestrated by the activity and stability of hyaluronan synthases (HAS1, HAS2, HAS3), which are differentially expressed and regulated in a tissue specific- and stimuli-dependent manner. In particular, HAS2 is often upregulated in breast cancer and is associated with tumor progression and neovascularization [51].

CD44 has been comprehensively characterized and plays a critical role in physiological processes such as hematopoiesis and lymphocyte homing. It is a highly ubiquitous molecule, expressed in a wide variety of cells, and is encoded by a single, highly conserved gene with twenty exons. The standard isoform of CD44 (CD44s) includes exons 1-5 and 16-20 in all its variants. This isoform was initially isolated from hematopoietic cells and forms the simplest version of CD44, which is predominantly expressed in mesenchymal or hematopoietic cells.

The CD44s core protein is a 37 kDa polypeptide that undergoes extensive O- and N-glycosylation, resulting in an 85-90 kDa glycoprotein. Exons 6-15 are subject to alternative splicing, leading to multiple CD44 variants (CD44v), which are more commonly expressed in epithelial cells. The N-terminal domain of the standard isoform, encoded by the first five exons, contains a 'Link' domain that binds to hyaluronan. Additionally, there is another hyaluronan binding site outside this region. The extracellular domain of CD44 is completed by exons 16 and 17, along with part of exon 5, forming the 'stem' region, which is adjacent to the cell membrane and includes sites for alternative splicing and proteolytic cleavage.

CD44 functions as a cell adhesion molecule that binds to various extracellular matrix (ECM) components, such as collagen, fibronectin, and osteopontin. It also serves as a signaling hub, interacting with cytoskeleton-related proteins and acting as a co-receptor for various kinases, thereby modulating signaling pathways crucial for cell growth, migration, and survival. Notably,
CD44 variants can enhance the binding repertoire to other glycosaminoglycans (GAGs) like chondroitin sulfate and heparin.

In cancer biology, CD44 expression has been associated with tumor progression and is considered a marker for cancer stem cells (CSCs). It promotes epithelial-mesenchymal transition (EMT) and has been implicated in the regulation of signaling pathways involved in tumor growth and metastasis. For instance, CD44 has been linked to the modulation of receptor tyrosine kinases (RTKs) and serine/threonine kinase receptors, influencing cell signaling in various cancers including those of the bladder, liver, lungs, and pancreas.

CD44 is a multifunctional glycoprotein involved in cell adhesion and signaling across various physiological and pathological contexts. Its role in cancer progression underscores its potential as a therapeutic target for intervention strategies aimed at modulating its expression or function.

1.6.2 Regulation and function of HAS2 in cancer

The three members of hyaluronan synthases (HAS1, HAS2, HAS3) are multi-transmembrane proteins that catalyze hyaluronan synthesis in their cytoplasmic part. Hyaluronan synthesis occurs in almost all vertebrate cells and is stimulated by growth factors (GFs) and cytokines, including PDGF-BB, TGFβ, and tumor necrosis factor alpha (TNFα). HAS activity depends on factors such as substrate availability and post-translational modifications. Mono-ubiquitination and dimerization of HAS2 are necessary for hyaluronan synthesis, and enzymes like USP4 and USP17 are involved in regulating this process. Hyaluronan biosynthesis is elevated in pathological conditions, and the activity of enzymes involved in these processes needs to be tightly regulated. In cancer progression, HAS2 plays a crucial role in breast cancer cell invasion and positively regulates the expression of tissue inhibitor of metalloproteinases-1 (TIMP-1). The interaction between HAS2-synthesized hyaluronan and its cell surface receptor CD44 is important for the adhesion of these cells to microvascular endothelium. HAS2 is also involved in epithelial-mesenchymal transition (EMT) and the maintenance and expansion of cancer stem cells (CSCs). The hyaluronan-CD44 axis affects metabolic processes and contributes to the Warburg effect in cancer cells. Additionally, metabolic reprogramming in hyaluronan-overproducing breast cancer cells is a potential mechanism for maintaining cancer stem-like properties.

1.6.3 TGFβ-induced EMT and CD44-hyaluronan cooperation

Our previous research has demonstrated that HAS2 is important for the transforming growth factor β (TGFβ)-mediated epithelial-mesenchymal transition (EMT), a critical process in development that is often hijacked during cancer progression to enhance invasiveness and resistance to chemotherapy [52]. In
the context of stem cell biology, cells that have undergone EMT acquire stem cell-like properties, and in metastatic breast cancer, induction of HAS2 is crucial for the interaction between cancer stem cells (CSCs) and their microenvironment, promoting CSC self-renewal and stromal cell activation [53]. Dual role of TGFβ as a tumor suppressor and promoter in cancer progression is mediated through complex signaling pathways involving TβRI and TβRII receptors and their downstream SMAD and non-SMAD signaling pathways [54]. Furthermore, TGFβ co-operates with RAS, involving ΔNp63 transcription, enhancing cancer progression [55]. TGFβ signaling also converges with EGF, preferentially in HER+ and EGFR+ breast cancer cells, promoting invasion [56].

Several studies have demonstrated that HAS2 plays a prominent role in the invasion of breast cancer cells [57, 58], by reversely modulating the expression of the tissue inhibitor of metalloproteinases-1 (TIMP-1) [59]. Interestingly, HAS2-deficient mice are embryonically lethal because of severe defects in the migratory capacity of endocardial cells during heart development [60]. Cells undergoing EMT are enriched with stem cell properties [61], and HAS2-synthesized hyaluronan and CD44 are important for the self-renewal capacity of cancer cells [53, 62, 63]. Furthermore, the regulation of the HAS2 gene by the natural antisense transcript HAS2-AS1 adds an additional layer of complexity to its expression control, with implications for fibrosis, cancer invasiveness, and potentially other pathologies [64]. However, the specific roles of HAS2-AS1/HAS2-AS in breast cancer remain unclear. We aimed to elucidate the roles of HAS2-AS, HMGA2, HAS2, and CD44 as significant mediators of tumorigenic effects of TGFβ in breast cancer.

1.6.4 Role of iASPP in p53-mediated apoptosis

The inhibitor of apoptosis-stimulating protein p53 (iASPP) was initially described as an inhibitor of NF-κB subunit p65 transcriptional activity [65]. It belongs to the ASPP family, consisting of ASPP1 and ASPP2 which bind to p53 and aid the transcriptional induction of proapoptotic genes, whereas iASPP represses the apoptotic function of p53 [66, 67]. ASPP1 and ASPP2 cooperate synergistically with p300 to enhance p53 transcriptional activity, whereas iASPP-mediated inhibition of p53 was less affected by its interaction with p300 [68]. Of note, the CD44ICD translocation to the nucleus activates transcriptional responses by cooperation with CREB-binding protein (CBP)/p300 [69]. Interestingly, iASPP's interaction with p53 is complex; it can inhibit or promote apoptosis depending on the context and is involved in the regulation of autophagy [70]. iASPP is overexpressed in a variety of cancers and accumulates in the nucleus of aggressive prostate cancer cells and melanomas, and is associated with poor patient outcomes [71]. iASPP is an oncoprotein that cooperates with other oncogenic-inducing molecules such as RAS, E7 and wild-type p53 to transform cells. Previous studies in our group
revealed that the cytoplasmic domain of CD44 interacts with iASPP, however, the functional significance of iASPP-CD44 complexes remains in its infancy [72].

p53 is the first suppressor gene to be identified and key mediator of cellular responses [73, 74]. p53 mutations are the most common changes during tumor progression and its mutant status is correlated with elevated CD44 expression [75]. Most interestingly, p53 binds to a noncanonical p53-binding sequence in the CD44 promoter, repressing \(CD44\) expression [74]. Around 12x10^6 people live with cancers containing wt p53, but non functioning. Whether blocking the entrance of iASPP in the nucleus most likely hijacks p53 suppressing its proapoptotic function, is an approach of great therapeutic interest.
2 Present studies

2.1 Present investigation (1) – High levels of serum hyaluronan is an early predictor of dengue warning signs and perturbs vascular integrity

2.1.1 Methods
The clinical study protocol of patient enrolment criteria, diagnostic procedures, and clinical definitions received approval from the IRB of Kaohsiung Medical University Hospital, ensuring ethical standards were met. Informed consent was obtained from all participants who were adults (≥20 years) presenting with dengue fever symptoms during the specified outbreak periods. Patients provided blood samples initially and on the third and seventh days of their hospital stay, if applicable, to minimize recall bias and to track the progression of the disease.

Only patients who provided multiple samples were included in the study. In addition to dengue patients, individuals with gram-negative bacilli (GNB) and gram-positive cocci (GPC) bacteremia, as well as those with influenza A virus (Flu A) infection confirmed by nasopharyngeal swab, were enrolled for comparative analysis. Control subjects included healthy adults undergoing routine health checks at the hospital.

Laboratory confirmation of dengue virus infection was carried out at the CDC in Taiwan, using NS1 antigen detection and real-time RT-PCR. Serotyping was performed with specific primers and probes. The distinction between primary and secondary dengue infections was made based on IgG/IgM antibody ratios according to WHO guidelines.

Clinical severity was categorized following the WHO's 2009 guidelines into three levels: without warning signs, with warning signs, and severe dengue. Daily evaluations of hospitalized patients were based on specific symptoms and signs listed in the guidelines. Ultrasound confirmed clinical fluid accumulations.

Plasma cytokine and NS1 levels were quantified using commercial kits. Cell cultures included human dermal fibroblasts and TIME cells, which were tested for mycoplasma contamination and used in various assays.

RNA isolation and real-time RT-PCR analysis were performed on cells cultured under different conditions to assess gene expression levels. Hyaluronan levels in patient serum and conditioned media from cell cultures were
also measured, though details of this process are omitted from the summary provided.

2.1.2 Main results

**Aberrant increase of serum hyaluronan characterizes moderate-to-severe dengue virus infection**

In our study of 250 dengue-infected patients, it was found that serum hyaluronan levels increased significantly during the febrile phase and remained elevated during the recovery phase. These elevated levels correlated with increased hematocrit and low platelet counts. Dengue patients exhibited significantly higher hyaluronan levels compared to patients infected with other agents such as gram-negative bacilli, gram-positive cocci, and influenza A virus. Additionally, high plasma NS1 levels in dengue patients correlated with higher circulating hyaluronan levels.

**Increased serum hyaluronan level is an early biomarker for development of warning signs for severe dengue**

Among 108 dengue patients, it was found that elevated serum hyaluronan levels during early infection were independently predictive of the occurrence of warning signs and severe dengue fever. Our study also revealed that high levels of the viral protein NS1, which indicate disease severity, correlated with increased concentrations of serum hyaluronan. Furthermore, the serum hyaluronan level was identified as a useful biomarker for predicting the occurrence of warning signs throughout the entire course of the disease, suggesting its potential for determining which dengue patients may require hospitalization. These findings underscore the importance of serum hyaluronan as an early predictor of dengue severity and its potential impact on vascular integrity.

This result highlights the potential significance of serum hyaluronan levels as a prognostic biomarker for dengue fever, particularly in identifying patients at risk of developing severe manifestations of the disease. The correlation between hyaluronan levels and disease severity underscores its potential as a valuable tool in guiding clinical decision-making and resource allocation for dengue patients.

**Exposure to dengue protein NS1 differentially modulates hyaluronan biosynthesis and CD44 expression in dermal fibroblasts and microvascular endothelial cells**

We found that treatment with the viral NS1 protein stimulated the expression of HAS1, HAS2, and HAS3 in dermal fibroblasts and endothelial cells. This treatment also led to an increase in hyaluronan concentrations in the cell culture media, particularly in fibroblasts. Our study suggests that the NS1 protein plays a role in promoting the synthesis of hyaluronan, particularly through the
induction of \textit{HAS2} transcription and subsequent increased hyaluronan production.

\textbf{Organization of hyaluronan matrix around human dermal fibroblasts and microvascular endothelial cells}

We compared the organization of hyaluronan matrices in dermal fibroblast and \textit{TIME} cell cultures in response to \textit{NS1} treatment. Interestingly, in \textit{NS1} treated fibroblast cultures, hyaluronan matrices formed a fibrous network spanning multiple cells, in comparison to untreated cultures. Hyaluronan-rich fibrous cables, longer than 20 \textmu m weaving over cell areas and higher hyaluronan intensity, were predominantly seen in \textit{NS1}-treated fibroblast cultures, compared to untreated control cells. Hyaluronan-rich fibrous network structures were formed to a much lesser extent around \textit{TIME} cells compared to fibroblasts; instead the hyaluronan-based matrices in microvascular endothelial cultures exhibited dot-like structures. The specificity for hyaluronan staining was verified by saturation of \textit{b-HABP} with excess of hyaluronan prior its addition, leading to abrogation of hyaluronan staining.

\textbf{Macrophage-like cell adhesion to \textit{NS1}-induced hyaluronan-rich structures is mediated by CD44}

In an effort to understand the increased immune response associated with dengue infection, we investigated how PMA-differentiated THP-1 macrophage-like cells adhered to hyaluronan matrices surrounding dermal fibroblasts and microvascular endothelial cells. We observed an increase in adhesion of CD44 expressing macrophage-like cells onto hyaluronan cables and hyaluronan-rich dot-like structures around \textit{NS1}-treated fibroblasts and endothelial cultures, respectively. Blocking of CD44-hyaluronan interactions with Hermes 1 antibodies resulted in about 30\% and 70\% suppression of macrophage adhesion to \textit{NS1}-treated dermal fibroblasts and endothelial cells, respectively. These findings support the notion that \textit{NS1}-induced hyaluronan matrices enhance the recruitment of inflammatory cells.

\textbf{\textit{NS1}-induced impairment of vessel-like networks correlates to increased levels of hyaluronan and CD44 suppression}

\textit{TIME} cells grown on Matrigel differentiated to tubular structures within 16 h, forming a network. Because hyaluronidase-dependent hyaluronan fragmentation and CD44 expression \cite{42, 76} are involved in the differentiation of microvascular endothelial cells, we investigated the effect of \textit{NS1} treatment on expressions of hyaluronan synthases and hyaluronidases, as well as \textit{CD44}, during tubular morphogenesis of \textit{TIME} cells. CD44 can occur as a standard form as well as several variant forms as a result of differential splicing; however, the \textit{TIME} cells contain almost exclusively the standard CD44 isoform. Interestingly, the transcriptional activities of \textit{HAS1}, 2, 3, and \textit{HYAL1}, as well as the constitutively highly expressed \textit{HYAL2}, were significantly induced after
treatment of the vessel-like structures for 9 h with NS1. Importantly, NS1 treatment significantly suppressed the expression of the \textit{CD44} transcripts during endothelial differentiation. In accordance, the protein levels of HAS2 and HYAL2 were markedly increased, whereas that of CD44 was decreased by about 30%, after 9 h of NS1 treatment of the endothelial cell vessel-like structures. Interestingly, exposure of differentiated TIME cell to NS1 resulted in the production of increased levels of fragmented hyaluronan in the conditioned medium.

**Dengue virus-mediated activation of circulating pro-inflammatory cytokines contribute to the increased hyaluronan levels**

Examination of immune responses among 108 patients, revealed significant increases in the levels of several cytokines already during the early febrile phase, that was significantly further increased during the critical phase. The MCP-1 and IL-6 levels increased initially during the febrile phase and then decreased during the critical phase, whereas the levels of TGFβ, TNFα, IL-8 and IL-10 increased significantly during the febrile phase of dengue infection, and remained high during the critical phase.

Because TGFβ and TNFα are potent stimulators of HAS2-synthesized hyaluronan in several cell types [77, 78], we investigated the effects of these mediators on NS1-induced hyaluronan production in fibroblast and endothelial cultures. The analysis demonstrated that fibroblasts stimulated with TGFβ or TNFα in combination with NS1 exhibited about 2-fold and 4-fold increases in \textit{HAS2} expression, respectively, compared to cell cultures treated with NS1 only. In TIME cells, TNFα stimulation also significantly increased the NS1-induced \textit{HAS2} expression, however, TGFβ did not. In addition, we investigated the effects of TGFβ and TNFα alone or in combination with NS1 on the expression levels of \textit{CD44} and \textit{TLR4}, encoding a receptor for NS1, in these cultures. Neither TGFβ nor TNFα, alone or in combination with NS1, significantly affected \textit{CD44} mRNA expression, whereas TNFα in combination with NS1, synergistically induced the expression of \textit{TLR4} in fibroblast cultures. In microvascular endothelial cultures, NS1 alone markedly induced the expression of \textit{TLR4}, which was partially decreased when NS1 was combined with TGFβ or TNFα. Neither TGFβ nor TNFα, alone or in combination with NS1, significantly affected the transcription of \textit{CD44} in endothelial cells. The hyaluronan levels increased significantly in both fibroblasts and endothelial cell cultures treated with TGFβ and TNFα in combination with NS1. Thus, circulating cytokines induced during dengue virus infection further enhanced the hyaluronan production induced by NS1.

**Signaling pathways involved in NS1-induced hyaluronan production**

To gain insights into the signaling pathways evoked by NS1 which can mediate increased hyaluronan production and signaling, we investigated the activation status of key signaling molecules in fibroblast and endothelial cultures.
Whereas NS1-treatment of fibroblasts did not affect the NF-κB signaling pathway, in endothelial cultures an increase in NF-κB activation was observed within 15 min of NS1 treatment that lasted, with a slight decrease, for up to 4 h. Other signaling pathways, including Akt, and Erk1/2 and p38 MAP-kinases, were activated within 15 min after NS1-treatment, followed by a similar attenuation pattern, both in fibroblasts and endothelial cultures.

### Hyaluronan-CD44 interactions regulate NS1-mediated endothelial hyper-permeability

Recently, we demonstrated a critical role of the hyaluronan/HYAL2/CD44 axis in normal vessel formation [42]. Therefore, we investigated the effect of CD44 and hyaluronan on the integrity of untreated and NS1-treated confluent endothelial cell monolayers, using an *in vitro* endothelium permeability assay. Confluent monolayers not exposed to NS1 exhibited a basal permeability, which was increased by about 3-fold when hyaluronan was added to the culture media or when cells were depleted of CD44. Treatment with NS1 increased the permeability by about 2-fold compared to non-treated cells. Importantly, hyaluronan stimulation, or silencing of CD44, further enhanced the NS1-induced endothelial permeability. Interestingly, if confluent endothelial cell monolayers were pre-treated by Hermes 1 antibody, which specifically blocks the binding of hyaluronan to CD44, before NS1-treatment, or if the cells were treated with *Streptomyces* hyaluronidase together with NS1, the endothelial hyper-permeability was prevented. These observations suggest a key regulatory role of hyaluronan-engaged CD44 in NS1-induced vascular hyper-permeability.

The NS1-induced endothelial permeability was accompanied by an increase in the number and size of paracellular gaps, as monitored by immunofluorescence staining. In response to stimulation with hyaluronan or knock-down of CD44, morphological changes occurred leading to formation of paracellular gaps, and enlargement of NS1-induced gaps, which was reverted by Hermes1 antibodies or treatment with *Streptomyces* hyaluronidase.

CD44-mediated regulation of vascular function occurs partly through modulation of VE-cadherin in adherens junctions [79, 80]. Under basal conditions, VE-cadherin was distributed in a striped or jagged fashion delineating confluent and resting endothelial cells, and colocalized with F-actin. There was no significant change in the total VE-cadherin protein expression levels in NS1-treated cells, with or without combination with hyaluronan or Hermes1 pre-treatment, compared to non-treated endothelial cells, neither did depletion of CD44 affect its expression, suggesting that hyaluronan/CD44 does not affect VE-cadherin expression. Importantly, stimulation with NS1 or hyaluronan, or CD44 depletion, caused re-distribution and fragility of VE-cadherin. Immunofluorescence analysis of TRITC-phalloidin-stained endothelial cells revealed that hyaluronan stimulation induced both longer and thicker F-actin stress fibers compared to control cells; CD44 depletion also promoted the
formation of longer and thicker F-actin stress fibers. Notably, a co-localization of VE-cadherin with the ends of F-actin stress fibers at sites of cell-cell junctions, was observed. Our observations support the notion that NS1, or treatment with hyaluronan or CD44 suppression, affects VE-cadherin and cytoskeletal associations, modulating endothelial-endothelial intercellular junctions.
2.2 Present investigation (2) – Has2 natural antisense RNA and Hmga2 promote Has2 expression during TGFβ-induced EMT in breast cancer

2.2.1 Methods
Namru mammary epithelial NMuMG cells [117], and tumorigenic Py2T [118], 4 T1 [119] and EpRas [120] mouse mammary cells were cultured in high glucose DMEM (Life Technologies Europe BV, Stockholm, Sweden), supplemented with 10% fetal bovine serum (FBS). The experimental method used in study II was involving HA-tagged Hmga2 overexpressing clones of NMuMG cells. Those methods include cell transfection, viral transduction, immunoblotting, RNA extraction and real-time qPCR, chromatin immunoprecipitation (ChIP), nuclear and cytoplasmic RNA fractionation, and phase contrast microscopy and immunofluorescence for visualization. The study involved various molecular and cellular biology techniques to investigate the effects of TGFβ1 stimulation and other experimental manipulations on gene expression, protein levels, and cellular localization.

2.2.2 Main results

**HAS2 expression in human invasive breast carcinomas correlates to HAS2-AS1, HMG A2, TGFβ1 expression and poor prognosis**
Our recent observations that TGFβ-dependent HAS2 expression is important for efficient TGFβ-induced mammary EMT [95], implies a novel function for HAS2. In an effort to resolve the mechanism of TGFβ-mediated regulation of HAS2 mRNA, we analyzed TCGA gene expression data. In the 20% of invasive breast carcinoma cases where HAS2 was over-expressed, HAS2 mRNA was highly correlated to the expression levels of HAS2-AS1, HMG A2 and TGFβ1 with Spearman's correlations of 0.74, 0.43 and 0.46, respectively [121, 122]. Interestingly, a decrease in survival was observed in patients with invasive breast carcinomas with high expression levels of HAS2, HAS2-AS1 and HMG A2. We depicts the gene organization of Has2 and cis-encoded Has2as, which shares complementary sequences with Has2 exon 1; through alternative splicing a short (S) or two longer (L1 and L2) Has2as transcripts are generated.

**TGFβ-dependent induction of Has2, Has2as and Hmg a2 transcripts in normal and malignant mammary epithelial cells**
In normal mammary epithelial NMuMG cells, TGFβ potently induced Has2, Has2as and Hmga2 transcripts. In the malignant Py2T cells the relatively high transcript levels of Has2, Has2as and Hmga2 were further enhanced by TGFβ. Similar expression patterns were also observed in malignant EpRas and 4 T1 cell cultures. In agreement with the response of Has2 to TGFβ stimulation,
Has2-mediated hyaluronan synthesis was enhanced in normal mammary cells, as well as in the cancer cell lines Py2T and EpRas, although not in 4 T1 cells. Thus, the induction of Has2, Has2as and Hmga2 transcripts occurs simultaneously in response to TGFβ in NMuMG cells, and in the three aggressive breast cancer cell lines examined.

**Has2as and Hmga2 mediate the transcriptional induction of Has2 by TGFβ**

The role of Has2as and Hmga2 in TGFβ-mediated transcriptional induction of Has2 was investigated in non-tumorigenic and malignant mammary cells. Knocking down Has2as or Hmga2 led to a significant suppression of TGFβ-induced Has2 expression, resulting in diminished hyaluronan production in both cell types. Additionally, Hmga2 overexpression was found to promote the expression of Has2 and all three Has2as transcripts, with TGFβ stimulation further increasing Has2 expression and hyaluronan production. The study also revealed that the TGFβ-mediated induction of Has2, Has2as, and Hmga2 involves both Smad-dependent and Smad-independent pathways, as inhibition of TβRI, MEK1, and p38 kinases strongly suppressed their induction.

These findings provide insights into the regulatory mechanisms involved in TGFβ-mediated transcriptional induction of Has2, shedding light on the roles of Has2as and Hmga2 in this process. The involvement of both Smad-dependent and Smad-independent pathways underscores the complexity of the regulatory network governing these molecular events.

**Has2as/Has2 and Hmga2 promote TGFβ-mediated EMT downstream of Cd44 in a Cd44–dependent manner**

We investigated the role of Has2as in TGFβ-mediated EMT in both non-malignant NMuMG and malignant Py2T cells. Depletion of Has2as resulted in the suppression of TGFβ-induced EMT, as evidenced by reduced expression of EMT markers and maintenance of an epithelial phenotype. Additionally, down-regulation of Snai1, Hmga2, and fibronectin (Fn1) was observed in Has2as-depleted cells, further emphasizing the importance of Has2as in efficient TGFβ-mediated EMT.

Furthermore, the study explored the involvement of Cd44 and Hmmr in the expression of Has2as/Hmga2/Has2 during TGFβ-mediated EMT. The findings revealed that Cd44, but not Hmmr, significantly influenced the TGFβ-mediated EMT phenotype and the transcriptional induction of Has2, Has2as, Fn1, Snai1, and Hmga2. Knockdown of Cd44 led to a strong inhibition of the TGFβ-induced EMT phenotype and a decrease in hyaluronan synthesis.
Hyaluronan-mediated migration is promoted by Akt, Erk and Snail signaling

We examined the effect of Has2as knock-down on cell migration and its relationship with hyaluronan production. Cells with Has2as knock-down exhibited reduced migration, and TGFβ-stimulation had no effect on cell motility under these conditions. Inhibition of hyaluronan synthesis and MEK1 also resulted in decreased cell motility. Rescue experiments using human HAS2 cDNA transfection in Has2as-deprived cells restored motility, indicating that Has2as promoted migration via induction of HAS2 and production of hyaluronan. Additionally, the study investigated the activation of key regulatory molecules triggered by hyaluronan signaling and found that disruption of Has2as led to decreased phosphorylation of Akt and Erk1/2 MAP-kinase. Re-expression of HAS2 restored the activation of Akt and Erk1/2, and increased the protein levels of the transcription factor Snail, demonstrating the importance of HAS2 for cell migration and Snail regulation.

Subcellular localization of Has2as expression and effect on Has2 mRNA levels

Because the functions of natural antisense transcripts and other lncRNAs are associated with their subcellular localization [127], we performed nuclear and cytoplasmic fractionation. The analysis revealed a predominantly nuclear (56%) localization of Has2as S, but a mainly cytoplasmic localization of Has2as L1 and L2 (>60%).

In order to explore the mechanisms by which Has2as regulates Has2 in Py2T cells, we investigated whether Has2as affected the stability of Has2 mRNA. The decrease of Has2 mRNA levels, after Act D treatment, was not affected by the absence or presence of Has2as. Thus, Has2as RNA does not significantly affect Has2 mRNA stability, rather it is likely that interactions between Has2as RNA and the Has2 gene occur.

Has2as recruits Smad2/3, but not Hmga2, during TGFβ-mediated Has2 transcriptional induction

We explored the link between Hmga2 and Has2 further by employing the mapper 2.0 software [128, 129], predicting potential binding elements for chromatin regulators and transcription factors. We identified Hmga2-binding elements (HBE) and Smad-binding elements (SBE) in the Has2 gene locus, both exhibiting high relative scores (>3). ChIP analyses using antibodies against Smad2/3 or Hmga2, identified new target sequences both upstream and downstream of the Has2 translational start site (TSS) in mouse mammary NMuMG and Py2T cells. Maximum binding of Smad2/3 occurred within 12 h of TGFβ treatment, at the predicted SBEs; the murine Serpine1 (encoding Pai1) promoter known to contain SBEs [130] served as a positive control. TGFβ is known to enhance the expression levels of Hmga2 in NMuMG cells.
[131]; it also significantly promoted Hmga2 binding to Has2. Interestingly, Has2as knockdown diminished the TGFβ-induced binding of Smad2/3 to Has2 nearly down to the basal level, independent of Smad2/3 activation status. The binding of Hmga2 to the −2.3 kb, and −250 regions of the Has2 promoter was independent of both TGFβ and Has2as expression. However, its binding to the +500 region of Has2 was dependent on TGFβ stimulation but not on Has2as expression.

**Has2as contributes to the maintenance of self-renewal capacity**

Because activation of HAS2 and excessive hyaluronan production promotes the CSC phenotype [132], we investigated the importance of Has2as for CSC features, using a sphere-forming assay. Py2T clones depleted of Has2as and stimulated with TGFβ created fewer and smaller spheres compared to wild-type Py2T cells, demonstrating that Has2as is important for the CSC phenotype, with a self-renewal ability.

The expression of major stem cell markers, such as Oct4, Sox2 and Nanog has been correlated to expansion of CSCs [133], therefore we assessed the expression of the stem cells markers in Py2T cells expressing Has2as, or not. Significant suppression of the Oct4 mouse homolog Pou5f1, Sox2 and Nanog expression were observed in Has2as-depleted cells compared to the control cells; also the pluripotency stem cell transcription factor gene Rex1 mouse homolog Zfp42 was decreased. Thus, Has2as is needed for the maintenance of stem cell transcription factors and breast cancer stemness.
2.3 Present investigation (3) – Hyaluronan-induced CD44-iASPP interaction affects fibroblast migration and survival

2.3.1 Methods

In order to study the role of CD44 and iASPP in cell biology, with a focus on understanding their functions in cancer cell growth and response to treatment, we used several molecular biology techniques to manipulate the CD44 gene. The CD44 standard cDNA was cloned into the pMSCV hygro vector using BglII and XhoI restriction sites. Various point mutations were introduced into CD44 using site-directed mutagenesis, and specific sequences were altered to study their effects on protein function, such as the putative nuclear localization signal (NLS) and the ankyrin binding domain. Additionally, a CD44 mutant lacking the 40 C-terminal amino acids was created.

The iASPPwt-v5 construct was received from Professor Xin Lu for further study. Various cell lines, including HEK293, MCF7, and others, were cultured under standard conditions and used for experiments. Cells were subjected to different treatments to stimulate certain pathways and then lysed for analysis.

Transfections were performed with wild-type and mutant CD44 and iASPP constructs to study their effects. Specific antibodies were used for immunoprecipitation and immunoblotting to analyze protein interactions and expression.

2.3.2 Main results

The Standard Isoform of CD44 Binds to iASPP in Mesenchymal and Epithelial Cells

We previously showed that an immobilized C-terminal part of CD44 bound iASPP in a cell-free system [72]. Here, we evaluated their interaction in living cells. We first determined the expression of CD44, iASPP, and p53 by immunoblotting of cell lysates from normal and transformed cells of both mesenchymal and epithelial origin, including AG1523 fibroblasts, immortalized hTERT-BJ fibroblasts, immortalized normal mammary epithelial cells MCF10A, metastatic breast cancer cells MCF-7, HEK293 cells, non-small cell lung carcinoma cells H1299, osteosarcoma U2OS, primary dermal fibroblasts MTS64, and hepatocellular HepG2 cells. We observed that iASPP was abundantly expressed in H1299 and HepG2 cells, while low expression was evident in MCF-7 and MTS64. The CD44 standard (CD44s) isoform was expressed in all cell types, abundantly in H1299, MTS64, hTERT-BJ, and AG1523, and at low levels in MCF-7, HEK293T, and HepG2 cells. CD44 variant isoforms (CD44v) were observed in U2OS, H1299, and MCF-7. p53 was expressed at high levels in HEK293 and HepG2 cells and at lower level
in H1299 cells. Two isoforms of p53 (P72 and R72) were expressed in MCF-7 cells and HEK293. To determine whether CD44 and iASPP interact, cell lysates from AG1523, hTERT-BJ, MCF10A, HepG2, MTS64, and H1299 cells were subjected to immunoprecipitation (IP) using an anti-iASPP antibody and IgG isotype as control, followed by immunoblotting (IB) with antibodies against CD44 and iASPP; complexes between iASPP and CD44 standard isoform (CD44s), but not with the variant form (CD44v), were seen in all cell types but at different amounts.

Hyaluronan Enhanced the Amount of iASPP-CD44s Complexes in Mesenchymal Cells but Not in Epithelial Cells
Stimulation with high molecular weight hyaluronan (HMW HA; Mw $1 \times 10^6$) enhanced the formation of an iASPP-CD44 complex in mesenchymal (hTERT-BJ) cells but not in epithelial (MCF10A) cells, suggesting that CD44-iASPP complex formation may be induced differently in different cell types in response to external stimuli. The hyaluronan-mediated increase in the interaction between iASPP and CD44 was also confirmed in situ by proximity ligation assay.

Characterization of the Epitopes Involved in the Interaction between CD44 and iASPP
The phosphorylation status of CD44 affects its association with the cytoskeleton and cell migration. Different mutations in CD44 influence its interaction with iASPP. Overexpression of p53 reduces CD44-iASPP complex formation. In silico analysis suggests that full-length CD44 might interact with the C-terminus of iASPP, where the ankyrin domain is located.

The Level of p53 in hTERT-BJ Cells Affects the Formation of the iASPP-CD44 Complex
Next, we addressed the question how the expression of p53 influenced the interaction between iASPP and CD44. Overexpression of p53 in hTERT-BJ cells strongly suppressed the complexes between CD44 and iASPP and considerably repressed the expression of CD44. To further investigate a possible role of p53 in iASPP-CD44 complex formation, we depleted hTERT-BJ cells of p53 using siRNA or treated cells with Nutlin, which inhibits MDM2-p53 interaction leading to stabilization of p53. Interestingly, a stronger interaction between iASPP and CD44 was observed in cells in which p53 had been knocked-down, compared with Nutlin-treated cells expressing high levels of p53.

CD44 Expression Modulates the Sub-Cellular Localization of iASPP-p53 Complexes
iASPP and p53 are found in both the nucleus and cytoplasm. The nuclear expression of iASPP and p53 may promote tumor progression. Silencing CD44
led to increased cytoplasmic localization of iASPP and p53, as well as an increase in iASPP-p53 complexes in the cytoplasmic fraction and a decrease in the nuclear fraction. This suggests that CD44 affects the cellular localization of iASPP and p53, as well as the formation of iASPP-p53 complexes.

**Differential Expression of CD44, iASPP, and p53 in Different Tumor Types**

Two types of gene interaction, co-occurrence and mutual exclusivity, impact somatic alterations in tumor growth. In invasive breast carcinoma, CD44-iASPP and CD44-p53 were mutually exclusive, while in liver hepatocellular carcinoma, they co-occurred with positive co-expression. Unaltered co-expression of CD44, iASPP, and p53 favored patient survival in certain cancers, while altered expression was associated with patient survival in others. Notably, CD44, iASPP, and p53 did not significantly impact survival in lung squamous cell carcinoma and adult soft tissue sarcoma.

**CD44 Suppresses Cell Growth**

CD44 promotes cell survival, while iASPP inhibits the apoptotic transactivation potential of p53. Depletion of CD44 in non-immortalized human fibroblasts led to faster cell growth and increased cell proliferation, with a decrease in p53 expression and an upregulation of p27. CD44 was found to suppress the growth and survival of these cells. Additionally, external stimuli such as FBS and PDGF-BB enhanced the interaction between CD44 and iASPP, while TGFβ stimulation did not have a significant effect.

**iASPP Is Required for Hyaluronan-Activated CD44-Induced Fibroblast Migration**

To address the role of iASPP in hyaluronan-induced CD44-dependent cell migration, we studied the effect of silencing iASPP on migration of hTERT-BJ cells, treated or not, with hyaluronan or PDGF-BB. iASPP-silenced cells were unable to migrate in response to hyaluronan. Notably, PDGF-BB-mediated cell migration was only partly dependent on iASPP and was independent of CD44. Moreover, the addition of Hermes 1 antibodies, which block the binding of hyaluronan to CD44, suppressed hyaluronan-induced cell migration to control levels but had no effect on PDGF-BB-induced migration. Thus, iASPP is needed for hyaluronan-engaged CD44-induced, but not for PDGF-BB-induced, migration of hTERT-BJ fibroblasts.

**p53 Is Required for CD44-Dependent Cell Adhesion**

We then investigated the role of hyaluronan-engaged CD44 in AG1523 cells for the adhesion of cells to hyaluronan. Cells depleted of iASPP anchored to a hyaluronan substratum more strongly than cells expressing iASPP, suggesting that iASPP binding to the C-terminus of CD44 may decrease the binding of CD44 to ERM proteins and prevent actin polymerization and, thus, reduce
cell adhesiveness. Treatment with the blocking antibody Hermes1 completely abolished cell adhesion, demonstrating a central role of hyaluronan binding to CD44 for fibroblast adhesion. Importantly, p53-depleted cells exhibited a reduced adhesiveness independently of the external stimuli, suggesting a role of p53 in CD44-dependent fibroblast adhesion.

**CD44 Increases p53-Mediated ROS Production**

Given that ROS inhibited the proliferation of normal cells in vitro through replicative senescence [186] and that the NADPH oxidase-generated ROS regulates the expression of CD44 [187], we investigated the role of CD44 and iASPP in the induction of ROS.

We transiently transfected CD44wt and iASPPwt-v5, individually and in combination, in HEK293 cells expressing high level of endogenous p53wt but negligible CD44s and moderate level of endogenous iASPP. We noticed that CD44wt, iASPPwt-v5, and endogenous p53wt formed complexes and that the ectopic transfection of iASPPwt-v5 in the presence and absence of CD44wt reduced ROS production significantly. However, CD44wt expression elevated the p53-induced ROS level in the absence of iASPP in HEK293 cells.
3 Discussion and future perspectives

3.1 Discussion

**High levels of serum hyaluronan is an early predictor of dengue warning signs and perturbs vascular integrity**

Our first research establishes that the rise in hyaluronan levels in the bloodstream, driven by the dengue virus NS1 protein, serves as an early indicator for the onset of warning signs in dengue infection. This increase is linked to enhanced permeability of blood vessels due to disruptions in the interactions between CD44 and hyaluronan, as well as changes in the organization of F-actin stress fibers and VE-cadherin. The NS1 protein levels in dengue patients' blood can vary from 0.02 to 15 μg/ml, with higher levels signifying more severe disease outcomes. Although circulating NS1 is recognized for its central role in dengue pathology, understanding the mechanisms behind severe vascular leakage has been challenging due to the absence of an animal model that replicates the transient capillary permeability syndrome observed in patients.

Previous studies have noted a surge in serum hyaluronan during the later stages of infection and a possible link to the severity of dengue, but the molecular underpinnings remained unclear. Our expanded research on a larger group of patients enrolled early in their infection confirms that hyaluronan levels above 70 ng/ml are an independent predictor for the development of warning signs and more severe forms of dengue fever. Our findings also reveal that NS1 does not alter VE-cadherin amounts in endothelial cells but does cause a reorganization that disrupts cell-cell junctions, increasing gaps between cells. This effect seems to be mediated by altered CD44-hyaluronan interactions, as similar structural changes are observed with hyaluronan stimulation or CD44 knockdown.

Further investigation showed that NS1 influences genes related to hyaluronan biosynthesis and signaling. Fibroblasts increased their hyaluronan production by 5-10 times compared to endothelial cells, suggesting a significant contribution to elevated hyaluronan levels during infection. NS1 exposure also led to different hyaluronan-rich matrix formations in cell cultures, attracting immune cells and potentially affecting tissue immunological properties. Our study also delved into the roles of CD44 in endothelial cell differentiation and tubulogenesis, with findings suggesting that CD44 interactions with hyaluronan are crucial for these processes. Moreover, NS1's induction of enzymes
for hyaluronan synthesis and degradation may contribute to the disorganization of the endothelial glycocalyx layer.

Cytokines from monocytes/macrophages, T cells, and endothelial cells play a critical role in dengue pathogenesis. Our data indicate that cytokine levels rise during different phases of dengue infection and contribute to endothelial damage. NS1 appears to activate multiple signaling pathways, resulting in increased hyaluronan production. The interaction of NS1 with TLR4 on monocytes triggers proinflammatory cytokine release, leading to vascular leakage and damage to endothelial glycocalyx and cell junctions. Our observations suggest that NS1-induced changes in CD44 expression are linked with increased vascular permeability.

In our patient cohort, predominantly infected by dengue virus serotype 2, we observed that high NS1 levels correlate with increased hyaluronan levels. Ongoing studies aim to understand the effects of different NS1 proteins from various serotypes on hyaluronan levels.

In study I, our data indicate that NS1 and pro-inflammatory factors stimulate hyaluronan biosynthesis and disrupt CD44 interactions, leading to junction disorganization and vascular leakage. Understanding these molecular mechanisms could pave the way for strategies to prevent plasma leakage during dengue infections.

Has2 natural antisense RNA and Hmga2 promote Has2 expression during TGFβ-induced EMT in breast cancer

Our second study provides new insights into the connection between HAS2-synthesized hyaluronan engaged Cd44 and breast cancer progression. It defines a pivotal role of Has2as and Hmga2 in TGFβ-induced Has2 expression during EMT and maintenance of cancer cell stemness. Gene structure analysis identified several transcripts for HAS1/Has1 and HAS3/Has3; whereas only one transcript encodes for HAS2/Has2, its anti-sense HAS2-AS/Has2as occurs in several transcripts. The expression of HAS mRNAs correlates to hyaluronan production, and thus their induction appears to be the primary control mechanism to regulate hyaluronan levels.

HAS2/Has2 transcripts dominate and can be rapidly repressed or induced through the binding of transcriptions factors, including Sp1, Sp3, NF-κB, YY1, and CREB, to the HAS2 promoter. The promoter of Has2as possesses binding sites for response elements such as O-GlcNAcylated NF-κB subunit p65, HIF-1, Sp1 and Sp3, and similar to the Has2 gene, it is epigenetically modified by DNA methylation. Our data demonstrate a negative correlation between HAS2-AS, HAS2 and HMGA2 expression and survival of patients with invasive breast carcinomas. Coordinated expression of HAS2-AS and HAS2 transcripts has also been reported in oral squamous cell carcinoma and metabolic disorders. The functions of natural antisense transcripts are associated with their subcellular localization and involve modulation of expression of target genes by formation of RNA/RNA or DNA/RNA duplexes. Our previous
results demonstrated that TGFβ-dependent Has2 expression, but not extracellular hyaluronan-Cd44 interactions, plays an important role in TGFβ-mediated EMT in NMuMG normal mammary epithelial cells.

We have demonstrated that Has2, Has2as and Hmga2 transcriptional patterns are tightly regulated during TGFβ-mediated EMT. Expression of Has2as, but not Hmga2, was required for the recruitment of Smad2/3 to selective areas in the Has2 gene locus. However, the induction of Has2as transcripts by Hmga2 was enough to drive constitutive Has2 expression, and TGFβ stimulation further promoted Has2 expression and hyaluronan production. Our findings establish that TGFβ activates Smad and non-Smad signaling pathways, resulting in the transcriptional induction of Hmga2, Has2as and Has2. The newly synthesized hyaluronan signals through Cd44 by activation of Akt and Erk1/2 MAP-kinase signaling, enhancing breast cancer cell motility and acquisition of stemness.

**Hyaluronan-induced CD44-iASPP interaction affects fibroblast migration and survival**

The third study reveals a new role of CD44 in regulating p53-mediated apoptosis and cell growth through interaction with the p53 inhibitor iASPP. The CD44s isoform binds iASPP and inhibits cell growth, while hyaluronan stimulation enhances the formation of iASPP-CD44 complexes in fibroblasts but not in epithelial cells. We did not observe any complex between iASPP and CD44 variant (CD44v) isoforms, indicating that different CD44 isoforms affect intracellular ROS levels and cell growth differently.

Our findings suggest that hyaluronan might induce more iASPP-CD44 complexes in mesenchymal cells than in epithelial cells due to differential expression levels of CD44v isoforms. Additionally, CD44 acts as a connector, regulating clustering of membrane receptors to elicit biological signaling. The interaction between iASPP and CD44 is crucial for regulating cell adhesion, migration, and downstream signaling.

Furthermore, our study demonstrates that hyaluronan stimulation of CD44 enhances fibroblast migration in an iASPP-dependent manner, consistent with the role of iASPP in promoting migration. Additionally, the interaction between CD44 and iASPP suppresses the generation of ROS, suggesting their potential as therapeutic targets to suppress ROS generation in transformed cells. Our observations also indicate that p53-dependent apoptotic signals depend on the repression of CD44, and the translocation of p53 and iASPP from the nucleus to the cytoplasm in CD44-silenced cells highlights the regulatory role of CD44 in cellular processes.

In third study, we have reported that hyaluronan induces a complex between the standard isoform of CD44 and iASPP, preferentially in fibroblasts. Knockdown of CD44 resulted in an increased cell growth and cell density and to an increased amount of p53-iASPP complexes in the cytoplasm. We also found that iASPP is needed for hyaluronan/CD44-induced cell migration and
adhesion. Our findings suggest that the balance between iASPP-CD44 and iASPP-p53 interactions affects cell migration and survival.

3.2 Future perspectives

3.2.1 Dengue treatment strategy by hyaluronan modulation

The following scheme shows our further working hypothesis.

Molecular weight distribution of hyaluronan and the roles of hyaluronidase

Using the clinical samples, we plan to analyze the molecular-weight distribution of hyaluronan in the plasma of patients (both from DVI and other bacterial infection). These analyses will provide us clues whether hyaluronan under stress, contributes to protection or pathogenesis of DVI. For that the activities of hyaluronidases during the different phases of dengue virus progression will be studied.

Therapeutic regimens [4-MU (4-Methylumbelliferone)] treatment in vitro

The idea is to decrease the recruitment of immune cells by NS1-treated dermal fibroblasts. Data from us and others suggest that stromal cells with cable-like hyaluronan structures might recruit immune cells to the local sites of infection, and then lead to the more chances of viral infection into the immune cells. Therefore, an “immune cells binding assay” without or with 4-MU treatment will be carried out on the NS1-treated dermal fibroblasts. Our aim is to provide
a pharmaceutical prophylaxis of DVI, i.e. to lower the possibility of progression from asymptomatic infection of dengue virus into symptomatic diseases. If successful, this will also diminish the reservoir of dengue virus and allow control of outbreaks.

**Analysis of a huge databank in Taiwan (epidemiologic study) to investigate whether viral infection affects tumor progression**

We will also work more on the epidemiologic analysis. There is a huge health insurance databank in Taiwan. We want to retrospectively enroll those patients with any kinds of stage 2 or 3 malignancy (majorly solid organs) diagnosed since 2000. These patients will be divided into DVI-positive or DVI-negative groups. We will compare the length of metastases (stage 4)-free period to know whether transient vascular leakage in DVI will affect distant metastases. We will also expose normal cells and tumor cells of various malignant phenotypes, *in vitro* to the dengue virus NS1 protein and analyze changes in mRNA expression by a microarray analysis to monitor effects on cancer biomarkers, such as estrogen receptors or p53.

**The relationship between hyaluronan-CD44 in Zika virus-infected glioma cells**

Zika virus (another mosquito-borne flavivirus) infection cause only mild illness in non-pregnant patients. However, it is harmful to the fetus and will result in microcephaly, an abnormality of central nervous system development, if pregnant women are infected. Based on our first manuscript, we will investigate the long-term effects of Zika virus infection on astrocytes and how it affects hyaluronan-CD44 signaling.

**Phase I clinical trial will be initiated**

It is expected that there will be an outbreak(s) of DVI in 2018 or 2019 based on the epidemiologic analyses in past 2 decades. If a pro-inflammatory role of hyaluronan in dengue progression was confirmed furthermore, we would like to conduct a phase I, open label therapeutic trial with recombinant human PH-20. The inclusion criteria are (1) laboratory-confirmed DVI symptomatic patients; (2) enrollment within 72 hours after symptoms onset; (3) febrile phase serum hyaluronan ≥70 ng/ml and also critical phase serum hyaluronan ≥2700 ng/ml; (4) primary DVI; and (5) did not have any WS before enrollment. The exclusion criteria include (1) pregnant women; (2) patients age ≥80 years; (3) HIV-infected persons. The primary endpoint will be the occurrence of warning signs and severe dengue. This drug (both traditional formula and the pegylated one) will be injected subcutaneously in three doses (traditional formula) in 12 hours or only once (pegylated formula). Patients will be followed up to measure the serum hyaluronan levels and the development of clinical WS for 10 days after treatment. Our goal is to enroll 10 patients in the treatment group and the other 10 in the placebo group.
3.2.2 Rescue experiments to modulate hyaluronan biosynthesis, signaling through CD44, and the implications for chronic inflammation and cancer treatment

We will explore the link between HAS2-synthesized hyaluronan, CD44, and cancer progression. Investigating the role of different transcripts of HAS1/Has1 and HAS3/Has3, as well as the antisense transcript HAS2-AS/Has2as, in hyaluronan production and regulation will be carried out. We will conduct rescue experiments (with small-molecule drugs or inhibitors as treatment) to investigate the role of Has2as in hyaluronan signaling and its impact or effect on cell growth and metastasis. Investigating the relationship between HAS2 regulation, HMGA2, let-7 miRNA, and other factors such as hyperglycemic stress, IL-1α, and PDGF-BB could also be performed. The purpose is to obtain a deeper understanding of the transcriptional regulation of the HAS2 gene and its impact on hyaluronan synthesis.

3.2.3 Expand the scope in CD44-iASPP interaction

We plan to investigate the specific roles of iASPP-CD44 signaling in the progression of different types of cancers, as iASPP and CD44 expression levels vary in different cancer types. Additionally, it may be beneficial to conduct more detailed analyses to elucidate the mechanisms by which CD44 and iASPP suppress ROS generation in transformed cells. Further studies could also explore the potential therapeutic applications of targeting CD44 and iASPP to suppress ROS production in cancer cells. Finally, it may be worth investigating the role of CD44 in regulating other biological signaling pathways beyond apoptosis, such as cell adhesion and migration.
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