Regulation of WNT5A Expression in Malignant Melanoma: Role in Tumor Progression

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Regulation of WNT5A Expression in Malignant Melanoma: Role in Tumor Progression

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Regulation of WNT5A Expression in Malignant Melanoma Progression

Abstract

Malignant melanoma is the most aggressive type of skin cancer, which initiates in the pigment-producing cells called melanocytes. Exposure to ultra violet radiation (UVR) of the sun and family history increases the risk of developing melanoma. Early detection of tumors improves survival. However, for patients with metastatic melanoma, there are few treatment options and a 5-year survival rate of less than 5%. Therefore, it is of importance to identify the molecular mechansims that drive metastasis of melanoma cells.

High expression of wingless-type MMTV integration site family 5A (WNT5A) has been reported in melanoma and correlates with increased migration and invasion of melanoma cells. In this thesis, we investigate that WNT5A and interleukin-6 (IL-6) form a positive feedback loop, which accelerates the invasive property of melanoma cells. In addition, we demonstrate that combined targeting of IL-6 signaling and WNT5A expression, using an anti-IL-6 antibody and a WNT5A antagonist peptide (Box5), respectively, more effectively reduces melanoma cell dissemination, as compared to each factor alone. Moreover, we show that the RNA-binding protein human antigen R (HuR) drives melanoma cell motility, and targeting HuR with a specific HuR inhibitor, MS-444, significantly reduces melanoma cell migration. Further analyses revealed that simultaneously targeting HuR, with MS-444, and WNT5A signaling, with Box5, reduces melanoma cell migration and invasion *via* two different and partially overlapping signaling pathways, which are PKC and PI3K-AKT. Our results also show that acquired BRAF-inhibitor (BRAFi) resistance leads to increased expression of HuR and WNT5A in malignant melanoma cells, and simultaneous therapeutic inhibition of HuR function and WNT5A signaling could be an efficient treatment strategy to impair the invasive migration of BRAFi-resistant melanomas.

Taken together, our results extend our knowledge about WNT5A regulation in melanoma and highlight the importance of HuR and WNT5A in melanoma cell migration and invasion.

Key words: Malignant Melanoma, WNT5A, IL-6, HuR, BRAF-inhibitir-resistance melanoma, Box5, MS-444

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Regulation of WNT5A Expression in Malignant Melanoma: Role in Tumor Progression

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Cover: Confocal image of actin (red)-, HuR (green)- and nucleus (blue)-stained human melanoma HTB63 cells.

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To My Family
&
My Dear Mahboubeh

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This thesis is based on the following papers, referred to in the text by their roman numerals:

I. Demonstration of a WNT5A-IL-6 positive feedback loop in melanoma cells: Dual interference of this loop more effectively impairs melanoma cell invasion
Rickard Linnskog, Purusottam Mohapatra, **Farnaz Moradi**, Chandra Prakash Prasad, Tommy Andersson, Oncotarget. 2016 Jun 21;7(25):37790-37802. doi: 10.18632/oncotarget.9332

II. Dual mechanisms of action of the RNA-binding protein human antigen R explains its regulatory effect on melanoma cell migration
Farnaz Moradi, Pontus Berglund, Rickard Linnskog, Karin Leandersson, Tommy Andersson, Chandra Prakash Prasad
Transl Res. 2016 Jun;172:45-60. doi: 10.1016/j.trsl.2016.02.007. Epub 2016 Feb 23

III. Combining a WNT5A- antagonist and a HuR-inhibitor results in a significant decrease in the invasiveness of malignant melanoma cells *(Manuscript)*
Farnaz Moradi, Purusottam Mohapatra, Tommy Andersson

IV. Inhibition of the invasive migration of BRAF- inhibitor-resistant melanoma cells by interfering with HuR function and WNT5A signalling *(Manuscript)*
Farnaz Moradi, Purusottam Mohapatra, Tommy Andersson

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List of Abbreviations

ABCB1	ATP-binding cassette sub-family B
AKT	v-akt murine thymoma viral oncogene homolog
A-P	Anterior-Posterior
APC	Adenomatous polyposis coli
APT-1	Acyl-protein thioesterase-1
ATP	Adenosine tri-phosphate
ARF6	Adenosine diphosphate ribosylation factor 6
Bad	Bcl-2- associated death promoter
BCC	Basal cell carcinoma
Bcl2	B-cell lymphoma 2
BMP	Bone morphogenic protein
BRAF	v-raf murine sarcoma viral oncogene homolog B
BRAFi	BRAF-inhibitor
BRAFi-R	BRAF-inhibitor resistant
Ca^{2+}	Calcium
CaMKII	Ca^{2+}-calmodulin-dependent protein kinase II
CD44	Cluster of differentiation 44
Cdc42	Cell division control protein 42 homolog
CDK4/6	Cyclin-dependent kinase 4/6
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CK	Casein kinase
CRD	Cysteine rich domain
CTF1	Cardiotrophin 1
DAG	Diacylglycerol
Dkk	Dickkopf
DNA	Deoxyribonucleic acid
DVL	Disheveled
EDNRB	Endothelin receptor type B
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase

FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridization
FOX	Forkhead box
FZD	Frizzled
GLI2	GLI family zinc finger 2
GPCR	G protein-coupled receptor
GSK3	Glycogen synthase kinase 3
HDAC	Histone deacetylase
Hh-Gli	Hedgehog-Glioma-associated oncogene homolog zinc finger protein
HOPP	Heuristic online phenotype prediction algorithm
HSPGs	Heparin sulfate proteoglycans
HuR	Human antigen R
IHC	Immunohistochemical
IL-6	Interleukin-6
IP_3	Inositol-1,4,5-thriphosphate
Int1	Integration site 1 gene
Jak/STAT	Janus kinase/signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
LEF	lymphoid-enhancing factor
LIF	Leukemia inhibitory factor
LRP	Low-density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MARCKS	Myrisotylated alanine-rich C kinase substrate
MCAM	Melanoma cell adhesion molecule
MEK	Mitogen-activated protein kinase kinase
MITF	Microphthalmia-associated transcription factor
MLANA	Melan-A
MMP	Matrix metalloproteinases
MMTV	Mouse mammary tumor virus
mTOR	The mechanistic target of rapamycin
MUSK	Muscle skeletal receptor tyrosine kinase
NFAT	Nuclear factor associated with T cells

NFATc2	Nuclear factor of activated T cells c2
NF-κB	Nuclear factor kappa B
NRAS	Neuroblastoma RAS viral oncogene homolog
p38-MAPK	p38 mitogen-activated protein kinase
Par6	Partitioning-defective protein 3
PAX3	paired box transcription factor
PCP	Planar cell polarity
PIP_2	Phosphatidylinositol (4,5) bisphosphate
PIP_3	Phosphatidylinositol (3,4,5) triphosphate
PI3K	Phosphoinositide 3-kinase
PDK1	Pyruvate dehydrogenase kinase isozyme 1
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
Porcn	Porcupine O-acyltransferase
PTEN	Phosphatase and tensin homolog
Rab	Ras-related proteins in brain
Rac	RAS-related c3 botulinum toxin substrate
RB	Retinoblastoma protein
RGP	Radial growth phase
Rho	RAS homolog gene family
RNA	Ribonucleic acid
Rock	Rho kinase
ROR	Receptor tyrosine kinase-like protein receptor
RTK	Receptor tyrosine kinase
RYK	Related to receptor tyrosine kinase
SA-β-gal	Senescence-associated-beta-galactosidase
SAHF	Senescence-associated heterochromatin foci
SCC	Squamous cell carcinoma
Ser	Serine
SFRP	Secreted frizzled-related proteins
Shc	Src homology 2 domain containing transforming protein 1

SNP	Single-nucleotide polymorphism
SOX10	SRY-related high-mobility-group box
Src	v-src sarcoma viral oncogene homolog
TCF	T-cell factor
TGF-β	Transforming growth factor-β
Thr	Threonine
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TYR	Tyrosine
UVR	Ultra violet radiation
VEGF	Vascular endothelial growth factor
VGP	Vertical growth phase
Wg	Wingless
WIF	WNT inhibitory factor
Wls	Wntless
WNT	Wingless-type mammary tumor virus integration site
W-RAMP	WNT5A-mediated-receptor-actin-myosin polarity
ZEB	Zinc finger E-box-binding homeobox
3'UTR	3'-untranslated region
β-TrCP	β-transducin-repeat-containing protein

Introduction

Cancer is the general name of about 200 different diseases. When cancer occurs, a perfect balance in cell division is disturbed. A cancer cell has the ability to divide in an uncontrollable manner and ultimately forms a tumor. The features a normal cell obtains, as it gradually changes into a precancerous and ultimately into cancer, are called the "Hallmarks of Cancer" [1]. The cancer cells use multiple mechanisms to escape apoptosis; have the ability to divide to infinity; stimulate their own growth; are insensitive to signals inhibiting growth; stimulate the growth of blood vessels into and from the tumor; and in most cases, have the ability to spread through blood and lymph to other organs (metastasis) [2]. The latter of these hallmarks is the main cause of cancer related death. There are a number of different dysregulated signaling pathways in cancer that induce the invasive property of cancer cells. The present thesis focuses on the role of a protein, called WNT5A, in malignant melanoma progression. More specifically, we explore our understanding about underlying mechanisms that regulate WNT5A expression in malignant melanoma cells. Moreover, we investigate how different approaches can reduce melanoma cell migration and invasion in melanoma.

Background

Overview of human skin

The skin is one of the largest organs in the body; in an adult it is about 2 square meters and represents 10-15% of total body weight. The skin has many functions: it regulates body temperature, protects the body from external attacks such as microorganisms (e.g. bacteria and viruses), ultra violet radiation (UVR), dehydration, chemicals and other harmful substances. In addition, the skin is a sensory device that feels touch, pressure and pain. The skin is made up of three different layers: the epidermis, dermis and the subcutaneous tissue (Figure 1) [3].

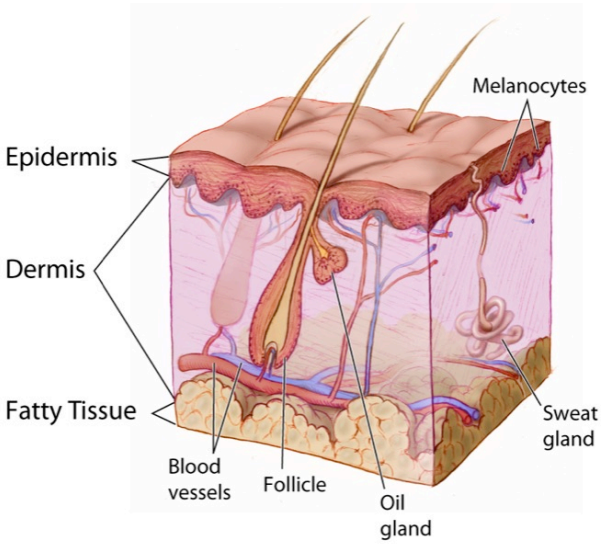

Figure 1. The basic anatomy of human skin. *Used with permission from National Institutes of Health, USA*

During the blastula stage and upon activation of different signaling pathways such as Wingless-type mammary tumor virus integration site (WNT), Fibroblast Growth Factor (FGF) and Bone Morphogenic Protein (BMP), the embryonic surface ectoderm gives rise to the formation of the epidermis, which is the topmost layer of the skin [3,4]. In the epidermis, there are numerous cell types, the most abundant being keratinocytes, which form 95% of epidermis. Other cells that are present in the epidermis are Langerhan's cells, Merkel cells, sensory nerves and the melanin-producing cells called melanocytes. Each melanocyte is associated with surrounding keratinocytes. Melanin is transferred from melanocyte into the neighboring keratinocyte where it determines skin color and protects the skin from ultraviolet radiation [4].

Melanocyte development is a complex process in which melanoblasts, a subset of the neural crest cells, undergoes an epithelial-to-mesenchymal transition (EMT) and migrates through the dermis into the epidermis where the cells develop into mature melanocytes [5]. Melanocytes are also found in the hair follicles, central nervous system, inner ear, and in the vascular middle layer of the eye [6]. Several signaling pathways such as WNT/β-catenin, endothelin receptor type B (EDNRB) and the tyrosine protein kinase, KIT, control the activity of enzymes and genes of receptors that are involved in melanocyte development and function [3,6-8]. Briefly, upon activation of WNT/β-catenin signaling, the transcription factor, lymphoid enhancer factor 1 (LEF1), binds to the promoter of microphthalmia-associated transcription factor (MITF) and regulates its transcription. MITF activation regulates the expression of various target genes that are essential for melanocyte development [9]. Likewise, activation of ENDRB and KIT, and their subsequent activation of transcription factors such as paired box transcription

factor (PAX3), SRY-related high-mobility-group box (SOX10) and MITF, regulates downstream genes essential for melanocyte proliferation, differentiation and survival [10]. The epidermis is resting on a thin extracellular matrix of tissue that function as an adherent connection and separates it from the dermis. This mat-like meshwork called the basement membrane is made up of members of the protein families of laminin, type II collagen, perlecan and nidogen [11].

The dermis is the middle of the skin's three main layers. It is a connective tissue that is rich in both collagen, which makes the tissue sturdy, and elastin, which makes the skin smooth, tight and elastic. The dermis is also rich in blood vessels that nourish the skin with nutrients and oxygen, as well as maintain body temperature. It also contains sensory structures, hair follicles and various glands [3,4]. The lowermost layer of the skin is the subcutaneous tissue, also known as hypodermis, which consists of collagen and elastin fibers, and attaches the hypodermis to the dermis. It also contains fat, blood and lymphatic vessels, hair follicle roots, nerves, as well as the glandular part of various glands [3,4].

The abnormal growth of skin cells causes skin cancer. There are two main types of skin cancer; melanoma and non-melanoma skin cancer. Non-melanoma skin cancers are either basal cell carcinoma (BCC) or squamous cell carcinoma (SCC) and are less likely to metastasize to other organs, while melanoma is often fast growing with increased chance of metastasis. All types of skin cancer can form anywhere on the body. Various genes and signaling pathways involved in melanocyte development are altered during skin cancer progression, suggesting that melanoma cells utilize the properties of their normal function to progress into cancer [12]. One such signaling cascade is WNT signaling.

The WNT signaling pathway

WNT signaling is one of the key signaling pathways involved in embryonic development and stemness. Aberrant WNT transduction has been implicated in a wide range of diseases, most notably cancer [13]. The discovery of WNT signaling was in the late 1970's, when it was reported that a mutation in the wingless (wg) gene in drosophila caused a defect in wing development [14]. The name Wnt originates from a combination of the wg gene and its homologous gene in mice, Integration site 1 gene (Int1), whose mutation was associated with increased chance of mammary carcinoma, when used as a target for mouse mammary tumor virus (MMTV) [15]. The WNT family of proteins contains 19 cysteine-rich glycoproteins that generate embryonic pattern in all species, from worms to humans [16]. Activation of WNT signaling is a necessary step in gastrulation and tissue patterning in the anterior-posterior (A-P) axis development and mutation in WNT genes leads to dramatic phenotypic defects, such as early gastrulation defects, abnormalities in lung morphogenesis and kidney hypoplasia. WNT signaling is also essential for tissue maintenance and regulation, as well as stem cell self-renewal in adult organs [16].

WNT maturation and secretion

WNT proteins are hydrophobic and have an insoluble nature due to palmitoylation, which is covalent attachment of palmitic acid by the membrane bound O-acyltransferase porcupine (Porcn) to N-terminal cysteine residues of WNT proteins as part of its post-translational modification in the endoplasmic reticulum (ER). The cysteines are also critical for protein conformation, as they form disulfide bonds [17].

Enzymatic protein depalmitoylation, using acyl-protein thioesterase-1 (APT-1), leads to loss of WNT secretion, suggesting that palmitoylation is necessary for WNT secretion and its activity [18]. WNT proteins are also subjected to N-linked glycosylation. However, the role of glycosylation in the molecular functions of WNT proteins is not fully understood. Although some studies suggest that a single mutation in cysteine residues does not eliminate WNT activity [19], other investigators found that glycosylation is an essential step for WNT signaling activity [20]. Therefore, more research is required to explain these contradictory findings. Apart from palmitoylation and glycosylation, several other post-transcriptional modifications of WNTs have been identified. For example, WNT5A and WNT11 are sulfated on their tyrosine residue. The tyrosine sulfation is essential for the formation of a WNT5A-WNT11 complex, which is required for dorsal axis formation in Xenopus [21].

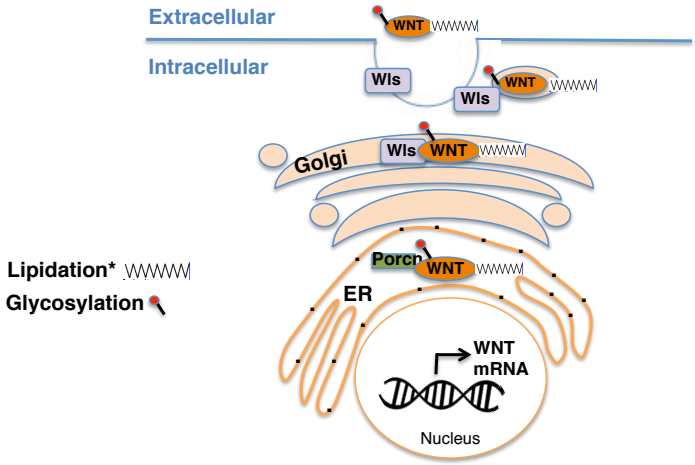

Figure 2. Model of WNT maturation and secretion. After lipid modification in the ER, Wls carries WNT to the plasma membrane where they are secreted by secretory vesicles. *Palmitoylation and/or acetylation. *The image is modified from Mikels and Nusse (2006) [22]*

After lipid modification in ER, the WNT proteins are transported into the Golgi apparatus, where a seven-pass membrane protein called Wntless (Wls), interacts with WNT proteins. The Wls protein carries WNTs to the plasma membrane and are secreted by secretory vesicles [23]. After transporting the WNT proteins Wls are recycled back to the Golgi by endocytosis to interact with a newly processed WNT protein (Figure 2) [24].

Once WNTs are secreted, several extracellular components, such as heparin sulfate proteoglycans (HSPGs), modulate WNT availability and regulate WNTs-receptor interaction [17]. In addition, a number of other binding partners can regulate the activity of WNTs. For example, WNT inhibitory factor (WIF), the members of secreted frizzled-related proteins (SFRPs), Cerberus and members of the Dickkopf (Dkk) family all function as WNT antagonists [25-29].

WNT receptors

To date, more than 15 WNT receptors and co-receptors have been identified [30]. The main WNT receptors are the members of Frizzled (FZD) family. Frizzled receptors are G protein-coupled receptor (GPCR) proteins, which consist of an intracellular C-terminus and an extracellular N-terminus. The external part contains a cysteine rich domain (CRD) to which WNTs are assumed to bind and activate different intracellular signaling cascades [31]. Thus far, 10 human FZD receptors have been identified. Aberrant expression of FZD receptors has been reported in cancers such as sporadic colon cancer, which suggests that FZD could be a prognostic or diagnostic marker [32,33]. Moreover, targeting selected members of FZD receptors

with a specific monoclonal antibody reduced the growth of a range of tumor types [34]. In addition to the frizzled receptors, a number of other receptors and/or co-receptors are involved in the activation of WNT signaling pathways, including receptor tyrosine kinase-like protein receptor 1 and 2 (ROR1/2), low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6), protein tyrosine kinase 7 (RTK7), receptor tyrosine kinase (RYK) and muscle skeletal receptor tyrosine kinase (MUSK) [30]. The interaction between WNT ligands and receptors triggers the activation of WNT signaling pathways that are divided into WNT/β-catenin signaling and β-catenin-independent signaling. Proteins such as WNT1 and WNT3A activate WNT/β-catenin signaling, while β-catenin-independent signaling is primarily activated by other WNTs such as WNT5A and WNT11 [35]

WNT/β-catenin signaling

The WNT/β-catenin signaling pathway is highly conserved during the evolutionary process. In the absence of WNT ligands (off-state), the scaffolding protein axin interacts with a protein complex consisting of glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC), Casein kinase CK-1 and β-catenin. This interaction leads to phosphorylation of β-catenin, APC and axin by CK1 and GSK3. Phosphorylation of β-catenin leads to its interaction with β-transducin-repeat-containing protein (β-TrCP) and, consequently, β-catenin ubiquitination and destruction by proteasomes (Figure 3, off-state). However, once secreted WNTs bind to receptors and co-receptors, such as FZD and LRP5/6, the destruction complex gets inactivated (on-state). This in turns leads to β-catenin accumulation in the nucleus, where it binds to LEF and T-cell factor (TCF), and consecutively, regulation of

various target genes such as MITF, cMYC and Cyclin D1, which are essential in distinct cellular processes, including proliferation and growth. (Figure 3, On-state) [16,36-38]

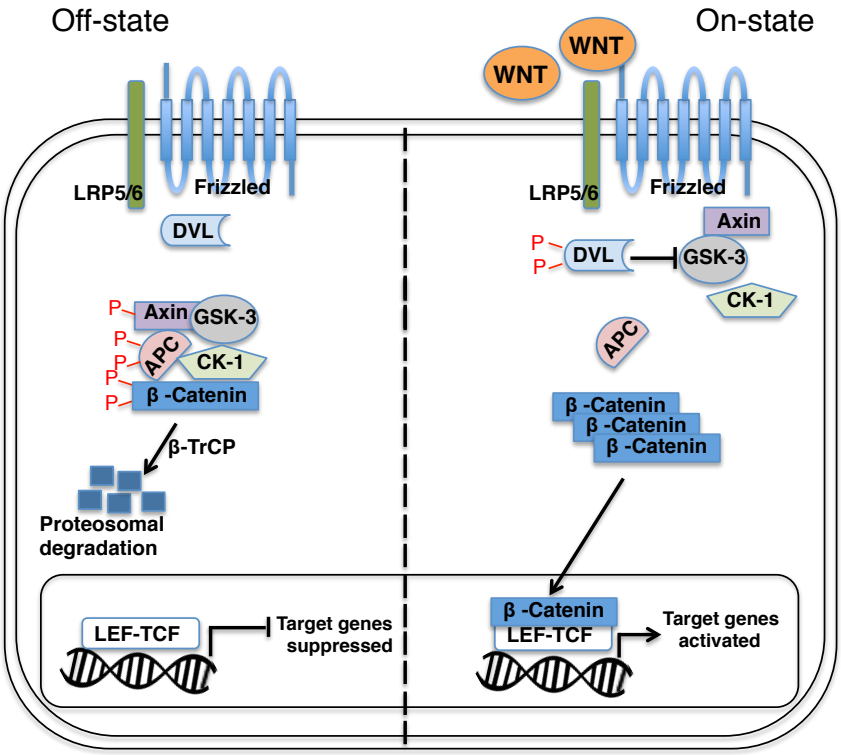

Figure 3. Schematic overview of WNT/β-catenin signaling in the absence of WNT ligands (Off-state) or in the presence of WNT ligands (On-state). *The figure is modified from Yu and Virshup (2014)[39]*

β-catenin-independent WNT signaling

Unlike β-catenin dependent signaling, which acts as a linear signaling cascade, the β-catenin-independent WNT signaling activates several downstream signaling cascades. The two best described β-catenin-

independent WNT signaling are the WNT/Ca^{2+} and WNT/planar cell polarity, also known as WNT/PCP pathway (Figure 4) [40].

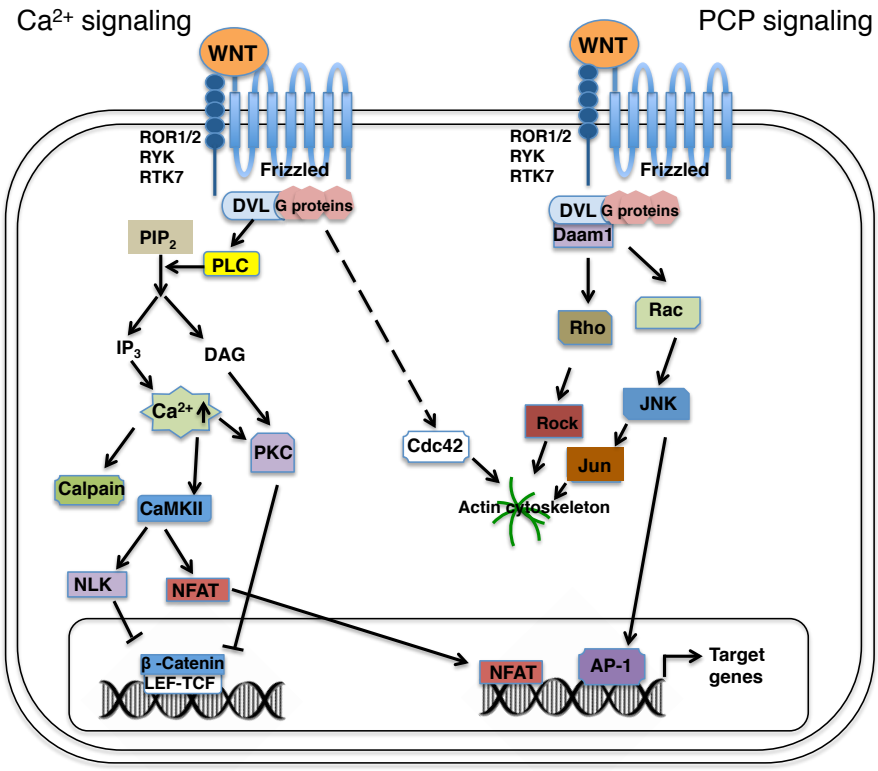

Figure 4. Schematic overview of β-catenin-independent WNT signaling. β-catenin-independent WNT ligands activate several signaling pathways such as WNT/Ca^{2+} and WNT/PCP involved in migration and cellular orientation. *The figure is modified from Shi et al. (2016)* [41]

Upon binding of certain WNTs, such as WNT5A to FZD receptors, the heterotrimeric G-protein activates phospholipase C (PLC), which consequently stimulates diacylglycerol (DAG) and inositol-1,4,5-thriphosphate (IP_3), leading to Ca^{2+} release and activation of several

downstream effectors, including protein kinase C (PKC), Calpain and Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII). The latter, CaMKII, activates the transcriptional factor, nuclear factor associated with T cells (NFAT), which directly affects target genes involved in cell fate and migration (Figure 4, left) [42]

The WNT/PCP pathway is activated upon WNT binding to FZD receptors and/or to receptor tyrosine kinases, such as ROR1/2 and RYK, which activates cytoplasmic phosphoprotein Disheveled (DVL). This in turn activates various downstream proteins, such as cell division control protein 42 homolog (Cdc42), RAS-related c3 botulinum toxin substrate (Rac) and RAS homolog gene family member (Rho) and their downstream effectors, which include c-Jun N-terminal kinase (JNK) and Rho kinase (Rock). The WNT/PCP pathway controls cellular orientation and tissue polarity. Moreover, it has been shown that the PCP signaling cascade controls cell migration during embryonic development in both vertebrates and invertebrates. For instance, in zebrafish, PCP pathway genes regulate oriented cell movement of a certain population of the facial branchiomotor neurons (Figure 4, right) [42,43].

WNT signaling in cancer

Deregulated WNT/β-catenin signaling has been implicated in carcinogenesis. Somatic and inherited mutations in APC has been linked to polyps formation in colon cancer [44]. Similarly, ectopic expression of axin1 induces apoptosis in hepatocellular carcinoma cells, which contain a mutation in their axin1 genes [45]. Mutations in β-catenin also leads to constitutive expression of cyclin D1, which then leads to activation of cyclin-dependent kinase 4/6

(CDK4/6) and cell growth in colon cancer [46]. Nuclear and cytosolic localization of β-catenin is correlated with poor prognosis in breast cancer patients [47]. Mutations in APC, axin and β-catenin have been also reported in thyroid cancer [48]. Moreover, aberrant β-catenin-independent WNT signaling has been implicated in various cancers. For example, in prostate cancer, androgen-dependent tumor growth is regulated via WNT5A-mediated, β-catenin-independent WNT signaling [49]. Another example of a WNT driven tumor is lung cancer. Gene expression analysis of patient samples revealed that normal β-catenin expression in lung cancer cells strongly correlates with β-catenin-independent components, including WNT5A and FZD2 [50]. Moreover, the Rho protein, a downstream effector of WNT/PCP signaling pathway, enhances metastasis of lung cancer via certain matrix metalloproteinases (MMPs), suggesting that targeting WNT signaling could act as a therapeutic strategy at multiple levels [51]. In melanoma, the role of WNT signaling is complex. While in some studies, β-catenin expression has been shown to be favorable for melanoma patients [52] other studies have shown that β-catenin expression has been linked with melanoma metastasis [53]. In the recent years, several investigators have highlighted the role of the WNT5A-mediated β-catenin-independent pathway in malignant melanoma progression. In our study, we focused on WNT5A regulation and signaling, in cutaneous melanoma, as well as potential use of WNT5A as a therapeutic target, leading to subsequent reduction in melanoma cell migration and invasion.

WNT5A

WNT5A is a member of WNT family of proteins, which initially activates β-catenin-independent WNT signaling. Human WNT5A gene is located at chromosome 3p14-p21 [54], and contains at least two WNT5A transcriptional start sites, which suggests that the WNT5A gene encodes two WNT5A transcripts. Bauer *et al*. recently showed that WNT5A mRNA encodes two distinct isoforms, WNT5A-S (Short) and WNT5A-L (Long) [55]. They demonstrated that these two isoforms exhibit various functions. For example, WNT5A-L may inhibit cell proliferation, whereas WNT5A-S was shown to enhance cell proliferation. WNT5A has been linked to a number of different cancers, however, so far, no mutation in the WNT5A gene has been reported in cancer patients. WNT5A has a crucial role in embryonic development. WNT5A is highly expressed during gastrulation and its expression is necessary for the formation of the limb, face, A-P axis and the development of the reproductive system. WNT5A knock out mice show abnormal morphogenesis of all these tissues and structures, and die shortly after birth, due to asphyxia [56]. As previously stated, WNT proteins, including WNT5A, are post-translationally modified. WNT5A is subjected to glycosylation on asparagine 114, 120, 311 and 325 residues [57]. Moreover, WNT5A is palmitoylated at its cysteine residue, which is necessary for its secretion [18]. It has also been shown that heparan sulfate proteoglycan modulation of WNT5A promotes WNT5A signal transduction [58]. By analyzing DVL phosphorylation as an indicator of WNT pathway activity, Takada *et al.* demonstrated that WNT5A signals through FZD3, FZD4, FZD5 and FZD8 [59]. There are also evidences that WNT5A signals via FZD7 and FZD2 in mouse fibroblast and zebrafish embryos respectively, suggesting that WNT5A functions by binding to several FZDs [60,61]. In addition to

FZD receptors, WNT5A can also trigger its signaling via ROR1/2, RYK or RTK7 [62-64]. For example, WNT5A, through FZD5, induces axis duplication in *xenopus* embryo [65]. Moreover, WNT5A, via FZD2, LRP and ROR2, can affect canonical WNT signaling [66]. WNT5A has also been shown to induce internalization of FZD2. This action also requires ROR1, ROR2 and DVL, and leads to Rac activation [67]. WNT5A can also inhibit β-catenin dependent WNT signaling through ROR2 [68]. Also, by binding to ROR2, WNT5A triggers JNK signaling and thereby regulates convergent extension [69]. Moreover, WNT5A, through FZD and DVL, triggers Rac and Rho and their downstream activator ROCK and JNK to regulate cell movement [70].

Regulation of WNT5A expression

Despite the extensive research on the role of WNT5A in cancer progression, the mechanisms behind aberrant WNT5A expression is not fully understood. To our knowledge, there is no mutation in the WNT5A encoding gene [71]. Deregulation of WNT5A expression occurs most likely at the transcriptional and/or the translational level, or through different mechanisms during cancer progression. Roatry *et al*. demonstrated that transforming growth factor-β (TGF-β) regulates the expression of WNT5A in mice mammary glands [72]. Moreover, Ca^{2+} was shown to induce WNT5A expression and secretion in human colon adenocarcinoma [73]. In addition, metabolic agents that selectively block PKC or protein tyrosine kinases reduce WNT5A expression [74]. In gastric cancer cells, tumor necrosis factor-α (TNFα) up-regulates WNT5A expression [75], whereas in endothelial progenitor cells, Notch signaling was shown to induce the expression of non-canonical WNTs, such as WNT5A and WNT11, suggesting that Notch signaling might crosstalk

with non-canonical signaling [76]. Genomic analysis of WNT5A confirms the existence of two WNT5A isoforms, with a conserved binding site for nuclear factor κB (NF-κB) within WNT5A promoter B, in mammals. TNFα and toll-like receptor (TLR) were shown to upregulate the expression of WNT5A via NF-κB [77]. Moreover, WNT5A has a conserved binding site for forkhead box (FOXs) within WNT5A intron 1. FOX family members are target genes of Hedgehog- Glioma-associated oncogene homolog zinc finger protein (Hh-Gli) signaling cascade, which indirectly upregulates the expression of WNT5A [77,78]. In addition, leukemia inhibitory factor (LIF) and cardiotrophin 1 (CTF1) upregulate the expression of WNT5A in an IL-6-JAK-STAT3-dependent manner [79]. We have recently reported that interleukin-6 (IL-6) regulates the expression of WNT5A in malignant melanoma cells *via* the activation of p38α-mitogen-activated protein kinase (p38α-MAPK) [80]. The RNA binding protein human antigen R (HuR), is also involved in WNT5A expression. In breast cancer cells, HuR was shown to suppress the expression of WNT5A [81].

Malignant melanoma

Cutaneous malignant melanoma is an aggressive and therapy-resistant form of cancer that starts in the pigment-forming melanocytes, at the bottom of the epidermis [82]. While it accounts for only 5% of all skin cancer, it causes more than 80% of skin cancer related death [83]. Melanocytes produce melanin, which gives color to the skin and hair and protect the skin from ultraviolet irradiation [84]. The most common symptom of malignant melanoma is a birthmark (nevus) that begins to grow, itch or change color and shape. The appearance of new moles that have not previously existed

may occur. The major risk factor for melanoma development is the exposure to UV, which causes DNA damage, and leads to accumulation of genetic mutations in melanocytes and melanoma initiations [85]. Other risk factors for melanoma development include geographical location, weak immune system and familial history of melanoma. Caucasians with red or blond hair and fair skin, who sunburn easily, have a higher risk of developing melanoma. When it is detected early, the tumor can be removed by surgery. These patients have a good prognosis with an overall 5-year survival rate of ~95%. However, patients with metastatic melanoma have a poor prognosis with a 5-year survival rate of less than 5% [86]. Melanoma progression is a stepwise process (Figure 5). The first step towards tumor progression is increased cell proliferation in normal melanocytes and the formation of benign nevi. The majority of these nevi are not harmful, and never progress to cancer, however abnormal proliferation of melanocytes could lead to the formation of a pre-malignant lesion, called dysplastic nevi [83,87].

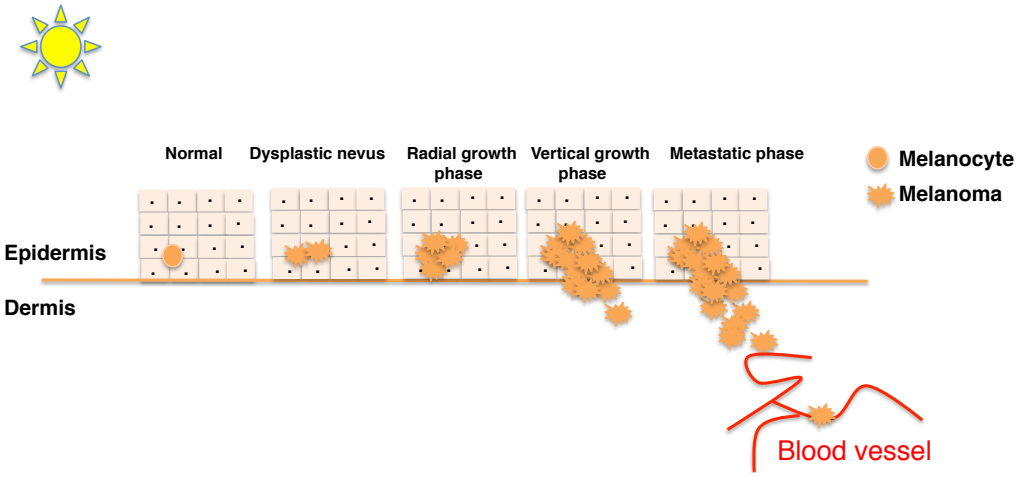

Figure 5. Hypothetical model of melanoma development. *The figure is modified from Paluncic et al. (2016)* [88]

Further proliferation, in combination with tumorigenic damage, causes the formation of a radial growth phase (RGP) melanoma, whereby the cells are horizontally spread within the epidermis [87]. Although E-cadherin mediates cell-cell adhesion, a few cells can invade the basement membrane and, subsequently, the dermis. Loss of E-cadherin and expression of N-cadherin, in combination with several genetic and epigenetic alterations, progress melanoma cells into a vertical growth phase (VGP) [83,89]. During these phases, melanoma cells undergo an EMT-like process and accumulate several hallmarks that enable them to lose adhesion and dissociate from the primary tumor, in order to migrate to distant organs in the body, in a process called metastasis. Lymph nodes, lungs, liver and brain are often affected by metastases.

WNT5A in malignant melanoma

The first evidence that connected WNT5A to melanoma came from the study made by Iozzo *et al.* in 1995, in which the authors analyzed the expression of WNT5A in several tumors and compared them with normal tissue. The results from their study demonstrated that, though there is no mutation in the WNT5A-expressing gene, there were elevated levels of WNT5A transcript in melanoma samples, as compared to normal skin [71]. Later, several independent studies confirmed the correlation between high WNT5A expression and invasiveness, in melanoma patients [90-92]. WNT5A has been shown to directly induce melanoma cell migration and invasion via activation of the PKC signaling pathway. In agreement, the author showed that blocking WNT5A signaling with a specific FZD5-receptor antibody inhibits WNT5A/PKC signaling and thereby reduces cellular invasion [91].

WNT5A overexpression induces activation of several pro-metastatic genes, such as vimentin and Snail, whereas it reduces the expression of the metastasis suppressor KISS-1 gene [93]. In addition, by using melanoma tissue microarrays, O'connell *et al*. showed that WNT5A overexpression is correlated with ROR2 expression and these two molecules act synergistically to increase melanoma cell metastasis [94]. Furthermore, it has been shown that HSPGs, in particular syndecan 1 and 4, modulate WNT5A expression and thereby increase melanoma cell migration and invasion [58]. Witze *et al*. demonstrated that WNT5A induces the internalization of the melanoma cell adhesion molecule (MCAM) and other receptors such as FZD3, actin and myosin IIB via mechanisms involving RhoB and ras-related proteins in brain (Rabs). This leads to the formation of a polarized structure called WNT5A-mediated-receptor-actin-myosin polarity (W-RAMP), which controls the coordination between membrane retraction and directional cell movement [95]. Supporting this finding, in a recent study, Connacher *et al*. demonstrated that in both melanoma and non-melanoma fibrosarcoma cells, WNT5A treatment induces W-RAMP structure formation, which in turn controls directional cell migration [96]. Furthermore, WNT5A, *via* FZD4-LRP6 receptor complex, activates the guanosine triphosphatase adenosine diphosphate ribosylation factor 6 (ARF6), which in turn disassociates β-catenin from N-cadherin. The free β-catenin pool increases transcription activity and invasion [97]. Conversely, it has been shown that in melanoma cells the transmembrane protein Klotho antagonizes WNT5A signaling, most likely via interfering with post-translational modification of WNT5A [98]. Likewise, loss of function of WNT5A antagonist, SFRP3, due to methylation, promotes melanoma cell migration and invasion [29]. The expression of WNT5A and MITF are inversely correlated in melanoma cells.

While MITF expression is correlated with a proliferative phenotype, high WNT5A expression is associated with an invasive phenotype [99]. WNT5A suppresses the expression of LEF-1, which is a transcriptional regulator of MITF and thereby accelerates the melanoma cell phenotype, switching from a more proliferative phenotype to an invasive phenotype. The cells can switch back and forth between these phenotypes, which might clarify the heterogenetic nature of melanoma cells [100]. Phenotypic switching may explain the contradictory findings that the expression of WNT5A is low in melanomas, which was shown by the authors [101]. This finding, along with that of Hoek *et al.,* where the authors suggest that WNT5A may not be important in the proliferative state of melanomas, explains the difficulty in melanoma treatment [100]. Moreover, WNT5A-high expressing cells exposed to therapeutic stress maintain their invasive capacity and continue to metastasize, regardless of the expression of senescence markers such as senescence-associated heterochromatin foci (SAHF) and senescence-associated-beta-galactosidase (SA-β-gal) [102]. This finding could further explain the role of WNT5A in maintaining resistance to targeted therapy. In addition, WNT5A, *via* phosphoinositide 3-kinas-v-akt murine thymoma viral oncogene homolog (PI3K-AKT) signaling activation, induces aerobic glycolysis and metabolic reprogramming in melanoma cells [103].

WNT5A and other cancers

Aberrant expression of WNT5A has been linked to several types of cancers. The role of WNT5A in cancer progression is complex as it can act as both tumor suppressor and tumor promoter. For instance, WNT5A functions as a tumor suppressor in thyroid, breast, colon and prostate cancers [104-107],

while in other types of cancers, including malignant melanoma, gastric, pancreatic and lung, it acts as a tumor promoter [92,108-110]. Loss of function of WNT5A signaling has been linked to poor prognosis in breast cancer patients. By analyzing the WNT5A transcript levels from 120 malignant breast tumors and 33 normal breast tissues, Leris *et al.* demonstrated that the expression level of WNT5A mRNA was higher in normal breast tissue as compared to malignant breast tumors [111]. Similarly, immunohistochemical (IHC) staining of WNT5A in triple negative breast cancer tissue showed that the lower expression of WNT5A is associated with lymph node metastasis and poor patient survival [112]. In line with these findings, Jönsson *et al.* reported that loss of WNT5A expression is correlated with early recurrence in invasive ductal breast carcinoma [105]. Moreover, it has been shown that WNT5A reduces the migration of invasive breast cancer cells in a CD44-AKT dependent manner [113]. In addition, via activation of Cdc42, WNT5A reduces extracellular-signal-regulated kinase 1/2 (ERK1/2) and MMP9 activities, which are essential for breast cancer cell metastasis [114]. WNT5A acts also as a tumor suppressor in prostate cancer. Immunohistochemical expression of WNT5A in tissue microarray from 503 patients revealed that elevated WNT5A expression was correlated with a better outcome compared with patients with low WNT5A expression [115]. In a similar study, Thiele *et al*. demonstrated that prostate cancer patients with high expression levels of WNT5A have a longer median survival rate [107]. On the contrary, High WNT5A expression is correlated with poor prognosis in gastric cancer patients. WNT5A was shown to drive metastasis via activation of focal adhesion kinase (FAK) and Rac in these patients [116]. Moreover, WNT5A was shown to promote gastric cancer metastasis via activation of RhoA in a PI3K/AKT dependent manner [117]. Likewise,

overexpression of WNT5A induces tumor cell viability and colony formation of pancreatic cancer cells [109]. WNT5A can also increase pancreatic cell migration in a JNK-dependent manner. WNT5A-mediated JNK phosphorylation activates key proteins involved in an EMT process, such as CD44 and MMP2 [118]. In oral squamous cell carcinoma, WNT5A was shown to regulate cancer progression via activation of Ca^{2+}/PKC signaling pathway [119]. In summary, WNT5A plays an important role during carcinogenesis and regulating WNT5A signaling could act as a strategy to improve cancer patient survival.

WNT5A and drug resistance

A major problem that cancer patients face is developing drug resistance. Several factors have been implicated in drug resistance, such as activation of oncogenic pathways and reactivation of targeted molecules. Some cancer cells develop mechanisms that enable them to inactivate the drugs [120]. In the past decade, several studies have highlighted the role of WNT5A in drug resistance in different cancer types. WNT5A was shown to be upregulated in colon cancer cells, which exhibit resistance to histone deacetylase (HDAC) inhibitors as compared to the HDAC inhibitor sensitive cells. WNT5A/ROR2 signaling was found to activate AKT/PKB survival pathway and induces the resistance to apoptosis [121]. Recently, it was shown that WNT5A enhances resistance of pancreatic cancer. *In vivo*, WNT5A, through activation of the transcription factor nuclear factor of activated T cells c2 (NFATc2), mediates resistance to apoptosis in pancreatic cancer [122]. Likewise, high WNT5A expression is correlated with chemoresistance in ovarian cancer patients [123]. High expression of WNT5A was found to activate protein kinase A

(PKA), which in turn phosphorylates GSK3β in multidrug-resistant cancer cells. This leads to the activation of ATP-binding cassette sub-family B member (ABCB1), which pumps out the drugs from the cancer cells and reduces the efficiency of the treatment [124]. High expression of WNT5A transcript and protein has been reported in a subset of v-raf murine sarcoma viral oncogene homolog B (BRAF)-inhibitor resistant (BRAFi-R) melanoma cells from patients showing resistance towards BRAFi. Loss of WNT5A function by either silencing WNT5A expression or its receptors such as FZD7 and RYK reduced cell growth, survival and AKT activation. Conversely, WNT5A gain of function promoted cell growth and AKT activation [125].

Other altered genetic and signaling pathways in melanoma

Genetic and epigenetic changes alter the expression of the genes that are crucial in the biological process such as proliferation, survival, apoptosis and metabolism. These alterations change the behavior of the cell, leading to the formation of a malignant tumor.

The majority of melanoma patients harbor mutation in their neuroblastoma RAS viral oncogene homolog (NRAS) or BRAF genes, which affect the RAS/RAF/MEK/ERK signaling cascade, leading to abnormal cell division, proliferation, and survival and ultimately to cancer progression. Approximately 15-30% of melanoma patients contain a mutation in their NRAS gene [89]. The most common NRAS mutation occurs in the amino acid substitution in codon 61 from glutamine to lysine. This mutation acts as a central motor, driving melanoma cell proliferation. Genetic alteration of

NRAS also results in the hyperactivation of PI3K-AKT signaling pathway, affecting cell growth and survival [126]. The BRAF V600E point mutation results in an amino acid substitution at codon 600, where the amino acid valine is replaced with glutamic acid [127]. This in turns results in increased kinase activity, followed by constitutive hyperactivation MAPK, PI3K- AKT and NF-κB signaling pathways, which leads to gene expression involved in melanoma progression [89,127]. For instance, NF-κB is essential for the direct maintenance of EMT through upregulation of Snail, and downregulation of E-cadherin [127]. Moreover, BRAF mutation has been shown to promote melanoma development by inducing angiogenesis *via* the induction of vascular endothelial growth factor (VEGF) expression [128]. Nevertheless, Michaloglou *et al*. demonstrated that while BRAF mutation triggers melanogenesis, this mutation alone promotes senescence in melanocytes and cannot transform melanocytes [129]. In support of this observation, other investigators showed that although BRAF mutations stimulate nevus formation, additional genetic alterations are required for melanocyte transformation, such as loss of function of p53 [130], or phosphatase and tensin homolog (PTEN) [131]. One of the most significant deregulated signaling pathways in melanoma is PI3K/AKT/mTOR pathway [132], which is also activated by WNT5A. The proto-oncogene serine/threonine kinase, AKT, is a regulator of several cellular functions such as proliferation, metabolism, growth and survival, and disruption of this pathway has been reported in a number of cancers such as melanoma, breast, pancreas and ovarian cancer [133-136]. Binding of growth factors to the RTK phosphorylates tyrosine residues on the cytoplasmic face of the membrane. PI3K bind to the phosphorylated RTK and converts phosphatidylinositol (4,5) bisphosphate (PIP_2) to phosphatidylinositol (3,4,5) bisphosphate (PIP_3),

this leads to the activation of AKT by either pyruvate dehydrogenase kinase isozyme 1 (PDK1) at Thr^{308}, or by mTOR at Ser^{473} residue [137]. AKT activation leads to activation or inhibition of a wide range of cancer-associated targets, such as NF-κB and mTOR and B-cell lymphoma 2 (Bcl-2)- associated death promoter (Bad) [137,138]. Taken together, these genetic alterations accelerate metastatic melanoma progression, most likely, due to the induction of a process referred to as epithelial-to-mesenchymal transition.

Another early event towards melanoma progression is a mutation in the tumor suppressor gene, PTEN. Inactivation of PTEN has been observed in several cancer types such as lung, thyroid and melanoma [139]. PTEN loss of function has been reported in 10-20% of melanomas [140]. PTEN has lipid phosphatase enzymatic activity and dephosphorylates PIP_3, which leads to inhibition of AKT signaling pathway. Moreover, overexpression of AKT due to a PTEN mutation, results in upregulation of apoptosis regulator protein Bcl-2 [141] and downregulation of caspase-depending apoptotic pathway [142]. PTEN loss of function leads to phosphorylation of FAK and src homology 2 domain containing transforming protein 1 (Shc,) thereby inducing melanoma cell migration and invasion [143]. Another event that takes place in melanoma is the loss of function of the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, which is essential for cellular senescence. CDKN2A encodes tumor suppressor proteins $p16^{INK4a}$ and ARF tumor suppressor ($p14^{ARF}$). The $p16^{INK4a}$ inhibits phosphorylation of the retinoblastoma protein (RB) in a CDK4/6-cyclin D1-dependent manner, thereby regulating transition from G1 to S phase in the cell cycle. The $p14^{ARF}$ protects another tumor suppressor protein called P53 from degradation [144]. A loss of function mutation in the CDKN2A gene has been reported in 20% of familial melanoma [145], and according to a recent study, the researchers

at Karolinska University Hospital in Sweden showed that in the Swedish population, familial melanoma patients with CDKN2A mutation were on average 10 years younger at the time of diagnosis, with worse survival, in comparison with non-mutated familial melanoma cases [146]. Another key factor that is involved in melanoma progression is MITF, which is a downstream target of canonical WNT signaling [147]. By using single-nucleotide polymorphism (SNP) array-based genetic maps, Garraway *et al.* identified MITF as a master regulator of melanocytes. Fluorescence *in situ* hybridization (FISH) analyses of 200 tissue samples derived from primary and metastatic melanoma detected MITF amplification in metastatic samples, which was correlated with a poor outcome. BRAF mutation and loss of function of p16 gene were shown to cooperate with MITF amplification to transform melanocytes, thereby suggesting that MITF acts as a melanoma oncogene [148]. Moreover, overexpression of MITF has been shown to regulate the expression of CDK2 and subsequently induce melanoma cell proliferation [149]. Unexpectedly, the expression of MITF and its target genes, such as tyrosinase (TYR) and melan-A (MLANA), are lost in other samples [150]. The authors suggest that the variation between MITF expressions across specimens is most likely due to differences between classes of melanoma, which may explain the observed effects of MITF expression during melanoma progression.

Epithelial to mesenchymal transitions in tumor metastasis

A key step in cancer cell metastases is a reversible switch, wherein the cells change from an epithelial to a mesenchymal phenotype, and initiate migration. There are various factors and signaling pathways that promote the

EMT-like process in malignant melanoma (Figure 6). Among these factors, cadherins play a crucial role in the initiation of EMT-like process. The normal epithelial cells express E-cadherin, which plays an important role in the cell-junction complex [151]. However, during cancer progression, the expression of E-cadherin is lost. The major EMT-inducing transcription factors belong to zinc finger E-box-binding homeobox (ZEB), Snail and Twist families of proteins. These proteins are regulated by the activation of various signaling pathways including PI3K/PTEN, WNT, TGF-β and MAPK [152-154].

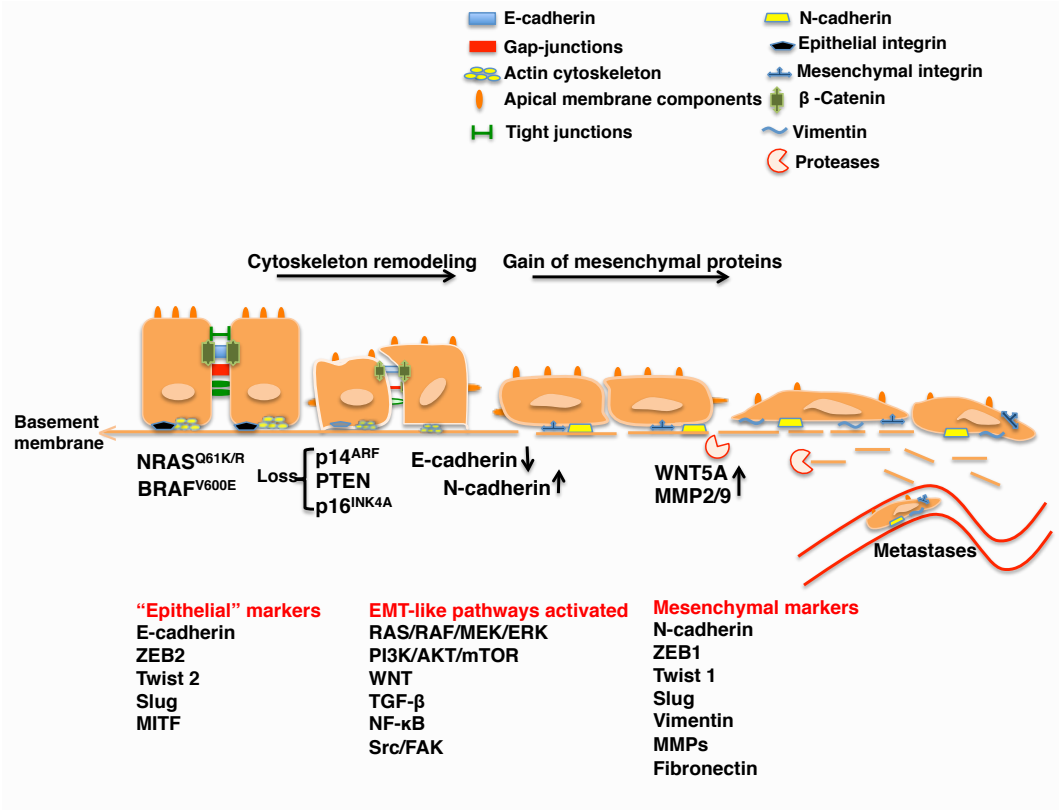

Figure 6. Schematic overview of the "epithelial" to mesenchymal transition. *The figure is modified from Heerboth et al. (2015)* [155]

Using a panel of melanoma cell lines with different progressive phases, L-Hao *et al*. demonstrated that the expression of E-cadherin was lost in the cells located in the radial and vertical growth phases. The loss of E-cadherin in these cells was correlated with loss of PTEN and a corresponding increase of PI3K/AKT activity. Furthermore, the author demonstrated that PI3K/PTEN pathway transcriptionally regulates the expression of Twist and Snail. This in turn controls the switch from E-cadherin to N-cadherin in melanoma cells [156].

Another signaling cascade that has been linked to induction of EMT-like changes is the TGF-β pathway. In normal epithelial cells, TGF-β activates a heterocomplex of SMAD2/3/4 proteins, which regulate the expression of various target genes, including several pro-apoptotic genes [157,158]. However, in various cancers, TGF-β signaling acts as a pro-oncogene. For example, in melanoma cells, TGF-β/SMAD signaling pathway increases melanoma cell motility by regulating the expression of the transcription factor GLI family zinc finger 2 (GLI2), which leads to downregulation of E-cadherin and upregulation of matrix metalloproteinases [159]. Loss of E-cadherin induces several transcription factors that are essential for EMT induction such as Twist1 and ZEB1 [160]. Furthermore, by inducing the expression of Snail and ZEB, and downregulation of Par6 expression, TGF-β contributes to the loss of cell polarity during EMT progression [161]. Constitutive BRAF activation in both murine and human species has also been linked to the expression of Twist1 and ZEB1 [12]. BRAF mutation is involved in the activation of NF-κB, which promotes the expression of matrix metalloproteinases and drives melanoma cell motility. Likewise, the activation of PI3K/AKT/mTOR pathway has been linked to EMT progression. AKT phosphorylation leads to activation of mTOR, which

induces cytoskeletal rearrangements and cell migration [162]. Similarly, the constitutive expression of cytoplasmic signaling protein, v-src sarcoma viral oncogene homolog (Src), promotes EMT progression. FAK activation through Src stimulates the c-JUN amino terminal kinase (JNK) signaling, which increases the expression of MMP2 and MMP9, and thereby promotes cell motility [163]. The activation of Src/FAK activates the p38MAPK, which controls the expression of several genes essential for melanoma cell migration and invasion [164]. Another signaling pathway that has been linked to EMT-like change is WNT signaling. High levels of β-catenin leads to a proliferative phenotype with the expression of proliferative marker such as cyclinD1 and Ki67 [38,153], whereas low expression of β-catenin is correlated with a more invasive phenotype, in addition to the expression of EMT-driving proteins, such as MMPs and VEGF [165,166]. Moreover, immunohistochemical analysis of melanoma samples show that β-catenin is mainly localized in tumor areas with high expression of E-cadherin and low expression of N-Cadherin [153]. In addition, WNT5A through PKC signaling initiates EMT by affacting snail expression [93]. These events along with expression of mesenchymal proteins, such as vimentin and fibronectin [167], leads to cell detachment and metastasis, which is a major cause of cancer related deaths.

Additional considerations

The role of WNT5A in cancer progression is complex. A better understanding of WNT5A regulation and signaling can shed light on the molecular mechanisms that drive aberrant cell behavior. Recent investigations on the role of WNT5A in drug resistance, in various cancers,

show the importance of further research on WNT5A. As summarized above, WNT5A acts as both tumor suppressor and tumor promoter. In light of this, our laboratory has developed a WNT5A antagonist peptide named Box5. By treating melanoma cells with Box5, Jenei *et al.* demonstrated that Box5 antagonizes the WNT5A induced protein kinase C and Ca^{2+} signaling, leading to an inhibition of basal migration and invasion of melanoma cells [168]. Moreover, in another study, it was shown that Box5 inhibits IL-6-induced melanoma cell migration, in HTB63 and A375 melanoma cells [80]. Box5, in combination with other WNT5A-targeting molecules, such as the HuR inhibitor MS-444 or IL-6 neutralizing antibody, could act as a better strategy to inhibit WNT5A-mediated melanoma cell migration and invasion.

The present investigation

Aims

The general aim of this thesis was to investigate the mechanisms that regulate the expression of WNT5A in malignant melanoma cells. In addition, we wanted to investigate the possibilities by which WNT5A expression, and subsequent melanoma cell migration and invasion, could be inhibited.

The specific aims of this thesis were:

I) To investigate the possible existence of a WNT5A-IL-6 positive feedback loop and investigate if dual interference of this loop could act as a better strategy to obstruct melanoma cell migration and invasion

II) To investigate whether the RNA binding protein HuR can regulate the expression of WNT5A in malignant melanoma cells

III) To analyze whether dual targeting of WNT5A signaling and expression, using the WNT5A antagonist Box5, and the HuR inhibitor, MS-444, respectively, could inhibit melanoma cell migration and invasion, in parental and BRAF-inhibitor resistant melanoma cells.

Results

Paper I

Demonstration of a WNT5A-IL-6 positive feedback loop in melanoma cells: Dual interference of this loop more effectively impairs melanoma cell invasion.

High WNT5A expression is correlated with high aggressive behavior of melanoma tumors [91,93]. We have recently demonstrated that IL-6 positively regulates the expression of WNT5A via P38α -MAPK signaling cascade [80]. Moreover, it has been shown that WNT5A promotes Ca^{2+}-dependent secretion of exosomes containing IL-6 [169]. In addition, loss of function of WNT5A significantly reduced IL-6 secretion in cell culture medium from rheumatoid arthritis patients [170]. Based on these observations, and our finding that IL-6 regulates the expression of WNT5A, we hypothesized the existence of a WNT5A-IL-6 positive feedback loop in malignant melanoma cells, which could accelerate melanoma metastasis.

To test our hypothesis, we first analyzed the phenotypic characterization of multiple melanoma cell lines based on their WNT5A and IL-6 expression. Recently, an online microarray data set, called heuristic online phenotype prediction (HOPP), was designed to identify the phenotype of melanoma cell lines using a standard signature, defined by 97 genes corresponding to a proliferative and/or an invasive phenotype [171]. Interestingly, elevated WNT5A and IL-6 expression levels were correlated with an invasive phenotype in melanoma cells. We then chose three invasive melanoma cell lines, WM852, HTB63 and A375 cells, and analyzed the mRNA and protein expression of WNT5A and IL-6 in them. All three cell lines were shown to

express WNT5A and IL-6 mRNA, as well as protein. However, no correlation between the expression of WNT5A and IL-6 mRNA was observed.

Next, we selected WM852 cells, as they show a higher expression level of both IL-6 and WNT5A. We have previously demonstrated that in HTB63 and A375 melanoma cells, recombinant IL-6 (rIL-6) increases the expression of WNT5A. However, rIL-6 treatment showed no effect on the expression of WNT5A in WM852 cells. The fact that WM852 cells secrete a relative higher level of IL-6, in comparison to HTB63 and A375 cells, may explain the ineffectiveness of rIL-6 on WNT5A expression, in WM852 cells. Taking these findings into consideration, it was of interest to study the effect of siRNA-mediated knockdown of IL-6 expression on WNT5A protein levels, in WM852 cells. We transfected WM852 cells with two independent IL-6-targeting siRNA, and analyzed the effect of IL-6 silencing on WNT5A protein expression and release. IL-6 silencing resulted in a pronounced reduction in WNT5A protein expression and release. These results were confirmed using IL-6 neutralizing antibody, which inhibits IL-6 signaling. In agreement, targeting IL-6 signaling with anti-IL-6 antibody resulted in a decrease in WNT5A protein expression. However, this approach has no effect on WNT5A mRNA expression, which suggests that IL-6 regulates the expression of WNT5A at the translational level. Conversely, silencing WNT5A with two independent WNT5A-targeting siRNA oligonucleotides reduced IL-6 secretion. To further confirm these results, WM852 cells were treated with a WNT5A antagonist peptide, called Box5. Initial experiments demonstrated that Box5 inhibits WNT5A-inducing melanoma cell migration and invasion [80]. In accordance, cells treated with Box5 reduced IL-6 secretion, which suggests that WNT5A regulates the expression of IL-6 in

WM852 cells. Interestingly, Box5 treatment has no effect on IL-6 mRNA expression, suggesting that WNT5A regulates the expression of IL-6, and this regulation happens at the translational level.

With these results in hand and the fact that WNT5A and IL-6 have been shown to promote melanoma cell motility [91,172], we investigated whether simultaneously targeting WNT5A and IL-6 could more effectively impair melanoma cell migration and invasion. We transfected WM852 cells with WNT5A-targeting siRNA alone, IL-6-targeting siRNA alone, or a combination of both siRNAs, and then we assessed the effect of these transfections on melanoma cell invasion. The results clearly demonstrated that simultaneously targeting endogenous WNT5A and IL-6 expression more effectively reduces melanoma cell invasion, as compared with each factor alone.

To confirm these finding in a more sufficient therapeutic approach, we targeted WNT5A signaling with Box5, in combination with targeting IL-6 signaling using IL-6 neutralizing antibody, and we evaluated their combined effect on melanoma cell migration and invasion. In accordance to our previous experiment, we observed a more pronounced reduction of melanoma cell migration and invasion in WM852 and HTB63 cells, when the cells were simultaneously treated with Box5 and IL-6 antibody, as compared with each compound alone. These findings reveal that WNT5A and IL-6 interact to accelerate melanoma cell motility, and that the combined therapeutic inhibition of both WNT5A and IL-6 signaling acts as an effective treatment approach to inhibit melanoma spreading.

Taken together, the results from this study exhibited the existence of a WNT5A-IL-6 positive feedback loop, in malignant melanoma cells, whereby

WNT5A regulates its own expression via this loop. Moreover, our study demonstrates that simultaneous targeting of both WNT5A signaling and IL-6 signaling acts as a novel and efficient strategy to pharmaceutically target the metastatic spread of melanoma cells.

Paper II

Dual mechanisms of action of the RNA-binding protein human antigen R explains its regulatory effect on melanoma cell migration

Elevated WNT5A expression promotes melanoma cell migration and invasion [93,173]. However, the mechanism behind high WNT5A expression in melanoma cells is not fully understood. In 2007, we reported that in human breast epithelial cells, the RNA binding protein HuR binds to the 3'-untranslated region (3'UTR) of the WNT5A transcript, and suppresses WNT5A translation [81]. HuR belongs to the embryonic lethal abnormal vision (ELAV) family of proteins, which is ubiquitously expressed in many cells [174,175]. Recently, extensive research has highlighted the role of HuR in the development of many cancer types, as it regulates the expression of several cancer related proteins that are essential for tumor cells growth, invasion and metastasis [174]. HuR has been shown to shuttle back and forth between the nucleus and cytoplasm. Once it binds to the target transcripts, the HuR-mRNA complex goes to the cytoplasm, where HuR protects mRNA from rapid degradation [176,177]. However, the role of HuR in melanoma progression has not yet been studied. Therefore, in paper II, we decided to investigate whether HuR binds to the WNT5A transcript in melanoma cells. Furthermore, we studied the possible functional consequence of WNT5A-HuR interaction.

Initially, we selected 4 melanoma cell lines: HTB63, MM170, MM380 and WM852, and analyzed the expression level of endogenous HuR protein in these cells. Our results showed that all these cell lines expressed high levels of HuR protein. As the function of HuR is related to its cytoplasmic localization, we decided to analyze the cytoplasmic HuR expression in these cells. We found that HTB63 and WM852 cells expressed higher levels of cytoplasmic HuR fraction, as compared to MM170 and MM383 cells. Next, we analyzed the correlation between cytoplasmic HuR levels and WNT5A mRNA and protein expression. Interestingly, we observed a high expression of WNT5A mRNA in HTB63 and WM852 cells, as compared to MM170 and MM383 cells. More importantly, the high cytoplasmic HuR expression was positively correlated with WNT5A protein levels, in HTB63 and WM852 cells, whereas MM170 and MM383 lacked endogenous WNT5A protein expression. These results prompted us to examine the phenotypic differences between high WNT5A expressing cells, HTB63 and WM852, and MM170 and MM383, which do not express WNT5A. HOPP algorithm characterized HTB63 and WM852 as invasive cells, while MM170 and MM383 were described as proliferative cells. This finding clearly suggests that high WNT5A expressing cells are more invasive, as compared to the cells with no WNT5A expression. Taken together, these results suggest that increased cytoplasmic HuR expression correlates with WNT5A protein expression and, consequently, an invasive phenotype in HTB63 and WM852 melanoma cells.

As HuR has been shown to bind to adenylate/uridylate (AU)-rich sequences, in target transcripts [174], we examined if such an interaction between HuR and WNT5A exists in HTB63 and WM852 cells. Using an RNA binding protein immunoprecipitation (IP) kit, we successfully pulled down the HuR-complex from HTB63 and WM852 cells. Then, we analyzed the WNT5A

mRNA expression in the HuR complex. Interestingly, WNT5A enrichment in HuR-IP was confirmed in both cell lines.

We then studied the functional consequences of HuR-WNT5A interaction. HTB63 and WM852 cells were transfected with two independent HuR-targeting siRNA oligonucleotides, and the WNT5A mRNA and protein expression levels were analyzed after 72 hours of transfection. In HTB63 cells, HuR silencing reduced WNT5A mRNA and protein expression levels, however, surprisingly, there was no significant reduction in WNT5A mRNA and protein expression, in WM852 cells. These results suggest that HuR regulates the expression of WNT5A in HTB63 cells, but not in WM852 cells.

To further validate the WNT5A binding site of HuR, we employed two 3'UTR luciferase reporter construct, which contain overlapping parts of WNT5A 3'UTR. The regulatory regions of WNT5A were cloned downstream of the luciferase reporter gene. The HTB63 cells were first transfected with control and HuR siRNA, and later they were transfected with control or WNT5A 3'UTR reporter plasmid. We observed lower relative reporter activity from the WNT5A 3'UTR reporter plasmid in cells that were transfected with HuR-siRNA, compared to the control siRNA transfected cells. These results indicate that HuR binds to the WNT5A 3'UTR in melanoma cells.

To confirm the regulatory effect of HuR in WNT5A expression, we used a specific low-molecular-weight HuR inhibitor, called MS-444. This component has been shown to interfere with HuR dimerization, and therefore inhibit HuR function [178]. Time- and dose-dependent analyses revealed that, in HTB63 cells, 20-50 μM of MS-444 for 6 h and 12 h treatments, was sufficient to reduce WNT5A mRNA and protein expression, respectively.

Therefore, we selected 30 μM of MS-444, and treated HTB63 and WM852 cells to analyze the effect of MS-444 on WNT5A mRNA and protein expression levels. In line with our previous findings, HuR inhibition reduces WNT5A mRNA and protein expression in HTB63 cells, however, MS-444 had no effect on WNT5A expression in WM852 cells.

Next, we tested whether HuR inhibition could downregulate melanoma cell migration, in HTB63 and WM852 cells. Inhibition of HuR function with MS-444 resulted in a significant reduction in HTB63 cell migration. Importantly, the inhibition of migration, in HTB63 cells, was partially restored by the addition of recombinant WNT5A (rWNT5A). Surprisingly, HuR inhibition significantly reduced WM852 cell migration, however, addition of rWNT5A did not restore this inhibition. Therefore, we investigated another HuR-dependent regulator, which could explain the observed inhibitory effect in WM852 cells. One such candidate was MMP-9, as several investigators have shown that MMP-9 is involved in tumor cell metastasis [179,180]. We analyzed the effect of MS-444 treatment on MMP-9 expression, in HTB63 and WM852 cells. Interestingly, MS-444 reduces MMP-9 protein expression in WM852 cells, however, no significant reduction of MMP-9 was observed in HTB63 cells. Further analysis revealed that HuR inhibition reduces WM852 melanoma cell migration, in an MMP-9-dependent manner, as the inhibition of migration was partially rescued by addition of recombinant MMP-9 (rMMP9).

Taken together, our results demonstrate that HuR inhibition impairs melanoma cell migration *via* at least two distinct mechanisms.

Paper III

Combining a WNT5A- antagonist and a HuR-inhibitor results in a significant decrease in the invasiveness of malignant melanoma cells

Given the results from paper I and II, we investigated an alternative therapeutic strategy for the treatment of melanoma patients. In particular, we focused on targeting WNT5A signaling, with Box5, in combination with targeting HuR function, with MS-444, in the invasive melanoma cell lines, HTB63 and WM852.

Initially, we examined the cytotoxic effect of Box5 and MS-444 on melanoma cell lines, HTB63 and WM852. MTT cell viability assay showed no significant toxic effect of these compounds on melanoma cell viability. We have previously demonstrated that HuR promotes melanoma cell migration, in both HTB63 and WM852 cells, *via* distinct mechanisms [181]. Moreover, we have shown that Box5 reduces melanoma cell migration and invasion in both HTB63 and WM852 cells [80,182]. In light of these findings, we speculated that targeting HuR function, in combination with Box5, could more effectively inhibit melanoma cell migration and invasion. Interestingly, though individual treatment of the cells, with MS-444 or Box5, significantly reduces melanoma cell migration and invasion in both cell lines, the combination of both factors had a greater effect on melanoma cell migration and invasion. These findings suggest that targeting WNT5A signaling, as well as HuR function, could act as an effective strategy to obstruct melanoma cell dissemination.

Next, we attempted to identify the molecular mechanisms by which Box5 and MS-444 reduce melanoma cell migration and invasion. WNT5A has been shown to directly affect melanoma cell motility *via* PKC activation [91,93].

To evaluate the effect of Box5 and MS-444 on PKC signaling, we treated HTB63 cells with Box5 alone, MS-444 alone or a combination of both compounds, and analyzed the effect of these treatments on the phosphorylation of the endogenous PKC substrate, MARCKS, using Western blotting. We found that the treatment of HTB63 melanoma cells, with Box5 or MS-444, leads to a significant decrease in MARCKS phosphorylation. However, no further increase in MARCKS phosphorylation was observed when cells were treated with a combination of both factors.

Since the combination of Box5 and MS-444 resulted in a more pronounced inhibition of migration and invasion, as compared to the effect of each factor alone, we examined the involvement of an additional signaling mechanism, which, in addition to the PKC pathway, may be responsible for the combinatorial effect of Box5 and MS-444 in the reduction of melanoma cell invasiveness. We have previously demonstrated that WNT5A, through the PI3K-AKT signaling cascade, increases LDH expression and lactate secretion. This in turn leads to aerobic glycolysis and aberrant cellular energy metabolism, in melanoma cells [103]. To evaluate the possible involvement of PI3K-AKT signaling in the observed melanoma cell migration and invasion, we treated HTB63 melanoma cells with Box5 alone, MS-444 alone and a combination of both compounds. Interestingly, only MS-444 caused a significant reduction in AKT phosphorylation, whereas Box5 had no effect, not even when the cells were treated with Box5 for shorter and longer treatment periods. These results demonstrate that Box5 inhibits WNT5A-mediated cell migration and invasion, mainly through a reduction in PKC activity, while MS-444 does so *via* two distinct signaling mechanisms. Since the activation of PI3K-AKT signaling alters cellular energy metabolism due to increased lactate secretion [103], we validate our finding by measuring the

effect of MS-444 on lactate secretion into the medium. In accordance, we found a significant reduction in the amount of lactate released into the culture medium, suggesting that MS-444, *via* inhibition of WNT5A, reduced AKT-dependent lactate secretion, which could also explain the inhibitory effect of MS-444 on melanoma cell migration and invasion. We did not, however, observe any effect on lactate release when the cells were subjected to Box5 treatment. In this context, it is noteworthy that lactate has been shown to enhance tumor cell motility. For instance, in head and neck squamous cell carcinoma, cervical cancer and rectal adenocarcinomas, there is a correlation between lactate levels and metastatic spread of the tumors [183-185]. Ectopic expression of lactate significantly increases head and neck squamous cell migration and invasion in a dose-dependent manner [186]. In another study, lactate was shown to enhance glioma migration *via* TGF-β2-dependent regulation of MMP2 [187].

Taken together, we show that simultaneously targeting WNT5A signaling, with Box5, and HuR function, with MS-444, leads to a significant reduction in the invasive capacity of malignant melanoma cells.

Paper IV

Inhibition of the invasive migration of BRAF- inhibitor-resistant melanoma cells by interfering with HuR function and WNT5A signaling

Recent studies, on melanoma, have highlighted the role of WNT5A in the development of resistance to BRAF inhibitors (BRAFi) [125,188]. The exact mechanisms behind acquiring resistance are not fully understood. In paper II, we showed that HuR regulates WNT5A expression in melanoma cells. In paper III, we also demonstrated that HuR and WNT5A drive melanoma cell migration and invasion, presumably *via* PKC and PI3I-AKT signaling, and

that combining MS-444 and Box5 more effectively reduces the invasiveness of melanoma cells. These findings prompted us to hypothesize that acquired resistance to the BRAF-I, PLX4032 could lead to elevated expression of HuR and WNT5A, and consequently, a more invasive phenotype in BRAFi-resistant (BRAFi-R) melanoma cells.

We first established the BRAFi-R melanoma cell line. HTB63 cells were regularly treated with 2 μM of PLX4032 for 4 weeks, and then the concentration of PLX4032 was increased to 5 μM, for a further 8 weeks. After 12 weeks, we tested BRAFi-R characteristics by performing an MTT cell viability assay. Since the HTB63 BRAFi-R cells had a higher IC_{50} than HTB63 parental cells, we used them as an experimental model in our study. Interestingly, HTB63 BRAFi-R cells have a higher migration potential, compared to their parental cells (data not shown).

Recently, we demonstrated that HuR regulates the expression of WNT5A, in HTB63 cells [181]. However, to the best of our knowledge, there is no study about the expression of HuR in BRAFi-R cells. To test our hypothesis that acquired resistance to PLX4032 leads to elevated level of HuR and WNT5A, we analyzed the endogenous expression of HuR and WNT5A, in HTB63 BRAFi-R cells, and compared them to their parental cells. Indeed, we observed a higher expression of HuR and WNT5A in BRAFi-R cells, suggesting that acquired BRAFi-resistance enhances the expression of HuR and WNT5A.

We and other investigators have demonstrated that WNT5A activates several downstream signaling cascades, including PI3K-AKT and PKC [93,103]. To evaluate if the high expression of WNT5A leads to an increased expression of its downstream signaling molecules, we analyzed the phosphorylation of

PI3K-AKT and MARCKS (the endogenous PKC substrate) in our cells. We observed a higher expression of phospho-AKT in BRAFi-R cells, as compared to their parental cells. However, no change in the phosphorylation levels of MARCKS was detected. These results suggest that acquired BRAFi-resistance may increase PI3K-AKT activity, and, therefore, enhance migration of melanoma cells.

In paper III, we demonstrated that simultaneously targeting WNT5A signaling and HuR function, with Box5 and MS-444, causes a significant reduction in the invasive capacity of malignant melanoma cells, as a result, we studied the combinatorial effect of Box5 and MS-444 on the BRAFi-R cell migration and invasion. Here, we treated the cells with Box5 alone, MS-444 alone, or a combination of both factors. Interestingly, the combination of Box5 and MS444 had a greater effect on the migratory and invasive properties of HTB63-R cells, when compared to each factor alone, demonstrating that simultaneously targeting WNT5A signaling and HuR function more effectively reduces melanoma cell migration and invasion.

To investigate the molecular mechanisms by which Box5 and MS-444 reduce the invasiveness of BRAFi-R cells, and based on our observation that acquired BRAFi-resistance increases PI3K-AKT activity, we analyzed the effect of Box5 and MS-444 on phospho-AKT levels, in HTB63 BRAFi-R cells. To this end, we treated HTB63-R cells with Box5, MS-444 or a combination of both treatments. We observed a significant reduction of phospho-AKT when the cells were treated with a combination of Box5 and MS-444, which suggests that simultaneous Box5 and MS444 treatment reduces AKT signaling in BRAFi-R HTB63 melanoma cells.

In summary, our results show the importance of WNT5A and HuR as major regulators of enhanced invasiveness of BRAFi-R melanoma cells. Our results demonstrate that simultaneously targeting HuR function and WNT5A signaling is a potential therapeutic approach to impair BRAFi-R melanoma cell invasive migration.

Discussion

Aberrant expression of WNT5A has been implicated in several cancers, including melanoma [71,90,91,94]. However, the mechanism behind this is not fully understood. The main focus of this thesis was to investigate the molecular mechanisms responsible for elevated WNT5A expression, in malignant melanoma cells, and subsequently, investigate different approaches that result in the reduction of the invasive capacity of melanoma cells.

We showed that WNT5A and IL-6 regulate each other's expression at a translational level, as we did not observe any effect on WNT5A mRNA expression, neither on IL6-mRNA levels, following inhibition of WNT5A and IL-6 signaling. Elevated levels of WNT5A and IL-6 have been reported in melanoma. High levels of IL-6 serum correlates with poor prognosis in melanoma patients [172,189]. In addition, WNT5A has also been reported to promote melanoma tumor metastasis [91,93]. Based on these findings, our present data supports the notion that WNT5A and IL-6 cooperates to accelerate melanoma cell invasiveness.

Furthermore, we demonstrate that simultaneously targeting WNT5A and IL-6 more effectively reduces melanoma cell migration and invasion, as compared with each factor alone. There are a number of different positive feedback loops that accelerate cancer cell metastasis. Further understanding of such positive feedback loops, which accelerate tumor metastasis, increases the possibility of developing combinatorial treatment alternatives for cancer patients, in order to improve therapeutic efficacy. Box5 has been shown to significantly reduce melanoma cell migration and invasion [80,168]. Our study clearly shows that targeting both WNT5A expression and signaling more effectively reduce melanoma cell migration and invasion. Moreover,

combinatorial treatment may lead to the use of lower Box5 concentrations, which may reduce the risk of possible side effects.

The fact that melanoma cells comprise different cell types, explains the difficulty of treatment options for melanoma patients. In order to validate which therapy is most suitable therapy for each patient, a better understanding of the underlying factors that drive melanoma cell motility is required. In search of the factors that regulate WNT5A expression in malignant melanoma cells, we focused on HuR, as it has been reported that in breast cancer cells, where WNT5A acts as a tumor suppressor, HuR suppresses WNT5A translation [81]. In paper II, we explored whether HuR regulates the expression of WNT5A in malignant melanoma cells.

Our results showed that high cytoplasmic expression of HuR displayed a high WNT5A mRNA and protein expression in two invasive melanoma cells, HTB63 and WM852. Cytoplasmic HuR expression has been reported in many cancer cells, and is associated with larger tumor size and tumor grade [190-195]. Taken together, this suggests that high cytoplasmic HuR expression may play a role in regulating WNT5A expression in invasive melanoma cells. Further, we demonstrate that HuR binds to the 3'UTR of WNT5A transcript. These results are in good agreement with previous studies, wherein HuR was found to interact with WNT5A mRNA [81,196]. Further analysis revealed that in HTB63 cells, HuR drives melanoma cell migration via WNT5A, whereas in WM852 cells HuR promotes melanoma cell migration through MMP-9. In this context, it deserves to be mentioned that melanoma has a heterogeneous property, and various pathways function cohesively to promote melanoma progression. Although HTB63 and WM852 cells harbor different mutations, targeting HuR inhibits melanoma cell migration in both cell lines. In addition, a previous study on MS-444 showed

promising pharmacokinetics *in vivo* with low toxicity [178,197]. These findings suggest that MS-444 may not affect the biological processes in adult organisms. The results from this study suggest that HuR is an attractive anti-invasive target for halting melanoma dissemination.

In paper III, we explored the hypothesis that a combination of MS444 and Box5 can more effectively, than each substance alone, reduce the invasive capacity of melanoma cells. In accordance, we demonstrated that Box5, in combination with MS-444, more effectively reduces melanoma cell migration and invasion. Furthermore, we showed that individual Box5 and MS-444 treatment significantly reduces the phosphorylation of MARCKS, which suggests that targeting WNT5A signaling and expression can lead to a reduction in WNT5A mediated PKC activity, and therefore reduce melanoma cell migration and invasion. Several investigators have shown that WNT5A promotes melanoma cell motility *via* activation of this pathway [91,103]. Further analysis showed that MS-444, but not Box5, reduces AKT-phosphorylation, in HTB63 cells. This finding is in agreement with those published by Sherwood *et al*., in which WNT5A was shown to activate PI3K-AKT signaling pathway, which in turn increased aerobic glycolysis [103]. These findings suggest that Box5 and MS-444 inhibit melanoma cell migration and invasion *via* two overlapping signaling pathways, suggesting that simultaneous targeting of HuR function and WNT5A signaling acts as a more effective therapeutic approach in order to inhibit melanoma cell migration and invasion.

A major problem that cancer patients face is the development of drug resistance. Recently, WNT5A was shown to enhance melanoma cell BRAF-inhibitor resistance [125]. In paper IV, we included BRAF-inhibitor resistant (BRAFi-R) melanoma cells to test whether simultaneously targeting HuR

function and WNT5A signaling could act as an additional effective strategy to inhibit melanoma cell invasion and migration. We demonstrate for the first time that acquired melanoma BRAFi-resistance results in a significant increase in HuR expression. The high expression levels of HuR were accompanied by a significant increase in WNT5A and PI3K-AKT cascade. High WNT5A expression has been reported in BRAFi-R cells. In a recent study, Anastas *et al.* demonstrated that WNT5A expression is increased when the cells were subjected to BRAF inhibitors, leading to the activation of the PI3k-AKT signaling pathway [125].

Moreover, our results showed that combined inhibition of HuR function and WNT5A signaling, with MS-444 and Box5, respectively, more effectively decreases the invasive migration of HTB63-R melanoma cells. We found a statistically significant reduction in AKT-activity in HTB63-R cells simultaneously treated with Box5 and MS444. However, in BRAFi-R melanoma cells AKT-activity was not significantly reduced by individual Box5 treatment. Our findings suggest that it is important to simultaneously inhibit WNT5A and HuR signaling, in order to achieve a better inhibitory effect in BRAFi-R cell migration and invasion. Recent HuR findings have suggested a role in acquiring resistance to tamoxifen, in estrogen positive breast cancer cells [198], and treatment resistance in glioma [199]. This suggests that targeting HuR may induce drug sensitivity in patients displaying resistance towards targeted therapy.

Although the results from our studies provide novel insights into the role of WNT5A in melanoma progression, further analyses are required. For example, in order to evaluate that PI3K-AKT and PKC signaling cascades are responsible for WNT5A-dependent melanoma cell migration and invasion, one should include the experiments in which the cells are treated with

inhibitors towards these two signaling pathways. In addition, immunohistochemical analysis of melanoma samples that explore the possible association between WNT5A and HuR expression, in addition to WNT5A and IL-6 expression, would further support the clinical relevance of these findings, as well as the correlation between these molecules with tumor burden. The anti-metastatic effect of Box5 and MS-444 should also be studied in *in vivo* models.

Conclusions

I. WNT5A and IL-6 interact *via* a positive feedback loop to increase melanoma cell migration and invasion and dual interference of this loop impairs melanoma cell motility.

II. HuR positively regulates the expression of different pro-migratory proteins such as WNT5A and MMP-9. Inhibition of HuR function with MS-444 reduces melanoma cell migration

III. Simultaneously targeting WNT5A expression and HuR function with Box5 and MS-444, respectively, more effectively impairs melanoma cell migration and invasion *via* distinct overlapping signaling pathways such as PKC and PI3K-AKT.

IV. BRAFi-R melanoma cells express increased levels of HuR and WNT5A, which may subsequently lead to increased levels of AKT phosphorylation. Dual targeting of HuR function and WNT5A signaling significantly reduces BRAFi-R melanoma cell migration and invasion. This highlights the important role of WNT5A and HuR in BRAFi-resistance.

Populärvetenskaplig sammanfattning

Malignt melanom är en aggressiv form av hudcancer som uppkommer från specialiserade hudceller som kallas melanocyter och har som uppgift att producera det skyddande hudförmörkande pigmentet melanin som skyddar huden från skada av ultraviolet strålning. Även om malignt melanom en gång ansågs ovanligt, har den årliga incidensen ökat dramatiskt de senaste decennierna. De allra vanligaste riskfaktorn som leder till malignt melanom är solexponering som orsakar förändringar i arvsmassan, DNA. Flera studier rapporterar att artificiellt UV-ljus som till exempel i solarium också kan vara en melanomrelaterad riskfaktor. Andra riskfaktorer är medfödda födelsemärken, nedsatt immunförsvar och familjehistoria av malignt melanom. Malignt melanom är mindre vanlig än andra typer av hudcancer, men det är mer sannolikt att den växer och sprider sig till andra delar av kroppen, en process som kallas metastaser. I tidigt stadium brukar man operera bort tumören med kirurgi. Dessa patienter har en god prognos men för patienter med metastaser är prognosen betydligt sämre och de flesta patienter dör inom ett år. Därför är det viktigt att förstå vilka mekanismer som ökar cellernas förmåga att invadera sin omgivning och ta sig till andra organ och bilda metastaser.

Den här avhandlingen fokuserar på rollen av ett protein, WNT5A, vid malign melanomutveckling. Mer specifikt utforskar vi vår förståelse för underliggande mekanismer som reglerar WNT5A-uttrycket i malignt melanomceller. Dessutom undersöker vi hur olika metoder kan minska melanomcellers migration och invasion.

Vi har tidigare rapporterat att ett protein vid namn interleukin 6 (IL-6) reglerar uttrycket av WNT5A. Det finns flera studier som visar att WNT5A i

sin tur kan reglera IL-6 uttrycket. Därför spekulerade vi att WNT5A och IL-6 samarbetar med varandra via en positiv feedback loop för att öka melanomcellers rörelse. Våra resultat visar att WNT5A och IL-6 reglerar varandras uttryck och att en kombination av Box5 (WNT5A antagonist) och en IL-6 blockerande antikropp, minskar melanomcellers förmåga att invadera.

I andra studien visar vi att det RNA bindande proteinet HuR driver melanomcellers migration genom att reglera uttrycket av olika pro-migratoriska proteiner såsom WNT5A och MMP9. När vi hämmade HuR med en specifik HuR hämmare, MS-444, lyckades vi minska melanomcellers migration. Baserad på våra resultat tror vi att angripa HuR-mRNA-interaktioner med HuR hämmare kan undersökas som potentiella läkemedelskandidater vid behandling av malignt melanom.

Mot bakgrunden av våra resultat från de två första studierna undersökte vi om Box5 i kombination med MS-444 på ett effektivare sätt kan minska melanomcellers migration och invasion. Dessutom undersökte vi de bakomliggande mekanismer genom vilka WNT5A och HuR driver melanomcellers invasion. I denna studie visade vi att Box5 i kombination med MS-444 minskar melanomcellers förmåga att invadera. För övrigt, tyder våra resultat på att WNT5A och HuR reglerar melanomcellers invasion via olika överlappande signalvägar, såsom PKC och PI3K-AKT.

Ett stort problem som cancerpatienter möter är att de utvecklar läkemedelsresistens. Under det senaste decenniet har flera studier belyst rollen av WNT5A i läkemedelsresistens i olika cancertyper, bland annat malignt melanom. Många patienter som svarar positivt till läkemedel som blockerar BRAF signalväg, utvecklar senare resistans mot dessa läkemedel

vilket leder till att läkemedlet inte längre hämmar tumörutvecklingen och att patienter i förlängningen avlider.

Eftersom vi har visat att HuR reglerar uttrycket av WNT5A i melanomceller, spekulerade vi om resistens mot BRAF-hämmaren PLX4032 leder till förhöjda nivåer av HuR och WNT5A. Våra resultat visar att celler som är resistenta mot BRAF hämmare har ett ökat uttryck av både HuR och WNT5A vilket kan leda till ökade nivåer av AKT-fosforylering som i sin tur kan öka cellernas rörelse. Dubbel blockering av HuR-funktion och WNT5A-signaleringen minskar rörelsen hos celler som är resistenta mot BRAF hämmaren. Detta framhäver den viktiga rollen som WNT5A och HuR har i att driva melanomcellers migration och invasion.

Sammanfattningsvis, den här avhandlingen ökar vår kunskap om WNT5A-reglering i malignt melanom och framhäver betydelsen av Box5 och MS-444 som potentiella läkemedel hos malignt melanompatienter.

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Research Paper

Demonstration of a WNT5A-IL-6 positive feedback loop in melanoma cells: Dual interference of this loop more effectively impairs melanoma cell invasion

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ABSTRACT

Increased expression and signalling of WNT5A and interleukin-6 (IL-6) have both been shown to promote melanoma progression. Here, we investigated the proposed existence of a WNT5A-IL-6 positive feedback loop that drives melanoma migration and invasion. First, the HOPP algorithm revealed that the invasive phenotype of cultured melanoma cells was significantly correlated with increased expression of *WNT5A* or IL-6. In three invasive melanoma cell lines, endogenous WNT5A protein expression was related to IL-6 protein secretion. Knockdown with anti-IL-6 siRNAs or treating WM852 melanoma cells with a neutralising anti-IL-6 antibody reduced WNT5A protein expression. Conversely, the silencing of WNT5A expression by WNT5A siRNAs or treating WM852 melanoma cells with Box5 (a WNT5A antagonist) significantly reduced IL-6 secretion. Interestingly, these effects occurred at the protein level but not at the transcriptional levels. Functionally, we demonstrated that combined siRNA knockdown of WNT5A and IL-6 expression or the simultaneous inhibition of WNT5A and IL-6 signalling inhibited melanoma cell invasion more effectively than suppressing each factor individually. Together, our results demonstrate that WNT5A and IL-6 are connected through a positive feedback loop in melanoma cells and that the combined targeting of both molecules could serve as an effective therapeutic means to reduce melanoma metastasis.

INTRODUCTION

Deregulation of *WNT5A* is often linked to the development and progression of various cancers [1]. While the loss of WNT5A expression is correlated with poor prognosis in breast [2] and colorectal cancer [3], the opposite trend was observed for cutaneous melanoma [4]. Increased WNT5A expression is associated with a higher invasive and metastatic potential of melanoma cells [5, 6]. Similar to WNT5A, the pro-inflammatory cytokine IL-6 promotes melanoma cell invasion, and its increased expression is correlated with reduced overall patient survival [7–10]. Two recent studies have demonstrated a link between IL-6 secretion and WNT5A expression in melanoma cells [11, 12], suggesting that the combined therapeutic interference with this link might be beneficial for preventing disease progression and metastatic spread.

WNT5A is a lipid-modified secreted glycoprotein that is regarded as a non-canonical WNT ligand, which means that it elicits the activation of β-catenin-independent WNT signalling pathways [13]. In turn, these pathways can be subdivided depending on the major downstream signalling molecule involved (e.g., Ca^{2+}, JNK and small GTPases such as Rho, Rac and Cdc42), and their selective activation is largely dictated by the cell surface context of different non-canonical WNT receptors [14, 15]. Certain members of the Frizzled family of GPCRs and tyrosine kinase receptors such as ROR2 and RYK have been demonstrated to mediate WNT5A-induced β-catenin-independent signalling [1, 16, 17]. In melanoma, many of these pathways have been directly shown to participate in WNT5A-driven cell migration and invasion [5, 18, 19].

Considering all of these factors, we have developed a WNT5A-derived antagonistic peptide that could be used to inhibit WNT5A signalling and subsequently reduce melanoma cell invasion [20].

Apart from WNT5A, there are other regulators of melanoma cell invasion that promote metastasis; IL-6 is one of these regulators. In cutaneous melanoma, IL-6 expression is detectable at the early nevi stage, and its level dramatically increases as the tumour invades deeper into the underlying dermis [10]. Similar to the IL-6 level, the expression of the IL-6 receptor (IL-6R) also increases with melanoma progression, indicating an autocrine or paracrine function for IL-6 during melanoma progression [10]. In the classical signalling pathway, IL-6 acts by binding to IL-6R, a receptor complex of IL-6Rα and glycoprotein 130 (gp130) receptors. IL-6 binding to IL-6R induces JAK-mediated phosphorylation of several tyrosine receptor motifs within the cytosolic domain of gp130, which activates the transcription factors of the STAT-family and also mediates the activation of RAS/RAF/MEK/MAPK and PI3K/AKT-signalling [21]. In agreement to these classical pathways, we have recently shown that IL-6 can induce p38α-MAPK activation in melanoma cells. More importantly, we demonstrated that the IL-6-induced p38α-MAPK activation promoted melanoma cell migration and invasion through increased WNT5A expression [12].

The aim of the current study was to explore the existence of a WNT5A-IL-6 positive feedback loop in malignant melanoma cells and to investigate whether dual interference with this loop would be a more effective therapeutic means to obstruct melanoma cell migration and invasion.

RESULTS

Elevated WNT5A and IL-6 expressions in invasive melanoma

To test our hypothesis that WNT5A and IL-6 could co-operate to accelerate melanoma metastasis, we first analysed whether their gene expression levels correlated with the invasive potential of melanoma cell lines. This investigation was possible due to the Heuristic Online Phenotype Prediction (HOPP) algorithm developed by Hoek and colleagues. The algorithm phenotypically stratifies publicly available microarray data sets to classify individual melanoma cell lines as either proliferative or invasive [22]. As previously demonstrated [12], extracted data revealed that significantly increased mRNA expression of *WNT5A* (Figure 1A) is associated with an invasive phenotype signature of melanoma cells. Interestingly, the same association was discovered for the mRNA expression of *IL-6* (Figure 1B). We also performed a correlation analysis between the two ligands on an individual cell line basis. However, we found only a poor correlation (Pearson correlation = 0.194) between *WNT5A* and *IL-6* mRNA expression (data not shown) in the invasive melanoma cell lines. In proliferative cell lines, our analyses revealed a similar weak correlation (Pearson correlation = 0.254) between *WNT5A* and *IL-6* mRNA expression (data not shown). We also analysed the co-expression of *WNT5A* and *IL-6* mRNA in different melanoma tumour tissue data sets by using the TCGA melanoma data set (www.cancergenome.nih.gov) and Oncomine melanoma data sets (www.oncomine.org). Our results showed either poor or no correlation between *WNT5A* and *IL-6* mRNA expression in patient-derived melanoma samples (data not shown).

Next, we selected three melanoma cell lines—WM852, HTB63 and A375—that are phenotypically classified as invasive by the HOPP algorithm (Figure S1), and compared their levels of *WNT5A* and *IL-6* mRNA/protein expression. In good agreement with our previous report [12], all three cell lines expressed *WNT5A* mRNA and protein, although WM852 cells displayed a significantly higher level of expression compared to HTB63 and A375 cells (Figure 1C and 1D). Similarly, QPCR and ELISA-based analysis of the three cell lines and their conditioned cell culture media demonstrated that the level of *IL-6* mRNA and IL-6 secretion was several magnitudes higher in WM852 cells compared to HTB63 and A375 cells (Figure 1E and 1F). However, because we also observed a difference in WNT5A protein expression between HTB63 and A375 cells (Figure 1D), we anticipated a similar difference when comparing their levels of IL-6 secretion. The reason why no difference was detected (Figure 1F) is presently unknown. Interestingly, as presented in Figure 1C and 1E, we did not observe any correlation between *WNT5A* and *IL-6* mRNA expression. Furthermore, the quantitative PCR analysis revealed a significantly higher transcriptional level of the IL-6 receptor (IL-6R) in HTB63 cells compared to WM852 and A375 cells (Figure S2). Together, these analyses and our cell line data suggest that *WNT5A* and *IL-6* mRNA expression is poorly correlated in melanoma cells, although their increased expression is characteristic of an invasive melanoma cell phenotype. The three melanoma cell lines (WM852, HTB63 and A375) studied expressed different levels of WNT5A protein and secreted different amounts of IL-6 (Figure 1D and 1F). However, due to the limited number of cell lines used in our study, their WNT5A protein levels and IL-6 secretion could not be correlated. Therefore, we investigated their possible relationship by different means.

IL-6 increases the expression and release of WNT5A in WM852 cells

We previously demonstrated that treatment with recombinant IL-6 (rIL-6) significantly increases the expression of WNT5A in HTB63 and A375 cells, but it had no effect on WNT5A expression in WM852 cells [12].

Based on the observations made by Terai *et al.*, in which rIL-6-induced IL-10 secretion was much more pronounced in melanoma cells with low compared to high endogenous IL-6 secretion [23], our results might have been due to a high level of endogenous IL-6 secretion in WM852 cells (Figure 1F). Consequently, to investigate whether WNT5A expression is regulated by IL-6 in WM852 cells, we explored how knockdown of IL-6 by two independent siRNAs affected WNT5A expression. Compared to NC siRNA-transfected cells, the two different IL-6 siRNAs significantly reduced IL-6 secretion 72 h post-transfection (Figure 2A). Interestingly, the reduced IL-6 secretion was concomitantly associated with significantly lowered WNT5A expression (Figure 2B) and release (Figure 2C), thus indicating that their high level of IL-6 secretion maintained the relatively high WNT5A expression in WM852 cells. To validate these findings, we studied whether the recombinant-IL-6 stimulation of IL-6 siRNA-depleted WM852 cells could regain WNT5A release after 48 h. In agreement with our assumption of a WNT5A-IL-6 positive feedback loop, we observed an increased release of WNT5A in the concentrated medium of recombinant-IL-6-stimulated IL-6-silenced WM852 cells after 48 h (Figure S3). To confirm these results, we treated WM852 cells for 24 h with increasing concentrations of a monoclonal anti-IL-6 antibody to neutralise endogenous IL-6 signalling. In accordance with our previous results, increasing concentrations of the neutralising IL-6 antibody reduced WNT5A expression in a dose-dependent manner, whereas equal concentrations of an IgG_1 isotype control antibody had no effect (Figure 3A). Cell viability studies did not reveal any significant effect of the human anti-IL-6 antibody on WM852 cell viability (Figure S4). To determine whether the IL-6-induced effect on WNT5A expression occurs at the transcriptional or translational level, the *WNT5A* mRNA level was also evaluated in WM852 cells after monoclonal anti-IL-6 antibody treatment. Interestingly, there was no significant reduction in the *WNT5A* mRNA level after treating the cells with increasing doses of the IL-6 neutralising antibody (Figure 3B). These results confirm that IL-6 positively regulates WNT5A expression in WM852 melanoma cells, and that this regulation occurs at the translational level.

WNT5A increases the release of IL-6 in WM852 cells

Having ascertained that IL-6 increases WNT5A expression in WM852 cells, we next investigated whether a reciprocal relationship exists between the two factors. Transient transfection (72 h) using two independent WNT5A-siRNAs resulted in significantly reduced WNT5A expression (Figure 4A) and release (Figure 4B) compared to NC siRNA-transfected cells. Notably, when the conditioned media from the same cells was analysed for the content of IL-6, both of the WNT5A-siRNAs caused a statistically significant reduction in IL-6 release compared to the NC siRNA-transfected cells (Figure 4C), indicating that WNT5A increases IL-6 release in WM852 cells. These results are in good agreement with previously published observations in which siRNA silencing of WNT5A reduced the exosomal release of IL-6 in HTB63 cells [11]. To confirm our results and also take a more therapeutic approach, we then treated WM852 cells for 24 h with 100 or 500 μM of the WNT5A-derived antagonistic peptide 'Box5' to inhibit endogenous WNT5A signalling. Although no significant difference was observed between the two different concentrations of Box5, they both mimicked the results obtained through siRNA-silenced WNT5A expression, lowering the IL-6 release compared to that in vehicle-treated cells (Figure 4D). To investigate whether the WNT5A-induced IL-6 secretion is regulated at the transcriptional level, the *IL-6* mRNA level was evaluated by QPCR in WM852 cells after Box5 (100 and 500 μM) treatment. Interestingly, there was no reduction in the *IL-6* mRNA expression which favours the non-transcriptional regulation of IL-6 after antagonising the effect of WNT5A by Box5 treatment (Figure 4E). There was no significant inhibition of WM852 cell viability after treatment with 100 μM of Box5. However, limited toxicity was observed after treatment with 500 μM of Box5, mainly due to the increased DMSO concentration (Figure S5). These data demonstrate that WNT5A increases IL-6 secretion in WM852 cells. Together, our observations provide evidence for the existence of an endogenous WNT5A-IL-6 positive feedback loop mechanism that facilitates the increased expression of these two pro-metastatic factors in WM852 melanoma cells.

Dual interference of the WNT5A-IL-6 positive feedback loop more effectively impairs melanoma cell migration and invasion

Several studies have highlighted the ability of either IL-6 or WNT5A to promote melanoma cell motility [5, 7, 9, 18–20]. As these findings and our present data demonstrate a WNT5A-IL-6 positive feedback loop in WM852 melanoma cells, we investigated whether targeting both these factors simultaneously could be a more efficient way to reduce or inhibit melanoma cell migration and invasion.

To investigate this phenomenon, we first transiently transfected WM852 cells for 72 h with anti-WNT5A siRNA #2 alone, anti-IL-6 siRNA #1 alone, or a combination of both, and we assessed their invasive capability relative to NC siRNA-transfected cells. Strikingly, although siRNA silencing of either WNT5A or IL-6 (Figure 5) alone reduced WM852 cell invasion by approximately 40%, the combined silenced expression of both WNT5A and IL-6 further reduced WM852 cell invasion to approximately 60% (Figure 5). Taking a more

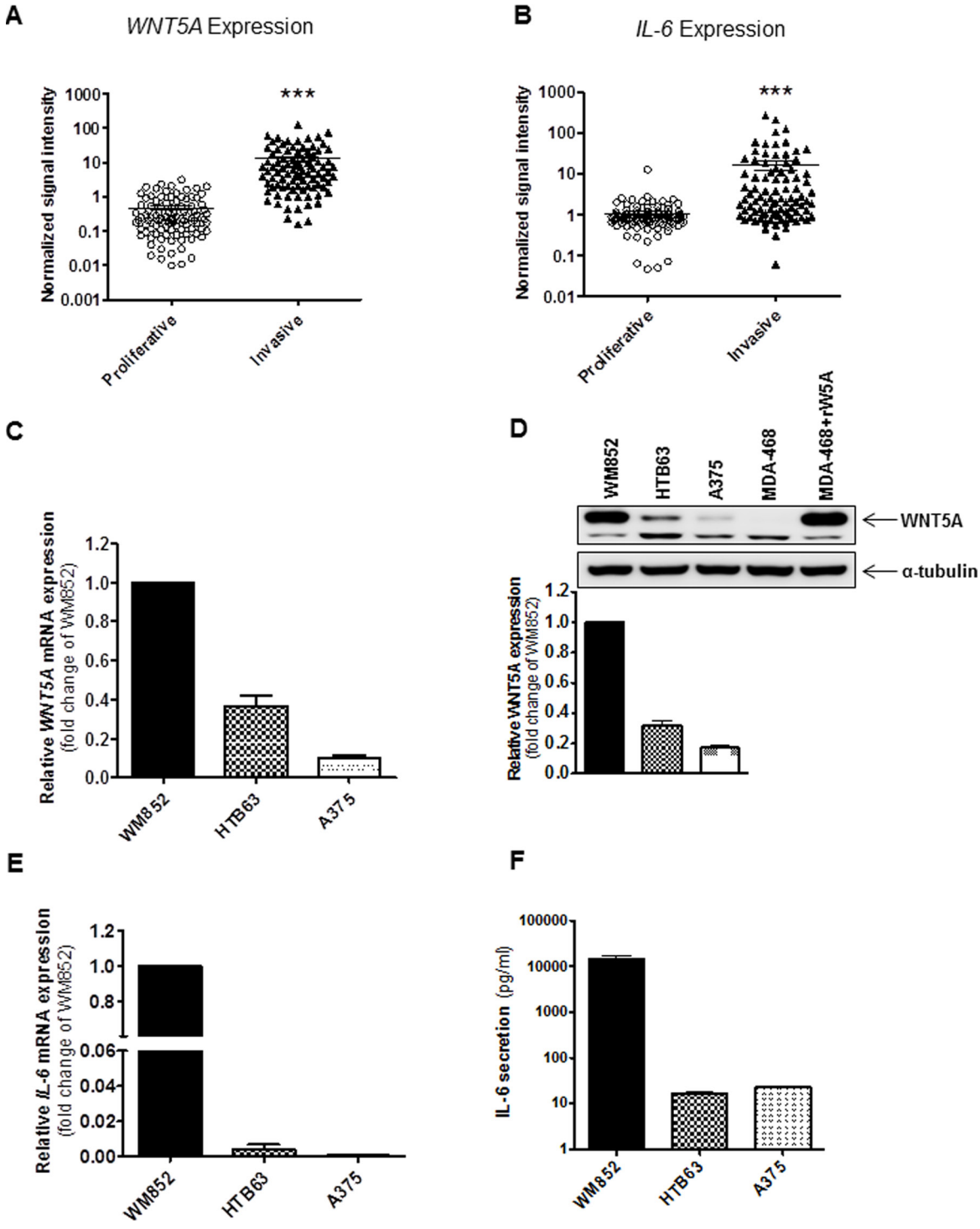

Figure 1: Increased expression of WNT5A and IL-6 is associated with an invasive melanoma cell phenotype. (**A**, **B**) The panels show the gene expression of *WNT5A* and *IL-6* in multiple microarray data sets of melanoma cell lines classified by the Heuristic Online Phenotype Prediction (HOPP) algorithm as proliferative versus invasive. (**C**) QPCR analyses of endogenous *WNT5A* mRNA expression levels in human WM852, HTB63, and A375 cells. Samples were normalised against *TATA-binding protein (TBP)* mRNA expression, and the results are presented relative to the *WNT5A* mRNA expression of WM852 cells ($n = 5$). (**D**) Western blot analysis showing the levels of endogenous WNT5A protein expression in the human melanoma cell lines WM852, HTB63, and A375. α-tubulin was used as a loading control. A representative blot from four separate experiments is shown. The lower panel shows densitometric analyses of WNT5A protein expression normalised against α-tubulin; the results are presented relative to WNT5A protein expression of WM852 cells ($n = 4$). (**E**) QPCR analysis of endogenous *IL-6* mRNA expression levels in human WM852, HTB63, and A375 cells. Samples were normalised against *TATA-binding protein (TBP)* mRNA expression; the results are presented relative to the *IL-6* mRNA expression of WM852 cells ($n = 5$). (**F**) ELISA analysis showing the quantification of endogenous IL-6 protein secretion in human WM852, HTB63, and A375 cells ($n = 5$). The results are presented as the means and S.E.Ms; ***$p < 0.001$.

feasible therapeutic approach, we treated two different melanoma cell lines (WM852 and HTB63) with Box5 (100 μM) and/or a neutralising anti-IL-6 antibody (1 μg/ml) and checked their migration/invasion. In both cell lines, we observed that combined blockage of WNT5A and IL-6 signalling more efficiently impaired cell migration/invasion (Figures 6 and S6), similar to the results obtained with combined siRNA transfection (Figure 5). We did not observe any significant effects on WM852 cell viability after Box5 (100 μM) and/or anti-IL-6 antibody (1 μg/ml) treatment (Figure S7). Our data clearly indicate that WNT5A and IL-6 co-operate to facilitate melanoma cell migration and invasion and that the combined therapeutic inhibition of both WNT5A and IL-6 signalling stands out as an effective treatment strategy to prevent melanoma dissemination.

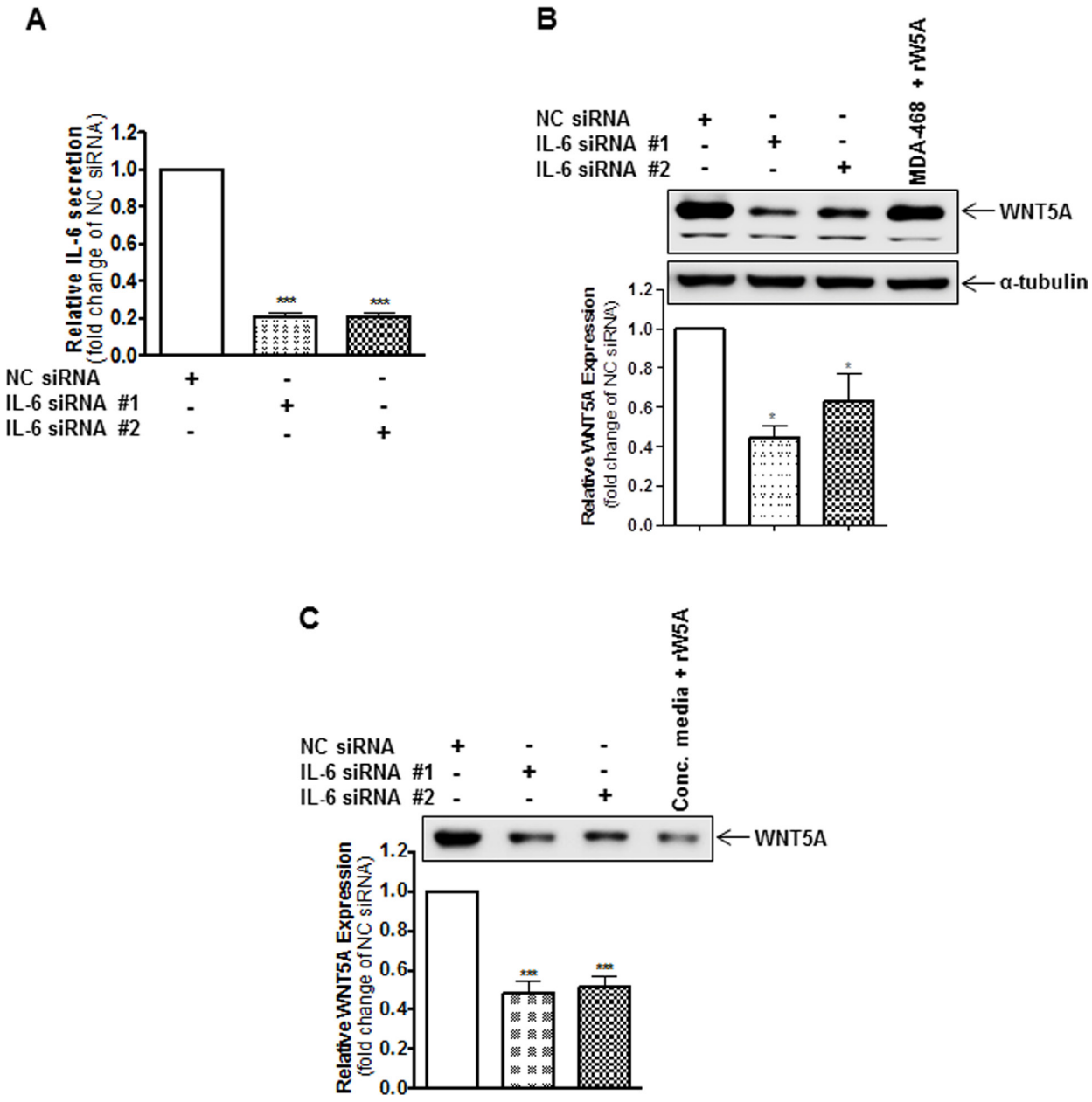

Figure 2: IL-6 knockdown reduces the endogenous protein expression of WNT5A in WM852 cells. (**A**) ELISA analysis showing siRNA-based silencing of endogenous IL-6 in WM852 cells. Cells were transfected with either negative control siRNA (NC siRNA; 50 nM), anti-IL-6-siRNA #1 (IL-6 siRNA #1; 50 nM) or anti-IL-6-siRNA #2 (IL-6 siRNA #2; 50 nM) and incubated for 72 h. The results are presented relative to the IL-6 secretion of NC siRNA-transfected cells (n = 4). (**B**) Western blot analysis of WNT5A expression in WM852 cells treated as described in (A). Densitometric analyses were performed, and the data were then normalised to α-tubulin, which was used as a loading control. The results are presented relative to the WNT5A expression of NC siRNA-transfected cells (n = 4). (**C**) Western blot analysis of WNT5A release from WM852 cells treated as described in (A). A representative blot from four separate experiments is shown. The results are presented as the means and S.E.Ms; *$p < 0.05$; ***$p < 0.001$.

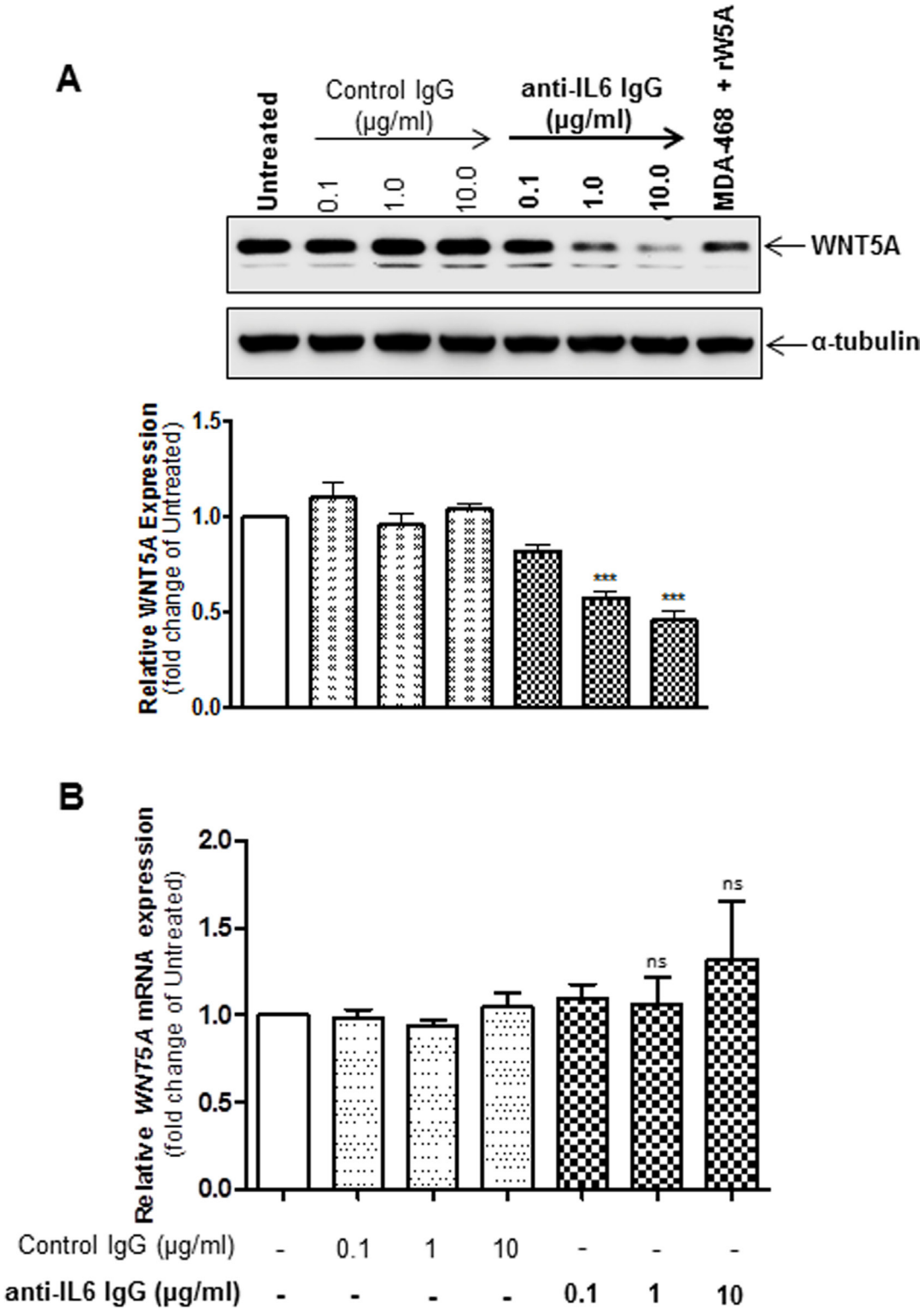

Figure 3: Inhibition of IL-6 signalling reduces WNT5A proteins expression but not *WNT5A* mRNA expression in WM852 cells. (**A**) Western blot analysis of WNT5A expression in WM852 cells treated with the indicated concentrations of a neutralising anti-IL-6 antibody or equal concentrations of an IgG1 isotype control antibody. Densitometric analyses were performed, and the data were then normalised to α-tubulin used as a loading control. The results are presented relative to the WNT5A expression of untreated WM852 cells ($n = 5$). (**B**) QPCR analyses of endogenous *WNT5A* mRNA expression levels in human WM852 treated with the indicated concentrations of a neutralising IL-6 antibody or equal concentrations of an IgG1 isotype control antibody. Samples were normalised against *TATA-binding protein (TBP)* mRNA expression, and the results are presented relative to the *WNT5A* mRNA expression of untreated cells (n = 5). The results are presented as the means and S.E.Ms; ***$p < 0.001$.

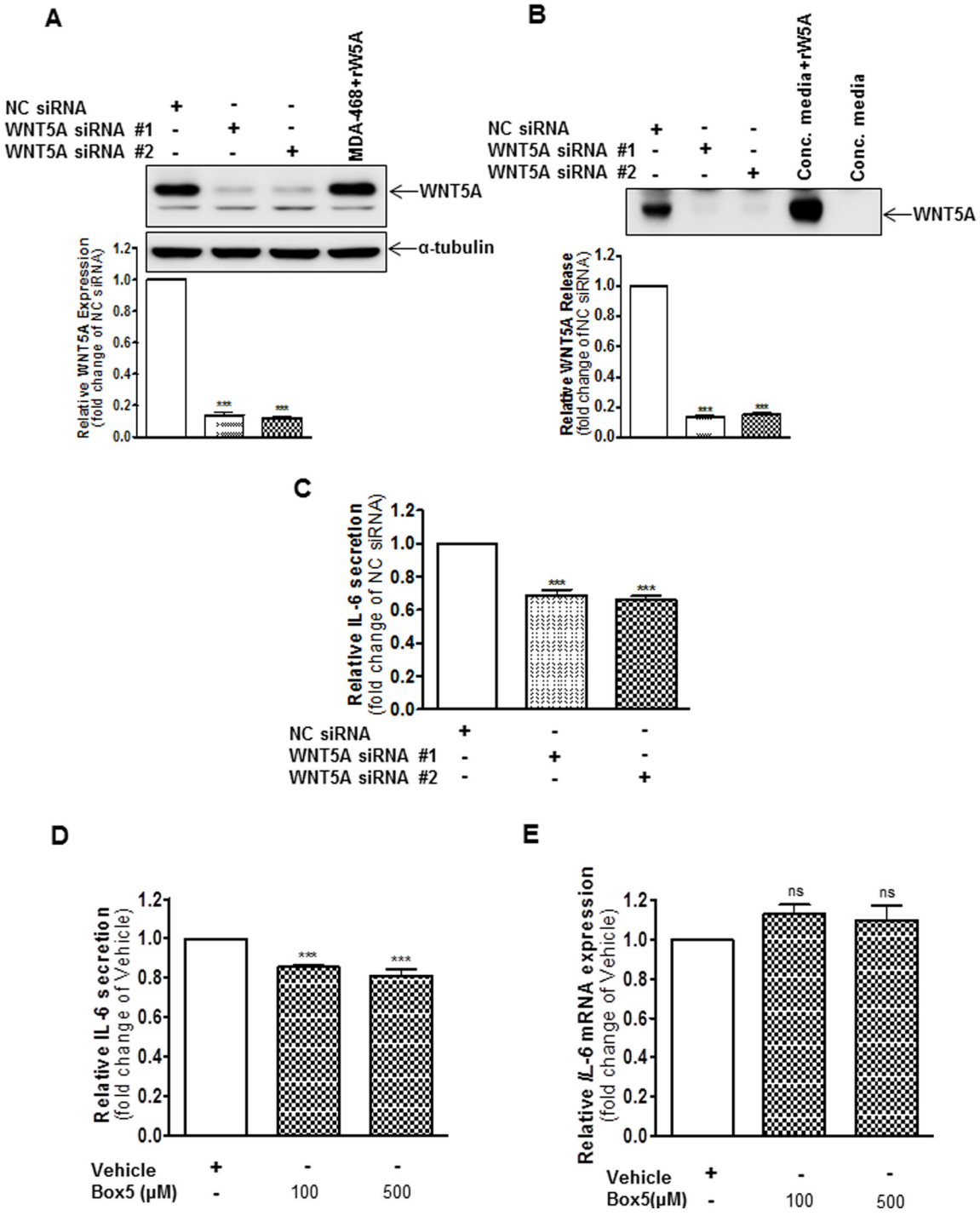

Figure 4: WNT5A increases IL-6 secretion from WM852 cells. (**A**) Western blot analysis showing siRNA silencing of endogenous WNT5A expression in the human melanoma cell line WM852. Cells were transfected with either negative control siRNA (NC siRNA; 100 nM), anti-*WNT5A*-siRNA #1 (WNT5A siRNA #1; 100 nM) or anti-*WNT5A*-siRNA #2 (WNT5A siRNA #2; 100 nM) and were incubated for 72 h. Densitometric analyses were performed, and the data were then normalised to α-tubulin, which was used as a loading control. The results are presented relative to the WNT5A expression of NC siRNA-transfected cells ($n = 4$). (**B**) Western blot analysis of WNT5A release from WM852 cells treated as described in (A). A representative blot from four separate experiments is shown. (**C**) ELISA analysis of IL-6 secretion from WM852 cells treated as described in (A). The results are presented as relative to IL-6 secretion of NC siRNA-transfected cells ($n = 4$). (**D**) ELISA analysis of IL-6 secretion from WM852 cells treated with the indicated concentrations of Box5 or vehicle control ($NaHCO_3$ buffer, pH ~7). The results are presented relative to IL-6 secretion of vehicle-treated cells ($n = 4$). (**E**) QPCR analyses of *IL-6* mRNA expression levels in human WM852 treated with the indicated concentrations of Box5 or vehicle control ($NaHCO_3$ buffer, pH ~7). Samples were normalised against *TATA-binding protein (TBP)* mRNA expression, and the results are presented relative to the *IL-6* mRNA expression of vehicle-treated cells ($n = 5$). The results are presented as the means and S.E.Ms; ***$p < 0.001$.

DISCUSSION

In a study on rheumatoid arthritis (RA), Sen et al. showed that WNT5A could induce the expression of the pro-inflammatory cytokine IL-6 in fibroblast-like synoviocytes (FLS) and the inhibition of WNT5A either with antisense or a dominant negative vector, reducing IL-6 levels in FLS obtained from patients with RA [24]. Moreover, it has been demonstrated that WNT5A can positively regulate the exosomal release of IL-6 [11], and we have shown that stimulation with exogenous IL-6 can induce WNT5A expression in malignant melanoma cells [12]. Taking into account these findings, we set out to investigate whether WNT5A can regulate IL-6 expression and *vice versa* in a feedback loop in melanoma cells. In the context of melanoma, WNT5A is an important player, whose increased expression could facilitate cell invasion and metastasis, thus promoting the progression of this disease [5, 19, 25, 26]. In support of our finding of a WNT5A-IL-6 positive feedback loop, both WNT5A [1, 5, 6] and IL-6 [7–10, 12] have been reported to promote tumour cell metastasis, although the role of IL-6 has not been completely explored in melanoma. However, an interesting study conducted by Hoejberg et al. demonstrated increased serum levels of IL-6 in melanoma patients, and these elevated levels of IL-6 could be linked to poor patient survival [8]. Further support for the existence of a WNT5A-IL-6 positive feedback loop comes from our analysis of a publically available online database [Heuristic Online Phenotype Prediction (HOPP) algorithm] [22]. In this analysis, we found elevated expression of both *WNT5A* and *IL-6* transcripts in invasive melanoma cells.

For the present study, we selected WM852 cells as an ideal system, first for their invasive nature (according to the HOPP database) and second for the increased levels of both WNT5A and IL-6. Although HTB63 and A375 cells are also stratified as invasive phenotypes (by HOPP), they express relatively low levels of WNT5A and IL-6 in comparison to the expression in WM852 cells. Using WM852 cells, we showed that either siRNA silencing of IL-6 or the neutralisation of endogenous IL-6 signalling

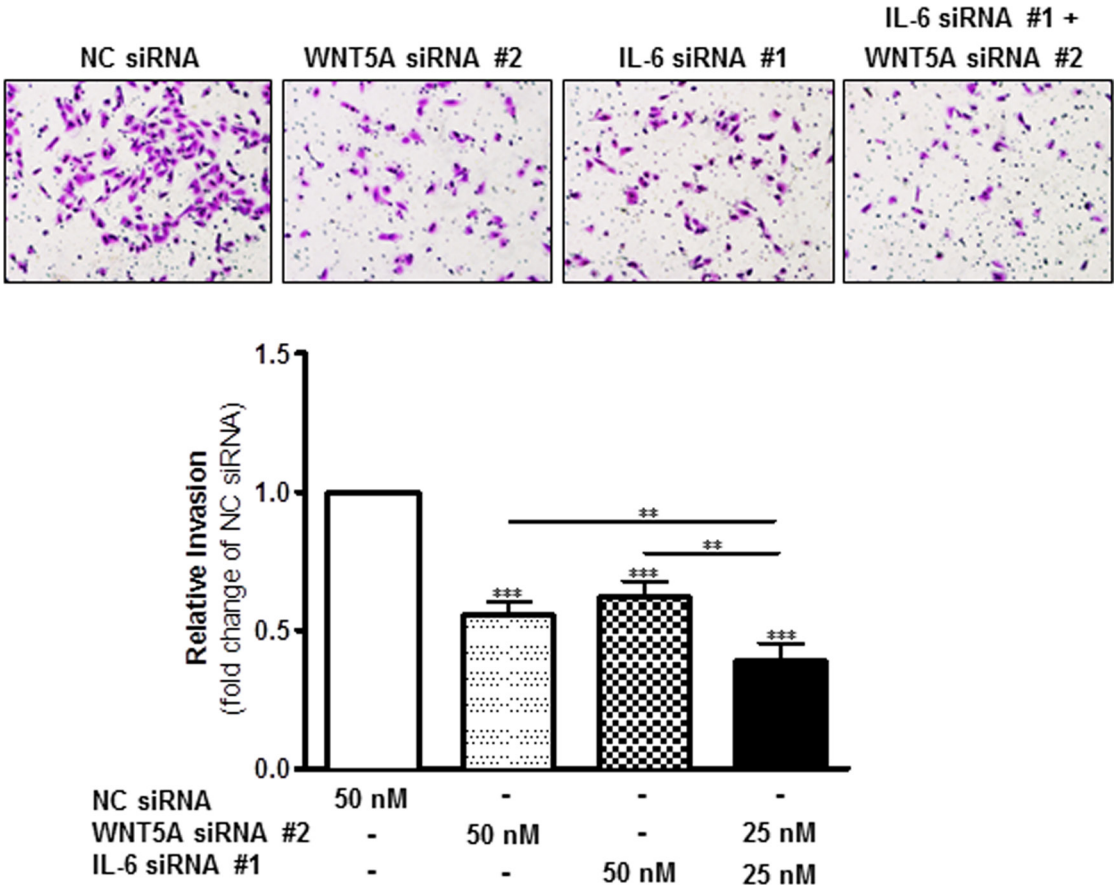

Figure 5: Combined knockdown of WNT5A and IL-6 protein expression more effectively inhibits WM852 cell invasion. In a Matrigel-based invasion assay system, the human melanoma cell line WM852 was transiently transfected for 72 h with either negative control siRNA (NC siRNA; 50 nM), anti-WNT5A-siRNA #2 (WNT5A siRNA #2; 50 nM), anti-IL-6-siRNA #1 (IL-6 siRNA #1; 50 nM) or anti-WNT5A-siRNA #2 and anti-IL-6-siRNA #1 (25 nM each). The invasion assays were performed during the last 24 h of each transient transfection and analysed as described in the Materials and Methods section. The results are presented as the relative invasion of NC siRNA transfected cells ($n = 4$) and are presented as the means and S.E.Ms; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

could significantly reduce WNT5A expression in WM852 cells. In addition, siRNA silencing of WNT5A expression and the inhibition of endogenous WNT5A signalling using Box5 significantly reduced IL-6 secretion in WM852 melanoma cells. However, there was no reduction in *WNT5A* or *IL-6* mRNA levels following treatment with either an IL-6 neutralising antibody or Box5, which excludes the possibility that the WNT5A-IL-6 loop in WM852 cells is regulated at the transcriptional level.

Many examples of different positive feedback loops operating at the molecular/cellular levels that facilitate cancer cell invasion and metastasis exist. For example, in glioma cells, the pro-metastatic fibroblast growth factor-inducible 14 (Fn14) can induce its own expression through the activation of Rac1 and NF-κB, thereby promoting tumour cell migration and invasion [27]. Moreover, in breast cancer, invasive mesenchymal-like cancer cells can secrete GM-CSF, which stimulates the differentiation of macrophages into tumour-associated macrophages (TAMs). Reciprocally, these TAMs provide the cancer cells with increased amounts of the chemokine CCL18, which maintain the mesenchymal phenotype of the breast

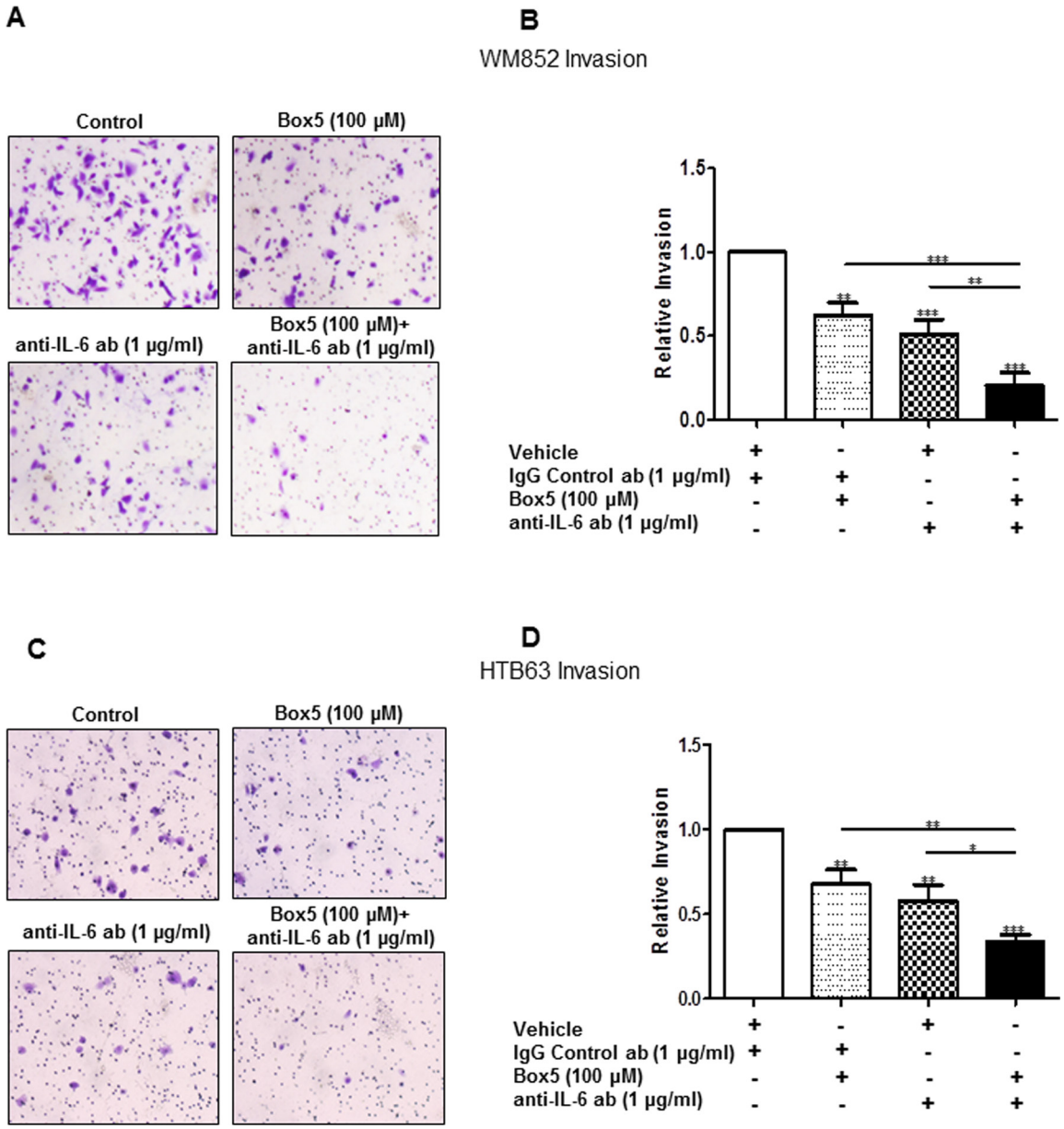

Figure 6: Combined inhibition of WNT5A and IL-6 signalling with Box5 and an anti-IL-6 antibody more effectively impairs WM852 and HTB63 cell invasion. Matrigel invasion of WM852 (**A** and **B**) and HTB63 (**C** and **D**) cells treated with either Box5 or neutralising anti-IL-6 antibody alone and their combination was evaluated as described under the Materials and Methods section. Proper controls (DMSO for Box5 treatment or IgG_1 isotype antibody for neutralising anti-IL-6-antibody treatment) were used to compare the treatment effect on the cell invasions. The invasion assays ($n = 5$) were performed over 24 h and analysed as described in the Materials and Methods section. The results are presented as the means and S.E.Ms; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

cancer cells, thus promoting breast cancer metastasis [28]. Although such mechanisms provide the tumours with an advantage in supporting cancer progression, they also widen the possibility of effective combinatorial treatment options to improve therapeutic efficacy. Considering the pro-metastatic role of both WNT5A and IL-6 in melanoma progression, a combined treatment that effectively inhibits the presently demonstrated WNT5A-IL-6 positive feedback loop is an attractive strategy to successfully impair melanoma cell invasion and metastasis.

Next, we investigated the efficacy of combined interference in the WNT5A-IL-6 positive feedback loop by inhibiting both WNT5A signalling and IL-6 signalling. As a proof of concept, we first demonstrated that combined siRNA knockdown of WNT5A and IL-6 more effectively inhibited the invasion of WM852 cells compared with the effects of individual siRNA knockdown of either WNT5A or IL-6. In the next set of experiments, we employed a more feasible therapeutic approach by combining Box5 (a WNT5A antagonist) and a neutralising anti-IL-6 antibody to investigate the efficacy of such a combined treatment on melanoma cell migration and invasion. Our results showed that the combined inhibition of WNT5A signalling (Box5) and IL-6 signalling (neutralising anti-IL-6 antibody) mimicked the results of the combined siRNA knockdown of WNT5A and IL-6 by more effectively reducing the migration and invasion of two different invasive melanoma cell lines, compared with the results from individual treatments with either Box5 or a neutralising anti-IL-6 antibody. These observations not only strengthen our demonstration of a WNT5A-IL-6 positive feedback loop but also, and more importantly, revealed the advantage of combined therapeutic inhibition as an attractive treatment strategy to prevent melanoma dissemination.

In summary, the present study demonstrates that WNT5A regulates its own expression in melanoma cells via a WNT5A-IL-6 positive feedback loop and that combined inhibition of both WNT5A signalling and IL-6 signalling could be an effective strategy to obstruct the dissemination of melanoma cells and thus slow or prevent disease progression.

MATERIALS AND METHODS

Microarray data analysis

The Zürich, Philadelphia, Mannheim, Wagner, Johansson and Augustine melanoma cell line microarray data sets were analysed online (http://www.jurmo.ch/hopp/hopp_mpse.php) using the Heuristic Online Phenotype Prediction (HOPP) algorithm. Retrieved normalised gene expression data of *WNT5A* (probe set 205990_s-at) and *IL-6* (probe set 205207_at) from melanoma cell lines that scored as either proliferative or invasive by the HOPP algorithm were plotted and statistically analysed using Student's *t*-test. Melanoma phenotype signatures of WM852 (GSM109044), HTB63 (GSM162902) and A375 (GSM29663) cells were retrieved online (http://jurmo.ch/hopp/hopp_default_data.php) and presented as Widmer plots [22] (Figure S1).

Cell culture and treatment

The WM852 human melanoma cell line originates from a metastatic site and its genetic background includes $NRAS^{Q61R}/BRAF^{WT}/PTEN^{+/-}/TP53^{mutated}$. The WM852 human melanoma cell line (Cat#WC00065) was procured in February 2015 from CORIELL Cell Repositories, Camden, NJ, USA. The identity of the WM852 cell line was confirmed by the supplier by short tandem repeat (STR) profiling using the AmpFlSTR®Identifiler® PCR Amplification Kit (Cat#4322288) from Life Technologies, USA using loci consistent with all major worldwide STR standards. The HTB63 human melanoma cell line originates from a metastatic site and its genetic background includes $BRAF^{V600E}/NRAS^{WT}/PTEN^{-/-}/TP53^{WT}$. The A375 human melanoma cell line originates from a metastatic site and its genetic background includes $BRAF^{V600E}/NRAS^{WT}/PTEN^{WT}/TP53^{WT}$. Both the HTB63 human melanoma cell line (Cat#HT-144, LOT: 59550354) and the A375 human melanoma cell line (Cat#CRL-1619, LOT:61573377) were obtained from American Type Culture Collection (ATCC, VA, USA) in August 2014. The identities of the HTB63 and A375 cell lines were confirmed by ATCC by using multiplex PCR, which amplifies the amelogenin gene and eight of the most informative polymorphic markers in the human genome. The analysis of the amplicons was performed by Promega PowerPlex® 1.2 system and the Applied Biosystems Genotyper 2.0 software. The WM852 cell line was purchased on the 23rd of February 2015, whereas the HTB63 and A375 cell lines were purchased on the 12th August 2014. Immediately upon arrival, all three cell lines were grown for 2 passages. Then, they were frozen in smaller aliquots that were thawed every fourth month as a fresh sample. Therefore, no cell line was used for more than 4 months.

All cell lines were regularly tested for the absence of mycoplasma contamination (EZ-PCR kit by Biological Industries, Bet Haemek, Israel) and grown as previously described. All cell lines were routinely serum starved prior to each experiment in 1% FBS-supplemented media for 24 h. For the neutralisation of secreted IL-6, required concentrations of mouse monoclonal anti-human IL-6 IgG_1 antibody (Clone #6708, R&D Systems, Minneapolis, Minnesota, USA) were used and compared against equal concentrations of mouse IgG_1 isotype control antibody (Clone #11711, R&D Systems, Minneapolis, Minnesota, USA). For the inhibition of WNT5A signalling, the indicated doses of Box5 (Calbiochem, San Diego, CA, USA) were used and compared to equal volumes of vehicle ($NaHCO_3$ buffer, pH of approximately 7 or cell culture grade DMSO with a final concentration below 0.1%).

Western blotting

Cells were lysed in 1 M Tris-HCl pH 7.5, 0.5 M NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.5 M EDTA, 1.5 mM $MgCl_2$, 10% Glycerol, and 1% Triton X-100, supplemented with complete Mini EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, Indiana, USA; 1 tablet/10 ml lysis buffer) and spun at 12,000 × *g* for 10 min at 4°C. Following the estimation of sample protein concentration using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, Illinois, USA), equal amounts of total protein (15 µg) were prepared in 4x Laemmli buffer and heated to 95°C for 5 min prior to loading on a SDS-PAGE gel. We used MDA-MB-468 breast cancer cell lysates as negative controls because they do not express endogenous WNT5A protein [12]. As positive controls, we used MDA-468 cell lysates supplemented with recombinant WNT5A protein (MDA-468 + rW5A). After the separation and transfer of the proteins to PVDF membranes, immuno-detection was performed using the following antibodies: primary antibodies, goat anti-WNT5A (1:100, R&D Systems, Minneapolis, Minnesota, USA) and mouse anti-α-tubulin (1:30000, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), secondary HRP-conjugated rabbit-anti-goat or goat anti-mouse antibodies (1:10000, Dako, Glostrup, Denmark). Chemiluminescent acquisitions of protein band intensities were obtained using the ChemiDoc™ imaging system (Bio-Rad Laboratories Inc., San Francisco, CA, USA) and densitometric quantification of relative protein expression was performed using Image Lab 3.0 software (Bio-Rad laboratories Inc., San Francisco, CA, USA).

IL-6 ELISA

IL-6 release was measured in conditioned low serum cell culture media (only supplemented with 1% FBS) using the Human IL-6 ELISA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All experiments were conducted in 6-well plates, and collected cell culture supernatants were routinely centrifuged at 1000 × *g* for 5 min to remove cell debris and frozen at – 80°C prior to analysis. For the quantification of endogenous IL-6 release, plated WM852, HTB63 and A375 cells were maintained in cell culture media supplemented with 10% FBS for approximately 2 days until confluent. After reaching confluence, the cells were washed in PBS and 1.5 ml low serum media was added to each well. The cells were then allowed to condition the low serum cell culture media for 48 h. For analysis of IL-6 release following transient siRNA silencing of either IL-6 or WNT5A (72 h), treated cells were washed in PBS 24 h post-transfection and subsequently allowed to condition the low serum cell culture media for the remaining 48 h of each transient siRNA transfection. The amount of IL-6 release was then presented relative to negative control siRNA-transfected cells.

RNA extraction, reverse transcriptase PCR and quantitative real-time PCR

Cells were washed twice with PBS, and total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Equal amounts (1 µg) of RNA from each sample were used for cDNA synthesis using random primers and the M-MuLV reverse transcriptase enzyme (Thermo Scientific, Rockford, Illinois, USA). Quantification of the mRNA expression levels of *WNT5A, IL-6* and *IL-6R* and the endogenous TATA box binding protein (TBP) in the samples was carried out on a Stratagene Mx3005P system (Agilent Technologies, Santa Clara, CA, USA) using Maxima Probe/ROX QPCR Master Mix (Thermo Scientific) and primers, TaqMan Gene Expression Assays Hs01075666_m1 and Hs00427620_m1, respectively (Thermo Fisher Scientific, Waltham, MA, USA). For the relative quantification of *WNT5A, IL-6* and *IL-6R* levels, the comparative Ct method was performed using MxPro 4.10 software (Agilent Technologies, Santa Clara, CA, USA) and normalised against TBP.

siRNA transfections

Transient siRNA transfections were performed as previously described [12] using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 400,000 cells/well seeded in 6-well plates were transfected with the indicated concentrations of the following siRNA oligonucleotides (all from Invitrogen): Negative Control (NC) siRNA (#4390843), anti-IL-6 siRNA #1 (s7311), anti-IL-6 siRNA #2 (s7312), anti-WNT5A siRNA #1 (s14871), and anti-WNT5A siRNA #2 (s14872).

Cell invasion

Cell invasion assays were performed as previously described [12] using Matrigel pre-coated cell culture inserts (Corning, Bedford, MA, USA) with 8-µm-pore-size membranes and manually quantified by counting the number of invaded cells using an inverted light microscope (Nikon TMS, Japan). For experiments assessing the invasive capacity following either independent or combined siRNA silencing of WNT5A and IL-6, treated WM852 cells were allowed to invade during the last 24 h of each 72 h transient transfection. The results were presented as the relative invasion compared to negative control siRNA-transfected cells. To investigate how the inhibition of WNT5A signalling, the neutralisation of secreted IL-6 or a combination of both phenomena affected the invasive capacity of melanoma cells, WM852 and HTB63 cells were first pre-treated with Box5 (100 µM/ml) and/or anti-human IL-6 IgG_1 antibody (1 µg/ml) alone or in combination for 24 h. After pre-treatment, invasion assays

were performed over 24 h in the presence of the same dose of fresh Box5 and/or anti-human IL-6 IgG_1 antibody. The results were presented as the relative invasion compared to cells treated with IgG_1 isotype control antibody and vehicle (DMSO with a final concentration of 0.025%).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0 and IBM-SPSS software. Significant differences between two groups were determined by Student's *t*-tests, and those between multiple groups were determined using an analysis of variance (ANOVA). Multiple group analyses were checked for significance after an ANOVA using the Newman-Keuls multiple comparison test. The Pearson correlation test was used for correlation analysis of *WNT5A* and *IL-6* mRNA expression in melanoma cell/tissue based data sets. All the experiments were repeated in at least triplicate, and the data were expressed as the mean ± S.E.M. Differences were considered to be significant if the *p* value was less than 0.05.

CONFLICTS OF INTEREST

Tommy Andersson is a shareholder of WntResearch and is the part-time Chief Scientific Officer of WntResearch. This circumstance does not alter the authors' adherence to all guidelines for publication in *Oncotarget.*

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Demonstration of a WNT5A-IL-6 positive feedback loop in melanoma cells: Dual interference of this loop more effectively impairs melanoma cell invasion

Supplementary Materials

SUPPLEMENTARY MATERIALS AND METHODS

MTT cell viability assay

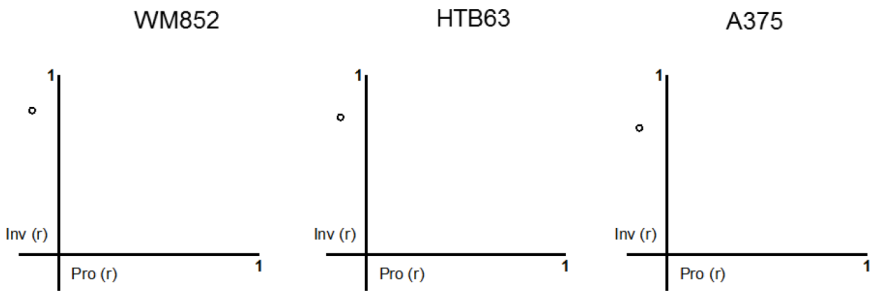
The MTT assay was performed to check the cytotoxicity of anti-human IL-6 IgG_1 antibody or Box5 in WM852 cells. Briefly, 15000 cells/well of WM852 cells were grown in 96-well flat-bottom tissue culture plates and treated with increasing concentrations of anti-human IL-6 IgG_1 antibody or Box5 for 48 h. In a separate treatment set, cells were exposed to fixed doses of either Box5 (100 μM) or anti-human IL-6 IgG_1 antibody (1 μg/ml) and their combination. For each treatment, cells exposed to the IgG_1 isotype control antibody or DMSO was considered to be the experimental control. After treatment, the media was removed and the wells were washed once with 1X PBS. Approximately 100 μL of 0.05% MTT reagent was added to each well followed by incubation at 37°C for 4 h to allow the formation of formazan crystals. Formazan crystals were dissolved, and colour intensity was measured spectrophotometrically at 570 nm.

Recombinant-IL-6 stimulation

To check for the presence of a WNT5A-IL-6 positive feedback loop, WM852 cells were first transiently transfected with *IL-6*-siRNA #1 for *IL-6* knockdown. The *IL-6* silenced cells were stimulated with recombinant-IL-6 for 48 h, and WNT-5A release was checked through western blotting.

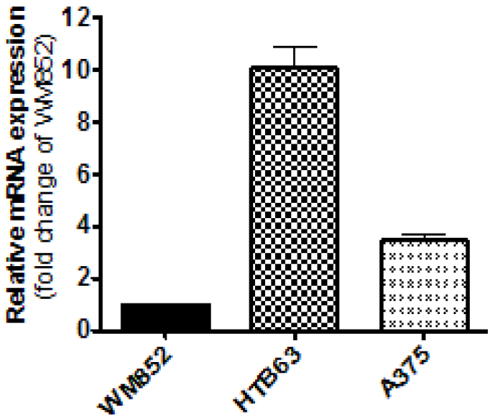
Migration assay

Cell migration experiments were performed in both WM852 and HTB63 cells following 24 h pretreatment with individual or combination of Box5 or/and anti-human IL-6 IgG_1 antibody. After pretreatment, the cells were detached by versene application and suspended in 1% FBS- containing media. Approximately 50,000 cells/insert were seeded with fresh Box5 or/and anti-human IL-6 IgG_1 antibody (individually or in combination) treatment in the respective samples and allowed to migrate over a time period of 24 h. For quantification, the cells were fixed and stained with crystal violet. The quantification of cell migration was based on the cell numbers of each cell line, which was calculated either by counting the numbers of cells using NIH IMAGEJ© software or by spectroscopic methods.

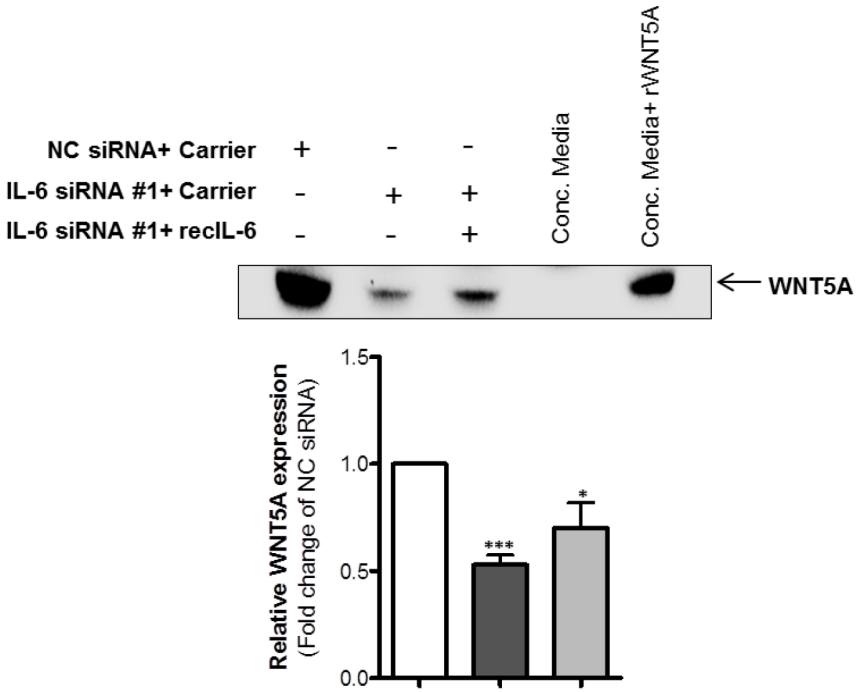


Accession ID	Cell Line	Pro (r)	Inv (r)
GSM109044	WM852	-0.1337	0.8041
GSM162902	HTB63	-0.1276	0.766
GSM29663	A375	-0.1379	0.7073

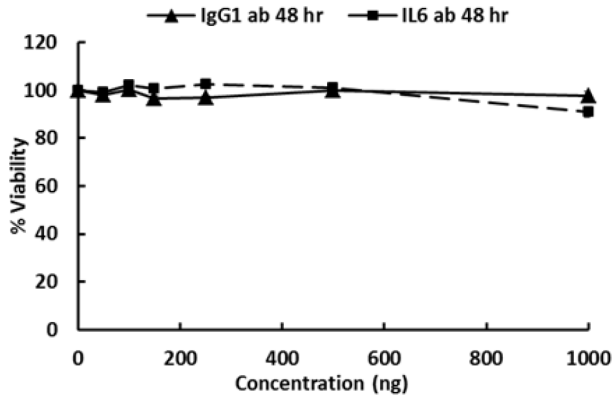
Supplementary Figure S1: HOPP phenotype signature of WM852, HTB63 and A375 cells. Widmer plots showing the phenotype signatures of the human melanoma cell lines WM852, HTB63 and A375 as analysed by the Heuristic Online Phenotype Prediction (HOPP) algorithm.



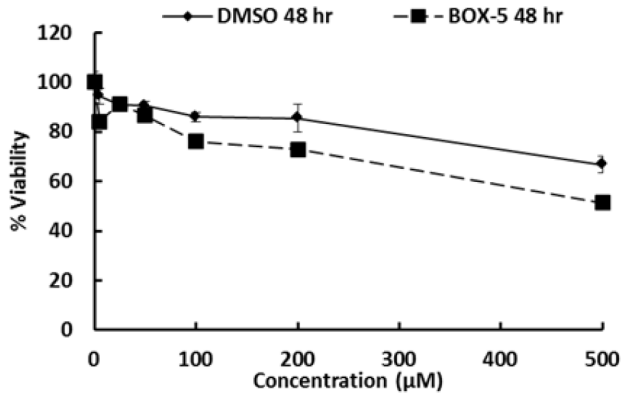
Supplementary Figure S2: IL-6R mRNA expression in WM852, HTB63 and A375 cells. QPCR analysis of endogenous *IL-6R* mRNA expression levels in human WM852, HTB63, and A375 cells. Samples were normalised against *TATA-binding protein (TBP)* mRNA expression, and the results are presented relative to the *IL-6R* mRNA expression of WM852 cells.



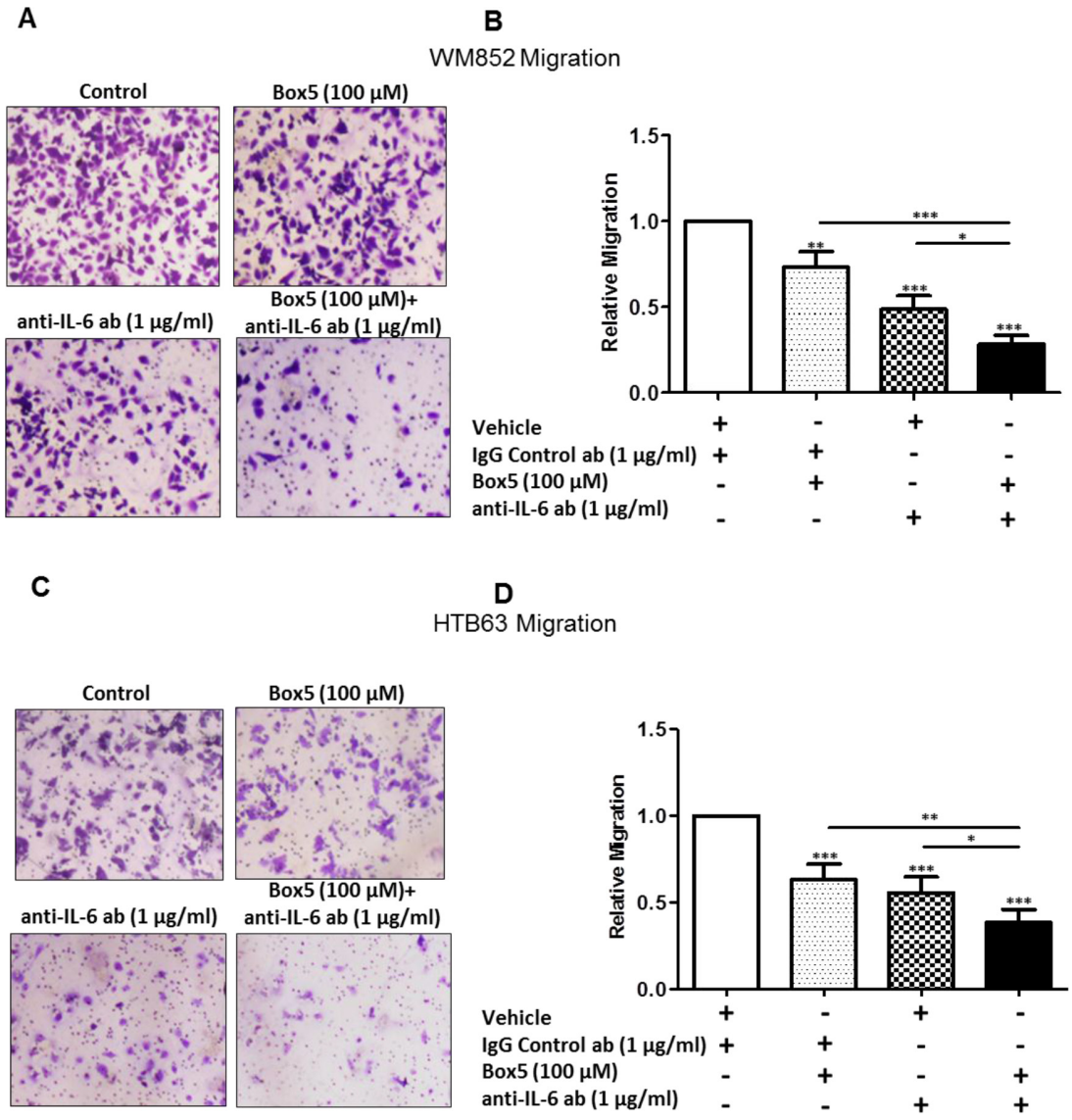
Supplementary Figure S3: Recombinant IL-6 stimulation recovers WNT5A release in IL-6-silenced WM852 cells. The release of WNT5A in WM852 cells post-*IL-6*-siRNA transfection was evaluated through immunoblotting. Briefly, equal volumes of the treatment medium were collected from NC siRNA, *IL-6*-siRNA #1 transfected and recombinant IL-6-stimulated (48 h) WM852 cells, and WNT5A release was determined in the concentrated sample. Recombinant WNT5A was used as a positive control for WNT5A detection in the blots.



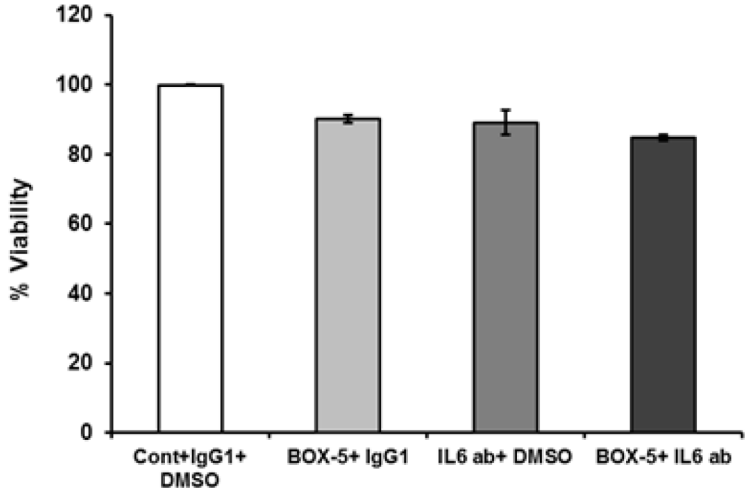
Supplementary Figure S4: Effect of IL-6 neutralising antibody on the viability of WM852 cells. An MTT cell viability assay was performed after WM852 cells were exposed to increasing doses of human anti-IL-6 antibody for 48 h as described in the Supplementary Materials and Methods section. The results were evaluated at 570 nm using a multi-well plate reader.



Supplementary Figure S5: Effect of the WNT5A-derived antagonistic peptide Box5 on WM852 cell viability. An MTT cell viability assay was performed after WM852 cells were exposed to increasing doses of human Box5 for 48 h as described in the Supplementary Materials and Methods section. Readings were taken at 570 nm using a multi-well plate reader.



Supplementary Figure S6: The combination of Box5 and anti-IL-6 antibody more effectively impairs WM852 and HTB63 cell migration. (**A**) transwell-based migration assay was performed to evaluate the cell migration efficiency of (**A** and **B**) WM852 and (**C** and **D**) HTB63 treated with either Box5 and neutralising anti-IL-6-antibody alone or their combination as described in the Supplementary Materials and Methods section. Proper controls (DMSO for Box5 treatment or IgG_1 Isotype antibody for neutralising anti-IL-6-antibody treatment) were used to compare the effect of the treatments on the cell migration. The results are given as means and S.E.Ms; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



Supplementary Figure S7: Combinatorial effect of Box5 and anti-IL-6-antibody on WM852 cell viability. WM852 cells were exposed to either individual Box5 or anti-IL-6 antibody for 48 h as described in the Supplementary Materials and Methods section. Readings were taken at 570 nm using a multi-well plate reader.

Dual mechanisms of action of the RNA-binding protein human antigen R explains its regulatory effect on melanoma cell migration

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Overexpression of wingless-type MMTV integration site family 5A (WNT5A) plays a significant role in melanoma cancer progression; however, the mechanism(s) involved remains unknown. In breast cancer, the human antigen R (HuR) has been implicated in the regulation of WNT5A expression. Here, we demonstrate that endogenous expression of WNT5A correlates with levels of active HuR in HTB63 and WM852 melanoma cells and that HuR binds to WNT5A messenger RNA in both cell lines. Although the HuR inhibitor MS-444 significantly impaired migration in both melanoma cell lines, it reduced WNT5A expression only in HTB63 cells, as did small interfering RNA knockdown of HuR. Consistent with this finding, MS-444-induced inhibition of HTB63 cell migration was restored by the addition of recombinant WNT5A, whereas MS-444-induced inhibition of WM852 cell migration was restored by the addition of recombinant matrix metalloproteinase-9, another HuR-regulated protein. Clearly, HuR positively regulates melanoma cell migration via at least 2 distinct mechanisms making HuR an attractive therapeutic target for halting melanoma dissemination. (Translational Research 2016;172:45–60)

Abbreviations: AREs = Adenylate uridylate rich elements; HOPP = Heuristic online phenotype prediction; MMP-9 = Matrix metalloproteinase-9; RBP = RNA binding protein; RIP = RNA binding protein immunoprecipitation; WNT5A = Wingless-type MMTV integration site family 5A

INTRODUCTION

Wingless-type MMTV integration site family 5A (WNT5A) is a non-canonical WNT family member initially recognized for its involvement in embryonic development.[1] WNT5A is now broadly accepted as playing a key role in promoting melanoma cell invasion through complex receptor-dependent mechanisms.[2-4] In support of a functional involvement of WNT5A in melanoma invasion, gene expression profiling studies of melanoma tissues, primary melanoma cell cultures, and established cell lines have revealed that high WNT5A expression is significantly associated with an invasive phenotype.[5-7] Furthermore, in a cohort of patients with metastatic melanomas, elevated WNT5A protein expression was shown to correlate significantly with a poor outcome.[8] Although a considerable amount of work has been published on the function of the WNT5A protein in malignant melanoma cells, several questions about WNT5A biology remain to be addressed. One such fundamental issue that has not been fully explored is related to the mechanisms by which WNT5A expression is regulated.

To our knowledge, there are no reports on mutations in the gene encoding for WNT5A in human

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AT A GLANCE COMMENTARY

Moradi F, et al.

Background

Elevated wingless-type MMTV integration site family 5A (WNT5A) expression has been positively associated with melanoma progression, but it remains unclear how WNT5A expression is upregulated in melanoma. In the present study, we investigated whether human antigen R (HuR) controls WNT5A expression and thereby promotes melanoma metastasis.

Translational Significance

The present study demonstrates that the coexpression of HuR and WNT5A is characteristic of the invasive melanoma cells HTB63 and WM852 but not of the proliferative melanoma cells MM170 and MM383. HuR-inhibition differentially disrupts WNT5A expression in HTB63 and matrix metalloproteinase-9 in WM852 cells, thereby impairing migration of both cell lines. These findings make HuR an attractive anti-invasive target for restricting metastasis of invasive melanoma cells.

malignancies. Therefore, the increased WNT5A expression in invasive melanomas is most likely due to altered regulation(s) at the transcriptional and/or the translational level. Regarding the transcriptional regulation of WNT5A expression, we have recently reported that interleukin (IL)-6 stimulation increases WNT5A expression in malignant melanoma in a p38α-MAPK–dependent manner.[9] Similar to IL-6, transforming growth factor-beta has also been implicated in the transcriptional regulation of WNT5A in melanoma[10] as well as other cancer types.[11-13] In one of the few detailed studies on the transcriptional regulation of WNT5A, Ripka et al[12] provided substantial support for CUTL1 acting as a transcription factor that could mediate transforming growth factor-beta–induced WNT5A expression. In addition to the transcriptional regulation, several other mechanisms can control protein expression including the post-transcriptional regulation of messenger RNA (mRNAs) by RNA-binding proteins (RBPs) and microRNAs, which in an orchestrated way regulate transcript stability and translational availability.[14]

Using cutting-edge photoactivatable ribonucleoside enhanced crosslinking and immunoprecipitation technique, Lebedeva et al[15] demonstrated human antigen R (HuR) binding sites in WNT5A mRNA in human cells. HuR is a member of the embryonic lethal abnormal vision (*ELAV*) family of RBPs that contains 3 RNA-recognition motifs with the ability to bind adenylate- and uridylate-rich elements (AREs) in a target mRNA.[16] These AREs are usually located in the 3′UTR of target transcripts, and the binding of HuR often results in mRNA stabilization and/or increased translation.[17,18] In this context, it should be noted that WNT5A mRNA has an extended 3′UTR sequence that contains several putative AREs to which HuR can bind.[19]

The impact of HuR on a specific target mRNA is regulated by both post-translational modifications of HuR itself and the presence of other RBPs and miRNAs.[20-22] Among the many established HuR targets are the transcripts of genes involved in cancer and inflammation, including COX-2 and matrix metalloproteinase-9 (MMP-9).[23] Indeed, aberrant HuR expression has been reported in malignancies such as colon, breast, and prostate cancer,[24-26] and altered HuR expression/activity is believed to contribute to tumor onset and progression.[27] In contrast to other cancers, the role of HuR in malignant melanoma has not yet been comprehensively studied. However, Serini et al[28] did report that HuR stabilizes COX-2 mRNA and mediates constitutive COX-2 expression in WM266-4 melanoma cells.

In the present study, we investigated if the RBP HuR interacts with WNT5A mRNA to regulate WNT5A protein expression and the functional consequences of such an interaction in melanoma cells.

METHODS

Cell culture. All melanoma cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2. Initially, WM852, MM170, and MM383 cells were kindly provided by Drs Göran Jönsson and Jill Howlin of the Department of Oncology, Lund University, Sweden and were later procured directly from ATCC & The Wistar Institute. HTB63 cells were procured from ATCC. HTB63 cells were maintained in McCOY's 5A modified medium, whereas MM170, MM383, and WM852 were maintained in RPMI-1640 medium. The growth media were supplemented with 10% fetal bovine serum (FBS), 2-mM L-glutamine, 0.5-U/mL penicillin, and 0.5-U/mL streptomycin. All cell lines were regulatory tested for the absence of mycoplasma infection.

HuR RNA-binding protein immunoprecipitation analyses. The RNA-binding protein immunoprecipitation (RIP) analysis was performed using the Magna RIP kit (Millipore Corporation, Billerica, Massachusetts) according to the manufacturer instructions. Briefly, $\sim 2.0 \times 10^6$ HTB63 and WM852 cells were lysed in 250-μL RIP lysis buffer. After

centrifugation, 10 μL of the supernatant was used each as RNA and protein input samples. A 100-μL aliquot of the remaining supernatant was added to protein A/G magnetic beads precoated with either 5 μg of HuR antibody (sc-5261; Santa Cruz Biotechnology, Inc, Dallas, Texas) or control immunoglobulin G (IgG)1 antibody (ab81216; Abcam, Cambridge, UK), and the mixtures were subsequently incubated overnight at 4°C. After immunoprecipitation (IP), the bead suspensions were extensively washed, and 1/10th of the suspension was removed to test the efficiency of HuR-IP by Western blotting. The remaining bead samples together with the input RNA sample were then treated with proteinase K, and the RNA from each sample was purified using the recommended phenol:chloroform protocol, followed by RNA precipitation overnight at −80°C. The precipitated RNA samples were washed, dissolved in 20-μL RNase-free water used for complementary DNA synthesis, and analyzed by qPCR for WNT5A, COX-2, MMP-9, and GAPDH mRNA (primer sequences are provided in the qPCR section). The relative amounts of WNT5A, MMP-9, and COX-2 mRNA from the HuR- and IgG-IPs were quantified by using the comparative Ct method and normalized to the amounts of nonspecific GAPDH mRNA in each immunoprecipitate. The HuR RIP analysis was repeated 3 times.

Transient small interfering RNA transfections. At 24 hours after seeding, cells were transfected with 50-nM small interfering RNA (siRNA) prepared using serum-free medium and Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, California) according to the manufacturer's recommendations. At 6 hours after siRNA transfection, the medium was changed to fresh growth medium; after 24 hours, it was replaced with low-serum medium (with 1% FBS). After 72 hours, the cells were harvested for RNA and/or protein extraction. The siRNA sequences used to target HuR were as follows: HuR si #1, target sequence: 5′-GCU CAG AGG UGA UCA AAG A-3' (ON-TARGETplus siRNA, Thermo Scientific, Rockford, Illinois); HuR si #2, target sequence: 5′-ACC AGT TTC AAT GGT CAT AAA-3' (FlexiTube siRNA, Qiagen GmbH, Hilden, Germany). ON-TARGETplus Non-targeting siRNA #1 from Thermo Scientific was used as a negative control.

Real-time quantitative reverse transcription polymerase chain reaction. Total RNA was purified with an RNeasy kit (Qiagen GmbH) as described by the manufacturer. Complementary DNA was synthesized and used as the template in qPCR reactions with the Maxima SYBR Green/ROX qPCR master mix (Thermo Scientific). All qPCR reactions were performed using a Stratagene Mx3005P with the following settings: preheating for 10 minutes at 95°C, 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at either 60°C for 60 seconds (for WNT5A, COX-2, and housekeeping genes) or at 52°C for 60 seconds (for MMP-9). Relative quantification of the expression levels was performed using the comparative Ct method and normalized to the expression of either GAPDH or to the geometric mean of GAPDH, SDHA, and HPRT1. The primer sequences used were as follows: WNT5A forward, 5′-TCA GGA CCA CAT GCA GTA-3'; WNT5A reverse, 5′-CTC ATG GCG TTC ACC ACC-3'; COX-2 forward, 5′- CAG ACA ACA TAA ACT GCG CCT TTT-3'; COX-2 reverse, 5′- GAC TTC CTG CCC CAC AGC AA-3′; HuR forward, 5′-TTG AAT CTG CAA AAC TTA TTC GGG-3'; HuR reverse, 5′- ACG ACA CCT TAA TGG TTT TTG ACT-3′; GAPDH forward, 5′-AAT TCC ATG GCA CCG TCA AGG CTG-3′; GAPDH reverse, 5′-TCG CCC CAC TTG ATT TTG GAG GGA-3′; SDHA forward, 5′-TGG GAA CAA GAG GGC ATC TG-3′; SDHA reverse, 5′-CCA CCA CTG CAT CAA ATT CAT G-3′; HPRT1 forward, 5′-TGA CAC TGG CAA AAC AAT GCA GAC T-3′ HPRT1 reverse, 5′-CTT CGT GGG GTC CTT TTC ACC AGC-3′; MMP-9 forward 5′-TTG ACA GCG ACA AGA AGT GG-3′; MMP-9 reverse 5′-GCC ATT CAC GTC GTC CTT AT-3′.

Western blotting. For protein isolation, cells were dissolved in lysis buffer; 20-mM TRIS-HCl, 150-mM NaCl, 30-mM sodium pyrophosphate, 50-mM NaF, 1-mM EDTA (pH 8), 1.5-mM $MgCl_2$, 0.2-mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, and 1 tablet of Roche Complete-Mini protease inhibitor (from Roche Diagnostics, Indianapolis, Indiana), vortexed and incubated on ice for 30 minutes. The lysates were then centrifuged for 30 minutes at 14.000 rpm and 4°C, and the supernatants were collected and stored at −80°C. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Scientific). Protein samples were mixed with Laemmli buffer supplemented with DTT (final concentration 40 mM), boiled for 10 minutes, and subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The separated proteins were subsequently transferred to polyvinylidene fluoride membranes and incubated with antibodies against WNT5A (R&D Systems, Minneapolis, Minnesota), HuR (Santa Cruz Biotechnology, Inc), or MMP-9 (Abcam). After overnight incubation with the primary antibodies, the membranes were washed and further incubated with respective secondary antibodies (goat anti-mouse/or rabbit or rabbit-anti goat HRP from Dako, Glostrup,

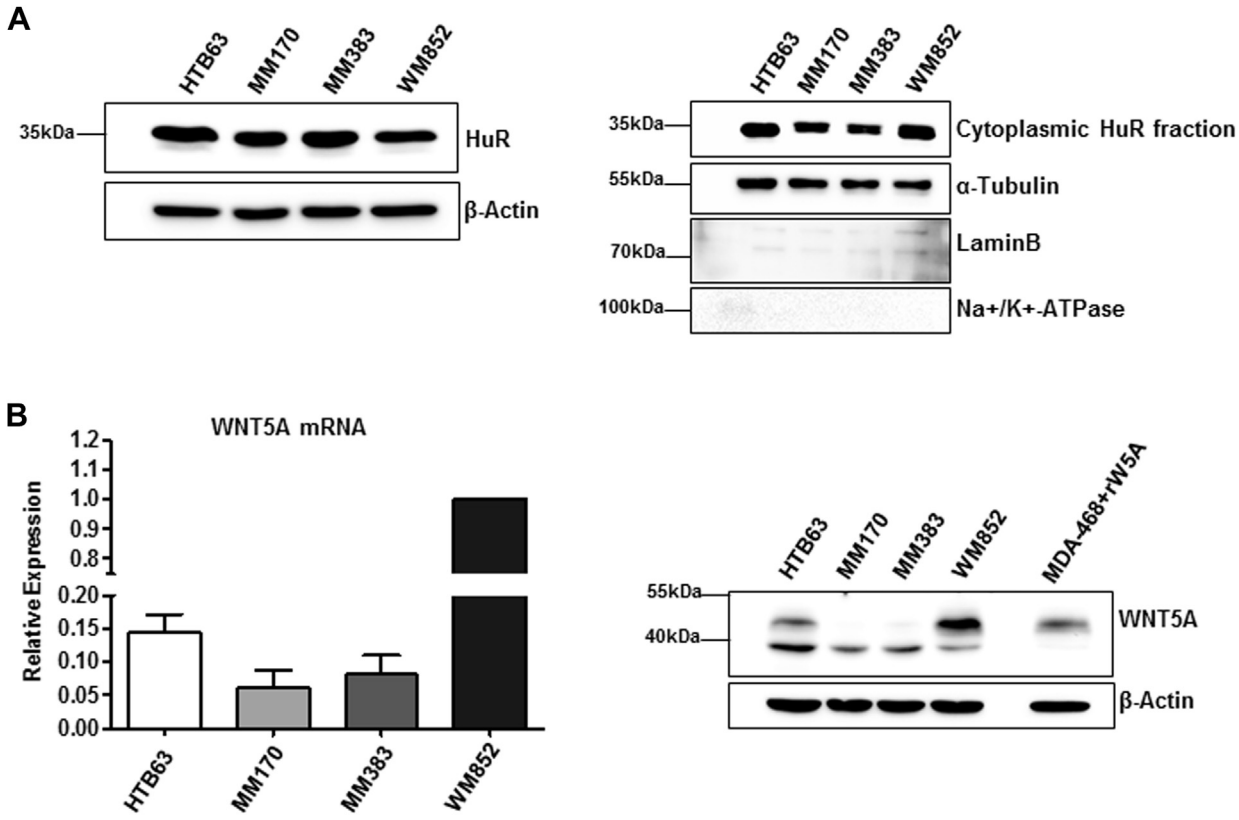

Fig 1. Cytoplasmic HuR accumulation is correlated with an increase in endogenous WNT5A expression. (A) Whole-cell lysate (left panel) or the cytosolic fraction (right panel) of HTB63, MM170, MM383, and WM852 melanoma cells was analyzed for HuR protein expression by western blotting. To assess the purity of the cytoplasmic HuR extract, the membrane was re-probed with α-tubulin. Moreover, the membranes were stripped and probed with anti-Lamin B and -Na,K-ATPase antibodies to detect possible cross-contamination. (B) The relative WNT5A mRNA expression level was determined in our panel of melanoma cell lines by qPCR (left panel). The relative WNT5A mRNA expression level was normalized against the mRNA expression of the housekeeping gene GAPDH. Endogenous WNT5A protein expression was analyzed in melanoma cell lines by western blotting (right panel) using β-actin as a loading control. HuR, human antigen R; mRNA, messenger RNA; WNT5A, wingless-type MMTV integration site family 5A.

Denmark) for 1 hour at room temperature. The antigen-antibody complexes were visualized using a chemiluminescent ECL reagent (Millipore Corporation), and densitometric analysis was carried out using Image Lab 3.0 software (Bio-Rad, Hercules, California). An antibody against β-actin (Sigma Aldrich, St. Louis, Missouri) was used to verify equal protein loading.

The subcellular protein fractionation kit (Cat. No. 78840; Thermo Scientific) was used to separate cytoplasmic protein extracts from melanoma cells according to the manufacturer's instructions. Protein concentrations were determined, and the samples were prepared for Western blotting as described previously. To determine the purity of the cytoplasmic HuR extract, the membrane was re-probed with anti-α-tubulin (1:30.000; from Abcam), -Lamin B (1:1000; from Santa Cruz Biotechnology, Inc), and -Na,K-ATPase (1:5000; from Abcam) antibodies.

WNT5A 3′UTR luciferase reporter assay. HTB63 cells were seeded in 12-well plates and transiently transfected with negative control or HuR siRNA (HuR si#1), as described previously. At 48 hours after siRNA transfection, the cells were transfected with 25-ng empty luciferase reporter vector or 1 of 2 WNT5A 3′UTR luciferase reporter vectors (vectors A and B) prepared using serum-free medium and Lipofectamine 2000 (Invitrogen Corporation) according to the manufacturer's recommendations. The WNT5A 3′UTR sequences corresponded to nucleotides 1-2543 (vector A) and 2394-4376 (vector B), with the numbers referring to nucleotides 3′ of the stop codon (accession no. NM.003392.3). All reporter vectors were derived from the pEZX-MT01 vector

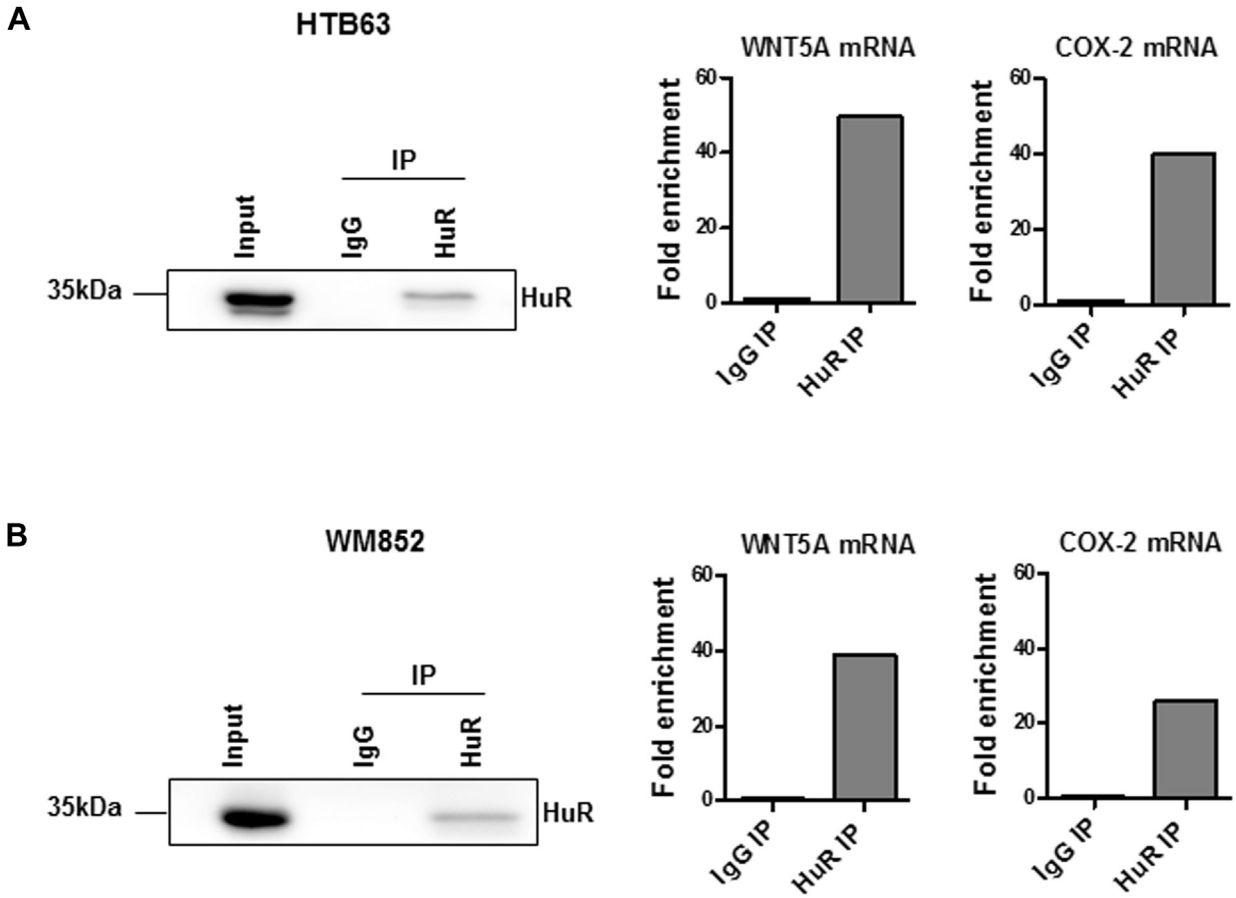

Fig 2. Interaction between the RNA-binding protein HuR and WNT5A mRNA. Whole-cell lysates from untreated HTB63 and WM852 melanoma cells were immunoprecipitated with a specific HuR antibody, as described in methods. The left panels (A and B) show specific pull-down of the HuR protein. Quantitative qPCR was performed to determine the relative fold expression of WNT5A and COX-2 enrichment in the HuR and IgG immunoprecipitates (A and B, right panels). We then normalized the relative amounts of WNT5A and COX-2 mRNA in the HuR- and IgG-IPs by the amount of GAPDH mRNA in each IP. A representative blot and qPCR analyses of 3 independent experiments are shown. HuR, human antigen R; IgG, immunoglobulin G; IP, immunoprecipitation; mRNA, messenger RNA; WNT5A, wingless-type MMTV integration site family 5A.

that includes a control Renilla luciferase gene (purchased from GeneCopoeia, Rockville, Maryland). At 24 hours after reporter vector transfection, the cells were harvested and analyzed for firefly and Renilla luciferase activities using Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin). For each sample, the 3′UTR-coupled firefly luciferase activity was normalized against the control Renilla luciferase activity.

MS-444 treatments. The compound MS-444 was kindly provided by N.C. Meisner, Novartis Institutes for Biomedical Research, Basel, Switzerland, diluted in DMSO at a stock concentration of 10 mM and stored at −20°C. Cells were seeded in 12-well plates and cultured for 24 hours in growth medium. The growth medium was then replaced with serum-free medium supplemented with MS-444 or an equivalent volume of the solvent alone (DMSO). In the initial dose-response experiments, HTB63 cells were incubated for 3–24 hours with MS-444 concentrations ranging from 2 to 50 μM. In subsequent experiments, both HTB6 and WM852 cell lines were incubated for 6–12 hours with 30-μM MS-444 (the same amount of DMSO was used in the control). After 6 hours of MS-444 treatment, total RNA was extracted; total proteins were isolated after 12 hours of MS-444 treatment. The levels of mRNA and protein expression were analyzed by qPCR and Western blotting.

Cell migration. Cell migration was evaluated in cell culture inserts (BD Falcon, New York) with a pore size of 8 μm. After 24 hours, the cells were washed and pretreated with either DMSO or MS-444 for 6 hours. After incubation, the cells were detached with Versene and resuspended in growth medium

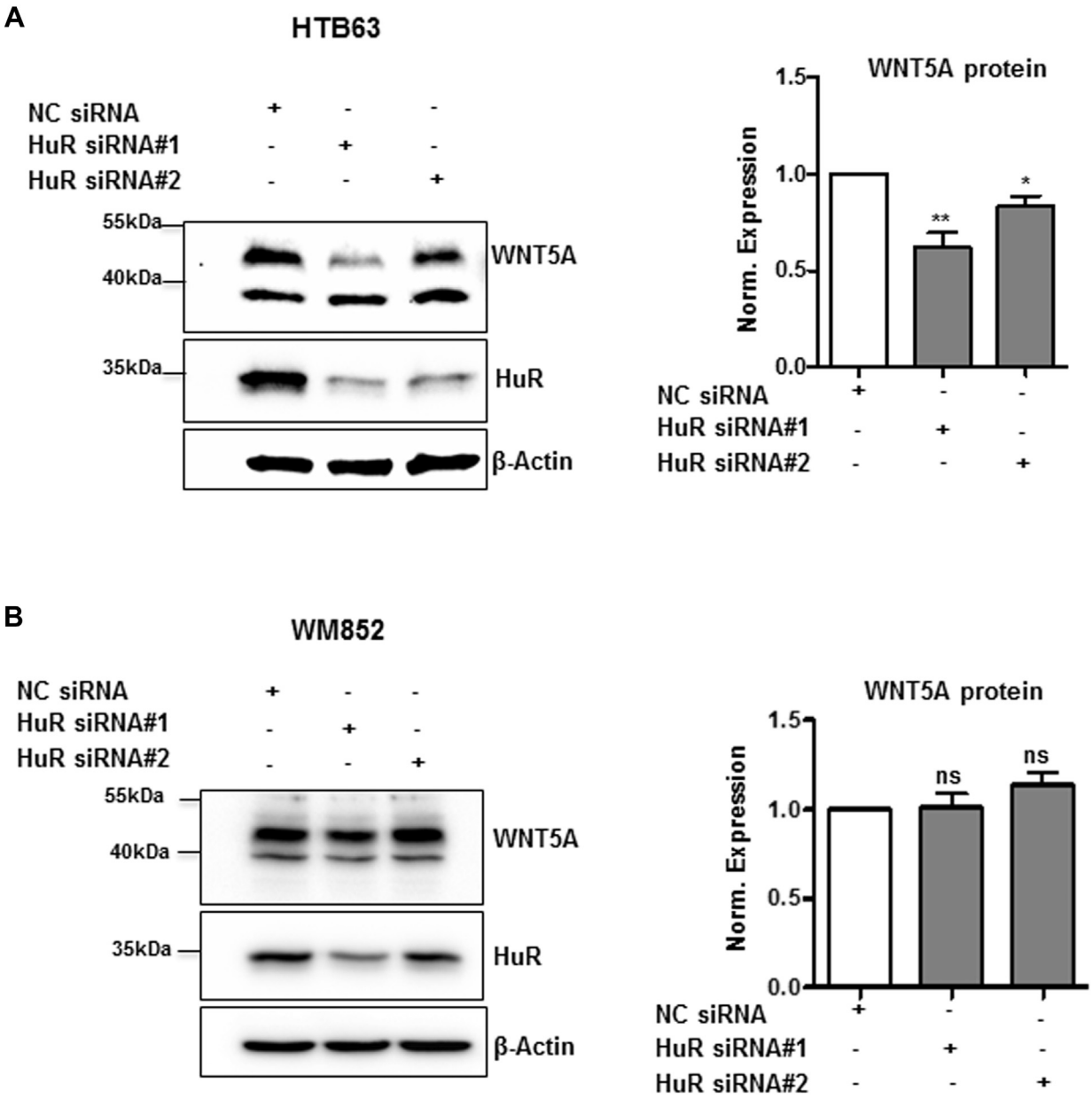

Fig 3. Effect of HuR silencing on WNT5A protein expression in HTB63 and WM852 cells. HTB63 and WM852 cells were transfected with either negative control (NC) siRNA or HuR-targeting siRNAs (#1 and #2). After 72 h of transfection, the efficiency of HuR knockdown and its effect on WNT5A protein levels were analyzed. Left panels show western blots analyses of WNT5A and HuR in the human melanoma cell lines HTB63 (A) and WM852 (B). β-Actin was used to ensure equal loading of all samples. A representative blot of 4 independent experiments is shown. Right panels show densitometric analyses of WNT5A protein expression, with the data normalized to β-actin. The results are presented as the means and SEM; $^{*}P < .05$; $^{**}P < .01$. HuR, human antigen R; SEM, standard error of the mean; siRNA, small interfering RNA; WNT5A, wingless-type MMTV integration site family 5A.

supplemented with 1% FBS. The cells were counted using an automated cell counter (Countess; Invitrogen Corporation), and 50,000 cells were suspended in 0.5-mL cell medium (supplemented with 1% FBS) and added to the upper chamber of cell culture inserts in either the absence or presence of recombinant WNT5A (rWNT5A; R&D Systems) and/or recombinant MMP-9 (rMMP-9; Calbiochem, EMD Chemicals, Gibbstown, USA). The lower chamber was filled with 0.75 ml of medium containing 10%

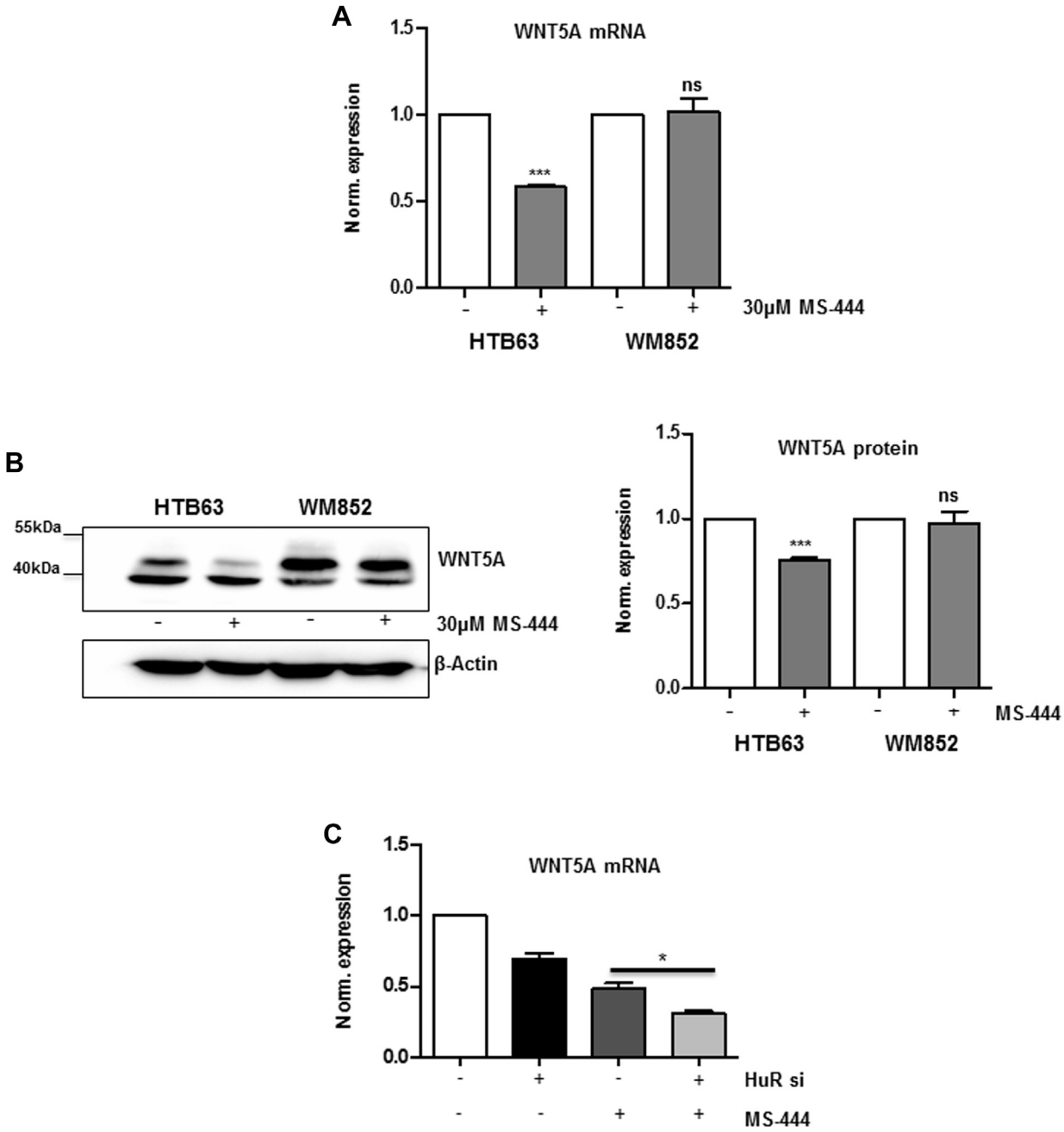

Fig 4. Effect of MS-444 on WNT5A mRNA and protein expression in melanoma cell lines HTB63 and WM852. At 24 h after seeding, the indicated melanoma cell lines were incubated with DMSO (control) or 30-μM MS-444 for 6 or 12 h. After treatment, total RNA and protein were harvested as described in methods. (A) qPCR analysis for WNT5A mRNA expression in HTB63 and WM852 cells was performed after 6 h of treatment with MS-444. The samples were normalized according to the expression of 3 housekeeping genes: GAPDH, SDHA, and HPRT1. (B) Western blot analysis showing WNT5A protein expression in HTB63 and WM852 cells treated with either 30-μM MS-444 or DMSO control for 12 h. β-Actin was used to ensure equal loading of all samples. Densitometric analyses were performed, and the data were normalized against β-actin. Symbols denoting statistical significance are as follows: ***$P < .001$. (C) HTB63 melanoma cells were transfected with control or HuR siRNA and cultured for ~66 h. The siRNA-transfected cells were then treated with either DMSO or 30-μM MS-444 for the next 6 h. The panel shows the response of each cell line to HuR silencing, MS-444 treatment, and the combination of HuR siRNA and MS-444 relative to the control-treated cells at the mRNA level. The data represent the means and standard deviations from 4 independent experiments; the data were analyzed by 1-way ANOVA together with Bonferroni's multiple comparison tests. Symbols denoting statistical significance are as follows: *$P < 0.05$. ANOVA, analysis of variance; HuR, human antigen R; mRNA, messenger RNA; ns, not significant; siRNA, small interfering RNA; WNT5A, wingless-type MMTV integration site family 5A.

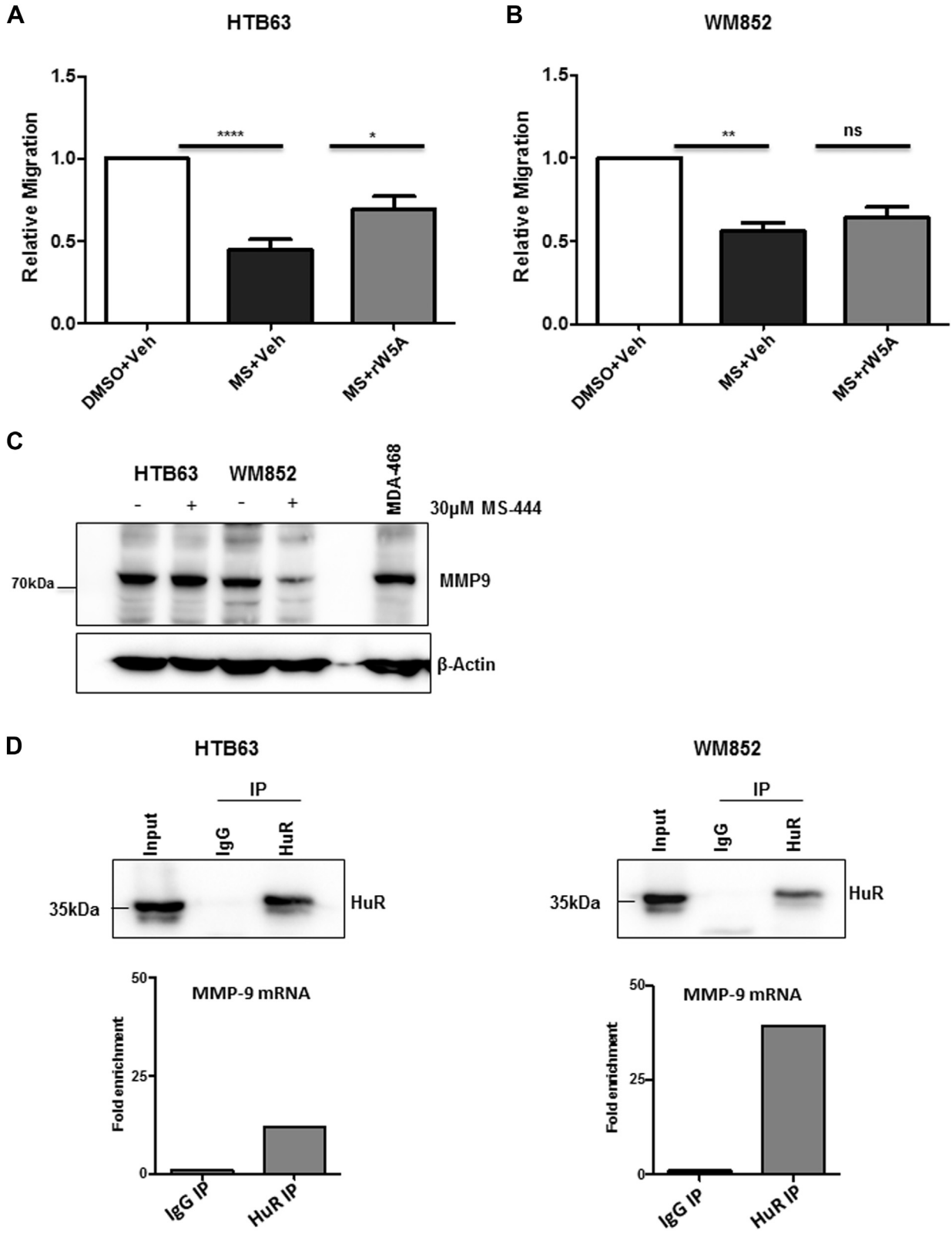

Fig 5. The HuR inhibitor MS-444 reduces melanoma cell migration in both HTB63 and WM852 melanoma cells. The panels show transwell migration assay results for HTB63 (A) and WM852 (B) cells treated either with DMSO, MS-444, or MS-444 in combination with rWNT5A. Migration assay was performed over 24 h and analyzed as

FBS. The cells were allowed to migrate for 24 h at 37°C in a humidified atmosphere with 5% CO_2. The rate of migration was analyzed either by counting the cells or as described by Prasad *et al*.[29]

Statistical analysis. All the experiments described herein were repeated more than three times. Means and standard deviations (SD; indicated as error bars on all graphs) were calculated and plotted using GraphPad Prism 5. Two-tailed, paired *t*-tests were used to determine the significance of two experimental data. All multiple group analyses were performed using one-way ANOVA together with Bonferroni multiple comparison test. The symbols denoting statistical significance were as follows: *, $P < .05$; **, $P < .01$; ***, $P < .001$, ns; non significant.

RESULTS

Increased cytoplasmic localization of HuR correlates with WNT5A protein expression in melanoma cells. Although the RBP HuR is predominantly nuclear,[30] its function is linked to its cytoplasmic accumulation.[31] To investigate the possible role of HuR in stabilizing and regulating WNT5A expression in melanoma, we analyzed endogenous HuR protein expression in a panel of melanoma cell lines (HTB63, MM170, MM383 and WM852) by western blotting (using total cell lysates). As shown in (Fig 1, A; left panel), all of these melanoma cell lines expressed HuR; however, cell fraction analysis revealed high cytoplasmic (active) HuR levels in HTB63 and WM852 melanoma cells in comparison with MM170 and MM383 cells (right panel). To examine whether HuR expression levels correlate with WNT5A expression in melanoma cells, we carried out quantitative reverse transcription polymerase chain reaction and western blot analyses for WNT5A mRNA and protein in our panel of melanoma cells. qPCR analysis showed that all tested cell lines were positive for WNT5A mRNA expression (Fig 1, B; left panel), though only HTB63 and WM852 cells exhibited WNT5A protein expression. These findings correlate with the relatively high cytoplasmic HuR (active) expression observed (Fig 1, B; right panel) and demonstrate that the cytoplasmic expression of the RBP HuR (active) might be important for the post-transcriptional regulation of WNT5A expression in melanoma cells.

These findings are also in good agreement with the phenotypical stratification by HOPP algorithm[7] of HTB63 and WM852 melanoma cells (Supplementary data Fig S1), as it characterizes them as invasive, in contrast to MM170 and MM383 cells, which are characterized as proliferative (Supplementary data Fig S1).

The RBP HuR physically interacts with WNT5A mRNA. For HuR to directly regulate WNT5A post-transcriptionally, HuR needs to interact physically with WNT5A transcripts. To test for this interaction, we subjected whole-cell lysates from cell lines HTB63 and WM852 to RIP either in the presence of an antibody specific for HuR or with a control IgG antibody. After RIP, total RNA was isolated from the HuR/IgG IPs and subsequently analyzed by qPCR using a WNT5A-specific primer. COX-2 mRNA, which is known to bind HuR protein, was used as a positive control.[28] To compensate for the nonspecific pull-down of RNA, we normalized the relative amounts of WNT5A and COX-2 mRNA in the HuR- and IgG-IPs according to the amount of GAPDH mRNA in each IP. Fig 2, A and B (left panel) demonstrate successful pull-downs of HuR from lysates of both HTB63 and WM852 cells. Further analysis showed specific enrichment of WNT5A mRNA in the HuR-IPs based on both melanoma cells lines compared with the control IgG-IPs (Fig 2, A and B; right panel). The results in Fig 2 are derived from three separate experiments; the results were similar, though the levels varied among the experiments (Supplementary data Fig S2). These data show that WNT5A mRNA is indeed a binding partner for the RBP HuR. In the next set of experiments, we investigated the functional consequences of this HuR-WNT5A interaction.

HuR knockdown results in reduced WNT5A expression in HTB63 cells but not in WM852 cells. To test whether the HuR protein is involved in the post-transcriptional regulation of WNT5A, we transiently transfected melanoma cells (HTB63 and WM852) with two

described in methods. The data represent the means and standard deviations; the data were analyzed by 1-way ANOVA together with Bonferroni's multiple comparison tests. (C) Western blot analysis showing MMP-9 protein expression in HTB63 and WM852 cells treated with 30-μM MS-444 for 12 h. β-Actin was used to ensure equal loading. (D) A specific HuR antibody and a control IgG antibody were used for RIP from whole-cell lysates of untreated HTB63 and WM852 melanoma cells, as described in methods. The upper panels show specific pull-down of the HuR protein. qPCR analyses were performed to determine the relative presence of MMP-9 mRNA in the HuR and IgG immunoprecipitates. Representative blots and qPCR analyses of 3 independent experiments are shown. Symbols denoting statistical significance are as follows: *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$. ANOVA, analysis of variance; HuR, human antigen R; IgG, immunoglobulin G; IP, immunoprecipitation; mRNA, messenger RNA; MMP-9, matrix metalloproteinase-9.

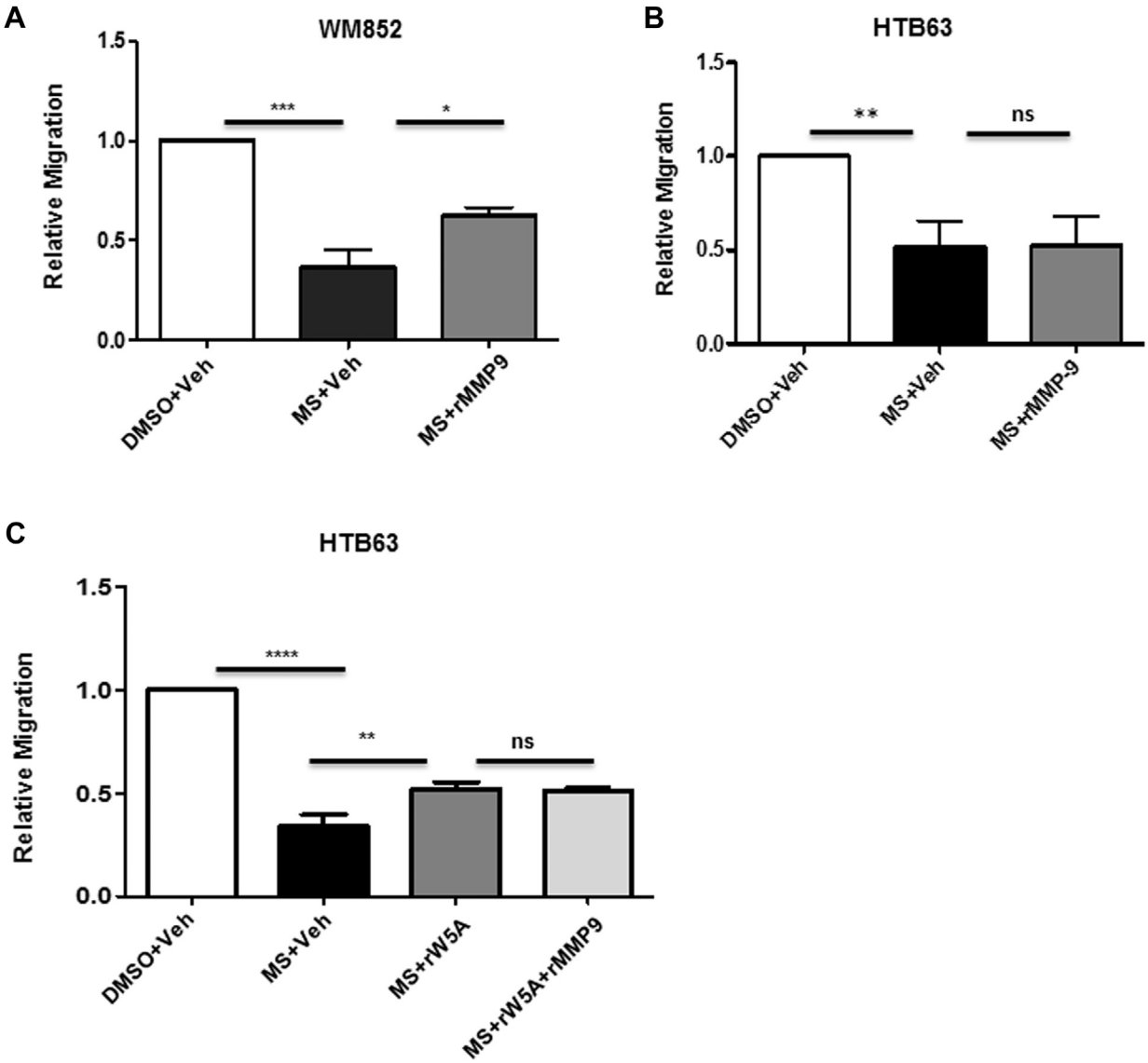

Fig 6. The inhibition of migration in WM852 cells is restored by human rMMP-9 but not in HTB63 cells. The panels show transwell migration assay results of (A) WM852 and (B) HTB63 melanoma cells treated with either DMSO or MS-444 for 6 h. After counting, the cells were added to the migration insert either in the absence or presence of rMMP-9, and the migration assays were performed over 24 h and analyzed as described in methods. The data represent the means and standard deviations; the data were analyzed by 1-way ANOVA together with Bonferroni's multiple comparison tests. (C) Human melanoma HTB63 cells were pretreated with either DMSO or MS-444 for 6 h. After counting, the cells were added to the migration insert in the absence or presence of rWNT5A alone or in combination with rMMP-9. The data represent the means and standard deviations; the data were analyzed by 1-way ANOVA together with Bonferroni's multiple comparison tests. Symbols denoting statistical significance are as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. ANOVA, analysis of variance; MMP-9, matrix metalloproteinase-9.

independent HuR-targeting siRNA oligonucleotides (see Methods); western blot analysis was performed using total cell lysates 72 h after HuR siRNA transfection. HuR silencing resulted in a significant decrease in WNT5A protein expression in HTB63 cells (up to 40%; Fig 3, A) but had no such effect in WM852 cells (Fig 3, B), even when the cells were transfected with a double dose (100 nM) of HuR siRNA for 72 h or 96 h (data not shown). Thereafter, we used only HuR siRNA #1 because the results from the knockdown showed that this siRNA produces more consistent and reproducible results

than HuR siRNA#2. The effects of HuR knockdown were also confirmed at the mRNA level. Similar to the effects on protein expression, HuR siRNA knockdown resulted in a significant decrease in HuR (*ELAVL1*) mRNA in both HTB63 and WM852 cells (Supplementary data Fig S3, A), though decreased WNT5A mRNA was observed only in HTB63 cells (Supplementary data Fig S3, B). These results suggest that HuR binds to and stabilizes WNT5A mRNA, thereby regulating WNT5A protein expression, in HTB63 cells. In contrast, no effect on WNT5A protein expression was observed in WM852 cells.

To further investigate how HuR binds to WNT5A mRNA, we utilized two 3′UTR luciferase reporter constructs containing overlapping parts of the WNT5A 3′UTR cloned downstream of a luciferase reporter gene (Supplementary data Fig S4, A; left panel). Control and HuR siRNA-transfected HTB63 cells were subjected to a second transfection with either a control reporter plasmid (pEZX-MT01 EV) or WNT5A 3′UTR reporter plasmid (pEZX-MT01 A-3′UTR or pEZX-MT01 B-3′UTR). Measurement of the relative reporter activity revealed significantly lower luciferase activity from the 3′UTR reporter plasmids in the HuR-silenced cells compared with the control siRNA-transfected cells (Supplementary data Fig S4, A; right panel). Importantly, HuR silencing did not significantly affect the luciferase activity from the control plasmid lacking a 3′UTR sequence. Control experiments demonstrated the successful knockdown of HuR (*ELAVL1*) mRNA in the analyzed cells (Supplementary data Fig S4, B). These results suggest that the RBP HuR binds to the 3′UTR of WNT5A mRNA in melanoma cells.

The low-molecular-weight HuR inhibitor MS-444 reduces WNT5A mRNA and protein expression. Initially in our study we tried to analyze how HuR siRNA silencing affected melanoma cell migration. However, initial transfection experiment with siRNA for 72 h seriously affected the basal migration of these cells, whereas treatment with small molecular HuR inhibitor MS-444 for 6 h did not. This compound was described as interfering with HuR function, thereby resulting in decreased mRNA expression of HuR target genes, e.g., IL-1β and IL-6.[32] In time- and dose-response experiments, we observed that MS-444 treatment of HTB63 cells resulted in decreased WNT5A mRNA levels, with maximal effects between 6-12 h and with a dose ranging between 20 and 50 μM (Supplementary data Fig S5, A and B). Based on these results, we treated melanoma cells with 30 μM MS-444 for 6 h and analyzed the effects on WNT5A mRNA levels. As shown in Fig 4, A; MS-444 treatment of HTB63 cells resulted in a significant decrease in WNT5A mRNA, but had no effect on the expression of HuR (Supplementary data Fig S6). In addition, the effects of MS-444 on WNT5A protein expression were analyzed after 12 h of treatment with the HuR inhibitor. In HTB63 cells, we found significant inhibition of WNT5A protein expression after 12 h of MS-444 treatment (Fig 4, B). However, similar to the HuR silencing experiments, MS-444 inhibition of HuR in WM852 cells did not result in any change in either WNT5A mRNA or protein expression (Fig 4, A and B). Because the siRNA-mediated knockdown of HuR and the MS-444-mediated inhibition of HuR operate at different levels, we sought to investigate whether a combination of both methods could result in a more potent reduction in WNT5A mRNA and protein in HTB63 cells. The data shown in Fig 4, C demonstrate that combined treatment with HuR siRNA and MS-444 potentiated the reduction in WNT5A mRNA expression. However, this combined effect was not observed at the protein level (data not shown). These complementary data further support a direct role of HuR in the regulation of WNT5A expression in HTB63 cells.

Inhibition of HuR reduces migration in both HTB63 and WM852 melanoma cells. WNT5A expression has been positively correlated with increases in melanoma cell motility and invasion. In the current study, we demonstrated that HuR can regulate WNT5A expression in HTB63 melanoma cells. Thus, to investigate whether the direct targeting of HuR (by MS-444) can impair migration in HTB63 cells, we performed a transwell cell migration experiment with HTB63 cells pretreated in the absence or presence of MS-444 for 6 h. Treatment with MS-444 resulted in a significant decrease in HTB63 cell migration, which could be partially restored by the addition of rWNT5A (Fig 5, A). Surprisingly, identical MS-444 inhibition of HuR in WM852 cells also resulted in the significant inhibition of their migration. However, in accordance with our previous results (Fig 4), this inhibition was not restored by rWNT5A (Fig 5, B). Therefore, the ability of MS-444 to inhibit WM852 cell migration suggests the involvement of another HuR-dependent regulator of WM852 melanoma cell migration. Within this context, it is interesting to note that the HuR protein has also been shown to stabilize and control the translation of MMP-9, a well-known regulator involved in tumor cell migration and invasion.[33] Accordingly, we next assessed the presence of MMP-9 in HTB63 and WM852 cells in the presence or absence of MS-444. Treatment with MS-444 resulted in a marked decrease in MMP-9 protein in WM852 cells, whereas no effect on MMP-9 was observed in HTB63 cells (Fig 5, C).

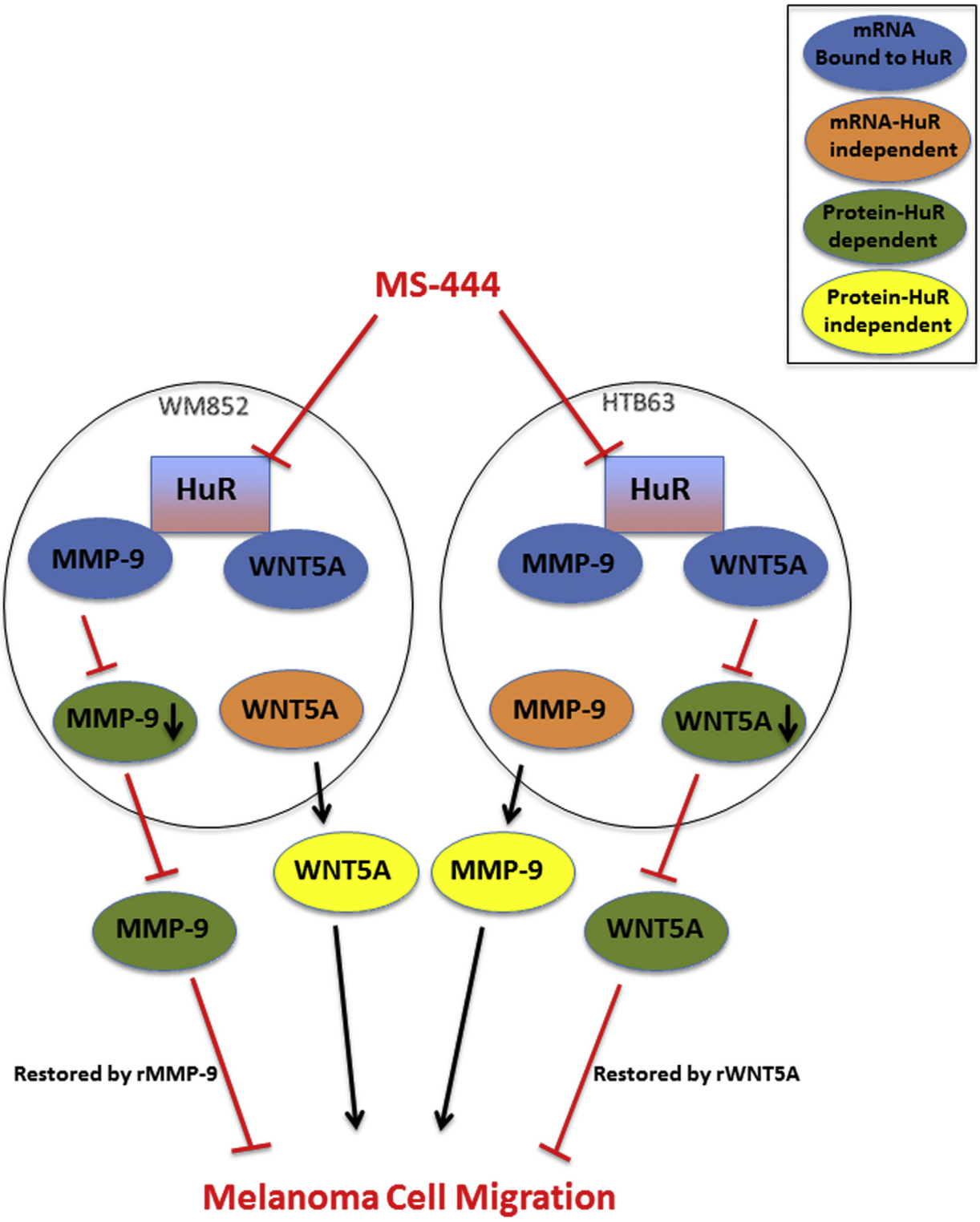

Fig 7. A schematic overview of the signaling network explaining the dual mechanisms of HuR in melanoma cell migration. The RNA-binding protein HuR interacts with WNT5A and MMP-9 mRNA (blue) in melanoma cells (HTB63 and WM852), thus stabilizing the mRNAs resulting in increased protein levels. MS-444 inhibits the HuR protein in melanoma cells (as indicated by the red lines). In WM852 cells with high endogenous WNT5A expression, MS-444 inhibition of HuR did not cause any detectable changes in either WNT5A mRNA (orange) or protein (yellow) levels. However, in these cells, MS-444 caused a significant reduction of MMP-9 (green) expression

These results prompted us to further investigate whether HuR binds physically to MMP-9 transcripts in melanoma cell lines. We performed RIP with either a HuR-specific antibody or a control IgG antibody. The HuR protein was successfully pulled-down in both cell lines (Fig 5, D; upper panel) and MMP-9 mRNA was readily detectable in these HuR-IP, but not in the control IgG-IP (Fig 5, D; lower panel). Notably, the MMP-9 mRNA fold enrichment in HTB63 cells was relatively lower than that in WM852 cells. The results in Fig 5, D are representative of 3 separate experiments. All 3 experiments are shown in Supplementary data Fig S7. Additionally, transfections with 2 independent HuR-targeting siRNA oligos reduced HuR protein levels in both cell lines (Supplementary data Fig S8, A and B; left panels). In good agreement with our previous results (Fig 5, C), HuR silencing resulted in a significant decrease in MMP-9 protein expression in WM852 cells, although the effect was only statistically significant for 1 of the 2 HuR siRNAs used (Supplementary data Fig S8, B). However, no change was detected in the MMP-9 protein level in HuR siRNA-transfected HTB63 cells (Supplementary data Fig S8, A).

In further experiments, we demonstrated that the inhibition of WM852 cell migration by MS-444 (Fig 5, B) depends on MMP-9, as the impairment of migration by MS-444 was partially reversed by the addition of human rMMP-9 (Fig 6, A). In contrast and consistent with the data presented in Fig 5, C, exogenous addition of human rMMP-9 did not restore HTB63 cell migration in the presence of MS-444. (Fig 6, B). Furthermore, we also demonstrate that addition of exogenous rMMP-9 did not further restore HTB63 cell migration in the presence of rWNT5A (Fig 6, C). Overall, these data show that HuR can regulate melanoma cell migration by affecting different regulatory proteins such as WNT5A and MMP-9.

DISCUSSION

WNT5A, a secreted glycoprotein and member of the WNT family ligands, has been shown to act as either a tumor suppressor or a tumor promoter.[34] WNT5A has been implicated as a tumor promoter in melanomas, and its expression has also been positively associated with melanoma progression and metastasis.[4] However, the regulatory mechanisms responsible for WNT5A upregulation in melanomas are still unclear. In breast cancer, where WNT5A has a tumor suppressor function, HuR has been shown to negatively regulate WNT5A protein expression by suppressing translation of its mRNA.[35] Important functions of the HuR protein have also been implicated in other types of cancer, regulating the expression levels of many tumor-promoting factors (eg, Cox-2, VEGF and so forth) by stabilizing their respective mRNAs.[36] In the present study, we explored the possibility that HuR protein can similarly regulate WNT5A expression in malignant melanoma. First, we investigated the HuR-WNT5A association by analyzing HuR and WNT5A protein expression in 4 melanoma cell lines: HTB63, MM170, MM383, and WM852. We demonstrated that of the 4 melanoma cell lines tested, 2 (HTB63 and WM852 cells) display high cytoplasmic (active) HuR expression as well as WNT5A protein expression in comparison with the other 2 cell lines (MM170 and MM383). Interestingly, phenotypical stratification (by HOPP algorithm) defined the HuR- and WNT5A-expressing HTB63 and WM852 cells as having an invasive phenotype, whereas the MM170 and MM383 cells were characterized as proliferative. These findings suggest that HuR is involved in positively regulating WNT5A protein expression in HuR-expressing invasive melanoma cells, perhaps by directly interacting with WNT5A mRNA.

We explored the mentioned possibility by performing HuR RIP experiments, revealing a direct interaction between the HuR protein and WNT5A mRNA. Furthermore, in separate experiments, we also defined the site of HuR protein binding by demonstrating that the 3′UTR region of WNT5A mRNA is responsible for binding the HuR protein. The results clearly demonstrate by different approaches that the HuR protein binds to WNT5A mRNA in HTB63 and WM852 melanoma cells. These findings are consistent with the results of 2 previous studies in which HuR was found to interact with WNT5A mRNA.[15,35]

We then assessed the functional consequences of this HuR-WNT5A mRNA association via siRNA knockdown of HuR in HTB63 and WM852 cells. HuR

leading to impaired melanoma cell migration restored by exogenous addition of rMMP-9. In HTB63 cells with a limited binding of MMP-9 mRNA to HuR, MS-444 inhibition of HuR did not cause any detectable changes in MMP-9 expression (orange and yellow). However, in these cells, MS-444 caused a significant reduction of WNT5A mRNA and protein (green) expression leading to impaired melanoma cell migration restored by exogenous addition of rWNT5A. HuR, human antigen R; mRNA, messenger RNA; MMP-9, matrix metalloproteinase-9; rMMP-9, recombinant MMP-9; WNT5A, wingless-type MMTV integration site family 5A. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

silencing significantly reduced both the WNT5A protein level as well as the mRNA level in HTB63 cells. Surprisingly, HuR knockdown in WM852 cells had no effect on either the WNT5A mRNA or protein level, despite the fact that the HuR protein was confirmed to bind to WNT5A mRNA in this cell line. Similar findings were observed after short-term treatment with the low-molecular-weight HuR inhibitor MS-444 in both HTB63 and WM852 cells. We speculate that the much higher expression of WNT5A mRNA in WM852 melanoma cells is the reason why HuR inhibition in these cells did not affect WNT5A mRNA or protein levels. This suggests that WNT5A mRNA bound to HuR protein constitutes only a minor part of the total amount of WNT5A mRNA in WM852 cells.

Several studies have demonstrated that high WNT5A expression in melanoma is correlated with increased cell migration and metastasis[4,37] and that inhibition of WNT5A signaling reduces the migration of melanoma cells.[38] In the present study, we also found relatively high active HuR and WNT5A expression in invasive melanoma cells compared with proliferative melanoma cells. Interestingly, direct and indirect evidence support a promigratory role for the HuR protein in various cancers.[39-42] Consequently, we tested whether HuR inhibition could hinder the migration of melanoma cell lines. To our surprise, despite the fact that treating WM852 cells with MS-444 had no effect on WNT5A levels, we did observe that MS-444 treatment led to a significant decrease in migration of both HTB63 and WM852 melanoma cells. Furthermore, the addition of rWNT5A significantly restored migration in MS-444–treated HTB63 cells, but not in WM852 cells. As HuR can stabilize and regulate different target mRNAs, we hypothesized that HuR regulates another promigratory factor in WM852 melanoma cells, which could explain the decreased migration of these cells on MS-444 treatment. MMP-9 mRNA has been documented as being regulated by HuR and also as promoting the migration and metastasis of different types of cancer, including melanoma, breast, and colon.[42-44] To evaluate whether the MS-444-induced inhibition of WM852 cell migration was mediated by MMP-9 downregulation, we analyzed MMP-9 protein expression in both HTB63 and WM852 melanoma cells after treatment with MS-444. We observed reduction in MMP-9 protein expression only in WM852 cells, but not in HTB63 cells. Similar results for MMP-9 expression in WM852 cells were obtained in our validation experiments using a HuR siRNA approach. Our RNA immunoprecipitation data revealed a direct interaction between the RBP HuR and MMP-9 mRNA in both HTB63 and WM852 melanoma cells. However, we observed a higher enrichment of MMP-9 mRNA in the HuR pull-down from WM852 cells compared with the HuR pull-down from HTB63 cells. This is a reasonable explanation for our observation that HuR inhibition had no effect on the MMP-9 expression in HTB63 cells. Our data are in good agreement with those published by Akool et al,[45] who demonstrated that HuR binds to ARE motifs of the 3′UTR of MMP-9 mRNA.

In further support of this finding, our reconstitution experiments revealed that active rMMP-9 restored impaired migration in MS-444-treated WM852 cells. These findings are in good agreement with those presented by Yuan et al,[46] who demonstrated that HuR silencing resulted in the reduction of breast cancer cell growth and invasion via the downregulation of MMP-9, and a study performed on hepatocellular carcinoma showing that inhibition of cytosolic HuR by N-benzylcantharidinamide suppresses cell invasion by inhibiting MMP-9 expression.[33] Furthermore, RNAi-mediated MMP-9 silencing resulted in the inhibition of migration and invasion in mouse melanoma cells both *in vitro* and *in vivo*.[43] Our current view of how HuR regulates melanoma cell migration has been schematically outlined in Fig 7.

Melanoma comprises different cell types in which different signaling pathways function cohesively toward disease progression. Accordingly, the differences in molecular profiles pose a large hurdle for therapeutic treatment. Based on the present results, we believe that targeting the HuR protein might be an effective strategy to prevent melanoma progression, as HuR stabilizes important promigratory molecules such as WNT5A and MMP-9, which are well-known regulators of melanoma cell migration. Disruption of HuR-mRNA interactions with small molecules such as MS-444 and recently developed CMLD-2[47] constitutes valuable tools for studying the role of HuR in cancer cell biology, but it is possible that they might also be investigated as potential drug candidates in the treatment of melanomas.

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Conflicts of interest: All authors have read the journal's policy on conflict of interest. T.A. is a shareholder of WNT Research and is the part-time Chief Scientific

Officer of WNT Research. This does not alter the authors' adherence to all guidelines for publication in *Translational Research*. F.M., P.B., R.L., K.L., and C.P.P. have no potential conflicts of interest to disclose.

Supplementary Data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.trsl.2016.02.007.

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SUPPLEMENTARY FIGURES

Fig S1. The HTB63, MM170, MM383 and WM852 melanoma cell lines were categorized as either proliferative or invasive using the Heuristic Online Phenotype Prediction (HOPP) algorithm. (http://jurmo.ch/hopp/hopp_default_data.php).

Fig S2. Interaction between the RNA-binding protein HuR and WNT5A mRNA. The figure provides details of all the three independent experiments performed as described in Fig 2.

Fig S3. Quantitative qPCR analysis of HuR (*ELAVL1*) **(A)** and WNT5A **(B)** mRNA expression in HuR-silenced melanoma cell lines HTB63 and WM852. The samples were normalized against GAPDH mRNA expression, and the results are presented as the mean and S.E.M; ***, $P<0.001$; ns, not significant.

Fig S4. HuR knockdown reduces WNT5A 3'UTR reporter activity. A control luciferase reporter vector (EV) or vectors containing WNT5A 3'UTR sequences cloned downstream of the luciferase reporter gene were transfected into control and HuR-silenced HTB63 cells. **(A)** The upper panel (left) represents the reporter constructs with 3'UTR nucleotide numbers relative to the WNT5A mRNA stop codon. The bars show the mean and standard deviation of normalized luciferase activity in the HuR-silenced cells relative to the negative control siRNA-transfected cells (right panel). **(B)** Successful knockdown of HuR (*ELAVL1*) mRNA in the analyzed cells, as normalized to the housekeeping gene GAPDH. The luciferase reporter assays were repeated independently five times.

Fig S5. The effect of the HuR inhibitor MS-444 on WNT5A mRNA expression was tested by performing a dose-response experiment using HTB63 cells. **(A)** Cells were incubated for 3, 6, 12 and 24 h with the indicated concentrations of MS-444 or equivalent volumes of DMSO. RNA was isolated, and the expression of WNT5A mRNA was analyzed by qPCR. The bars in each panel represent the means and standard deviations of WNT5A mRNA expression relative to the respective controls. Each treatment was repeated three times. **(B)** Summarized graphical representation of WNT5A mRNA expression with different doses and treatment times of the HuR inhibitor MS-444.

Fig S6. The effect of the HuR inhibitor MS-444 on HuR (*ELAVL1*) mRNA and protein expression in melanoma cell line HTB63. 24 h after seeding, the indicated melanoma cell line was incubated with DMSO (control) or 30 μM MS-444 for 6 or 12 h. After treatment, total RNA and protein were harvested *as described in methods*. **(A)** qPCR analysis for HuR (*ELAVL1*) mRNA expression in HTB63 cells was performed after 6 h of treatment with MS-444. The samples were normalized according to the expression of three housekeeping genes: GAPDH, SDHA and HPRT1. The results are presented as the mean and S.E.M; ns, not significant. **(B)** Western blot analysis showing HuR protein expression in HTB63 cells

treated with either 30 μM MS-444 or DMSO control for 12 h. β-Actin was used to ensure equal loading of all samples.

Fig S7. Interaction between the RNA-binding protein HuR and MMP-9 mRNA. The figure provides details of all the three independent experiments performed as described in Fig. 5, D.

Fig S8. Effect of HuR silencing on MMP-9 protein expression in HTB63 and WM852 cells. **(A)** HTB63 and **(B)** WM852 cells were transfected with either negative control (NC) siRNA or HuR-targeting siRNAs (#1 and #2). After 72 h of transfection, the efficiency of HuR knockdown and its effect on WNT5A protein levels were analyzed. Left panels **(A, B)** show western blots analyses of MMP-9 and HuR in the human melanoma cell lines HTB63 and WM852. β-Actin was used to ensure equal loading of all samples. A representative blot of three independent experiments is shown. Right panels **(A, B)** show densitometric analyses of MMP-9 protein expression in HTB63 and WM852 melanoma cells, with the data normalized to β-actin. The results are presented as the means and S.E.M; *, $P<0.05$; ns, not significant.

Figure S1.

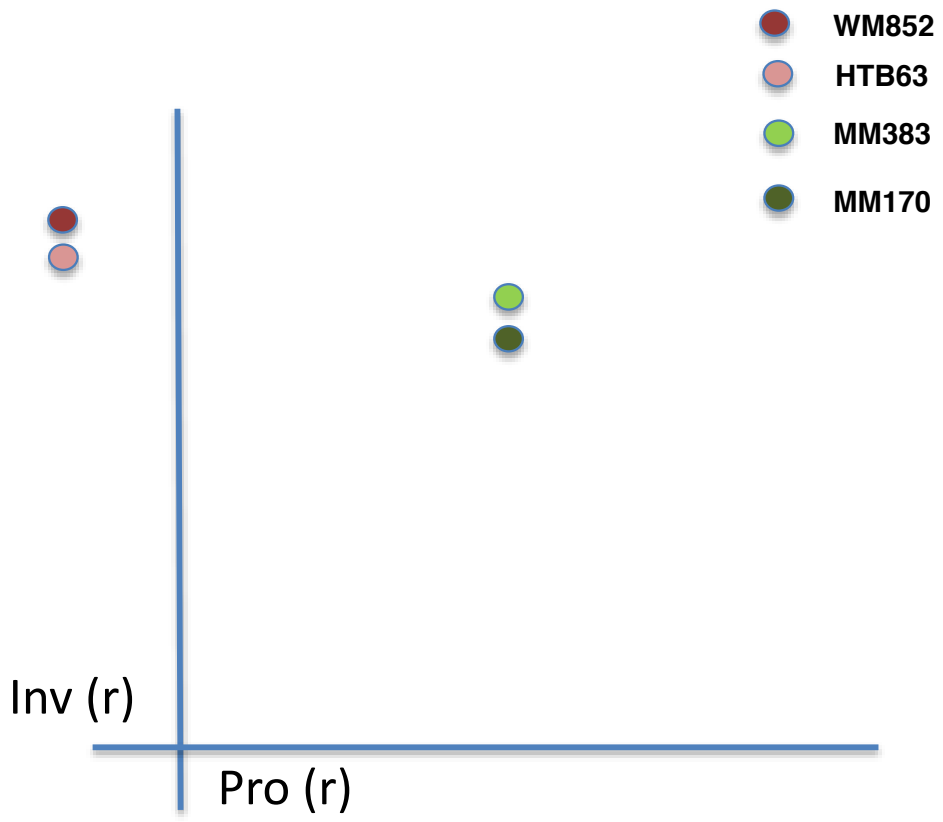


Figure S2.

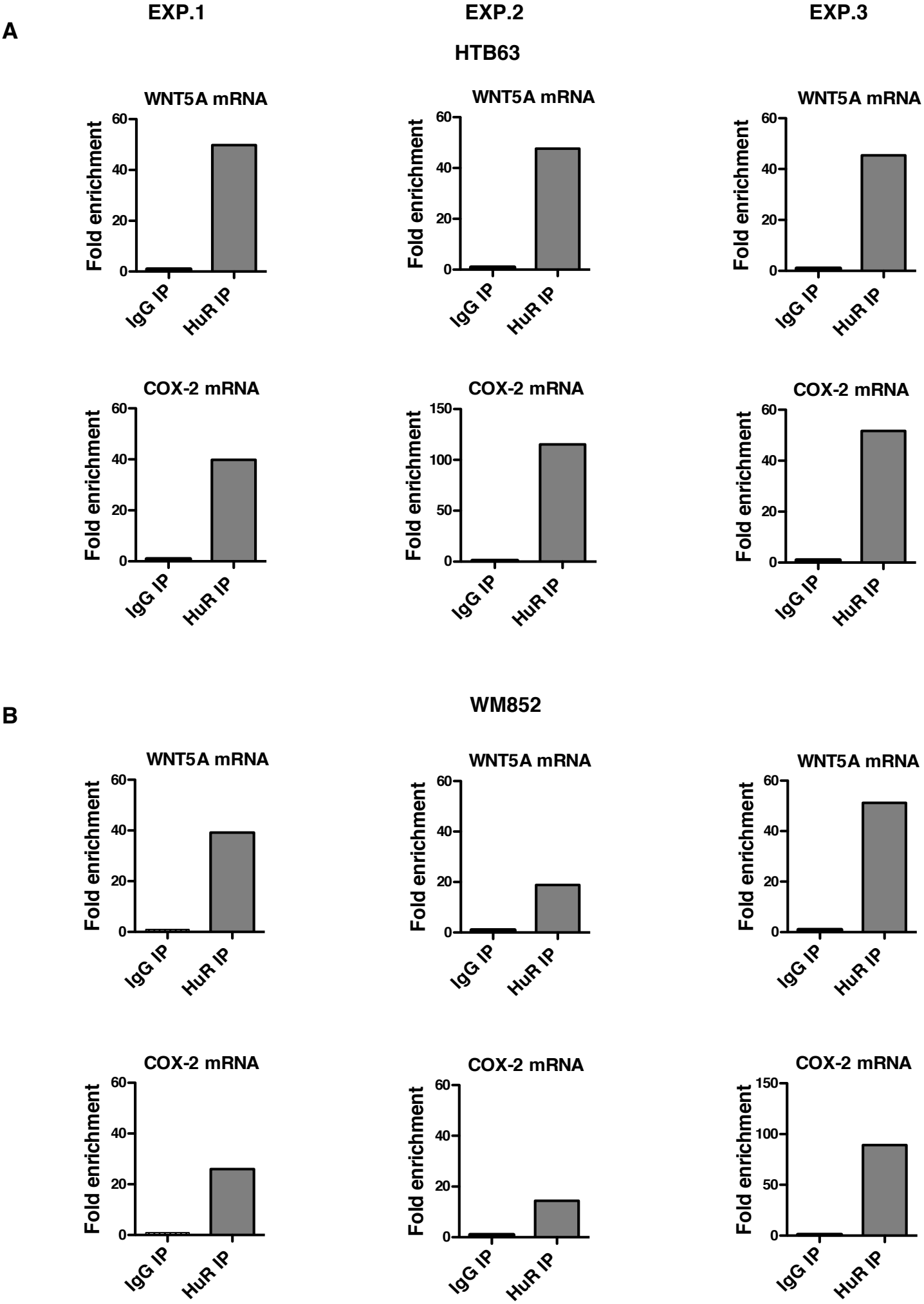
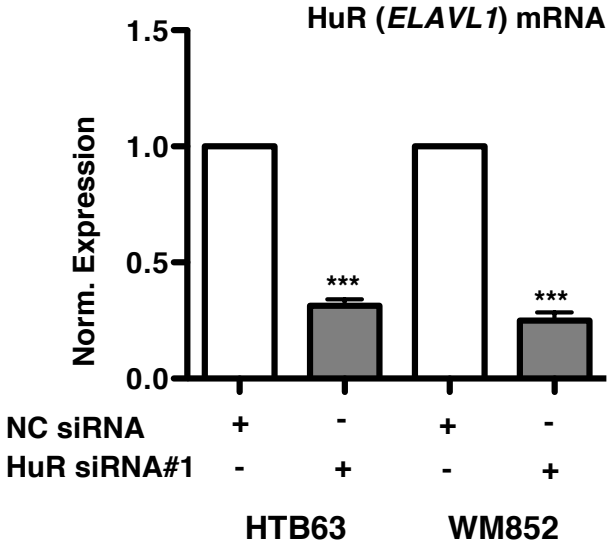

A
EXP.1
EXP.2
EXP.3
HTB63
WNT5A mRNA
COX-2 mRNA
Fold enrichment
IgG IP
HuR IP
B
WM852

Figure S3.

A


HuR (*ELAVL1*) mRNA
Norm. Expression
1.5
1.0
0.5
0.0
***
***
NC siRNA + - + -
HuR siRNA#1 - + - +
HTB63
WM852

B

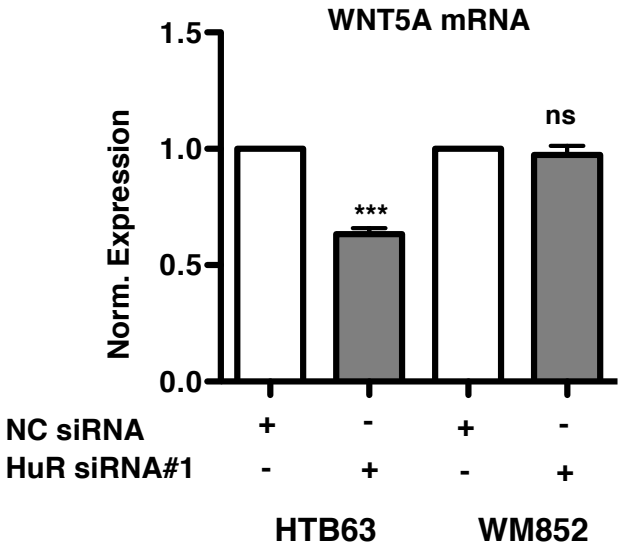

WNT5A mRNA
Norm. Expression
1.5
1.0
0.5
0.0
***
ns
NC siRNA + - + -
HuR siRNA#1 - + - +
HTB63
WM852

Figure S4.

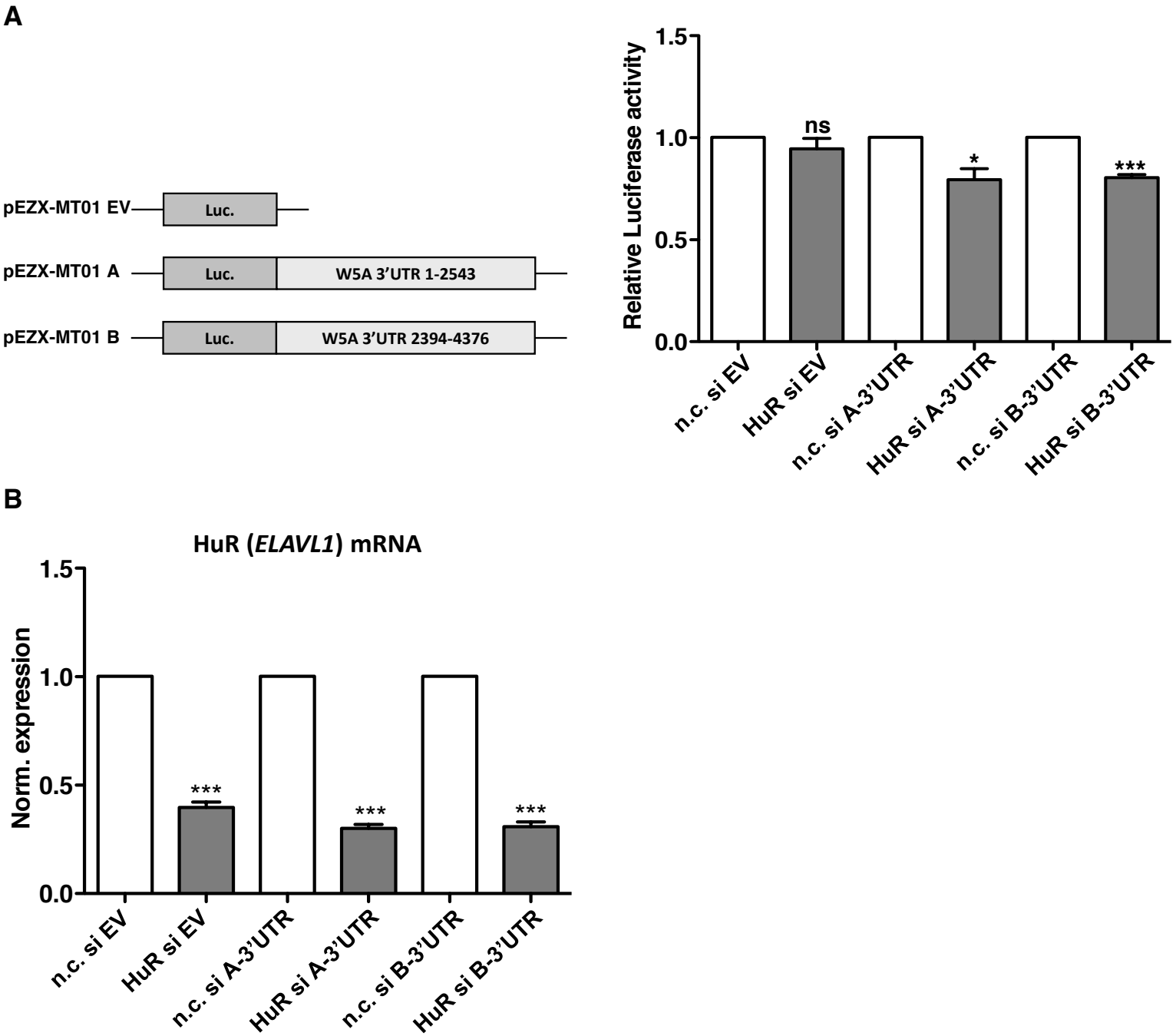
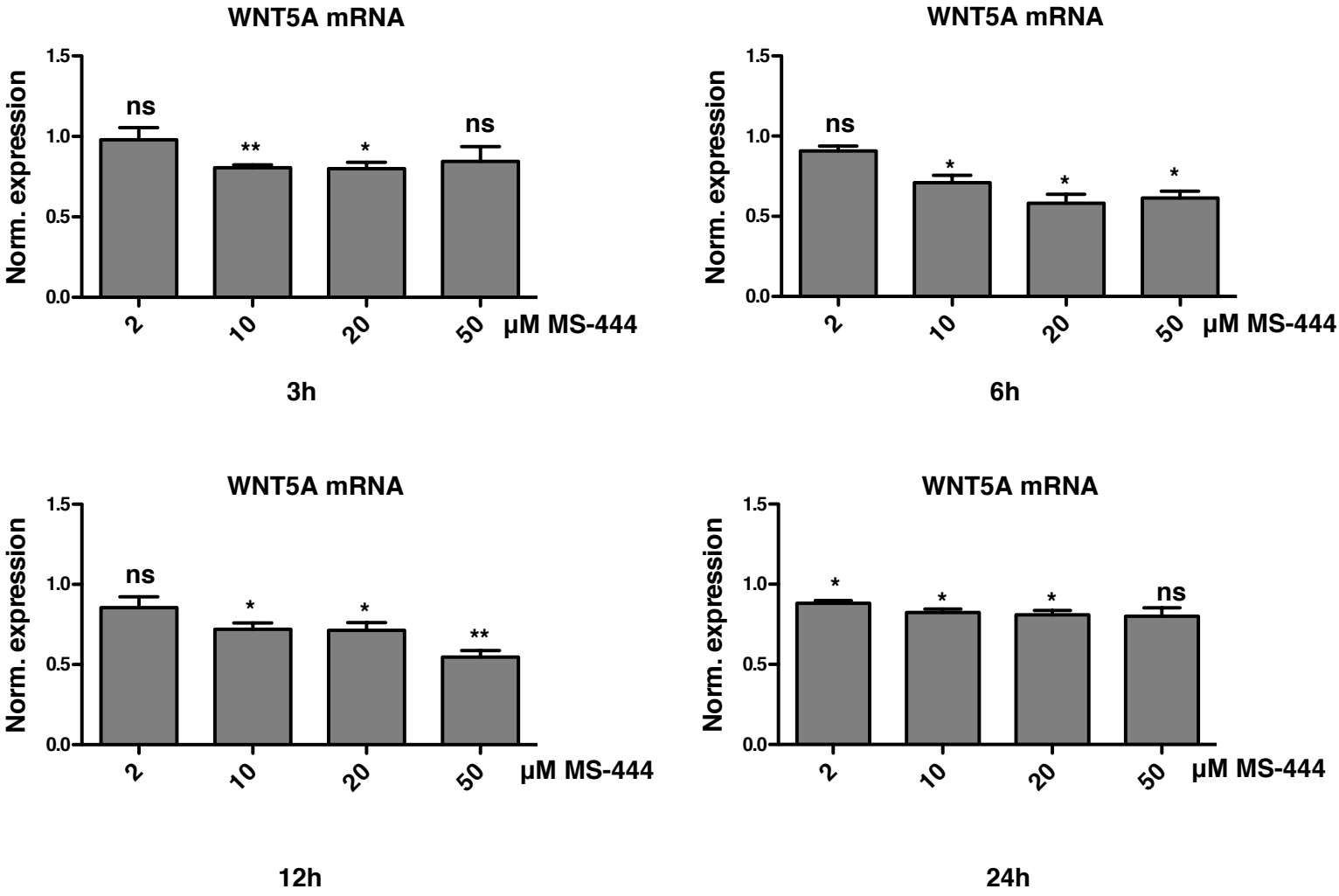

A
pEZX-MT01 EV
Luc.
pEZX-MT01 A
Luc.
W5A 3'UTR 1-2543
pEZX-MT01 B
Luc.
W5A 3'UTR 2394-4376
Relative Luciferase activity
1.5
1.0
0.5
0.0
ns
*
***
n.c. si EV
HuR si EV
n.c. si A-3'UTR
HuR si A-3'UTR
n.c. si B-3'UTR
HuR si B-3'UTR
B
HuR (ELAVL1) mRNA
Norm. expression
1.5
1.0
0.5
0.0
***
***
***
n.c. si EV
HuR si EV
n.c. si A-3'UTR
HuR si A-3'UTR
n.c. si B-3'UTR
HuR si B-3'UTR

Figure S5.

A


WNT5A mRNA
Norm. expression
1.5
1.0
0.5
0.0
ns
**
*
ns
2
10
20
50
μM MS-444
3h
WNT5A mRNA
Norm. expression
ns
*
*
*
6h
WNT5A mRNA
Norm. expression
ns
*
*
**
12h
WNT5A mRNA
Norm. expression
*
*
*
ns
24h

B

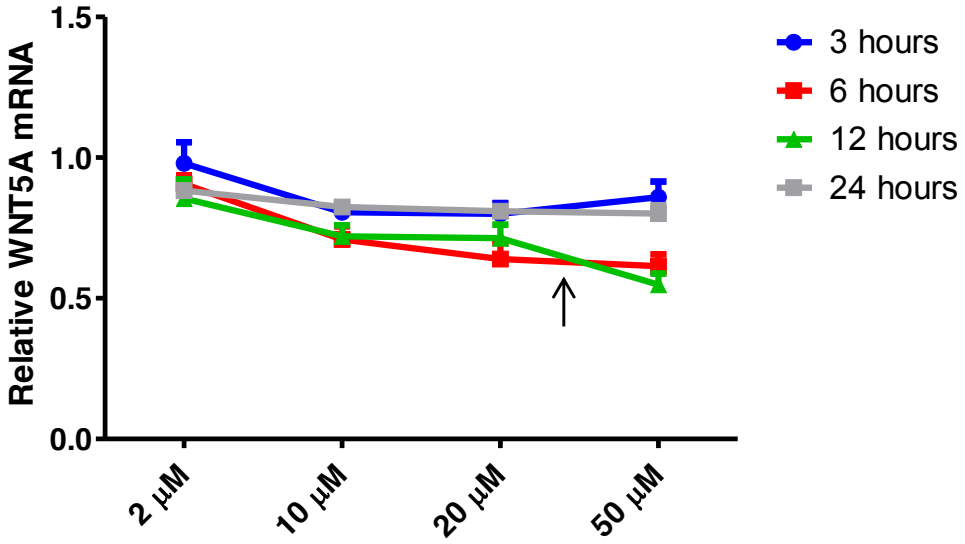

Relative WNT5A mRNA
1.5
1.0
0.5
0.0
2 μM
10 μM
20 μM
50 μM
3 hours
6 hours
12 hours
24 hours

Figure S6.

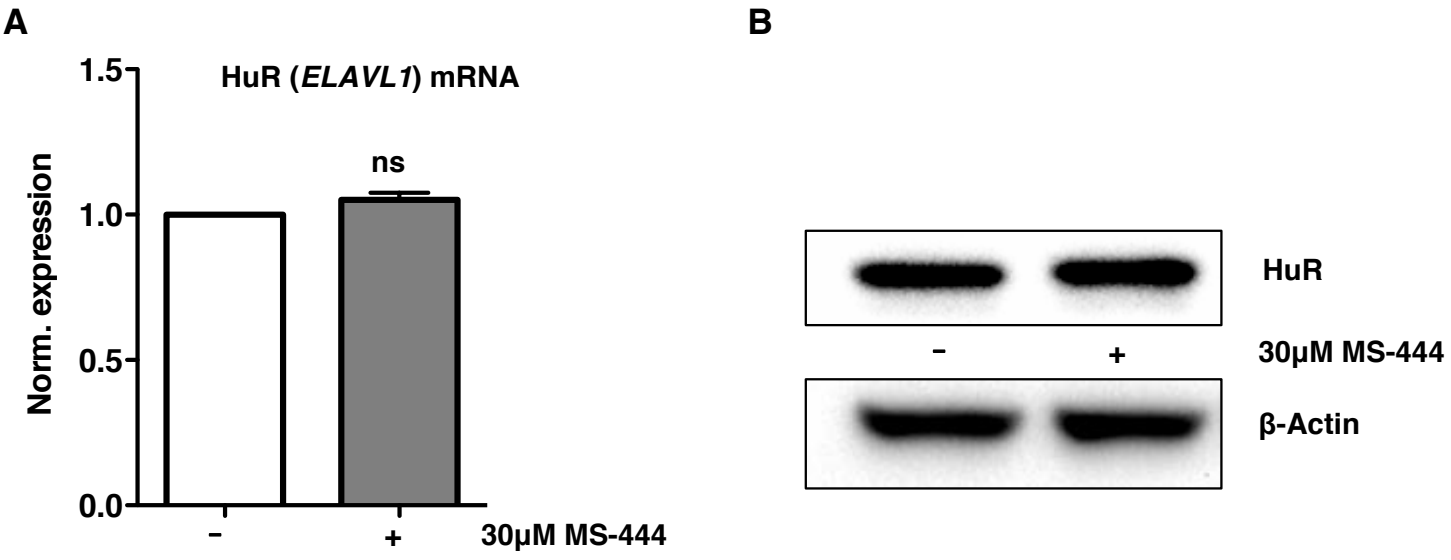

A
HuR (*ELAVL1*) mRNA
Norm. expression
1.5
1.0
0.5
0.0
ns
-
+
30μM MS-444
B
HuR
-
+
30μM MS-444
β-Actin

Figure S7.

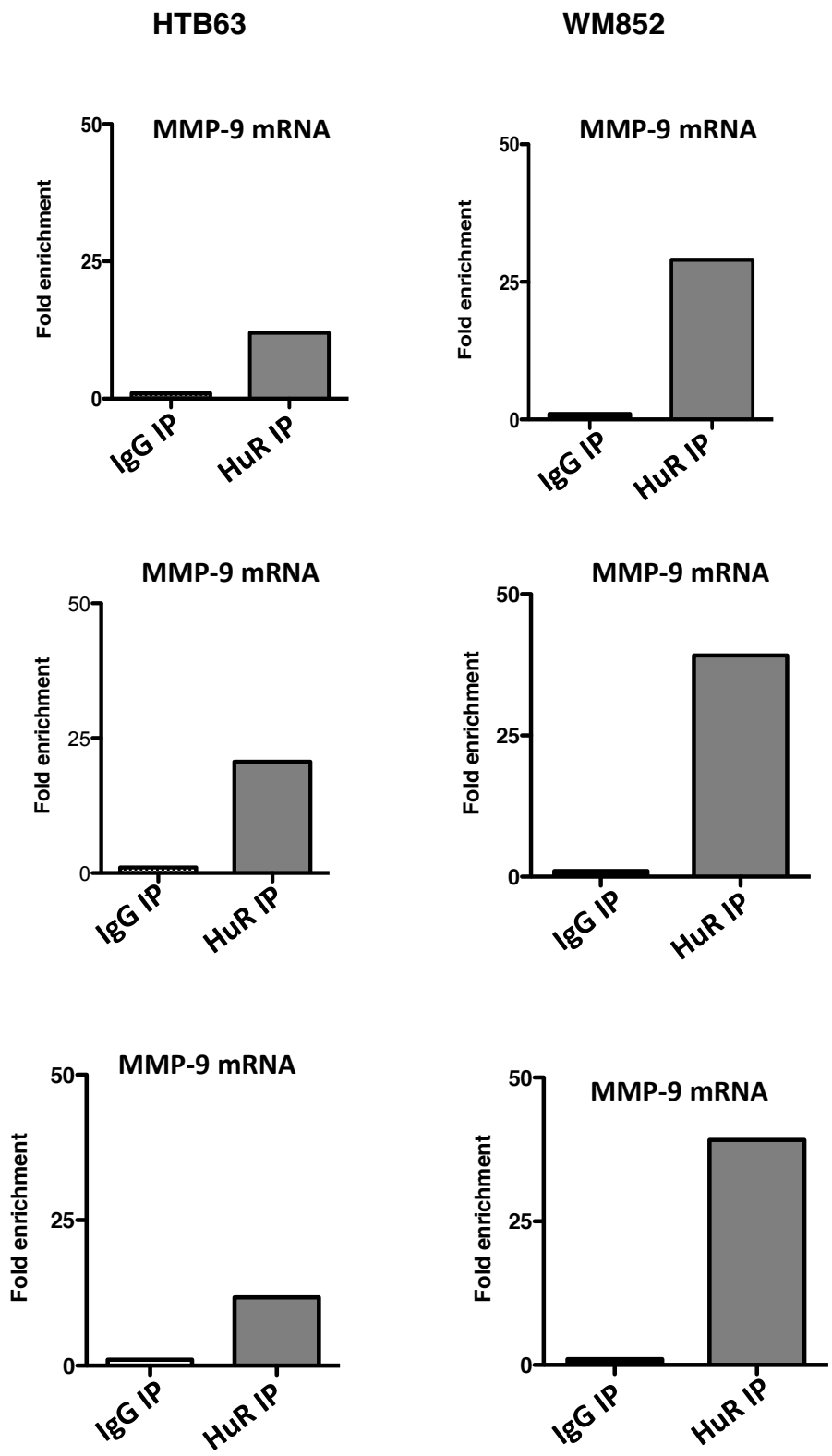
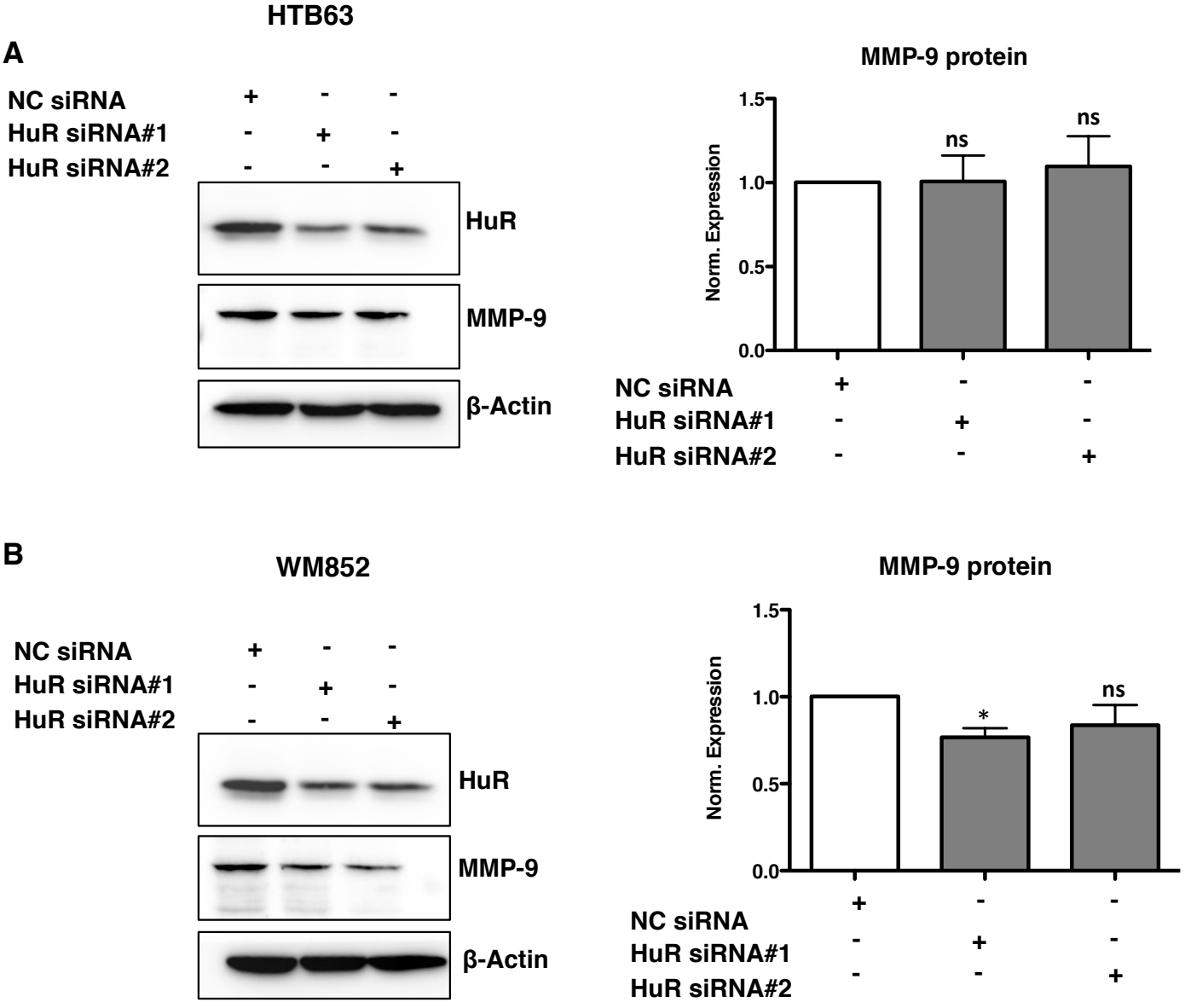


Figure S8.


A
HTB63
NC siRNA + - -
HuR siRNA#1 - + -
HuR siRNA#2 - - +
HuR
MMP-9
β-Actin
MMP-9 protein
Norm. Expression
1.5
1.0
0.5
0.0
ns
ns
NC siRNA + - -
HuR siRNA#1 - + -
HuR siRNA#2 - - +
B
WM852
NC siRNA + - -
HuR siRNA#1 - + -
HuR siRNA#2 - - +
HuR
MMP-9
β-Actin
MMP-9 protein
Norm. Expression
1.5
1.0
0.5
0.0
*
ns
NC siRNA + - -
HuR siRNA#1 - + -
HuR siRNA#2 - - +